ECOLOGY OF INTERTIDAL MICROORGANISMS AND MULTIVARIATE ANALYSIS OF ANTIBIOTIC AND HEAVY METAL SUSCEPTIBILITY OF DEEP-SEA BACTERIA

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In the Name of Allah, the Benificent,
the Merciful

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SUMMARY ABSTRACT

Deep-sea bacterial isolates from 5 sites in the N. E. Atlantic have been classified on the basis of their ability to grow in metal and antibiotic containing media at various concentrations, using a replica plating technique. The minimum inhibitory concentrations of antimicrobial agents for bacteria were determined and data submitted to cluster analysis.

Cluster analysis was applied to split up a total of 843 deep-sea isolates into a number of groups depending on their antibiotic and metal salt resistance. Boundary lines were drawn across the dendrogram at different similarity levels and a number of groups were obtained. All groups clustered at or above 72% similarity level were drawn as a shaded triangles in my final diagram of the cluster analysis. The distance measure used in cluster analysis was the Euclidean distance squared. It was also transformed into percentage similarity (%S).

The maximum number of isolates (107) occurred in cluster 12, while the minimum number (11) occurred in cluster 11. Only 1/3 of the clusters contained isolates from all the sampling sites. The number of isolates in each of the groups between sites and between depths was compared. Significant correlations were found between sites 2 and 4 and between sites 4 and 5. However, a large number of significant correlations were found between the sampling depths.

A total of 561 isolates were obtained from the sediment samples in contrast to 143 and 139 isolates from burrow linings and faecal pellets respectively. The difference between the number of isolates/group in sediment, burrow linings and faecal pellets was analysed. A highly significant variation was found. 11 of the 21 clusters could be presumptively identified as they clustered with the reference cultures used.

Cluster analysis was also applied to antibiotics alone (7 variables), to the heavy metals alone (6 variables) and to the antibiotics and heavy metals together (13 variables). The results showed that antibiotics classified into two distinct clusters due to their mode of action on Gram-positive or Gram-negative bacteria. Heavy metals calssified into one distinct cluster based on the mode of action on bacterial cell. Clustering of antibiotics and heavy metals together showed three distinct clusters and metal resistance was associated with the resistance to antibiotics.

A bacteriological survey of Ardmore Point (Clyde Estuary) has been carried out over a period of 13 months. Samples have been taken monthly using a coring technique. Heterotrophic bacterial, fungal and yeast counts have been made on different media using the spread-plating method.

API ZYM test-kits have been applied to intertidal sediments from Ardmore in an attempt to classify them biochemically by their enzyme reactions. API 20 E and API 20 NE have been applied to marine and non-marine isolates in order to test their accuracy in identifying marine bacteria.

GENERAL SUMMARY

SECTION 1

1- The aim of my work in this section was to study the toxicity effects of heavy metals and antibiotics on deep-sea sedimentary bacteria and to apply a cluster analysis to the data in order to classify the isolates into groups.

2- Deep-sea bacteria used in this study were obtained from deep-sea sediment collected at 5 sites around the Rockall Trough area of the North East Atlantic. Isolates were grown on ZoBell agar medium at 10°C.

A number of standard reference cultures were also used. These cultures were obtained from the National Collection of Marine Bacteria (NCMB), the National Collection of Industrial Bacteria (NCIB), and the National Collection of Type Cultures (NCTC). Additional isolates were supplied by Professor A. Wardlaw of the department of microbiology at Glasgow University.

3- Preliminary experiments were conducted to select the suitable concentrations of antimicrobial agents in the definitive experiments.

4- All antimicrobial agents tested were able to suppress the growth of some members of the bacterial population.

5- Increasing concentration of the antimicrobial agents were found to decrease the number of isolates which grew.

6- Mercury was the most toxic metal and manganese the least toxic.

7- Chloramphenicol and polymyxin B were the most toxic antibiotics and nalidixic acid the least toxic.

8- The relative toxicity of antibiotics and the relative toxicity of heavy metals were studied using student's t-tests. The

results showed that 5 out of 21 comparisons showed a significant difference in the toxicity between antibiotics while 12 out of 15 comparisons showed a significant difference in the toxicity between metals. Therefore, in general, heavy metals showed more significant difference in the toxicity between pairs of metals than antibiotics.

9- When the dye methylene blue was used as a selective medium, no consistent trend in the decrease in number of isolates with increasing concentrations was obtained.

10- Unlike antibiotics, with heavy metals there was no apparent general increase in bacterial growth on selective media with increasing incubation time.

11- For each isolate, the minimum inhibitory concentration (MIC) with each antimicrobial agent was defined as the lowest concentration of antimicrobial agent that inhibited growth. It varied from one antimicrobial agent to another.

12- Cluster analysis was applied to split up a total of 843 deep-sea isolates into a number of groups depending on their antibiotic and metal salt resistance.

13- A total of 27 standard reference cultures were also put through the antibiotic and metal salt system and a series of "Reference Resistance Profiles" were generated. It was hoped to broadly classify the previously grouped unknown isolates on the basis of their relationship to the reference cultures.

14- At each similarity level, a number of groups were obtained. For example, at 50% and 72% similarity levels, 11 and 21 groups were obtained respectively.

15- The results of Chi-square tests to compare the number of isolates between groups for each site, between sites for each group, between groups for each depth, and between depths for each

group at 50% S showed highly significant variation in each case.

16- The homogeneity of variances between groups for sites and depths was statistically analysed using Bartlett's and F-max tests. The results showed that there were no significant differences between overall site variances and overall depth variances for the 11 groups.

17- When groups or clusters were compared (using F-ratio) with respect to the variance in the number of isolates present between sites, it was found that only 2 groups were significant (2 and 4).

18- Similarly when groups were compared (using F-ratio) with respect to depth variances, it was found that significant differences occurred in 10 out of 55 comparisons.

19- The variances of number of isolates/site with variances of number of isolates/depth were compared using F-ratio tests. The results showed that in general, there was no significant differences between site and depth variances for each of the 11 groups (only 2 out of 11 tests were significant).

20- 72% similarity level was selected to produce my final dendrogram. All groups (21) clustered at or above this level were drawn as shaded triangles of the cluster analysis.

21- The maximum number of isolates (107) occurred in cluster 12, while the minimum number (11) occurred in cluster 11.

22- Only 1/3 of the clusters contained isolates from all the sampling sites.

23- 11 of the 21 clusters could be presumptively identified as they clustered with the reference cultures used.

24- Chi-square tests were used to test the variation in the number of isolates between sites for each group, groups for each site, depths for each group, and groups for each depth at 72%

similarity level. The results showed that there was statistically significant variation in most of cases.

25- The number of isolates in each of the groups between sites and between depths was compared using Spearman's rank order correlation tests. The results showed that only significant correlation were found between sites 2 and 4 and between sites 4 and 5. However, a large number of significant correlations were found between the sampling depths.

26- A total of 561 isolates were obtained from the sediment samples in contrast to 143 and 139 isolates from burrow linings and faecal pellets respectively.

27- The difference between the number of isolates/group in the sediment, burrow linings, faecal pellets, and data combined (total) was analysed using Chi-square tests. The results showed that all tests were highly significant.

28- The difference in the number of isolates between groups for sediment and burrow linings + faecal pellets was analysed using Chi-square tests. A highly significant variation in the number of isolates was found.

29- Similarly, the difference in the number of isolates between groups for burrow linings and faecal pellets was analysed using Chi-square tests. A highly significant variation in the number of isolates was found.

30- The distance measure used in cluster analysis was the Euclidean distance squared. It has been transformed into percentage similarity (%S). The difference between these two measures is that "similarity" take values between 1 (i.e. 100% similarity) and 0 (i.e. 0% similarity), and "distance measure" can take any positive value from 0 (i.e. no difference) upwards as difference increases.

31- The relationships between the percentage similarity and number of isolates/group, the number of groups (including single isolates), the number of groups (excluding single isolates), the number of single isolates and the coefficient of variation of number of isolates/group were studied and the following conclusions were made:

(a) As the percentage similarity increased, the number of isolates/group decreased.

(b) The number of groups including single isolates increased exponentially with increasing percentage similarity.

(c) The number of groups excluding single isolates increased with increasing percentage similarity to 99.9%, after which it decreased again.

(d) The number of single isolates increased with increasing percentage similarity.

(e) The coefficient of variation of number of isolates/group fluctuated with percentage similarity.

32- Similar conclusions can be drawn from the Euclidean distance measure.

(a) As the Euclidean distance increased, the number of isolates/group increased.

(b) Conversely, the Euclidean distance decreased exponentially with the number of groups including single isolates.

(c) Excluding single isolates, the number of groups clustered increased with decreasing Euclidean distance to 0.001, after which it decreased again.

(d) The number of single isolates decreased with increasing Euclidean distance measure.

(e) The coefficient of variation of number of isolates/group

fluctuated with Euclidean distance.

33- Cluster analysis was applied to the antibiotics alone (7 variables), to the heavy metals alone (6 variables), and to the antibiotics and heavy metals together (13 variables).

(a) Clustering of antibiotics, showed that antibiotics classified into two distinct clusters due to their mode of action on Gram-positive or Gram-negative bacteria. Two antimicrobial agents remained unclustered.

(b) Clustering of heavy metals, showed that three metals were clustered in one distinct cluster. The other metals used remained unclustered. Heavy metals were clustered based on the mode of action on bacterial cell.

(c) When cluster analysis was applied to all antimicrobial agents together, it was concluded that there were three distinct clusters and metal resistance was associated with the resistance to antibiotics.

SECTION 2

The aims of my work in this section were to estimate the seasonal viable counts of heterotrophic bacteria, yeasts and fungi in overlying, interstitial waters and sediments (vertical profiles) at Ardmore, Clyde Estuary.

BACTERIA

1- The relationship between numbers of bacteria in overlying and interstitial water at Ardmore Point over a period of thirteen months was studied. The results showed that the numbers of bacteria in interstitial water were higher than that of overlying water.

2- In overlying water, a peak in bacterial numbers was shown in February 1984, while in interstitial water, number of marked peaks of bacterial numbers were in March and between April-November

1984.

3- The variation in the numbers of bacteria at different sediment depths was studied. The following conclusions were made.

(a) The numbers of bacteria were greatest at the surface sediment and tended to decrease with depth.

(b) At the surface sediment, peaks of high levels of bacteria occurred in February, April, August and November 1984 and in January 1985.

(c) At 5 and 10cm sediment depth, similar ranges of bacterial numbers occurred.

(i)- At 5cm depth, three peaks occurred in February-March, and August 1984 and in January 1985.

(ii)- At 10cm depth, two peaks occurred in March and June 1984.

(d) At 20 and 35cm depth, much lower numbers of bacteria were found than at 5 and 10cm depth.

(i)- At 20cm depth, three minor peaks occurred in March-April, July-August and in January 1985.

(ii)- At 35cm depth, only one peak of bacterial numbers occurred in August 1984.

4- The variation in the numbers of bacteria with depth for each month from February 1984 to February 1985 was studied. The results showed that in general, the patterns of decreasing numbers of bacteria with depth was similar for all months except for surface sediment which showed high levels of bacteria in February, April, August, November 1984 and in January 1985.

5- The bacterial numbers at each sampling depth were compared using all data collected over thirteen months. This was carried out using regression analyses and sudent's t-tests. The bacterial

numbers at each depth were also plotted against the other depths (e.g. surface against the other depths). The results showed the following.

(i) A direct relationship in bacterial numbers occurred in all cases with only a few exceptions as follows.

(ii) An indirect relationship was found in the comparisons of surface-10cm and surface-35cm depth.

(iii) No significant relationship was found in the 5-10cm comparison.

(iv) The t-tests showed a significant difference in bacterial numbers between all depths with the exception of 5-10cm, where no significant difference was found.

6- The relationship between the numbers of bacteria (C.F.U.) in overlying and interstitial waters and incubation time (days) for the month of July 1984 was investigated. The results showed that bacteria in overlying water reached a maximum growth after 20 days, while in interstitial water, the maximum growth was after only 7 days.

7- The relationship between the numbers of bacteria (C.F.U.) in sediment from different depths, and incubation time (days) was also studied. The results showed that in general, there was a decrease in bacterial numbers with sediment depth at each incubation time. In addition at greater sediment depths there was a less pronounced increase in bacterial growth with increasing incubation time. The maximum growth was always reached approximatley after 15 days incubation.

YEASTS

1- The relationship between numbers of yeasts in overlying and interstitial waters at Ardmore Point over a period of thirteen months was studied. The results showed that interstitial water

contained significantly higher numbers of yeasts than overlying water.

2- In overlying water, 4 peaks of yeast numbers were shown in February, April and August 1984, and in January 1985, while in interstitial water, 3 peaks of yeasts were shown in May, August and October 1984.

3- The variation in numbers of yeasts in sediment at different depths was studied. The results showed the following conclusions.

(a) The numbers of yeasts were greatest at the surface sediment and in general, tended to decrease with depth.

(b) At the surface sediment, peaks of high levels of yeasts occurred in November with lesser peaks in March and September 1984 and in January 1985.

(c) At 5cm depth, maximum numbers of yeasts were found in February 1985.

(d) At 10cm depth, maximum numbers were found in June 1984.

(e) At 20cm and 35cm depth, similar curves were found although peaks occurred at slightly different times of the year (August at 20cm and July at 35cm).

4- The variation in the numbers of yeasts with depth for each month from February 1984 to February 1985 was studied. The results showed that in general, the patterns of decreasing numbers of yeasts with depths was similar for all months with the exception of February, March, June, July and August which showed peaks of numbers of yeasts at 10cm and 20cm depth.

5- The yeast numbers at each sampling depth were compared using all data collected over thirteen months. This was carried out using regression analysis and student's t-tests. the yeast numbers at each depth were also plotted against the other depths (e.g.

surface against the other depths). The results showed the following.

(a) Highly inversely significant relationships were found with all the surface comparisons.

(b) There were no significant relationships between all the other depths with exception of 20cm-35cm depth which showed a direct significant relationship.

FUNGI

1- The relationship between numbers of fungi in overlying and interstitial waters at Ardmore Point over a period of thirteen months was studied. The results showed similar seasonal abundance with overlying and interstitial waters and the ranges in fungal abundance were close in both samples.

2- In general, there were 3 peaks of fungal growth for both overlying water (February, July and November 1984) and interstitial water (February-March, and November 1984).

3- The variation in the numbers of fungi at different sediment depths was studied. There was no clear decrease in the numbers of fungi with depth. The results also showed at all depths, there were 2 peaks of high numbers of fungi (February 1984 and May-August).

4- The variation in the numbers of fungi with depth for each month from February 1984 to February 1985 was studied. The results showed no clear pattern of decreasing numbers of fungi with depth.

SECTION 3

1- The aim of my work in this section was to apply the API ZYM, API 20E, and API 20NE systems to marine and non-marine samples in a number of experiments. The samples consisted of intertidal sediment and marine and non-marine named bacterial isolates.

2- An experiment was conducted to determine the quantity of

sediment dispensed from a pasteur pipette into each API ZYM cupule. Statistical analysis using two-way analyses of variance showed that in both the first and second replicate experiments there was no significant variation between drops or pipettes.

3- An experiment was carried out to determine the optimum incubation time of inoculated API ZYM strips using intertidal surface sediment. The results of this experiment suggested that the highest amount of hydrolysed substrate in most cupules occurred in the time between 10-20 hours of the first incubation and between 5-20 hours of the second incubation time.

Student's t-tests were applied to the data and showed that there were differences between the first incubation times in most cupules. However, in the second incubation times, there was no significant variation.

4- An experiment was conducted to determine how soon after addition of API ZYM reagents colour developed and reached a maximum intensity. A two-way analysis of variance applied to the data showed possible statistical interaction. Subsequent one-way analyses showed a highly significant variation between cupules at a depth of 5cm. However, there was no significant variation between cupules at 10cm, 20cm and 35cm depth. At the surface, there was only possible variation between cupules. One-way analyses of variance also showed a significant variation between depths with cupules 2 and 6. However, with cupules 7 and 11, there was no significant variation between depths.

The variation in time at the which maximum score was reached was studied. Two-way analysis of variance showed that there was a significant variation between depths, but that there was no significant variation between cupules.

5- An experiment was conducted to test whether the colour noticed in API ZYM cupules was produced by membrane filterable products and if so, whether these products were heat stable or heat labile. The results showed that for both overlying and interstitial water, there is no difference between control and membrane filtered seawater and no difference between boiled and autoclaved seawater. The results also showed that there are significant differences between the cupules, and the activity is not affected by membrane filtration but is abolished by boiling and autoclaving.

Statistical analysis comparing overlying water with interstitial water (control and membrane filtered) for each cupule, showed a highly significant variation between overlying and interstitial water samples in 3 out of 5 cupules. In these 3 cupules, the activity in the interstitial water was greater than that in the overlying water.

6- An experiment was conducted to determine the growth rate of marine and non-marine bacteria. The results showed the following conclusions:

(a) The number of cells increased with increasing incubation time up to 12 hours and the non-marine strains showed a greater increase in the number of cells than marine strains.

(b) The log phase of all strains started just before 6 hours, with the exception of Escherichia coli which reached slightly earlier.

(c) The stationary phase of all strains examined was always reached after 12 hours, with the exception of Aeromonas hydrophila which reached the stationary phase after 18 hours.

7- An experiment was conducted to test how well the API 20E and API 20NE systems work with marine and non-marine bacteria. The

effect of bacterial cell concentration on the kit-system reaction was also tested. The results showed the following conclusions.

(a) With the API 20E system and after 24 hours, all the strains gave some positive results and in general, the colour intensity decreased with increasing dilutions of cell suspension. The exception to this was Micrococcus sp. which gave negative results with all the tests.

(b) The results with Vibrio fischeri, Aeromonas hydrophila and Escherichia coli showed little change after 48, 72 and 96 hours except in the carbohydrate tests which were affected by the added reagents.

(c) With the API 20NE system and after 24 hours, the marine strains Vibrio fischeri and Micrococcus sp. gave two and one positive result respectively. The non-marine strains Aeromonas hydrophila and Esherichia coli gave numerous positive results.

(d) After 48, 72 and 96 hours of incubation, the strains Vibrio fischeri and Micrococcus sp. showed only a few positive results in the conventional tests. However, after 72 and 96 hours, there were many positive results (growth) in the assimilation tests at the high cell concentrations.

(e) With the strains Aeromonas hydrophila and Esherichia coli, there were more positive results after 48, 72 and 96 hours than at 24 hours. The majority of these positive results were from the assimilation tests.

8- Preliminary and definitive experiments were conducted to test the effect of the number of Escherichia coli cells and incubation time on the API 20E system. The results demonstrated that colour intensity decreased with increasing dillutions after 24 hours.

GENERAL INTRODUCTION

Microorganisms in soil and sediments belong to many different groups in the plant and animal Kingdoms (ZoBell, 1946; Burges, 1958; Wood, 1965; Alexander, 1977). In the plant Kingdom fungi, algae, bacteria and actinomycetes are the most abundant. In the animal Kingdom protozoa, flagellates and minute metazoan organisms are widely distributed in marine deposits. This study is mainly concerned with marine bacteria. Bacteria living in the sea are different from those in fresh water, and bacteria of the rivers are different from those in lakes (Rheinheimer, 1985). Most of the aquatic bacteria are heterotrophic (i.e. live on organic substances). Bacteria may exist in the water unattached, or attached to solid materials (Meadows, 1964; 1965; Goulder, 1977). Bacteria and fungi play an important role in the food cycle by synthesising cell substances and converting waste or dissolved organic matter into a particular form which can be used as food for the sea bed fauna (ZoBell, 1938; 1946; Wood, 1965; Rheinheimer, 1985).

Observations made during the Galathea Expedition of 1950-1952 demonstrated for the first time the presence of living bacteria in sediment samples obtained at depths greater than 6000m (ZoBell, 1952 a; 1954). Because of the sampling difficulties, bacteria at this depth have not been extensively studied. At shallower depths, many investigators have reported the presence of living bacteria (Bortholomew and Rittenberg, 1949; Morita and ZoBell, 1955; Meadows and Tait, 1985). ZoBell, (1952a,b) reported that in the period 1948-1950, bacteria were isolated from sediment obtained from the floor of the Atlantic Ocean and the mid-Pacific Ocean at depths of 5800 and 5300m respectively. Bortholomew and Rittenberg (1949) reported the presence of Gram-positive, spore-forming, rod-shaped thermophilic bacteria (growth at 60^{o}C) in deep-ocean bottom cores.

In contrast, in inshore environments, a great deal of work has been done on the bacterial population (Lloyd, 1931; Meadows, 1964; 1965; 1966; Meadows and Anderson, 1968; Anderson and Meadows, 1969; Dale, 1974; Ezura et al., 1974; Litchfield and Floodgate, 1975; Goulder, 1977; Goulder et al., 1979; Anderson et al., 1980; Meadows et al., 1980; Kennedy, 1984).

Bacterial flora have been studied since early times and occurs not only in seawater, but in the mud and sand of the sea bottom. Drew (1911) found that shallow coastal water in the tropics was rich with bacteria. This was because the mud flats below were unusually rich in bacteria. Lloyd (1931) investigated the bacterial content of mud deposits in the Clyde sea area. The author found the numbers of bacteria in mud decreased with increasing sediment depth, and these numbers fluctuated very much in the top mud layers. More recently, Meadows and Tait (1985) found this phenomenon in deep-sea sediments.

Meadows (1964) observed that distilled water induced many bacteria to separate from the surfaces of particles of marine sand. Meadows (1965) continued examining the attachment of aquatic bacteria to surfaces under experimental conditions. The author found that in rivers and estuaries, marine bacteria will only remain attached to solid surfaces when tidal flow produces water of high salinity, while fresh water bacteria will only remain attached up-rivers where salinity is low.

Wright and Coffin (1983) found that the total number of bacteria in the tidal areas of the river estuaries on the East coast of North America, was much higher than in the adjacent limnic and marine areas. The authors deduced that in the warmer seasons, a concentration of nutrients caused by mixing processes, led to this phenomenon.

The total bacterial numbers in coastal waters of the North and Baltic seas, were investigated and were found to range from one hundred thousand to several millions (Rheinheimer, 1985).

Fuller accounts and a more detailed literature review are given in the introduction to sections 1, 2 and 3.

Plan of thesis

The work reported in this thesis is divided into three sections as follows:

Section 1

Deep-sea bacterial isolates from 5 sites in the N. E. Atlantic have been classified on the basis of their ability to grow in metal and antibiotic containing media at various concentrations, using a replica plating technique. The minimum inhibitory concentrations of antimicrobial agents for bacteria were determined and data submitted to cluster analysis using the University main frame computer.

Section 2

A bacteriological survey of Ardmore Point (Clyde Estuary) has been carried out over a period of 13 months. Samples have been taken monthly using a coring technique. Heterotrophic bacterial, fungal and yeast counts have been made on different media using the spread-plating method.

Section 3

API ZYM test-kits have been applied to intertidal sediments from Ardmore in an attempt to classify them biochemically by their enzyme reactions. API 20E and API 20NE have been applied to marine and non-marine isolates in order to test their accuracy in identifying marine bacteria.

In each section, the figures, tables and plates are numbered from one onwards. (For example, the reference to figure 5 in section 1, refers to figure 5 of that particular section).

SECTION 1

Cluster analysis of antibiotic and heavy metal susceptibility of deep-sea bacteria

combining with cellular proteins and denaturing them (Pelczar and Reid, 1972; Summers and Silver, 1972; Vallee and Ulmer, 1972; Cole, 1977). For example, mercuric chloride inhibits enzymes containing the sulfhydryl grouping. Salts of heavy metals are also protein precipitants, and in high concentrations such salts could cause the death of a cell.

The mode of action of dyes is obscure. however, a reasonable assumption is that their action is through combination with cellular macromolecules (i.e. acridine dyes are known to bind to nucleic acids). In general, Gram-positive bacteria are more susceptible to dyes than Gram-negative. This is probably due to the difference in the cell wall structure of both kinds of bacteria.

A detailed accounts of the action of antibiotics and heavy metals on bacterial cells is given in appendix A p. 217-235.

The aim of my work was to classify by cluster analysis bacterial populations that had been isolated from deep-sea sediments into a number of groups depending on their antibiotic and metal salt resistance. Two series of experiments were conducted for this purpose. The first are called the preliminary series of experiments, the second are called the definitive series. The results of the preliminary experiments were used to select more suitable concentrations of antimicrobial agents in the definitive experiments.

A number of standard reference strains have also been included, so that a series of reference resistance profiles can be generated. It would then be possible to identify the unknown isolates previously grouped on the basis of their relateness to standard reference strains.

For each of the 843 deep-sea isolates and 27 reference cultures, the minimum inhibitory concentration (MIC) with each

antimicrobial agent was determined in order to submit this data to a cluster analysis using the University main frame computer.

One could in future compare deep-sea results with that of shallow water bacteria.

Replica plating technique

The technique used for the antimicrobial agent experiments was the replication of pure cultures onto ZoBell agar plates containing wide ranges of antimicrobial agent concentrations. The technique of replica plating was introduced by Lederberg and Lederberg (1952). It is based on the transfer of isolates from one initial agar plate to a series of selective agar media. Replica plating was used in this study in order to estimate the frequency of antibiotic and metal resistant profiles of the bacteria recovered from deep-sea sediments.

Several studies have been carried out using this rapid and economic technique (Harris, 1963; Corlett et al., 1965; Shiaris and Cooney, 1983). Corlett et al. (1965) used the replica plating technique for a quantitative identification of microbial flora in food. The authors have used the technique in order to allow a large number of isolates examined without an accompanying increase in labour and inoculate 12 selective media for the identification purpose. Shiaris and Cooney (1983) found that the replica plating method was useful as a tool to screen large number of colonies and to estimate the relative numbers of potential phenanthrene utilizers and cometabolizers in natural populations.

Cluster analysis

In this account, I firstly make some general statments about cluster analysis and its efficiency in identifying bacterial isolates. I then describe the two major parts of any cluster analysis:

similarity coefficients (similarity and distance measure) and the method of clustering (analysis of data matrix). Finally I review some important papers in the field.

Cluster analysis is a branch of numerical taxonomy used to sort out a number of cases into groups depending on the degree of association between members. The method suggested by Sneath (1957a,b) for analysing a large bulk of data was to count the number of similar and dissimilar characters between isolates, and then to sort the isolates into groups whose members have a high percentage of similarities. The method has been used with great success in recent years in microbial classification. The first who applied numerical taxonomy to microorganisms was Sneath (1957a,b) and Sokal and Sneath (1963). Since then remarkable progress has been made by many investigators (Pfister and Burkholder, 1965; Sneath and Sokal, 1973; Bonde, 1975; Hauxhurst et al., 1980; Austin et al., 1981; Holder-Franklin et al., 1981; Lee et al., 1981; Sneath et al., 1981; Austin, 1982; Allen et al., 1983; Bridge and Sneath, 1983; Knivett et al., 1983; West et al., 1983; Williams et al., 1983; West et al., 1984; Austin and Moss, 1986; Baya et al., 1986; Gil et al., 1986; Marquez et al., 1987).

Beers et al. (1962) attempted to determine the relationships among 54 strains of bacteria including the genera Achromobacter, Aerobacter, Alcaligenes, Escherichia, Mima, Pseudomonas, Serratia and Streptococcus using computer methods. They suggested that the use of distance rather than similarity values may be better for elucidating relationships among groups of organisms. Bascomb et al. (1973) also applied a computer program to identify 1079 reference cultures of Gram-negative aerobic bateria. A comparison of conventional methods and the computer identification showed that 90.8% of fermentative and

82% Of non-fermentative isolates could be identified. Friedman et al. (1973) used a fortran computer program to assist in the identification of dextrose-fermenting Gram-negative rods isolated from clinical sources. The program correctly identified the unknown clinical isolates in more than 99%. The work of Bascomb et al. (1973) and Friedman et al. (1973), therefore, show clearly that between 80% and 99% of bacteria are likely to be correctly identified using the methods of numerical taxonomy.

Measurements of similarity and distance (similarity coefficent). Most clustering techniques begin with the calculation of a matrix of similarities or distances between individuals. In numerical taxonomy, different types of similarity or dissimilarity measures were employed to estimate the relationship between individuals. Clifford & Stephenson (1975) discussed many of these measures, such as coefficient measures "similarity measures" and Euclidean distance "dissimilarity distance measure". The difference between the two measures is that "similarities" take values between 1 (i.e. 100% similarity) and 0 (i.e. 0% similarity), and "distance measure" can take any positive value from 0 (i.e. no difference) upwards as difference increases.

The coefficient measure used in my study was the squared Euclidean distance as a dissimilarity measure (see materials and methods, p. 47).

Although the Euclidean distance is widely used in clustering techniques, there are many other possible distances. Clifford and Stephenson (1975) state that the similarity measure, correlation coefficient has been used with considerable success in clustering techniques. Wishart (1978) illustrate 40 options of similarity coefficients under the procedure CORREL of the CLUSTAN package. Two options are given for some coefficients, one for binary and the other

for continuous data. The reason is that some coefficient measures are restricted only to binary data. Wishart (1978, p. 32, 114) and Everitt (1980, p. 17) state that the Euclidean distance is the most commonly used and familiar measure of dissimilarity between cases or clusters. West et al. (1983) applied a numerical study on species of Vibrio isolated from the aquatic environment and birds in Kent (England). The authors clustered strains by three methods and used Euclidean distance coefficient to calculate the similarity between species. Calculations of the similarity profiles were performed using the CLUSTAN 1C release 2 package (Wishart, 1978) and the three methods gave similar results.

Analysis of data matrix (clustering method). Once the data matrix has been obtained using one of the above methods (distance or similarity measures), there are a number of agglomerative hierarchical and non-hierarchical clustering techniques that can be applied. The hierarchical techniques form an initial partition of N clusters (each individual is a cluster) and in a stagewise manner proceed to reduce the number of clusters one at a time until all N individuals are in one cluster. The non-hierarchical techniques involve the isolation and representation of groups of similar data, the groups not necessarly being conjoined. The following techniques as explained in detail in Everitt (1980, p. 25-34) are the most common hierarchical clustering models:

1- Single linkage (Nearest neighbour).

2- Complete linkage (Furthest neighbour).

3- Centroid cluster analysis.

4- Group average method.

5- Ward's method.

The technique of single linkage fuses the individuals according to the distance between their nearest neighbours (minimum distance or maximum similarity). Complete linkage is the opposite of

single linkage technique. It fuses the individuals according to the distance between their furthest neighbours (maximum distance or minimum similarity). Centroid cluster analysis fuses the individuals according to the distance between their centroids, the groups with the smallest distance being fused first. The distance between groups is defined as the distance between the group centroids. Group average linkage defines distance between groups as the average of the distances (average distance or similarity) between all possible pairings of individuals in the two groups.

Ward's method, which was the method I used, has been investigated by many authors (Ward, 1963; Paykel and Rossaby, 1978; Lee et al., 1981) and is described in the materials and methods (p. 48).

Wishart (1978) listed 40 options of similarity coefficients under the procedure CORREL of the CLUSTAN package as mentioned above. If the coefficient has not been specified (for example, ICOEF = 24 i.e. error sum of squares), then the procedure CORREL substitutes the appropriate distance coefficient (ICOEF = 1 or 2 i.e. squared Euclidean distance). This is regarded by CLUSTAN as the standard default option. Wishart also states that Ward's method is only meaningful when distance coefficient (squared Euclidean distance) has been computed with the procedure CORREL. Paykel and Rassaby (1978) applied four agglomerative hierarchical methods (including Ward's method) on a sample of 236 suicide attempters. The authors found that Ward's method was the most satisfactory clustering technique.

Much of the literature on cluster analysis has been covered above. However, the following are some additional important papers.

Bacterial populations from different aquatic sources have been studied using different similarity coefficients. Allen et al.

(1983) collected 722 strains from freshwater fish farm and examined the similarity between these isolates and 124 characters using simple matching and Jaccard coefficients. The authors applied the average linkage clustering technique and showed that a similarity level of 70% or above as calculated with the Jaccard coefficient, 82% of the isolates were recovered in 14 major and 56 minor clusters. Austin et al. (1978) analysed data of 600 isolates from green leaves of Lolium perenne S24 using the simple matching coefficient. The authors concluded from the cluster analysis of average linkage that at a similarity of 80% or above, 74% of the isolates were recovered in six major and 45 minor clusters. Hauxhurst et al. (1980) applied a numerical taxonomic analysis on large population of isolates from the Northwest and Northeast Gulf of Alaska. The workers used the Jaccard similarity coefficient and the single linkage clustering technique. They found that at 70% similarity level, 24 clusters from the Northeast Gulf isolates and 12 clusters from the Northwest Gulf isolates were recovered. Lee et al. (1981) carried out a numerical taxonomic study to evaluate the relationship of group F bacteria to other biochemically similar organisms within the family Vibrionaceae. Lee and his associates clustered their isolates for 114 characters using four clustering methods (single linkage, complete linkage, average linkage and Ward's method). All calculations were carried out using the CLUSTAN 1A (Wishart, 1969) package and results showed very similar pattern. Pfister and Burkholder (1965) examined bacterial isolates from Antarctic and tropical seawaters with a series of characteres. The authors computed the similarity values of 88 isolates from Antarctic, six from tropical water and two yeast cultures according to the simple matching coefficient which includes negative matches. They used the single linkage and results were plotted for different percent similarities at 80% or above. The organisms tested

by Pfister and his co-worker were grouped into nine major clusters. West et al. (1984) examined 18 cultures of phenanthrene degrading isolates from Chesapeake Bay and 21 reference cultures for 123 characteres. The authors analysed their data numerically by the use of simple matching and Jaccard coefficients. They constructed seven clusters by the single and average linkage. Hudson et al. (1986) performed a numerical taxonomy on 45 New Zealand isolates and six non-New Zealand type isolates. Similarity matrices were calculated with the simple matching (Sokal & Michener, 1958) and Jaccard (Sneath, 1957) coefficients using the single and the average linkage. The authors constructed seven clusters and found that the majority of the New Zealand isolates did not cluster with non- New Zealand isolates. They also showed that there was a relationship between the composition of the clusters and the temperature and pH of the source of the isolates. Hudson and his co-workers concluded that within New Zealand isolates, the geographical source of the isolates had no bearing on the clusters formed.

SECTION 1

MATERIALS AND METHODS

Collection and culture of samples

Deep-sea work was divided into two parts as shown in (Figure 1).

1- Work conducted on ship.

2- Work conducted in laboratory.

1- On ship

Samples were collected at five sites around the Rockall Trough area of the North East Atlantic. Details of the bacteriological sampling at the sites are given in figure 2 and table 1. Table 1 also shows the final number of isolates obtained (column 9) and the main frame computer library file name used for storing data on the responses of the isolates to antimicrobial agents (Appendix table 31).

Site 1 (995 m) was on the edge of the Hebridean Shelf. Site 2 (2170 m) was adjacent to the Anton Dohrn seamount on the Abyssal plain. Site 3 (2000 m) was on the North East of the Rockall Bank (South of George Bligh Bank) Site 4 (2200 m) was on the North Feni Ridge just South of Bill Bailey's Bank . Site 5 (1660 m) was on the North Feni Ridge South of Lousy Bank.

Sediment samples were collected using a spade box corer (Plates 1 and 2). Subcoring of the samples was carried out immediately the spade box corer was brought inboard. Subcores were extruded and divided up into the following depth sections: 0 to 1cm, 2.5 to 3.5cm, 5.0 to 6.0cm, 10 to 11cm, 15 to 16cm and 20 to 21cm. These are refered to from now onwards (Table 1) as the following average depths: 00.5, 03.0, 05.5, 10.5, 15.5, and 20.5 cm. Samples were also taken from invertebrate burrow linings and faecal pellets at some sites. The faecal pellets and the burrow linings depths are exact (Table 1). Samples were processed (plating out) immediately they were brought inboard. Following plating out using a standard spread plate

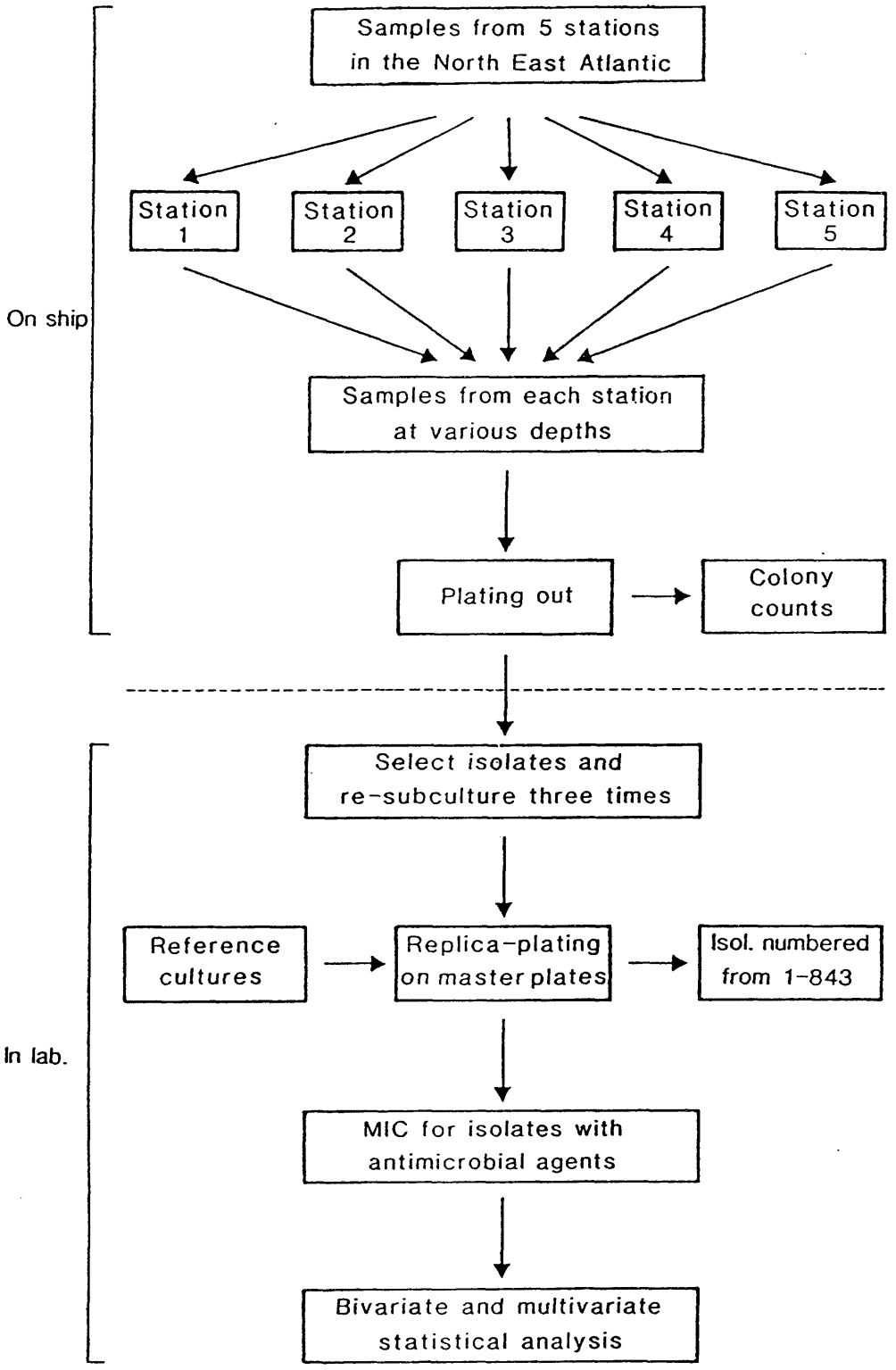


Figure 1

Procedure adapted to process the deep-sea samples on ship and in laboratory (see materials and methods p. 10).

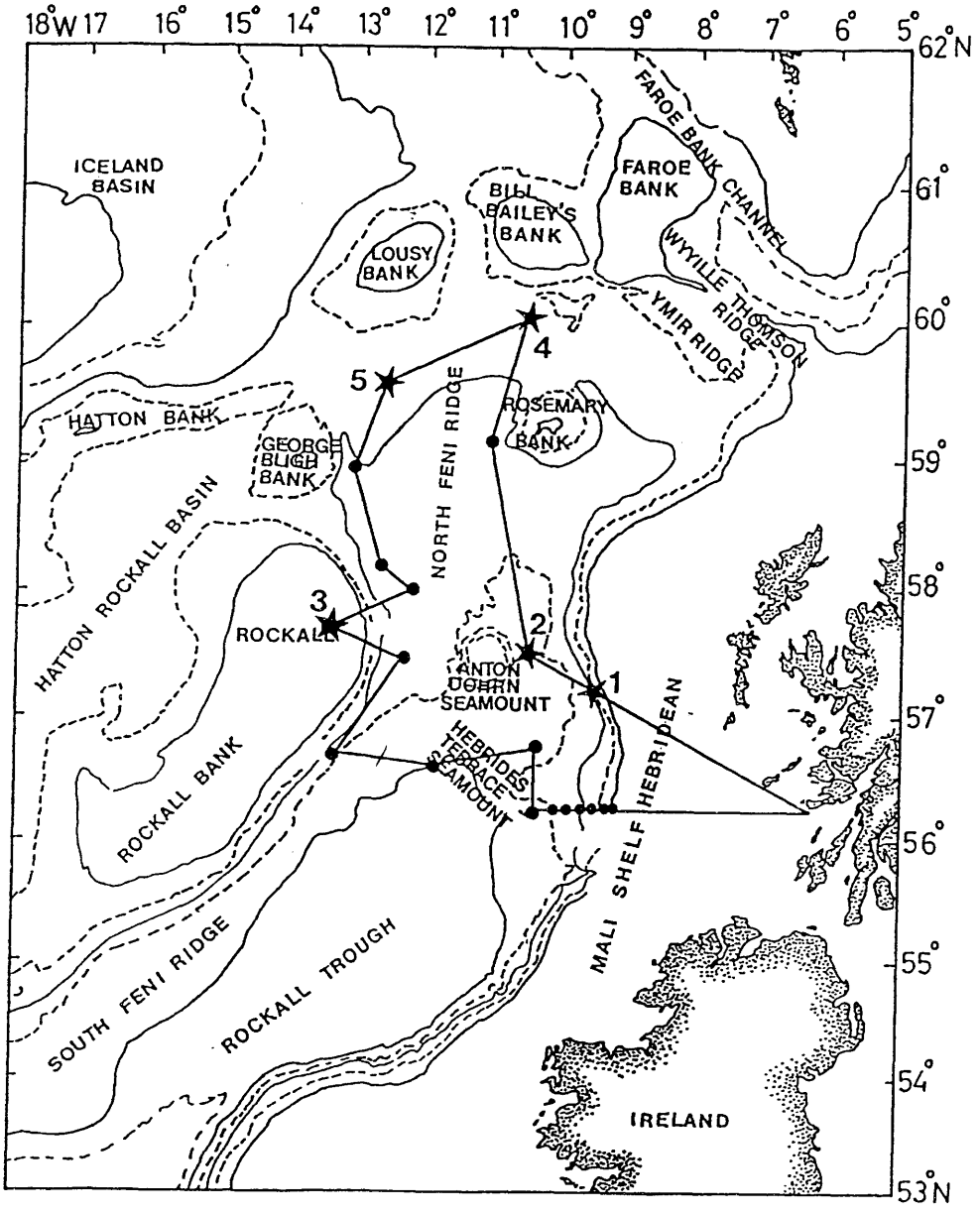


Figure 2 Deep-sea sampling sites. Continuous line shows Cruise route.

* = Stations from which bacterial isolates were obtained.

● = Other sampling sites on Cruise.

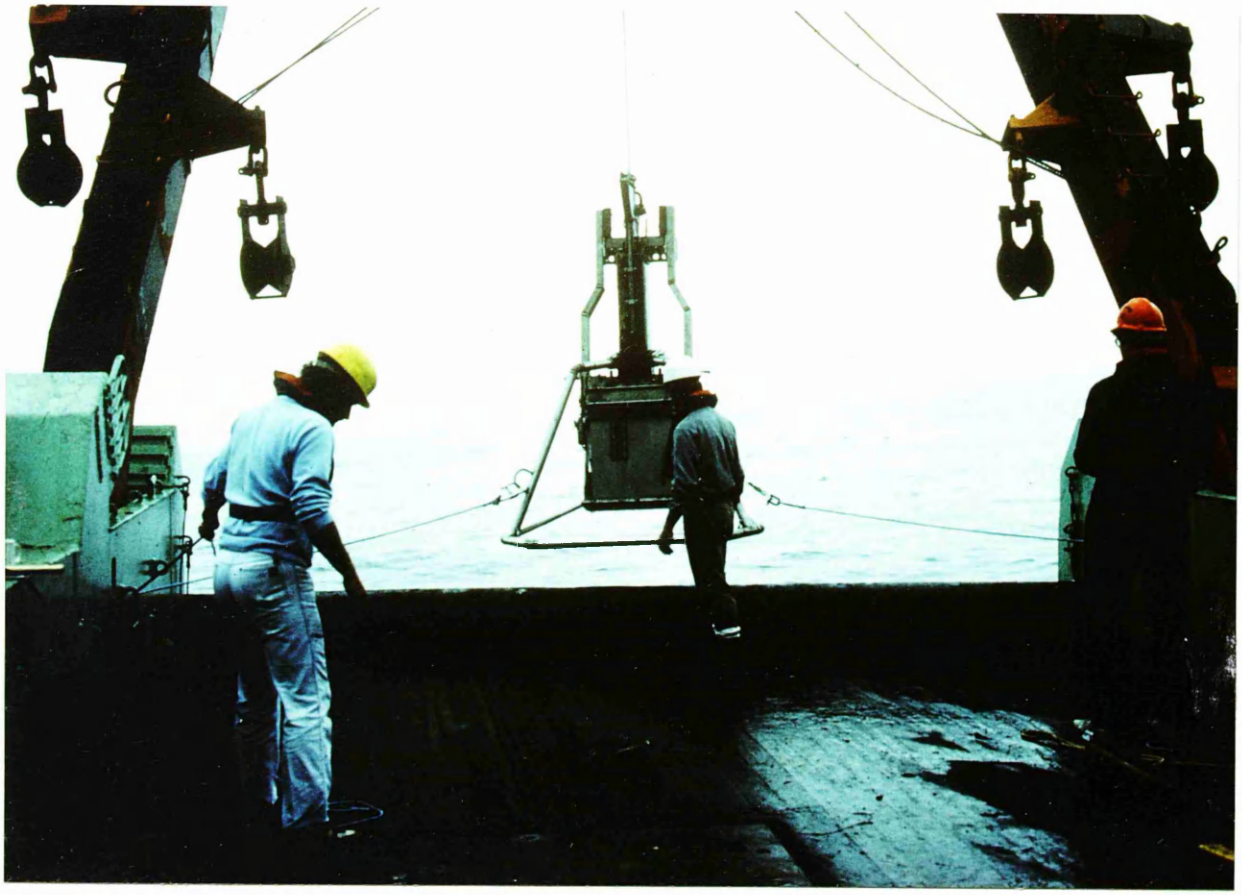
Site no.	Position of sites	Water depth (m)	Source of bacteria	Depth code	Sample depth (cm)	Incub. temp. (°C)	Computer library file name	Final no. isolates
			sediment	1	00.5	10	DSBACT.ONE 1	33
			sediment	2	03.0	10	DSBACT.ONE 2	23
1	57° 06.790'	995	sediment	3	05.5	10	DSBACT.ONE 3	06
	09° 22.200'		sediment	4	10.5	10	DSBACT.ONE 4	08
			sediment	5	15.5	10	DSBACT.ONE 5	04
			sediment	6	20.5	10	DSBACT.ONE 6	01
			sediment	1	00.5	10	DSBACT.TWO 1	22
			sediment	2	03.0	10	DSBACT.TWO 2	33
			sediment	3	05.5	10	DSBACT.TWO 3	31
			sediment	4	10.5	10	DSBACT.TWO 4	09
			sediment	5	15.5	10	DSBACT.TWO 5	21
2	57° 21.510'	2170	sediment	6	20.5	10	DSBACT.TWO 6	20
	10° 19.080'		faecal pell.	A	11.0	10	DSBACT.TWO 7	42
			faecal pell.	A	11.0	04	DSBACT.TWO 8	38
			bside burr.	B	11.0	10	DSBACT.TWO 9	14
			bside burr.	B	11.0	04	DSBACT.TWO 10	39
			surface sed.	D	00.0	10	DSBACT.THREE1	26
			surface sed.	D	00.0	04	DSBACT.THREE2	27
			burrow lin.	B	07.5	10	DSBACT.THREE3	16
3	-	2000	burrow lin.	J	07.5	10	DSBACT.THREE4	05
			faecal pell.	L	07.5	10	DSBACT.THREE5	38
			faecal pell.	L	07.5	04	DSBACT.THREE6	07
			faecal pell.	H	07.5	04	DSBACT.THREE7	14
			subsurf.sed.	F	07.5	10	- -	-
			sediment	1	00.5	10	DSBACT.FOUR 1	32
			sediment	2	03.0	10	DSBACT.FOUR 2	57
			sediment	3	05.5	10	DSBACT.FOUR 3	01
			sediment	4	10.5	10	DSBACT.FOUR 4	06
4	56° 40. 896'	2200	sediment	5	15.5	10	DSBACT.FOUR 5	03
	10° 29. 293'		sediment	6	20.5	10	- -	-
			burrow lin.	mg3	04.0	10	DSBACT.FOUR 6	35
			sediment	mg3	04.0	10	DSBACT.FOUR 7	25
			burrow lin.	st4	13.0	10	DSBACT.FOUR 8	39
			sediment	st4	13.0	10	DSBACT.FOUR 9	32
			sediment	1	00.5	10	DSBACT.FIVE 1	57
			sediment	2	03.0	10	DSBACT.FIVE 2	30
5	56° 14.790'	1660	sediment	3	05.5	10	DSBACT.FIVE 3	33
	09° 44.390'		sediment	4	10.5	10	DSBACT.FIVE 4	14
			sediment	5	15.5	10	DSBACT.FIVE 5	02
			sediment	6	20.5	10	- -	-

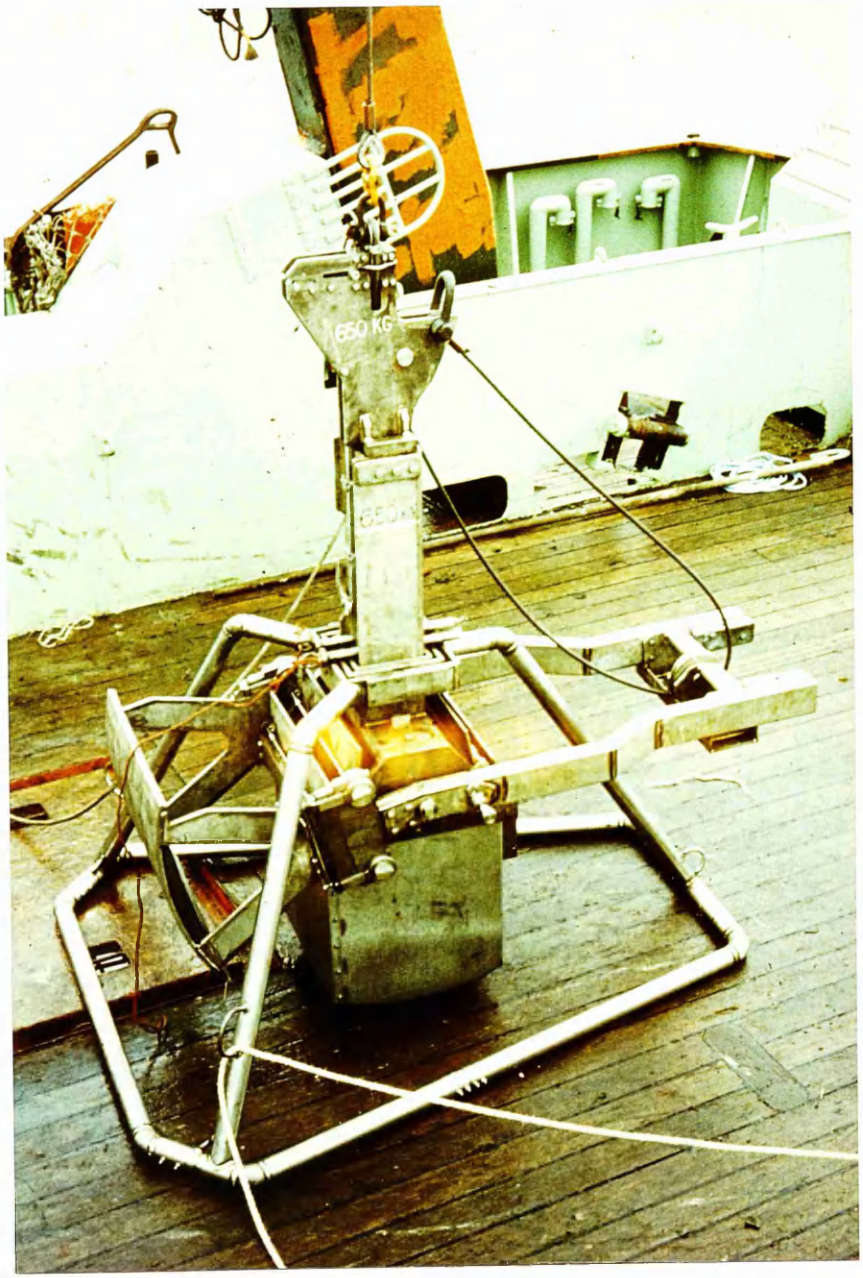
Table 1

Details of the bacteriological sampling at sites 1-5. It shows the depths, the final number of isolates obtained (column 9) and the mainframe computer library file name used for storing data on responses of the isolates to antimicrobial agents.

Plates 1 and 2

Spade-box corer used to collect undisturbed deep-sea sediment.




650 KG

method (Cruickshank, et al., 1975) on ZoBell agar plates, the plates were incubated at 4°C or 10° for 7 days. The composition of the ZoBell medium was as follows:

Bacteriological peptone 5g

Ferric orthophosphate 0.1g

Bacteriological agar No.1 .....15g

85% Artificial seawater 1L

Sterilization of this medium was achieved by autoclaving at 121°C for 15 minutes.

Heterotrophic bacterial counts were obtained for each core depth. Counts were also taken from the invertebrate burrow linings and faecal pellets. These counts together with physical and chemical data have been published separately (Meadows & Tait, 1985). The counts were obtained as follows:

All of the ship board work was conducted by Mr P. S. Meadows and Dr J. Tait because it was not possible for me to join the cruise.

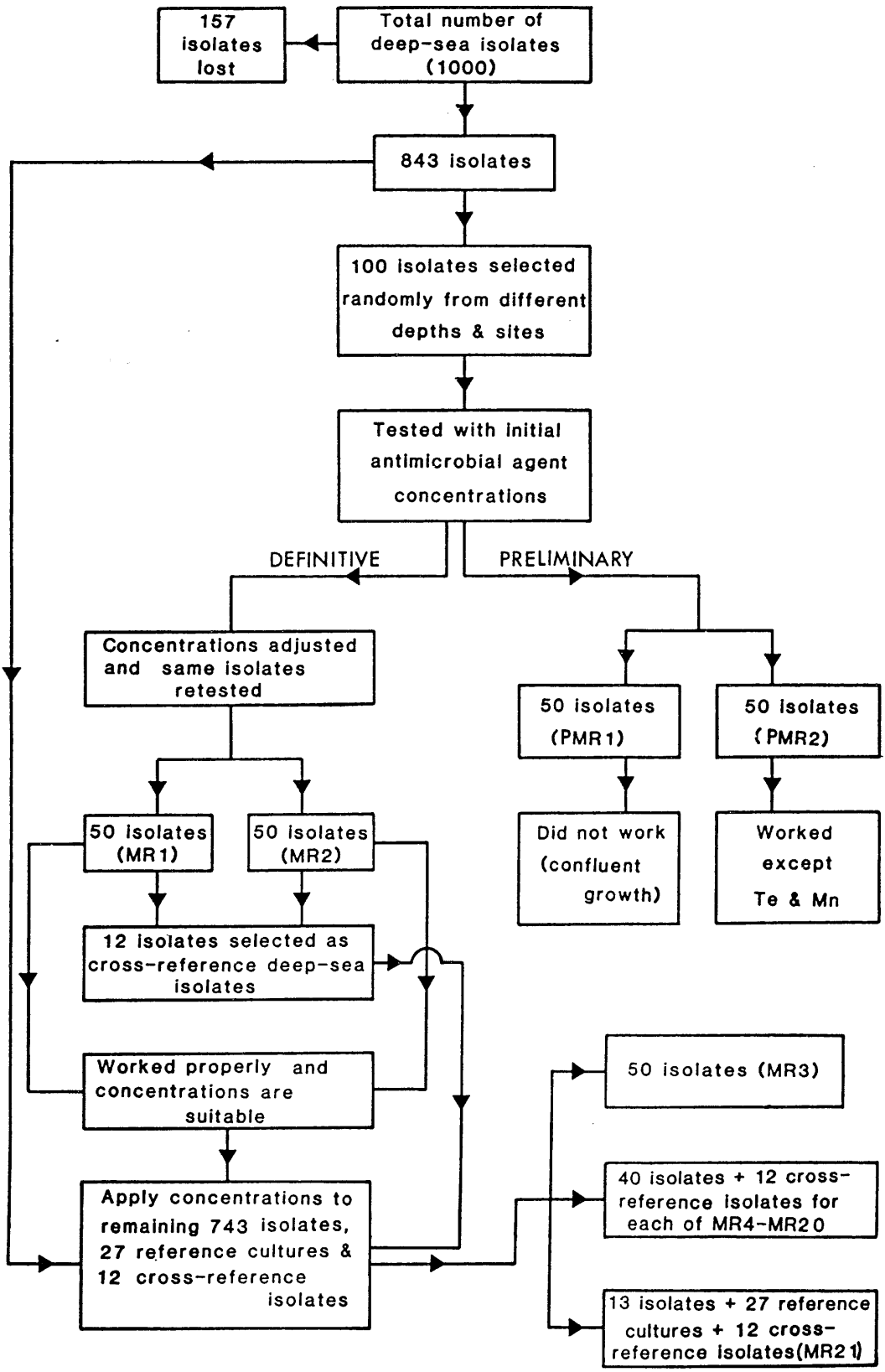
2- **In laboratory**

At the end of the cruise, the plates were transported to the Zoology Department, Glasgow University. 40 isolates were selected randomly from each depth using a marked grid and random number tables choosing the best serial dilution plates. For example, at station 5 at depth 00.5 cm, 20 colonies from each of the 10^{-2} dilutions were selected and then streaked out onto ZoBell 2216 agar plates. Plates were then stored in polythene bags at 4°C or 10°C for 7 days. The resubculturing process was carried out 3 times in order to obtain pure cultures.

A series of replica plating techniques on master plates was then carried out on a total of 843 isolates plus 27 reference cultures (Figure 3). These tests fell into two parts - preliminary and

Figure 3

Procedure adapted in the preliminary and definitive experiments to test deep-sea isolates using a range of concentrations of antimicrobial agents. PMR1=preliminary master replica 1, PMR2=preliminary master replica 2, Te = tetracycline, Mn = manganese.


157 isolates lost
Total number of deep-sea isolates (1000)
843 isolates
100 isolates selected randomly from different depths & sites
Tested with initial antimicrobial agent concentrations
DEFINITIVE
PRELIMINARY
Concentrations adjusted and same isolates retested
50 isolates (PMR1)
50 isolates (PMR2)
50 isolates (MR1)
50 isolates (MR2)
Did not work (confluent growth)
Worked except Te & Mn
12 isolates selected as cross-reference deep-sea isolates
Worked properly and concentrations are suitable
50 isolates (MR3)
Apply concentrations to remaining 743 isolates, 27 reference cultures & 12 cross-reference isolates
40 isolates + 12 cross-reference isolates for each of MR4-MR20
13 isolates + 27 reference cultures + 12 cross-reference isolates(MR21)

definitive experiments. Full details are given below.

The minimum inhibitory concentration (MIC) for each isolate with each antimicrobial agent was determined and the results then analysed by bivariate and multivariate statistics. A full explanation of these procedures are given below.

Source of bacteria

Deep-sea isolates were obtained from the spread plates described above.

Twenty seven standard reference cultures were included in the definitive experiments. These cultures were obtained from the National Collection of Marine Bacteria (NCMB), the National Collection of Industrial Bacteria (NCIB), and the National Collection of Type Cultures (NCTC). Additional isolates were supplied by Professor A. Wardlaw of the Department of Microbiology at Glasgow University and by Dr R. Millar, Zoology department, Glasgow University. The standard reference cultures were:

Source of culture	number of culture	Name of culture
*NCMB	1493	Planococcus citreus
*NCMB	628	Planococcus sp.
NCMB	308	Moraxella
*NCMB	13	Micrococcus sp.
*NCMB	365	Micrococcus sp.
*NCMB	35	Coryneform strain
NCMB	8	Coryneform strain
NCMB	1274	Vibrio fischeri
NCMB	9046	Pseudomonas fluorescens
*NCMB	320	Pseudomonas sp.
NCMB	19	Alteromonas haloplanktis
NCMB	292	Cytophaga lytica
NCIB	8806	Klebsiella pneumonia
NCIB	9261	Klebsiella pneumonia
NCIB	8805	Klebsiella pneumonia
NCIB	2847	Serratia marcescens
NCIB	8508	Bacillus megaterium
NCIB	8250	Acinetobacter calcoaceticus
NCIB	4175	Proteus vulgaris
NCIB	9240	Aeromonas hydrophila
NCIB	6576	Pseudomonas cleovorans
NCIB	9255	Corynebacterium xerosis
*NCTC	10331	Pediococcus cerevisiae
NCTC	6571	Staphylococcus aureus
NCTC	2665	Micrococcus luteus
**----	----	Staphylococcus albus
**----	----	Bacillus cereus

* = Cultures from Microbiology Department at Glasgow University.
**= Cultures from Dr R. Millar, Zoology Department, Glasgow University

Overall plan of laboratory experiments

As shown in figure 3, a total of 1000 isolates were obtained from the deep-sea sediment samples. 157 isolates were lost during the time of resubculturing mainly due to the fungal contamination. This left 843 isolates.

(i) Preliminary experiments

The purpose of the preliminary experiments was to determine the best range of concentrations of heavy metals and antibiotics. In planning this I considered the concentrations used by other workers. A computer survey was therefore conducted. Representative papers of this survey showing the antibiotic and heavy metal concentrations at which they were used are shown in table 2 and table 3 respectively. The concentration ranges chosen should broadly fulfill two criteria. They should be able to discriminate between different isolates (i.e. results must not be all plus or all minus). Secondly, they should subdivide the bacterial isolates into sensitive and resistant strains.

The most appropriate range of concentrations were ascertained as follows. A 100 of the 843 isolates were randomly selected from different depths and sites using a marked grid under the petri dishes and random number tables. These isolates were inoculated onto two master replica plates called PMR1 (Preliminary Master Replica 1) and PMR2 (Preliminary Master Replica 2) (i.e. 50 onto PMR1 and 50 onto PMR2) in order to test the concentrations of the antimicrobial agents initially selected.

The concentrations of antimicrobial agents tested on the 50 isolates in PMR1 and PMR2 were the same and are shown in table 4. Unfortunately the selective media plates were not dried before being inoculated. Because of this, the isolates tested from PMR1 showed confluent growth. This data was therefore discarded. The plates used

Author'sname & year of publication	Microorganisms tested	Technique used	Antibiotics tested										
			tested in this study						not tested in this study				
			Ch	St	Te	Am	Po	Na	G	K	R	Su	F
Datta (1969)	E.coli	Disk-diffusion	25	15	10	25	25	25	-	25	-	100	25
Davidson & Summers (1983)	Thiobacilli	Replica-plate	25	-	25	25	-	50	20	50	100	-	-
Groves & Young (1975)	Staphylococci	Disk-diffusion	30	-	30	10	-	-	-	-	-	-	-
Kelch & Lee (1978)	Pseudomonas Moraxella Acinetobacter Flavobacterium cytophaga	30-colony nichrome wire stab replicator	25	10	25	10	-	25	-	25	-	-	-
Koditschek & Guyre (1974a)	Coliform	Disk-diffusion	30	10	30	-	-	30	-	30	-	-	-
Marques et.al (1979)	Pseudomonas aeruginosa	Disk-diffusion	30	10	30	10	-	30	30	30	-	-	-
Pratt & Reynolds (1974)	Vibrio Pseudomonas	Spread-plate method	-	-	-	-	0 50 100	-	-	-	-	-	-
Quigley & Colwell (1968)	Pseudomonas aeromonas	Disk-diffusion	30	-	30	-	30	-	-	30	-	-	-
Stewart & Koditschek (1980)	E.coli	Donor-recipient antibiotic resistance transfer	-	-	40	-	-	40	-	-	-	-	-
Timoney et.al (1978)	Bacillus sp.	Replica-plate	30	50	30	50	-	-	-	50	-	-	-

Table 2

Concentrations of antibiotics (mg/l) and techniques used to test the effect of antibiotics on microorganisms. Representative papers.

Ch=Chloramphenicol St=Streptomycin Te=Tetracycline

Am=Ampicillin Po=Polymyxin B Na=Nalidixic acid

G =Gentamicin K =Kanamycin R =Rifampin

Su=Sulphathiazole F =Nitrofurantion

Author's name & year of publication	Microorganisms tested	Technique used	Metals tested											
			tested in this study						not tested in this study					
			Cd	Cr	Cu	Pb	Mn	Hg	Co	Fe	Te	Zn	Ni	Ag
Austin et.al(1977)	Bacillus Erwinia Mycobacterium Pseudomonas Coryneforms	Spread-plate	-	-	-	100 mg/l	-	10 mg/l	100 mg/l	-	-	-	-	-
Calomiris et.al (1984)	393 isolates of drinking water (e.g Acinetobacter)	Replica-plate	25 50 100 200 400 ug/ml	-	200 400 800 1600 3200 ug/ml	800 1600 2400 3200 ug/ml	-	-	-	-	-	200 400 800 1600 3200 ug/ml	-	-
Fisher & Frood (1980)	Marine diatoms	Batch-culture	2 10 25 ug/l	-	5 10 25 ug/l	-	-	-	-	-	-	20 40 60 ug/l	-	-
Lester et.al (1979)	Brevibacterium Alcaligenes Pseudomonas unidentified g(-) rod	Continous culture	50 mg/l	50 mg/l	50 mg/l	50 mg/l	-	-	-	-	-	-	-	-
Marques et.al (1979)	Pseudomonas-aeroginossa	Disk-method	1600 ug/ml	1600 ug/ml	-	3200 ug/ml	-	10 ug/ml	-	-	64 ug/ml	1600 ug/ml	-	128 ug/ml
Timony et.al (1978)	Bacillus sp.	Replica-plate	8 11 28 56 84 112 ug/ml	-	-	-	-	1.5 2 5 10 15 20 50 ug/ml	-	-	-	32 49 65 162 324 488 650 ug/ml	-	-

Table 3

Concentrations of metals and techniques used to test the effect of metals on microorganisms. Representative papers.

Cd=Cadmium Cr=Chromium Cu=Copper

Pb=Lead Mn=Manganese Hg=Mercury

Co=Cobalt Fe=Iron Te=Tellurium

Zn=Zinc Ni=Nickel Ag=Silver

Antimicrobial agent	Concentration range(mg/l)					
Cadmium	128	64	32	16	8	4
Chromium	128	64	32	16	8	4
Copper	128	64	32	16	8	4
Lead	800	400	200	100	50	25
Manganese	128	64	32	16	8	4
* Mercury	32	16	8	4	2	1
Ampicillin	64	32	16	8	4	2
Chloramphenicol	64	32	16	8	4	2
Polymyxin B	64	32	16	8	4	2
Streptomycin	64	32	16	8	4	2
Tetracycline	64	32	16	8	4	2
Nalidixic acid	64	32	16	8	4	2
Methylene blue	32	16	8	4	2	1

Table 4
Concentrations of antimicrobial agents used in the preliminary experiments.

* After the thesis had prepared it was found that the quoted concentrations of mercury in the preliminary experiments had been calculated incorrectly. The correct concentrations are as follows. 36.8, 18.4, 9.2, 4.6, 2.3 and 1.1mg/l. This does not affect the definitive experiments (see figure 7).

to test the isolates from PMR2 were just dry enough for good results to be obtained except on the plates containing tetracycline and manganese. Two problems arose in the data from PMR2. In some cases the concentrations of antimicrobial agents were not high enough to inhibit a significant proportion of the isolates (ampicillin, streptomycin, nalidixic acid, methylene blue, and chromium). In other cases, there was a very sharp reduction in growth between one concentration and the next (cadmium, copper and lead).

These two problems were solved by increasing the concentrations of antimicrobial agents or expanding the range of concentrations where appropriate. Figures 4 A and 4 B illustrate the two problems and their solutions as models (4 A) and actual results (4 B).

(ii) **Definitive experiments**

The concentrations used were adjusted as described above, and used to retest the same 100 isolates. These isolates were tested using two master replica plates called MR1 and MR2 (50 onto MR1 and 50 onto MR2). The adjusted concentrations of the antimicrobial agents (Table 5) worked well on the isolates from both master replica plates, and gave good results. Before applying these concentrations to the remaining isolates, 27 reference cultures and 12 isolates from MR1 and MR2 were selected. The 27 reference cultures were included to aid in the final taxonomic description of the clusters. The 12 isolates from MR1 and MR2 were included to allow cross-referencing and accurate comparison between results from different replica plates. The 12 cross-reference isolates were included on master replica plates MR4 to MR21 (i.e. 40 isolates + 12 cross-reference isolates per master replica plate). In addition the 27 reference cultures were also included on MR21.

Figure 4 A

Models of bad and good results of the experiments to determine the range and scale of antimicrobial agent concentrations.

A- Preliminary - range is too low (little inhibitory effect).

B- Preliminary - scale needs expansion (sharp reduction in numbers between one concentration and the next).

C- Definitive - range and scale are suitable.

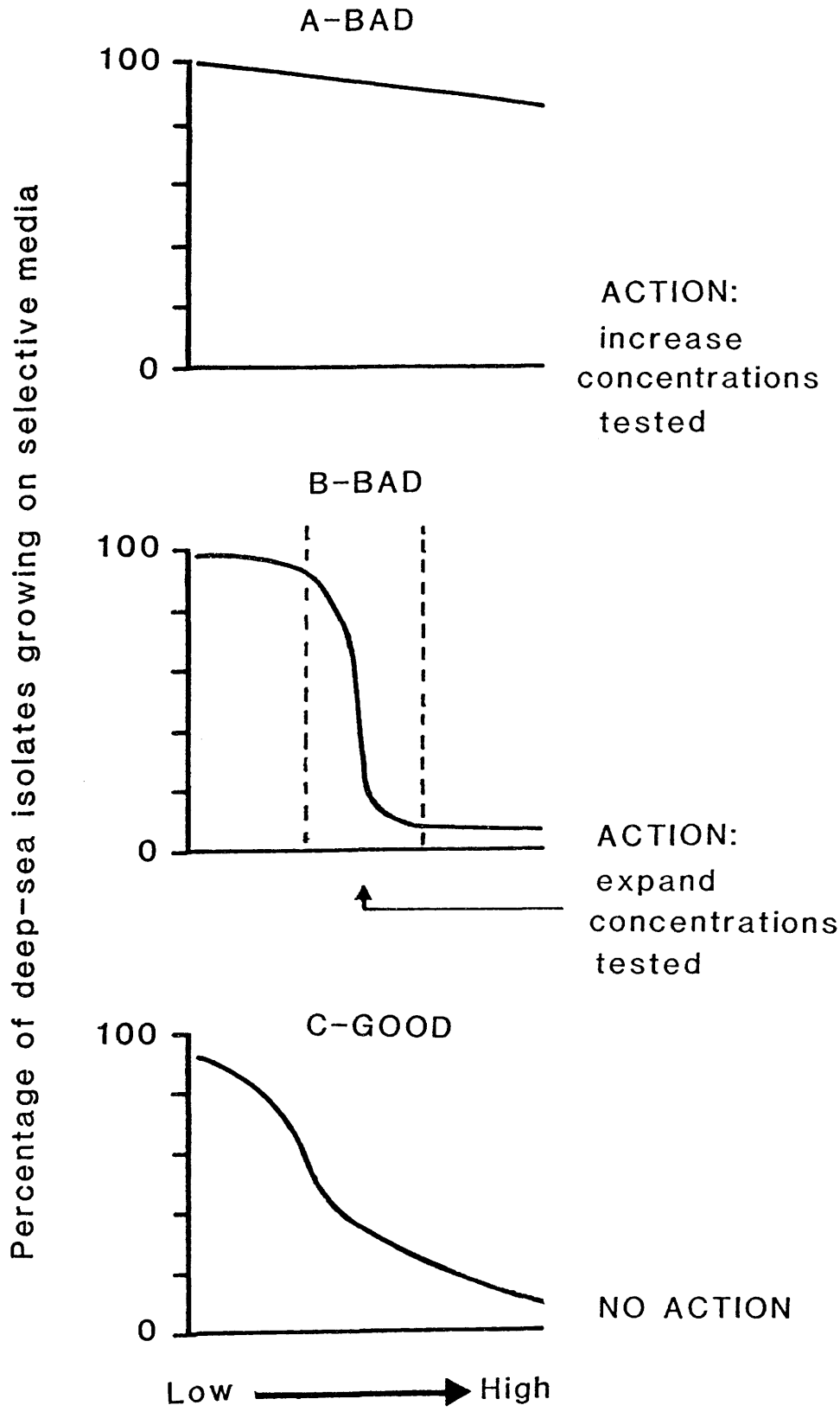

A-BAD
100
0
ACTION:
increase
concentrations
tested
B-BAD
100
0
ACTION:
expand
concentrations
tested
C-GOOD
100
0
NO ACTION
Percentage of deep-sea isolates growing on selective media
Low
High
Concentrations of antimicrobial agent

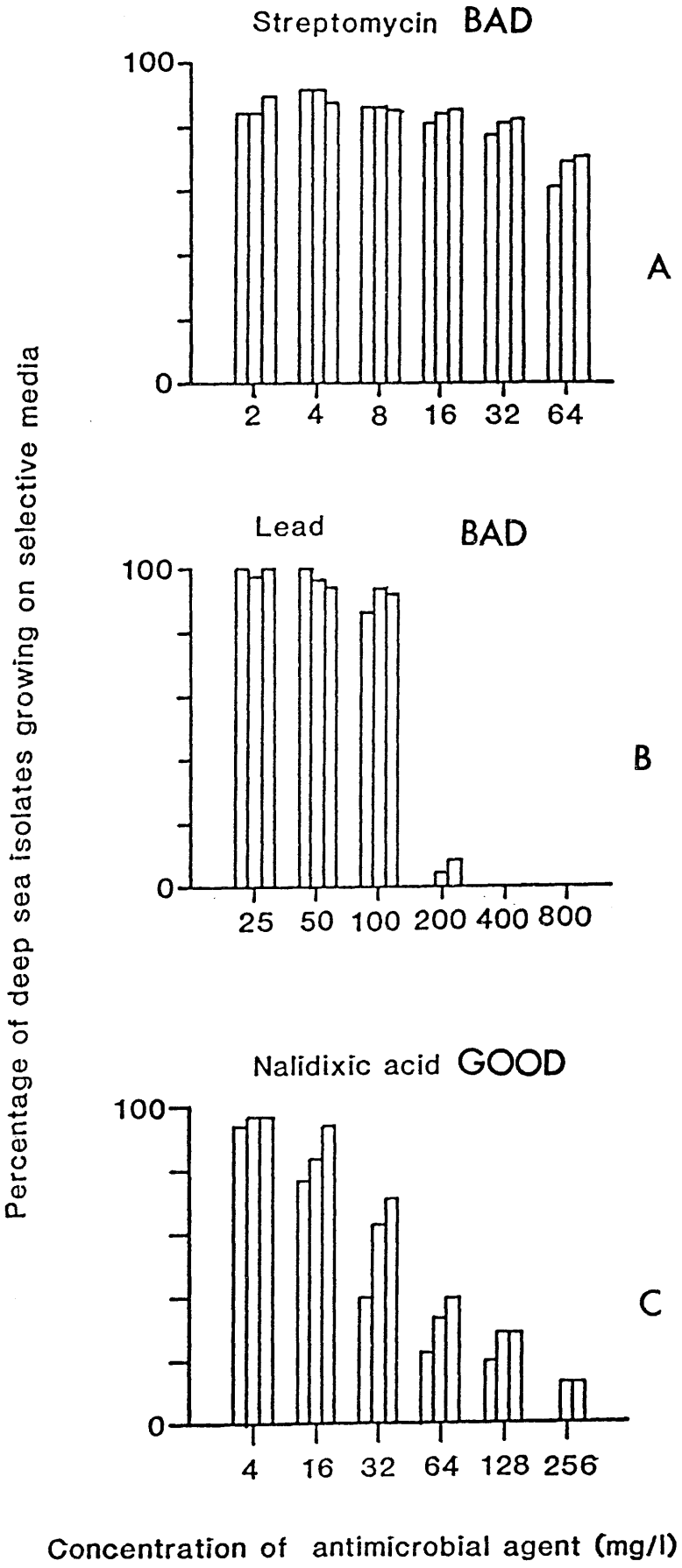
Figure 4 B

Illustrating the results of the experiments to determine the range and scale of antimicrobial agent concentrations.

A- Preliminary - range is too low (little inhibitory effect).

B- Preliminary - scale needs expansion (sharp reduction in numbers between 100 and 200mg/l).

C- Definitive - range and scale are suitable.


Streptomycin BAD
100
0
2 4 8 16 32 64
A
Lead BAD
100
0
25 50 100 200 400 800
B
Nalidixic acid GOOD
100
0
4 16 32 64 128 256
C
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Antimicrobial agent	Concentration range(mg/l)					
Cadmium	128	64	48	32	16	8
Chromium	256	128	64	32	16	8
Copper	128	64	48	32	16	8
Lead	512	256	192	160	128	64
Manganese	4096	2048	1024	512	256	128
Mercury	64	32	16	8	4	2
Ampicillin	256	128	64	32	8	2
Chloramphenicol	64	32	16	8	4	2
Polymyxin B	128	64	32	16	8	4
Streptomycin	256	128	64	32	16	4
Tetracycline	128	64	32	16	8	4
Nalidixic acid	256	128	64	32	16	4
Methylene blue	256	128	64	32	8	2

Table 5

Concentrations of the antimicrobial agents used in the definitive experiments.

In summary, the bacteria on MR1 to MR21 were as follows.

MR1 ..... 50 deep-sea isolates.

MR2 ..... 50 deep-sea isolates.

MR3 ..... 50 deep-sea isolates.

MR4-MR20 ..... 40 deep-sea isolates + 12 cross-reference deep-sea isolates from MR1 and MR2.

MR21 ..... 13 deep-sea isolates + 12 cross-reference deep-sea isolates from MR1 and MR2 + 27 reference cultures.

The total number of deep-sea isolates is: (3x50) + (17x40) + 13 =843 (see above).

Replica plating

In the preliminary experiments, two master replica plates (PMR1 and PMR2) were prepared by inoculating 50 pure cultures previously obtained by repetitive streaking on ZoBell agar plates. In the definitive experiments, the master plates MR1 to MR21 were used to test the deep-sea isolates. These master replica plates were taken two plates at a time (e.g. MR1 and MR2, MR3 and MR4, Figure 5). The isolates on each master replica plate were then subcultured onto 7 further plates in exactly the same position as on the master replica plate (e.g. MR1.a, MR1.b, MR1.c, MR1.d, MR1.e, MR1.f and MR1.g, Figure 5). 6 of these 7 plates (e.g. MR1a, b, c, e, f, g) were used to inoculate 2 separate antimicrobial agent media using 2 sterilised velvets. The seventh plate (e.g. MR1d) was used to inoculate 1 antimicrobial agent medium. These procedure was necessary because there were an odd number of antimicrobial agents - 13. The master plates in the definitive experiments were prepared by inoculating 50 (MR1-MR3) or 52 (MR4-MR21) isolates onto the plate (Plate 3) and

Figure 5

Diagram shows the method used to print master replica plates (MR1 and MR2) on velvets, and how to "stamp" a series of selective media plates on inoculated velvets starting from the highest concentrations and finishing with control plate (C). I - VI = highest - lowest concentration.

MR1 = Master replica plate No. 1

MR2 = Master replica plate No. 2

MR1 = MR1.a to MR1.g

MR2 = MR2.a to MR2.g

Am = Ampicillin	Cd = Cadmium
Ch = Chloramphenicol	Cr = Chromium
Po = Polymyxin B	Cu = Copper
St = Streptomycin	Pb = Lead
Te = Tetracycline	Mn = Manganese
Na = Nalidixic acid	Hg = Mercury

Me = Methylene blue

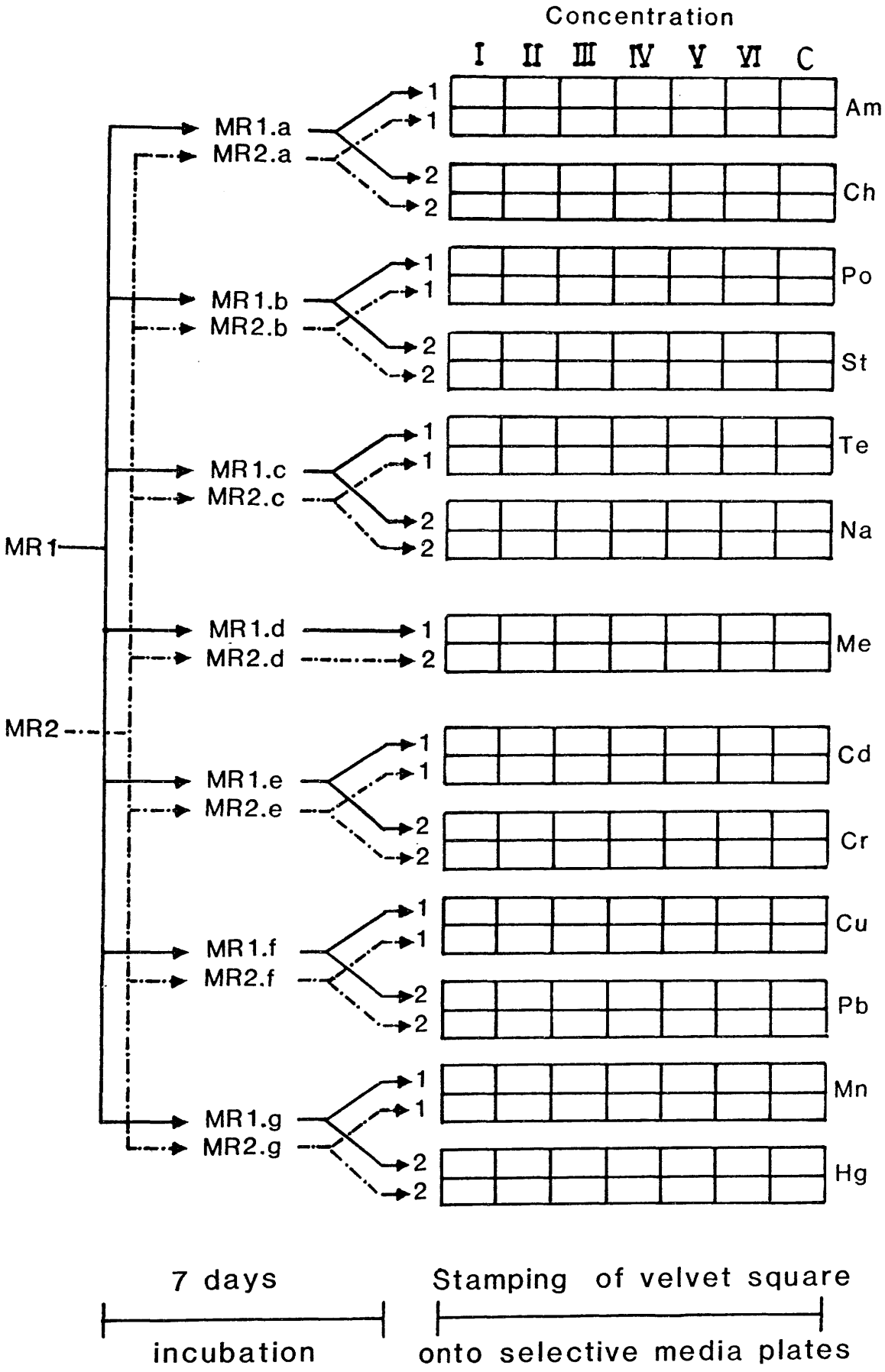
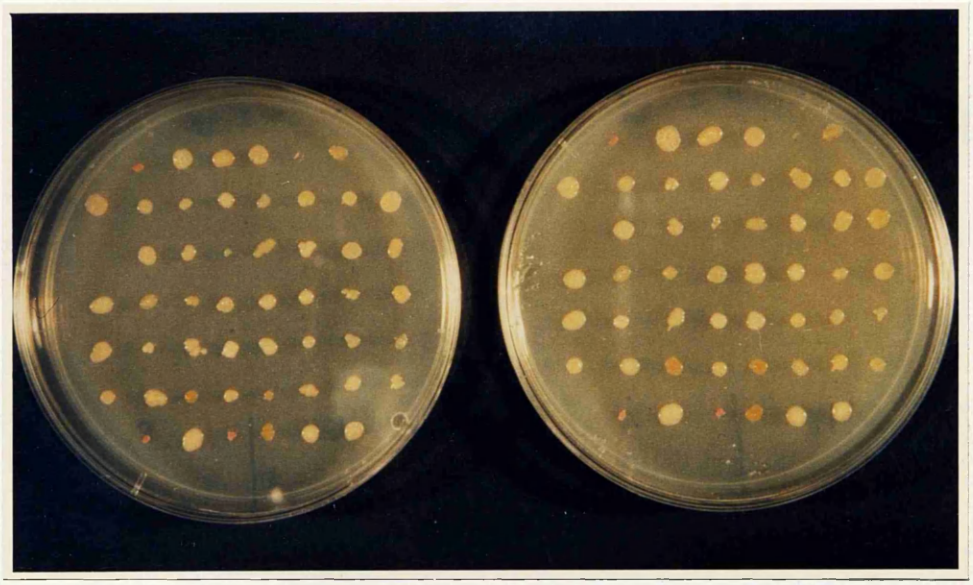

Concentration
I II III IV V VI C
MR1
MR2
MR1.a
MR2.a
MR1.b
MR2.b
MR1.c
MR2.c
MR1.d
MR2.d
MR1.e
MR2.e
MR1.f
MR2.f
MR1.g
MR2.g
1
1
2
2
Am
Ch
Po
St
Te
Na
Me
Cd
Cr
Cu
Pb
Mn
Hg
7 days
incubation
Stamping of velvet square
onto selective media plates

Plate 3

Master replica plates prepared by inoculating 52 isolates and incubated at 10°C for 7 days.



incubating it at 10°C for seven days. Following incubation, each master plate was "stamped" onto a velvet square, which had been previously sterilized in a hot air oven at 160°C for two hours. The velvet was then used to re-inoculate a series of selective media plates. The order of printing plates started at the highest concentration, worked down the concentrations, and finished with the control plate (Plate 4). The reason for this was to ensure that the higher concentration plates received a good inoculum. Plate printing finished with the control plate in order to make sure that there were still bacterial cells left after stamping the last selective media plate, in other words to insure that all the selective media plates received an inoculum. Plates were then incubated at 10°C, and results recorded after 7, 14 and 21 days. Cluster analysis was performed using the minimum inhibitory concentration after 14 days. The reasons for 14 days were:

1- All control isolates had grown by 14 days.

2- The potency of antimicrobial agents may have decreased after 14 days.

3- Contamination of some plates with fungi occured after 14 days.

4- Some isolates became dry and changed the readings.

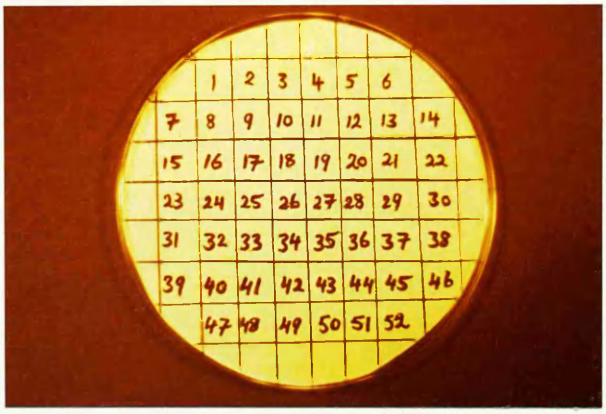
5- Colonies started to spread after 14 days which made it difficult to read the plates.

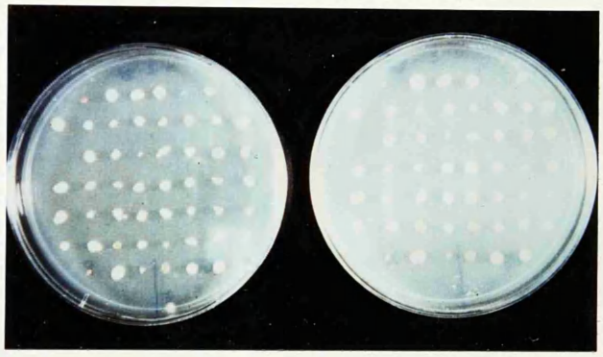
The replica plating method used in the definitive experiments was the same as that of the preliminary experiments, except that the selective media plates were dried at 37°C for one hour (Cruickshank et al., 1975).

Two examples of the recording sheets for the preliminary experiments are shown in table 6. The recording sheets for the definitive experiments were exactly the same.

Plate 4

The method used to print master replica plates on velvet square, and how to "stamp" a series of selective media plates on inoculated velvets.





Control plate

Table 6

Two examples of how the antimicrobial agent results were recorded, using polymyxin B and chromium data after 7, 14 and 21 days. Data for the other antimicrobial agents were recorded in the same way. Each sheet represents one master replica plate. -*+ = no growth after 7 days, intermediate growth after 14 days and extensive growth after 21 days respectively.

	Chromium concentration mg/l						
	128	64	32	16	8	4	C
01	---	---	--*	+++	+++	+++	+++
02	+++	+++	+++	+++	+++	+++	+++
03	+++	+++	+++	+++	+++	+++	+++
04	---	-++	-++	-++	+++	+++	+++
05	*++	+++	+++	+++	+++	+++	+++
06	+++	+++	+++	+++	+++	+++	+++
07	*++	+++	+++	+++	+++	+++	+++
08	*++	+++	+++	+++	+++	+++	+++
09	---	---	-++	+++	+++	+++	+++
10	---	---	---	***	+++	+++	+++
11	+++	+++	+++	+++	+++	+++	+++
12	---	---	-++	+++	+++	+++	+++
13	---	---	--*	-++	+++	+++	+++
14	---	---	-++	+++	+++	+++	+++
15	---	**+	*++	+++	+++	+++	+++
16	---	***	*++	+++	+++	+++	+++
17	*++	+++	+++	+++	+++	+++	+++
18	+++	+++	+++	+++	+++	+++	+++
19	*++	+++	+++	+++	+++	+++	+++
20	---	---	---	---	---	---	+++
21	--*	--*	---	---	---	+++	+++
22	-++	+++	+++	+++	+++	+++	+++
23	--+	--+	---	---	---	---	---
24	**+	-*+	-**	-**	+++	+++	+++
25	-*+	-*+	-++	-++	+++	+++	+++
26	-*+	-*+	-++	+++	+++	+++	+++
27	---	--*	-**	-++	-++	+++	+++
28	---	---	--*	-**	+++	+++	+++
29	-*+	-*+	-++	+++	+++	+++	+++
30	-++	-++	-++	-++	+++	+++	+++
31	-++	-++	-++	-++	+++	+++	+++
32	--+	-*+	-++	-++	+++	+++	+++
33	-*+	-++	-++	-++	+++	+++	+++
34	-*+	-++	-++	-**	---	---	***
35	--*	---	--*	-**	--*	--*	***
36	+++	+++	*++	**+	-**	**+	+++
37	+++	+++	+++	+*+	**+	*++	+++
38	+++	+++	+++	+*+	**+	*++	+++
39	--*	-**	-++	-++	+++	+++	+++
40	--*	-*+	---	---	---	---	---
41	-*+	-**	**+	**+	-++	-++	-*+
42	--*	-*+	+++	+++	+++	+++	+++
43	*++	*++	+++	+++	+++	+++	+++
44	-*+	-++	+++	+++	+++	+++	+++
45	---	---	---	---	+++	+++	+++
46	---	---	+++	+++	+++	+++	+++
47	--+	-++	+++	+++	+++	+++	+++
48	---	--*	-**	-**	---	***	+++
49	---	---	+++	+++	+++	+++	+++
50	---	-++	+++	+++	+++	+++	+++

	Polymyxin.B concentration mg/l						
	64	32	16	8	4	2	C
01	---	---	---	---	+++	---	+++
02	+++	---	+++	+++	+++	+++	+++
03	---	---	+++	+++	+++	+++	+++
04	---	---	+++	+++	+++	+++	+++
05	---	---	+++	+++	+++	+++	+++
06	---	---	+++	+++	+++	+++	+++
07	-++	+++	+++	+++	+++	+++	+++
08	---	---	--*	+++	+++	+++	+++
09	---	---	---	+++	+++	---	+++
10	---	---	---	---	---	---	+++
11	---	---	---	+++	+++	+++	+++
12	---	---	---	---	---	---	+++
13	---	---	---	---	---	---	+++
14	---	---	---	+++	+++	+++	+++
15	---	---	---	+++	+++	+++	+++
16	---	---	---	+++	+++	---	+++
17	---	---	---	+++	+++	+++	+++
18	---	---	---	+++	+++	+++	+++
19	---	---	---	+++	+++	+++	+++
20	---	---	---	---	---	---	+++
21	---	---	---	---	---	---	---
22	---	+++	+++	+++	+++	+++	+++
23	---	+++	+++	+++	+++	+++	+++
24	+++	+++	+++	+++	+++	+++	+++
25	+++	+++	+*+	**+	**+	+++	+++
26	---	+++	+*+	+++	+++	--*	+++
27	---	---	---	+++	+++	+++	+++
28	---	---	---	---	---	---	+++
29	---	*++	*++	+++	+++	+++	+++
30	---	*++	+++	*++	+++	---	+++
31	+++	+++	**+	+++	+++	+++	+++
32	**+	+++	+++	*++	+++	+++	+++
33	**+	---	*++	**+	---	+++	+++
34	---	+++	+++	+++	+++	+++	+++
35	---	---	---	---	--*	-**	+++
36	+++	+++	+++	+++	+++	+++	+++
37	+++	+++	+++	*++	+++	+++	+++
38	+++	+++	+++	*++	*++	+++	+++
39	--*	*++	*++	+++	+++	+++	+++
40	---	**+	**+	--+	---	-++	---
41	+++	+++	+++	+++	+++	+++	*++
42	---	+++	+++	+++	+++	+++	+++
43	-*+	+++	+++	+++	+++	+++	+++
44	---	---	-*+	-*+	--+	--+	+++
45	---	---	---	---	---	---	+++
46	---	---	---	---	---	---	+++
47	---	---	---	+++	+++	+++	+++
48	---	--+	+++	+++	+++	+++	+++
49	---	---	---	---	---	---	+++
50	---	---	---	---	+++	+++	+++

Cross-reference cultures

Twelve isolates of deep-sea bacteria previously tested with the antimicrobial agents in master replica 1 and 2, were selected and incorporated as controls for "typing" the remaining isolates of the definitive experiments. The criteria used in selecting these isolates were that they formed reasonable size colonies on replica plates, were resistant to some antimicrobial agents and sensitive to others, and grew within 14 days. The control cultures were distributed on different positions on the master plate as shown in figure 6.

Calculation of % isolates growing on selective media

The effect of the antimicrobial agents on growth of each isolate was classified as positive (+), negative (-), or intermediate (*) when compared with the same isolate on the control plate. The scoring system used in the experiments is shown in table 7. The number of organisms growing on a given selective medium at a given concentration was expressed as a percentage of isolates growing on the control plate (Appendix tables 1-6). For example, with ampicillin (Figure 8) at a concentration of 2mg/l, the percentage was calculated as follows. 39 isolates grew on the plate containing 2mg/l of ampicillin, while 46 isolates grew on the control plate. Therefore, 39/46 x 100 = 84.8% of the 50 isolates on the control grew on the ampicillin plate, at 2mg/l.

Minimum inhibitory concentration (MIC)

For each of the 843 environmental isolates and 27 reference cultures, the minimum inhibitory concentration (MIC) for each of the 13 antimicrobial agents was defined as the lowest concentration of the antimicrobial agent that inhibited growth. For each antimicrobial agent, if the control plate showed growth (+) then the first concentration which showed intermediate (*) or no growth (-) was

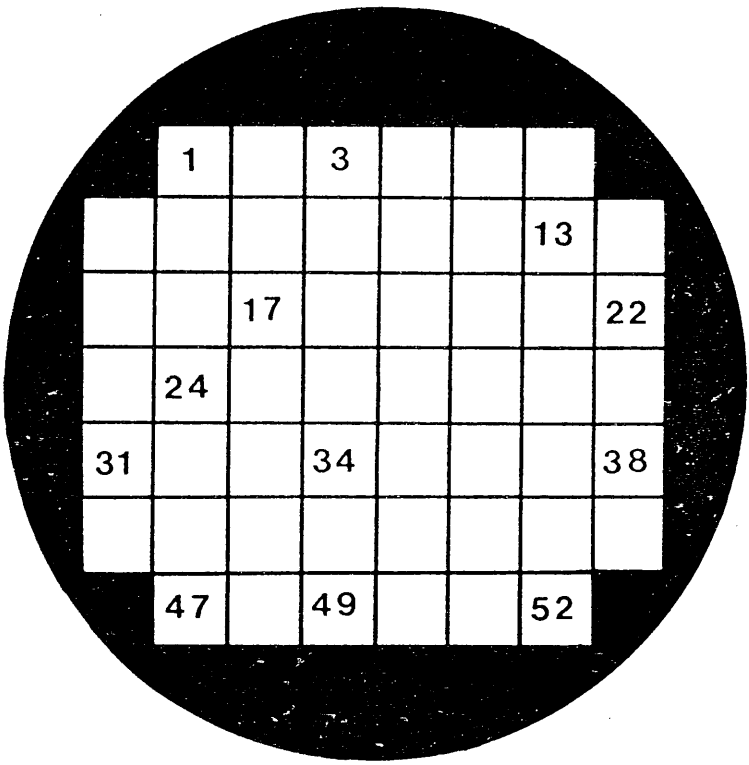


Figure 6

The position of twelve isolates of deep-sea bacteria phenotyped from master replica 1 and 2 and incorporated as controls on master replica plates for "typing" the remaining isolates of the definitive experiments.

Notation		Score	
Control	Experimental	Control	Experimental
+	+	1	1
+	*	1	0
+	-	1	0
*	+	1	1
*	*	1	1
*	-	1	0
-	+	0	0
-	*	0	0
-	-	0	0

Table 7

Relationship between notation and scoring system used for reading plates after 7, 14 and 21 days incubation.

The following notations were used:

+ = Vigorous growth (Resistant).

- = No growth (Sensitive).

* = Little growth (Intermediate).

The follow scores were used: 1 , 0 .

Control: plates with no antimicrobial agents.

Experimental: plates containing antimicrobial agents.

taken to be the minimum inhibitory concentration. If the control plate showed intermediate growth (*) then the first concentration of that antimicrobial agent which showed no growth (-) was taken.

Preparation of selective media

Selective media were prepared for the preliminary and definitive experiments by incorporating appropriate quantities of antibiotic and heavy metal stock solutions into molten agar maintained at 50°C in a waterbath. The antimicrobial stock solutions were prepared as follows.

A- **Preliminary experiments**

Ampicillin

Weigh out 0.32g of ampicillin and dissolve in 100 ml of sterile distilled water.

Chloramphenicol

Weigh out 0.32g of chloramphenicol and add directly to 100 ml of sterile distilled water.

Polymyxin B

Weigh out 0.32g of polymyxin B and add directly to 100 ml of sterile distilled water and dissolve.

Streptomycin Sulphate

Weigh out 0.32g of streptomycin sulphate and add directly to 100 ml of sterile distilled water.

Tetracycline Hydrochloride

Weigh out 0.32g of tetracycline hydrochloride and add directly to 100 ml of sterile distilled water.

Nalidixic acid

Weigh out 0.064g of nalidixic acid and dissolve in 2 ml of N NaOH to form the first stock solution. Additional stock

solutions are prepared by serial dilutions with N NaOH (e.g. 1ml of the first stock solution is added to 1ml of N NaOH to form the second stock solution, this step is repeated to give 6 concentrations of nalidixic acid).

Methylene Blue

Weigh out 0.16g of methylene blue and dissolve in 100 ml of sterile distilled water.

Cadmium Chloride ($CdCl_2.2.5H_2O$)

Stock solution. Weigh out 1.30g of cadmium chloride and dissolve in 100 ml of sterile distilled water.

Potassium Chromate (K_2CrO_4)

Stock solution. Add 2.390g of potassium chromate to 100 ml of sterile distilled water and dissolve.

Copper Sulphate ($CuSO_4.5H_2O$)

Stock solution. Add 0.503g of copper salt to 100 ml of sterile distilled water and dissolve.

Lead Acetate $Pb(OOCCH_3)_2.3H_2O$

Stock solution. Add 7.32g of lead acetate to 100 ml of sterile distilled water and dissolve.

Manganous Chloride ($MnCl_2.4H_2O$)

Stock solution. Add 2.30g of manganous chloride to 100 ml of sterile distilled water.

Mercuric Chloride ($HgCl_2$)

Stock solution. Weigh out 5.0g of mercuric chloride and dissolve in 100 ml of sterile distilled water. (SAFETY CAUTION: DO NOT AUTOCLAVE ! Never heat to dissolve faster).

B- **Definitive experiments**

Ampicillin

Weigh out 0.64g of ampicillin and dissolve in 100 ml of sterile distilled water.

Chloramphenicol

Weigh out 0.32g of chloramphenicol and add directly to 100 ml of sterile distilled water.

Polymyxin B

Weigh out 0.32g of polymyxin B, add directly to 100 ml of sterile distilled water and dissolve.

Streptomycin Sulphate

Weigh out 0.64g of streptomycin sulphate and add directly to 100 ml of sterile distilled water.

Tetracycline Hydrochloride

Weigh out 0.32g of tetracycline hydrochloride and add directly to 100 ml of sterile distilled water.

Nalidixic acid

Weigh out 0.256g of nalidixic acid and dissolve in 2 ml of N NaOH to form the first stock solution. Additional stock solutions are prepared by serial dilutions with N NaOH (e.g. 1ml of the first stock solution is added to 1ml of N NaOH to form the second stock solution, this step is repeated to give 6 concentrations of nalidixic acid).

Methylene Blue

Weigh out 0.64g of methylene blue and dissolve in 100 ml of sterile distilled water.

Cadmium Chloride ($CdCl_2.2.5H_2O$)

Stock solution. Weigh out 1.30g of cadmium chloride and dissolve in 100 ml of sterile distilled water.

Potassium Chromate (K_2CrO_4)

Stock solution. Add 2.390g of potassium chromate to 100 ml of sterile distilled water and dissolve.

Copper Sulphate ($CuSO_4.5H_2O$)

Stock solution. Add 2.515g of copper salt to 100 ml of sterile distilled water and dissolve.

Lead Acetate $Pb(OOCCH_3)_2.3H_2O$

Stock solution. Add 4.680g of lead acetate to 100 ml of sterile distilled water and dissolve.

Manganous Chloride ($MnCl_2.4H_2O$)

Stock solution. Add 18.45g of manganous chloride to 100 ml of sterile distilled water.

Mercuric Chloride ($HgCl_2$)

Stock solution. Weigh out 0.43g of mercuric chloride and dissolve in 100 ml of sterile distilled water. (SAFETY CAUTION: DO NOT AUTOCLAVE ! Never heat to dissolve faster).

The volume of stock solutions (ml) of the antimicrobial agents added to the volume of molten agar and the final concentrations (mg/l) used in the preliminary and definitive experiments are given in appendix tables 7-8 and 9-10 respectively.

The following example (copper sulphate in definitive experiment) shows the way in which the volume of stock solution was calculated.

The molecular weight of $CuSO_4.5H_2O$ =249.7g/l

The molecular weight of Cu =63.54g/l

If I need 128 mg/l in the agar. This equivalent to:

12.8 mg/100 ml agar.

If I put 2ml of stock solution into 100ml, I will need 12.8mg of Cu^{2+} in 2ml stock solution.

$12.8mg\ Cu^{2+}/2ml = 6.4mg\ Cu^{2+}/ml$

$= 6.4x1000mg\ Cu^{2+}/l$

$= 6400mg\ Cu^{2+}/l$

$= 6.4g\ Cu^{2+}/l$

Let x = concentration in $CuSO_4.5H_2O/l$ which is equivalent to 6.4g Cu^{2+}/l.

If $249.7g/l\ CuSo_4.5H_2O$ -------- $63.54g/l\ Cu^{2+}$

x ---------- 6.4g/l

Therefore, $x = \frac{249.7}{63.54} x6.4$

$= 25.15g\ CuSO_4.5H_2Og/l$

$=2.515g\ CuSO_4.5H_2Og/100ml$ This is the concentration of stock solution if I need 2ml of it contains 12.8mg Cu^{2+}.

Procedure used in applying cluster analysis to the deep-sea isolates

To apply numerical taxonomy to a bacterial population, there are several stages needed to reach the point where all the bacterial isolates are classified into taxa. These stages include strain and test selection, coding data and their entry into the computer, data analysis and interpretation of results.

1- **Isolates selection**

1000 deep-sea isolates were selected from deep-sea sediments as shown in figure 3. During the re-subculturing processes, 157 isolates were lost mainly due to fungal contamination. This left 843 pure deep-sea isolates. A Full explanation on how the bacterial isolates were selected is given in the materials and methods (p. 16).

2- **Test selection**

The tests used can be observational tests such as colony size, colour, shape, biochemical tests such as fermentation of carbohydrate, indole and nitrate reduction, or sensitivity tests such as susceptibility to antibiotics and metals. I used the sensitivity of isolates to a range of antimicrobial agents.

Six antibiotics, six heavy metals and one dye with six concentrations were selected to test the sensitivity of the deep-sea isolates. The names and concentrations of the antimicrobial agents used in the preliminary and definitive experiments are shown in tables 4 and 5 respectively. The criteria used to select the antibiotics and heavy metals were as follows.

Antibiotics were used because they inhibit the growth of some bacteria and do not completely affect others due to their resistance. This enabled me to classify deep-sea isolates into groups according to their sensitivity and resistant. It also gave me information regarding the relative sensitivity of deep-sea bacteria from different depths and sites to selected antibiotics. Antibiotics were selected by their specific mode of action on cell wall (ampicillin), cell membrane (polymyxin B), nucleic acid (nalidixic acid) or cell protein (chloramphenicol, streptomycin, tetracycline) synthesis (Appendix A p. 225-235).

Heavy metals were used because they are essential to microorganisms in trace quantities, but at higher concentrations have antibacterial properties. They are also of economic importance, and with the exception of manganese, are not normally found in large quantities in the deep-sea. In addition, heavy metals have been used by a number of other workers for taxonomic analysis (Austin et al. 1977; Mallory et al. 1977; Timoney et al. 1978).

3- **Recording data**

Sensitivity profiles of all isolates were recorded as extensive growth (+), intermediate growth (*) or no growth (-). An example of the recording sheets are shown in table 6 pages 33 and 34. As mentioned above, the minimum inhibitory concentration (MIC) was defined for each isolate with each antimicrobial agent. This data was recorded in an n (row) x t (column) table, where n is the isolate number and t is the MIC for each of the antimicrobial agents (Appendix table 31).

4- **Coding of data**

Deep-sea isolates were coded from number 1 to 843. Site number, source of sample and the incubation temperatures were also recorded with the isolate number (Appendix table 31). There were three sources of sample - sediment, burrow linings and faecal pellets. These were coded respectively as 1, 2, 3. Not all this information was used by the computer. The only categories used by the computer were the isolate number and the minimum inhibitory concentration of antimicrobial agents. The final n (rows) x t (columns) matrix contained 870 strains (843 deep-sea isolates + 27 reference cultures = 870 see p.28) and 13 unit characters (6 antibiotics, 6 metals and 1 dye).

Each of the 870 strains is a row. Each of the 13 characters is a column. Each row is sometimes called a case, and each column is sometimes called a variable.

Missing values present difficulties in the interpretation of the classification of the results. It is not however, worthwhile excluding a whole case just because of one or two missing values. There are several acceptable ways of dealing with this problem. The first is to exclude the isolates with missing data from the analysis.

This seems to me to be a rather inefficient way of proceeding because it is not efficient to exclude many cases due to few missing characters.

The second way is to include the isolates with missing data and to use some sort of mean value for the missing data. There are two ways of doing this. In the first, the mean value which takes the place of the missing value is the mean of all the data in that column for all the isolates. However, I have been slightly more restrictive than this: I have replaced missing data in a particular strain by the mean value of the data for the other isolates from the same site and depth.

I realised after I had conducted the cluster analysis that 5 isolates as shown in the table below, had all 13 missing values replaced by mean values and should not therefore have been included. However, these isolates only represent 0.59% of the 843 isolates and this small percentage will not significantly affect the weight of the clusters obtained.

The number of isolates, the number of missing values and the percentage of information (MIC) missing per isolate are shown as follows.

No. isolates	No. missing values	Total No. missing values	% of information (MIC) missing per isolate
178	1	178	1/13 x 100 = 7.7
43	2	86	2/13 x 100 = 15.4
25	3	75	3/13 x 100 = 23.1
21	4	84	4/13 x 100 = 30.8
7	5	35	5/13 x 100 = 38.5
9	6	54	6/13 x 100 = 46.2
12	7	84	7/13 x 100 = 53.8
13	8	104	8/13 x 100 = 61.5
8	9	72	9/13 x 100 = 69.2
13	10	130	10/13 x 100 = 76.9
16	11	176	11/13 x 100 = 84.6
8	12	96	12/13 x 100 = 92.3
5	13	65	13/13 x 100 = 100

Total number of isolates have missing data (column 1) = 358

Total number of missing values (column 3) = 1239

Total number of information units (MIC) including missing values

= 843 (case) x13 (variable)

=10959

The percentage of missing values = 1239/10959 x 100

= 11.3%

5- **Computer analysis**

Many different programs have been used to sort Operational Taxonomic Units (OUT's) into groups or clusters. In microbiology these OUT's are usually isolates. Data for these analyses are arranged in a table of n rows and t columns. Each row represents an OUT. Each column represents a variable. The value of the variable in the table

is either continuous or discontinuous data (binary data). An example of continuous data is the minimum inhibitory concentration presented in my data. An example of discontinuous data is presence/absence (+/-).

The initial stage in the computer analysis is to compare the data for each OUT (isolate) with every other OUT. This results in the calculation of a similarity or dissimilarity value for each pair of isolates. This value can take a number of forms. For example, it can be a Euclidean distance, a matching coefficient, error sum of squares and so on. I used the Euclidean distance squared for three reasons. Firstly, it was readily available on the Clustan 1 C release 2 package Wishart (1978). Secondly, Wishart regards the Euclidean distance squared as the most commonly used measure of dissimilarity between clusters. Thirdly, the Euclidean distance squared is one of the distance measures that can be used in Ward's method which Wishart regards as "possibly the best of the hierarchical options" available.

The Euclidean distance is defined as the square-root of the sum of squares of the differences between the values of the variables for two cases. For example, if the variables of two cases x and y as the following:

case x	2	1	3	4	
case y	4	6	3	2	
(x-y)	2	5	0	2	
$(x-y)^2$	4	25	0	4	=33

Therefore, Euclidean distance = $\sqrt{33}$ = 5.745

Once all the squared Euclidean distances between every pair of isolates has been obtained, they are entered into a triangular table or matrix. Each element of the matrix measures the similarity between two individuals. Although there may be initial confusion as to

the reasons for a triangular and not a square table, if a square table is used, data are included twice. The following example shows this.

square

	1	2	3
1	-	4.6	2.3
2	4.6	-	7.9
3	2.3	7.9	-

triangular

	1	2	3
1	-		
2	4.6	-	
3	2.3	7.9	-

Ward's hierarchical clustering method was then applied to the data in the triangular similarity matrix to obtain cluster dendrograms. The application of Ward's method to my data resulted in a dendrogram containing a number of groups including all the isolates.

Ward's method employs the sum of squared distances from each individual to the centroids of its cluster. For example, given a set of values of a single variable (1, 2, 7, 9, 12) for 5 individuals (A, B, C, D, E), to use the mean value to represent all the scores rather than to consider each individual separately. Ward (1963) proposed that at each stage of an analysis the loss of information that results from the grouping of individuals into one cluster with a mean of 6.2 can be quantified by the error sum of squares.

At each stage in the analysis, every two clusters whose fusion yields the least increase in the error sum of squares are joined together. For example, at stage one eacn individual is regarded as a single member group and its E.S.S. is zero. At stage two the two individuals whose fusion results in the minimum increase in E.S.S. form the first group. For the data shown in the example above these are individuals A and B and their E.S.S. is 0.5. At the next stage individuals C and D fuse to form the second group, where E.S.S. is 2. This increases the total E.S.S. to 2.5. Next, individual E joines the group formed by C and D, and the E.S.S. of this group is 8. The total

E.S.S. is now 2.5 + 8 = 10.5. Finally, the two remaining groups (A, B) and (C, D, E) are fused and their E.S.S. is 302. The E.S.S. of all the individuals as one group is thus 10.5 + 302 = 312.5. The following calculations show the procedure carried out by the computer in order to fuse the individuals into groups.

A	B	C	D	E
1	2	7	9	12

The error sum of squares (E.S.S.) is given by

$$\text{E.S.S.} = \sum_{i=1}^{n} (x_i - \bar{x})^2$$

where x_i is the score of the ith individual.

A , B $= (1 - 1.5)^2 + (2 - 1.5)^2 = 0.5$

A , C $= (1 - 4)^2 + (7 - 4)^2 = 18$

A , D $= (1 - 5)^2 + (9 - 5)^2 = 32$

A , E $= (1 - 6.5)^2 + (12 - 6.5)^2 = 60.5$

B , C $= (2 - 4.5)^2 + (7 - 4.5)^2 = 12.5$

B , D $= (2 - 5.5)^2 + (9 - 5.5)^2 = 24.5$

B , E $= (2 - 7)^2 + (12 - 7)^2 = 50$

C , D $= (7 - 8)^2 + (9 - 8)^2 = 2$

C , E $= (7 - 9.5)^2 + (12 - 9.5)^2 = 12.5$

D , E $= (9 - 10.5)^2 + (12 - 10.5)^2 = 4.5$

	A	B	C	D	E
A	-				
B	0.5*	-			
C	18	12.5	-		
D	32	24.5	2	-	
E	60.5	50	12.5	4.5	-

A+B	C	D	E
3	7	9	12

$A+B\ ,\ C = (3 - 5)^2 + (7 - 5)^2 = 8$

$A+B\ ,\ D = (3 - 6)^2 + (9 - 6)^2 = 18$

$A+B\ ,\ E = (3 - 7.5)^2 + (12 - 7.5)^2 = 40.5$

	A+B	C	D	E
A+B	-			
C	8	-		
D	18	2*	-	
E	40.5	12.5	4.5	-

A+B	C+D	E
3	16	12

$C+D\ ,\ E = (16 - 14)^2 + (12 - 14)^2 = 8$

$C+D\ ,\ A+B = (16 - 9.5)^2 + (3 - 9.5)^2 = 84.5$

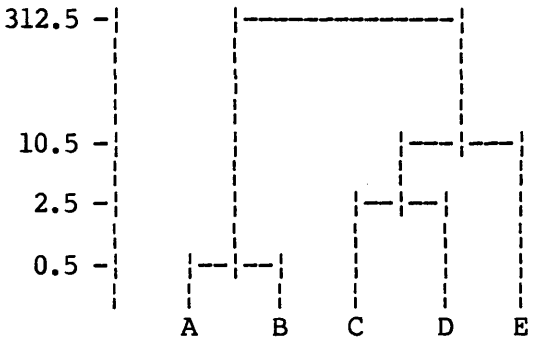
	A+B	C+D	E
A+B	-		
C+D	84.5	-	
E	40.5	8*	-

A+B	C+D+E
3	28

$A+B\ ,\ C+D+E = (3 - 15.5)^2 + (28 - 15.5)^2 = 312.5$

	A+B	C+D+E
A+B	-	
C+D+E	312.5	-

* = The minimum error sum of squares.



6- Interpretation of results

Once cluster analysis had been applied to the data (870 isolates: 870 cases (rows) and 13 antimicrobial agents were 13 variables (columns)) I obtained a 5 metre x 4 metre dendrogram by

sellotaping together sheets from the computer print out. This contained isolates on the x-axis and the distance measure on the y-axis. The distance measure was transformed into a corresponding set of values for a similarity function using the equation %S = 1/(1+d) x 100. Similarity levels of 50, 60, 72, 80, 90, 96, 97, 98, 99, 99.5, 99.9 and 100% were selected and marked on the dendrogram using a marker pen. The number of groups and members of each group were defined at each of these levels. The number of single isolates not clustered with any other members at each similarity level was also obtained.

One of the main difficulties facing me in this study was the presentation of results from the very large dendrogram that I obtained. The dendrogram - being 5m x 4m - needed a large room to view it. I used the floor of the Zoology department museum.

A boundary line was drawn across the dendrogram at 72% similarity level. All groups clustered at or above this similarity level were drawn as a shaded triangles in my final diagram of the cluster analysis. Clusters or groups were labelled "1" to "21". Numbers of isolates in each group including standard reference cultures were also included in the shaded dendrogram as shown in the results.

I have justified the choice of similarity level of 72% on page 193.

SECTION 1

RESULTS

The results of this section are divided into the following parts:

1- Antimicrobial agents sensitivity (p. 54 to 75).

A- Preliminary experiments

B- Definitive experiments

2- Analysis of clusters formed at 50% and 72% similarity levels (p. 76 to 112).

A- Analysis of clusters formed at 50% similarity level.

B- Clusters formed at 72% similarity level.

3- Statistical analysis of clusters formed at 72% similarity level (p. 113 to 137).

A- Cluster by sites.

(i) Variation in number of isolates between groups for each site.

(ii) Variation in number of isolates between sites for each group.

(iii) Comparison of the number of isolates between sampling sites.

B- Cluster by depths.

(i) Variation in number of isolates between groups for each depth.

(ii) Variation in number of isolates between depths for each group.

(iii) Comparison of the number of isolates between sampling depths.

C- Relationship between isolates of each group obtained from sediment, burrow linings and faecal pellets.

4- Description and interpretation of figures 18 to 23 drawn from 72% similarity level data (Tables 28 and 29).

A- Variation in the number of sediment isolates between groups at each sampling site (p.138 to 139).

B- Variation in the number of sediment isolates of each group, between sampling sites (p. 140 to 145).

C- Variation in the number of sediment isolates between groups at each sampling depth (p. 145 to 149).

D- Variation in the number of sediment isolates of each group, between sampling depths (p. 149 to 156).

5- Interrelationships between similarity measure, number of isolates/group, number of groups, the coefficient of variation, and the number of single isolates (p. 157 to 174).

6- Clustering of variables (p. 175 to 178).

****

1-Antimicrobial agents sensitivity

A- Preliminary experiments

The results obtained in the preliminary experiments (with 100 isolates) show that different patterns of resistance to metals were observed. For example, cadmium, copper and lead showed a sharp reduction between one concentration and the next (e.g. 32-64, 32-64 and 100-200mg/l respectively) whereas chromium and mercury showed a gradual decline in the number of bacteria growing at higher concentration (Figure 7). Only two of the antibiotics, polymyxin B and chloramphenicol were able to discriminate clearly between sensitive and resistant groups of bacteria (Figure 8). The dye methylene blue showed no consistent results between different concentrations (Figure 8).

With the antimicrobial agents, cadmium, chromium, ampicillin,

Figure 7

Histograms from the preliminary experiments showing the percentage of deep-sea isolates growing on media containing cadmium ($CdCl_2.2.5H_2O$), chromium (K_2CrO_4), copper ($CuSO_4.5H_2O$), lead $Pb(OOCCH_3)_2.3H_2O$, and mercury ($HgCl_2$) at a range of concentrations (mg/l). Data from master replica 2 only-50 isolates (materials and methods, p.17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 1, p. 236).

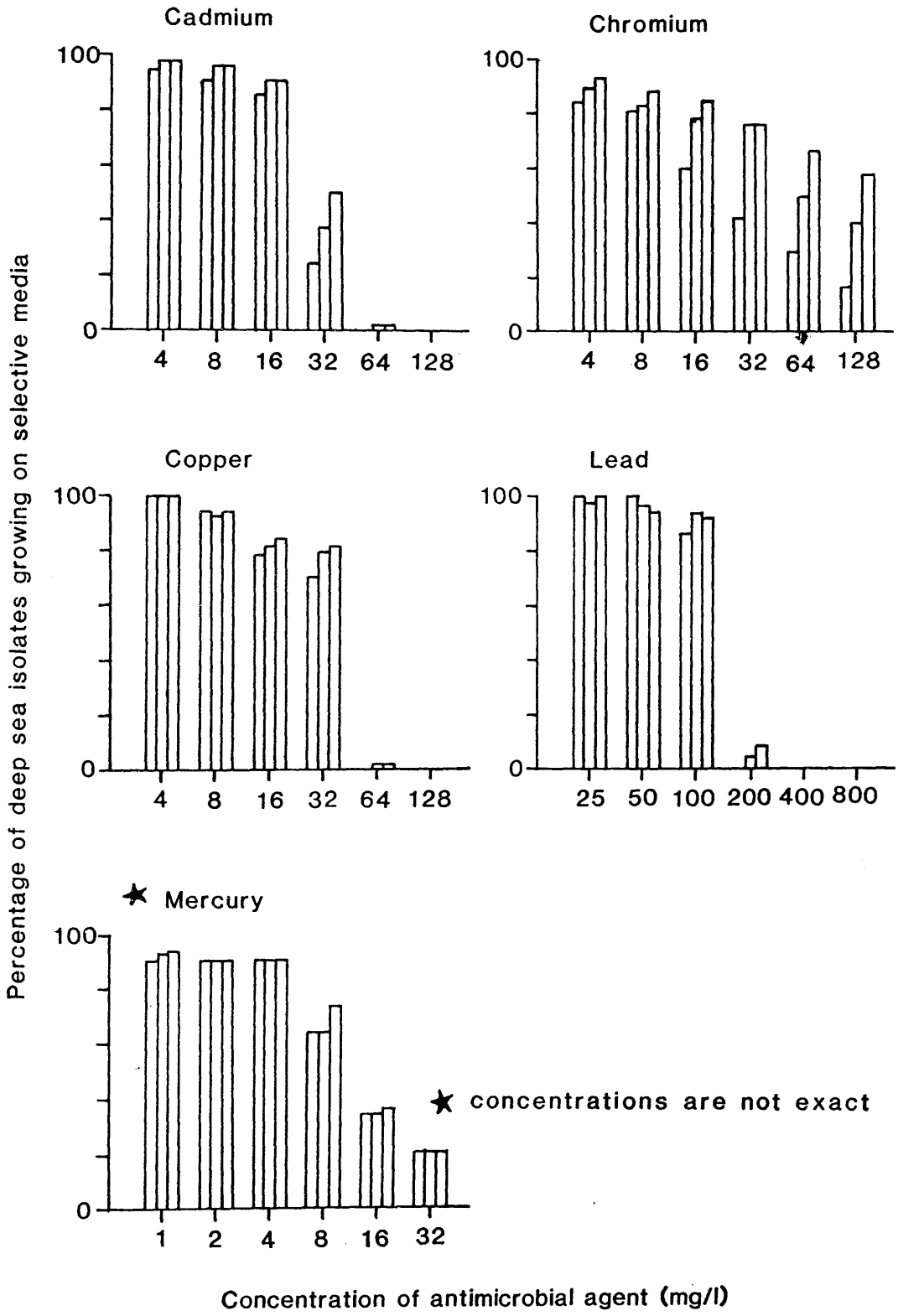
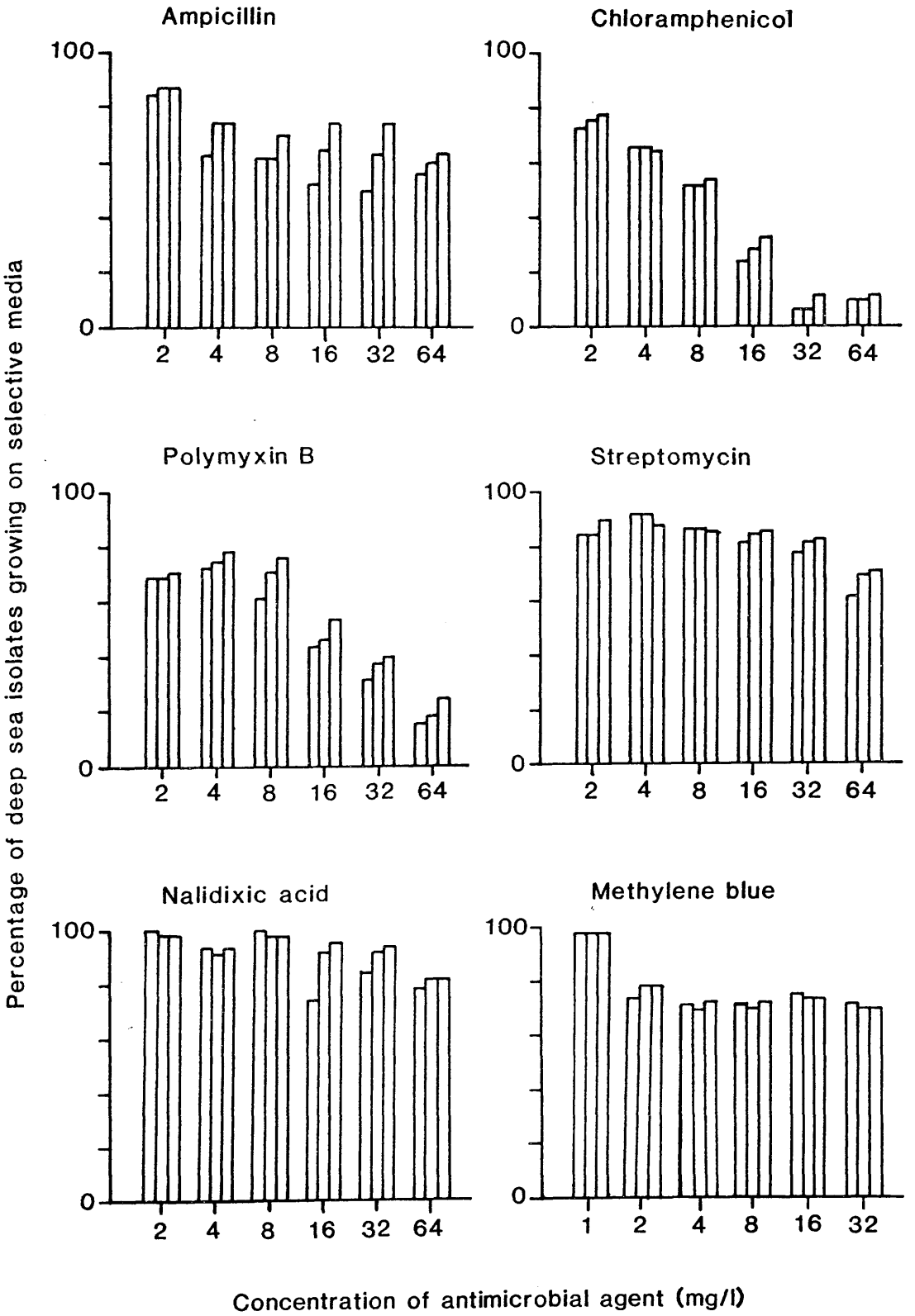

Cadmium
Chromium
Copper
Lead
Mercury
100
0
4 8 16 32 64 128
25 50 100 200 400 800
1 2 4 8 16 32
★ concentrations are not exact
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Figure 8

Histograms from the preliminary experiments showing the percentage of deep-sea isolates growing on media containing ampicillin, chloramphenicol, polymyxin B, streptomycin, nalidixic acid, and methylene blue at a range of concentrations (mg/l). Data from master replica 2 only-50 isolates (materials and methods, p. 17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 2, p. 237).


Ampicillin
Chloramphenicol
Polymyxin B
Streptomycin
Nalidixic acid
Methylene blue
100
0
2 4 8 16 32 64
1 2 4 8 16 32
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

polymyxin B and nalidixic acid, it was found that increasing the incubation time led to an increase in the number of isolates growing on selective media. For example, in (Figure 7, chromium) at concentrations 16, 32, 64 and 128mg/l, there is difference in the bacterial growth between 7, 14 and 21 days.

B- Definitive experiments

The results of the preliminary experiments recorded in figures 7 and 8 enabled me to choose a more appropriate range of concentrations for the antimicrobial agents used in the definitive experiments (see materials and methods p. 24). The concentrations used in the definitive experiments are shown in table 5. The original data are shown in appendix table 31.

I will now describe in detail the results of part of the definitive experiments based on 100 of the 843 isolates, which demonstrate the percentage of deep-sea bacteria growing on media containing antibiotics and heavy metals at various concentrations. This is followed by a short comment on methylene blue and by an account of the relative toxicity of the antibiotics and heavy metals.

Antibiotics

The results shown in figures 9 and 10 (MR1), 11 and 12 (MR2) show a consistent trend in that the number of isolates grown on media containing antibiotics decreased with increasing antibiotic concentrations except in the case of methylene blue.

It can also be seen that the percentage of isolates growing increased with increasing incubation time. However, this increase in growth did not always occur in the presence of low concentrations of antibiotics. For example, with ampicillin (2 and 8mg/l), tetracycline (4, 8 and 16mg/l), and nalidixic acid (4mg/l) in master replica 1 and

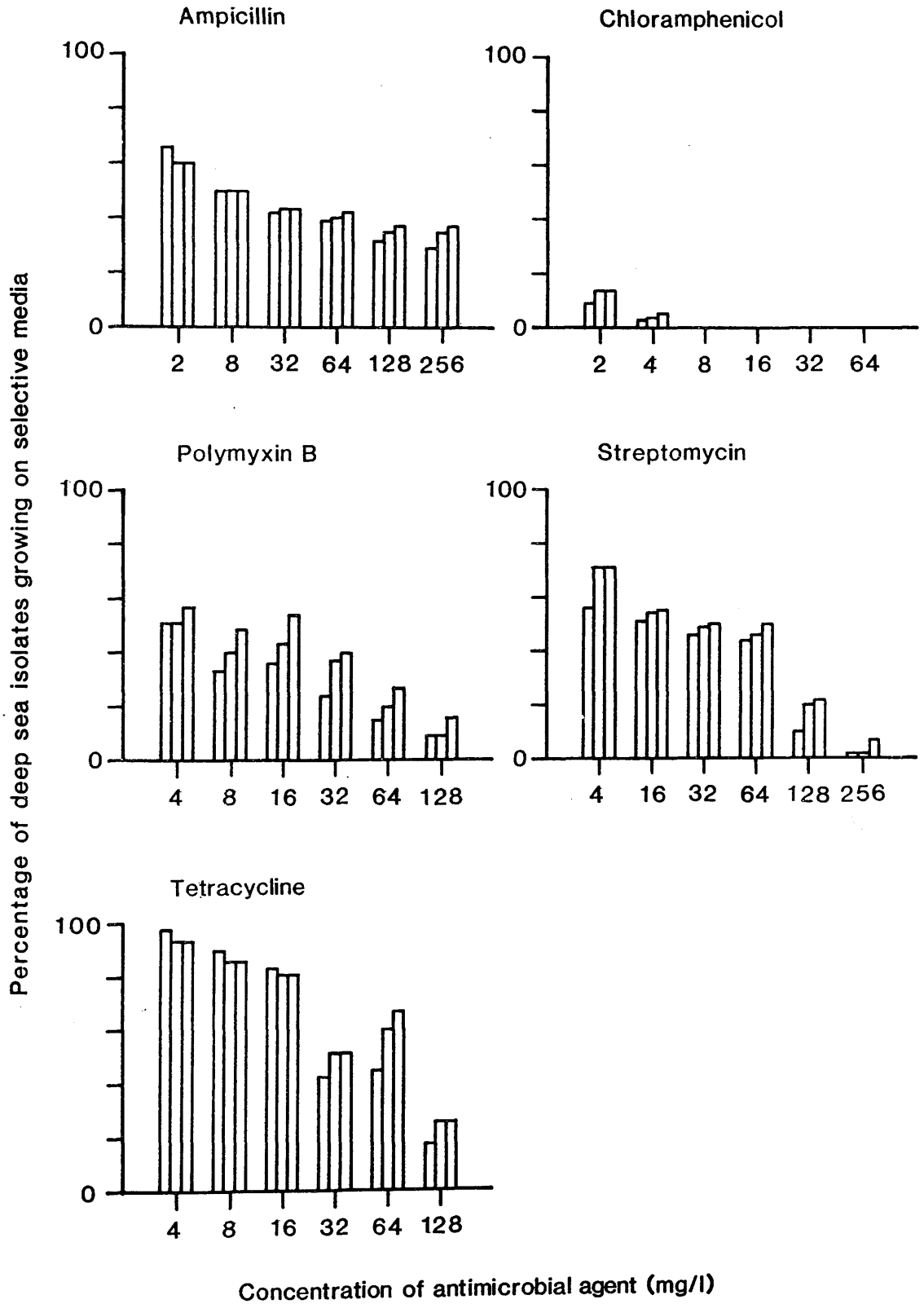

Ampicillin
100
0
2 8 32 64 128 256
Chloramphenicol
100
0
2 4 8 16 32 64
Polymyxin B
100
0
4 8 16 32 64 128
Streptomycin
100
0
4 16 32 64 128 256
Tetracycline
100
0
4 8 16 32 64 128
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Figure 9

Histograms from the definitive experiments showing the percentage of deep-sea isolates growing on media containing ampicillin, chloromphenicol, polymyxin B, streptomycin, and tetracycline at a range of concentrations (mg/l). Data from master replica 1 only-50 isolates (materials and methods, p. 17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 3, p. 238).

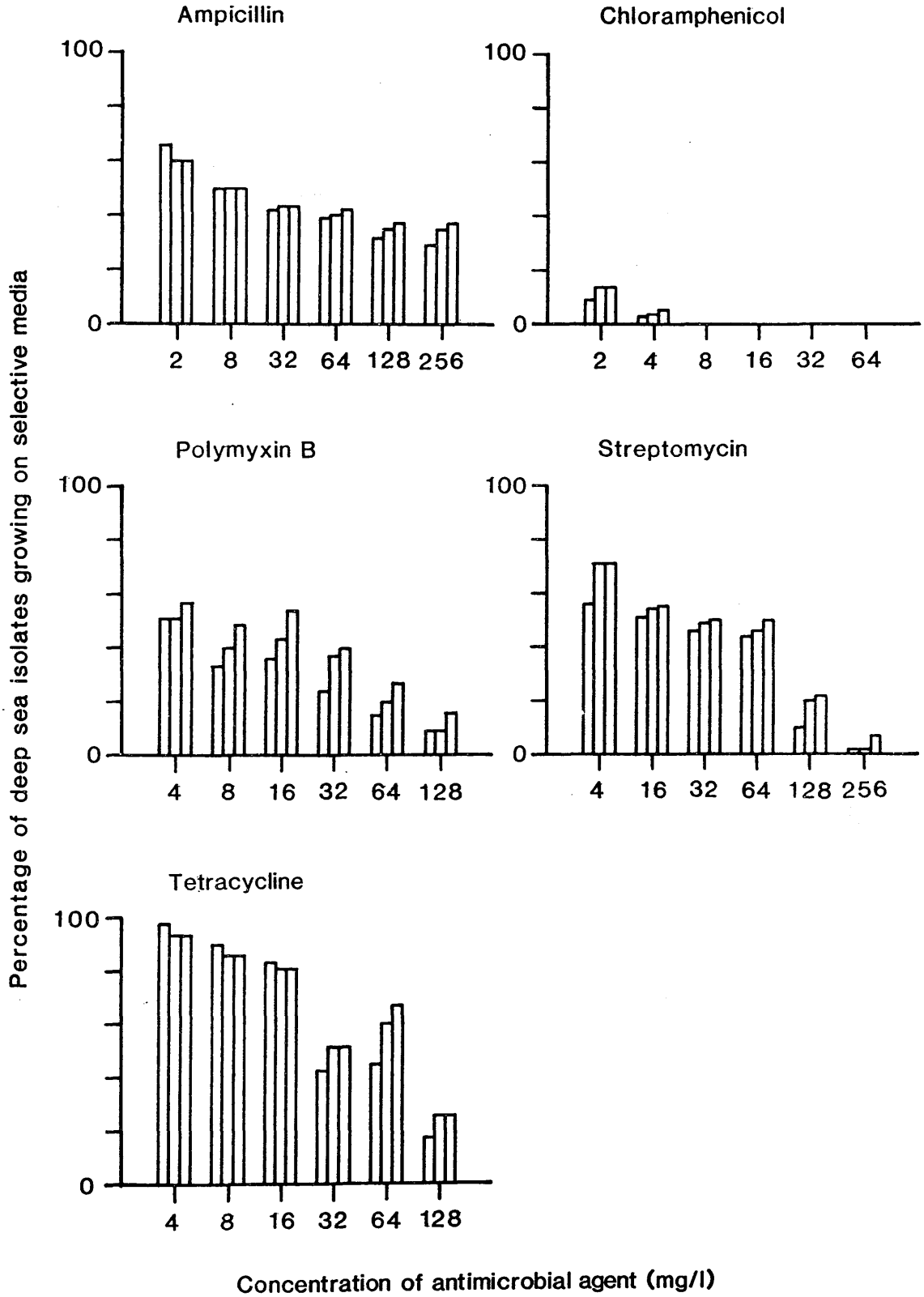

Ampicillin
100
0
2 8 32 64 128 256
Chloramphenicol
100
0
2 4 8 16 32 64
Polymyxin B
100
0
4 8 16 32 64 128
Streptomycin
100
0
4 16 32 64 128 256
Tetracycline
100
0
4 8 16 32 64 128
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Figure 10

Histograms from the definitive experiments showing the percentage of deep-sea isolates growing on media containing nalidixic acid and the dye methylene blue at a range of concentrations (mg/l). Data from master replica 1 only-50 isolates (materials and methods, p.17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 3, p. 238).

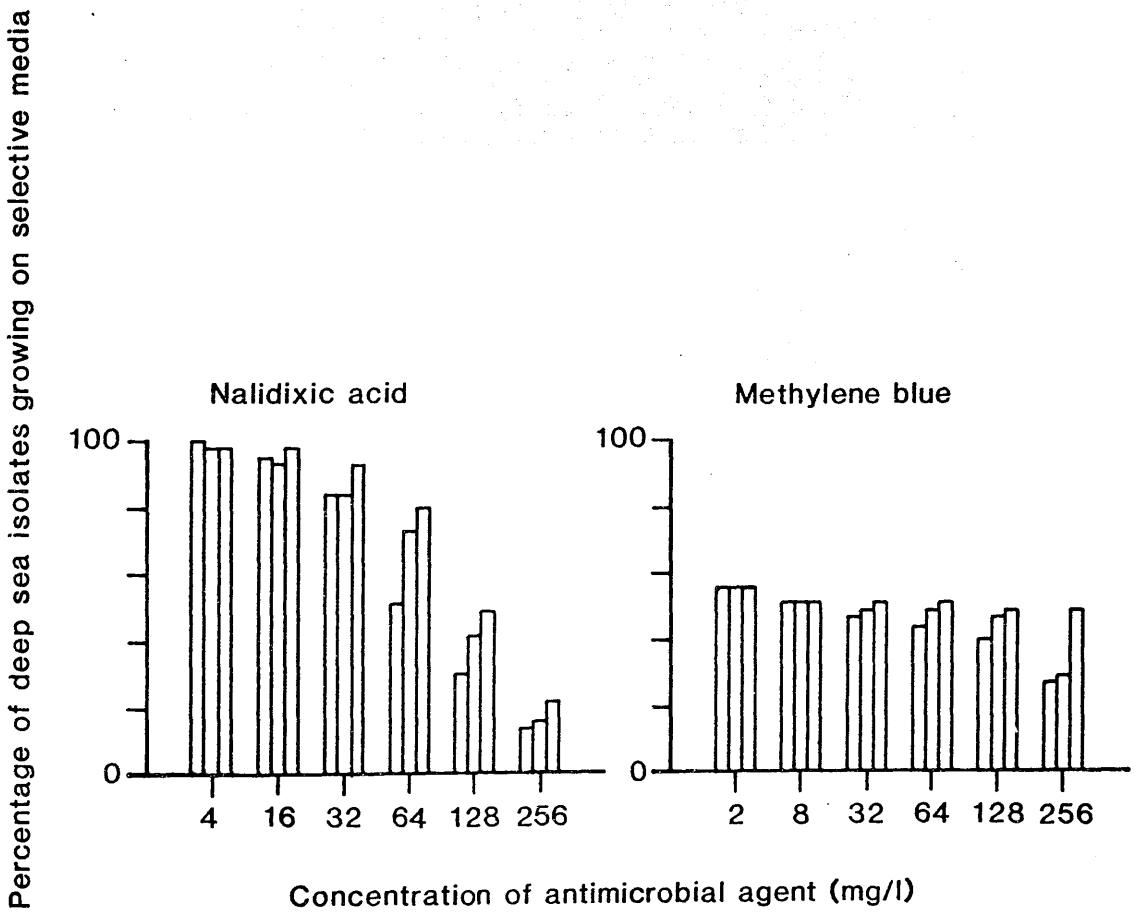

Percentage of deep sea isolates growing on selective media
Nalidixic acid
Methylene blue
100
0
100
0
4
16
32
64
128
256
2
8
32
64
128
256
Concentration of antimicrobial agent (mg/l)

Figure 11

Histograms from the definitive experiments showing the percentage of deep-sea isolates growing on media containing ampicillin, chloramphenicol, polymyxin B, streptomycin and tetracycline at a range of concentrations (mg/l). Data from master replica 2 only-50 isolates (materials and methods, p. 17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 4, p. 239).

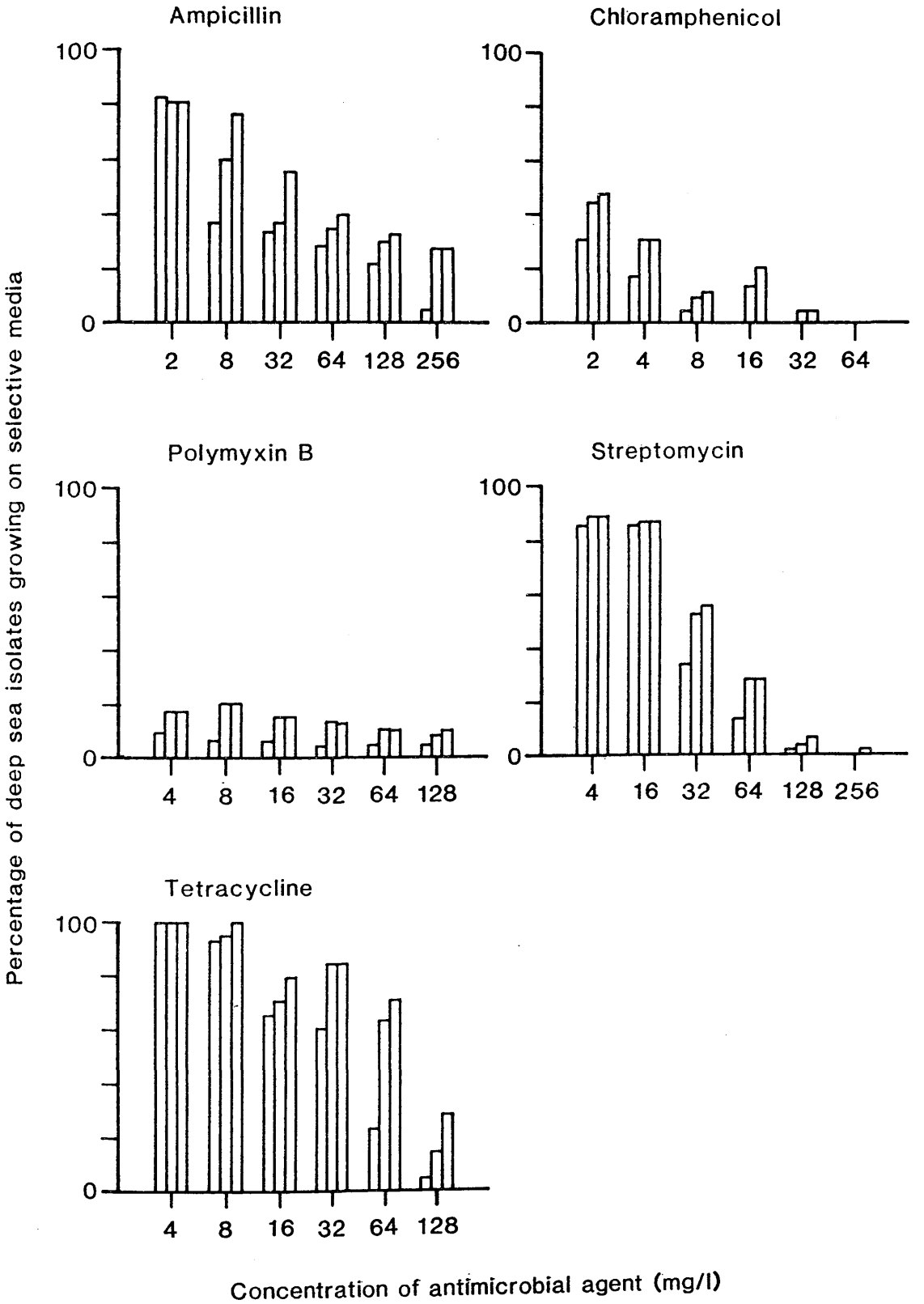
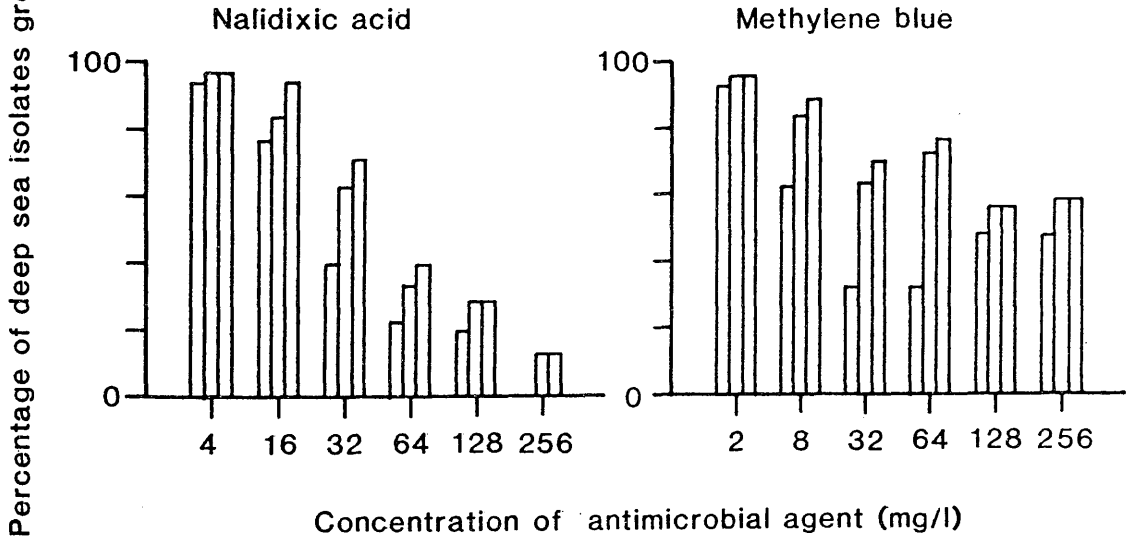

Ampicillin
100
0
2 8 32 64 128 256
Chloramphenicol
100
0
2 4 8 16 32 64
Polymyxin B
100
0
4 8 16 32 64 128
Streptomycin
100
0
4 16 32 64 128 256
Tetracycline
100
0
4 8 16 32 64 128
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Figure 12

Histograms from the definitive experiments showing the percentage of deep-sea isolates growing on media containing the antibiotic, nalidixic acid and the dye, methylene blue at a range of concentrations (mg/l). Data from master replica 2 only-50 isolates (materials and methods, p. 17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 4, p. 239).


Percentage of deep sea isolates growing on selective media
Nalidixic acid
Methylene blue
100
0
4
16
32
64
128
256
2
8
32
64
128
256
Concentration of antimicrobial agent (mg/l)

with ampicillin (2mg/l) and tetracycline (4mg/l) in master replica 2. there is little difference between the 7, 14 and 21 day counts. This lack of difference should be contrasted with the very marked differences between the counts on days 7, 14 and 21 at the higher concentrations of the same antimicrobial agent. The reasons of the differences in growth between incubation times with antibiotics and heavy metals are discussed in the discussion on p. 187, 188.

The antibiotics chloramphenicol and polymyxin B inhibited a larger number of isolates from growing than the other antibiotics used. These two antibiotics are therefore the most effective antibiotics, and this agrees with the reports in the literature (Washington, 1969; McNicol, 1980).

Heavy metals

Figures 13 (MR1) and 14 (MR2) show the percentage of deep-sea isolates growing on media containing the 6 metals tested. In general, as with antibiotics there was a decrease in percentage of isolates growing with increasing heavy metal concentrations. The decrease in growth of deep-sea isolates with increasing metal concentrations appeared to be greatest with cadmium, manganese and mercury. Of these metals, mercury was the most effective metal. In contrast, at low test concentrations of chromium, copper and lead, there was only a slight decrease in the number of isolates growing on selective media. However, when high concentrations were reached, a significant decrease in growth was observed even with these metals.

Unlike antibiotics, there was no apparent general increase in the number of isolates grown on selective media with increasing incubation time. This difference between the growth at 7, 14 and 21 days in the presence of antibiotics and heavy metals is shown in tables 8 and 9 respectively. Here I have expressed each percentage

Figure 13

Histograms from the definitive experiments showing the percentage of deep-sea isolates growing on media containing cadmium ($CdCl_2.2.5H_2O$), chromium (K_2CrO_4), copper ($CuSO_4.5H_2O$), lead $Pb(OOCCH_3)_2.3H_2O$, manganese ($MnCl_2.4H_2O$), and mercury ($HgCl_2$) at a range of concentrations (mg/l). Data from master replica 1 only-50 isolates (materials and methods, p.17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 5, p. 240).

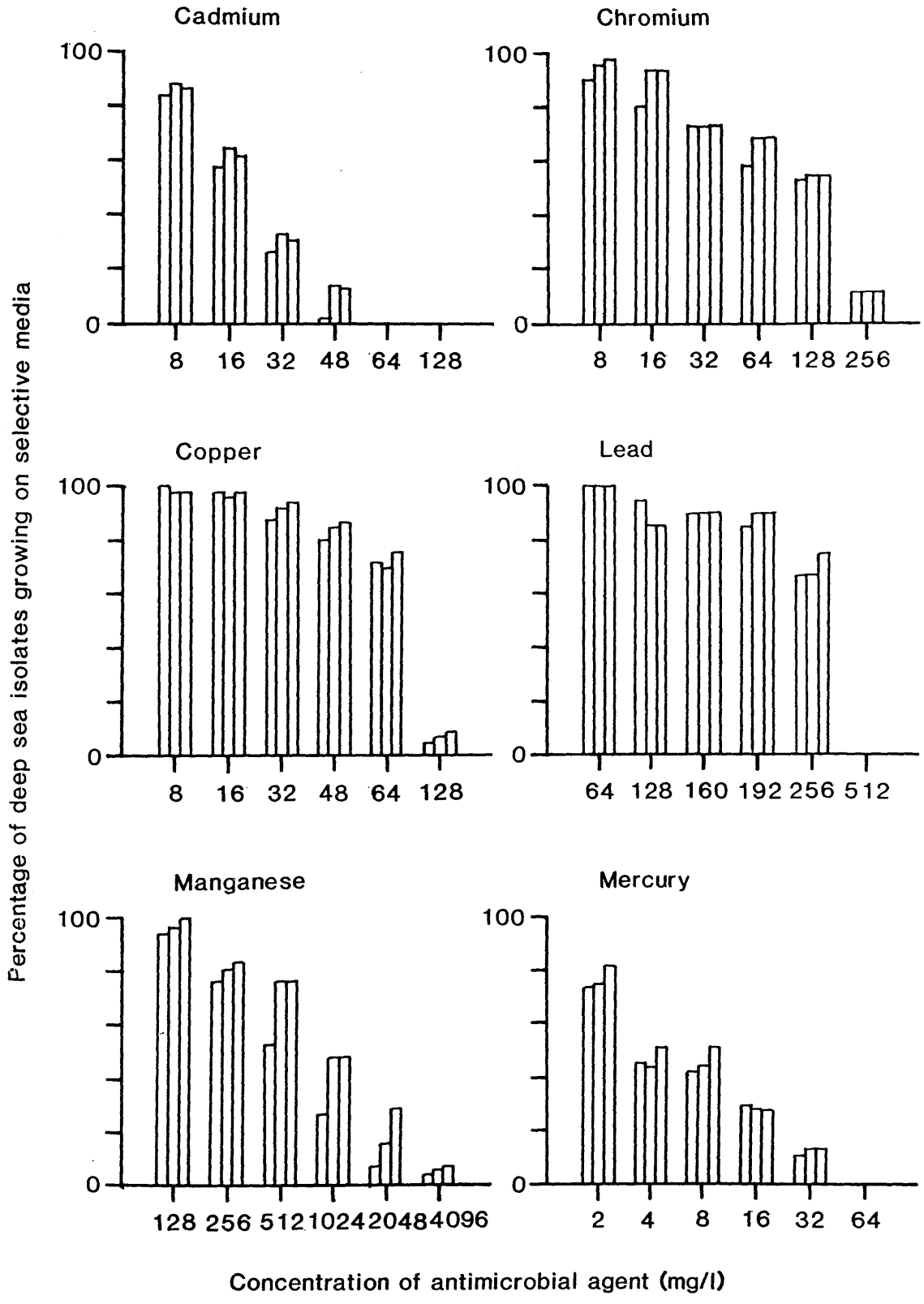

Cadmium
100
0
8 16 32 48 64 128
Chromium
100
0
8 16 32 64 128 256
Copper
100
0
8 16 32 48 64 128
Lead
100
0
64 128 160 192 256 512
Manganese
100
0
128 256 512 1024 2048 4096
Mercury
100
0
2 4 8 16 32 64
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Figure 14

Histograms from the definitive experiments showing the percentage of deep-sea isolates growing on media containing cadmium ($CdCl_2.2.5H_2O$), chromium (K_2CrO_4), copper ($CuSO_4.5H_2O$), lead $Pb(OOCCH_3)_2.3H_2O$, manganese ($MnCl_2.4H_2O$), and mercury ($HgCl_2$) at a range of concentrations(mg/l). Data from master replica 2 only-50 isolates (materials and methods, p. 17). % are calculated as % of number of isolates growing on control plate for each antimicrobial agent tested (Appendix Table 6, p. 241).

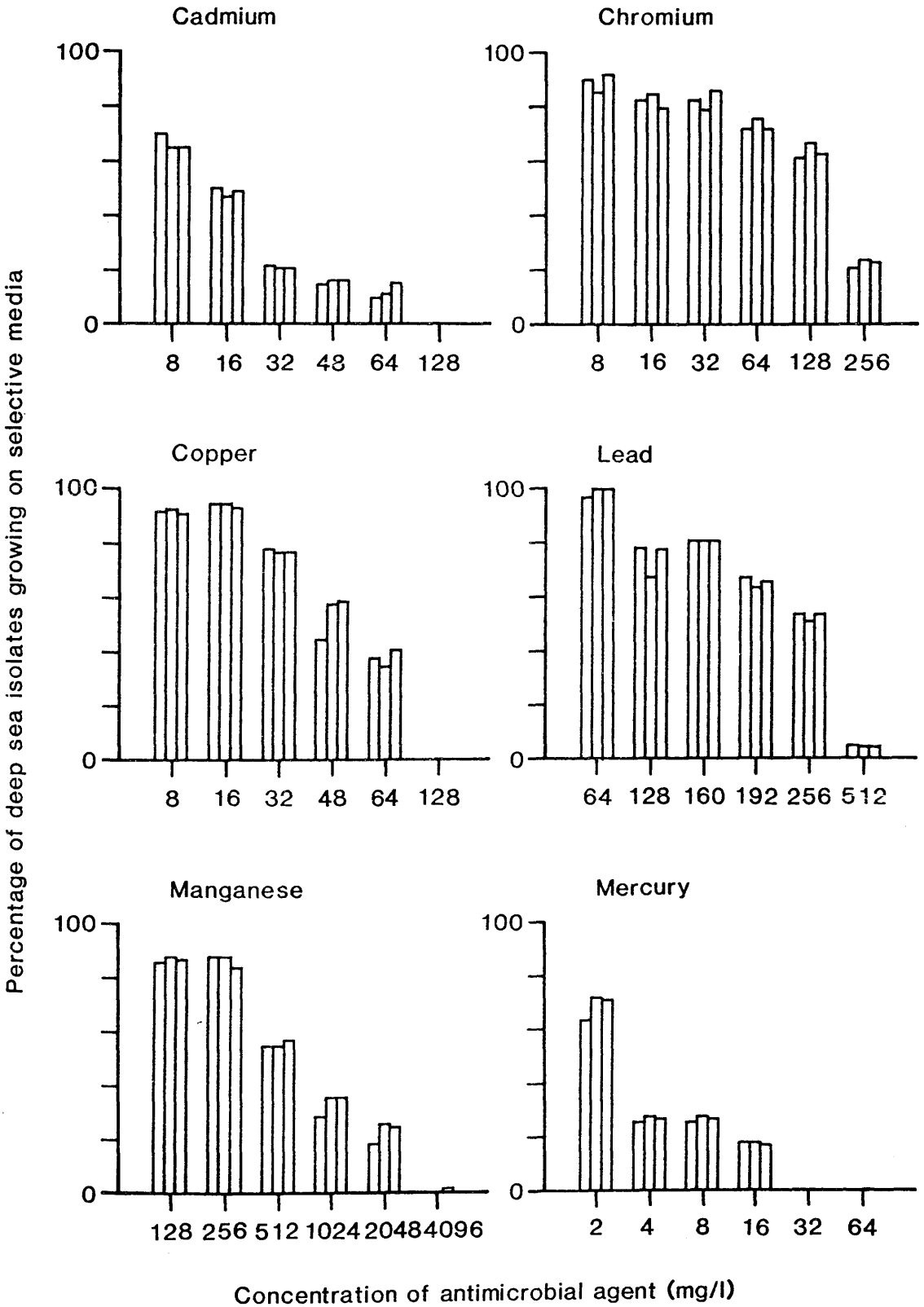

Cadmium
100
0
8 16 32 48 64 128
Chromium
100
0
8 16 32 64 128 256
Copper
100
0
8 16 32 48 64 128
Lead
100
0
64 128 160 192 256 512
Manganese
100
0
128 256 512 1024 2048 4096
Mercury
100
0
2 4 8 16 32 64
Percentage of deep sea isolates growing on selective media
Concentration of antimicrobial agent (mg/l)

Table 8

The percentage of number of isolates growing at various concentrations (mg/l) of antibiotics and methylene blue dye after 7, 14 and 21 days.For each master replica plate, the 14 and 21 day counts were calculated as a percentage of the 7 day count which was regarded as being 100% (see P.71). Blanks shown in the table, mean that no growth was obtained at these concentrations.

Antimicrobial agent	Master replica	Plate count	Concentration (mg/l)					
			2	8	32	64	128	256
Ampicillin	1	7	100	100	100	100	100	100
		14	95	100	101	101	111	121
		21	95	100	101	106	119	130
	2	7	100	100	100	100	100	100
		14	98	165	109	119	137	572
		21	98	210	164	135	148	572
			2	4	8	16	32	64
Chloramphenicol	1	7	100	100	-	-	-	-
		14	153	93	-	-	-	-
		21	148	180	-	-	-	-
	2	7	100	100	100	-	-	-
		14	147	173	186	-	-	-
		21	155	173	232	-	-	-

Table 8 cont'd.

Antimicrobial agent	Master replica	Plate count	Concentration (mg/l)					
			4	8	16	32	64	128
Polymyxin B	1	7	100	100	100	100	100	100
		14	100	120	118	153	132	95
		21	110	146	149	167	178	178
	2	7	100	100	100	100	100	100
		14	191	287	223	286	239	191
		21	187	280	218	279	233	233
			4	16	32	64	128	256
Streptomycin	1	7	100	100	100	100	100	100
		14	126	105	105	105	200	100
		21	127	107	108	114	219	292
	2	7	100	100	100	100	100	-
		14	103	100	156	213	191	-
		21	103	100	163	213	290	-
			4	8	16	32	64	128
Tetracycline	1	7	100	100	100	100	100	100
		14	95	95	98	123	132	150
		21	95	95	98	123	148	150
	2	7	100	100	100	100	100	100
		14	100	102	107	140	260	299
		21	100	108	122	140	290	201

Table 8 cont'd.

Antimicrobial agent	Master replica	Plate count	Concentration (mg/l)					
			4	8	16	32	64	128
Nalidixic acid	1	7	100	100	100	100	100	100
		14	98	98	101	143	140	111
		21	98	103	111	156	162	159
	2	7	100	100	100	100	100	-
		14	103	147	158	149	145	-
		21	103	166	178	172	145	-
			2	8	32	64	128	256
Methylene blue	1	7	100	100	100	100	100	100
		14	100	100	105	110	117	108
		21	100	100	109	115	122	183
	2	7	100	100	100	100	100	100
		14	103	134	195	223	116	121
		21	103	141	216	237	116	121

Table 9

The percentage of number of isolates growing at various concentrations (mg/l) of heavy metals after 7, 14 and 21 days. For each master replica plate, the 14 and 21 day counts were calculated as a percentage of the 7 day count which was regarded as being 100% (see P. 71). Blanks shown in the table, mean that no growth was obtained at these concentrations.

Antimicrobial agent	Master replica	Plate count	Concentration (mg/l)					
			8	16	32	48	64	128
Cadmium	1	7	100	100	100	100	100	-
		14	93	93	93	109	116	-
		21	93	98	93	93	163	-
	2	7	100	100	100	100	-	-
		14	106	112	127	601	-	-
		21	104	107	121	571	-	-
			8	16	32	64	128	256
Chromium	1	7	100	100	100	100	100	100
		14	95	102	95	105	107	117
		21	102	97	104	99	101	111
	2	7	100	100	100	100	100	100
		14	107	118	100	117	104	100
		21	109	118	100	117	104	100

Table 9 cont'd.

Antimicrobial agent	Master replica	Plate count	Concentration (mg/l)					
			8	16	32	48	64	128
Copper	1	7	100	100	100	100	100	-
		14	101	100	99	129	93	-
		21	98	98	99	131	109	-
	2	7	100	100	100	100	100	100
		14	98	98	104	106	96	87
		21	98	100	107	109	105	174
			64	128	160	192	256	512
Lead	1	7	100	100	100	100	100	100
		14	103	87	99	94	95	91
		21	103	99	99	97	99	91
	2	7	100	100	100	100	100	-
		14	100	89	100	106	100	-
		21	100	89	100	106	112	-
			128	256	512	1024	2048	4096
Manganese	1	7	100	100	100	100	100	-
		14	103	100	100	125	138	-
		21	101	95	104	127	132	-
	2	7	100	100	100	100	100	100
		14	104	105	145	181	241	194
		21	107	109	145	181	435	195

Table 9 cont'd.

Antimicrobial agent	Master replica	Plate count	Concentration (mg/l)					
			2	4	8	16	32	64
Mercury	1	7	100	100	100	100	-	-
		14	112	110	110	100	-	-
		21	110	105	105	96	-	-
	2	7	100	100	100	100	100	-
		14	101	98	104	98	122	-
		21	111	115	122	98	122	-

growth as a percentage of the 7 day value in appendix tables 3-6. For example, consider the 7, 14 and 21 days, percentages for ampicillin at a concentration of 2mg/l. These are 24/38 = 63.2%, 24/40 =60% and 24/40 = 60% (Appendix table 3). The percentages in table 8 for these data were then calculated as: 63.2/63.2 x 100 = 100% (7days)

60/63.2 x 100 = 95% (14 days)

60/63.2 x 100 = 95% (21 days)

Wilcoxon's matched pairs tests were applied to the data in tables 8 and 9 to compare the percentage of bacterial growth found at 14 days high concentrations with that at 14 days low concentrations firstly for antibiotics (Appendix table 11) then for heavy metals (Appendix table 12). The same procedure was carried out with the 21 day counts (Appendix tables 13 and 14). The results of these tests are summarised in table 10. With the antibiotics, significant differences were found between high and low concentrations for both incubation times. However, With the heavy metals, there was only a significant difference at 21 days, and this was close to being non-significant ($0.05>P>0.025$).

Methylene blue dye

When the dye methylene blue was used as selective media, no consistent trend in the decrease in number of isolates with increasing concentrations was obtained. For example, with master replica plate 1 (Figure 10), there was not a clear decrease in the percentage of deep-sea isolates with increasing methylene blue concentration. In master replica 2 (Figure 12), the decrease in percentage of isolates grown was more appearent.

Subject	Comparison	Negative ranks(R-)	Size of sample	P
Antibiotics	14 days (high concentrations) / 14 days (low concentrations)	4.5	9	0.025>P>0.01
Antibiotics	21 days (high concentrations) / 21 days (low concentrations)	0	10	P<0.001
Heavy metals	14 days (high concentrations) / 14 days (low concentrations)	15	11	P<0.05
Heavy metals	21 days (high concentrations) / 21 days (low concentrations)	14	12	0.05>P>0.025

Table 10

Summary of the results of Wilcoxon's matched pairs test (appendix tables 11, 12, 13 and 14p. 246, 247, 248 and 249 respectively) to compare bacterial growth found at high and low concentrations of antimicrobial agents, at both 14 and 21 day counts.

Relative toxicity of antibiotics and heavy metals

The order of toxicity of antibiotics and heavy metals was investigated by obtaining the overall mean and standard deviation of the minimum inhibitory concentration (MIC) of the antibiotics and heavy metals for the 843 isolates tested (Table 11). The lower the mean of MIC, the more toxic antibiotic or metal. The relative toxicity of antibiotics and the relative toxicity of heavy metals were studied using student's t-tests. Table 12 (i) and (ii) shows the t-test values between pairs of antibiotics or pairs of metals and letters showing the significance levels of t-test values. 5 out of 21 comparisons showed a significant difference in the toxicity between antibiotics while 12 out of 15 comparisons showed a significant difference in the toxicity between metals. Therefore, in general, heavy metals showed more significant difference in the toxicity between pairs of metals than antibiotics.

Antimicrobial agent	Minimum inhibitory concentration (MIC) (mg/l)	
	Mean	Standard deviation
Chloramphenicol	6.668	9.252
Polymyxin B	58.339	84.053
Tetracycline	109.037	87.022
Ampicillin	115.971	183.788
Streptomycin	138.759	152.107
Methylene blue	182.566	213.822
Nalidixic acid	186.669	179.874
Mercury	24.131	31.287
Cadmium	29.453	31.562
Copper	81.798	52.247
Chromium	188.745	188.728
Lead	321.634	169.859
Manganese	2041.398	1963.746

Table 11

Overall mean and standard deviation of minimum inhibitory concentrations of antibiotics and heavy metals for the 843 isolates tested (see appendix table 31).

(i)

ANTIBIOTICS

	Ch	Po	Te	Am	St	MB	Na
Ch	-	J	N*	J	M*	L*	M*
Po	1.833	-	H	G	I	I	J
Te	3.509	1.257	-	A	D	G	H
Am	1.782	0.8555	0.1023	-	C	F	F
St	2.600	1.388	0.5088	0.2866	-	D	E
MB	2.466	1.622	0.9555	0.7086	0.5008	-	N*
Na	2.998	1.939	1.166	0.8247	0.6102	4.406	-

(ii)

HEAVY METALS

	Hg	Cd	Cu	Cr	Pb	Mn
Hg	-	C	M*	M*	N*	M*
Cd	0.3592	-	L*	L*	N*	M*
Cu	2.841	2.573	-	I	N*	M*
Cr	2.581	2.497	1.638	-	I	M*
Pb	5.167	5.074	4.049	1.570	-	M*
Mn	3.081	3.073	2.993	2.817	2.617	-

Table 12

Student's t-tests on the relative toxicity of antimicrobial agents. Lower triangles: Student's t-test values between pairs of antibiotics (i) and heavy metals (ii). Upper triangles: letters showing the significance level of t-test values in the lower triangles. * =toxicity is significantly different.

Significance levels:

A = P>0.90
B = 0.90>P>0.80
C = 0.80>P>0.70
D = 0.70>P>0.60
E = 0.60>P>0.50
F = 0.50>P>0.40
G = 0.40>P>0.30
H = 0.30>P>0.20
I = 0.20>P>0.10
J = 0.10>P>0.05
*K = 0.05>P>0.025
*L = 0.025>P>0.01
*M = 0.01>P>0.001
*N = P<0.001

2- Analysis of clusters formed at 50% and 72% similarity levels

A numerical analysis was applied on 843 deep-sea isolates in order to classify them into taxa. By drawing a boundary line across the dendrogram at different levels of similarity, the OUTs (isolates) were clustered in different groups or clusters. For example, at a similarity level of 50% or above, 11 clusters were obtained while at a similarity level of 72% or above, the isolates were recovered in 21 clusters. I shall deal with the 50% similarity level data first, and the 72% similarity level data second.

A- Analysis of clusters formed at 50% similarity level

It is important to note that throughout the following analysis, the original data being analysed is the number of isolates in each group obtained from the cluster analysis, at different sites and different depths.

The 843 isolates were classified into 11 groups at the 50% similarity level. The number of isolates/group are shown in table 13 and range from 19 in group 11 to 201 in group 5. The groups were then further subdivided by distinguishing the number of isolates at each site and the number of isolates at each depth. For successive groups in turn these data are shown in table 14 for the sites and table 15 for the depths. For example, of the 51 isolates in group 1, 10 occurred at site 1 (all depths combined), 5 at site 2 (all depths combined), and so on to site 5. Similarly, of the 51 isolates in group 1, 19 occurred at the surface (all sites combined), 10 occurred at 0.5cm (all sites combined), and so on to depth 20.5cm.

Data on the number of isolates/group/site and the number of isolates/group/depth were analysed statistically. Before statistical analysis were conducted, the data on sites (Table 14) and depths (Table 15) were tested for normality by a graphical method (Sokal and

Group	Total no. isolates in group
1	51
2	140
3	38
4	68
5	201
6	87
7	34
8	54
9	68
10	83
11	19

Table 13 Total number of isolates/group clustered at 50% similarity level.

Group	Site					Total No. of isolates in group
	1	2	3	4	5	
1	10	5	25	4	7	51
2	0	65	30	32	13	140
3	0	12	5	15	6	38
4	11	9	29	12	7	68
5	7	92	22	43	37	201
6	0	36	10	27	14	87
7	6	4	0	4	20	34
8	0	23	0	25	6	54
9	34	6	9	6	13	68
10	3	15	1	50	14	83
11	3	2	1	13	0	19

Table 14: Number of bacterial isolates/group at each site (e.g. no. isolates/group/site). Untransformed data. Cluster analysis at 50% similarity level.

Group	Depth (cm)											Total no. of isolates in group
	00.0	00.5	03.0	04.0	05.5	07.5	10.5	11.0	13.0	15.5	20.5	
1	19	10	10	0	1	6	0	1	0	3	1	51
2	3	14	21	5	4	27	0	53	11	0	2	140
3	2	5	2	5	2	3	0	10	9	0	0	38
4	20	8	4	8	3	10	9	2	1	3	0	68
5	6	14	24	8	37	16	23	26	14	18	15	201
6	2	10	11	8	5	8	3	20	14	4	2	87
7	0	23	8	0	2	0	0	1	0	0	0	34
8	0	15	16	0	2	0	1	12	8	0	0	54
9	0	31	17	1	6	9	0	2	0	2	0	68
10	1	14	28	19	9	0	1	3	7	0	1	83
11	1	0	3	6	0	0	0	2	7	0	0	19

Table 15: Number of bacterial isolates/group at each depth (e.g. no. isolates/group/depth). Untransformed data. Cluster analysis at 50% similarity level.

Rohlf, 1981, p. 117-126) and by the relationship between the mean and standard deviation (Snedecor and Cochrane, 1967, p.325). When the data was plotted against percent cumulative frequency on a probability scale paper (Appendix figure 2A, sites; Appendix figure 3A, depths) and the relationship between mean and standard deviation was analysed (Appendix figure 4A, sites; Appendix figure 5A, depths), both sites and depths showed non-normal distributions. A series of transformations using square-root (Appendix table 16, sites; and Appendix table 17, depths) and $\log_{10}$ (Appendix table 18, sites; Appendix table 19, depths) were therefore applied to the data in order to obtain a normal distribution. The graphs obtained are shown in appendix figures 2B and 2C, (sites) and appendix figures 3B and 3C, (depths). When regression analysis was applied to the untransformed, square-root and $\log_{10}$ transformed data on sites and on depths (Appendix tables 20 and 21 respectively), the highest correlation coefficient (r) was found with the square-root data for both sites and depths (0.9871, (Appendix table 22) and 0.9963, (Appendix table 23) respectively). For the mean and standard deviation data, the lowest correlation coefficient was found with the square-root transformed data in most cases (Appendix tables 24 and 25). Therefore, the square-root transformation was selected as the most satisfactory transformation (Appendix B p.251). Parametric analyses of the data were conducted after this transformation had been applied, while non-parametric tests were applied only to the untransformed data. I shall firstly deal with the non-parametric tests and then with the parametric tests. A flow chart showing the procedure I adopted in these analyses is given in figure 15.

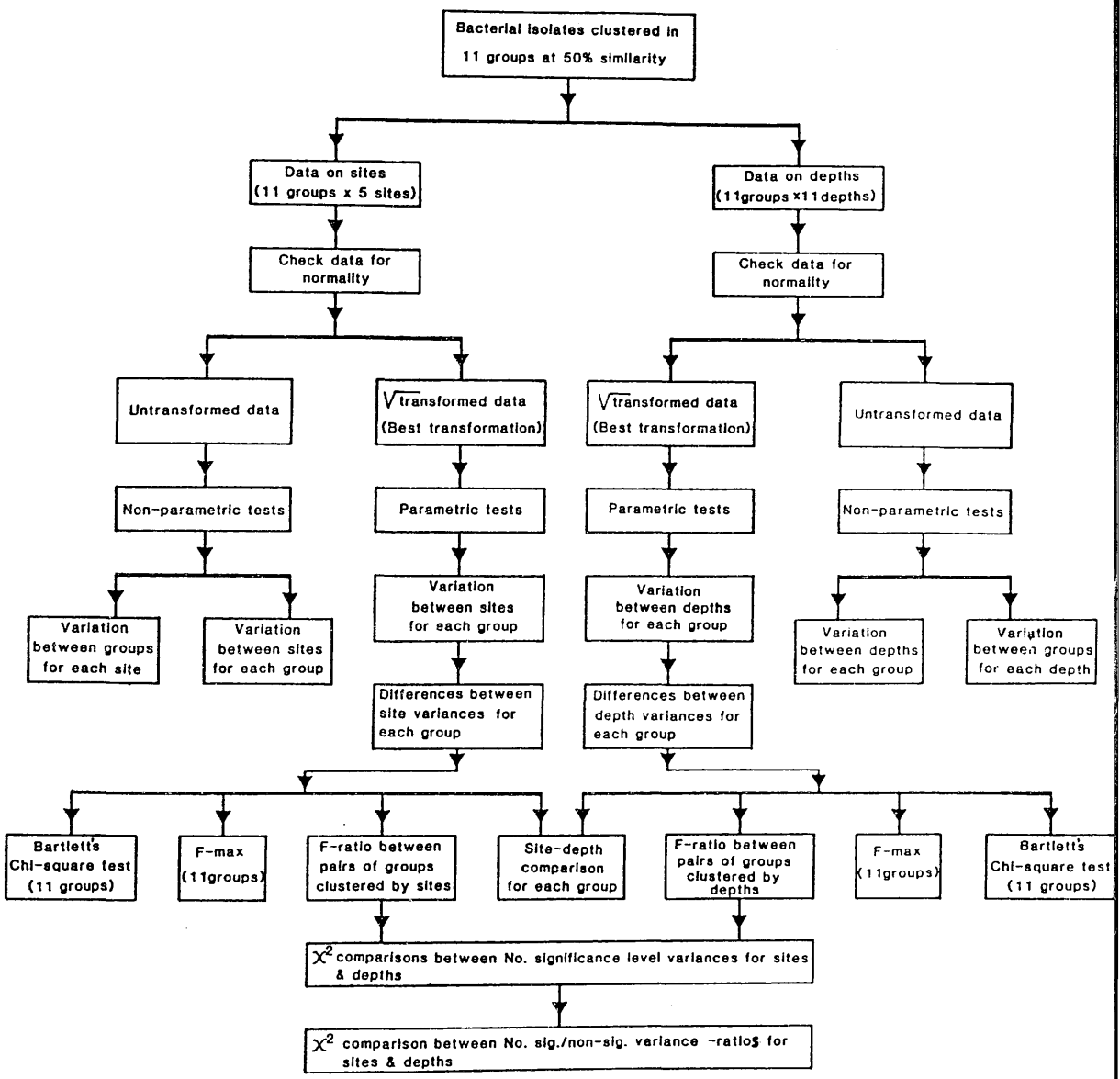


Figure 15

Flow chart outlining the statistical analyses applied to the data for the bacterial isolates clustered at 50% similarity level.

Non-parametric tests

(i)- **Number of isolates/group at each site** (Table 14)

The data in table 14 were analysed by a series of Chi-square tests to answer two questions. The first question was: is there any difference between the number of isolates at each site within a given group? In other words for group 1, are the numbers 10, 5, 25, 4 and 7 (Table 14 row 1) significantly different from each other? The results of these 11 Chi-square tests, one for each group, are shown in table 16 where it can be seen that all the Chi-square tests are highly significant. This means that the isolates in each of the 11 groups are not distributed randomly between the sites. The Chi-square values in table 16 show that the greatest variation between sites occurred in group 5 (X^2=102.9) and the least variation between sites occurred at group 3 (X^2=18.58). It is also interesting to note that some groups had no isolates at site 1 (e.g. groups 2, 3, 6 and 8), whereas other groups had a relatively large proportion of the isolates at site 1 (e.g. group 1, 10/51=20%; group4, 11/68=16%; group 9, 34/68=50%).

The second question was: is there any difference in the number of isolates between groups at each site? In other words for site 1 are the numbers 10, 0, 0, 11, ... 34, 3, 3 (Table 14 column 1) significantly different from each other? The results of these 5 Chi-square tests, one for each site, are shown in table 17 where it can be seen that all the Chi-square tests are highly significant. This means that the isolates at each of the sites are not distributed randomly between the groups. The Chi-square values in table 17 show that the greatest variation between groups occurred at site 2 (X^2=345.6) and the least variation between groups occurred at site 5 (X^2=77.02).

Group	Site					No. isolates in group	% of isolates in group	X^2	d.f.	P
	1	2	3	4	5					
1	10	5	25	4	7	51	6.0	28.90	4	P < 0.001
2	0	65	30	32	13	140	16.6	85.64	4	P < 0.001
3	0	12	5	15	6	38	4.5	18.58	4	P < 0.001
4	11	9	29	12	7	68	8.1	22.88	4	P < 0.001
5	7	92	22	43	37	201	23.8	102.9	4	P < 0.001
6	0	36	10	27	14	87	10.3	46.39	4	P < 0.001
7	6	4	0	4	20	34	4.0	34.82	4	P < 0.001
8	0	23	0	25	6	54	6.4	56.19	4	P < 0.001
9	34	6	9	6	13	68	8.1	40.68	4	P < 0.001
10	3	15	1	50	14	83	9.8	93.57	4	P < 0.001
11	3	2	1	13	0	19	2.3	29.16	4	P < 0.001

Table 16: Chi-square comparisons of the variation between sites for each group. Cluster analysis at 50% similarity level.

Site	Group											No. isolates at site	% of isolates at site	X^2	d.f.	P
	1	2	3	4	5	6	7	8	9	10	11					
1	10	0	0	11	7	0	6	0	34	3	3	74	8.8	146.6	10	P < 0.001
2	5	65	12	9	92	36	4	23	6	15	2	269	31.9	345.6	10	P < 0.001
3	25	30	5	29	22	10	0	0	9	1	1	132	15.7	122.8	10	P < 0.001
4	4	32	15	12	43	27	4	25	6	50	13	231	27.4	118.2	10	P < 0.001
5	7	13	6	7	37	14	20	6	13	14	0	137	16.3	77.02	10	P < 0.001

Table 17: Chi-square comparisons of the variation between groups for each site. Data as in table 16. Cluster analysis at 50% similarity level.

(ii)- **Number of isolates/group at each depth** (Table 15)

The data in table 15 were analysed by a series of Chi-square tests to answer a similar pair of questions. The first question was: is there any difference between the number of isolates at each depth within a given group? In other words, for group 1 are the numbers 19, 10, 10 , 0,..... 0, 3, 1 (Table 15 row 1) significantly different from each other? The results of these 11 Chi-square tests, one for each group, are shown in table 18 where it can be seen that all the Chi-square tests are highly significant. This means that the isolates in each of the 11 groups are not distributed randomly between the depths. The Chi-square values in table 18 show that the greatest variation between depths occurred at group 2 (X^2=202.2) and the least variation between depths occurred at group 3 (X^2=34.50). It is also interesting to note that some groups had no isolates at the sediment surface (0cm), (e.g. groups 7, 8 and 9), whereas other groups had a relatively large proportion of the isolates at the sediment (e.g. group 1, 19/51=37%; group 4, 20/68=29%).

The second question was: is there any difference in the number of isolates between groups at each depth? In other words, for surface sediment (0cm), are the numbers 19, 3, 2, ... 0, 1, 1 (Table 15 column 1) significantly different from each other? The results of these 11 Chi-square tests, one for each depth, are shown in table 19 where it can be seen that all the Chi-square tests are highly significant. This means that the isolates at each of the depths are not distributed randomly between the groups. The Chi-square values in table 19 show that the greatest variation between groups occurred at 11cm depth (X^2=214.0) and the least variation between groups occurred at 13cm depth (X^2=45.96).

Group	Depth (cm)											No. isolates in group	% of isolates in group	X^2	d.f.	P
	00.0	00.5	03.0	04.0	05.5	07.5	10.5	11.0	13.0	15.5	20.5					
1	19	10	10	0	1	6	0	1	0	3	1	51	6.0	80.99	10	P < 0.001
2	3	14	21	5	4	27	0	53	11	0	2	140	16.6	202.2	10	P < 0.001
3	2	5	2	5	2	3	0	10	9	0	0	38	4.5	34.50	10	P < 0.001
4	20	8	4	8	3	10	9	2	1	3	0	68	8,1	52.85	10	P < 0.001
5	6	14	24	8	37	16	23	26	14	18	15	201	23.8	42.31	10	P < 0.001
6	2	10	11	8	5	8	3	20	14	4	2	87	10.3	39.86	10	P < 0.001
7	0	23	8	0	2	0	0	1	0	0	0	34	4.0	159.0	10	P < 0.001
8	0	15	16	0	2	0	1	12	8	0	0	54	6.4	87.53	10	P < 0.001
9	0	31	17	1	6	9	0	2	0	2	0	68	8.1	154.1	10	P < 0.001
10	1	14	28	19	9	0	1	3	7	0	1	83	9.8	114.2	10	P < 0.001
11	1	0	3	6	0	0	0	2	7	0	0	19	2.3	38.94	10	P < 0.001

Table 18: Chi-square comparisons of the variation between depths for each group. Cluster analysis at 50% similarity level.

Depth	Group											No. isolates at depth	% of isolates at depth	X^2	d.f.	P
	1	2	3	4	5	6	7	8	9	10	11					
00.0	19	3	2	20	6	2	0	0	0	1	1	54	6.4	112.4	10	P < 0.001
00.5	10	14	5	8	14	10	23	15	31	14	0	144	17.1	53.96	10	P < 0.001
03.0	10	21	2	4	24	11	8	16	17	28	3	144	17.1	59.15	10	P < 0.001
04.0	0	5	5	8	8	8	0	0	1	19	6	60	7.1	56.86	10	P < 0.001
05.5	1	4	2	3	37	5	2	2	6	9	0	71	8.4	167.8	10	P < 0.001
07.5	6	27	3	10	16	8	0	0	9	0	0	79	9.4	98.28	10	P < 0.001
10.5	0	0	0	9	23	3	0	1	0	1	0	37	4.4	146.0	10	P < 0.001
11.0	1	53	10	2	26	20	1	12	2	3	2	132	15.7	214.0	10	P < 0.001
13.0	0	11	9	1	14	14	0	8	0	7	7	71	8.4	45.96	10	P < 0.001
15.5	3	0	0	3	18	4	0	0	2	0	0	30	3.6	103.8	10	P < 0.001
20.5	1	2	0	0	15	2	0	0	0	1	0	21	2.5	102.6	10	P < 0.001

Table 19: Chi-square comparisons of the variation between groups for each depth. Data as in table 18. Cluster analysis at 50% similarity level.

Parametric tests

(i)- Comparison of the number of isolates/group at each site and depth

The following parametric analyses were concerned with the variances in the number of isolates/group between sites or depths. It is important to note that the variance ratios used to analyse these variances are calculated from the larger variance/the smaller variance. A computer program "F-ratio" was developed to calculate the variance ratio test (Appendix table 26). The flow diagram used to develop the program is shown in appendix figure 7. An example of the calculation performed by the computer is given in appendix table 27.

Table 20A, shows the mean, standard deviation and the variance of the number of isolates/site for groups 1-11. The largest variance occurred in group 2 (7.546) and the smallest variance occurred in group 4 (1.100). The variances between sites for each group were analysed by Bartlett's and F-max tests in order to see whether they were homogeneous between the groups. In other words, for the 11 groups, are the numbers 1.371, 7.546, ... 5.272, 1.272 (Table 20A column 3) significantly different from each otner? The results of Bartlett's and F-max tests are shown in table 20B where it can be seen that with both tests, the difference between site variances for each group were not significant. This means that at each site, there is the same pattern of change in the number of isolates with depth (i.e. homogenous between sites with regard to depth distribution).

Similarly, table 21A shows the mean, standard deviation and the variance in the number of isolates/depth for groups 1-11. The largest variance occurred in group 2 (4.211) and the smallest variance occurred in group 11 (0.6153). The variances of depths for each group were analysed in the same way as with sites (Bartlett's and F-max tests) in order to determine the homogeneity of variances between

A

Group	Mean	Standard deviation	Variance	d.f.
1	3.099	1.171	1.371	4
2	4.740	2.747	7.546	4
3	2.615	1.256	1.578	4
4	3.636	1.049	1.100	4
5	5.964	2.533	6.416	4
6	3.808	2.061	4.248	4
7	2.405	1.376	1.893	4
8	2.772	2.126	4.520	4
9	3.546	1.382	1.910	4
10	3.589	2.296	5.272	4
11	1.812	1.128	1.272	4

B

Homogeneity of variance test	Test value	d.f.	P
Bartlett Chi-square	9.132	10	0.70 > P > 0.50
$F_{max} = \frac{\text{Large variance}}{\text{Small variance}}$	6.860	11,4	P > 0.05

Table 20: Table A: mean, standard deviation and variance of number of bacterial isolates/site for groups 1-11. Square-root (x + 0.5) transformed data from appendix table 16 p.267 . Cluster analysis at 50% similarity level. Table B: Bartlett's Chi-square and F_{max} tests (Sokal & Rohlf, 1981, Box 13.1, p. 404, 405) for the homogeneity of variance.

A

Group	Mean	Standard deviation	Variance	d.f.
1	1.919	1.264	1.598	10
2	3.066	2.052	4.211	10
3	1.795	0.8968	0.8043	10
4	2.369	1.085	1.177	10
5	4.223	1.018	1.036	10
6	2.754	0.9514	0.9052	10
7	1.411	1.327	1.761	10
8	1.890	1.421	2.019	10
9	2.058	1.640	2.690	10
10	2.395	1.594	2.541	10
11	1.292	0.7844	0.6153	10

B

Homogeneity of variance test	Test value	d.f.	P
Bartlett Chi-square	16.64	10	0.10 > P > 0.05
$F_{max} = \frac{\text{Large variance}}{\text{Small variance}}$	6.844	11,10	P > 0.05

Table 21: Table A: mean, standard deviation and variance of number of bacterial isolates/depth for groups 1-11. Square-root (x+0.5) transformed data from appendix table 17 p. 268. Cluster analysis at 50% similarity level. Table B: Bartlett's Chi-square and F_{max} tests (Sokal & Rohlf, 1981, Box 13.1, p. 404,405) for the homogeneity of variance.

groups. In other words for the 11 groups, are the numbers 1.598, 4.211, ... 2.541, 0.6153 (Table 21A, column 3) significantly different from each other ? The results of Bartlett's and F-max tests are shown in table 21B where it can be seen that with both tests, there were no significant differences between depth variances. This means that the depth variances for each group were homogeneous. In other words, if one select any sampling depth and looks at the variation in number of isolates between sites then it will be the same pattern of change at all depths.

(ii)- **Comparison of variances in the number of isolates/group between different groups: (a) for sites (b) for depths**

The variance in the number of isolates/group were compared beween different groups at all the sites. In other words in table 20A, the variance in the number of isolates in group 1 (1.371) was compared with the variance in the number of isolates in group 2 (7.546) and then the variance of group 1 (1.371) was compared with the variance of group 3 (1.578) and so on. These comparisons were carried out using the variance ratios for each pair of groups. For the above instance, the larger variance/the smaller variance; 7.546/1.371=5.504 and 1.578/1.371= 1.151.

Similarly, the variance in the number of isolates/group at all depths were compared between different groups. In other words in table 21A, the variance in the number of isolates in group 1 (1.598) was compared with the variance in the number of isolates in group 2 (4.211) and then the variance of group 1 (1.598) was compared with the variance of group 3 (0.8043) and so on. These comparisons were carried out using the variance ratios for each pairs of groups. For the above example, the larger variance/ the smaller variance; 4.211/1.598=2.635, 1.598/0.8043 =1.987. The results of all site and depth comparisons are

shown in table 22. This table consists of an upper and a lower table. The upper table shows variance ratios for the number of isolates/site along with letters showing the significance of these variance ratios, while the lower table shows variance ratios for the number of isolates/depth along with letters denoting levels of significance. With sites, there was only one significant variance ratio. This occurred between groups 4 and 2 (6.86). With depths however, there were ten significant differences between groups. These occurred between groups 4-2, 9-3, 10-3 and 8-11 (all $0.05>P>0.025$); 5-2, 6-2, 9-11 and 10-11 (all $0.025>P>0.01$); 3-2 ($0.01>P>0.001$) and 11-2 ($0.005>P>0.001$).

The number of letters showing the significance level; A, B, C,..... G, H were counted for both sites and depths. For example, as can be seen in the upper table 23, with sites there were no A letters, 25 B letters, 21 C letters and so on. Similarly, with depths, there were no A letters, 19 B letters, 17 C letters and so on. Chi-square tests were conducted to answer the question: is there any difference between the significance levels of variance ratios for the number of isolates/site and significance levels of variance ratios for the number of isolates/depth ? In other words, are the numbers 0, 25, 21,..... 0, 0 (upper table 23, row 1) significantly different from the numbers 0, 19, 17,..... 1, 1 (upper table 23, row 2) ? The results of the Chi-square test showed that there was no significant difference between sites and depths ($0.90>P>0.80$; upper table 23).

The total number of significant and non-significant variance ratios for sites and depths were also counted. With sites, there were 54 non-significant and only 1 significant variance ratio. With depths, there were 45 non-significant and 10 significant variance ratios. Chi-square tests were conducted in order to answer the question: is there

Table 22: Lower triangles: variance ratios between pairs of groups clustered by sites (i) and depths (ii) at 50% similarity level. Upper triangles: letters showing the significance level of the variances in the lower triangles.

Significance levels.

0.75 > P > 0.50 = A	0.05 > P > 0.025 = E
0.50 > P > 0.25 = B	0.025 > P > 0.01 = F
0.25 > P > 0.10 = C	0.01 > P > 0.005 = G
0.10 > P > 0.05 = D	0.005 > P > 0.001 = H

(i) Sites

Groups

	1	2	3	4	5	6	7	8	9	10	11
1		D	B	B	D	C	B	C	B	C	B
2	5.504		D	E*	B	B	C	B	C	B	D
3	1.151	4.782		B	C	C	B	C	B	C	B
4	1.246	6.86*	1.435		D	C	B	C	B	D	B
5	4.680	1.176	4.066	5.833		B	C	B	C	B	D
6	3.098	1.776	2.692	3.862	1.510		C	B	C	B	C
7	1.381	3.986	1.110	1.721	3.389	2.244		C	B	C	B
8	3.297	1.669	2.864	4.109	1.419	1.064	2.388		C	B	C
9	1.393	3.951	1.210	1.736	3.359	2.224	1.009	2.366		C	B
10	3.845	1.431	3.341	4.793	1.217	1.241	2.785	1.166	2.760		D
11	1.078	5.932	1.241	1.156	5.044	3.340	1.4882	3.553	1.502	4.145	

(ii) Depths

Groups

	1	2	3	4	5	6	7	8	9	10	11
1		D	C	B	B	C	B	B	C	C	D
2	2.635		G*	E*	F*	F*	D	C	C	C	H*
3	1.987	5.236*		B	B	B	C	D	E*	E*	B
4	1.358	3.578*	1.463		B	B	B	C	C	C	C
5	1.542	4.065*	1.288	1.136		B	C	C	D	D	C
6	1.765	4.652*	1.125	1.300	1.145		C	C	D	D	B
7	1.102	2.391	2.189	1.496	1.700	1.945		B	B	B	D
8	1.263	2.086	2.510	1.715	1.949	2.230	1.147		B	B	E*
9	1.683	1.565	3.345*	2.285	2.597	2.972	1.528	1.332		B	F*
10	1.590	1.657	3.159*	2.159	2.453	2.807	1.443	1.259	1.059		F*
11	2.597	6.844*	1.307	1.913	1.684	1.471	2.862	3.281*	4.372*	4.130*	

* Significant

	Probability (see legend)								X^2	d.f.	P
	A	B	C	D	E	F	G	H			
Sites	0	25	21	8	1	0	0	0	9.098	15	0.90>P>0.80
Depths	0	19	17	9	4	4	1	1			

	Total N.sig	Total sig.	X^2	d.f.	P
Sites	54	1	8.182	1	0.01>P>0.001
Depths	45	10			

Table 23: Upper table: Number of Chi-square comparisons at different significance levels (A,B,C,D,E,F,G,H) for sites and depths. Lower table: Chi-square comparisons between total number of significant and non-significant variance ratios for sites and depths (see table 22(i) and (ii)).

Significance levels.

0.75 > P > 0.50 = A	0.05 > P > 0.025 = E
0.50 > P > 0.25 = B	0.025 > P > 0.01 = F
0.25 > P > 0.10 = C	0.01 > P > 0.005 = G
0.10 > P > 0.05 = D	0.005 > P > 0.001 = H

any difference in the significant and non-significant variance ratios between sites and depths ? In other words, are the numbers 54 and 1 (lower table 23, row 1) significantly different from the numbers 45 and 10 (lower table 23, row 2) ? As expected, the results of Chi-square showed that there were significant differences between sites and depths with regard to the total number of significant and non-significant variance ratio tests (0.01>P>0.001; lower table 23).

(iii)- **Comparison of variances in the number of isolates/site with variances in the number of isolates/depth**

The variance ratio test was used to compare the variance in the number of isolates/site with the variance in the number of isolates/depth for each of the 11 groups clustered at 50% similarity level. Variances were calculated on square-root transformed data from appendix tables 16 and 17. The results of this comparison are shown in table 24. It is important to note that the variance ratio is equal to the larger variance/ the smaller variance and as shown in table 24, in some cases (e.g. group 2), the larger variance (7.546) is found in the site column while in other cases (e.g. group 1), it occurs in the depth column (4.211). Column 2 shows the site variances and column 4 shows the depth variances. In each of these columns there were 11 values. In the site column (column 2), there were eight groups with larger variances (2, 3, 5, 6, 7, 8, 10 and 11) and three groups with smaller variances (1, 4 and 9). When the variance ratios were calculated for the site and depth variances (Table 24), the results showed that groups 5 (6.193) and 6 (4.693) had the largest variance ratio and therefore they were significant (0.01>P>0.005; 0.025>P>0.01 respectively).

In the depth column (column 4), there were three groups with larger variances (1,4 and 9) and eight groups with smaller variances

Group	No. isolates/site		No. isolates/depth		F-ratio	d.f.		P
	Variance	d.f.	Variance	d.f.		Numerator	Denominator	
1	1.371	4	1.598	10	1.166	10	4	0.50 > P > 0.25
2	7.546	4	4.211	10	1.792	4	10	0.25 > P > 0.10
3	1.578	4	0.8043	10	1.962	4	10	0.25 > P > 0.10
4	1.100	4	1.177	10	1.070	10	4	0.75 > P > 0.50
5	6.416	4	1.036	10	6.193	4	10	0.01 > P > 0.005
6	4.248	4	0.9052	10	4.693	4	10	0.025 > P > 0.01
7	1.893	4	1.761	10	1.075	4	10	0.50 > P > 0.25
8	4.520	4	2.019	10	2.239	4	10	0.25 > P > 0.10
9	1.910	4	2.690	10	1.408	10	4	0.50 > P > 0.25
10	5.272	4	2.541	10	2.075	4	10	0.25 > P > 0.10
11	1.272	4	0.6153	10	2.067	4	10	0.25 > P > 0.10

Table 24: F-ratios comparing variances of No. isolates/site with variances of No. isolates/depth, for each of the 11 groups. Variances calculated on square-root (x + 0.5) transformed data from appendix tables 16 and 17. Groups obtained from cluster analysis at 50% similarity level. F-ratio = large variance/small variance. In some cases above (e.g. group 2), the larger variance is found in the site column while in others (e.g. group 1), it occurs in the depth column.

(2, 3, 5, 6, 7, 8, 10 and 11). According to the variance ratio values all these groups were not significant. Table 25 summarises the above findings.

B- **Clusters formed at 72% similarity level**

The 72% similarity level data are shown as original data in appendix table 28 and as a dendrogram in figure 16. The details of the clusters and the sites and depths from which the isolates come are shown in table 26. For example, in cluster 1, there were 11 isolates from site 1 and 5 isolates from site 2 and so on. Another example, 78% of the isolates in cluster 1 were obtained from sediment while 22% were from faecal pellets. These information were obtained from appendix table 28.

I shall now describe each of the clusters in the dendrogram using the information in table 26 and figure 16.

Cluster 1 was formed at 79.4% S. It contained 32 isolates and the reference culture of Planococcus citreus (NCMB 1493). The isolates in this group came from different sites and depths (site 1 (11 isolates) at depths of 3cm, 5.5cm and 7.5cm; site 2 (5 isolates) at depths of 0.5cm, 11cm and 20.5cm; site 3 (5 isolates) at a depth of 7.5cm; site 4 (4 isolates) at depths of 3cm and 15.5cm; and site 5 (7 isolates) at a depth of 0.5cm). 78% of the isolates in this group were obtained from sediment and 22% came from faecal pellets.

Cluster 2 contained 19 isolates formed at 94.6% S. None of the reference cultures were clustered in this group. The most important aspect of this cluster was that all the isolates came from the surface sediment (0cm) of site 3.

Cluster 3 contained 41 isolates clustered at 73.4% S. None of

	Site			Depth		
	Sig.	N. sig.	Total	Sig.	N. sig.	Total
Groups with larger variances	5,6	2,3,7, 8,10,11	-	-	1,4,9	-
Number of larger variances	2	6	8	0	3	3
Groups with smaller variances	-	1,4,9	-	-	2,3,5, 6,7,8, 10,11	-
Number of smaller variances	0	3	3	0	8	8

Table 25: Summary of table 24 representing the significant and non-significant groups and number of larger and smaller variances of No. isolates/site/depth for each of the 11 groups clustered at 50% similarity level.

Table 26

Details of clusters formed at 72% S. The similarity level, number of isolates in each cluster, site and depth from where isolates were obtained, the percentage of isolates from each source and reference cultures clustered with groups are shown. Three sources were used; S=sediment, B=burrow linings and F=faecal pellets. Numbers in parentheses are numbers of isolates clustered from sites (e.g. in cluster 1 there were 11 isolates from site 1, 5 isolates from site 2).

Cluster	S%	No. isolates	Site	Depth (cm)	Source	Reference culture
1	79.4	32	1(11) 2(5) 3(5) 4(4) 5(7)	3, 5.5, 7.5 0.5, 11, 20.5 7.5 3, 15.5 0.5	78%S 22%F	*Planococcus citreus*
2	94.6	19	3(19)	0	100%S	-
3	73.4	41	2(5) 3(29) 4(5) 5(2)	0.5, 3, 5.5, 11 0, 7.5 3, 4, 13 0.5	27%S 7%B 66%F	-
4	82.8	52	2(36) 3(1) 4(6) 5(9)	0.5, 3, 11, 20.5 0 0.5, 4, 13 0.5, 3, 5.5	35%S 35%B 30%F	-
5	88.4	47	2(24) 4(21) 5(2)	0.5, 3, 11, 20.5 0.5, 3, 4, 13 0.5, 3	57%S 9%B 34%F	*Pseudomonas fluorescens* *Alteromonas haloplanktis*

Table 26 cont'd.

Cluster	S%	No. isolates	Site	Depth (cm)	Source	Reference culture
6	80.6	24	2(9)	3, 11	50%S	-
			3(2)	0, 7.5	17%B	
			4(7)	0.5, 4, 13	33%F	
			5(6)	0.5, 5.5		
7	91.4	14	2(3)	11	7%S	-
			3(3)	0, 7.5	86%B	
			4(8)	4, 13	7%F	
8	85.6	23	1(4)	0.5, 3, 5.5, 15.5		
			2(5)	0.5, 5.5, 11	82%S	*Micrococcus* sp.
			3(4)	0, 7.5	9%B	*Micrococcus* sp.
			4(3)	0.5, 3,	9%F	Coryneform
			5(7)	0.5, 3, 5.5, 10.5		
9	87.6	45	1(7)	10.5	80%S	
			2(4)	0, 10.5, 15.5	4%B	*Pediococcus cerevisiae*
			3(25)	0, 7.5	16%F	
			4(9)	4, 13,		
10	87.3	59	1(1)	15.5		-
			2(35)	0.5, 3, 5.5, 11, 15.5, 20.5	75%S	
			3(5)	0, 7.5	12%B	
			4(11)	0.5, 4, 10.5, 13	13%F	
			5(7)	3, 5.5, 10.5		

Table 26 cont'd.

Cluster	S%	No. isolates	Site	Depth (cm)	Source	Reference culture
11	88.0	11	1(2)	0.5	64%S	*Cytophaga lytica*
			2(1)	5.5		
			3(7)	0, 7.5	36%F	
			4(1)	0.5		
12	72.4	107	1(4)	0.5, 10.5, 20.5	70%S	-
			2(51)	5.5, 10.5, 11, 15.5,20.5		
			3(8)	0, 7.5	22%B	
			4(24)	0.5, 3, 4, 10.5, 13	8%F	
			5(20)	0.5, 3, 5.5, 10.5, 15.5		
13	93.6	24	2(5)	5.5, 10.5, 11	83%S	*Serratia marcescens*
			3(2)	0, 7.5		
			4(7)	3	4%B	
			5(10)	0.5, 3, 5.5, 10.5, 15.5	13%F	
14	75.0	87	2(36)	0.5, 3, 5.5, 10.5, 11, 15.5, 20.5	55%S	*Aeromonas hydrophila*
			3(10)	0, 7.5	38%B	
			4(27)	0.5, 3, 4, 5.5, 11, 13	7%F	
			5(14)	0.5, 3, 5.5		
15	78.5	34	1(6)	0.5, 5.5	97%S	*Vibrio fischeri*
			2(4)	0.5, 3, 11		
			4(4)	0.5, 3	3%F	
			5(20)	0.5, 3		

Table 26 cont'd.

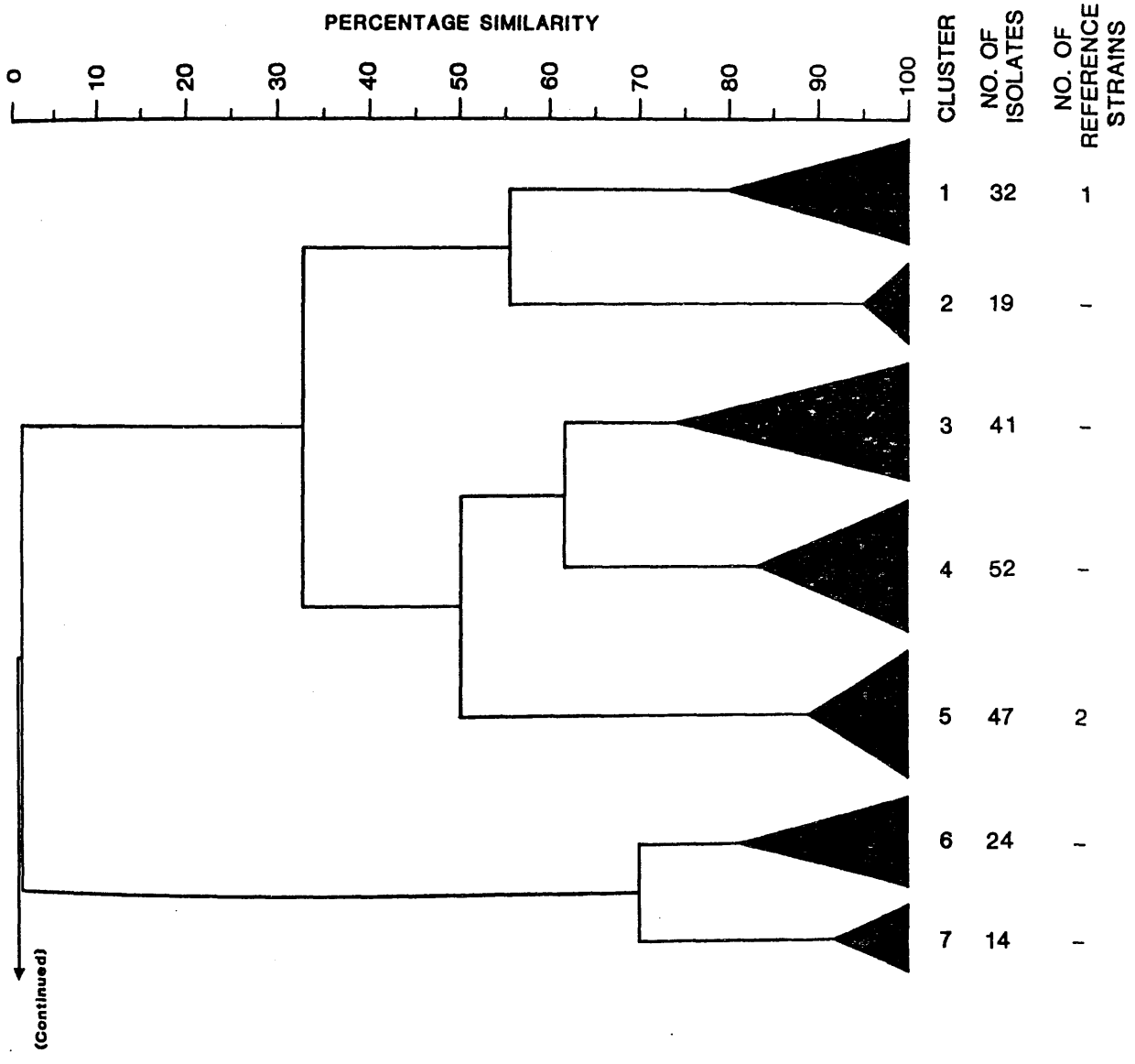
Cluster	S%	No. isolates	Site	Depth (cm)	Source	Reference culture
16	84.1	54	2(23)	0.5, 3, 10.5, 11	72%S	-
			4(25)	0.5, 3, 13	17%B	
			5(6)	0.5, 3, 5.5	11%F	
17	85.2	27	1(27)	0.5, 3	93%S	Pseudomonas cleovorans
			2(4)	0.5, 3	7%F	
			3(2)	7.5		
			4(2)	3		
			5(2)	0.5		
18	79.6	41	1(17)	0.5, 3, 5.5, 15.5	78%S	Coryneform
			2(2)	11	22%F	Pseudomonas
			3(7)	7.5		Proteus vulgaris
			4(4)	0.5, 3, 4		
			5(11)	0.5, 3, 5.5		
19	90.8	30	2(8)	3, 11	90%S	-
			4(21)	0.5, 3, 4, 13	10%B	
			5(1)	0.5		
20	86.3	53	1(3)	0.5	72%S	Planococcus sp.
			2(7)	0.5, 10.5, 11, 20.5	24%B	
			3(1)	0	4%F	
			4(29)	3, 4, 13		
			5(13)	0.5, 5.5		
21	82.1	19	1(3)	3	32%S	-
			2(2)	11	63%B	
			3(1)	0	5%F	
			4(13)	4, 13		

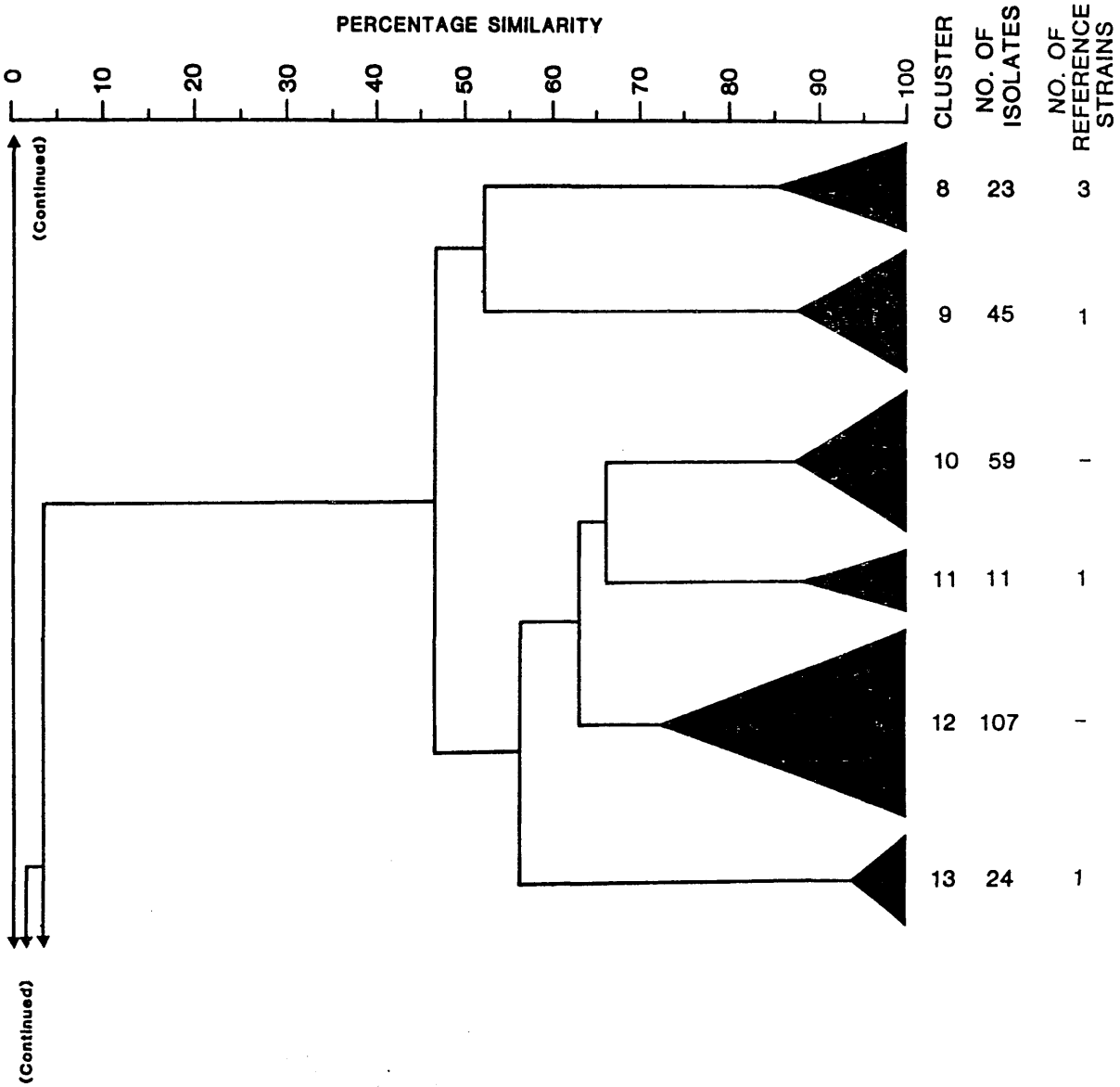
Figure 16

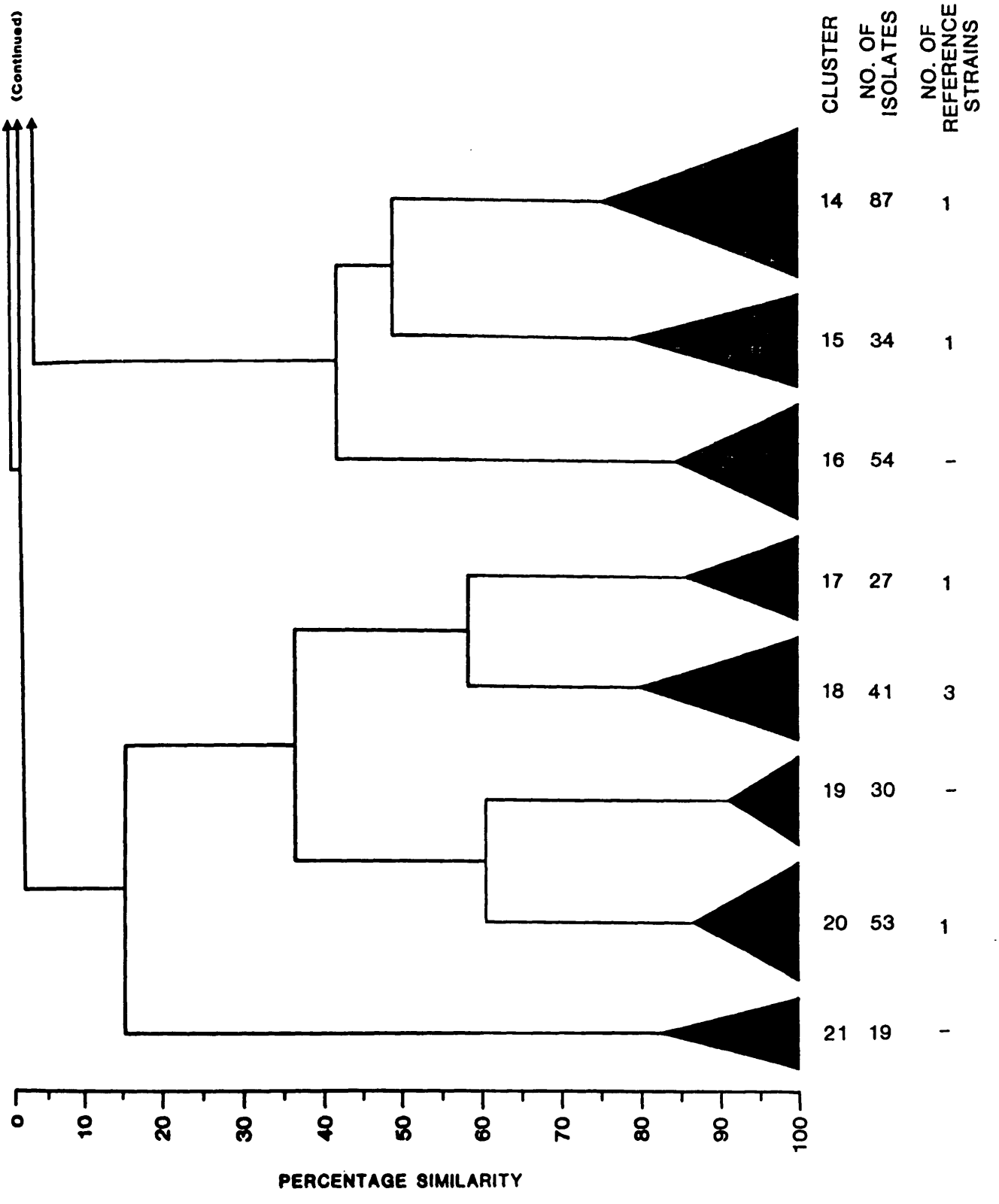
Simplified dendrogram (continued on next 3 pages) showing the relationship between clusters of deep-sea bacteria based on the Euclidean distance coefficient and Ward's method of clustering. Distance has been transformed into percentage similarity. Numbers of isolates in each cluster are shown (more details in appendix table 28). Reference cultures are also shown and their names are listed in table 45.

Figure 16

Simplified dendrogram (continued on next 3 pages) showing the relationship between clusters of deep-sea bacteria based on the Euclidean distance coefficient and Ward's method of clustering. Distance has been transformed into percentage similarity. Numbers of isolates in each cluster are shown (more details in appendix table 28). Reference cultures are also shown and their names are listed in table 45.


PERCENTAGE SIMILARITY
0
10
20
30
40
50
60
70
80
90
100
CLUSTER
NO. OF ISOLATES
NO. OF REFERENCE STRAINS
1 32 1
2 19 –
3 41 –
4 52 –
5 47 2
6 24 –
7 14 –
(Continued)


PERCENTAGE SIMILARITY
0
10
20
30
40
50
60
70
80
90
100
CLUSTER
NO. OF ISOLATES
NO. OF REFERENCE STRAINS
(Continued)
8 23 3
9 45 1
10 59 -
11 11 1
12 107 -
13 24 1
(Continued)


(Continued)
CLUSTER
NO. OF ISOLATES
NO. OF REFERENCE STRAINS
14 87 1
15 34 1
16 54 -
17 27 1
18 41 3
19 30 -
20 53 1
21 19 -
0 10 20 30 40 50 60 70 80 90 100
PERCENTAGE SIMILARITY

the reference cultures were clustered in this group. Most of these isolates came from site 3 (29 isolates) at depths of 0cm and 7.5cm. This was followed by site 2 (5 isolates) at depths of 0.5cm, 3cm, 5.5cm and 11cm; site 4 (5 isolates) at depths of 3cm, 4cm and 13cm; and site 5 (2 isolates) at a depth of 0.5cm. Most of the isolates (66%) in this group were obtained from faecal pellets, followed by 27% from sediment and only 7% from burrow linings.

Cluster 4 contained 52 isolates clustered together at 82.8% S. None of the reference cultures were clustered in this group. The isolates in this group came mainly from site 2 (36 isolates) at depths of 0.5cm, 3cm, 11cm and 20.5cm; followed by site 5 (9 isolates) at depths of 0.5cm, 3cm and 5.5cm; and then site 4 (6 isolates) at depths of 0.5cm, 4cm and 13cm; and site 3 (1 isolate) at surface sediment (0cm). 35% of the isolates in this group were obtained from sediment, 35% from burrow linings and 30% from faecal pellets.

Cluster 5 was formed at 88.4% S. It contained 47 isolates and the reference cultures of Pseudomonas fluorescens (NCMB 9046) and Alteromonas haloplanktis (NCMB 19). The isolates in this group came from site 2 (24 isolates) at depths of 0.5cm, 3cm, 11cm and 20.5cm; site 4 (21 isolates) at depths of 0.5cm, 3cm, 4cm and 13cm; and site 5 (2 isolates) at depths of 0.5cm and 3cm. Most of the isolates (57%) in this group were obtained from sediment, followed by 34% from faecal pellets and 9% from burrow linings.

Cluster 6 contained 24 isolates clustered together at 80.6% S. None of the reference cultures were clustered with these isolates. The isolates in this group came from site 2 (9 isolates) at depths of 3cm and 11cm; site 3 (2 isolates) at depths of 0cm and 7.5cm; site 4 (7 isolates) at depths of 0.5cm, 4cm and 13cm; and site 5 (6 isolates)

at depths of 0.5cm and 5.5cm. 50% of the isolates were obtained from sediment samples and 33% were from faecal pellets. Only 17% were obtained from burrow linings.

Cluster 7 was formed at 91.4% S, and contained 14 isolates. None of the reference cultures clustered with it. The isolates in this group came from site 2 (3 isolates) at a depth of 11.0cm, site 3 (3 isolates) at depths of 0cm and 7.5cm, and site 4 (8 isolates) at depths of 4cm and 13.0cm. Most of the isolates (86%)in this group were obtained from burrow linings. Only 7% came from faecal pellets and the same percent came from burrow linings.

Cluster 8 was formed at 85.6% S. It contained 23 isolates and the reference cultures of Micrococcus sp. (two strains, NCMB 13, NCMB 365) and Coryneform strain NCMB 8. The isolates in this group came from all sites (site 1 (4 isolates) at depths of 0.5cm, 3cm, 5.5cm and 15.5cm; site 2 (5 isolates) at depths of 0.5cm, 5.5c and 11cm; site 3 (4 isolates) at depths of 0cm and 7.5cm; site 4 (3 isolates) at depths of 0.5cm and 3cm; and site 5 (7 isolates) at depths of 0.5cm, 3cm, 5.5cm and 10.5cm). 82% of the isolates in this group were recovered from sediment samples, while only 9% were recovered from burrow linings and the same percent from faecal pellets.

Cluster 9 was formed at 87.6% S. It contained 45 isolates and the reference culture of Pediococcus cerevisiae (NCTC 10331). The isolates in this group came from site 1 (7 isolates) at a depth of 10.5cm; site 2 (4 isolates) at depths of 0cm, 10.5cm and 15.5cm; site 3 (25 isolates) at depths 0cm and 7.5cm and site 4 (9 isolates) at depths of 4cm and 13cm. 80% of the isolates were obtained from sediment samples. 4% and 16% of the isolates were recovered from burrow linings and faecal pellets respectively.

Cluster 10 comprised 59 isolates clustered at 87.3% S. No reference cultures clustered with it. Most of the isolates in this group came from site 2 (35 isolates) at depth range 0.5cm-20.5cm. In order of decreasing abundance these were followed by site 4 (11 isolates) at depths of 0.5cm, 4cm, 10.5cm and 13cm; site 5 (7 isolates) at depths of 3cm, 5.5cm and 10.5cm; site 3 (5 isolates) at depths of 0cm and 7.5cm; and site 1 (1 isolate) at a depth of 15.5cm. Most of these isolates (75%) were obtained from sediment samples. 12% of the isolates were recovered from burrow linings and 13% from faecal pellets.

Cluster 11 was formed at 88% S. It contained 11 isolates and the reference culture of Cytophaga lytica (NCMB 292). Most of these isolates came from site 3 (7 isolates) at depths of 0cm and 7.5cm; followed by site 1 (2 isolates) at a depth of 0.5cm; and site 2 and 4 (both 1 isolate) at depths of 5.5cm and 0.5cm respectively. These isolates were obtained from sediment samples (64%) and faecal pellets (36%).

Cluster 12 comprised 107 isolates clustered together at 72.4% S. No reference cultures were clustered with this group. The isolates came from different sites and depths. Most isolates came from site 2 (51 isolates) at depth range of 5.5cm-20.5cm; followed by site 4 (24 isolates) at depth range of 0.5cm-13cm; and then site 5 (20 isolates) at depth range of 0.5cm-15.5cm. Other isolates from site 3 (8 isolates) at 0cm and 7.5cm; and site 1 (4 isolates) at 0.5cm, 10.5cm and 20.5cm were also clustered in this group. The isolates in this group were obtained from sediment samples (70%), burrow linings (22%) and faecal pellets (8%).

Cluster 13 was formed at 93.6% S. It contained 24 isolates

and the reference culture of Serratia marcescens (NCIB 2847). The isolates in this group came from site 2 (5 isolates) at depths of 5.5cm, 10.5cm and 11cm; site 3 (2 isolates) at depths of 0cm and 7.5cm; site 4 (7 isolates) at a depth of 3cm; and site 5 (10 isolates) at depth range 0.5cm-15.5cm. The isolates in this group were recovered from sediment samples (83%), followed by faecal pellets (13%) and then burrow linings (4%).

Cluster 14 was formed at 75% S. It comprised 87 isolates and the reference culture of Aeromonas hydrophila (NCIB 9240). The isolates in this group came from 4 sites; site 2 (36 isolates) at depth range 0.5cm-20.5cm; site 3 (10 isolates) at depths of 0cm and 7.5cm; site 4 (27 isolates) at depth range of 0.5cm-13.0cm; and site 5 (14 isolates) at depths of 0.5cm, 3cm and 5.5cm. 55% of the isolates in this group were obtained from sediment samples, while 38% and 7% were obtained from burrow linings and faecal pellets respectively.

Cluster 15 was formed at 78.5% S. It contained 34 isolates and the reference culture of Vibrio fischeri (NCMB 1274). Most of the isolates in this group came from site 5 (20 isolates) at depths of 0.5cm and 3cm; followed by site 1 (6 isolates) at depths of 0.5cm and 5.5cm, and then site 2 (4 isolates) at depths of 0.5cm, 3cm and 11cm; and then site 4 (4 isolates) at depths of 0.5cm and 3cm. Almost all the isolates (97%) in this group were obtained from sediment samples. Only one isolate (3%) came from faecal pellets.

Cluster 16 contained 54 isolates clustered together at 84.1% S. This group contained none of the reference cultures. The isolates in this group came from 3 sites. Most of the isolates came from sites 2 and 4. Site 2 (23 isolates) came from depths of 0.5cm, 3cm, 10.5cm and 11cm; site 4 (25 isolates) from depths of 0.5cm, 3cm and 13cm; and

site 5 (6 isolates) from depths of 0.5cm, 3cm and 5.5cm. A total number of 39 isolates (72%) were obtained from sediment samples. In addition, 9 (17%) and 6 (11%) isolates were obtained from burrow linings and faecal pellets respectively.

Cluster 17 was formed at 85.2% S. It contained 27 isolates and the reference culture of Pseudomonas cleovorans (NCIB 6576). The isolates in this group came mainly from site 1 (27 isolates) at depths of 0.5cm and 3cm; followed by site 2 (4 isolates) at the same depths, then sites 3, 4 and 5 (all 2 isolates) at 7.5cm, 3cm and 0.5cm depth respectively. Almost all the isolates (93%) were obtained from sediment samples, while only 7% came from faecal pellets.

Cluster 18 was formed at 79.6% S. It comprised 41 isolates and the reference cultures of Coryneform strain (NCMB 35), Pseudomonas sp. (NCMB 320) and Proteus vulgaris (NCIB 4175). The isolates came mainly from site 1 (17 isolates) at depths of 0.5cm, 3cm, 5.5cm and 15.5cm; followed by site 5 (11 isolates) at depths of 0.5cm, 3cm and 5.5cm; site 3 (7 isolates) at a depth of 7.5cm, site 4 (4 isolates) at depths of 0.5cm, 3cm and 4cm; and site 2 (2 isolates) at a depth of 11.0cm. The isolates (78%) from sites 1, 4 and 5 were obtained from sediment samples while the isolates (22%) recovered from sites 2 and 3 were from faecal pellets.

Cluster 19 contained 30 isolates clustered together at 90.8% S. This group contained none of the reference cultures. The isolates in this group came mainly from site 4 (21 isolates) at depths of 0.5cm, 3cm, 4cm and 13cm; and site 2 (8 isolates) at depths of 3cm and 11cm. Only 1 isolate came from site 5 and this was from 0.5cm depth. 90% of these isolates were obtained from sediment samples, while 3 isolates (10%) came from burrow linings.

Cluster 20 was formed at 86.3% S. It comprised 53 isolates and the reference culture of Planococcus sp. (NCMB 628). Most of the isolates in this group came from site 4 (29 isolates) at depths of 3cm, 4cm and 13cm; site 5 (13 isolates) at depths of 0.5cm, and 5.5cm. 7 isolates were recovered from site 2 at depths of 0.5cm, 10.5cm, 11cm and 20.5cm. A few isolates came from site 1 (3 isolates) at a depth of 0.5cm. 1 isolate from site 3 at a depth of 0cm were also clustered in this group. Most isolates in this group were obtained from sediment samples (72%) although some came from burrow linings (24%) and faecal pellets (4%).

Cluster 21 contained 19 isolates clustered together at 82.1% S. This group contained none of the reference cultures. The majority of the isolates in this group came from site 4 (13 isolates) at depths of 4cm and 13cm. 3 isolates were also collected from site 1 at a depth of 3cm, 2 isolates from site 2 at a depth of 11cm, and 1 isolate from site 3 at a depth of 0cm. In this group, 12 (63%) out of 19 isolates were obtained from burrow linings while 6 (32%) and 1 (5%) isolate were recovered from sediment samples and faecal pellets respectively.

The results of this cluster analysis showed that at 72% S or above, 11 of the reference cultures were clustered together in a separate group. These cultures are Morexalla (NCMB 308), Klebsiella pneumonia (3 strains NCIB 8805, 8806, 9261), Bacillus megaterium (NCIB 8508), Bacillus cereus, Corynebacterium xerosis (NCIB 9255), Acinetobacter calcoaceticus (NCIB 8250), Micrococcus luteus (NCTC 2665), Staphylococcus aureus (NCTC 6571) and Staphylococcus albus.

Having described each of the clusters in turn, I shall now describe the overall structure of the cluster dendrogram and the similarity level at which the clusters joined.

Cluster 1 was joined with cluster 2 at 55.2% S. Cluster 3 was joined with cluster 4 at 61.3% S. Cluster 5 was connected with clusters 3 and 4 at 49.8% S and these in turn joined up with clusters 1 and 2 at 32.4% S. Clusters 6 and 7 were joined at 69.7% S and these two clusters in turn joined up with clusters 1 to 5 at 0.45% S.

Cluster 8 was joined with cluster 9 at 52.2% S. Cluster 10 was connected with cluster 11 at 66.2% S. Cluster 12 was joined with clusters 10 and 11 at 63.1% S and these clusters (10, 11, 12) in turn connected up with cluster 13 at 56.3% S. These four clusters (10, 11, 12, 13) joined with clusters 8 and 9 at 46.7% S.

Cluster 14 was joined with cluster 15 at 48.7% S and these two clusters joined cluster 16 at 41.6% S. These three clusters (14, 15, 16) in turn joined up with clusters (8, 9, 10, 11, 12, 13) at 3.6% S. Cluster 17 was joined to cluster 18 at 58.2% S. Cluster 19 was joined to cluster 20 at 60.4% S. These four clusters were connected up with cluster 21 at 15.1% S.

Clusters 17 to 21 were joined to clusters 8 to 16 at 1.55% S and these in turn joined up with clusters 1 to 7 at 0.28% S.

3- Statistical analysis of clusters formed at 72% similarity level

The 843 isolates obtained from the different sites and different depths were classified into 21 groups at 72% similarity level. Of these, 561 were obtained from sediment, 143 from burrow linings and 139 from faecal pellets. Sections A and B (p. 113-133) deal with the 561 isolates from sediment. Section C (p. 133-137) deals with comparisons between the 561, 143 and 139 isolates from the three sources.

The 561 isolates are shown in table 27 and these range from 1 in group 7 to 75 in group 12. The groups were then further subdivided by distinguishing the number of isolates at each site and the number of isolates at each depth. For successive groups in turn these data are shown in table 28 for the sites and table 29 for the depths. For example, of the 25 isolates in group 1, 10 occurred at site 1 (all depths combined), 4 at site 2 (all depths combined), and so on to site 5 where 7 isolates occurred. Similarly, of the 25 isolates in group 1, none occurred at the surface sediment, 10 occurred at 0.5cm (all sites combined), 10 occurred at 3cm (all sites combined), and so on to depth 20.5cm. The results in this analysis are divided into two parts.

(A) Cluster by sites. (B) Cluster by depths.

A- Cluster by sites

Statistical analyses were carried out on the data to determine three main factors:

i) Whether there is a significant variation in the number of isolates between sites for each group.

ii) Whether there is a significant variation in the number of isolates between groups for each site.

iii) Whether there is a correlation in the number of isolates between the five sites.

Group	Total no. isolates in group
1	25
2	19
3	11
4	18
5	27
6	12
7	1
8	19
9	36
10	44
11	7
12	75
13	20
14	49
15	33
16	39
17	23
18	32
19	27
20	38
21	6

Table 27

Total number of isolates/group clustered at 72% similarity level. 561 isolates from sediment.

Group	Site					Total
	1	2	3	4	5	
1	10	4	0	4	7	25
2	0	0	19	0	0	19
3	0	3	2	4	2	11
4	0	4	1	4	9	18
5	0	6	0	19	2	27
6	0	2	1	3	6	12
7	0	0	1	0	0	1
8	4	3	2	3	7	19
9	7	4	17	8	0	36
10	1	28	1	7	7	44
11	2	1	3	1	0	7
12	4	35	1	15	20	75
13	0	2	1	7	10	20
14	0	17	2	16	14	49
15	6	3	0	4	20	33
16	0	11	0	22	6	39
17	17	4	0	2	0	23
18	17	0	0	4	11	32
19	0	7	0	19	1	27
20	3	5	1	11	18	38
21	3	1	1	1	0	6

Table 28 Number of bacterial isolates/group at each site (e.g. no. isolates/group/site). Cluster analysis at 72% similarity level. This data is also presented in figure 19. 561 isolates from sediment.

Group	Depth (cm)											Total
	0.0	0.5	3.0	4.0	5.5	7.5	10.5	11.0	13.0	15.5	20.5	
1	0	10	10	0	1	0	0	0	0	3	1	25
2	19	0	0	0	0	0	0	0	0	0	0	19
3	2	3	3	2	1	0	0	0	0	0	0	11
4	1	4	5	0	3	0	0	1	3	0	1	18
5	0	7	13	0	0	0	0	0	6	0	1	27
6	1	5	2	1	2	0	0	0	1	0	0	12
7	1	0	0	0	0	0	0	0	0	0	0	1
8	2	8	4	0	3	0	1	0	0	1	0	19
9	18	0	0	8	0	0	8	0	0	2	0	36
10	1	4	4	3	11	0	5	0	2	8	6	44
11	3	3	0	0	1	0	0	0	0	0	0	7
12	1	5	12	3	21	0	14	0	1	9	9	75
13	1	2	8	0	4	0	4	0	0	1	0	20
14	4	9	11	3	5	0	3	0	8	4	2	49
15	0	8	8	0	17	0	0	0	0	0	0	33
16	0	15	16	0	2	0	1	0	5	0	0	39
17	0	14	9	0	0	0	0	0	0	0	0	23
18	0	17	6	1	6	0	0	0	0	2	0	32
19	0	4	21	0	0	0	0	0	2	0	0	27
20	1	10	7	5	9	0	1	0	4	0	1	38
21	1	0	3	1	0	0	0	1	0	0	0	6

Table 29 Number of isolates/group at each depth (e.g. no. isolates/group/depth). Cluster analysis at 72% similarity level. 561 isolates from sediment.

(i)- **Variation in the number of isolates between sites for each group**

The data in table 28 were analysed by a series of Chi-square tests to answer the question: is there any difference between the number of isolates at each site within a given group ? In other words for group 1, are the numbers 10, 4, 0, 4 and 7 (Table 28, row 1) significantly different from each other ? The results of these 21 Chi-square tests, one for each group, are shown in table 30 where it can be seen that a highly significant variation between sites was found with most of the groups (15 out of 21 groups). However, a non-significant variation was found between sites in groups 6 ($0.10>P>0.05$) and 3, 7, 8, 11 and 21 (all $0.50>P>0.30$).

(ii) **Variation in the number of isolates between groups for each site**

The data in table 28 were analysed by a series of Chi-square tests to answer the question: is there any difference in the number of isolates between groups at each site ? In other words for site 1, are the numbers 10, 0, 0, ... 0, 3, 3 (Table 28, column 1) significantly different from each other ? The results of these 5 Chi-square tests, one for each site, are shown in table 31 where it can be seen that at all sites a highly significant variation was found between groups ($P<0.001$).

(iii) **Comparison of the number of isolates between sampling sites**

The difference in the number of isolates/group between different sites was studied. In other words in table 28, the number of isolates in each group at site 1 (10, 0, 0, ... 3, 3) was compared with the number of isolates in each group at site 2 (4, 0, 3, 4, ... 5, 1) and then the number of isolates in each group at site 1 was compared with the number of isolates in each group at site

Group	Site 1	2	3	4	5	Total	X^2	d.f.	P
1	10	4	0	4	7	25	11.20	4	0.05 > P > 0.02
2	0	0	19	0	0	19	76.00	4	P < 0.001
3	0	3	2	4	2	11	4.000	4	0.50 > P > 0.30
4	0	4	1	4	9	18	13.67	4	0.01 > P > 0.001
5	0	6	0	19	2	27	47.26	4	P < 0.001
6	0	2	1	3	6	12	8.833	4	0.10 > P > 0.05
7	0	0	1	0	0	1	4.000	4	0.50 > P > 0.30
8	4	3	2	3	7	19	3.895	4	0.50 > P > 0.30
9	7	4	17	8	0	36	22.06	4	P < 0.001
10	1	28	1	7	7	44	56.45	4	P < 0.001
11	2	1	3	1	0	7	3.714	4	0.50 > P > 0.30
12	4	35	1	15	20	75	49.47	4	P < 0.001
13	0	2	1	7	10	20	18.50	4	P < 0.001
14	0	17	2	16	14	49	27.02	4	P < 0.001
15	6	3	0	4	20	33	36.85	4	P < 0.001
16	0	11	0	22	6	39	43.18	4	P < 0.001
17	17	4	0	2	0	23	44.17	4	P < 0.001
18	17	0	0	4	11	32	34.56	4	P < 0.001
19	0	7	0	19	1	27	49.11	4	P < 0.001
20	3	5	1	11	18	38	25.16	4	P < 0.001
21	3	1	1	1	0	6	4.000	4	0.50 > P > 0.30

Table 30: Chi-square comparisons of the variation in number of isolates between sites for each group in turn using the sediment source only. Cluster analysis at 72% similarity level. 561 isolates from sediment.

Site	No. isolates in group																					Total	X^2	d.f.	P
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21				
1	10	0	0	0	0	0	0	4	7	1	2	4	0	0	6	0	17	17	0	3	3	74	159.2	20	P < 0.001
2	4	0	3	4	6	2	0	3	4	28	1	35	2	17	3	11	4	0	7	5	1	140	253.2	20	P < 0.001
3	0	19	2	1	0	1	1	2	17	1	3	1	1	2	0	0	0	0	0	1	1	53	218.1	20	P < 0.001
4	4	0	4	4	19	3	0	3	8	7	1	15	7	16	4	22	2	4	19	11	1	154	129.4	20	P < 0.001
5	7	0	2	9	2	6	0	7	0	7	0	20	10	14	20	6	0	11	1	18	0	140	136.8	20	P < 0.001

Table 31: Chi-square comparisons of the variation in number of isolates between groups for each site in turn using the sediment source only. Cluster analysis at 72% similarity level. Five 1 x 21 Chi-square tests. 561 isolates from sediment.

3 (0, 19, 2, ... 1, 1) and so on until all sites were compared with each other. These comparisons were carried out using the non-parametric Spearman's rank order correlation coefficient (r_s). The results of these comparisons are shown in table 32. This table consists of sites compared, Spearman's correlation coefficient and student's t-tests used to obtain the significance levels. A summary of the significance levels of these rank correlations are shown in table 33. A non-significant correlation was found between all sites with the exception of sites 2-4 and 4-5 which showed a significant correlation ($P<0.001$ and $0.05>P>0.02$ respectively).

B- Cluster by depths

Data on the number of isolates clustered by depths was statistically analysed to determine three main factors in a similar way to the analysis of clustering by sites.

i) Whether there is a significant variation in the number of isolates between depths for each group.

ii) Whether there is a significant variation in the number of isolates between groups for each depth.

iii) Whether there is a correlation in the number of isolates between the 11 depths.

(i)- **Variation in the number of isolates between depths for each group**

The data in table 29 were analysed by a series of Chi-square tests to answer the question: is there any difference between the number of isolates at each depth within a given group ? In other words for group 1, are the numbers 0, 10, 10, ... 3 and 1 (Table 29, row 1) significantly different from each other ? The results of these 21 Chi-square tests, one for each group, are shown in table 34 where it can be seen that a highly significant variation in the number

Sites compared	Spearman rank correlation (r_s)	t	d.f.	P
1 - 2	-0.0405	-0.1767	19	0.90 > P > 0.80
1 - 3	-0.2092	-0.9324	19	0.40 > P > 0.30
1 - 4	-0.1665	-0.7359	19	0.50 > P > 0.40
1 - 5	0.1413	0.6219	19	0.60 > P > 0.50
2 - 3	-0.2396	-1.076	19	0.30 > P > 0.20
2 - 4	0.8112	6.047	19	P < 0.001
2 - 5	0.3814	1.798	19	0.10 > P > 0.05
3 - 4	-0.3290	-1.519	19	0.20 > P > 0.10
3 - 5	-0.2462	-1.107	19	0.30 > P > 0.20
4 - 5	0.4613	2.267	19	0.05 > P > 0.02

Table 32: Spearman's rank correlation comparing between no. isolates in the 21 groups (table 28) for each pair of sites. Cluster analysis at 72% similarity level. t = student's t-test. 561 isolates from sediment.

		Sites				
		1	2	3	4	5
	1	-				
	2	B	-			
Sites	3	G	H	-		
	4	F	N*	G	-	
	5	E	J	H	K*	-

Table 33: Triangle showing the significance level of Spearman's rank correlation between no. isolates at each pair of sites (table 28. Cluster analysis at 72% similarity level. * = statistically significant result. 561 isolates from sediment.

Significance levels:

A	=	P > 0.90	H	=	0.30 > P > 0.20
B	=	0.90 > P > 0.80	I	=	0.20 > P > 0.10
C	=	0.80 > P > 0.70	J	=	0.10 > P > 0.05
D	=	0.70 > P > 0.60	*K	=	0.05 > P > 0.025
E	=	0.60 > P > 0.50	*L	=	0.025> P > 0.01
F	=	0.50 > P > 0.40	*M	=	0.01 > P > 0.001
G	=	0.40 > P > 0.30	*N	=	P < 0.001

Group	Depth (cm)											Total	X^2	d.f.	P
	00.0	00.5	03.0	04.0	05.5	07.5	10.5	11.0	13.0	15.5	20.5				
1	0	10	10	0	1	0	0	0	0	3	1	25	67.04	10	P<0.001
2	19	0	0	0	0	0	0	0	0	0	0	19	193.1	10	P<0.001
3	2	3	3	2	1	0	0	0	0	0	0	11	16.00	10	0.10>P> 0.05
4	1	4	5	0	3	0	0	1	3	0	1	18	20.35	10	0.05>P>0.02
5	0	7	13	0	0	0	0	0	6	0	1	27	75.50	10	P<0.001
6	1	5	2	1	2	0	0	0	1	0	0	12	20.83	10	0.05>P>0.02
7	1	0	0	0	0	0	0	0	0	0	0	1	10.10	10	0.50>P>0.30
8	2	8	4	0	3	0	1	0	0	1	0	19	36.58	10	P<0.001
9	18	0	0	8	0	0	8	0	0	2	0	36	102.5	10	P<0.001
10	1	4	4	3	11	0	5	0	2	8	6	44	29.00	10	0.01>P>0.001
11	3	3	0	0	1	0	0	0	0	0	0	7	24.27	10	0.01>P>0.001
12	1	5	12	3	21	0	14	0	1	9	9	75	68.77	10	P<0.001
13	1	2	8	0	4	0	4	0	0	1	0	20	36.47	10	P<0.001
14	4	9	11	3	5	0	3	0	8	4	2	49	28.17	10	0.01>P>0.001
15	0	8	8	0	17	0	0	0	0	0	0	33	106.0	10	P<0.001
16	0	15	16	0	2	0	1	0	5	0	0	39	106.5	10	P<0.001
17	0	14	9	0	0	0	0	0	0	0	0	23	109.0	10	P<0.001
18	0	17	6	1	6	0	0	0	0	2	0	32	94.11	10	P<0.001
19	0	4	21	0	0	0	0	0	2	0	0	27	157.9	10	P<0.001
20	1	10	7	5	9	0	1	0	4	0	1	38	40.79	10	P<0.001
21	1	0	3	1	0	0	0	1	0	0	0	6	17.50	10	0.10>P>0.05

Table 34: Chi-square comparisons[a] of the variation in number of isolates between depths for each group in turn using the sediment source only. Cluster analysis at 72% similarity level.

561 isolates from sediment.

of isolates occurred in most of the groups ($P<0.001$). Groups 4 and 6 showed a variation with a significance level of $0.05>P>0.02$ while groups 10, 11 and 14 showed a significance level of $0.01>P>0.001$. A non-significant variation in the number of isolates between depths was found only with groups 3 and 21 (both $0.10>P>0.05$) and 7 ($0.50>P>0.30$).

(ii)- **Variation in the number of isolates between groups for each depth**

The data in table 29 were analysed by a series of Chi-square tests to answer the question: is there any difference in the number of isolates between groups at each depth ? In other words for the surface sediment, are the numbers 0, 19, 2, ... 0, 1 and 1 (Table 29, column 1) significantly different from each other ? The results of these 11 Chi-square tests, one for each depth, are shown in table 35 where it can be seen that a highly significant variation in the number of isolates occurred between the groups ($P<0.001$) for all depths with the exception of the 11cm depth which showed a non-significant variation ($0.70>P>0.50$).

(iii)- **Comparison of the number of isolates between sampling depths**

The difference in the number of isolates/group between different depths was studied. In other words in table 29, the number of isolates in each group at 0cm depth (0, 19, 2,..... 1, 1) was compared with the number of isolates in each group at 0.5cm depth (10, 0, 3,..... 10, 0) and then the number of isolates in each group at 0cm depth was compared with the number of isolates in each group at 3cm depth (10, 0, 3,..... 7, 3) and so on until all depths were compared with each other. These comparisons were carried out using the non-parametric Spearman's rank order correlation coefficient (r_s). The

Depth	No. isolates in group																					Total	X^2	d.f.	P
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21				
00.0	0	19	2	1	0	1	1	2	18	1	3	1	1	4	0	0	0	0	0	1	1	56	213.6	20	P < 0.001
00.5	10	0	3	4	7	5	0	8	0	4	3	5	2	9	8	15	14	17	4	10	0	128	83.25	20	P < 0.001
03.0	10	0	3	5	13	2	0	4	0	4	0	12	8	11	8	16	9	6	21	7	3	142	94.68	20	P < 0.001
04.0	0	0	2	0	0	1	0	0	8	3	0	3	0	3	0	0	0	1	0	5	1	27	67.92	20	P < 0.001
05.5	1	0	1	3	0	2	0	3	0	11	1	21	4	5	17	2	0	6	0	9	0	86	167.3	20	P < 0.001
07.5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-	-
10.5	0	0	0	0	0	0	0	1	8	5	0	14	4	3	0	1	0	0	0	1	0	37	137.7	20	P < 0.001
11.0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	18.10	20	0.70>P>0.50
13.0	0	0	0	3	6	1	0	0	0	2	0	1	0	8	0	5	0	0	2	4	0	32	74.17	20	P < 0.001
15.5	3	0	0	0	0	0	0	1	2	8	0	9	1	4	0	0	0	2	0	0	0	30	97.97	20	P < 0.001
20.5	1	0	0	1	1	0	0	0	0	6	0	9	0	2	0	0	0	0	0	1	0	21	104.0	20	P < 0.001

Table 35: Chi-square comparisons of the variation in number of isolates between groups for each depth in turn using the sediment source only. Cluster analysis at 72% similarity level. 561 isolates from sediment.

results of these comparisons are shown in table 36. This table consists of depths compared, Spearman's correlation coefficient and student's t-tests used to obtain the exact significance levels. A summary of the significance levels of these correlations are shown in table 37. It is important to note that there was no comparison between the depth 7.5cm and the other depths because none of the isolates were clustered from this depth in any of the groups.

In general, 11 of the comparisons were significant and 34 were not significant. The details are as follows.

In the comparison between 0cm and the other depths (Table 36), there were non-significant correlations in all cases with the exception of 0 - 0.5 and 0 - 3cm depth which showed a significant correlation ($0.01>P>0.001$ and $P<0.001$ respectively).

In the comparison between 0.5cm and the other depths, non-significant correlations were found in all cases with the exception of 0.5 - 3cm depth which showed a significant correlation of $0.01>P>0.001$.

In the comparison between 3cm and the other depths, non-significant correlations were found in all cases with the exception of the depths 3 - 13cm which showed a significant correlation ($0.01>P>0.001$).

In the comparison between 4cm depth and the other depths, non-significant correlations were found an all cases with the exception of 4 - 10.5 and 4 - 15.5cm depth which showed a significant correlation ($0.01>P>0.001$ and $0.5>P>0.02$ respectively).

In the comparison between 5.5cm depth and the other depths, there were significant correlations between all depths with the exception of 5.5 - 11 and 5.5 - 13cm depth which showed a non-significant correlation ($0.60>P>0.50$ and $0.30>P>0.20$ respectively).

Depths compared	Spearman rank correlation (r_s)	t	d.f.	P
0 - 0.5	-0.5856	-3.149	19	0.01 > P > 0.001
0 - 3	-0.6664	-3.896	19	P < 0.001
0 - 4	-0.3757	1.767	19	0.10 > P > 0.05
0 - 5.5	-0.0595	-0.2596	19	0.80 > P > 0.70
0 - 7.5	-	-	-	-
0 - 10.5	0.3241	1.493	19	0.20 > P > 0.10
0 - 11	0.0281	0.1226	19	P > 0.90
0 - 13	-0.1609	-0.7108	19	0.50 > P > 0.40
0 - 15.5	0.1506	0.6640	19	0.60 > P > 0.50
0 - 20.5	-0.0196	-0.0853	19	P > 0.90

Table 36: Spearman's rank correlation comparing between no. isolates in the 21 groups (Table 29) for each pair of depths. Comparison tables are on 6 pages. Cluster analysis at 72% similarity level. t = Student's t test. 561 isolates from sediment.

Table 36 cont'd.

Depths compared	Spearman rank correlation (r_s)	t	d.f.	P
0.5 - 3	0.6350	3.583	19	0.01 > P > 0.001
0.5 - 4	-0.0334	-0.1457	19	0.90 > P > 0.80
0.5 - 5.5	0.4300	2.076	19	0.10 > P > 0.05
0.5 - 7.5	-	-	-	-
0.5 - 10.5	0.0127	0.0555	19	P > 0.90
0.5 - 11	-0.2830	-1.286	19	0.30 > P > 0.20
0.5 - 13	0.3430	1.592	19	0.20 > P > 0.10
0.5 - 15.5	0.1616	0.7137	19	0.50 > P > 0.40
0.5 - 20.5	0.2443	1.098	19	0.30 > P > 0.20

Table 36 cont'd.

Depths compared	Spearman rank correlation (r_s)	t	d.f.	P
3 - 4	-0.1602	-0.7075	19	0.50 > P > 0.40
3 - 5.5	0.2453	1.103	19	0.30 > P > 0.20
3 - 7.5	-	-	-	-
3 - 10.5	0.1570	0.6928	19	0.50 > P > 0.40
3 - 11	-0.1480	-0.6522	19	0.60 > P > 0.50
3 - 13	0.5540	2.901	19	0.01 > P > 0.001
3 - 15.5	0.1669	0.7380	19	0.50 > P > 0.40
3 - 20.5	0.3891	1.841	19	0.10 > P > 0.05

Table 36 cont'd.

Depths compared	Spearman rank correlation (r_s)	t	d.f.	P
4 - 5.5	0.3460	1.607	19	0.20 > P > 0.10
4 - 7.5	-	-	-	-
4 - 10.5	0.5503	2.873	19	0.01 > P > 0.001
4 - 11	-0.0447	-0.1949	19	0.90 > P > 0.80
4 - 13	0.2134	0.9520	19	0.40 > P > 0.30
4 - 15.5	0.4622	2.272	19	0.05 > P > 0.02
4 - 20.5	0.3836	1.811	19	0.10 > P > 0.05
5.5 - 7.5	-	-	-	-
5.5 - 10.5	0.4848	2.416	19	0.05 > P > 0.02
5.5 - 11	-0.1231	-0.5405	19	0.60 > P > 0.50
5.5 - 13	0.2551	1.150	19	0.30 > P > 0.20
5.5 - 15.5	0.4878	2.435	19	0.05 > P > 0.02
5.5 - 20.5	0.4712	2.329	19	0.05 > P > 0.02

Table 36 cont'd.

Depths compared	Spearman rank correlation (r_s)	t	d.f.	P
10.5 - 11	-0.2457	-1.105	19	0.30 > P > 0.20
10.5 - 13	0.2724	1.234	19	0.30 > P > 0.20
10.5 - 15.5	0.6854	4.103	19	P < 0.001
10.5 - 20.5	0.4134	1.979	19	0.10 > P > 0.05
11 - 13	0.0446	0.1945	19	0.90 > P > 0.80
11 - 15.5	-0.2455	-1.104	19	0.30 > P > 0.20
11 - 20.5	0.0641	0.2801	19	0.80 > P > 0.70
13 - 15.5	0.0181	0.0791	19	P > 0.90
13 - 20.5	0.6249	3.489	19	0.01 > P > 0.001
15.5 - 20.5	0.5243	2.684	19	0.02 > P > 0.01

Depths (cm)	00.0	00.5	03.0	04.0	05.5	07.5	10.5	11.0	13.0	15.5	20.5
00.0	-										
00.5	M*	-									
03.0	N*	M*	-								
04.0	J	B	F	-							
05.5	C	J	H	I	-						
07.5	-	-	-	-	-	-					
10.5	I	A	F	M*	K*	-	-				
11.0	A	H	E	B	E	-	H	-			
13.0	F	I	M*	G	H	-	H	B	-		
15.5	E	F	F	K*	K*	-	N*	H	D	-	
20.5	C	H	J	J	J	-	J	C	M*	L*	-

Table 37: Triangle showing the significance level of Spearman's rank correlation between no. isolates at each pair of depths (Table 29). Cluster analysis at 72% similarity level. Significance levels see Table 33. * = Statistically significant result. 561 isolates from sediment.

In the comparison between 10.5cm depth and the other depths, non-significant correlations were found in all cases with the exception of 10.5 - 15.5cm which showed a highly significant correlation ($P<0.001$).

In the comparison between 11cm depth and the other depths, non-significant correlations were found in all cases.

In the comparison between 13cm depth and the other depths, non-significant correlations were found between the depths 13 - 15.5cm ($P>0.90$). However, there was a significant correlation between the depths 13 - 20.5cm ($0.01>P>0.001$).

In the comparison between 15.5cm and 20.5cm depths, significant correlation was found between the two depths ($0.02>P>0.01$).

C- Relationship between isolates of each group obtained from sediment, burrow linings and faecal pellets

The deep-sea isolates used in my study were obtained from three different sources; sediment (561), burrow linings (143) and faecal pellets (139). Table 38 shows the number of isolates/group in each of these sources. The data are also plotted in figure 17 to make visual comparisons easy.

A total of 561 isolates obtained from the sediment source were clustered in 21 groups at 72% similarity level. The number of isolates/group was in the range 1 to 75 (Table 38, rowl). For example, group 7 showed the lowest number of isolates (1) and group 12 showed the highest number of isolates (75).

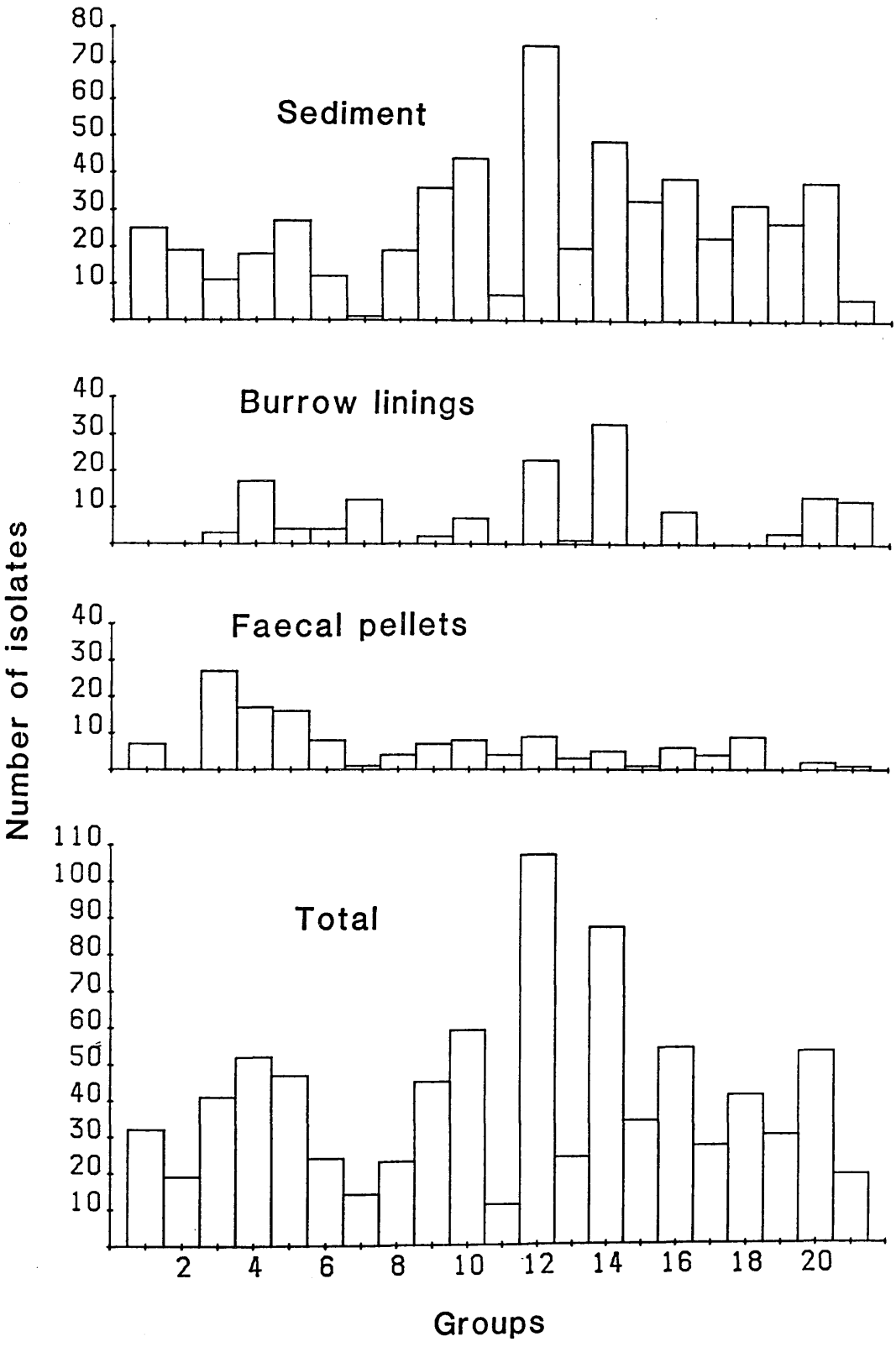
The 143 isolates obtained from animal burrows were clustered in 14 out of 21 groups. The number of isolates/group was in the range 1 to 33 (Table 38, row 2). A large number of isolates were clustered

	Groups	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Total
Number of isolates	Sediment 1	25	19	11	18	27	12	1	19	36	44	7	75	20	49	33	39	23	32	27	38	6	561
	Burrow linings 2	0	0	3	17	4	4	12	0	2	7	0	23	1	33	0	9	0	0	3	13	12	143
	Faecal pellet 3	7	0	27	17	16	8	1	4	7	8	4	9	3	5	1	6	4	9	0	2	1	139
	Total	32	19	41	52	47	24	14	23	45	59	11	107	24	87	34	54	27	41	30	53	19	843

Table 38: Number of bacterial isolates in sediment, burrow linings and faecal pellets for the 21 groups clustered at 72% similarity level. This data is also plotted in Figure 17, p.135.

Figure 17

Histograms showing the distribution of isolates in sediment, burrow linings and faecal pellets between the 21 groups obtained at 72% similarity level. This data is also presented in table 38.


Sediment
Burrow linings
Faecal pellets
Total
Number of isolates
Groups
80
70
60
50
40
30
20
10
110
100
90
2
4
6
8
10
12
14
16
18
20

in groups 4, 7, 10, 12, 14, 16, 20 and 21.

The 139 isolates collected from faecal pellets were clustered in almost all the groups (19 out of 21). The number of isolates/group was in the range 1 to 27 (Table 38, row 3).

The data in table 38 were firstly analysed to test whether there was any difference between the number of isolates/group in the sediment, burrow linings, faecal pellets, and data combined (total). This was done by applying four 1 x 21 Chi-square tests to the data. The results of these Chi-square tests are shown in table 39. All 4 tests were highly significant. The order of significance is data combined (X^2 =279.3), burrow linings (X^2 =231.3), sediment (X^2 =215.6), and faecal pellets (X^2 =131.0).

The data in table 38 were then analysed using Chi-square tests in order to answer the question: is there any difference in the number of isolates between groups for sediment and burrow linings + faecal pellets ? In other words, are the number 25, 19, 11, ... 38, 6 (Table 38, row 1) significantly different from the numbers 7, 0, 30, 34, 20 ..... 15, 13 (Table 38 row 2+3) ? The result of Chi-square tests are shown in table 40 where it can be seen that a highly significant variation in the number of isolates occurred between sediment samples and burrow linings+faecal pellets ($P<0.001$).

Similarly, the data in table 38 were analysed using Chi-square tests in order to answer the question: is there any difference in the number of isolates between groups for burrow linings and faecal pellets ? In other words, are the numbers 0, 0, 3, 17, ... 13, 12 (Table 38 row 2) significantly different from the numbers 7, 0, 27, 17, 16,..... 2, 1 (Table 38, row 3) ? The result of Chi-square tests are shown in table 40 where it can be seen that a highly significant variation in the number of isolates occurred between burrow linings and faecal pellets ($P<0.001$).

	X^2	d.f.	P
Sediment	215.6	20	P<0.001
Burrow linings	231.3	20	P<0.001
Faecal pellets	131.0	20	P<0.001
Total	279.3	20	P<0.001

Table 39

Statistical comparison of number of isolates in groups 1 to 21 for sediment, burrow linings, faecal pellets and data combined (total). Four 1 x 21 Chi-square tests.

Data compared	X^2	d.f.	P
Sediment vs Burrow linings + Faecal pellets	147.2	20	P<0.001
Burrow linings vs Faecal pellets	148.0	19	P<0.001

Table 40

Statistical comparison of number of isolates in groups 1 to 21 between sediment and burrow linings + faecal pellets and between burrow linings and faecal pellets. The number of isolates in group 2 for both burrow linings and faecal pellets were zero, therefore, group 2 was excluded from the analysis. One 2 x 21 and one 2 x 20 Chi-square tests.

4- **Description and interpretation of figures 18 to 23 drawn from 72% similarity level data** (Tables 28 and 29).

A- **Variation in the number of sediment isolates between groups at each sampling site**

The following refers only to the 561 isolates obtained from the sediment and not to isolates from burrow linings or faecal pellets. The number of isolates from each of the five sites (Table 28) was plotted against groups separately (Figure 18). Chi-square tests showed that at all sites, there was a highly significant variation between groups (all $P<0.001$, table 31). Most of the isolates from site 1 were clustered in groups 1, 9, 15, 17 and 18. The number of isolates was in the range 0 to 17.

At site 2, most of the isolates were clustered in groups 5, 10, 12, 14, 16, 19 and 20. The number of isolates was in the range 0 to 35. The highest number of isolates from this site was at groups 10 and 12.

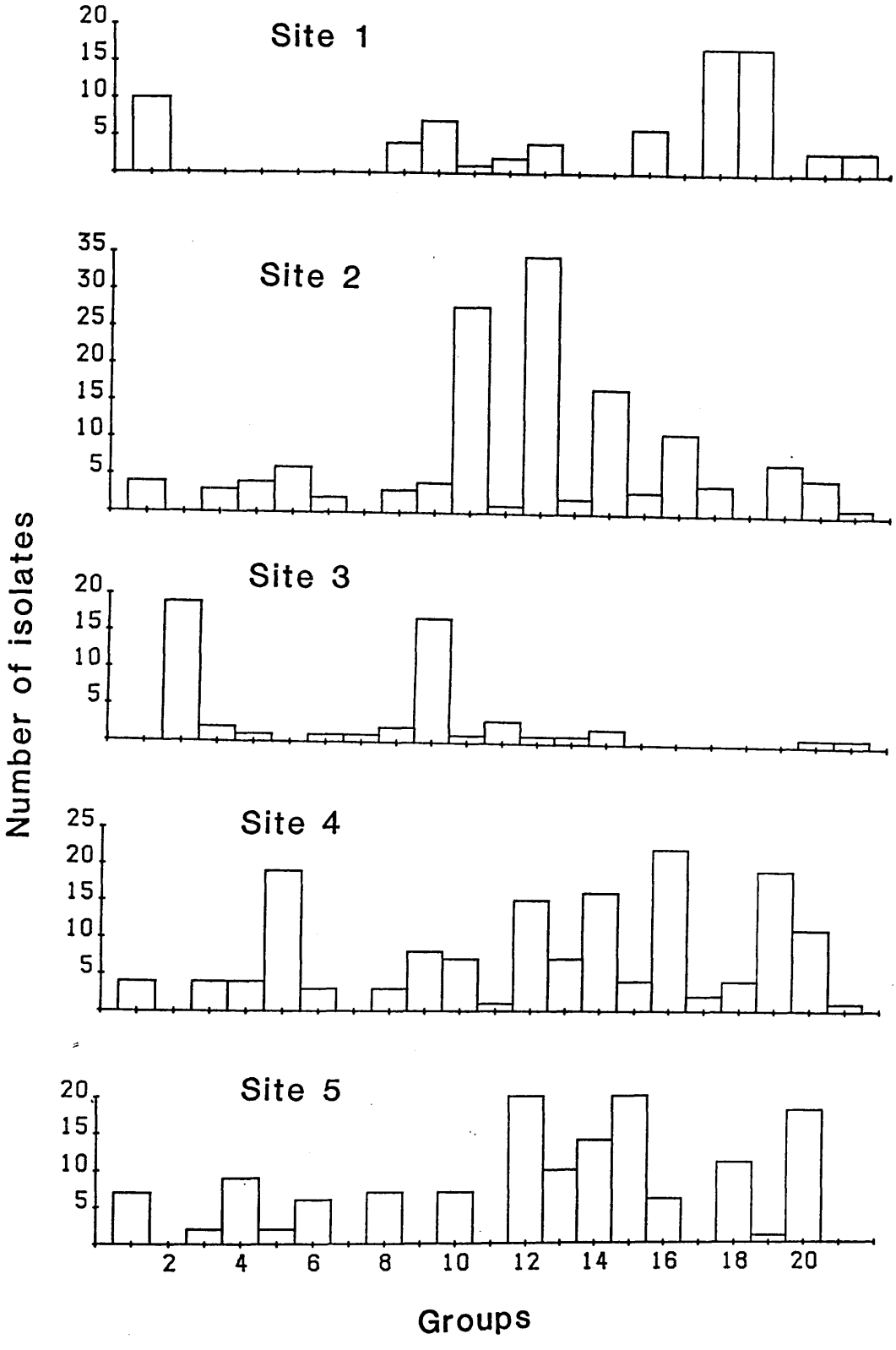
At site 3, there was a very small number of clustered isolates with the exception of groups 2 and 9 where 19 and 17 isolates were clustered respectively. The number of isolates was in the range 0-19.

At site 4, a large number of isolates were clustered in a wide range of groups (5, 9, 10, 12, 13, 14, 16, 19 and 20). The number of isolates was in the range 0-22.

At site 5, clustering occurred in most of the groups (1, 4, 6, 8, 10, 12, 13, 14, 15, 16, 18 and 20). The number of isolates was in the range 0-20.

Figure 18

Histograms showing the distribution of isolates in sediment between groups for each site at 72% similarity level.


Site 1
Site 2
Site 3
Site 4
Site 5
Number of isolates
Groups
20
15
10
5
35
30
25
2
4
6
8
10
12
14
16
18
20

B- **Variation in the number of sediment isolates of each group, between sampling sites**

The following refers only to the 561 isolates obtained from the sediment and not to isolates from burrow linings or faecal pellets. The number of isolates for each group at each site are presented in table 28 (p. 115) and plotted in figure 19. In general, for each group, isolates were found at 3 or more of the sites with the exception of groups 2 and 7 where isolates occurred only at site 3. Only 4 groups (8, 10, 12 and 20) had isolates that came from all 5 sites. The total number of isolates was greatest at site 4 (154), followed by sites 2 and 5 (both 140), then site 1 (74) and site 3 (53) (Table 31 p. 119) and figure 19). Chi-square tests applied to the data showed that significant variation occurred between sites for each group in 15 out of 21 cases (Table 30 p. 118).

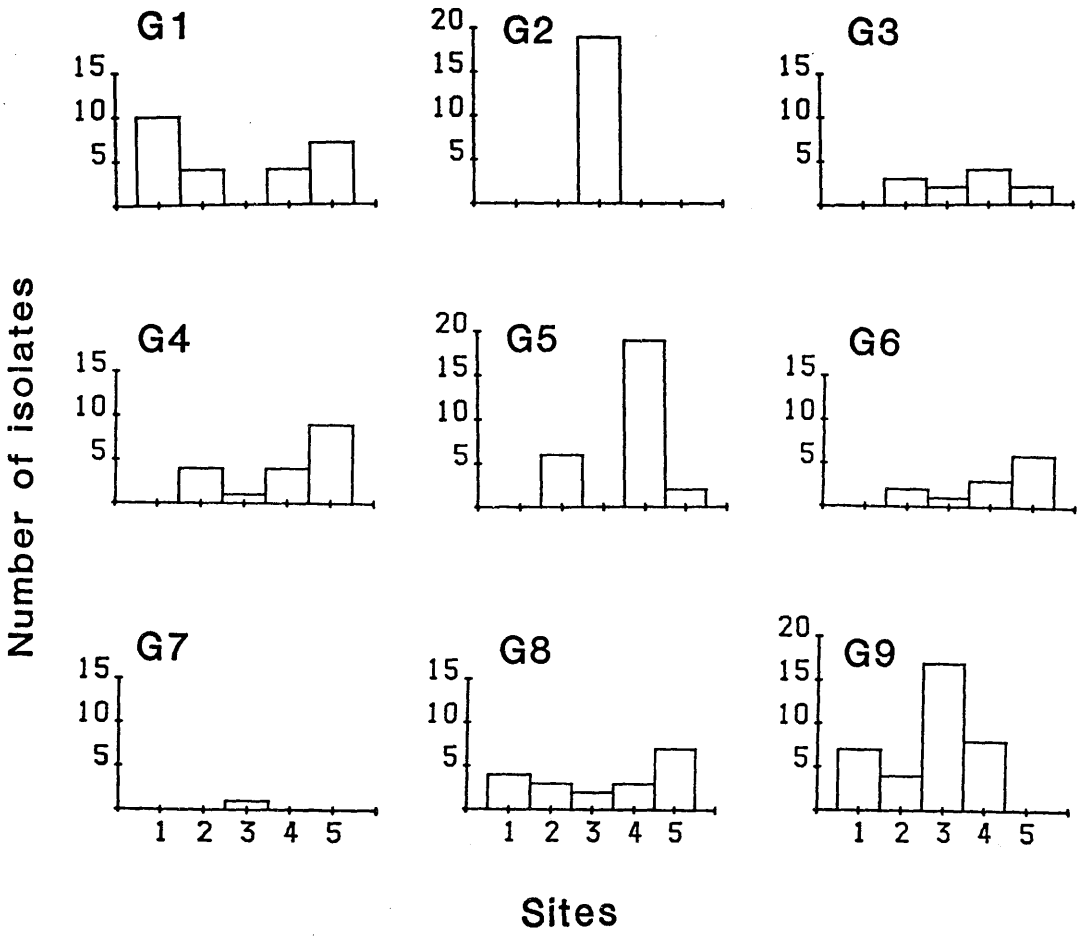
I then decided to analyse the distribution of the isolates in each group between the different sites and then to assess whether there was a relationship between the number of sites at which isolates were found in a particular group and the percentage similarity at which that group was formed.

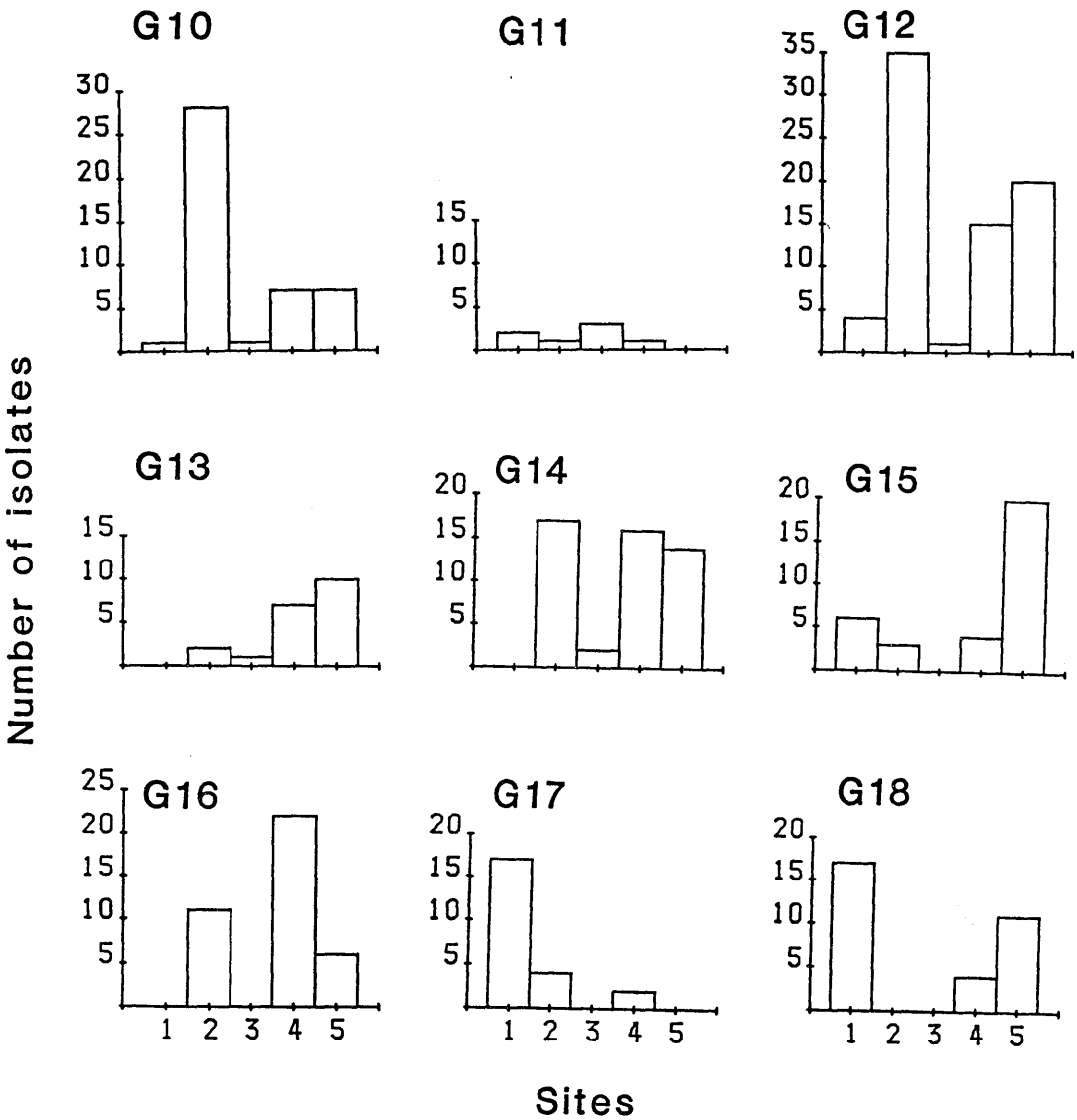
Table 41 gives a summary of the group number, percentage similarity, number of isolates in group and the number of sites at which isolates in group were found. According to sites, groups were classified into 4 categories. Category 1 contained 1 group with isolates found at 1 site. Category 2, contained 4 groups (5, 7, 16 and 19) with isolates obtained at 3 sites. Category 3, contained 9 groups (3, 4, 6, 9, 11, 13, 14, 15 and 21) with isolates obtained from 4 sites. Category 4, contained 7 groups (1, 8, 10, 12, 17, 18 and 20) all with isolates obtained at 5 sites.

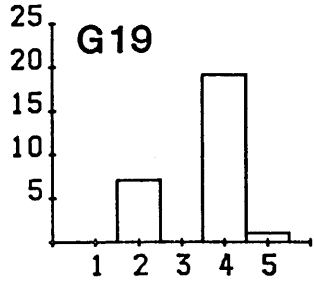
The relationship between the percentage similarity at which groups were clustered and the number of sites at which isolates of

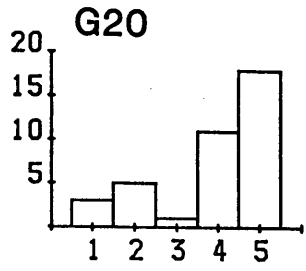
Figure 19

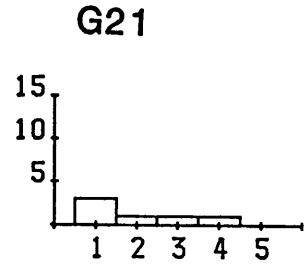
Histograms showing the distribution of isolates between sampling sites for each of the 21 groups at 72% similarity level. The data in this figure were taken from table 26.

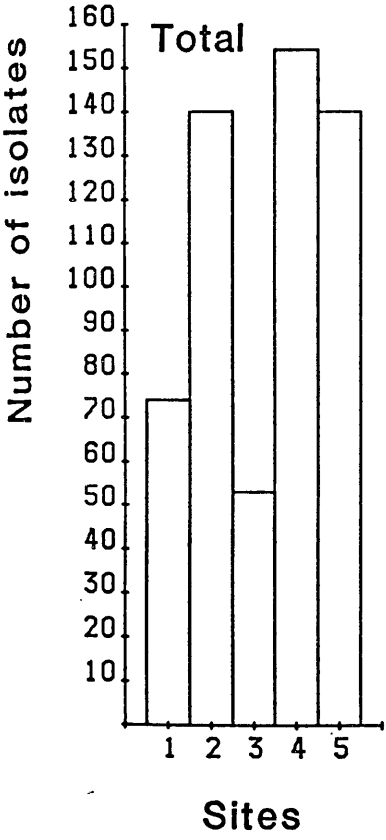

G1
G2
G3
G4
G5
G6
G7
G8
G9
Number of isolates
Sites
1 2 3 4 5
5
10
15
20


G10
G11
G12
G13
G14
G15
G16
G17
G18
Number of isolates
Sites
1 2 3 4 5


G19
25
20
15
10
5
1 2 3 4 5


G20
20
15
10
5
1 2 3 4 5


G21
15
10
5
1 2 3 4 5


Total
Number of isolates
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
1 2 3 4 5
Sites

Number of groups	Group number	percentage similarity	Number of isolates in group	Number of sites at which isolates were found	Sites 1	2	3	4	5
1	2	94.6	19	1			✓		
4	5	88.4	47	3		✓		✓	✓
	7	91.4	14	3		✓	✓	✓	
	16	84.1	54	3		✓		✓	✓
	19	90.8	30	3		✓		✓	✓
9	3	73.4	41	4		✓	✓	✓	✓
	4	82.8	52	4		✓	✓	✓	✓
	6	80.6	24	4		✓	✓	✓	✓
	9	87.6	45	4	✓	✓	✓	✓	
	11	88.0	11	4	✓	✓	✓	✓	
	13	93.6	24	4		✓	✓	✓	✓
	14	75.0	87	4		✓	✓	✓	✓
	15	78.5	34	4	✓	✓		✓	✓
	21	82.1	19	4	✓	✓	✓	✓	
7	1	79.4	32	5	✓	✓	✓	✓	✓
	8	85.6	23	5	✓	✓	✓	✓	✓
	10	87.3	59	5	✓	✓	✓	✓	✓
	12	72.4	107	5	✓	✓	✓	✓	✓
	17	85.2	27	5	✓	✓	✓	✓	✓
	18	79.6	41	5	✓	✓	✓	✓	✓
	20	86.3	53	5	✓	✓	✓	✓	✓

Table 41

A summary of the group number, percentage similarity, number of isolates in group and the number of sites at which isolates in group were found. ✓ = specific sites at which isolates were found.

groups were found was investigated by regression analysis, fitting the equation $y = bx + c$ to the data in table 41. The results are shown in figure 20. A significant negative relationship was found between the percentage similarity and the number of sites ($y = -3.1350x + 96.6686$, $r = -0.4991$, $0.05>P>0.02$). This means that the fewer the sites contributing isolates to a particular group, the higher the similarity at which that group was formed.

C- **Variation in the number of sediment isolates between groups at each sampling depth**

The following refers only to the 561 isolates obtained from the sediment and not to isolates from burrow linings or faecal pellets. The number of isolates obtained from various sediment depths were plotted against the groups (Figure 21 and table 29). In almost all cases (9 out of 10 groups) highly significant variation was found between groups at each depth (Table 35). With the surface sediment (0cm), the isolates were clustered in a narrow range of groups (Figure 21). A large number of isolates were clustered in groups 2 and 9. The number of isolates found at this depth ranged from 0 to 19.

The isolates from 0.5cm depth were scattered over almost all the groups, and ranged from 0 to 17.

The highest number of isolates occured at 3cm depth (142), and clustering was found in almost all the groups. The number of isolates found at this depth ranged from 0 to 21.

At 4cm depth, clustering occurred in only 9 of the 21 groups, and the number of isolates found was in the range from 0 to 8.

At 5.5cm depth, clustering occurred in more than half of the groups (14 out of 21). The number of isolates ranged from 0 to 21.

At 7.5cm depth, no clusters occurred, and no isolates were

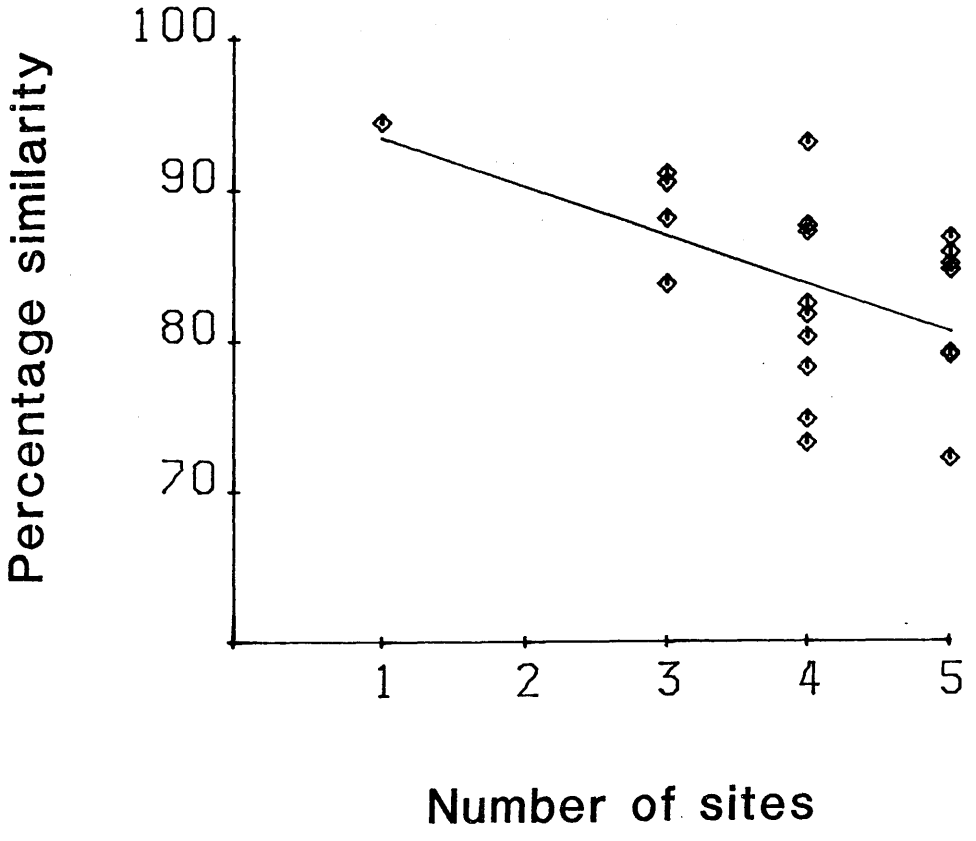
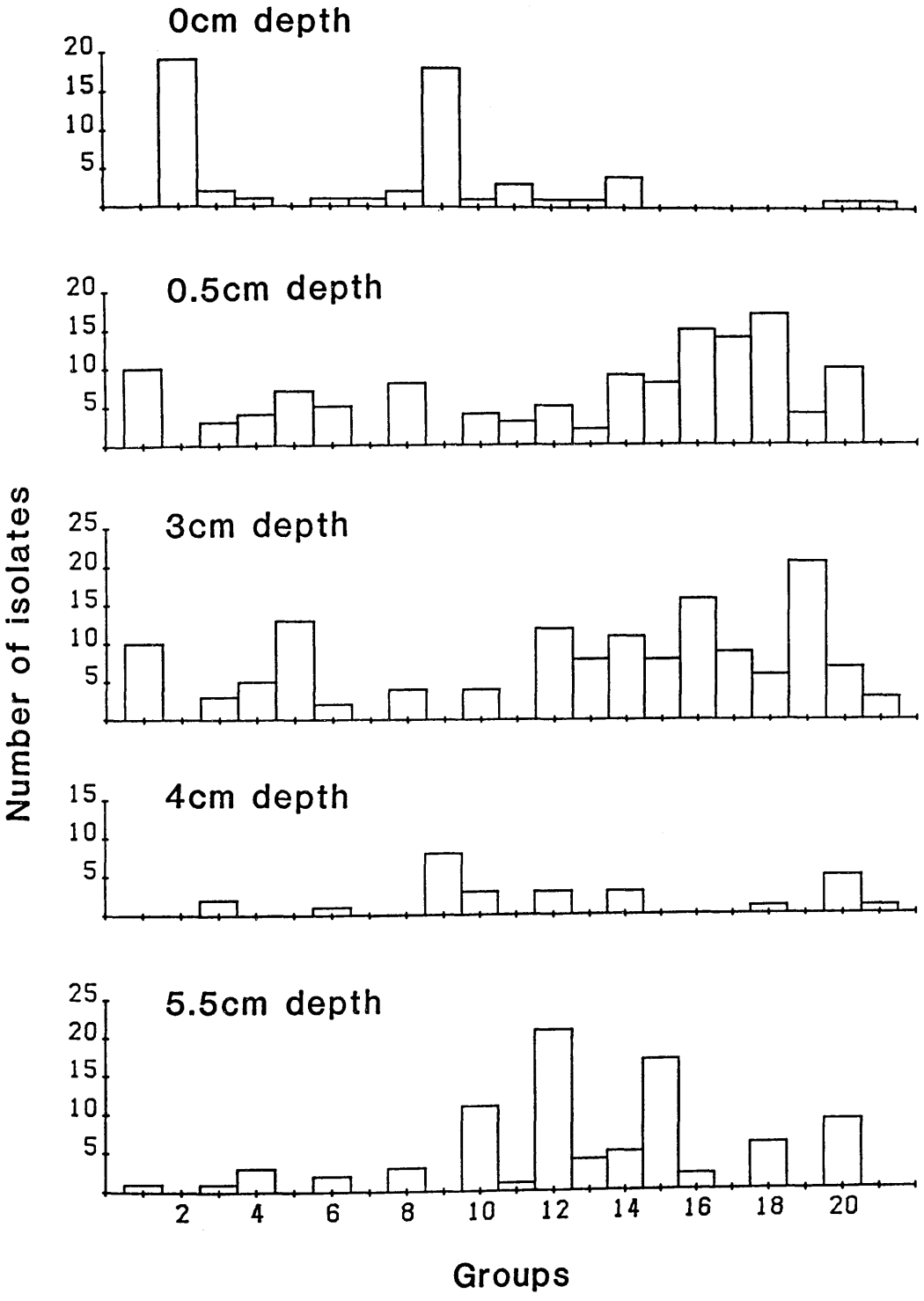


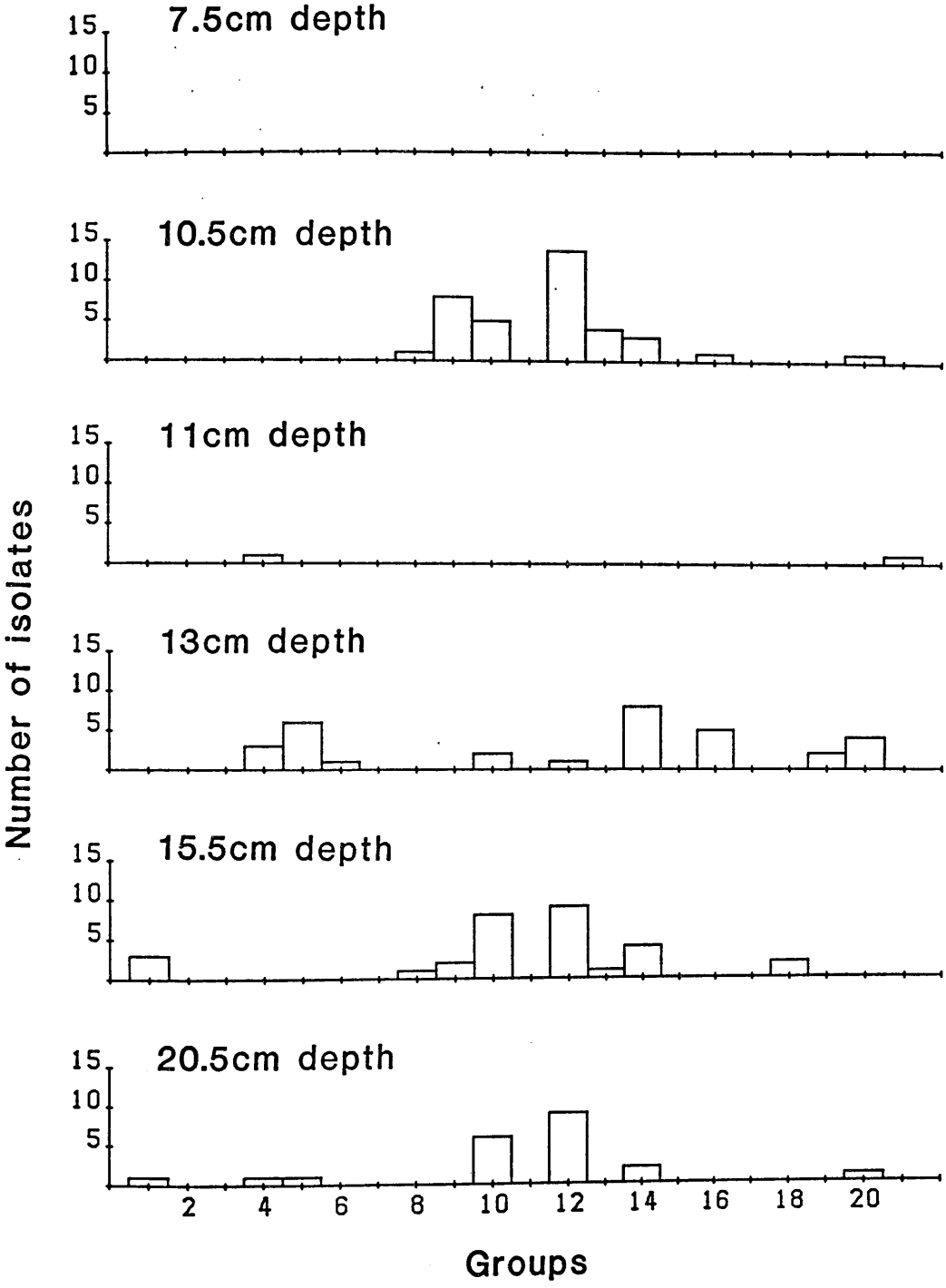
Figure 20

Relationship between the percentage similarity at which groups were clustered and the number of sites at which isolates in groups were found. Regression equation $y = -3.1350x + 96.6686$, Correlation coefficient $(r) = -0.4991$, $P = 0.05>P>0.02$.

Figure 21

Histograms showing the distribution of isolates between the 21 groups for each of the 11 depths at 72% similarity level.


0cm depth
0.5cm depth
3cm depth
4cm depth
5.5cm depth
Number of isolates
Groups
2
4
6
8
10
12
14
16
18
20


7.5cm depth
10.5cm depth
11cm depth
13cm depth
15.5cm depth
20.5cm depth
15
10
5
Number of isolates
2
4
6
8
10
12
14
16
18
20
Groups

found.

At 10.5cm depth, clustering occurred in 8 of the 21 groups, and the number of isolates ranged from 0 to 14.

At 11cm depth, clustering occurred in only 2 groups, with one isolate in each group.

At 13cm depth, clustering occurred in 9 of the 21 groups, and the number of isolates ranged from 0 to 8.

At 15.5cm depth, clustering occurred in 8 of the 21 groups, and the number of isolates ranged from 0 to 9.

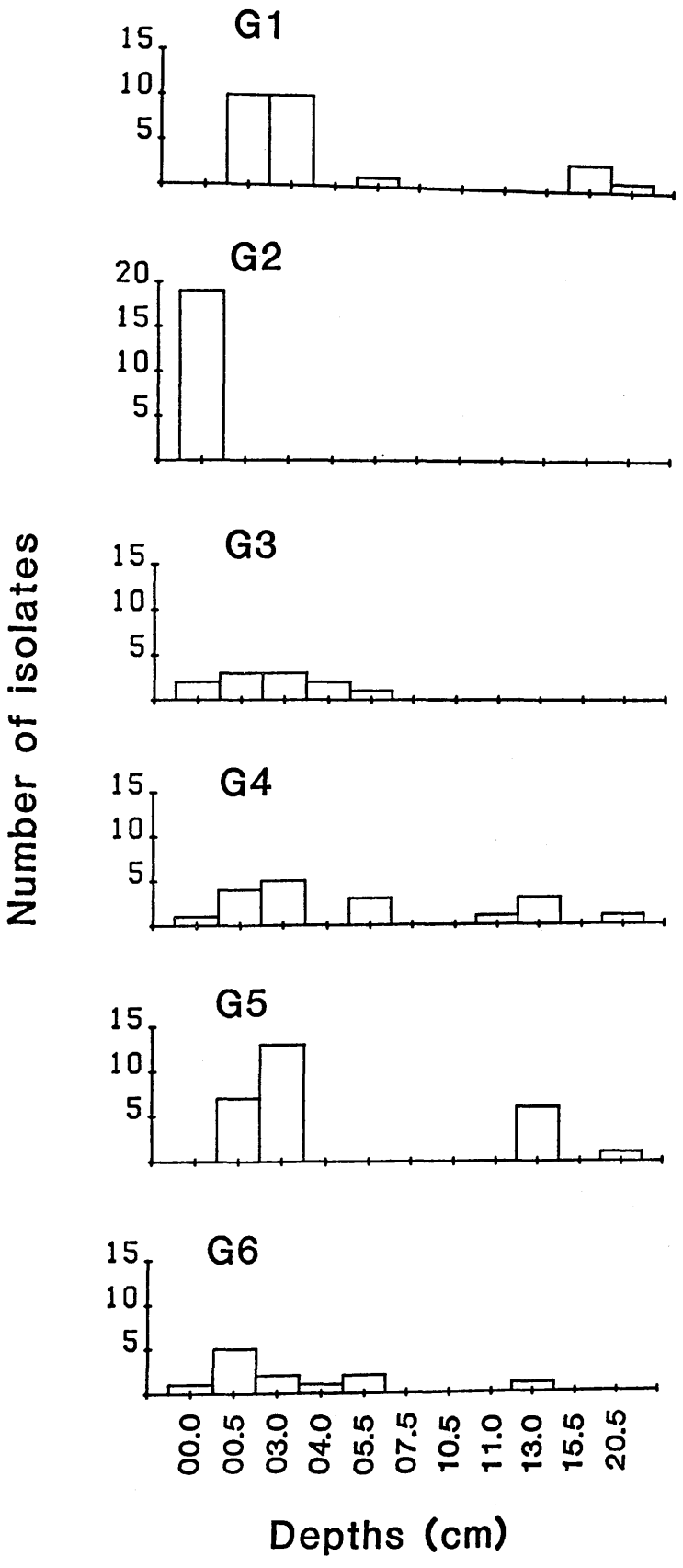
At the maximum depth of 20.5cm, clustering occurred in 7 of the 21 groups, and the number of isolates ranged from 0 to 9.

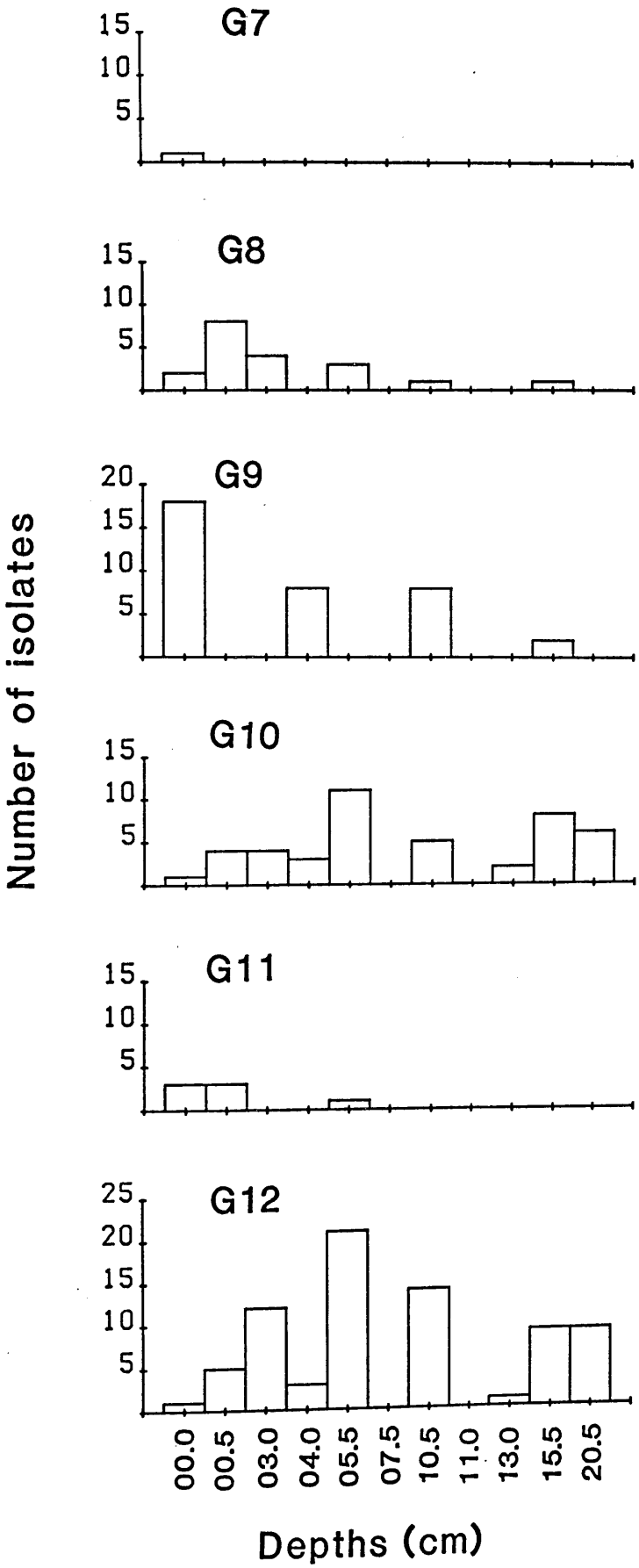
D- Variation in the number of sediment isolates of each group, between sampling depths

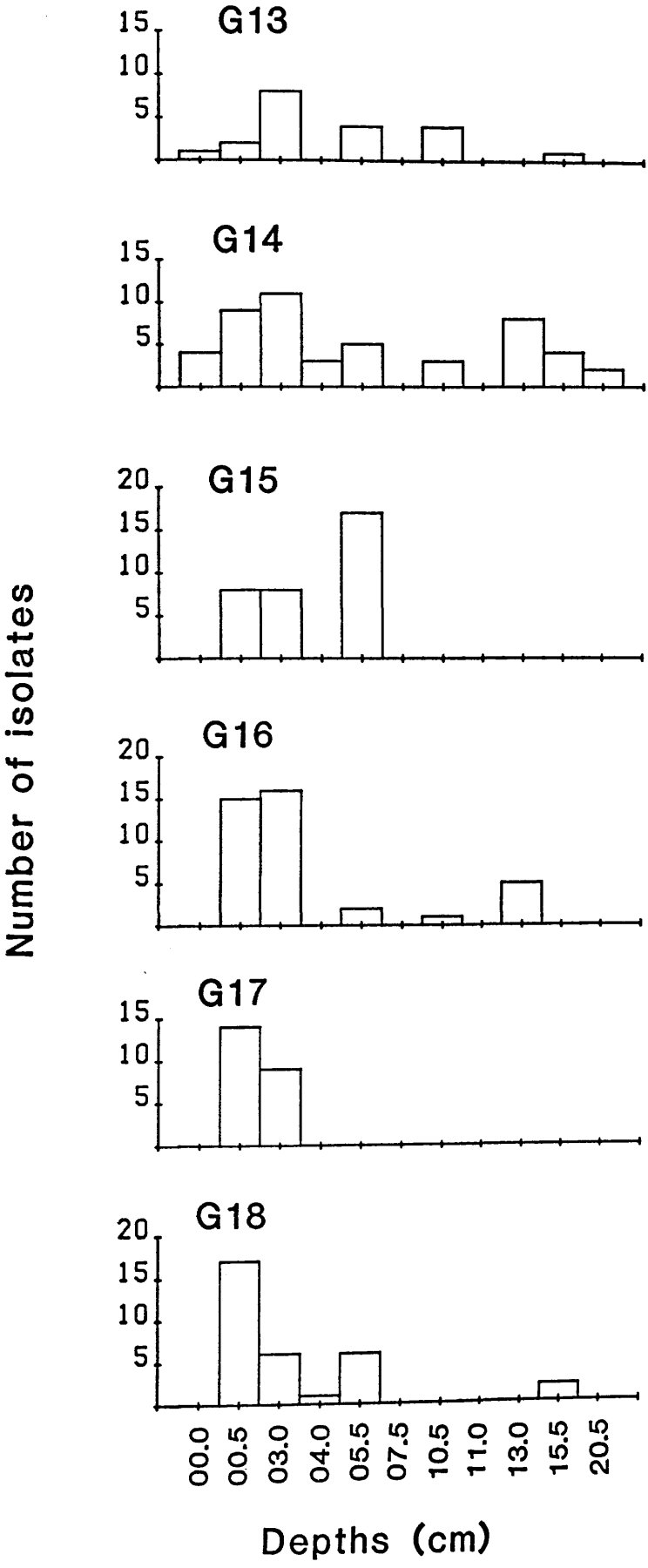
The following refers only to the 561 isolates obtained from sediment and not to isolates from burrow linings and faecal pellets. The numbers of isolates for each group at each sampling depth are presented in table 29 (p. 116) and plotted in figure 22. Significant variation was found between depths with each group in most cases (18 out of 21, table 34). For groups 2 and 7, isolates were found at the sediment surface only. At groups 10, 12, and 14, isolates were found at nine of the eleven sampling depths. With the other groups, isolates were found at depths between these two extremes (Figure 22). Figure 22 also shows the number of isolates plotted against sampling depths for pooled data from all the groups. The maximum and minimum number of isolates (142 and 0 respectively) were found at depths of 3cm and 7.5cm respectively. In general, however, there was a pronounced decrease in the number of isolates found with increasing depth below 3cm.

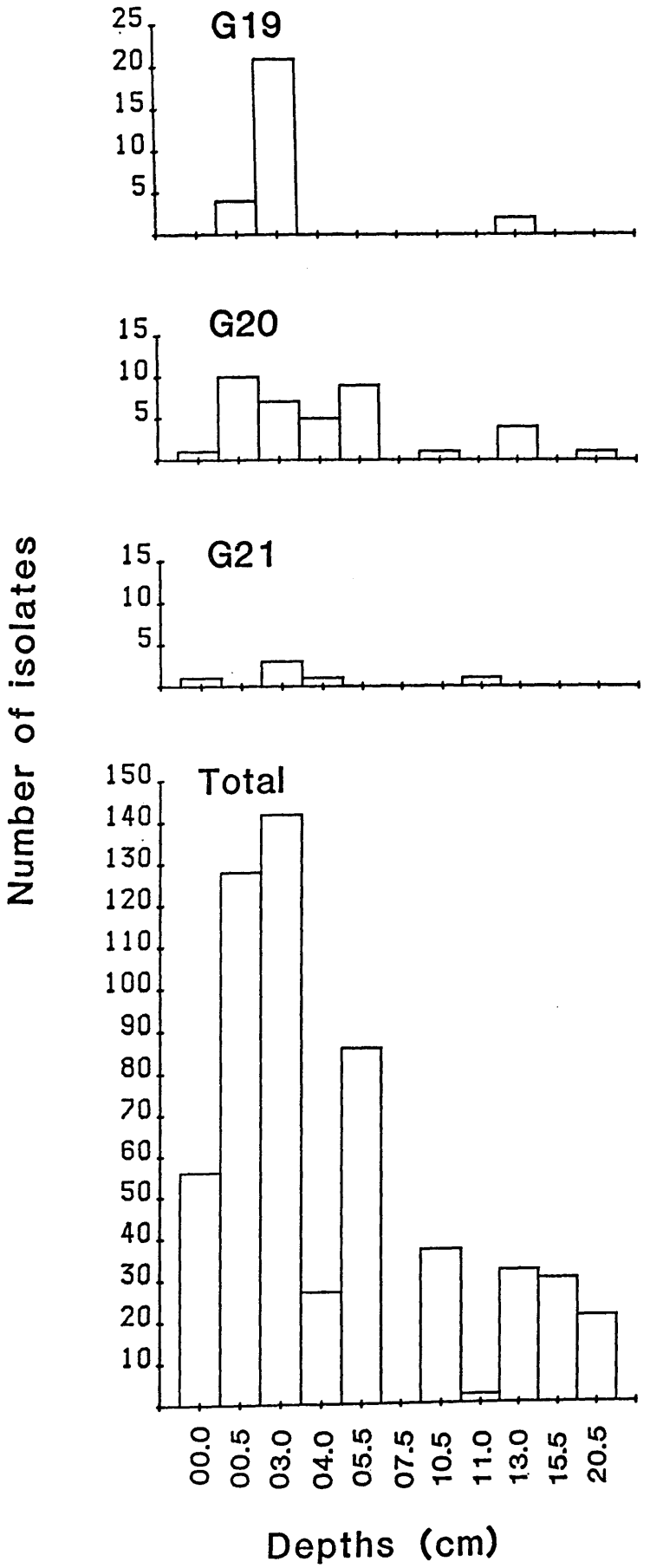
Figure 22

Histograms showing the distribution of isolates between depths for each of the 21 groups at 72% similarity level.


G1
15
10
5
G2
20
15
10
5
G3
15
10
5
G4
15
10
5
G5
15
10
5
G6
15
10
5
Number of isolates
00.0
00.5
03.0
04.0
05.5
07.5
10.5
11.0
13.0
15.5
20.5
Depths (cm)


G7
G8
G9
G10
G11
G12
Number of isolates
Depths (cm)
00.0
00.5
03.0
04.0
05.5
07.5
10.5
11.0
13.0
15.5
20.5


G13
G14
G15
G16
G17
G18
Number of isolates
Depths (cm)
00.0
00.5
03.0
04.0
05.5
07.5
10.5
11.0
13.0
15.5
20.5


G19
25
20
15
10
5
G20
15
10
5
G21
15
10
5
Total
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Number of isolates
00.0
00.5
03.0
04.0
05.5
07.5
10.5
11.0
13.0
15.5
20.5
Depths (cm)

As with the sites, I then decided to analyse the distribution of the isolates in each group between the different depths and then to assess whether there was a relationship between the number of depths at which isolates were found in a particular group and the percentage similarity at which that group was formed.

Table 42 gives a summary of the group number, percentage similarity, number of isolates in group and the number of depths at which isolates were found. According to depths, groups were classified into 8 categories. Category 1 contained group 2 with isolates found at 1 depth. Category 2, contained group 17 with isolates obtained at 3 depths. Category 3 contained groupe 11 and 15, both with isolates found at 4 depths. Categories 4, 5, 6 and 8 contained groups (7, 19, 21), (5, 9, 16), (1, 4, 18) and (10, 12, 14) respectively with isolates from 5, 6, 7, and 11 depths respectively. Category 7, contained groups 3, 6, 8, 13 and 20 with isolates obtained from 8 depths.

The relationship between the percentage similarity at which groups were clustered and the number of depths at which isolates of groups were found was investigated by regression analysis fitting the equation $y = bx + c$ to the data in table 42. The results are shown in figure 23. A significant negative correlation was found between the percentage similarity and the number of depths ($y = -1.2247x + 92.2347$, $r = -0.5065$, $0.02>P>0.01$). This means that, as with the sites, the fewer the depths contributing isolates to a particular group, the higher the similarity at which that group was formed.

Number of groups	Group number	percentage similarity	Number of isolates in group	Number of depths at which isolates were found	Depth (cm) 0	0.5	3	4	5.5	7.5	10.5	11	13.0	15.5	20.5
1	2	94.6	19	1	✓										
1	17	85.2	27	3		✓	✓			✓					
2	11	88.0	11	4	✓	✓			✓	✓					
	15	78.5	34	4		✓	✓		✓			✓			
3	7	91.4	14	5	✓			✓		✓		✓	✓		
	19	90.8	30	5		✓	✓	✓				✓	✓		
	21	82.1	19	5	✓		✓	✓				✓	✓		
3	5	88.4	47	6		✓	✓	✓				✓	✓		✓
	9	87.6	45	6	✓			✓		✓	✓		✓	✓	
	16	84.1	54	6		✓	✓		✓		✓	✓	✓		
3	1	79.4	32	7		✓	✓		✓	✓		✓		✓	✓
	4	82.8	52	7	✓	✓	✓	✓	✓			✓			✓
	18	79.6	41	7		✓	✓	✓	✓	✓		✓		✓	
5	3	73.4	41	8	✓	✓	✓	✓	✓	✓		✓	✓		
	6	80.6	24	8	✓	✓	✓	✓	✓	✓		✓	✓		
	8	85.6	23	8	✓	✓	✓		✓	✓	✓	✓		✓	
	13	93.6	24	8	✓	✓	✓		✓	✓	✓	✓		✓	
	20	86.3	53	8	✓	✓	✓	✓	✓		✓	✓	✓		
3	10	87.3	59	11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	12	72.4	107	11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	14	75.0	87	11	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table **42**
A summary of the group number, percentage similarity, number of isolates in group and the number of depths at which isolates were found. ✓ = specific depths at which isolates were found.

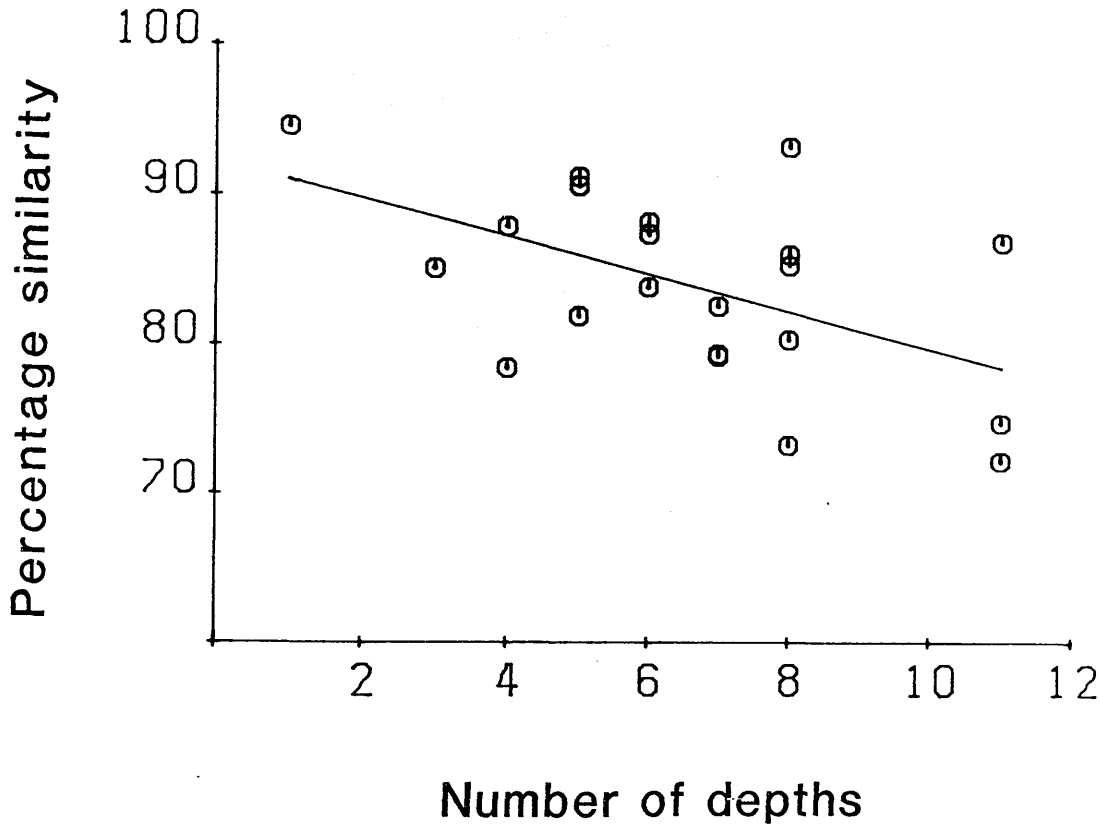


Figure 23

Relationship between the percentage similarity at which groups were clustered and the number of depths at which isolates in groups were found. Regression equation $y = -1.2249x + 92.2347$, Correlation coefficient (r) = -0.5065, P. = 0.02>P>0.01.

5- Interrelationships between similarity measure, number of isolates/group, number of groups, the coefficient of variation, and the number of single isolates

The distance measure used for computation in cluster analysis was the Euclidean distance squared. It has been transformed into percentage similarity (% S). The relationships between the percentage similarity and the number of isolates/group, the number of groups (including single isolates), the number of groups (excluding single isolates), the coefficient of variation and the number of single isolates were studied. The following paragraphs describe the results of these investigations.

Table 43 shows the percentage similarity of 50, 60, 72, 80, 90, 96, 97, 98, 99, 99.5, 99.9 and 100% and the Euclidean distance corresponding to each of these similarity levels. The total number of groups including single isolates (i.e. single isolates considered as groups) and the total number of groups excluding single isolates (i.e. single isolates not considered as groups) are also shown. It is important to note the effect of single isolates on the number of groups at each similarity level, therefore, the number of single isolates was determined at each level and these are also shown in this table.

As the percentage similarity increased, the number of isolates/group decreased (Figure 24, using data from table 44). This decrease occurred in almost linear way from 50 to 100% similarity. It was also interesting to note that standard deviation also decreased with an increase in percentage similarity.

The number of groups, including single isolates, increased exponentially with increasing percentage similarity (Figure 25, using

% S	Euclidean distance	Total No. groups including single isolates	No. groups excluding single isolates	No. single isolates
50	1.003	11	11	0
60	0.679	16	16	0
72	0.388	21	21	0
80	0.257	28	28	0
90	0.107	47	47	0
96	0.042	97	96	1
97	0.031	122	117	5
98	0.020	172	149	23
99	0.010	265	183	82
99.5	0.005	366	199	167
99.9	0.001	562	157	405
100	0.00	682	105	577

Table 43: Similarity levels ranging from 50 to 100% and equivalent Euclidean distances. The total number of groups, both excluding and including single isolates, is also shown as are the number of single isolates at each % similarity.

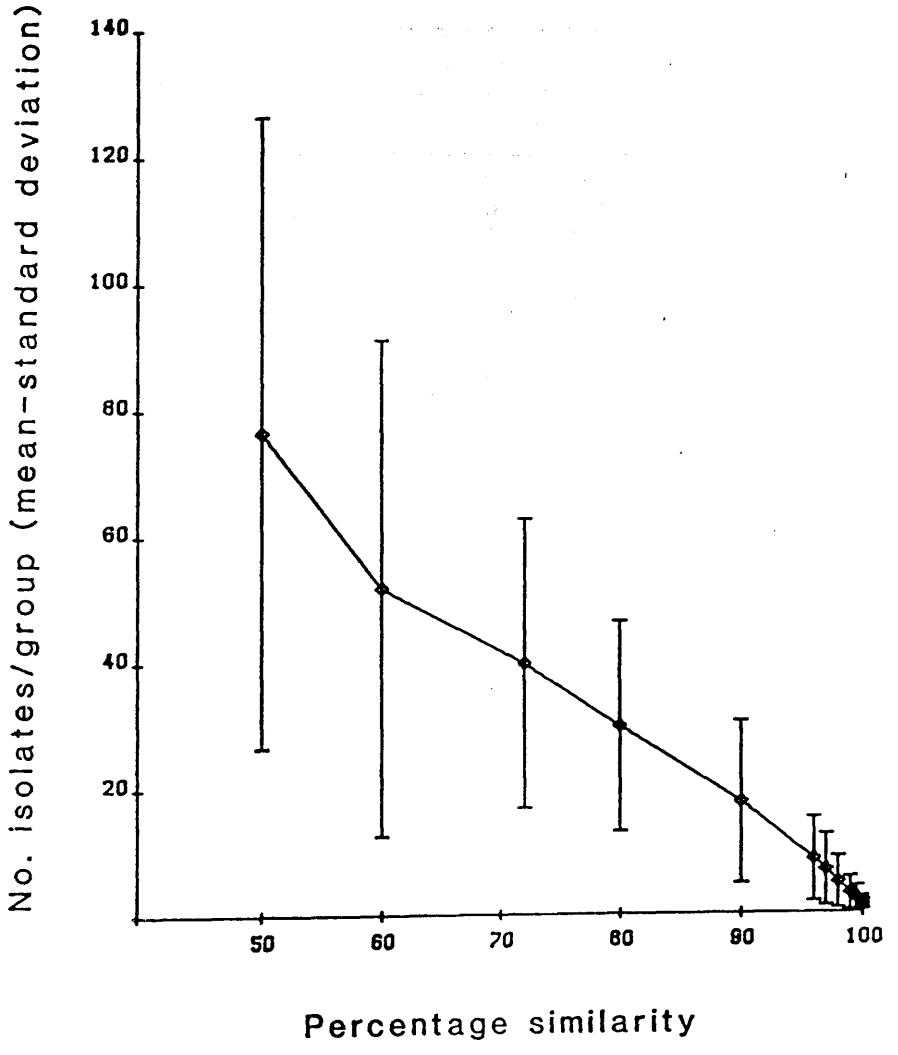


FIGURE 24

Relationship between No. bacterial isolates/ group (mean ± standard deviation) and percentage similarity.

% S	Euclidean distance	Log_{10} % S	Log_{10}(E.d+1)	Total No. groups incl. single isol.	No. isol./ group ($\bar{x} \pm$ s.d.)	Coefficient of variation
50	1.003	1.699	0.3017	11	76.64 ± 50.07	65.33
60	0.679	1.778	0.2251	16	52.69 ± 39.55	75.06
72	0.388	1.857	0.1424	21	40.14 ± 23.11	57.57
80	0.257	1.903	0.0993	28	30.11 ± 16.99	56.43
90	0.107	1.954	0.0441	47	17.91 ± 13.15	73.42
96	0.042	1.982	0.0179	97	8.670 ± 6.829	78.77
97	0.031	1.987	0.0133	122	6.910 ± 5.863	84.85
98	0.020	1.991	0.0086	172	4.901 ± 4.238	86.47
99	0.010	1.996	0.0043	265	3.177 ± 2.724	85.74
99.5	0.005	1.998	0.00217	366	2.301 ± 2.001	86.96
99.9	0.001	2.000	0.00043	562	1.500 ± 1.140	76.00
100	0.00	2.000	0.000	682	1.235 ± 0.7682	62.20

Table 44: Similarity levels and log_{10} similarity levels from 50 to 100%, and equivalent Euclidean distances and log_{10} Euclidean distances. Total number of groups including single isolates, number of isolates/group ($\bar{x} \pm$ s.d.) and coeffient of variation are also shown at each % similarity.

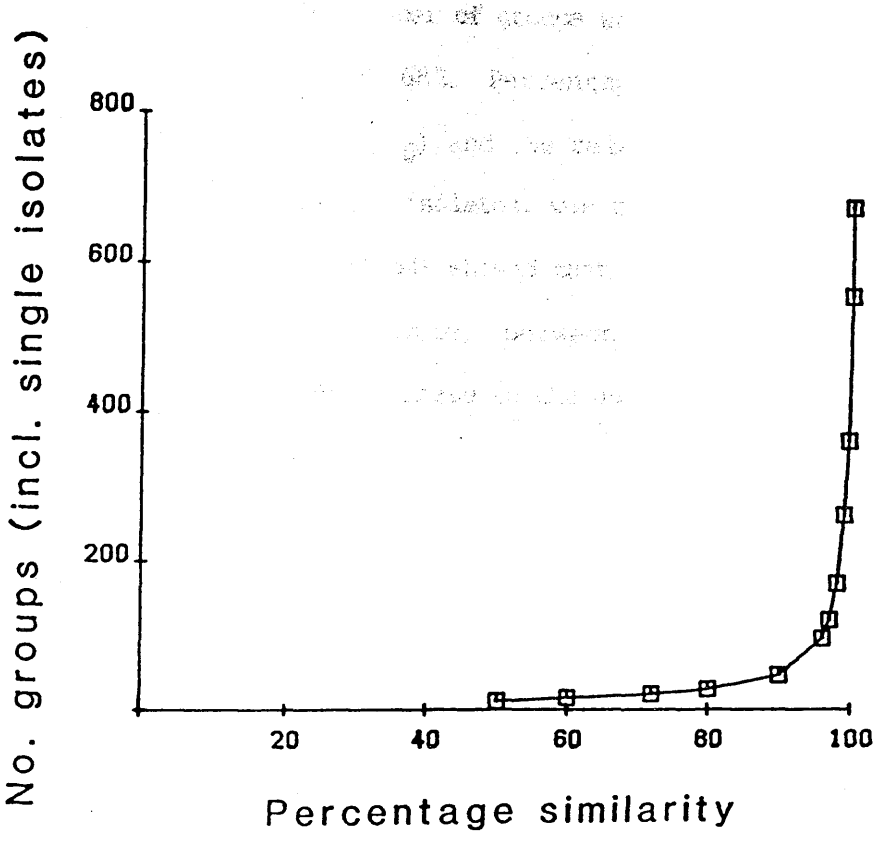


FIGURE 25

Relationship between No. groups clustered (including single isolates) and percentage similarity.

data from table 43). Between 50 and 90% similarity, the increase in the number of groups was gradual, but after 90% similarity it was very rapid. For instance, at 90% the number of groups was 47 but at 100% it had increased dramatically to 682. Percentage similarity was transformed into logarithm ($\log_{10}$) and the relationship with the number of groups, including single isolates, was plotted. The results (Figure 26, using data from table 44) showed that there was no change in this relationship. For example, between $\log_{10}$ percentage similarity 1.699 and 1.954 the increase in the number of groups was gradual but after 1.954 it was rapid.

The number of groups, excluding single isolates, increased with increasing percentage similarity to 99.5%, after which it decreased again (Figure 27A, using data from table 43). Between 50 and 90% similarity, the increase was relatively gradual but from 90 to 99.5% it was very dramatic. Figure 27B, shows the same data, but the percentage similarity axis has been expanded to allow the decrease in the number of groups after 99.5 to be examined more closely. It can be seen that the decrease in the number of groups occurring after 99.5% is even more dramatic than the increase to this similarity level.

The number of single isolates increased dramatically with increasing percentage similarity after 96% similarity (Figure 28A, using data from table 43). Up to 96% similarity, no single isolates were found. In figure 28B, the percentage similarity axis (x-axis) of figure 28A has been expanded to enable the increase in the number of [redacted] isolates after 96% similarity to be seen more clearly. Figure 28B, shows that between 96 and 98% the increase in the number of single isolates is gradual, but after 98% the increase becomes very rapid especially between 99.5 and 100% similarity.

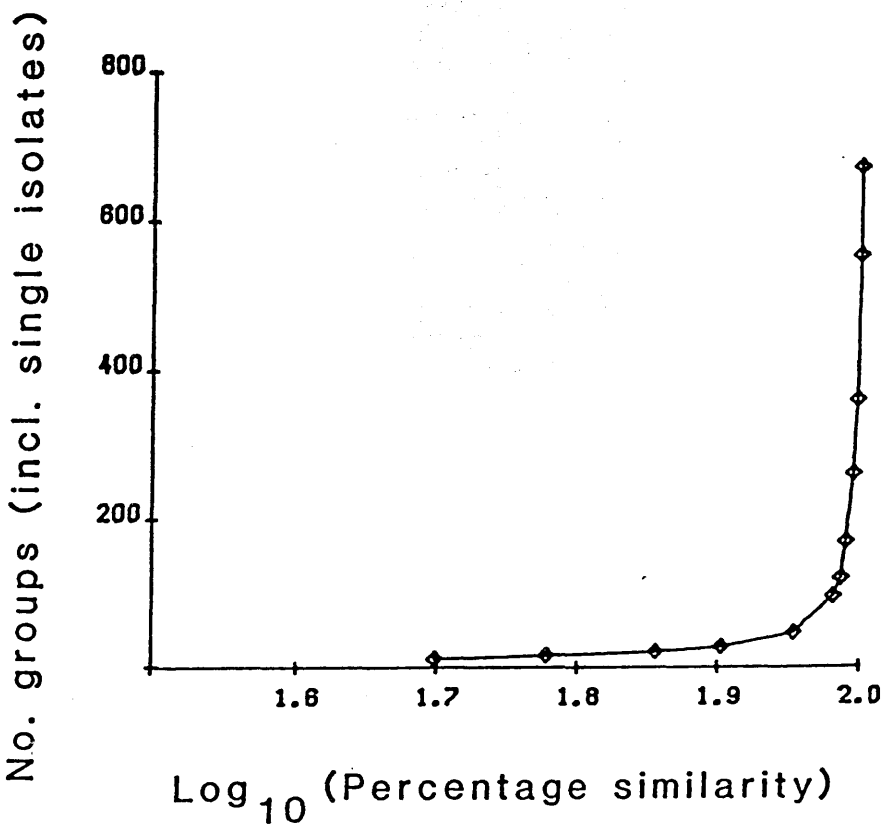


FIGURE 26

Relationship between No. groups clustered (including single isolates) and $\log_{10}$ percentage similarity.

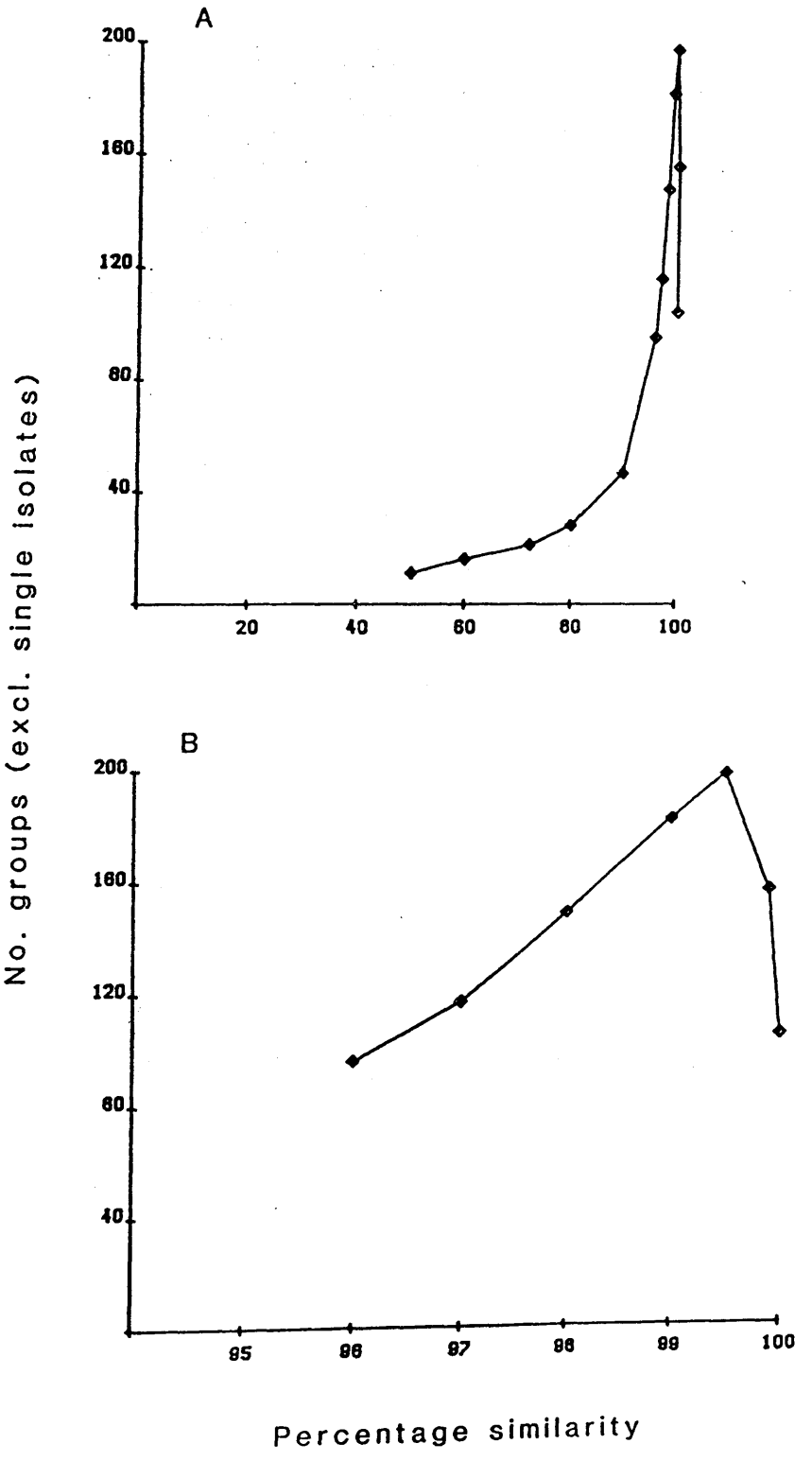


FIGURE 27

A- Relationship between No. groups (excluding single isolates) and percentage similarity.

B- Shows the peak on graph (A) on an expanded scale to improve resolution.

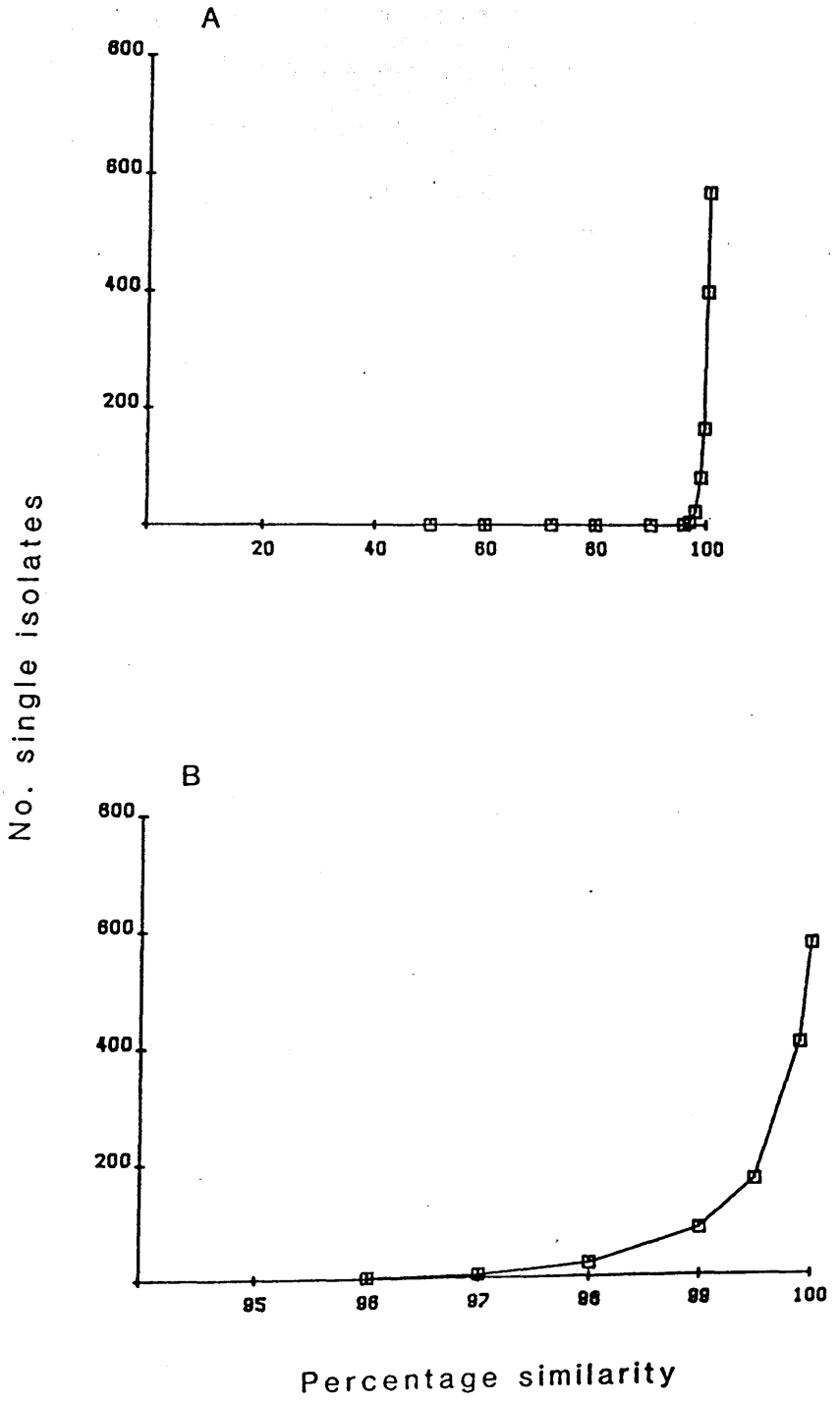


FIGURE 28

A- Relationship between No. single isolates and percentage similarity.

B- Shows the curve on graph (A) on an expanded scale to improve resolution.

The coefficient of variation fluctuated with percentage similarity (Figure 29, using data from table 44). In this figure, it can be seen that the data are bimodal i.e. have two peaks. These peaks occur at 60% and 99.5% similarity. After 99.5% there is a sharp decrease in the coefficient of variation to 100% similarity. At 72% and 80% similarity, there is a trough in the data.

Similar conclusions can be drawn from the Euclidean distance measure. This is to be expected because the similarity index is derived from the Euclidean distance squared. They are as follows.

As the Euclidean distance increased, the number of isolates/group increased (Figure 30, using data from table 44). This increase occurred in an almost linear way from 1.003 (50%S) to 0 (100%S). It was also interesting to note that standard deviation also increased with an increase in Euclidean distance.

Conversely, the Euclidean distance decreased exponentially with an increase in the total number of groups including single isolates (Figure 31, using data from table 43). Between 1.003 and 0.107 Euclidean distance, the increase in the number of groups was gradual, but after 0.107 it was very rapid. For example, at 0.107 the number of groups was 47, but at 0 Euclidean distance it had increased dramatically to 682. Euclidean distance was transformed into logarithm ($\log_{10}$) and the relationship with the number of groups including single isolates was plotted. The results (Figure 32, using data from table 44) showed that there was no change in the relationship. For instance, between $\log_{10}$ Euclidean distance (0.3017 and 0.0441), the increase in the number of groups was gradual but after 0.0441 it was very rapid.

Excluding single isolates, the number of groups clustered

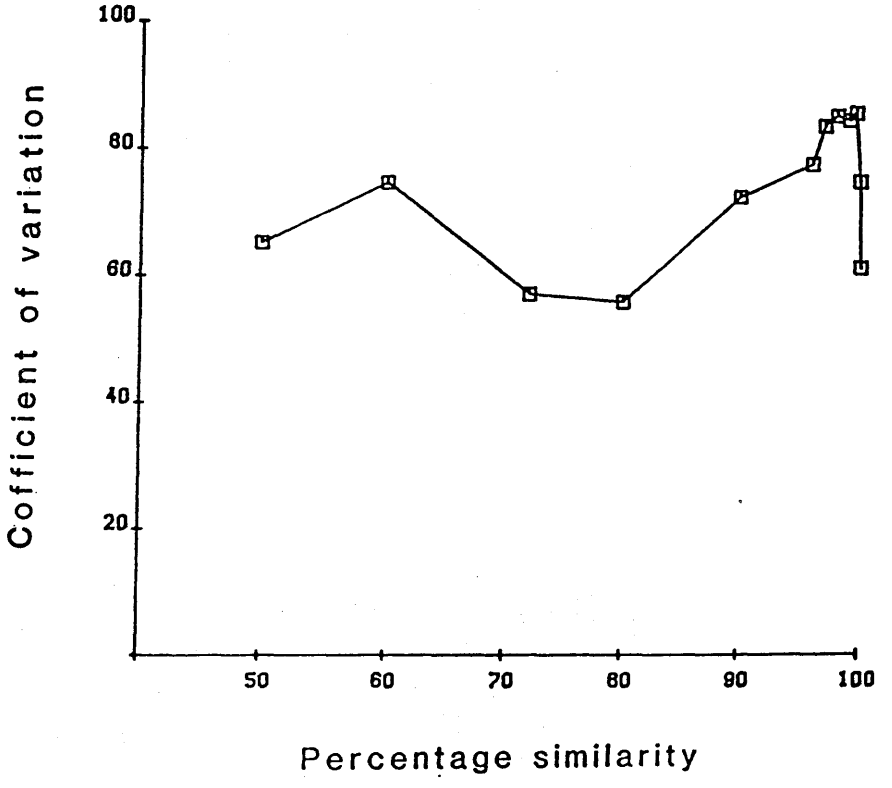


FIGURE 29

Relationship between coefficient of variation of No. isolates/ group and percentage similarity.

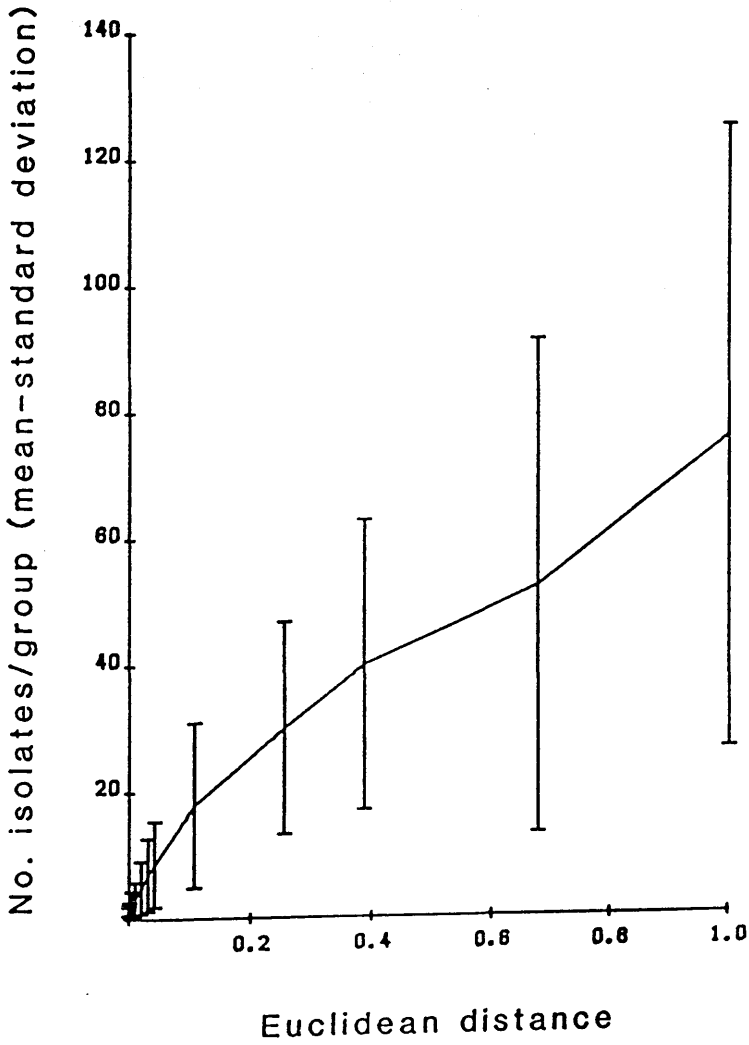


FIGURE 30

Relationship between No. bacterial isolates/ group (mean ± standard deviation) and Euclidean distance.

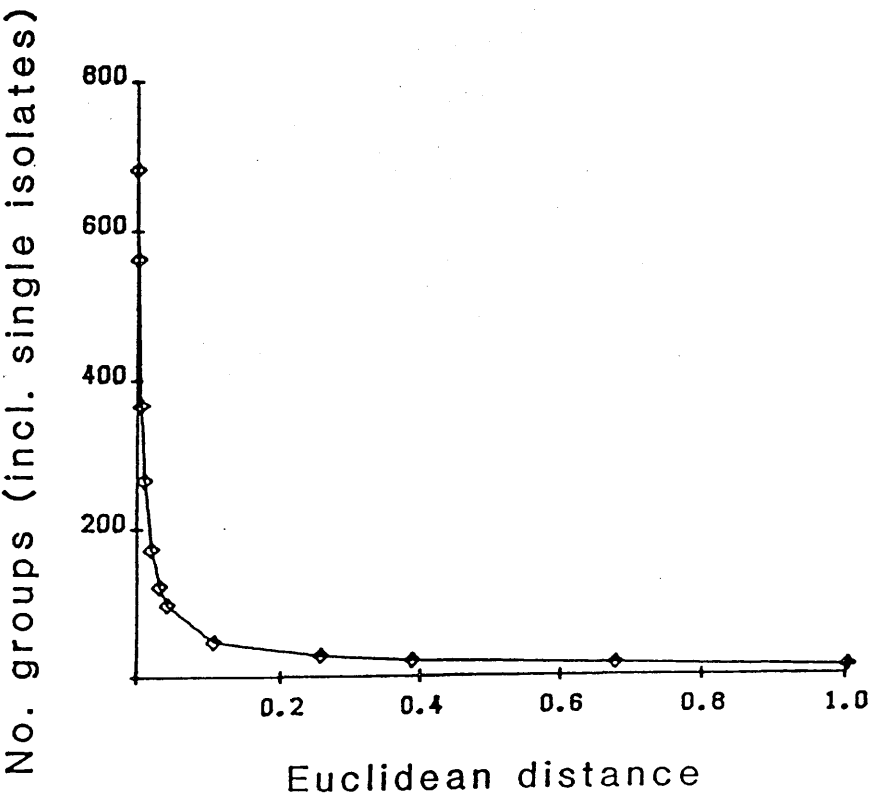


FIGURE 31

Relationship between No. groups clustered (including single isolates) and Euclidean distance.

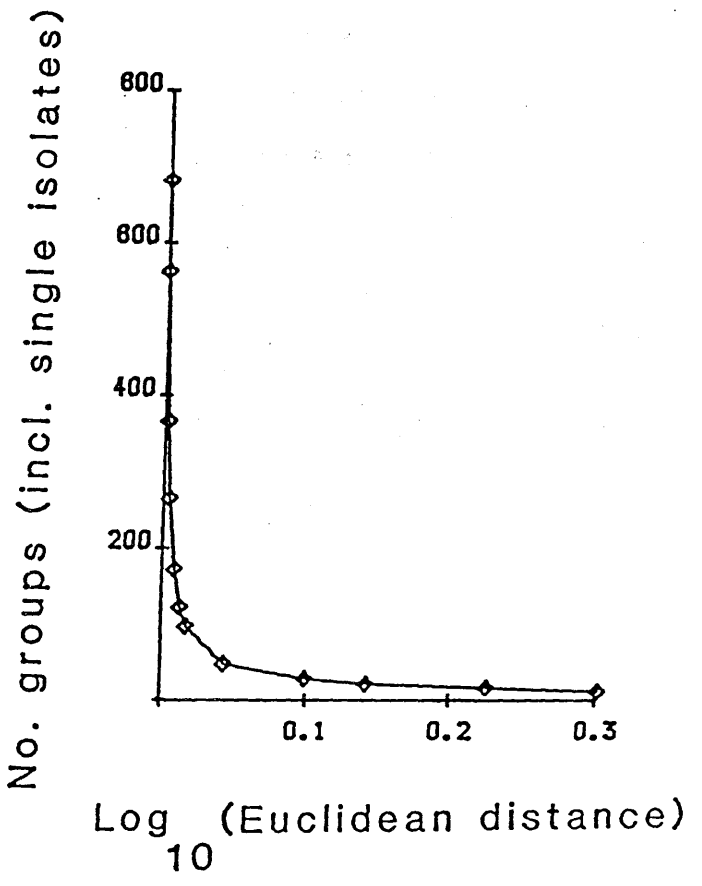


FIGURE 32

Relationship between No. groups clustered (including single isolates and $\log_{10}$ Euclidean distance.

increased with decreasing Euclidean distance to 0.005, after which it decreased again (Figure 33, using data from table 43). Between 1.003 and 0.107 Euclidean distance, the increase in number of groups was relatively gradual but from 0.107 to 0.005, it was very dramatic. Figure 33B, shows tha same data, but the Euclidean distance axis (x-axis) of figure 33A has been expanded to allow the decrease in the number of groups after 0.005 to be examined more closely. It can be seen that the decrease in the number of groups occurring after 0.005 is oven more dramatic than the increase to this Euclidean distance value.

The number of single isolates increased with decreasing Euclidean distance measure after 0.107 as shown in figure 34 and table 43. Up to 0.042 Euclidean distance, no single isolates were found. In figure 34B, the Euclidean distance axis (x-axis) of figure 32A has been expanded to enable the increase in the number of single isolates after 0.042 to be seen more clearly. Figure 34B, shows that Between 0.042 and 0.020, the increase becomes very rapid especially between 0.005 and 0.

The coefficient of variation fluctuated with Euclidean distance (Figure 35 and table 44). In this figure, it can be seen that the data are bimodal i.e. have two peaks. These peaks occur at the Euclidean distances 0.679 and 1.998. After 1.998 there is a sharp decrease in the coefficient of variation to 0 Euclidean distance. At The Euclidean distances 0.388 and 0.257, there is a trough in the data.

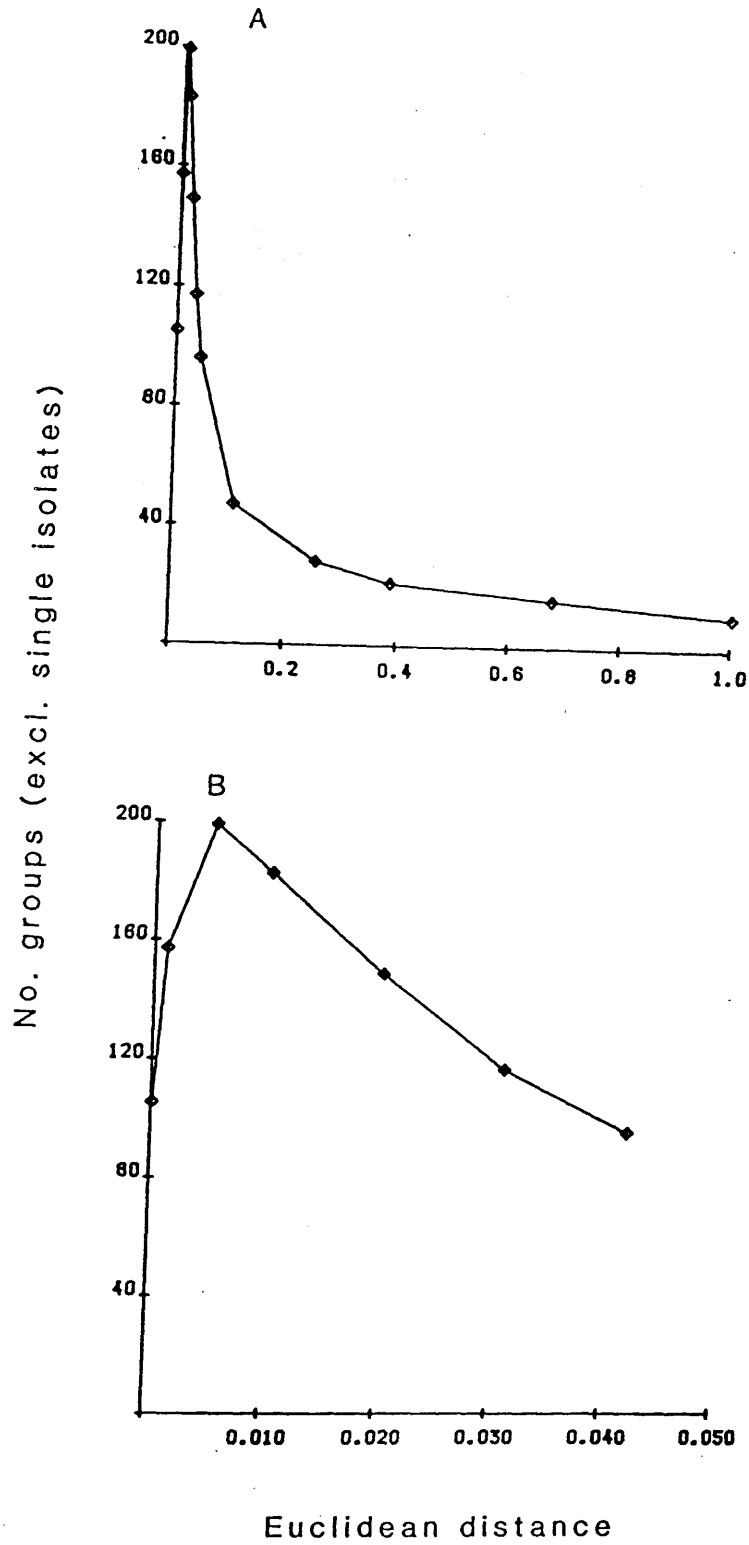


FIGURE 33

A- Relationship between No. groups (excluding single isolates) and Euclidean distance.

B- Shows the peak on graph (A) on an expanded scale to improve resolution.

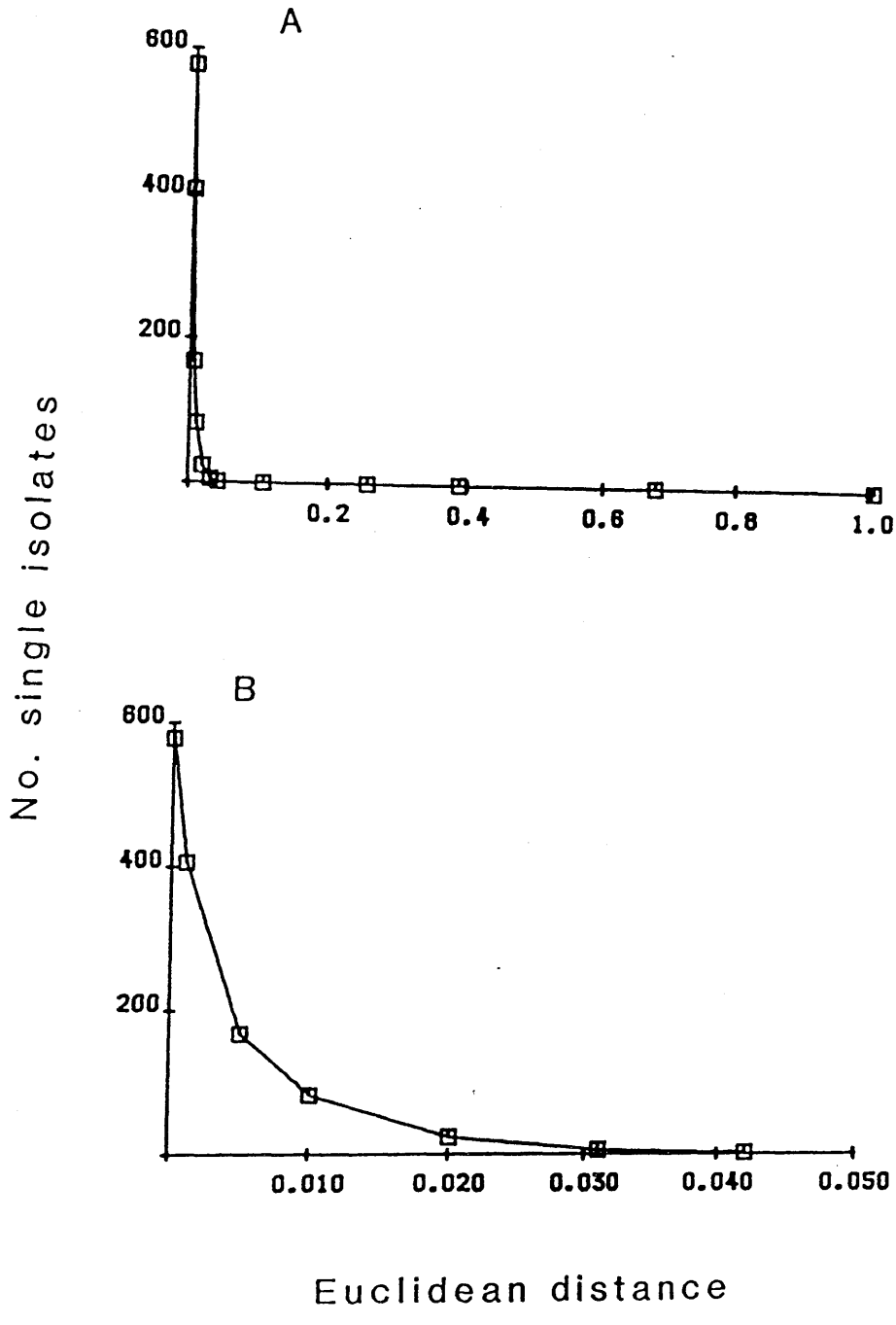


FIGURE 34

A- Relationship between No. single isolates and Euclidean distance.

B- Shows the curve on graph (A) on an expanded scale to improve resolution.

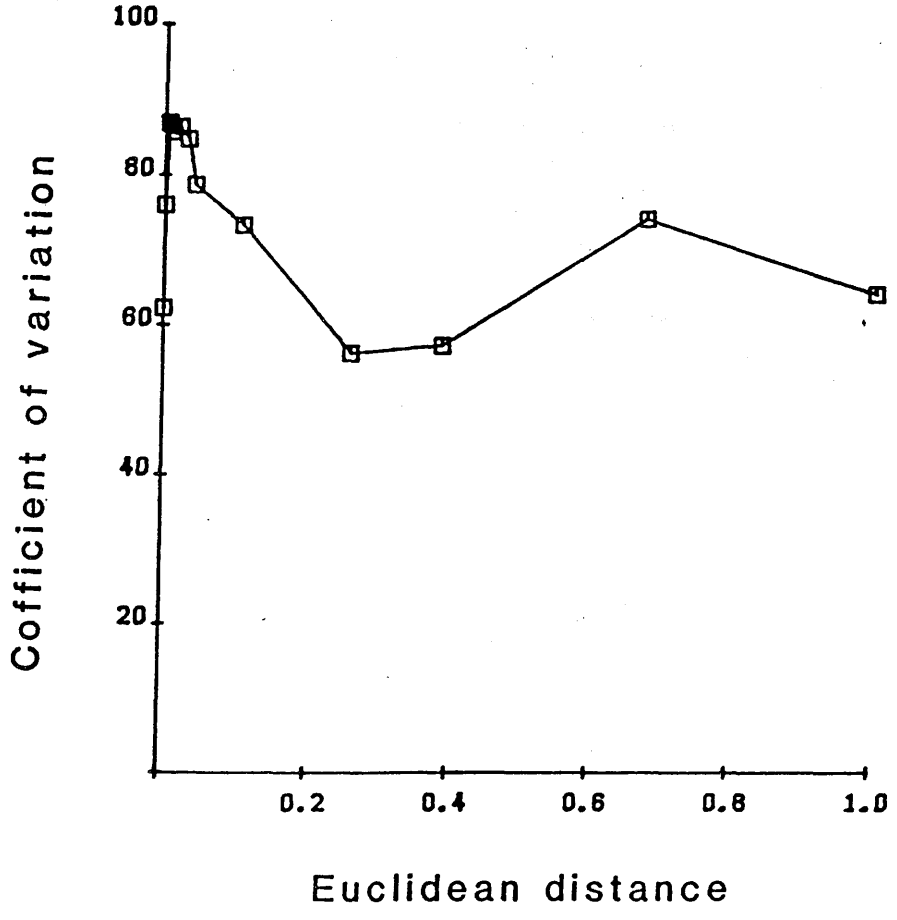


FIGURE 35

Relationship between coefficient of variation of No. isolates/ group and Euclidean distance.

6- Clustering of variables

The dendograms in figure 36 show clustering of antibiotics alone, metals alone and antibiotics and metals together. These dendrograms were drawn using the matrix data generated by the computer. An example of calculating the correlation coefficient matrix between clusters of heavy metals is shown in appendix table 29. Pictorial representaion of the relationship between clusters of antibiotics and heavy metals together is shown in figure 37. This was redrawn from the diagrams generated by the computer. An example of clustering of heavy metals alone is shown in appendix table 30. Clustering of antibiotics showed that there were two distinct clusters: cluster 1 contained two antibiotics, polymyxim B and nalidixic acid with an overall correlation of 0.4540; cluster 2 contained three antibiotics, streptomycin, tetracycline and ampicillin with an overall correlation of 0.3645. These two clusters joined together at a correlation of 0.3643. Methylene blue and chloramphomical were unclustered.

Cluster analysis of metals showed that there was probably one cluster formed at the 0.4023 correlation level, which contained three metals, mercury, copper and lead. Manganese, cadmium and chromium were unclustered.

It was concluded from the dendrogram showing cluster analysis of all antimicrobial agents, that there were three distinct clusters. Cluster 1 formed at correlation level of 0.3650 and contained polymyxin B, nalidixic acid and chromium. Cluster 2 included the antibiotics, streptomycin, tetracycline and ampicillin. These clusters formed at a correlation level of 0.3645, and joined cluster 1 at 0.3643 correlation level. Cluster 3 included five antimicrobial agents, mercury, copper, lead, methylene blue and manganese. This cluster formed at 0.3331 correlation level and joined the clusters 1

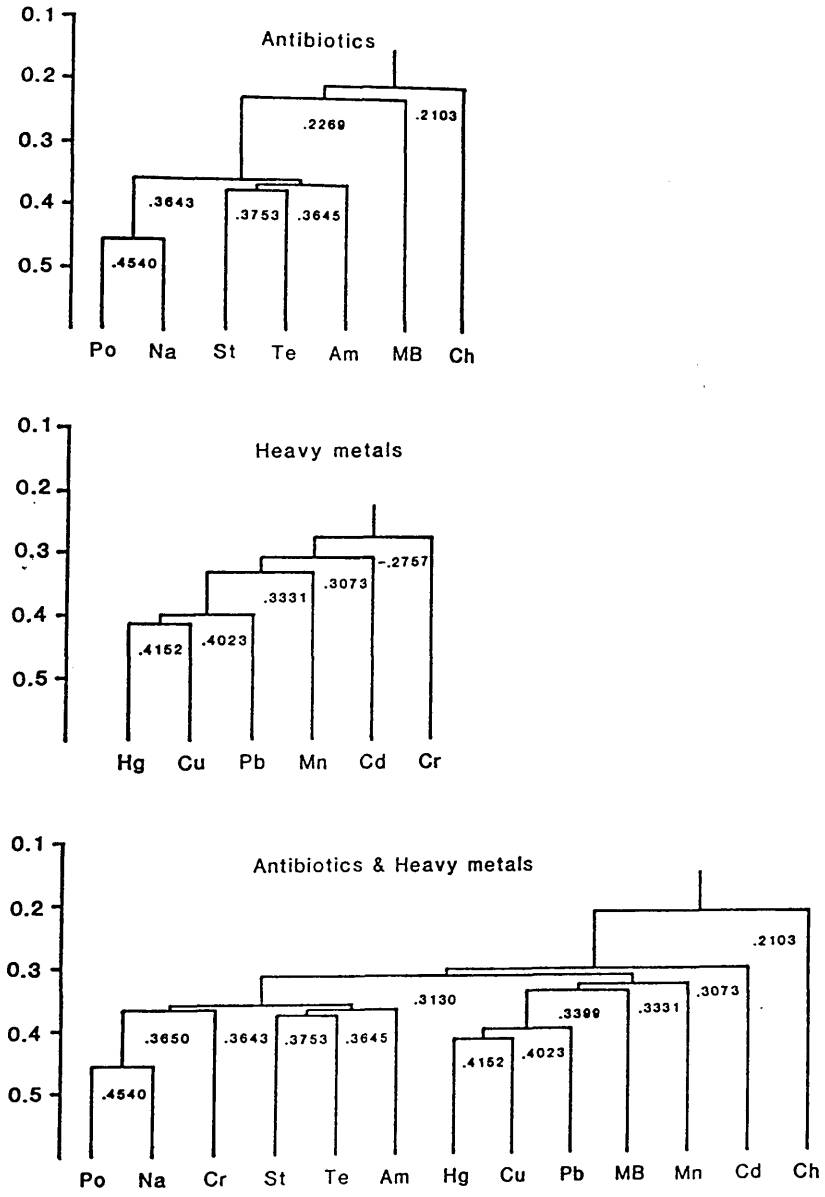
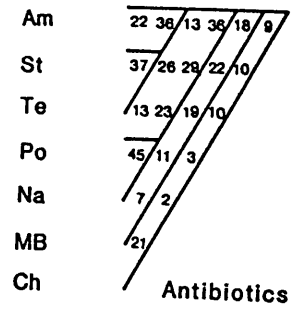
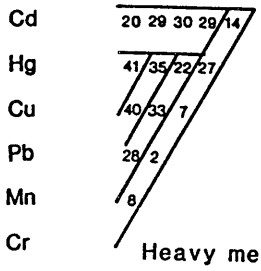


Figure 36

Simplified dendrogram showing the relationship between clusters of antibiotics alone, heavy metals alone and antibiotics and metals together based on the correlation coefficient and single linkage method of clustering (BMDP1M - cluster analysis of variables). These dendrogram were drawn using the matrix data generated by the computer. An example of calculating the correlation coefficient between clusters of heavy metals is shown in appendix table 29. Numbers on the dendrogram represent the correlation coefficients between clusters.





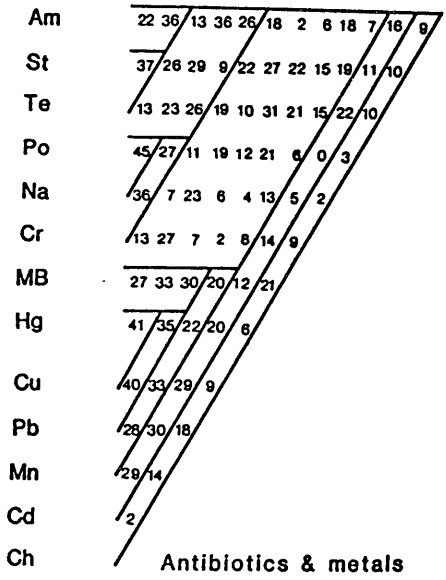


Figure 37

Pictorial representation of the relationship between clusters of antibiotics alone, heavy metals alone and antibiotics and metals together based on the correlation coefficient and single linkage method of clustering (BMDP1M-cluster analysis of variables). These were redrawn from the diagrams generated by the computer. An example of clustering of heavy metals alone is shown in appendix table 30. The values in these diagrams are the correlation coefficient scaled 0 to 100 according to the table shown on p. 302 .

and 2 at a correlation level of 0.3130. Only cadmium and chloramphenicol were unclustered.

SECTION 1

DISCUSSION

The discussion in this section is divided into the following parts:

1- Antimicrobial agents sensitivity.

A- Preliminary experiments.

B- definitive experiments.

2- Cluster analysis.

A- Cluster analysis at 50% similarity level.

(i) Variation in the number of isolates between groups, between sites, and between depths.

(ii) Comparison of variances between sampling sites and depths for each group.

B- Cluster analysis at 72% similarity level.

3- Statistical analysis of clusters formed at 72% similarity level.

A- Variation in the number of isolates between groups, between sites and between depths.

B- Comparison of the number of isolates between sampling sites and between depths.

C- Comparison of the number of isolates obtained from sediment, burrow linings and faecal pellets.

4- Clustering of variables.

1- Antimicrobial agents sensitivity

A- Preliminary experiments

Characterisation of bacteria according to their susceptibility to antimicrobial agents has been carried out by many investigators (Corlett et al., 1965; Gilardi, 1971; Colwell and Kettling, 1974; Friedman and Maclowry, 1973; Darland, 1975; Sielaff et al., 1976; Timoney et al., 1978; Jones, 1986). Resistance and sensitivity of microbial populations varies according to the sources. For example, Timoney et al. (1978) found that populations of resistant Bacillus sp. were much greater in sediments contaminated with high concentrations of mercury and other heavy metals than in sediments containing low levels of heavy metal pollutants.

Bacterial populations can be divided into groups on the basis of their relative susceptibility to various antimicrobial agents. For example, Pratt and Reynolds (1974) studied methylene blue and polymyxin B as potentially effective agents for characterising marine bacterial populations. The methylene blue selected for oxidative oxidase-positive motile rods and against fermentative oxidase-positive rods. The antibiotic polymyxin B selected for some non-pigmented fermentative bacteria and for pigmented (yellow, orange, pink) non-fermentative Gram-negative rods. Pratt and Reynolds (1974) also made the important observation that pigmented bacteria failed to grow on media without polymyxin and were inhibited by the growth of polymyxin-sensitive non-pigmented rods.

In general, the trend obtained with the metals and antibiotics was a decrease in the percentage of bacterial isolates growing on media containing increasing concentrations of antimicrobial agents (see p. 55 and 56, Figures 7 and 8). This trend was not always clearly evident in the preliminary study (e.g. ampicillin, streptomycin, nalidixic acid and methylene blue, figure 8), probably

for two reasons. Firstly, the order of inoculation was from the highest concentration to the lowest concentration; this probably resulted in a slightly higher inoculum being present on the former plates. Secondly, the presence of moisture on the plates may have had the effect of lifting more cells off of the velvet and hence led to a higher inoculum. These two factors probably caused variation in the density of the inoculum and this in turn produced inconsistent results (p. 56, Figure 8, ampicillin and nalidixic acid). To alleviate the effect of moisture, the plates were dried at 37°C for one hour in all the definitive experiments according to Cruickshank et al. (1975). Drying the plates produced reliable relationships between antimicrobial agent concentrations and the percentage of bacterial isolates growing. Therefore the first hypothesis (i.e. order of inoculation) was not important.

In the preliminary series of experiments, different patterns of resistance to metals were observed. For example, chromium and mercury showed a gradual decline in the number of isolates growing at higher concentrations of metal, while cadmium, copper and lead showed a rapid reduction between one concentration and the next (p. 55, Figure 7). These two problems were solved in the definitive experiments by increasing the concentration or expanding the range of concentrations of metals where appropriate (see materials and methods, p. 24).

Only two of the antibiotics in the preliminary study (polymyxin B and chloramphenicol) proved to be successful in discriminating between bacterial isolates under test conditions. It appears from the preliminary experiments that concentrations of most of the antibiotics were not high enough to inhibit a significant proportion of isolates. This problem was solved by extending the range

of concentrations showed no discrimination between the isolates (see materials and methods p. 24). When the antibiotic concentrations were increased in the definitive experiments, more discrimination between the isolates was shown (see figures 9, 10, 11, and 12).

The random fluctuation shown by the dye methylene blue might be interpreted as indicating that this selective agent is not suitable for differentiating between isolates in the replica plate method used in my study. Pratt and Reynolds (1974) noted a similar effect. They used a modified disk-diffusion method and showed that methylene blue inhibited the growth of 15 out of 20 cultures of marine bacteria and that the degree of inhibition varied between the cultures. The fact that Pratt and Reynolds and I both obtained similar effect using different techniques is important, because it shows variability is independent of a particular technique.

B- **Definitive experiments**

The choice of concentrations of antimicrobial agents used to divide bacterial communities varies considerably in the published literature (Tables 2 and 3). The concentrations of antibiotics used by different authors ranges from 10mg/l to 100mg/l, and of metals from 0.002mg/l to 3200mg/l. I chose concentrations in my experiments that fell within those reported in the literature (see materials and methods p. 21 and 22).

Antibiotics

The trend found in my experiments was that the number of isolates grown on selective media decreased with increasing antibiotic concentrations. McNicol (1980) studied isolates from samples collected at the Jones Falls, Eastern Bay, Bloody Point and Chesapeake Beach stations of upper Chesapeake Bay during 1978-79 cruises on the R. V. Ridgely Warfield. The author employed antibiotics and heavy

metals using three different methods: direct plating, replica plating, and minimum inhibitory concentration (MIC). All three methods showed similar qualitative results. Resistant and sensitive organisms could be distinguished by a concentration of 20ug/ml for the antibiotics ampicillin, chloramphenicol, nalidixic acid, penicillin, streptomycin and tetracycline. The concentration of 20ug/ml of antibiotics chosen by McNicol as the best dose to use to divide natural bacterial populations into sensitive and resistant strains is within the range of concentrations I used in my study.

Some bacteria grew even at the highest concentration of antibiotics that I used. These therefore, are by definition resistant isolates in my experiments. The reasons for resistance to antibiotics are discussed in the appendix A p. 221.

Figures 9, 10, 11, and 12 (p. 58, 59, 60, and 61 respectively) show that prolonged incubation time often led to an increase in the number of bacteria grown on antibiotic selective media. This increase in growth which is quantified in tables 8 and 10 (p. 66 and 72) may be due to the increase of the lag phase period caused by the effect of high concentrations of antibiotics.

The antibiotics chloramphenicol and polymyxin B inhibited the growth of large numbers of isolates, and appear to be the most toxic antibiotics. McNicol (1980) (see above) found that chloramphenicol and tetracycline were the most inhibitory drugs and streptomycin was the least inhibitory. This author did not study the antibiotic polymyxin B. I found that chloramphenicol and polymyxin B were the most effective antibiotics followed by tetracycline, ampicillin, streptomycin, and nalidixic acid (Table 11, p. 74). McNicol and I therefore agree that chloramphenicol is the most toxic antibiotic.

Washington (1969) studied the antimicrobial susceptibility of clinical Enterobacteriacea and non-fermenting Gram-negative bacilli

This author found that at 20ug/ml, chloramphenicol inhibited 90% or more of Escherichia coli, Salmonella, Citrobacter, Proteus mirabilis, Pseudomonas maltophilia, and Pseudomonas pseudomallei. I found that 16mg/l of chloramphenicol inhibited 100% of my isolates in master replica 1 (i.e. 0% growth). Washington (1969) also found that polymyxin B was very active against members of the family Enterobacteriaceae with the exception of Arizona, Serratia, and species of Proteus other than Proteus mirabilis and Proteus providence. 93% of Pseudomonas aeruginosa were inhibited by 5ug/ml and 52% of Pseudomonas maltophilia were inhibited by 20ug/ml. In contrast, I found that 16mg/l inhibited 57.1% of the isolates in master replica 1 (i.e. 42.9% growth) and 4mg/l inhibited 48.6% of the isolates (i.e. 51.4% growth) (see appendix table 3). From Washington's results, it appears that the degree of activity of polymyxin B varied between the species of Pseudomonas, because 5ug/ml inhibited 93% of Pseudomonas aeruginosa while 20ug/ml inhibited only 52% of Pseudomonas maltophilia.

Heavy metals

The actual concentrations at which the inhibition of microbial growth occurs varies according to the organism, the metal and the chemical and physical composition of the medium. For example, for any given metal the toxic concentration for different organisms may vary over a wide range. Thus with copper, the inhibitory concentration of the following species are: green algae ($1x10^{-7}M$); Pseudomonas, a common soil bacterium ($5x10^{-4}M$); Ferrobacillus (0.2M) and some strains of fungi (1.0M), (Sadler and Trudinger, 1967).

It is also well known that many bacterial isolates are resistant to heavy metals such as mercury, copper, cadmium, chromium, lead, nickel, cobalt and arsenic (Smith, 1967; Hedges and Baumberg,

1973; Summers and Lewis, 1973; Walker and Colwell, 1974; Doyle et al., 1975; Groves and Young, 1975; Mitra et al., 1975; Baldry et al., 1977; Fujiwara et al., 1977; Nakahara et al., 1977; Summers and Jacoby, 1978; Summers et al., 1978; Lighthart, 1980; Alking et al., 1982; Olson and Thornton, 1982; Bopp et al., 1983; McEntee et al., 1986; Trevors et al., 1986).

In general, mercury and silver are the most toxic metals and manganese and zinc are the least toxic. Sadler and Trudinger (1967) found that mercury, cadmium, lead, ferrous iron, copper and zinc have decreasing toxicity to Escherichia coli in that order. Duxbury (1981) tested the order of toxicity of several heavy metals toward bacteria obtained from soil situated in bushland 180km south of Sydney. The author had suggested that metals tested probably fall into broad categories of toxicity: high toxicity e.g. mercury, intermediate toxicity e.g. cadmium, and low toxicity e.g. cobalt, copper, nickel and zinc. Lester et al. (1979) studied the influence of 50mg/l of cadmium, chromium, copper, and lead on bacterial species originally isolated from activated sludge. The authors found that the response of population was found to vary for each metal and the most toxic metal was copper followed by cadmium, lead and chromium. Lighthart (1980) states that the extent to which cadmium inhibits growth is dependant on the bacterial and chemical species involved. This author found that Escherichia coli inhibition was a function of the cadmium-ion concentration and irrespective of the presence of citrate as a medium component. However, with Pseudomonas sp., the cadmium inhibition was a function of both the cadmium-ion and the presence of citrate. With no citrate present, inhibition of this organism occurred only at relatively high cadmium-ion concentrations (above 10^{-4}M). However, when citrate was added to the same cadmium-containing medium,

inhibition was observed at lower cadmium-ion concentrations (10^{-7}M).

My results show that the order of toxicity of heavy metals is: mercury, cadmium, copper, chromium, lead, and manganese (Table 11). This agrees with Sadler and Trudinger (1967) who found that mercury and cadmium are very toxic to microorganisms, but they and I disagree about the order of copper and lead.

My results also agree with Duxbury and Bicknell (1983) who tested the toxicity of various metals towards bacterial populations from natural and metal polluted soils. They found that mercury, cadmium and copper have decreasing toxicity in that order. This is the same order as in my experiments. Bitton and Freihofer (1978) found that copper was much more toxic to Klebsiella aerogenes than cadmium. This may have been due to the very strong binding of copper by yeast extract (Ramamoorthy and Kushner, 1975). Ramamoorthy and Kushner studied the binding of heavy metal ions to a number of bacterial growth media. They found that all media bound large amounts of Hg^{2+}, Pb^{2+}, and Cu^{2+}, but much less Cd^{2+}. 80ppb or less remained as free cations in the solution after adding 20ppm of Hg^{2+}, Pb^{2+}, or Cu^{2+}. This might suggest that such ions enter bacterial cells as organic complexes, or that bacterial cells can compete successfully with growth media for bound ions. An earlier study was made by Abelson and Aldous (1950) who reported that the order of decreasing toxicity of nickel, cobalt, cadmium, zinc and manganese for Escherichia coli was in that order. They also reported that the toxicity of these cations is lowered in the presence of magnesium. If magnesium is not present in the medium, these elements are toxic at very low levels.

Adaptation of bacteria to heavy metals is more likely than adaptation to antibiotics since the chances of previous exposure to these metals in the form of heavy metal pollutants are greater than the chances of previous exposure to antibiotic pollutants -even in the

deep-sea (Pfister and Burkholder, 1965).

Tornabene and Edwards (1972), Vaituzis et al. (1975), and Timoney et al. (1978) are examples of papers on the adaptation of bacteria to heavy metals. Tornabene and Edwards (1972) found that Micrococcus luteus and Azotobacter sp. immobilized large quantities of lead in media containing lead salt. The authors also found that fractions of cell wall and cell membrane contain 99.3 and 99.1% of the lead associated with Micrococcus luteus and Azotobacter sp. respectively. The remaining lead is found associated with the cytoplasmic fractions. Vaituzis et al. (1975) found that most cultures which adapt to growth in the presence of $HgCl_2$ exhibit extensive morphological changes. They suggested that in the presence of toxic levels of mercury, reduction of the mercury concentration to a definit threshold level, either by metabolic or chemical volatilization, permits growth of the organism. Timoney et al. (1978) found that the number of resistant Bacillus isolates was much greater in sediments containing high concentrations of mercury and other heavy metals than in sediments from areas where heavy metal concentrations were found to be low.

The bacterial isolates from natural environments may already be adapted to antimicrobial agents because they have encountered them in the field, or show progressive adaptation during exposure to antimicrobial agents in the laboratory. Since the chances of previous exposure to metals in the form of heavy metal pollutants are greater than that of antibiotics, I would expect my isolates to be adapted to heavy metals but not to antibiotics when collected. My results with heavy metals show that there was no general increase in the percentage of isolates growing with increasing incubation time (Figures 13 and 14). This suggests that bacteria may have adapted to heavy metals in

deep-sea (Pfister and Burkholder, 1965).

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the field. In contrast, the extended lag phase observed in my results with antibiotics (Figures 9, 10, 11, and 12) suggests that bacteria may have adapted to these antibiotics in the laboratory.

Zevenhuizen et al. (1979) explained the extended lag phase as a process of competition between adaptation and death. Mitra et al. (1975) found high accumulations of the cadmium ion in Escherichia coli cells when a concentration of 3×10^{-6}M was used. They observed that Escherichia coli exhibited a physiological adaptation mechanism to cadmium, which is indicated by an extended lag phase. Mitra and his associates explaned lag phase as a reversible process of accomodation to the presence of Cd^{2+} during which cells required viability involving the exclusion of the ion from the cell and reversal of damage caused by prior exposure to the ion. More recently, Alking et al. (1982) made similar observations on Klebsiella aerogenes. The authors concluded that on solid media containing different concentrations of cadmium, adapted cells of Klebsiella aerogenes showed a far greater tolerance to cadmium than did unadapted cells (not previously exposed to cadmium). Kim (1985) observed that the inhibition of growth of natural bacterial populations was stronger in 1m subsurface water than in the neuston (neuston are those plankters that live attached within about 100-1000 um of the air-sea interface; includes some bacteria, protozoa, phytoplankters, and insects; Levinton, 1982; Meadows and Campbell, 1987). Several possible reasons were given for this, one reason was the enrichment of organic substances in the surface microlayers may have resulted in the formation of complexes with heavy metals and thus decreased their toxicity. A second reason was the bacteria may have adapted to the continuous presence of heavy metals in the surface microlayers.

2- Cluster analysis

The taxonomy of ecologically important species can be described by application of a number of numerical procedures to taxonomic data gathered for bacteria isolated from the natural environment. Cluster analysis is one procedure that is being used more frequently in recent studies, and has been found to be very useful for studying large collections of unidentified bacteria. Most studies have been concerned with medically important species (Fontaine et al., 1986; Pretorius et al., 1986). However, a number of workers have used multivariate methods (e.g. cluster analysis) to group together bacteria from a range of aquatic environments.

Colwell and Liston (1961) were the first workers applied adansonian principles to marine bacteria. Adansonian principles that based on the early ideas of Adanson (1727-1806) (Everitt, 1980 p. 4). In this method all characteristics are given equal weight and division into taxa is based on the correlated features. Colwell and Liston (1961) applied this technique to derive a taxonomy of the Pseudomonas-Achromobacter group of Gram-negative, asporogenous , rodlike bacteria. They found that 54 out of 58 Pseudomonas strains tested in an analysis of 80 bacterial cultures clustered into 4 large groups. The validity of the general method was tested by an analysis of 40 well defined strains representative of different genera and families of the Pseudomonadales and the Eubacteriales. The analysis clearly separated groups which are recognized to be taxonomically distinct. Floodgate and Hayes (1963) applied adansonian classification to analyse the morphological, cultural, and biochemical features of 62 yellow pigmented marine bacteria. The authors found that in terms of the overall similarity, the organisms examined fell into two large groups. Hansen et al. (1965) used cluster analysis to perform a

quantitative taxonomic analysis for 20 strains of marine Pseudomonas piscicida bacteria. Pfister and Burkholder (1965) applied cluster analysis to bacteria isolated from Antarctic and tropical seawaters near Puerto Rico. The results of these analyses showed that 9 groups were distinguished. Quigley and Colwell (1968) used cluster analysis to identify bacteria from deep-sea sediment samples collected at depths from 9,400 to 10,400m in the Philippine and Marianas Trench of the Pacific Ocean. Colwell and Kettling (1974) used cluster analysis to study the hydrostatic pressure tolerance of deep-sea bacteria isolated from a sediment collected in the Virgin Island Basin of the Atlantic Ocean at a depth of 4000m. The taxonomic analysis showed that isolates clustered into two groups and identified as Vibrio and Acinetobacter. Boeye and Aerts (1976) isolated 138 strains from North sea sediments and grouped them into six groups using cluster analysis. Austin et al. (1977) clustered and presumptively identified bacteria from Chesapeake Bay based on their resistance to specific heavy metals. Mallory et al. (1977) subjected 162 strains of bacteria from Chesapeake Bay water and sediment to taxonomic analysis and were able to assign some species to particular areas based on their nutrient requirements. Baya et al. (1986) clustered and identified bacteria from polluted and unpolluted Atlantic Ocean samples based on their resistance to antibiotics. These studies show that the application of cluster analysis and similar techniques to marine isolates gives realistic clusters, and that these clusters can be useful in analysing the different types of bacteria in a range of marine environments.

In my study, I combined the selective agents (antibiotics and heavy metals) of Austin et al. (1977) and Baya et al. (1986) and clustered my isolates with regard to their resistance to both antibiotics and heavy metals.

A- **Cluster analysis at 50% similarity**

(i) **Variation in the number of isolates between groups, between sites and between depths**

The results of Chi-square tests to compare the number of isolates between sites for each group (Table 16), groups for each site (Table 17), depths for each group (Table 18), and groups for each depth (Table 19) showed that, in each case, highly significant variation was found. This means that the isolates in each of the 11 groups are not distributed randomly between the sites and between the depths. This also means that isolates at each of the sites and depths are not distributed randomly between the groups. Cluster analysis of bacteria based on antibiotic and metal tolerance produces groups containing species that are physiologically similar with respect to these factors. One would expect to find that each bacterial group is adapted to different physico-chemical and nutritional parameters. These parameters are likely to be affected by differences in sediment properties such as particle size, organic carbon level, and Eh, between sites and between depths. It is known that these properties vary between sampling sites and with sediment depth, both in estuaries (Moshiri and Crumpton, 1978) and in the deep-sea (Meadows and Tait, 1985). The significant differences in the numbers of isolates between groups, sites and depths, found in my study, may have been due to the variation in these sediment properties.

(ii) **Comparison of variances between sampling sites and depths for each group**

Tables 20 A and 21 A show for each group the mean, standard deviation and the variance of number of isolates/site and number of isolates/depth respectively. The variances of number of isolates in both tables were analysed in two ways. Firstly, to see whether the

overall variances were homogeneous between the groups. Secondly, to compare the variances of number of isolates for each pair of groups for both sites and depths.

Firstly, I used Bartlett's and F-max tests in order to determine the homogeneity of variances between groups for sites and depths. In other words for the 11 groups, are the numbers 1.371, 7.546, ... 5.272, 1.272 (Table 20 A column 3, sites) significantly different from each other ? and also are the numbers 1.598, 4.211, ... 2.541, 0.6153 (Table 21 A column 3, depths) significantly different from each other ? The results of Bartlett's Chi-square and F-max tests for both sites and depths are shown in tables 20 B and 21 B respectively where it can be seen that there were no significant differences between overall site variances and between overall depth variances for the 11 groups.

Secondly, I used the variance ratios to compare the variances of number of isolates/site or /depth between different groups. For example, in table 20 A (sites), the variance in the number of isolates in group 1 (1.371) was compared with the variance in the number of isolates in group 2 (7.546) and then the variance of group 1 (1.371) was compared with the variance of group 3 (1.578) and so on. The same procedures were carried out with depths. The results of all site and depth comparisons are shown in table 22. With sites, it was found that only groups 2 and 4 were significantly different (2% of comparisons). With depths however, it was found that significant differences occurred in 10 out of 55 comparisons i.e. 18% (significant differences were found between groups 4 and 2; 5 and 2; 3 and 2; 9 and 3; 6 and 2; 11 and 2; 10 and 3; 9 and 11; 8 and 11; and 10 and 11). This means that the variance between depths is greater than between sites. This in turn may mean that the sediment is more heterogeneous vertically at each site than it is horizantally between sites. This is a little

surprising because the sites are many kilometres apart while the depths are only centimetres apart.

B- **Cluster analysis at 72% similarity**

The results of the numerical study of 843 deep-sea isolates and 27 reference cultures using the Euclidean distance coefficient and Ward's method of clustering produced a large dendrogram (5m x 4m). Boundary lines were drawn across the dendrogram at similarity levels of 50, 60, 72, 80, 90, 96, 97, 98, 99, 99.5, 99.9 and 100%, in order to obtain the number of groups at each level. 72% similarity level was selected to produce my final dendrogram. This similarity level was selected because it is within the range (70-85%) used by many authors (Quigley and Colwell, 1968; Austin et al., 1977; Mallory et al., 1977; Austin et al., 1978; Hauxhurst et al., 1980; Austin et al., 1981; Austin, 1982; Knivett et al., 1983; Allen et al., 1983; Hudson et al., 1986; Baya et al., 1986; Austin and Moss, 1986; Gil et al., 1986; Marquez et al., 1987) and produces reasonable numbers of groups and reasonable numbers of isolates/group. There was also an indication of good environmental separation according to sites and depths (e.g. the isolates in group 2 were all obtained from surface sediment of site 3). All groups clustered at or above this level were drawn as shaded triangles in my final dendrogram of the cluster analysis (Figure 16). 21 groups were defined at 72% similarity level from the 843 isolates. The results of the numerical study were summarised in table 26 (p. 99-102).

Some important points can be taken from this table. The maximum number of isolates found (107) occurred in cluster 12, while the minimum number (11) occurred in cluster 11. Only 1/3 of the clusters contained isolates from all the sampling sites.

I have ranked each site using (a) number of clusters and (b)

maximum number of isolates at each site. I have also summarised the data in table 26 to show the differences between the sites as follows.

Site number	number of clusters	maximum number of isolates in a cluster
1	11 (5)	27 (4)
2	20 (1=)	51 (1)
3	17 (3)	29 (2=)
4	20 (1=)	29 (2=)
5	16 (4)	20 (5)

Ranks = ( )

As can be seen in the table above, sites 1 to 5 can be ranked in the order 5, 1=, 3, 1=, and 4 according to the number of clusters at that site and in the order 4, 1, 2=, 2=, and 5 according to the maximum number of isolates in a cluster. The two rankings broadly agree with each other. Site 2 is ranked first in both rankings, either alone or with another site. This shows that isolates from this site occur in a high number of clusters (i.e. 20 out of 21) and have the highest number of isolates in a cluster. Isolates from site 4 occur in the same number of clusters as site 2 but site 4 has a lower number of isolates per cluster than site 2. Site 3 has a lower number of clusters (17 clusters) than sites 2 and 4 (both 20 clusters) but has the same number of isolates as site 4. Site 5, has a fairly moderate number of clusters (16 clusters) and the least number of isolates in a cluster.

The clusters can be grouped according to the source of isolates (sediment, burrow linings, and faecal pellets) in them as follows.

Sed.+burr. + faec.	Sed.+ Faec.	Sed.	Sed.+burr.	Burr.	Faec.	Burr+Faec.
14	5	1	1	0	0	0

sed. = sediment, burr. =burrow linings , faec. =faecal pellets

14 clusters contained isolates from sediment + burrow linings + faecal pellets, 5 clusters contained isolates from sediment + faecal pellets. Only 1 cluster contained isolates from sediment and sediment + burrow linings. No clusters were formed entirely of isolates from burrow linings, faecal pellets, or burrow linings + faecal pellets.

A total of 27 reference cultures were used in my study. The number of deep-sea isolates in each cluster with the reference cultures are shown in table 45. The reference cultures helped in the presumptive identification of 11 out of 21 clusters. The presumptive identification of these 11 clusters are shown in the table below. The clusters contain bacteria that have antibiotic and heavy metal sensitivities close to that of the reference cultures with which they cluster.

Some important points can be made from the table below. As an example, the isolates in groups 11 and 17 which are related to Cytophaga lytica and Pseudomonas cleovorans respectively were isolates clustered at close similarity levels (88 and 85 respectively) and obtained from a depth range of surface to 7.5cm. They also obtained from sediment and faecal pellets but not from burrow linings.

Cluster No.	Presumptive identification
1	Planococcus citreus
5	Pseudomonas fluorescens, Alteromonas hydrophila
8	Micrococcus, Corynform
9	Pediococcus cerevifiae
11	Cytophaga lytica
13	Serratia marcescens
14	Aeromonas hydrophila
15	Vibrio fischeri
17	Pseudomonas cleovorans
18	Coryneform, Pseudomonas, Proteus vulgaries
20	Planococcus sp.

3- **Statistical analysis of clusters formed at 72% similarity level**

A- **Variation in the number of isolates between groups, between sites and between depths**

Chi-square tests were used to test the significance of the variation in the following:

(i) The number of isolates between sites for each group in turn (Table 30).

(ii) The number of isolates between groups for each site in turn (Table 31).

(iii) The number of isolates between depths for each group in turn (Table 34).

(iv) The number of isolates between groups for each depth in turn (Table 35)

The results of these Chi-square tests showed that there was

Cluster No.	No. isolates	No. ref. strains	Name of reference cultures in cluster
1	32	1	Planococcus citreus (NCMB 1493)
2	19	-	
3	41	-	
4	52	-	
5	47	2	Pseudomonas fluorescens (NCMB 9046)/Alteromonas haloplanktis (NCMB 19)
6	24	-	
7	14	-	
8	23	3	Micrococcussp. (NCMB 13)/Micrococcussp. (NCMB 365)/Coryneform (NCMB 8)
9	45	1	Pediococcus cerevisiae (NCTC 10331)
10	59	-	
11	11	1	Cytophage lytica (NCMB 292)
12	107	-	
13	24	1	Serratia marcescens (NCIB 2847)
14	87	1	Aeromonas hydrophila (NCIB 9240)
15	34	1	Vibcio fischeri (NCMB 1274)
16	54	-	
17	27	1	Pseudomonas cleovocans (NCIB 6576)
18	41	3	Coryneform (NCMB 35)/Pseudomonassp. (NCMB 320)/Proteus valgaris (NCIB 4175)
19	30	-	
20	53	1	Planococcussp. (NCMB 628)
21	19	-	
Cluster contains only reference cultures			Morexalla (NCMB 308)/Klebsiella pneumonia (NCIB 8806)/Klebsiella pneumonia (NCIB 8805)/Klebsiella pneumonia (NCIB 9261)/Bacillus megaterium (NCIB 8508)/Corynebacterium xerosis (NCIB 9255)/Micrococcus luteus (NCTC 2665)/Bacillus cereus/Staphylococcus aureus (NCTC 6571)/Staphylococcus albus/Acinetobacter calcoaceticus (NCIB 8250).

Table 45
Clusters formed at 72% S and the number of isolates in each cluster. The numbers and names of reference cultures in each cluster are also shown.

statistically significant variation in most of cases. The number of significant Chi-square tests is summarised in the following table.

	Chi-square tests	
	Number of significant	Number of not significant
(i)	15	6
(ii)	5	0
(iii)	18	3
(iv)	9	1

The variation between groups, between sites, and between depths in my data probably indicate geographical and microtopographical changes in sediment properties. A wide variation in the local concentrations of the major biochemical fractions (proteins, carbohydrates and extractable lipids) exists within marine sediments (Brock, 1966; Krauskopf, 1979; White et al., 1979; Battersby and Brown, 1982; Maxwell and Wardroper, 1982; DeLong and Yayanos, 1986; Meadows and Campbell, 1987). Decreases in protein and carbohydrate levels, and increases in lipid levels with increasing sediment depth have been observed in deep-sea sediments from some areas (Lindblom, 1963). Similar changes in sediment physico-chemical properties such as Eh, chlorophyll, and sulphide levels have been reported as occurring over a few millimetres in intertidal and deep-sea sediments (Anderson and Meadows, 1978; Anderson et al., 1981; Meadows and Tait, 1985)). Anderson and Meadows (1978) found that intertidal sediment properties such as Eh, total carbon, organic carbon, total nitrogen, heterotrophic bacterial content and chlorophyll can change notably over short distances in the sediment. The authors also showed the effect of bioturbation structures such as animal burrows on these properties. More recently, Meadows and Tait

(1985) reported a decrease in bacterial abundance with sediment depth in deep-sea sediment from the North East Atlantic. They also found that Eh, and shear strength have been correlated with depth and with burrow systems. Bioturbation therefore has dramatic effects on the properties of deep-sea sediments. Decreases in easily utilizable organic matter occur below the sediment surface and this food limitation controls the basic depth distribution of microbial activity.

B- Comparison of the number of isolates between sampling sites and between depths

The difference in the number of isolates/group between different sites and depths was studied. For example, in table 28 (sites), the number of isolates in each group at site 1 (10, 0, 0, ... 3, 3) was compared with the number of isolates in each group at site 2 (4, 0, 3, 4, ... 5, 1) and then the number of isolates in each group at site 1 was compared with the number of isolates in each group at site 3 (0, 19, 2, ... 1, 1) and so on until all sites were compared with each other. The same procedures were carried out with depths. These comparisons are aimed at answering the question: is there any correlation between the number of isolates in each of the groups between sites and between depths ? They were conducted using the non-parametric Spearman's rank order correlation coefficient. The results of these comparisons between sampling sites and between depths are shown in tables 32 and 36 and summarised in figures 38 and 39 respectively.

Significant correlations were only found between sites 2 and 4 and between sites 4 and 5. However, a large number of significant correlations were found between sampling depths (11 out of 34 comparisons).

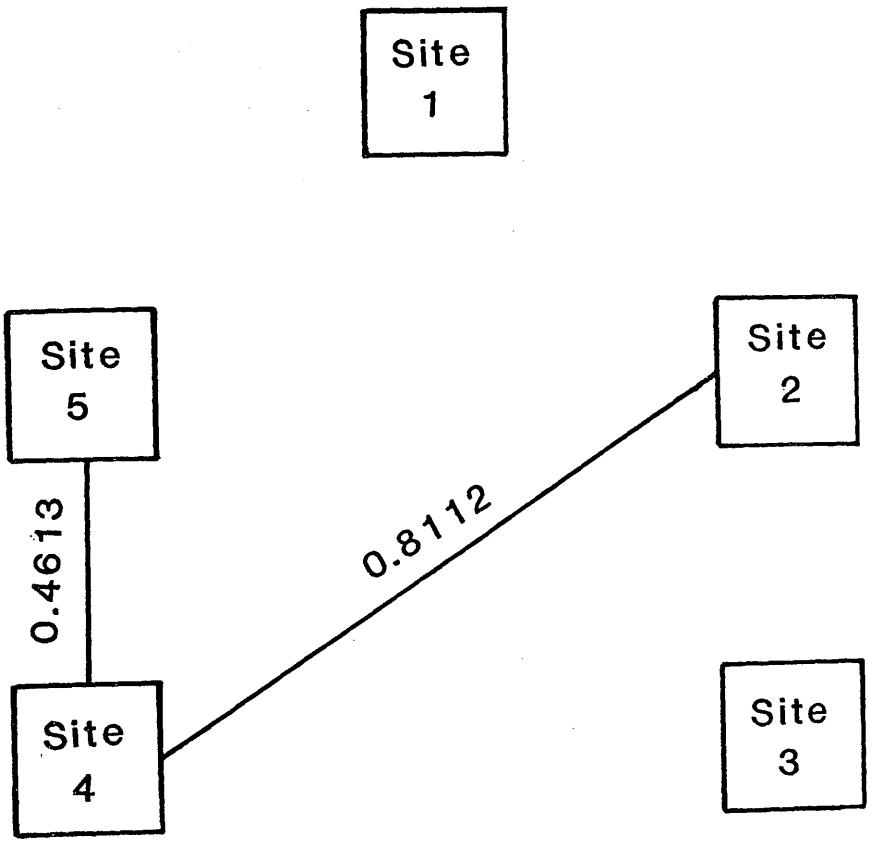


Figure 38

Diagram of the relationships in number of isolates between sampling sites. Significant correlations are indicated by solid lines. Values on the solid lines are Spearman's correlation coefficient. Significance levels as shown in table 32.

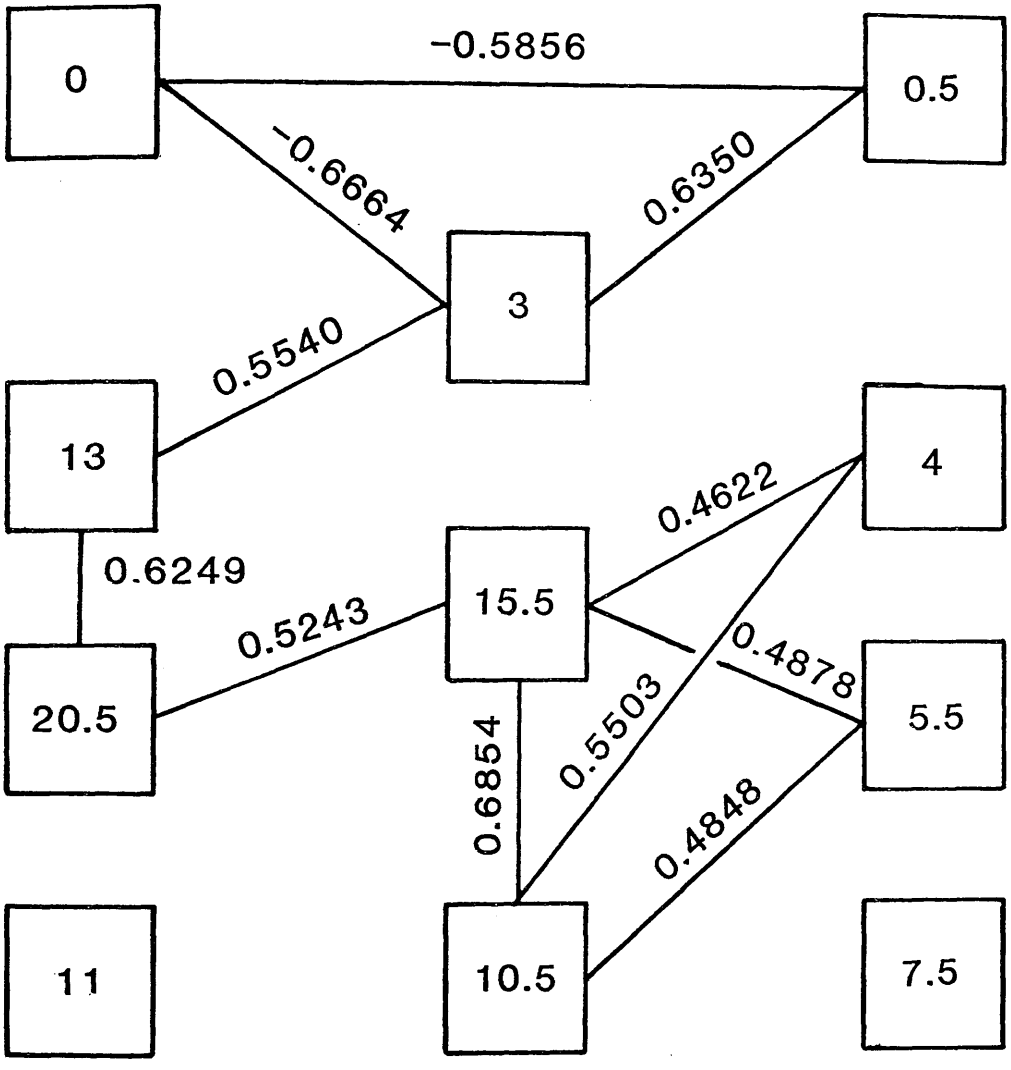


Figure 39

Diagram of the relationships in number of isolates between sediment depths (cm). Significant correlations are indicated by solid lines. Values on the solid lines are Spearman's correlation coefficient. Significance levels of correlations as shown in table 36.

Although there are a number of studies on marine isolates using multivariate methods such as cluster analysis (see p. 7-9), there have been no comparative studies similar to mine using heavy metals and antibiotics on deep-sea sedimentary isolates. Meadows and Tait (1985) sampled bacteria from the same sites in the North East Atlantic but did not use cluster analysis. They found variations between sites and between sampling depths. Bacterial numbers ranged from $2.8x10^2$ to $6.5x10^4$ c.f.u/g dry sediment (surface counts) between sites and decreased dramatically with increasing sediment depth (10^2-10^5 g^{-1} dry sediment to ca.1 g^{-1} dry sediment at depths of 20cm). Aller and Yingst (1980) found differences in sediment bacterial numbers between sites in Long Island Sound, USA, and also found dramatic decreases in bacterial numbers with increasing sediment depth at each site. They found corresponding decreases in sulphate reduction, ammonia production and ATP levels with increasing sediment depth. In conclusion, they suggested that bacterial distribution patterns were related to changes in chemical parameters and nutrient availability in the sediment.

C- Comparison of the number of isolates obtained from sediment, burrow linings and faecal pellets

Table 38 shows the number of isolates in sediment, burrow linings, and faecal pellets for the 21 groups clustered at 72% similarity level. As can be seen in this table, a total of 561 isolates were obtained from the sediment samples in contrast to 143 and 139 isolates from burrow linings and faecal pellets respectively.

The data in table 38 were analysed in three ways. Firstly, to test whether there was any difference between the number of isolates/group in sediment, burrow linings, faecal pellets, and data

combined (total). Secondly, to test whether there was any difference in the number of isolates between groups for sediment and burrow linings + faecal pellets. Thirdly, to test whether there was any difference in the number of isolates between groups for burrow linings and faecal pellets. These comparisons were carried out using Chi-square tests. The results show highly significant variation in each case (Tables 39 and 40).

High levels of organic material occur in the surface layers of marine sediments and these levels generally decrease exponentially with depth (Moshiri and Crumpton, 1978). Anderson, Boonruang and Meadows (1981) studied the relationships between chlorophylls, carbon, nitrogen, and heterotrophic bacteria in an intertidal sand sediment. The authors found a reduction in chlorophyll, carbon, nitrogen, and heterotrophic bacteria with depth. They also found that all these variables were strongly correlated with each other with the exception of heterotrophic bacteria. The authors think that the lack of correlation between bacterial counts and any other assayed parameter may reflect the limitations of dilution plate counting procedure which substantially underestimates numbers of viable heterotrophic bacteria in natural environments. Therefore, my sediment samples taken from near the sediment surface are likely to contain high numbers of bacteria. Fairly similar numbers of isolates were found between burrow linings and faecal pellets. Some of these samples were taken at depths where the surrounding sediment contained very low bacterial counts. The burrow linings and faecal pellets therefore stimulate the growth of bacteria at these depths. This may be caused by several factors including the availability of oxygen for growth resulting from irrigation of burrows by their inhabitants, and the high organic levels present in burrow linings and faecal pellets (Yingst and Rhoads, 1980). Some macrofauna such as Arenicola marina actively

stimulate the growth of bacterial populations in their burrow linings to augment their food supply (Hylleberg, 1975; Meadows and Campbell, 1987). The respiratory currents produced in the burrow by the animal encourage bacterial growth. Interestingly enough, there are often high numbers of particular species of meiofaunal organisms in the burrows of animals that may be feeding on these bacterial populations (Reise, 1985; Meadows and Campbell, 1987) although some burrowing organisms appear to produce antimicrobial agents (King, 1986; Meadows 1986). Saccoglossus kowalewskii produces high concentrations of 2, 4-dibromophenol (DBP) in its mucus which then becomes incorporated into the lining of the animal's burrow and inhibits aerobic bacterial growth. Faecal pellets are also expected to have high bacterial counts, because of the high bacterial biomass present in animal guts (Meadows and Tait, 1985; Velji and Albright, 1986).

Of the 21 clusters or groups obtained at 72% similarity level, all of these contained isolates from the sediment source. Only 14 and 19 clusters contained isolates from the burrow linings and faecal pellets respectively. These findings may reflect a more specialised and narrower range of bacteria living on burrow linings and faecal pellets. The high abundance found in the sediment is probably related to the higher nutritional and oxygen levels in the sediment, particularly at the sediment surface.

4- Clustering of variables

Firstly I applied cluster analysis in order to classify isolates (cases) according to their response to antimicrobial agents. I then carried out further cluster analysis to classify the antimicrobial agents (variables) themselves according to their overall minimum inhibitory concentrations. This numerical analysis was conducted in three different ways. Firstly, to the antibiotics alone (7 variables), secondly, to the heavy metals alone (6 variables), and thirdly, to the antibiotics and heavy metals together (13 variables).

Clustering of antibiotics showed that there were two distinct clusters: cluster 1 contained polymyxin B and nalidixic acid, cluster 2 contained streptomycin, tetracycline and ampicillin. Methylene blue and chloramphenicol remained unclustered.

I firstly consider possible explanations as to why polymyxin B and nalidixic acid clustered together in cluster 1. The mode of action of polymyxin B and nalidixic acid is different. However, most marine bacteria are Gram-negative, and both antibiotics are active antimicrobial agents against Gram-negative bacteria. Polymyxins are bactericidal against most Gram-negative bacteria (Viljanen et al., 1986). Feingold et al. (1974) studied the action of polymyxin B on cell membranes. They found that bacteria varied in their susceptibility to this antibiotic, depending on whether they were Gram-positive or Gram-negative. Gram-positive bacteria were relatively resistant to the lethal action of polymyxin B, whereas the cytoplasmic membrane of Gram-negative bacilli were damaged. Nalidixic acid is also an active antimicrobial agent against Gram-negative bacteria (Franklin and Snow, 1971). It acts by inhibiting DNA replication enzymes (Edwards, 1980). The above study shows the importance of Gram-reaction classification and the relative effect of antibiotics on bacteria depends on the Gram-reaction of the bacteria being studied

Cluster 2 contained streptomycin, tetracycline and ampicillin. Streptomycin and tetracycline act by inhibiting the protein synthesis of sensitive bacteria. Both antibiotics inhibit the 30s ribosomal subunit of both Gram-positive and Gram-negative bacteria. Ampicillin is an antibiotic known to inhibit the cell wall of Gram-positive bacteria. The reason for this is that the composition of the cell wall is different between the Gram-positive and Gram-negative bacteria. For example, Gram-negative bacteria possess a lipid-rich layer surrounding the cell wall called the outer membrane (Hammond and Lambert, 1978). The layer is not present in Gram-positive bacteria.

Streptomycin and tetracycline clustered together probably because they both inhibit the 30s ribosomal subunit of Gram-positive and Gram-negative bacteria. However, it is unclear why ampicillin clustered with streptomycin and tetracycline, since the mode of action of ampicillin is different from that of the other two antibiotics.

It was not surprising that methylene blue remained unclustered. It's behaviour as an antimicrobial agent was inconsistent throughout my study. The antibiotic chloramphenicol was also unclustered. There are probably two reasons for this. Firstly, the mode of action of chloramphenicol is different from the other antibiotics. It inhibits the 50s ribosomal subunit of both Gram-positive and Gram-negative bacteria. Secondly, the toxicity of chloramphenicol is significantly different from the other antibiotics (Table 12 i).

Clustering of heavy metals showed that there was one cluster formed at the 0.4023 correlation level. This cluster contained three metals; mercury, copper and lead. The metals manganese, cadmium and chromium remained unclustered.

The metals mercury, copper and lead were clustered together

possibly because they share a similar mode of action. Ramamoorthy and Kushner (1975) studied the binding of heavy metal ions to a number of bacterial growth media. They found that all media bound large amounts of Hg^{2+}, Pb^{2+}, and Cu^{2+}, but much less Cd^{2+}. 80 ppb or less remained as free cations in the solution after adding 20ppm of Hg^{2+}, Pb^{2+}, or Cu^{2+}. This might suggest that such ions enter bacterial cells as organic complexes, or that bacterial cells can compete successfully with growth media for the bound ions. Bubela (1970) studied chemical and morphological changes in Bacillus stearothermophilus induced by copper. The morphological changes were reversible when transformed to copper free media. The author concluded that the morphological changes may be indicative of alterations in the cell wall-membrane structure. Vaituzis et al. (1975) showed that growth of some strains of mercury resistant bacteria at high levels of mercury was associated with characteristic morphological changes. Onset of growth and cell division was delayed and there were irregularities associated with cell wall and cytoplasmic membrane synthesis and functions. Tornabene and Edwards (1972) found that Micrococcus luteus and Azotobacter sp. immobilized large quantities of lead in media containing lead salt. The authors also found that fractions of cell wall and cell membrane contain 99.3 and 99.1% of the lead associated with Micrococcus and Azotobacter respectively.

Cadmium is an important environmental pollutant exerts its toxic effects over a wide range of concentrations. In most cases, bacteria and fungi appear to be resistant to this element (Trevors et al., 1986). The reason that cadmium remained unclustered is probably because the ability of cadmium ions to bind organic molecules is different from the other metals (Ramamoorthy and kushner, 1975) (see above). Mitra et al. (1975) observed a prolonged lag phase in their

batch cultures of Escherichia coli supplemented with cadmium. They suggested that accommodation of Escherichia coli to the presence of Cd^{2+} involves exclusion of the ion from the cell and reversal of damage caused by prior exposure to the ion. Alking et al. (1982) reported similar results with the culture Klebsiella aerogenes. They suggested that the formation of CdS was probably the most important reason of detoxification in this organism.

The metal manganese also remained unclustered probably because of it's interference with physiological processes. Adams and Ghiorse (1985) determined the influence of Mn^{2+} on growth of Leptothrix discophora strain. The authors suggested that the decrease in cell yield observed at low and moderate concentrations of Mn^{2+} was related to the formation of manganese oxide, which may have bound cationic nutrients essential to the growth of Leptothrix discophora. These inhibitory effects could be caused by Mn^{2+} interference with physiological processes requiring divalent cations such as Mg^{2+}.

Chromium not being clustered with the other metals may be explained by its unusual valency states. Chromium exists in valencies from -2 to +6, but in the environment only Cr^{+3} and Cr^{+6} are of significance (National Research Council, Committee on Biologic Effects of Atmospheric Pollutants, 1974). There are several studies on the effect of chromium on microorganisms. However, these do not appear to have direct relevant to the importance of valency in determining the toxicity of chromium. Wong et al. (1982) studied the effect of chromium and manganese on Thiobacillus ferrooxidans. The author found that the growth of this culture at pH 2.5 is inhibited by concentrations of $Cr_2(SO_4)_3$ greater than 1.5×10^{-2} M or by concentrations of $MnSO_4$ greater than 0.6M. Earlier study on the effect of chromium and copper on Klebsilla aerogenes was conducted by Baldry et al. (1977). The authors found that the action of chromic chloride

on Klebsiella aerogenes (NCIB 418) was very slight but potassium chromate and cupric sulphate were much more toxic. In my study potassium chromate (K_2CrO_4) was used.

Clustering of all antimicrobial agents together. Cluster analysis of all the antimicrobial agents together was conducted because resistance to heavy metals is often associated with resistance to antibiotics (Varma et al., 1976; Summers et al., 1978; Calomeris et al., 1984). This analysis showed that there were three distinct clusters. Cluster 1 contained polymyxin B, nalidixic acid and chromium. Cluster 2 included streptomycin, tetracycline and ampicillin. Cluster 3 included mercury, copper, lead, methylene blue, and manganese.

There have been a number of previous studies in this field (see appendix A p. 219-225) but none of these studies have used multivariate methods such as clustre analysis. The following are examples. Nakahara et al. (1977) studied the linkage of mercury, cadmium and arsenate and drug resistance of clinical isolates of Pseudomonas aeruginosa. They found that 99.8% of the 787 isolates tested were metal resistant. Most of these metal resistant isolates were multiple metal and antibiotic resistant, whereas only 19% of the isolates were metal resistant but drug sensitive. Calomeris et al. (1984) studied the association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. They concluded that relation of metal tolerance to multiple antibiotic resistance varied among isolates from different distribution waters. Resistant bacillus populations have a greater frequency in sites polluted by high concentrations of mercury and other heavy metals than unpolluted sites, and ampicillin follows the same trend (Timoney et al., 1978). Baldry et al. (1977) studied the chromium and copper

sensitivity and tolerance in Klebsiella aerogenes. They found that Cu^{2+}-resistance was associated with increased resistance to chloramphenicol and increased sensitivity to streptomycin but the sensitivity to nalidixic acid was unchanged. In contrast, CrO_4^{2-}-resistant organisms were resistant to nalidixic acid but not to the other three antibiotics. This agreed with my results when chromium was clustered in the same group with polymyxin B and nalidixic acid.

SECTION 1

SUMMARY

1- The aim of my work in this section was to study the toxicity effects of heavy metals and antibiotics on deep-sea sedimentary bacteria and to apply a cluster analysis to the data in order to classify the isolates into groups.

2- Deep-sea bacteria used in this study were obtained from deep-sea sediment collected at 5 sites around the Rockall Trough area of the North East Atlantic. Isolates were grown on ZoBell agar medium at 10°C.

A number of standard reference cultures were also used. These cultures were obtained from the National Collection of Marine Bacteria (NCMB), the National Collection of Industrial Bacteria (NCIB), and the National Collection of Type Cultures (NCTC). Additional isolates were supplied by Professor A. Wardlaw of the department of microbiology at Glasgow University.

3- Preliminary experiments were conducted to select the suitable concentrations of antimicrobial agents in the definitive experiments.

4- All antimicrobial agents tested were able to suppress the growth of some members of the bacterial population.

5- Increasing concentration of the antimicrobial agents were found to decrease the number of isolates which grew.

6- Mercury was the most toxic metal and manganese the least toxic.

7- Chloramphenicol and polymyxin B were the most toxic antibiotics and nalidixic acid the least toxic.

8- The relative toxicity of antibiotics and the relative toxicity of heavy metals were studied using student's t-tests. The results showed that 5 out of 21 comparisons showed a significant

difference in the toxicity between antibiotics while 12 out of 15 comparisons showed a significant difference in the toxicity between metals. Therefore, in general, heavy metals showed more significant difference in the toxicity between pairs of metals than antibiotics.

9- When the dye methylene blue was used as a selective medium, no consistent trend in the decrease in number of isolates with increasing concentrations was obtained.

10- Unlike antibiotics, with heavy metals there was no apparent general increase in bacterial growth on selective media with increasing incubation time.

11- For each isolate, the minimum inhibitory concentration (MIC) with each antimicrobial agent was defined as the lowest concentration of antimicrobial agent that inhibited growth. It varied from one antimicrobial agent to another.

12- Cluster analysis was applied to split up a total of 843 deep-sea isolates into a number of groups depending on their antibiotic and metal salt resistance.

13- A total of 27 standard reference cultures were also put through the antibiotic and metal salt system and a series of "Reference Resistance profiles" were generated. It was hoped to broadly classify the previously grouped unknown isolates on the basis of their relationship to the reference cultures.

14- At each similarity level, a number of groups were obtained. For example, at 50% and 72% similarity levels, 11 and 21 groups were obtained respectively.

15- The results of Chi-square tests to compare the number of isolates between groups for each site, between sites for each group, between groups for each depth, and between depths for each

group at 50% S showed highly significant variation in each case.

16- The homogeneity of variances between groups for sites and depths was statistically analysed using Bartlett's and F-max tests. The results showed that there were no significant differences between overall site variances and overall depth variances for the 11 groups.

17- When groups or clusters were compared (using F-ratio) with respect to the variance in the number of isolates present between sites, it was found that only 2 groups were significant (2 and 4).

18- Similarly when groups were compared (using F-ratio) with respect to depth variances, it was found that significant differences occurred in 10 out of 55 comparisons.

19- The variances of number of isolates/site with variances of number of isolates/depth were compared using F-ratio tests. The results showed that in general, there was no significant differences between site and depth variances for each of the 11 groups (only 2 out of 11 tests were significant).

20- 72% similarity level was selected to produce my final dendrogram. All groups (21) clustered at or above this level were drawn as shaded triangles of the cluster analysis.

21- The maximum number of isolates (107) occurred in cluster 12, while the minimum number (11) occurred in cluster 11.

22- Only 1/3 of the clusters contained isolates from all the sampling sites.

23- 11 of the 21 clusters could be presumptively identified as they clustered with the reference cultures used.

24- Chi-square tests were used to test the variation in the number of isolates between sites for each group, groups for each site, depths for each group, and groups for each depth at 72% similarity level. The results showed that there was statistically

significant variation in most of cases.

25- The number of isolates in each of the groups between sites and between depths was compared using Spearman's rank order correlation tests. The results showed that only significant correlation were found between sites 2 and 4 and between sites 4 and 5. However, a large number of significant correlations were found between the sampling depths.

26- A total of 561 isolates were obtained from the sediment samples in contrast to 143 and 139 isolates from burrow linings and faecal pellets respectively.

27- The difference between the number of isolates/group in the sediment, burrow linings, faecal pellets, and data combined (total) was analysed using Chi-square tests. The results showed that all tests were highly significant.

28- The difference in the number of isolates between groups for sediment and burrow linings + faecal pellets was analysed using Chi-square tests. A highly significant variation in the number of isolates was found.

29- Similarly, the difference in the number of isolates between groups for burrow linings and faecal pellets was analysed using Chi-square tests. A highly significant variation in the number of isolates was found.

30- The distance measure used in cluster analysis was the Euclidean distance squared. It has been transformed into percentage similarity (%S). The difference between these two measures is that "similarity" take values between 1 (i.e. 100% similarity) and 0 (i.e. 0% similarity), and "distance measure" can take any positive value from 0 (i.e. no difference) upwards as difference increases.

31- The relationships between the percentage similarity and number of isolates/group, the number of groups (including single isolates), the number of groups (excluding single isolates), the number of single isolates and the coefficient of variation of number of isolates/group were studied and the following conclusions were made:

(a) As the percentage similarity increased, the number of isolates/group decreased.

(b) The number of groups including single isolates increased exponentially with increasing percentage similarity.

(c) The number of groups excluding single isolates increased with increasing percentage similarity to 99.9%, after which it decreased again.

(d) The number of single isolates increased with increasing percentage similarity.

(e) The coefficient of variation of number of isolates/group fluctuated with percentage similarity.

32- Similar conclusions can be drawn from the Euclidean distance measure.

(a) As the Euclidean distance increased, the number of isolates/group increased.

(b) Conversely, the Euclidean distance decreased exponentially with the number of groups including single isolates.

(c) Excluding single isolates, the number of groups clustered increased with decreasing Euclidean distance to 0.001, after which it decreased again.

(d) The number of single isolates decreased with increasing Euclidean distance measure.

(e) The coefficient of variation of number of isolates/group fluctuated with Euclidean distance.

33- Cluster analysis was applied to the antibiotics alone (7 variables), to the heavy metals alone (6 variables), and to the antibiotics and heavy metals together (13 variables).

(a) Clustering of antibiotics, showed that antibiotics classified into two distinct clusters due to their mode of action on Gram-positive or Gram-negative bacteria. Two antimicrobial agents remained unclustered.

(b) Clustering of heavy metals, showed that three metals were clustered in one distinct cluster. The other metals used remained unclustered. Heavy metals were clustered based on the mode of action on bacterial cell.

(c) When cluster analysis was applied to all antimicrobial agents together, it was concluded that there were three distinct clusters and metal resistance was associated with the resistance to antibiotics.

Section 1 appendices

APPENDIX A

Toxicity of heavy metals to bacteria

Heavy metals are well known to be toxic to microorganisms and to inhibit microbial growth as their concentration increases. There are a large number of reviews on various aspects of the effects of heavy metals on microorganisms (Sadler and Trudinger, 1967; Vallee and Ulmer, 1972; Austin et al., 1977; Gadd and Griffiths, 1978; Summers, 1978; Sterritt and Lester, 1980).

Heavy metals in general act as a result of their affinity for chelating agents. This affinity may indicate their comparative toxicity (Albert, 1975). In decreasing order of affinity this series is: Fe^{3+}, $Hg^{2+} > Cu^{2+}$, $Al^{3+} > Ni^{2+}$, $Pb^{2+} > Co^{2+}$, $Zn^{2+} > Fe^{2+}$, $Cd^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+} > Li^{+} > Na^{+} > K^{+}$.

Chelating agents are substances that form a soluble complex with metals and which thus prevent them from forming insoluble complexes with phosphates (Stanier et al., 1980).

The toxic concentration of metals varies from species to species. This is a well known phenomenon and has often been reported (Babich and Stotzky, 1977a,b; Zevenhuizen et al., 1979; Ligthart, 1980; Wong et al., 1982).

Generally, mercury and silver are the most toxic metals and manganese and zinc are the least toxic. For example, mercury, cadmium, lead, ferrous iron, copper and zinc have decreasing toxicity to Escherichia coli in that order (Sadler and Trudinger, 1967).

Certain metals have different toxicities in seawater and fresh water. For example, the toxicity of mercury to Aeromonas sp. and the bacteriophage 0 11M15 of Staphylococcus aureus was less in seawater than in lake water (Babich and Stotzky, 1979).

Within one species some strains are more sensitive to heavy

metals than others. Ross and Old (1973) studied the effect of mercuric chloride on isolates of the fungus Pyrenophora avenae obtained from oat seeds. 41 isolates were studied and appeared to separate into three groups - a resistant group of 18 isolates, a sensitive group of 13 isolates, and 10 isolates of intermediate resistance.

Some heavy metals are known to characteristic morphological and biochemical changes. Bubela(1970) studied chemical and morphological changes in Bacillus stearothermophilus induced by copper. The morphological changes were reversible when transformed to copper free media. Bubela compared copper-grown and normal cells by infrared spectra of freeze-dried whole cells and cellular fractions. He also examined cellular extracts by ultraviolet, infrared and mass spectroscopy, and by gas and thin layer chromatography. He concluded that "copper induces the production of an esterified aromatic dicarboxylic and probably phthalic or isophathalic acid in the cells and the morphological changes may be indicative of alterations in the cell wall-membrane structure". Vaituzis et al. (1975) showed that growth of some strains of mercury resistant bacteria at high levels of mercury was associated with characteristic morphological changes. Onset of growth and cell division was delayed and there were irregularities associated with cell wall and cytoplasmic membrane synthesis and functions.

The toxicity of heavy metals to bacteria may also depend on many environmental factors. For example, toxic effects on activated sludge processes were decreased by increasing the suspended solids concentration (Lamb and Tollefson, 1973). Babich and Stotzky (1977a,b) found that the toxicity of cadmium to the species they studied seemed to be pH dependent. These authors also reported that Actinomycetes were more tolerant to cadmium than Gram -negative bacteria which were more tolerant than Grame-positive bacteria. As a final example,

Calomiris et al. (1984) showed that positive correlations between tolerance to high levels of Cu^{2+}, Pb^{2+}, and Zn^{2+} and multiple antibiotic resistance occurred among bacteria from distribution waters but not among bacteria from raw waters.

Resistance of bacteria to heavy metals

Heavy metal salts are common in aquatic environments and can reach high concentration. They accumulate through (i) volcanism which is responsible for Oceanic and atmospheric concentration of mercury, cadmium, copper, zinc and silver (Boutron and Lorius, 1979; Mukherji and Kester, 1979); (ii) leaching of metal ora-rich soil (Tonomura and Kanzaki, 1969); and (iii) industrial plant wastes (Summers et al. 1978).

It is well known that many bacterial isolates are resistant to heavy metals (Summers and Silver, 1972; Nelson et al., 1973; Walker and Colwell, 1974; Hamdy and Noyes, 1975; Nakahara et al., 1977). Resistant strains can arise either by chromosomal mutation (Traub and Kleber, 1977) or by extrachromosomal genetic elements called plasmids (Smith, 1967; Dyke et al., 1970; Hedges and Baumberg, 1973; Bopp et al., 1983). Plasmids are small extrachromosomal circular DNA molecules capable of autonomous replication in the host cell. Plasmids also can be infectiously transferred by means of bacteriophages from a resistant bacterial species to sensitive species (Stanier et al., 1980, p. 462, 488).

Plasmid-specified chromate resistance has been reported for both Pseudomonas aeruginosa (Summers and Jacoby, 1978) and Streptococcus lactis (Efstathious and Mckay, 1977). In each case, the plasmid-bearing strain is approximately 10-fold more resistant to chromate than in the plasmidless strains. Capsular material in some species may also aid in metal resistance. Azotobacter sp. having a

large quantity of capsular material were found by Tornabene and Edwards (1972) to be more efficient in immobilising lead than Micrococcus luteus.

Resistance to heavy metals is often found associated with resistance to antibiotics (Varma et al., 1976; Summers et al., 1978; Calomeris et al., 1984). Nakahara et al. (1977) studied the linkage of mercury, cadmium, and arsenate and drug resistance of clinical isolates of Pseudomonas aeruginosa. They found that 99.8% of the 787 isolates were metal resistant. Most of these metal resistant isolates were multiple metal and antibiotic resistant, whereas only 19% of the isolates were metal resistant but drug sensitive. Calomiris et al. (1984) studied the association of metal tolerance with multiple antibiotic resistance of bacteria isolated from drinking water. They concluded that relation of metal tolerance to multiple antibiotic resistance varied among isolates from different distribution waters. They also reported that bacteria from distribution waters exhibited unique patterns of metal and antibiotic resistance. Resistant Bacillus populations have a greater frequency in sites polluted by high concentrations of mercury and other heavy metals then unpoluted sites, and ampicillin follows the same trend (Timoney et al., 1978).

The presence of one microorganism can greatly influence the activity of another under different conditions. For example, Escherichia coli caused a decrease in the sensitivity of Staphylococcus aureus to mercuric chloride when the two strains were mixed (Stutzenberger and Bennett, 1965). The protective effect carried by Escherichia coli was due to the production of extracellular glutathione and hydrogen sulphide in the medium, also to an unequal distribution of the inhibitor between the two species. Temple and Roux (1964) demonstrated that Desulphovibrio desulphuricans decreased the

sensitivity of Pseudomonas aeruginosa to the inhibitory concentration of mercury of the latter strain when both of them were mixed. These authors suggested that hydrogen sulphide produced by the sulphate reducer nutralized the toxicity of mercury to the Pseudomonas.

History of antibiotics

Antibiotics are substances produced by microorganisms that inhibit the growth of or kill other species of microorganisms (Edwards, 1980).

It is generally accepted that antibiotics were discovered in 1929, when Alexander Fleming investigated penicillin from fungal penicillium sp. (Fleming, 1929). Following that, in 1939, Rene Dubos isolated gramicidin and tyrocidin from Bacillus brevis (Dubos, 1939) and streptomycin was discovered by Schatz, Bugie and Waksman in 1944 (Waksman, 1949).

Many thousands of antibiotics have been isolated and identified since 1940 - the beginning of the antibiotic revelution. This still continues either as the production of antibiotics from microorganisms naturally or by the synthetic production of antibiotics from chemicals (Pelczar and Reid, 1972).

Resistance of microorganisms to antibiotics

The occurence of antibiotic resistant strains of bacteria has increased significantly in recent years, and antibiotic resistant mutants can occur spontaneously without prior exposure to antibiotics. This phenomenon is well known, and has been described by a number of workers (Luria and Delbruck, 1943; Demerec, 1948; Lederberg and Lederberg, 1952).

Microorganisms can exhibit resistance to antibiotics through biochemical, physiological and morphological modifications as follows.

Microorganisms can inactivate the drug by producing enzymes.

For example, Escherichia coli strains (Abraham and Chain, 1940), Bacillus cereus (Pollock et al., 1956), Staphylococcus eureus (Richmond, 1965) and Bacillus licheniformis (Pollock, 1965) carry chromosomal genes for the enzyme β-lactamase which hydrolyses the β-lactam ring present in penicillin (Appendix figure 1).

The following four paragraphs are based on Hammond and Lambert (1978, Ch.8) and on Kagan (1980, Ch.1).

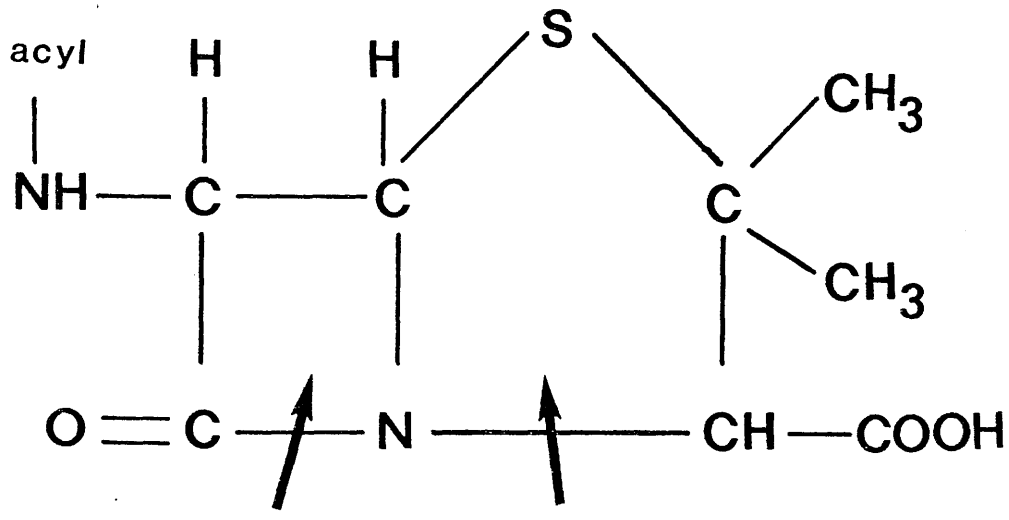
Microorganisms can resist drugs by changing their permeability to the drug. For example, tetracyclines accumulate in sensitive bacteria. Resistance to polymyxins is also associated with a change in permeability of the cell to the drugs.

Microorganisms may develop an altered structural target for the drug. For instance, chromosomal resistance to aminoglycosides is associated with the alteration of the target (receptor) on the 30s subunit of the bacterial ribosome.

Microorganisms may show enhancement of alternative metabolic pathways. For example, bacteria which are resistant to sulfonamide do not need extracellular para-amino benzoic acid, but can utilize preformed folic acid.

Microorganisms can develop new enzyme functions in the presence of antibiotics. For example, in some sulfonamide-sensitive bacteria, tetrahydropteroic acid synthetase has a much higer affinity for sulfonamide than for paraaminobenzoic acid, while in sulfonamide resistant mutants, the opposite is the case.

Several investigators have reported that the transposition of a discrete piece of DNA carrying an antibiotic-resistant gene between replicons in microorganisms can occur. (A replicon may be regarded as a genetic element of DNA which is capable of independent replication Hayes, 1964). Transposition of this sort between replicons has been



Appendix Figure 1

General structure of penicillin

reported in the structural genes of ampicillin resistance (Heffron et al., 1975; Bennett and Richmond, 1976; Rubens et al., 1976; Heffron et al., 1977) and for tetracycline resistance (Kleckner et al., 1975). The ampicillin resistant determinants of the plasmid RP4 can be transposed onto various other replicons genetically unrelated to RP4 (Hedges and Jacob, 1974).

Some microorganisms may change their characteristics under changing environmental conditions. For example, resistance to penicillin and tetracycline in Staphylococcus aureus is carried by two different plasmids (May et al., 1964; Asheshov, 1966). Only 12 of the 50 strains tested by Asheshov showed a loss of penicillin resistance when incubated at 43-44°C. In addition three of these 12 strains had lost tetracycline resistance. Both resistant determinants were lost independently. However, penicillin resistance was lost only in strains that were also resistant to tetracycline, and vice versa.

Polluted sites may play an important role in the transfer of antibiotic resistance genes. Koditschek and Guyre (1974) studied the antimicrobial resistant coliforms obtained from sediment and overlying water in a sewage sludge bed and found that some of the isolates were able to transfer antibiotic resistance (by conjugation) into recipient strains of Salmonella gallinarum. There are many other examples. Timoney et al. (1978) showed that ampicillin resistance in bacterial strains obtained from sediment near a sewage sludge dump was significantly greater than for strains obtained from control sediments. Marques et al. (1979) isolated Pseudomonas aeruginosa from polluted soils, and found these strains were able to transfer antibiotic resistance. In a later paper, Stewart and Koditschek (1980) showed that antibiotic resistance could be transferred from donor to recipient strains of Escherichia coli that had been inoculated into sterilized sewage sludge sediment from U.S. Environmental Protection

Agency Station NYB 44 (Lat: 40°25'54", Long: 73°45'00").

Resistance of bacteria to antibiotics may vary among isolates. Niemi and co-workers (1983) studied antibiotic resistance among fecal coliforms isolated from 14 water samples and found that there was a significant variation in the incidence of resistant strains among isolates. This variation was not connected with water source or pollution. They also concluded that there was a significant correlation between the frequency of Klebsiella species and the incidence of ampicillin resistance in water samples.

Mode of action of antibiotics

The effective agent of antibiotics must reach the pathogen in a high enough concentration either to inhibit its growth (bacteriostatic) or to kill it (bacteriocidal). Most antibiotics act in one of four major ways: inhibition of cell wall synthesis (e.g. Penicillins including Ampicillin), inhibition of cell membrane function (e.g. Gramicidins, Oligomycin, Polymyxin B), inhibition of nucleic acid synthesis (e.g. Nalidixic acid, Rifampicin) or inhibition of protein synthesis (e.g. Chloramphenicol, Streptomycin, Tetracycline). The antibiotics that I have used in typing deep-sea bacteria act in one of the four ways and are Ampicillin, Polymyxin B, Nalidixic acid, Chloramphenicol, Streptomycin and Tetracycline. The following four subsections outline the four major ways in which antibiotics act in more details.

Inhibition of cell wall synthesis (Ampicillin in this study)

The cell wall of bacteria is a rigid structure which maintains the shape of bacterial cell. The bacterial cell wall possesses a high internal osmotic pressure which is required for the cell to function. In Gram-positive bacteria the internal osmotic

pressure of the cell is higher than that of Gram-negative bacteria (Kagan, 1980). The composition of the cell wall is different between the two groups. for example, Gram-negative bacteria possess a lipid-rich layer surrounding the cell wall called the outer membrane (Hammond and Lambert, 1978). The layer is not present in Gram-positive bacteria (Plate 5). Hugo and Stretton (1966) showed that increasing the lipid content of Bacillus subtilis, Staphylococcus aureus and Streptococcus faecalis increased the resistance of these organisms to a number of penicillins.

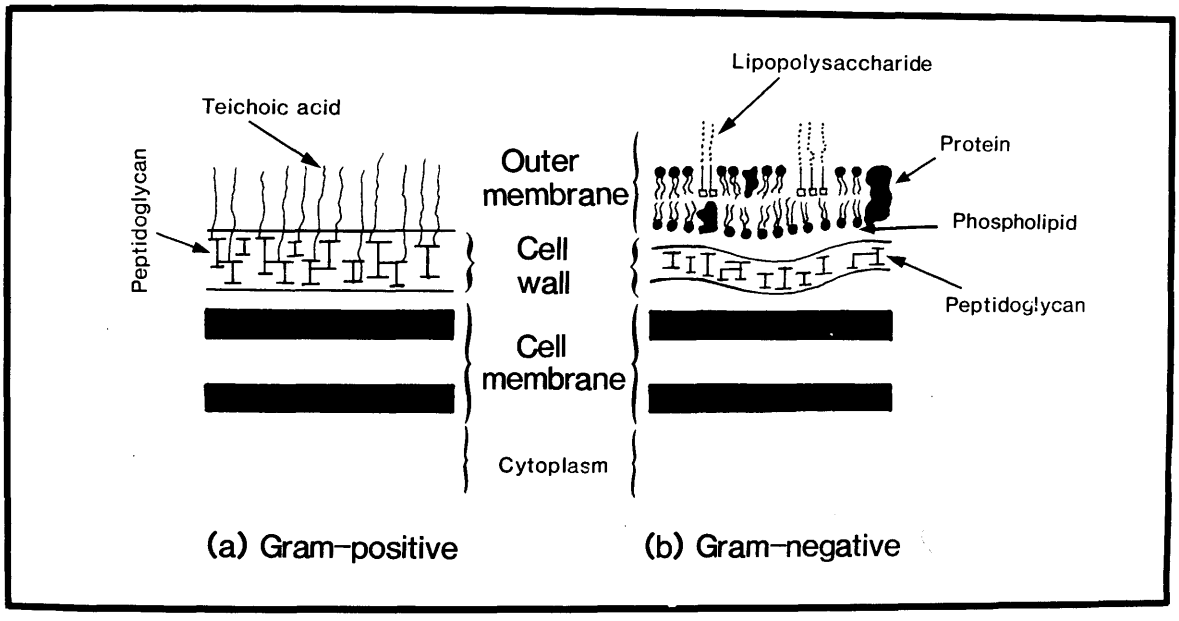
Many antibiotics are well known as inhibitors of bacterial cell wall synthesis, but the most selective inhibitors are penicillins and cephalosporins, which are bactericidal to growing cells (kagan, 1980). They inhibit the terminal cross-linking of the peptidoglycan in the cell wall. Peptidoglycan is a complex cross-linked network composed of sugar chains (glycans) which are made up of the amino sugars N-acetylglucosamine and N-acetylmuramic acid (Hammond and Lambert, 1978).

Benzylpenicillin and all derivatives of 6-aminopenicillanic acid (6-APA) are bactericidal antimicrobial agents. They cause spheroblast formation in bacteria in a similar way to that caused by lysozyme. However, unlike lysozyme which directly attacks the cell wall by its mucopeptidase activity, penicillins interfere with the formation of mucopeptide, thus inhibiting cell wall formation. This causes lysis of the bacterial cell and accounts for the bactericidal action of penicillins (Pitton, 1972).

The work of several experimenters supports this mode of action. Park (1952) detected an accumulation of N-acetylmuramic acid derivatives within Staphylococcus aureus cells growing in penicillin-containing media. Lederberg (1956) found that some bacteria

Plate 5

Structure of the cell wall of Gram-positive and Gram-negative bacteria (Hammond and Lambert, 1978).


Lipopolysaccharide
Teichoic acid
Protein
Peptidoglycan
Outer membrane
Phospholipid
Cell wall
Peptidoglycan
Cell membrane
Cytoplasm
(a) Gram-positive
(b) Gram-negative

(Escherichia coli and Salmonella typhimurium) formed spheroplasts when grown in a 10% sucrose media containing penicillin. Mandelstam and Rogers (1959) found that penicillin did not inhibit the synthesis of cytoplasmic proteins but inhibited the incorporation of specific amino acids into the cell wall.

Based on the above experiments, Pitton (1972, p.33) outlines the current hypothesis for the exact mode of action of penicillin, which the states is that penicillin prevents one of the final reactions in cell wall formation. Pitton (1972) suggests that penicillin inhibits polymerization of the mucopeptide peptidoglycan by stopping the linking between the pentaglycine parts of the molecules with the D-alanines in the tetrapeptides of adjacent glucosamine-muramic acid chains (Plate 6).

Inhibition of cell membrane function (Polymyxin B in this study)

One of the major problems in studying the mode of action of antibiotics on the cell membrane is that the structure and functions of normal membranes are still not fully known. Many types of model membranes have been proposed to show the distribution of protein components in the lipid bilayer (for example the Danielli and Davson mosaic membrane model (Edwards, 1980)).

In general, bacterial cell membranes can be regarded as asymmetric lipid bilayers in which synthesised protein are dissolved. The lipid molecule is composed of two parts - a polar hydrophilic group (water-loving) and nonpolar hydrophobic group (water hating). The structure of bacterial cell membrane is shown in Plate 7. It has been reported that the membranes are composed of approximately 30-40% lipid, 50-60% protein and 0-10% carbohydrate (Schmit et al., 1974).

Several investigators have reviewed the mode of action of antibiotics on cell membrane function (Few, 1955; Newton, 1956;

Plate 6

Diagram showing the structure of the peptidoglycan in the cell wall of Staphylococcus aureus. Glycan chains are composed of N-acetylglucosamine (G) and N-acetylmuramic acid (M). Tetrapeptide chains attached to each M are cross-linked by peptide bridges containing pentaglycine. The amino acids are L-alanine (Ala), D-glutamine (Glu), L-lysine (Lys), D-alanine (Ala), and glycine (Gly). Arrows indicate points where antibiotic breaking the linkage between the pentaglycine parts of the molecules with the D-alanines in the tetrapeptides of adjacent glucosamine-muramic acid chains (modefied from Hammond and Lambert, 1978).

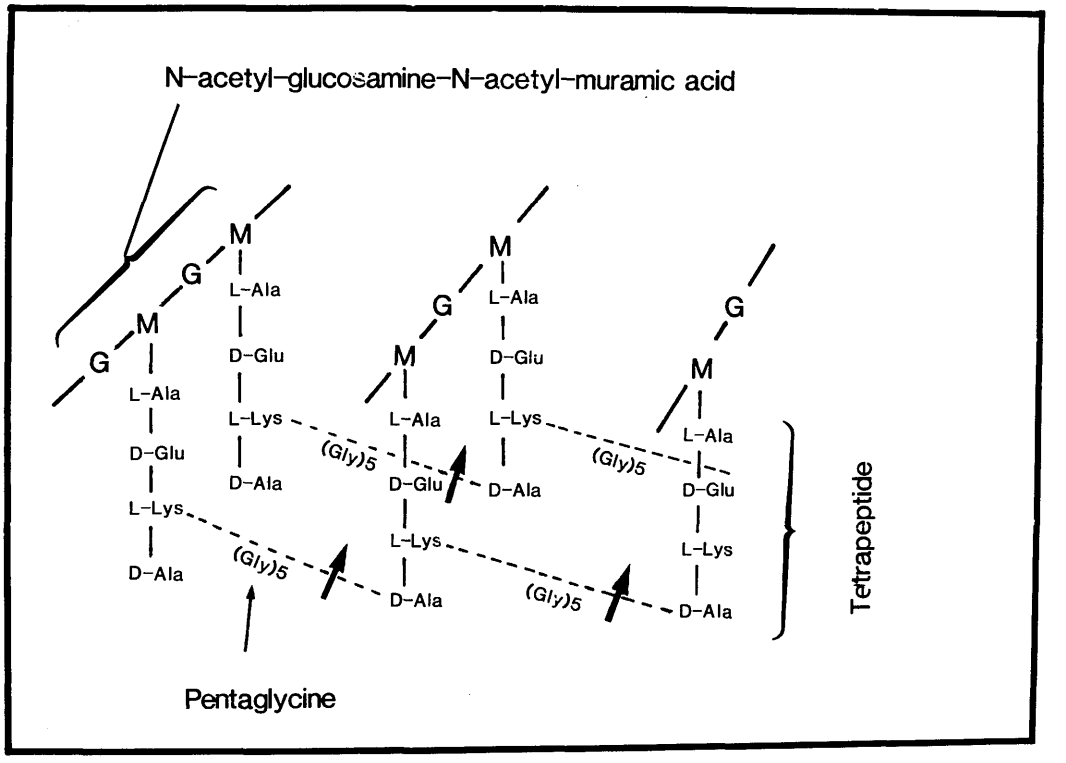
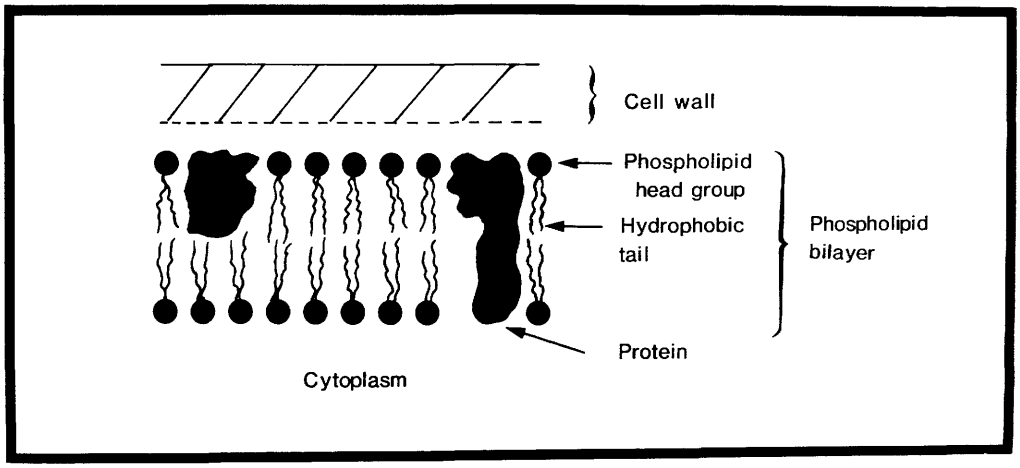

N-acetyl-glucosamine-N-acetyl-muramic acid
M
G
L-Ala
D-Glu
L-Lys
D-Ala
(Gly)5
Pentaglycine
Tetrapeptide

Plate 7

Diagram showing the generalised morphology of the microbial cell membrane (Hammond and Lambert, 1978).


Cell wall
Phospholipid
head group
Hydrophobic
tail
Phospholipid
bilayer
Protein
Cytoplasm

Feingold et al., 1974; Meyers et al., 1974). Antibiotics which effect the cell membrane may be divided into three groups (i) those which disorganise membrane structure (ii) those which alter membrane permeability, and (iii) those affecting membrane enzyme systems. Group (i) will be explained in detail because it contains the antibiotic polymyxin B used in this study. Group (ii) and (iii) will not be discussed here extensively, but full details can be found in Gottlieb and Shaw (1967), Hunter and Schwartz (1967) and Edwards, 1980).

Polymyxins are bactericidal against most Gram-negative bacilli. Feingold et al. (1974) studied the action of polymyxin B on cell membranes. They found that bacteria were varied in their susceptibility to this antibiotic. These workers also found that Gram-positive bacteria were relatively resistant to the lethal action of polymyxin B, whereas the cytoplasmic membrane of Gram-negative bacilli were damaged. HsuChen and Feingold (1973) studied the selectivity of polymyxin on bacteria. They concluded that the susceptibility of bacterial membranes to polymyxins depends on the presence of target molecules such as phosphatidyl ethanolamine, and on a threshold density of these molecules on the membrane surface. They also concluded that the effect of polymyxin B on liposomes was proportional to increase in the molar percent of Escherichia coli phospholipid percent. These workers suggested that the selective toxicity of polymyxins may depend on the absence of lecithin from the bacterial cell membrane. However Pache et al. (1972) studied the interaction of polymyxin B on artificial lecithin-water membrane systems and concluded that polymyxin interacts with lecithin, and does so by an electrostatic interaction between the amino group of the antibiotic and the phosphate group of the lecithin.

Several antibiotics can effect bacterial cells by altering

link DNA, some cause strand breaks in DNA, and some inhibit DNA replication and transcription enzymes.

An example is rifampicin which is very active against Gram-positive bacteria. This antibiotic is widely used clinically, especially in cases of tuberculosis. It inhibits RNA synthesis in susceptible cells but does not affect DNA or protein synthesis in vitro. Specifically, rifampicin inhibits bacterial growth by binding strongly to DNA-dependent RNA-polymerase of sensitive bacteria (Edwards, 1980).

Some drugs can affect DNA properties, such as ethidium and acridines. They increase the viscocity of DNA. Other effects of the drugs upon DNA include a decrease of the sedimentation coefficient and an increase in the thermal stability of DNA.

Nalidixic acid, which was used in my study, is an active antimicrobial agent for treatment of Gram-negative bacterial infections of the urinary tract (Franklin and Snow, 1971). It acts by inhibiting DNA replication enzymes (Edwards, 1980, p. 158, 185).

Inhibition of protein synthesis (Chloramphenicol, Streptomycin, Tetracycline in this study)

Proteins are defined as polymers made up of chains of amino acids joined together by peptide bonds (Hammond and Lambert, 1978). Synthesis of proteins occurs on ribosomes at a varied rate depending upon the system. (For example, β-galactosidase is synthesised in bacteria at a rate which approaches 15 amino acids polymerized per second (Edwards, 1980)).

Many drugs can inhibit protein synthesis in bacteria. Antibiotics inhibiting protein synthesis in bacteria can be divided into two types, inhibitors of the 30s ribosomal subunit, and inhibitors of the 50s ribosomal subunit. Garvin et al. (1974) studied

the effect of streptomycin or dihydro-streptomycin binding to 16s RNA or to 30s ribosomal subunits. They suggested that streptomycin binds to 16s RNA or to the 30s ribosomal subunit at the same site on the RNA chain. Dihyro-streptomycin, has the same bactericidal effect as streptomycin, binds to the same site, but has a lower affinity for that site. Garvin et al. (1974) also suggested that drug binding by streptomycin to 16s RNA was reversible, but that drug binding to the 30s subunit was irreversible even after withdrawal of the drug.

Other antibiotics, for example tetracycline, inhibit the 30s ribosomal subunit. A number of investigators have studied tetracyclines and their effects on bacterial cells (Gale and Folkes, 1953; Benbough and Morrison, 1965; Holmes and Wild, 1966; Suzuki et al., 1966; Kagan, 1980). Benbough and Morrison (1965) studied the bacteriostatic activities of some tetracyclines. They suggested that chlorinated tetracyclines could inhibit the growth of the organisms examined by acting on D-glutamate accumulation during aerobic conditions. De-Zeeuw (1968) found that the net accumulation of tetracyclines by Escherichia coli occurs in two separate stages as the concentration is increased. The first stage occurs at concentrations less than the bacteriostatic level and involves adsorption onto the surface of the cell. At concentrations above the bacteriostatic level, the second stage occurs and involves penetration of the cell membrane by the tetracycline molecules. Kagan (1980) states that tetracyclines bind to the 30s subunit of microbial ribosomes. They inhibit protein synthesis by preventing the attachment of aminoacyl tRNA to ribosomes.

Some antibiotics inhibit the 50s ribosomal subunit. One example is chlormphenicol, which is mainly used as a bacteriostatic drug. Addition of chloramphenicol to bacterial cultures at inhibitory concentrations results in inhibition of protein synthesis. This

phenomenon has been described by many investigators (Lacks and Gros, 1959; Vazquez, 1964; Das et al., 1966). Julian (1965) showed that chloramphenicol at a very high concentration strongly inhibited the synthesis of longer peptide chains, but formation of di-and tri-peptides were not affected. Das et al. (1966) studied the inhibition of protein synthesis by chloramphenicol in Escherichia coli cells. They found that the drug does not affect the association between mRNA and ribosomes. Its primary mode of action is to block the attachment of new amino acids to the growing protein chains that are attached to the ribosomes.

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
			128	64	32	16	8	4
Cadmium	2	7	0/44(0%)	0/44(0%)	11/44(25.0%)	38/44(86.4%)	40/44(90.9%)	42/44(95.5%)
		14	0/45(0%)	1/45(2.22%)	17/45(37.8%)	41/45(91.1%)	43/45(95.6%)	44/45(97.8%)
		21	0/45(0%)	1/45(2.22%)	23/45(51.1%)	41/45(91.1%)	43/45(95.6%)	44/45(97.8%)
Chromium	2	7	8/47(17.0%)	14/47(29.8%)	20/47(42.6%)	28/47(59.6%)	38/47(80.9%)	40/47(85.1%)
		14	19/48(39.6%)	24/48(50.0%)	37/48(77.1%)	38/48(79.2%)	40/48(83.3%)	43/48(89.6%)
		21	28/48(58.3%)	32/48(66.7%)	37/48(77.1%)	41/48(85.4%)	43/48(89.6%)	45/48(93.8%)
Copper	2	7	0/38(0%)	0/38(0%)	27/38(71.1%)	30/38(78.9%)	36/38(94.7%)	38/38(100%)
		14	0/39(0%)	1/39(2.56%)	30/39(76.9%)	32/39(82.1%)	36/39(92.3%)	39/39(100%)
		21	0/39(0%)	1/39(2.56%)	32/39(82.1%)	33/39(84.6%)	37/39(94.9%)	39/39(100%)
			800	400	200	100	50	25
Lead	2	7	0/38(0%)	0/38(0%)	0/38(0%)	33/38(86.8%)	38/38(100%)	38/38(100%)
		14	0/40(0%)	0/40(0%)	2/40(5.0%)	38/40(95.0%)	39/40(97.5%)	39/40(97.5%)
		21	0/43(0%)	0/43(0%)	4/43(9.30%)	40/43(93.0%)	41/43(95.3%)	43/43(100%)
			32	16	8	4	2	1
Mercury	2	7	9/43(20.9%)	15/43(34.9%)	28/43(65.1%)	39/43(90.7%)	39/43(90.7%)	39/43(90.7%)
		14	9/43(20.9%)	15/43(34.9%)	28/43(65.1%)	39/43(90.7%)	39/43(90.7%)	40/43(93.0%)
		21	9/43(20.9%)	16/43(37.2%)	32/43(74.4%)	39/43(90.7%)	39/43(90.7%)	41/43(95.3%)

Appendix Table 1: The number of isolates growing at various concentrations (mg/l) of heavy metals aftr 7, 14 and 21 days. The number is expressed as a percentage of the isolates growing on the control plate (see materials and methods, p. 35). Data from preliminary master replica two (PMR_2).

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
			64	32	16	8	4	2
Ampicillin	2	7	26/46 (56.5%)	23/46 (50.0%)	24/46 (52.2%)	28/46 (60.9%)	29/46 (63.0%)	39/46 (84.8%)
		14	28/46 (60.9%)	29/46 (63.0%)	30/46 (65.2%)	28/46 (60.9%)	34/46 (73.9%)	40/46 (87.0%)
		21	29/46 (63.0%)	34/46 (73.9%)	34/46 (73.9%)	32/46 (69.6%)	34/46 (73.9%)	40/46 (87.0%)
Chloram-phenicol	2	7	4/41 (9.76%)	3/41 (7.32%)	10/41 (24.4%)	21/41 (51.2%)	27/41 (65.9%)	30/41 (73.2%)
		14	4/41 (9.76%)	3/41 (7.32%)	12/41 (29.3%)	21/41 (51.2%)	27/41 (65.9%)	31/41 (75.6%)
		21	5/43 (11.6%)	5/43 (11.6%)	14/43 (32.6%)	23/43 (53.5%)	28/43 (65.1%)	34/43 (79.1%)
Polymyxin.B	2	7	8/48 (16.7%)	15/48 (31.3%)	21/48 (43.8%)	30/48 (62.5%)	35/48 (72.9%)	33/48 (68.8%)
		14	9/48 (18.8%)	18/48 (37.5%)	22/48 (45.8%)	34/48 (70.8%)	36/48 (75.0%)	33/48 (68.8%)
		21	12/48 (25.0%)	19/48 (39.6%)	26/48 (54.2%)	37/48 (77.1%)	38/48 (79.2%)	34/48 (70.8%)
Streptomycin	2	7	25/40 (62.5%)	31/40 (77.5%)	33/40 (82.5%)	35/40 (87.5%)	37/40 (92.5%)	34/40 (85.0%)
		14	28/40 (70.0%)	33/40 (82.5%)	34/40 (85.0%)	35/40 (87.5%)	37/40 (92.5%)	34/40 (85.0%)
		21	30/42 (71.4%)	35/42 (83.3%)	36/42 (85.7%)	36/42 (85.7%)	37/42 (88.1%)	38/42 (90.5%)
Nalidixic acid	2	7	38/48 (79.2%)	41/48 (85.4%)	36/48 (75.0%)	48/48 (100%)	45/48 (93.8%)	48/48 (100%)
		14	40/49 (81.6%)	45/49 (91.8%)	45/49 (91.8%)	48/49 (98.0%)	45/49 (91.8%)	48/49 (98.0%)
		21	40/49 (81.6%)	46/49 (93.9%)	47/49 (95.9%)	48/49 (98.0%)	46/49 (93.9%)	48/49 (98.0%)
			32	16	8	4	2	1
Methylene Blue	2	7	33/46 (71.7%)	35/46 (76.1%)	33/46 (71.7%)	33/46 (71.7%)	34/40 (73.9%)	45/46 (97.8%)
		14	33/47 (70.2%)	35/47 (74.5%)	33/47 (70.2%)	33/47 (70.2%)	37/47 (78.7%)	46/47 (97.9%)
		21	33/47 (70.2%)	35/47 (74.5%)	34/47 (72.3%)	34/47 (72.3%)	37/47 (78.7%)	46/47 (97.9%)

Appendix Table 2: The number of isolates growing at various concentrations (mg/l) of antibiotics and anethylene blue dye after 7, 14 and 21 days. The number is expressed as a percentage of the isolates growing on the control plate (see materials and methods, p.35). Data from preliminary maser replica (PMR_2).

Appendix Table 3

The number of isolates growing at various concentrations (mg/l) of antibiotics and methylene blue dye after 7, 14 and 21 days. The number is expressed as a percentage of the isolates growing on the control plate (see materials and methods p. 35). Data from definitive master replica 1 (MR1).

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
			256	128	64	32	8	2
Ampicillin	1	7	11/38(28.9%)	12/38(31.6%)	15/38(39.5%)	16/38(42.1%)	19/38(50.0%)	24/38(63.2%)
		14	14/40(35.0%)	14/40(35.0%)	16/40(40.0%)	17/40(42.5%)	20/40(50.0%)	24/40(60.0%)
		21	15/40(37.5%)	15/40(37.5%)	17/40(42.5%)	17/40(42.5%)	20/40(50.0%)	24/40(60.0%)
			64	32	16	8	4	2
Chloram-phenicol	1	7	0/33(0%)	0/33(0%)	0/33(0%)	0/33(0%)	1/33(3.0%)	3/33(9.1%)
		14	0/36(0%)	0/36(0%)	0/36(0%)	0/36(0%)	1/36(2.8%)	5/36(13.9%)
		21	0/37(0%)	0/37(0%)	0/37(0%)	0/37(0%)	2/37(5.4%)	5/37(13.5%)
			128	64	32	16	8	4
Polymyxin.B	1	7	3/33(9.1%)	5/33(15.2%)	8/33(24.2%)	12/33(36.3%)	11/33(33.3%)	17/33(51.5%)
		14	3/35(8.6%)	7/35(20.0%)	13/35(37.1%)	15/35(42.9%)	14/35(40.0%)	18/35(51.4%)
		21	6/37(16.2%)	10/37(27.0%)	15/37(40.5%)	20/37(54.1%)	18/37(48.6%)	21/37(56.8%)
			256	128	64	32	16	4
Streptomycin	1	7	1/41(2.43%)	4/41(9.75%)	18/41(43.9%)	19/41(46.3%)	21/41(51.2%)	23/41(56.1%)
		14	1/41(2.43%)	8/41(19.5%)	19/41(46.3%)	20/41(48.8%)	22/41(53.7%)	29/41(70.7%)
		21	3/42(7.1%)	9/42(21.4%)	21/42(50.0%)	21/42(50.0%)	23/42(54.8%)	30/42(71.4%)
			128	64	32	16	8	4
Tetracycline	1	7	7/40(17.5%)	18/40(45.0%)	17/40(42.5%)	33/40(82.5%)	36/40(90.0%)	39/40(97.5%)
		14	11/42(26.2%)	25/42(59.5%)	22/42(52.4%)	34/42(81.0%)	36/42(85.7%)	39/42(92.9%)
		21	11/42(26.2%)	28/42(66.7%)	22/42(52.4%)	34/42(81.0%)	36/42(85.7%)	39/42(92.9%)
			256	128	64	32	16	4
Nalidixic acid	1	7	6/43(14.0%)	13/43(30.2%)	22/43(51.2%)	36/43(83.7%)	41/43(95.3%)	43/43(100%)
		14	7/45(15.6%)	19/45(42.2%)	33/45(73.3%)	38/45(84.4%)	42/45(93.3%)	44/45(97.8%)
		21	10/45(22.2%)	22/45(48.9%)	36/45(80.0%)	42/45(93.3%)	44/45(97.8%)	44/45(97.8%)
			256	128	64	32	8	2
Methylene-Blue	1	7	12/45(26.7%)	18/45(40.0%)	20/45(44.4%)	21/45(46.7%)	23/45(51.1%)	25/45(55.6%)
		14	13/45(28.9%)	21/45(46.7%)	22/45(48.9%)	22/45(48.9%)	23/45(51.1%)	25/45(55.6%)
		21	22/45(48.9%)	22/45(48.9%)	23/45(51.1%)	23/45(51.1%)	23/45(51.1%)	25/45(55.6%)

Appendix Table 4

The number of isolates growing at various concentrations (mg/l) of antibiotics and methylene blue dye after 7, 14 and 21 days. he number is expressed as a percentage of isolates growing on the control plate (see materials and methods, p. 35). Data from definitive master replica 2 (MR1).

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
			256	128	64	32	8	2
Ampicillin	2	7	2/41(4.88%)	9/41(22%)	12/41(29.3%)	14/41(34.1%)	15/41(36.6%)	34/41(82.9%)
		14	12/43(27.9%)	13/43(30.2%)	15/43(34.9%)	16/43(37.2%)	26/43(60.5%)	35/43(81.4%)
		21	12/43(27.9%)	14/43(32.6%)	17/43(39.5%)	24/43(55.8%)	33/43(76.7%)	35/43(81.4%)
			64	32	16	8	4	2
Chloram-phenicol	2	7	0/39(0%)	0/39(0%)	0/39(0%)	2/39(5.13%)	7/39(17.9%)	12/39(30.8%)
		14	0/42(0%)	2/42(4.76%)	6/42(14.3%)	4/42(9.52%)	13/42(31%)	19/42(45.2%)
		21	0/42(0%)	2/42(4.8%)	9/42(21.4%)	5/42(11.9%)	13/42(31%)	20/42(47.6%)
			128	64	32	16	8	4
Polymyxin.B	2	7	2/42(4.76%)	2/42(4.76%)	2/42(4.76%)	3/42(7.14%)	3/42(7.14%)	4/42(9.52%)
		14	4/44(9.09%)	5/44(11.4%)	6/44(13.6%)	7/44(15.9%)	9/44(20.5%)	8/44(18.2%)
		21	5/45(11.1%)	5/45(11.1%)	6/45(13.3%)	7/45(15.6%)	9/45(20%)	8/45(17.8%)
			256	128	64	32	16	4
Streptomycin	2	7	0/44(0%)	1/44(2.3%)	6/44(13.6%)	15/44(34.1%)	38/44(86.4%)	38/44(86.4%)
		14	0/45(0%)	2/45(4.4%)	13/45(28.9%)	24/45(53.3%)	39/45(86.7%)	40/45(88.9%)
		21	1/45(2.22%)	3/45(6.67%)	13/45(28.9%)	25/45(55.6%)	39/45(86.7%)	40/45(88.9%)
			128	64	32	16	8	4
Tetracycline	2	7	2/41(4.88%)	10/41(24.4%)	25/41(61%)	27/41(65.9%)	38/41(92.7%)	41/41(100%)
		14	6/41(14.6%)	26/41(63.4%)	35/41(85.4%)	29/41(70.7%)	39/41(95%)	41/41(100%)
		21	12/41(29.3%)	29/41(70.7%)	35/41(85.4%)	33/41(80.5%)	41/41(100%)	41/41(100%)
			256	128	64	32	16	4
Nalidixic acid	2	7	0/35(0%)	7/35(20%)	8/35(22.9%)	14/35(40%)	20/35(57.1%)	33/35(94.3%)
		14	5/38(13.2%)	11/38(28.9%)	13/38(34.2%)	24/38(63.2%)	32/38(84.2%)	37/38(97.4%)
		21	5/38(13.2%)	11/38(28.9%)	15/38(39.5%)	27/38(71.1%)	36/38(94.7%)	37/38(97.4%)
			256	128	64	32	8	2
Methylene-Blue	2	7	21/43(48.8%)	21/43(48.8%)	14/43(32.6%)	14/43(32.6%)	27/43(62.8%)	40/43(93.0%)
		14	26/44(59.1%)	25/44(56.8%)	32/44(72.7%)	28/44(63.6%)	37/44(84.1%)	42/44(95.5%)
		21	26/44(59.1%)	25/44(56.8%)	34/44(77.3%)	31/44(70.5%)	39/44(88.6%)	42/44(95.5%)

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
			256	128	64	32	8	2
Ampicillin	2	7	2/41(4.88%)	9/41(22%)	12/41(29.3%)	14/41(34.1%)	15/41(36.6%)	34/41(82.9%)
		14	12/43(27.9%)	13/43(30.2%)	15/43(34.9%)	16/43(37.2%)	26/43(60.5%)	35/43(81.4%)
		21	12/43(27.9%)	14/43(32.6%)	17/43(39.5%)	24/43(55.8%)	33/43(76.7%)	35/43(81.4%)
			64	32	16	8	4	2
Chloram-phenicol	2	7	0/39(0%)	0/39(0%)	0/39(0%)	2/39(5.13%)	7/39(17.9%)	12/39(30.8%)
		14	0/42(0%)	2/42(4.76%)	6/42(14.3%)	4/42(9.52%)	13/42(31%)	19/42(45.2%)
		21	0/42(0%)	2/42(4.8%)	9/42(21.4%)	5/42(11.9%)	13/42(31%)	20/42(47.6%)
			128	64	32	16	8	4
Polymyxin.B	2	7	2/42(4.76%)	2/42(4.76%)	2/42(4.76%)	3/42(7.14%)	3/42(7.14%)	4/42(9.52%)
		14	4/44(9.09%)	5/44(11.4%)	6/44(13.6%)	7/44(15.9%)	9/44(20.5%)	8/44(18.2%)
		21	5/45(11.1%)	5/45(11.1%)	6/45(13.3%)	7/45(15.6%)	9/45(20%)	8/45(17.8%)
			256	128	64	32	16	4
Streptomycin	2	7	0/44(0%)	1/44(2.3%)	6/44(13.6%)	15/44(34.1%)	38/44(86.4%)	38/44(86.4%)
		14	0/45(0%)	2/45(4.4%)	13/45(28.9%)	24/45(53.3%)	39/45(86.7%)	40/45(88.9%)
		21	1/45(2.22%)	3/45(6.67%)	13/45(28.9%)	25/45(55.6%)	39/45(86.7%)	40/45(88.9%)
			128	64	32	16	8	4
Tetracycline	2	7	2/41(4.88%)	10/41(24.4%)	25/41(61%)	27/41(65.9%)	38/41(92.7%)	41/41(100%)
		14	6/41(14.6%)	26/41(63.4%)	35/41(85.4%)	29/41(70.7%)	39/41(95%)	41/41(100%)
		21	12/41(29.3%)	29/41(70.7%)	35/41(85.4%)	33/41(80.5%)	41/41(100%)	41/41(100%)
			256	128	64	32	16	4
Nalidixic acid	2	7	0/35(0%)	7/35(20%)	8/35(22.9%)	14/35(40%)	20/35(57.1%)	33/35(94.3%)
		14	5/38(13.2%)	11/38(28.9%)	13/38(34.2%)	24/38(63.2%)	32/38(84.2%)	37/38(97.4%)
		21	5/38(13.2%)	11/38(28.9%)	15/38(39.5%)	27/38(71.1%)	36/38(94.7%)	37/38(97.4%)
			256	128	64	32	8	2
Methylene-Blue	2	7	21/43(48.8%)	21/43(48.8%)	14/43(32.6%)	14/43(32.6%)	27/43(62.8%)	40/43(93.0%)
		14	26/44(59.1%)	25/44(56.8%)	32/44(72.7%)	28/44(63.6%)	37/44(84.1%)	42/44(95.5%)
		21	26/44(59.1%)	25/44(56.8%)	34/44(77.3%)	31/44(70.5%)	39/44(88.6%)	42/44(95.5%)

Appendix Table 5

The number of isolates growing at various concentrations (mg/l) of heavy metals after 7, 14 and 21 days. The number is expressed as a percentage of the isolates growing on control plate (see materials and methods, p. 35). Data from definitive master replica 1 (MR1).

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
Cadmium	1		128	64	48	32	16	8
		7	0/40(0%)	4/40(10.0%)	6/40(15.0%)	9/40(22.5%)	20/40(50.0%)	28/40(70.0%)
		14	0/43(0%)	5/43(11.6%)	7/43(16.3%)	9/43(20.9%)	20/43(46.5%)	28/43(65.1%)
		21	0/43(0%)	7/43(16.3%)	7/43(16.3%)	9/43(20.9%)	21/43(48.8%)	28/43(65.1%)
Chromium	1		256	128	64	32	16	8
		7	6/29(20.7%)	18/29(62.1%)	21/29(72.4%)	24/29(82.8%)	24/29(82.8%)	26/29(89.7%)
		14	8/33(24.2%)	22/33(66.7%)	25/33(75.8%)	26/33(78.8%)	28/33(84.8%)	28/33(84.8%)
		21	8/35(22.9%)	22/35(62.9%)	25/35(71.4%)	30/35(85.7%)	28/35(80.0%)	32/35(91.4%)
Copper	1		128	64	48	32	16	8
		7	0/40(0%)	15/40(37.5%)	18/40(45.0%)	31/40(77.5%)	38/40(95.0%)	37/40(92.5%)
		14	0/43(0%)	15/43(34.9%)	25/43(58.1%)	33/43(76.7%)	41/43(95.3%)	40/43(93.0%)
		21	0/44(0%)	18/44(40.9%)	26/44(59.1%)	33/44(76.7%)	41/44(93.2%)	40/44(91.0%)
Lead	1		512	256	192	160	128	64
		7	2/37(5.4%)	20/37(54.1%)	25/37(67.6%)	30/37(81.1%)	29/37(78.4%)	36/37(97.3%)
		14	2/41(4.9%)	21/41(51.2%)	26/41(63.4%)	33/41(80.5%)	28/41(68.3%)	41/41(100%)
		21	2/41(4.9%)	22/41(53.7%)	27/41(65.9%)	33/41(80.5%)	32/41(78.0%)	41/41(100%)
Manganese	1		4096	2048	1024	512	256	128
		7	0/42(0%)	8/42(19.0%)	12/42(28.6%)	23/42(54.8%)	37/42(88.1%)	36/42(85.7%)
		14	0/42(0%)	11/42(26.2%)	15/42(35.7%)	23/42(54.8%)	37/42(88.1%)	37/42(88.1%)
		21	1/44(2.3%)	11/44(25.0%)	16/44(36.4%)	25/44(56.8%)	37/44(84.1%)	38/44(86.4%)
Mercury	1		64	32	16	8	4	2
		7	0/39(0%)	0/39(0%)	7/39(17.9%)	10/39(25.6%)	10/39(25.6%)	25/39(64.1%)
		14	0/39(0%)	0/39(0%)	7/39(17.9%)	11/39(28.2%)	11/39(28.2%)	28/39(71.8%)
		21	0/41(0%)	0/41(0%)	7/41(17.1%)	11/41(26.8%)	11/41(26.8%)	29/41(70.7%)

Appendix Table 6

The number of isolates growing at various concentrations (mg/l) of heavy metals after 7, 14 and 21 days. The number is expressed as a percentage of the isolates growing on the control plate (see materials and methods, p. 35). Data from definitive master replica 2 (MR2).

Antimicrobial agent	Master replica plate	Plate count (days)	Concentration (mg/l)					
			128	64	48	32	16	8
Cadmium	2	7	0/43(0%)	0/43(0%)	1/43(2.33%)	11/43(25.6%)	25/43(58.1%)	36/43(83.7%)
		14	0/43(0%)	0/43(0%)	6/43(14%)	14/43(32.6%)	28/43(65.1%)	38/43(88.4%)
		21	0/45(0%)	0/45(0%)	6/45(13.3%)	14/45(31.1%)	28/45(62.2%)	39/45(86.7%)
			256	128	64	32	16	8
Chromium	2	7	6/49(12.2%)	26/49(53.1%)	29/49(59.2%)	36/49(73.5%)	39/49(79.6%)	44/49(89.8%)
		14	6/49(12.2%)	27/49(55.1%)	34/49(69.4%)	36/49(73.5%)	46/49(93.9%)	47/49(95.9%)
		21	6/49(12.2%)	27/49(55.1%)	34/49(69.4%)	36/49(73.5%)	46/49(93.9%)	48/49(98%)
			128	64	48	32	16	8
Copper	2	7	2/40(5.00%)	29/40(72.5%)	32/40(80%)	35/40(87.5%)	39/40(97.5%)	40/40(100%)
		14	2/46(4.35%)	32/46(69.6%)	39/46(84.8%)	42/46(91.3%)	44/46(95.7%)	45/46(97.8%)
		21	4/46(8.7%)	35/46(76.1%)	40/46(87%)	43/46(93.5%)	45/46(97.9%)	45/46(97.8%)
			512	256	192	160	128	64
Lead	2	7	0/39(0%)	26/39(66.7%)	33/39(84.6%)	35/39(89.7%)	37/39(94.9%)	39/39(100%)
		14	0/39(0%)	26/39(66.7%)	35/39(89.7%)	35/39(89.7%)	33/39(84.6%)	39/39(100%)
		21	0/39(0%)	29/39(74.4%)	35/39(89.7%)	35/39(89.7%)	33/39(84.6%)	39/39(100%)
			4096	2048	1024	512	256	128
Manganese	2	7	1/30(3.33%)	2/30(6.67%)	8/30(26.7%)	16/30(53.3%)	23/30(76.7%)	29/30(93.5%)
		14	2/31(6.45%)	5/31(16.1%)	15/31(48.4%)	24/31(77.4%)	25/31(80.6%)	30/31(96.8%)
		21	2/31(6.5%)	9/31(29.0%)	15/31(48.4%)	24/31(77.4%)	26/31(83.9%)	31/31(100%)
			64	32	16	8	4	2
Mercury	2	7	0/38(0%)	4/38(10.5%)	11/38(28.9%)	16/38(42.1%)	17/38(44.7%)	28/38(73.7%)
		14	0/39(0%)	5/39(12.8%)	11/39(28.2%)	17/39(43.6%)	17/39(43.6%)	29/39(74.4%)
		21	0/39(0%)	5/39(12.8%)	11/39(28.2%)	20/39(51.3%)	20/39(51.3%)	32/39(82.1%)

Antimicrobial agent	Vol. stock solution (ml)	Vol. agar (ml)	Final concentration (mg/l)
Ampicillin	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.06		2
Chloramphenicol	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.06		2
Polymyxin B	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.06		2
Streptomycin sulphate	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.06		2
Tetracycline hydrochloride	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.06		2
Nalidixic acid	0.2 from serial dilution of N NaOH	100	64
	0.2		32
	0.2		16
	0.2		8
	0.2		4
	0.2		2
Methylene blue	2	100	32
	1		16
	0.5		8
	0.25		4
	0.125		2
	0.06		1

Appendix Table 7

Volume of stock solutions (ml) of the antibiotics and dye added to the volume of molten agar and the final concentrations (mg/l) used in the preliminary experiments.

Antimicrobial agent	Vol. stock solution (ml)	Vol. agar (ml)	Final concentration (mg/l)
Cadmium chloride	2	100	128
	1		64
	0.5		32
	0.25		16
	0.125		8
	0.06		4
Potassium chromate	2	100	128
	1		64
	0.5		32
	0.25		16
	0.125		8
	0.06		4
Copper sulphate	2	100	128
	1		64
	0.5		32
	0.25		16
	0.125		8
	0.06		4
Lead acetate	2	100	800
	1		400
	0.5		200
	0.25		100
	0.125		50
	0.06		25
Manganous chloride	2	100	128
	1		64
	0.5		32
	0.25		16
	0.125		8
	0.06		4
* Mercuric chloride	0.1	100	32
	0.05		16
	0.1 of 25%		8
	0.05 of 25%		4
	0.1 of 6.25%		2
	0.05 of 6.25%		1

Appendix Table 8

Volume of stock solution (ml) of heavy metals added to the volume of molten agar and the final concentrations (mg/l) used in the preliminary experiments.

* See table 4 P. 23.

Antimicrobial agent	Vol. stock solution (ml)	Vol. agar (ml)	Final concentration (mg/l)
Ampicillin	4	100	256
	2		128
	1		64
	0.5		32
	0.125		8
	0.031		2
Chloramphenicol	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.062		2
Polymyxin B	4	100	128
	2		64
	1		32
	0.5		16
	0.25		8
	0.125		4
Streptomycin sulphate	4	100	256
	2		128
	1		64
	0.5		32
	0.25		16
	0.0625		4
Tetracycline hydrochloride	4	100	128
	2		64
	1		32
	0.5		16
	0.25		8
	0.125		4
Nalidixic acid	0.2 from serial dilution of N NaOH	100	256
	0.2		128
	0.2		64
	0.2		32
	0.2		16
	0.2		4
Methylene blue	4	100	256
	2		128
	1		64
	0.5		32
	0.125		8
	0.031		2

Appendix Table 9
Volume of stock solutions (ml) of the antibiotics and dye added to the volume of molten agar and the final concentrations (mg/l) used in the definitive experiments.

Antimicrobial agent	Vol. stock solution (ml)	Vol. agar (ml)	Final concentration (mg/l)
Cadmium chloride	2	100	128
	1		64
	0.75		48
	0.50		32
	0.25		16
	0.125		8
Potassium chromate	4	100	256
	2		128
	1		64
	0.50		32
	0.25		16
	0.125		8
Copper sulphate	2	100	128
	1		64
	0.75		48
	0.50		32
	0.25		16
	0.125		8
Lead acetate	2	100	512
	1		256
	0.75		192
	0.625		160
	0.50		128
	0.25		64
Manganous chloride	4	100	4096
	2		2048
	1		1024
	0.5		512
	0.25		256
	0.125		128
Mercuric chloride	2	100	64
	1		32
	0.5		16
	0.25		8
	0.125		4
	0.0625		2

Appendix Table 10

Volume of stock solutions (ml) of the heavy metals added to the volume of molten agar and the final concentrations (mg/l) used in the definitive experiments.

No.	Subject	Counts 14 days (high conc.)	Counts 14 days (low conc.)	Difference	Ranks +ve	Ranks -ve
1	Tetracycline	150	95	+ 55	6	
2		299	100	+199	8	
3	Nalidixic acid	111	98	+ 13	2	
4		145	103	+ 42	5	
5	Ampicillin	121	95	+ 26	3.5	
6		572	98	+474	9	
7	Chloramphenicol	-	-	-		
8		-	-	-		
9	Polymyxin B	95	100	- 5		1
10		191	191	-		
11	Streptomycin	100	126	- 26		3.5
12		191	103	+ 88	7	
Size of sample =9					R+ =40.5	R- =4.5

Appendix Table 11

Wilcoxon's matched pairs test for comparing the difference between 14 day counts of bacterial growth at low and high concentrations of antibiotics.

No.	Subject	Counts 14 days (high conc.)	Counts 14 days (low conc.)	Difference	Ranks +ve	Ranks -ve
1	Cadmium	116	93	+ 23	8	
2		601	106	+495	11	
3	Chromium	117	95	+ 22	7	
4		100	107	- 7		1
5	Copper	93	101	- 8		2
6		87	98	- 11		3
7	Lead	91	103	- 12		4.5
8		100	100	-		
9	Manganese	138	103	+ 35	9	
10		194	104	+ 90	10	
11	Mercury	100	112	- 12		4.5
12		122	101	+ 21	6	
Size of samples =11					R+ =51	R- =15

Appendix Table 12

Wilcoxon's matched pairs test for comparing the difference between 14 day counts of bacterial growth at low and high concentrations of heavy metals.

No.	Subject	Counts 21 days (high conc.)	Counts 21 days (low conc.)	Difference	Ranks +ve	Ranks -ve
1	Tetracycline	150	95	+ 55	4	
2		201	100	+101	7	
3	Nalidixic acid	159	98	+ 61	5	
4		145	113	+ 32	1	
5	Ampicillin	130	95	+ 35	2	
6		572	98	+474	10	
7	Chloramphenicol	-	148	-		
8		-	155	-		
9	Polymyxin B	178	110	+ 68	6	
10		233	187	+ 46	3	
11	Streptomycin	292	127	+165	8	
12		290	103	+187	9	
Size of sample =10					R+ =55	R- =0

Appendix Table 13

Wilcoxon's matched pairs test for comparing the difference between 21 day counts of bacterial growth at low and high concentrations of antibiotics.

NO.	Subject	Counts 21 days (high conc.)	21 days (low conc.)	Difference	Ranks +ve	-ve
1	Cadmium	163	93	+ 70	9	
2		571	104	+467	12	
3	Chromium	111	102	+ 9	1.5	
4		100	109	- 9		1.5
5	Copper	109	98	+ 11	3.5	
6		174	98	+ 76	10	
7	Lead	91	103	- 12		5.5
8		112	100	+ 12	5.5	
9	Manganese	132	101	+ 31	8	
10		195	107	+ 88	11	
11	Mercury	96	110	- 14		7
12		122	111	+ 11	3.5	
Size of sample =12					R+ =64	R- =14

Appendix Table 14

Wilcoxon's matched pairs test for comparing the difference between 21 day counts of bacterial growth at low and high concentrations of heavy metals.

APPENDIX B

Analysis of the normality distribution of the number of bacterial isolates clustered at 50% S and the selection of a suitable transformation method

1- Analysis of normality

There are several methods available for determining whether data is normally distributed. These include the following.

A- Graphic method for analysing normality

Two graphic methods can be used to analyse for normality. The first method is the cumulative percentage method. This is based on the frequency distribution table prepared prior to any step. This method is used when n is greater than 50. The second method is the rankits method. This is used when n is less than 50 (Sokal and Rholf, 1981, p. 117-126).

In this study, n was always greater than 50, and therefore the first method was applied. Sokal and Rholf (1981) give the procedure for the use of the cumulative percentage method in Box 6.3 p. 120 and 121.

After the frequency distribution table was prepared (Appendix tables 15 A and 15 B), the number of isolates/group/site or isolates/group/depth (x-axis) was plotted against the percent cumulative frequency (y-axis) on a propability scale. Although a straight line can be fitted to the points by eye (Sokal and Rholf, 1981, p. 120), it is more accurate to use regression analysis. I obtained the (x, y) pairs for the regression analysis as follows. The x value was read directly from the x-axis. The y value was obtained by measuring its hight above an arbitrary line drawn parallel to the x-axis. I only included points located between the cumulative

frequencies of 2% to 89%. This more conservative, and therefore safer than the 25% and 75% recommended by Sokal and Rholf (1981) who state that "in drawing the line, most weight should be given to the points between cumulative frequencies of 25% and 75%".

B- **The relationship between mean and standard deviation**

Snedecor and Cochrane (1967, p. 325) state that one of the features of non-normal distribution is that the variance is related to the mean. The mean (x-axis) and the standard deviation (y-axis) of the number of isolates/group/site or isolates/group/depth grouped by cluster analysis at 50% similarity level, were therefore plotted against each other. If the number of isolates is normaly distributed, there will be no significant relationship between the mean and standard deviation.

2- **The selection of a suitable transformation method**

The best transformation was chosen as follows. The untransformed data, the square-root (x+0.5) and the $\log_{10}$ (x+0.5) transformed data were put through procedure A and B above. The graphs obtained for A are shown in appendix figures 2 and 3 and for B in appendix figures 4 and 5. Firstly, consider the graphic analysis of the untransformed data. It can be seen that the "square-root" transformation is the best transformation because it gave the best fit of points on the regression line. Secondly, consider the relationship between the mean and the standard deviation; it can be seen that there is no significant relationship between the mean and standard deviation. Therefore, The square-root method was selected as the method of transformation before parametric statistical analysis was carried out.

Appendix Table 15 A

Frequency distribution table of the number of isolates/group/site. The transformations $\sqrt{x+0.5}$ and $\log_{10}(x+0.5)$ were applied to the data to determine the best fit.

No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
0	0.707	-0.301	7	7	12.7
1	1.225	0.176	2	9	16.4
2	1.581	0.398	1	10	18.2
3	1.871	0.544	2	12	21.8
4	2.121	0.653	3	15	27.3
5	2.345	0.740	2	17	30.9
6	2.550	0.813	5	22	40.0
7	2.739	0.875	3	25	45.5
8	2.915	0.929	0	25	45.5
9	3.082	0.978	2	27	49.1
10	3.240	1.021	2	29	52.7
11	3.391	1.061	1	30	54.5
12	3.536	1.097	2	32	58.2
13	3.674	1.130	3	35	63.6
14	3.808	1.161	2	37	67.3
15	3.937	1.190	2	39	70.9
16	4.062	1.217	0	39	70.9
17	4.183	1.243	0	39	70.9
18	4.301	1.267	0	39	70.9
19	4.416	1.290	0	39	70.9
20	4.528	1.312	1	40	72.7
21	4.637	1.332	0	40	72.7
22	4.743	1.352	1	41	74.5

Appendix Table 15 A cont'd.

No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
23	4.848	1.371	1	42	76.4
24	4.950	1.389	0	42	76.4
25	5.050	1.407	2	44	80.0
26	5.148	1.423	0	44	80.0
27	5.244	1.439	1	45	81.8
28	5.339	1.455	0	45	81.8
29	5.431	1.470	1	46	83.6
30	5.523	1.484	1	47	85.5
31	5.612	1.498	0	47	85.5
32	5.701	1.512	1	48	87.3
33	5.788	1.525	0	48	87.3
34	5.874	1.538	1	49	89.1
35	5.958	1.550	0	49	89.1
36	6.042	1.562	1	50	90.9
37	6.124	1.574	1	51	92.7
38	6.205	1.585	0	51	92.7
39	6.285	1.597	0	51	92.7
40	6.364	1.607	0	51	92.7
41	6.442	1.618	0	51	92.7
42	6.519	1.628	0	51	92.7
43	6.595	1.638	1	52	94.5
44	6.671	1.648	0	52	94.5
45	6.745	1.658	0	52	94.5
46	6.819	1.667	0	52	94.5
47	6.819	1.667	0	52	94.5
48	6.964	1.686	0	52	94.5

Appendix Table 15 A cont'd.

No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
49	7.036	1.695	0	52	94.5
50	7.106	1.703	1	53	96.4
51	7.176	1.712	0	53	96.4
52	7.246	1.720	0	53	96.4
53	7.314	1.728	0	53	96.4
54	7.382	1.736	0	53	96.4
55	7.450	1.744	0	53	96.4
56	7.517	1.752	0	53	96.4
57	7.583	1.760	0	53	96.4
58	7.649	1.767	0	53	96.4
59	7.714	1.775	0	53	96.4
60	7.778	1.782	0	53	96.4
61	7.842	1.789	0	53	96.4
62	7.906	1.796	0	53	96.4
63	7.969	1.803	0	53	96.4
64	8.031	1.810	0	53	96.4
65	8.093	1.816	1	54	98.2
66	8.155	1.823	0	54	98.2
67	8.216	1.829	0	54	98.2
68	8.276	1.836	0	54	98.2
69	8.337	1.842	0	54	98.2
70	8.396	1.848	0	54	98.2
71	8.456	1.854	0	54	98.2
72	8.515	1.860	0	54	98.2
73	8.573	1.866	0	54	98.2
74	8.631	1.872	0	54	98.2

Appendix Table 15 A cont'd.

No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
75	8.689	1.878	0	54	98.2
76	8.746	1.884	0	54	98.2
77	8.803	1.889	0	54	98.2
78	8.860	1.895	0	54	98.2
79	8.916	1.900	0	54	98.2
80	8.972	1.906	0	54	98.2
81	9.028	1.911	0	54	98.2
82	9.083	1.916	0	54	98.2
83	9.138	1.922	0	54	98.2
84	9.192	1.927	0	54	98.2
85	9.247	1.932	0	54	98.2
86	9.301	1.937	0	54	98.2
87	9.354	1.942	0	54	98.2
88	9.407	1.947	0	54	98.2
89	9.460	1.952	0	54	98.2
90	9.513	1.957	0	54	98.2
91	9.566	1.961	0	54	98.2
92	9.618	1.966	1	55	100.0

Appendix Table 15 B

Frequency distribution table of the number of isolates/group/depth. The transformations $\sqrt{x+0.5}$ and $\log_{10}(x+0.5)$ were applied to the data to determine the best fit.

No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
0	0.707	-0.301	33	33	27.3
1	1.225	0.176	11	44	36.4
2	1.581	0.398	12	56	46.3
3	1.871	0.544	8	64	52.9
4	2.121	0.653	3	67	55.4
5	2.345	0.740	4	71	58.7
6	2.550	0.813	4	75	62.0
7	2.739	0.875	2	77	63.6
8	2.915	0.929	7	84	69.4
9	3.082	0.978	4	88	72.7
10	3.240	1.021	5	93	76.9
11	3.391	1.061	2	95	78.5
12	3.536	1.097	1	96	79.3
13	3.674	1.130	0	96	79.3
14	3.808	1.161	5	101	83.5
15	3.937	1.190	2	103	85.1
16	4.062	1.217	2	105	86.8
17	4.183	1.243	1	106	87.6
18	4.301	1.267	1	107	88.4
19	4.416	1.290	2	109	90.1
20	4.528	1.312	2	111	91.7
21	4.637	1.332	1	112	92.6
22	4.743	1.352	0	112	92.6

Appendix Table 15 B cont'd.

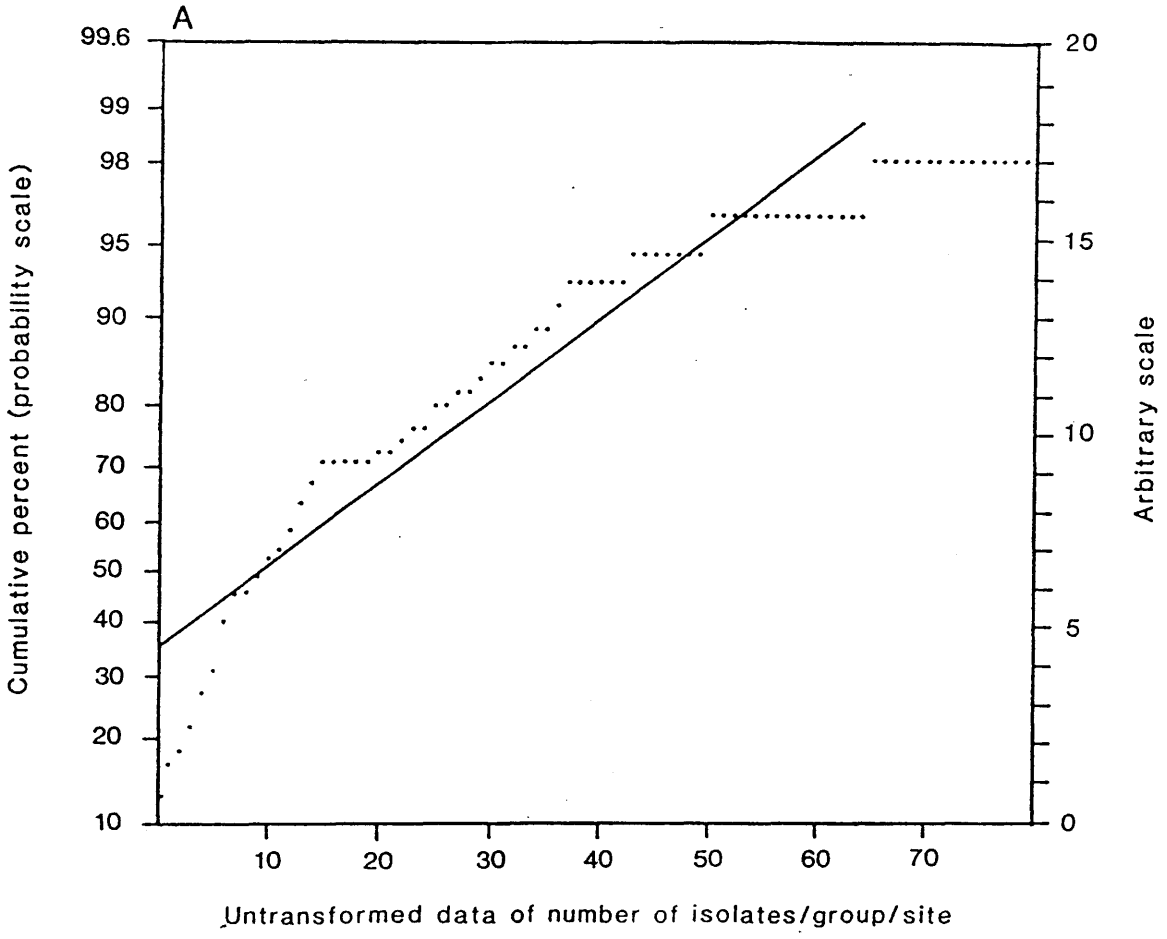
No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
23	4.848	1.371	2	114	94.2
24	4.950	1.389	1	115	95.0
25	5.050	1.407	0	115	95.0
26	5.148	1.423	1	116	95.9
27	5.244	1.439	1	117	96.7
28	5.339	1.455	1	118	97.5
29	5.431	1.470	0	118	97.5
30	5.523	1.484	0	118	97.5
31	5.612	1.498	1	119	98.3
32	5.701	1.512	0	119	98.3
33	5.788	1.525	0	119	98.3
34	5.874	1.538	0	119	98.3
35	5.958	1.550	0	119	98.3
36	6.042	1.562	0	199	98.3
37	6.124	1.574	1	120	99.2
38	6.205	1.585	0	120	99.2
39	6.285	1.597	0	120	99.2
40	6.364	1.607	0	120	99.2
41	6.442	1.618	0	120	99.2
42	6.519	1.628	0	120	99.2
43	6.595	1.638	0	120	99.2
44	6.671	1.648	0	120	99.2
45	6.745	1.658	0	120	99.2
46	6.819	1.667	0	120	99.2
47	6.892	1.677	0	120	99.2

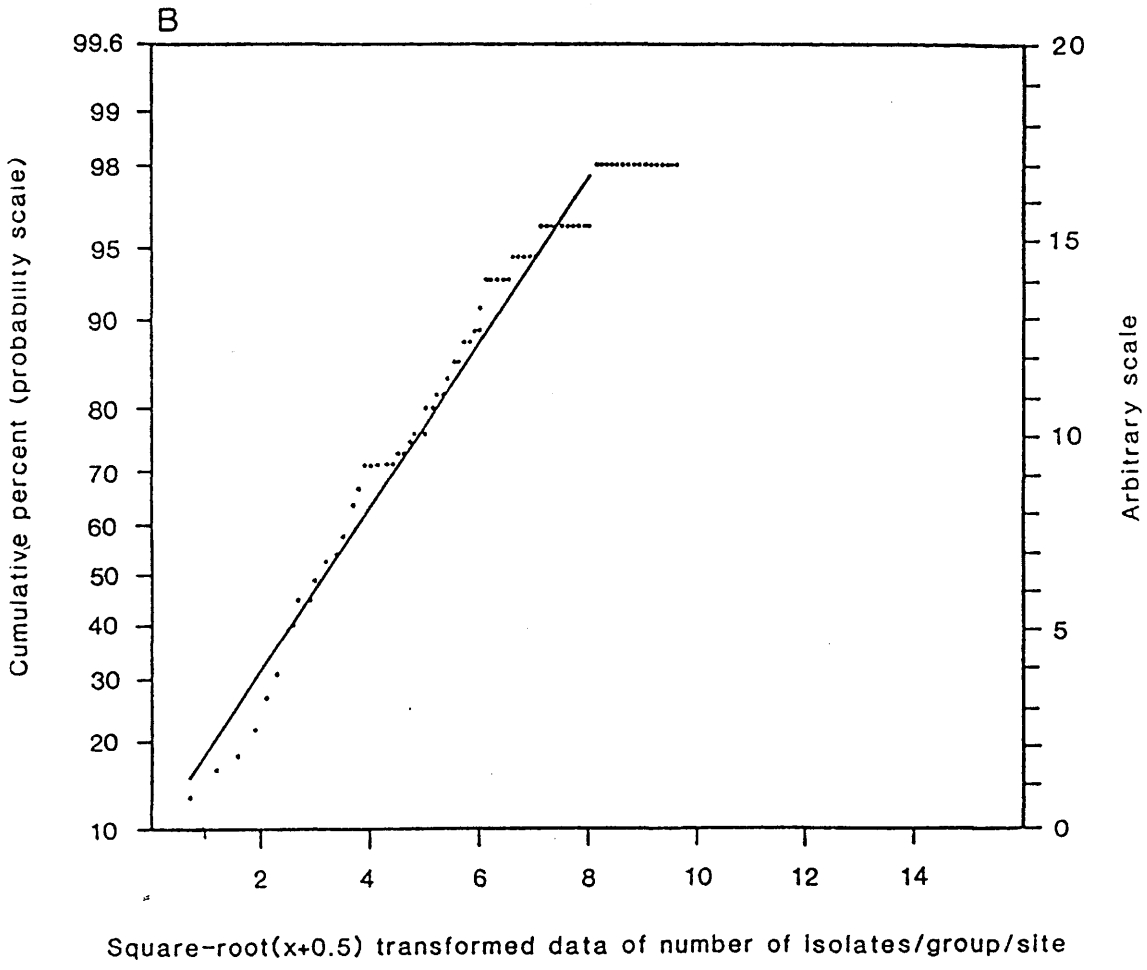
Appendix Table 15 B cont'd.

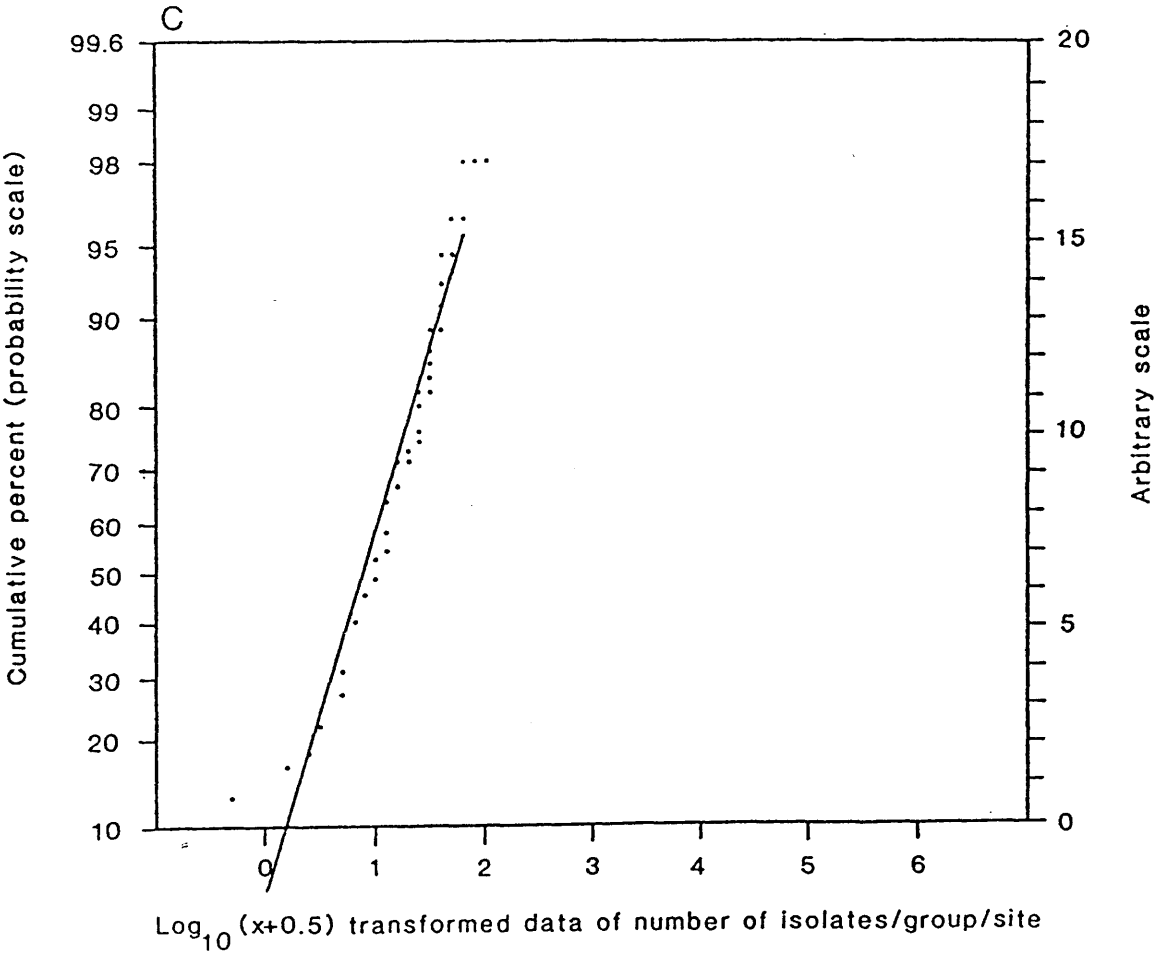
No. isolates/ group	$\sqrt{x+0.5}$	$\log_{10}(x+0.5)$	f	Cumulative frequencies F	Percent cumulative frequencies
48	6.964	1.686	0	120	99.2
49	7.036	1.695	0	120	99.2
50	7.106	1.703	0	120	99.2
51	7.176	1.712	0	120	99.2
52	7.246	1.720	0	120	99.2
53	7.314	1.728	1	121	100.0

Appendix Figure 2

Relationship between the number of bacterial isolates/group/site (Table 14) (x-axis), and their cumulative percent (y-axis on probability scale). A: Untransformed data, B:(square-root x+0.5) transformed data and C:($\log_{10}$ x+0.5) transformed data. Arbitrary scale on the right hand Y-axis=cm on original size of graph. This linear cm scale was used to obtain y values for the regression analysis. Cluster analysis at 50% similarity level.

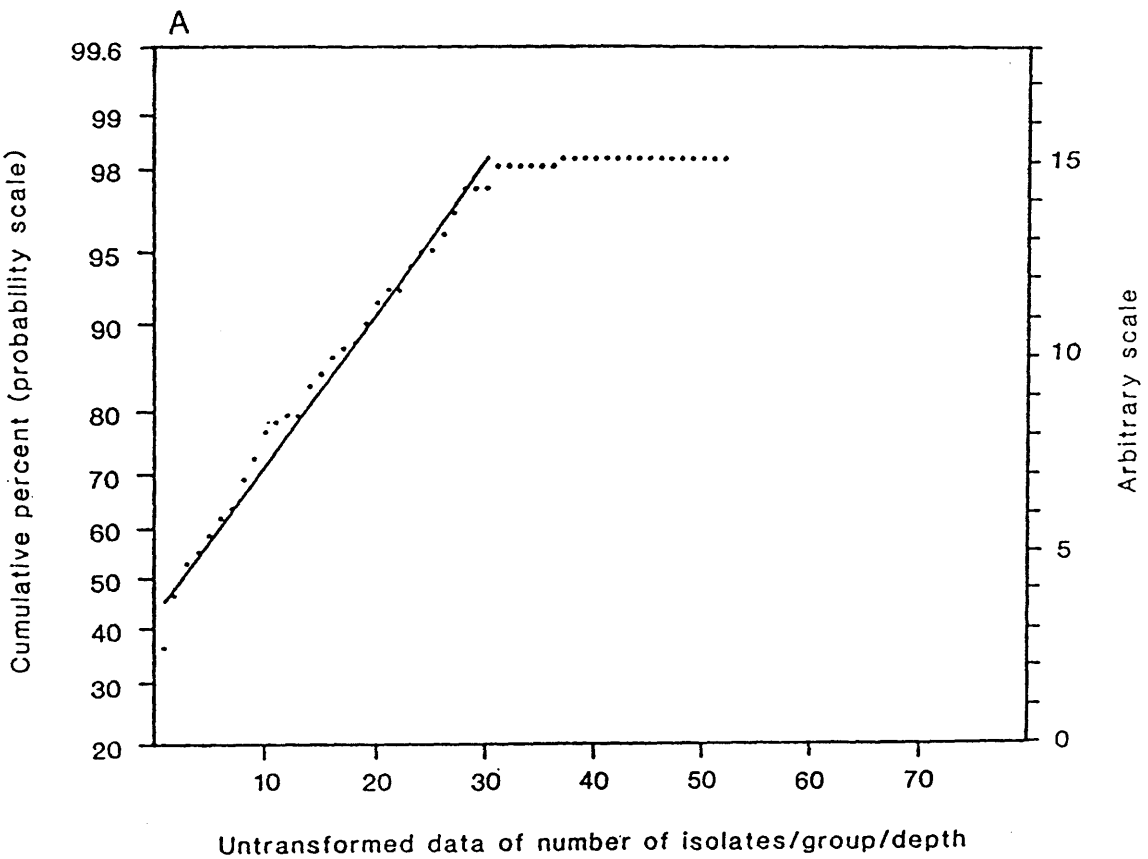

A
99.6
99
98
95
90
80
70
60
50
40
30
20
10
Cumulative percent (probability scale)
20
15
10
5
0
Arbitrary scale
10
20
30
40
50
60
70
Untransformed data of number of isolates/group/site

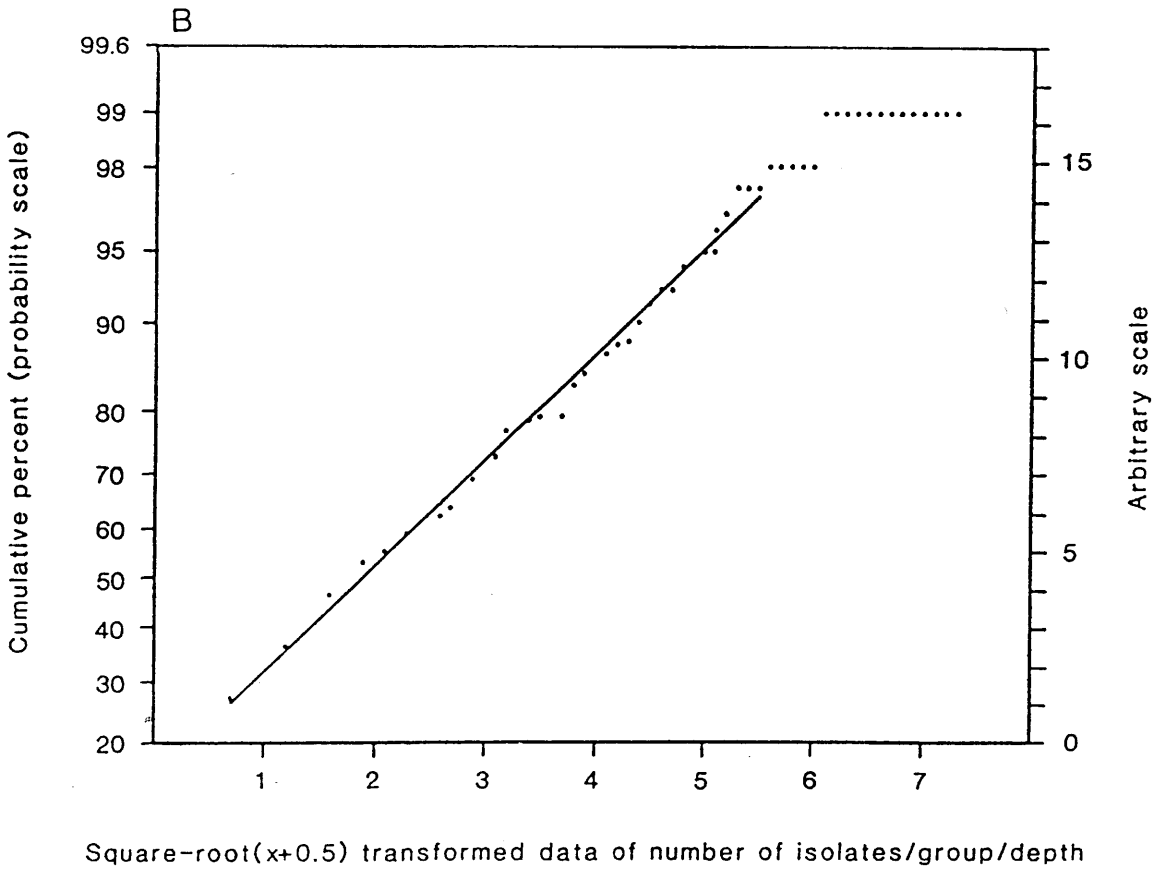

B
99.6
99
98
95
90
80
70
60
50
40
30
20
10
20
15
10
5
0
2
4
6
8
10
12
14
Cumulative percent (probability scale)
Arbitrary scale
Square-root(x+0.5) transformed data of number of isolates/group/site

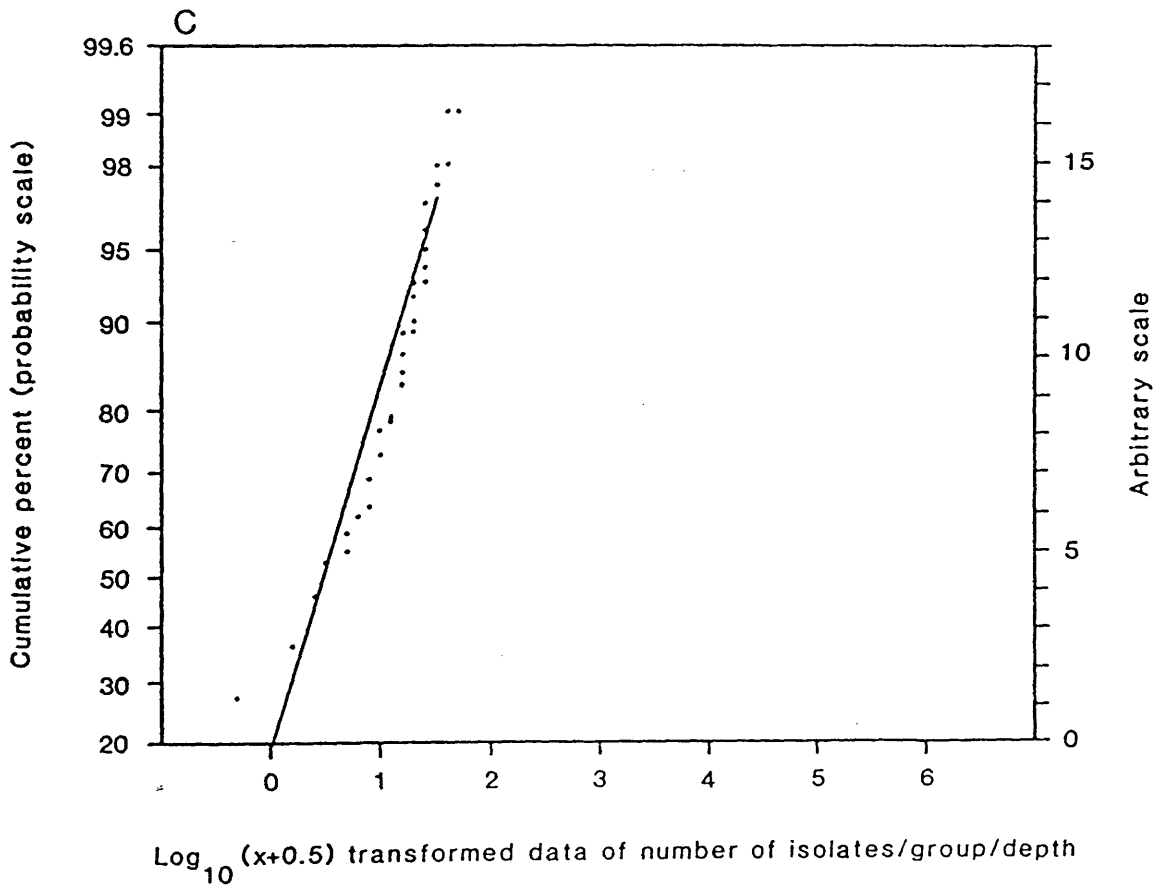

C
99.6
99
98
95
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
20
15
10
5
0
Cumulative percent (probability scale)
Arbitrary scale
Log10 (x+0.5) transformed data of number of isolates/group/site

Appendix Figure 3

Relationship between the number of bacterial isolates/group/depth (Table 15) (x-axis), and their cumulative percent (y-axis on probability scale). A: untransformed data, B: (square-root x+0.5) transformed data and C: ($\log_{10}$ x+0.5) transformed data. Arbitrary scale on the right hand y-axis=cm on original size of graph. This linear cm scale was used to obtain y values for the regression analysis. Cluster analysis at 50% similarity level.

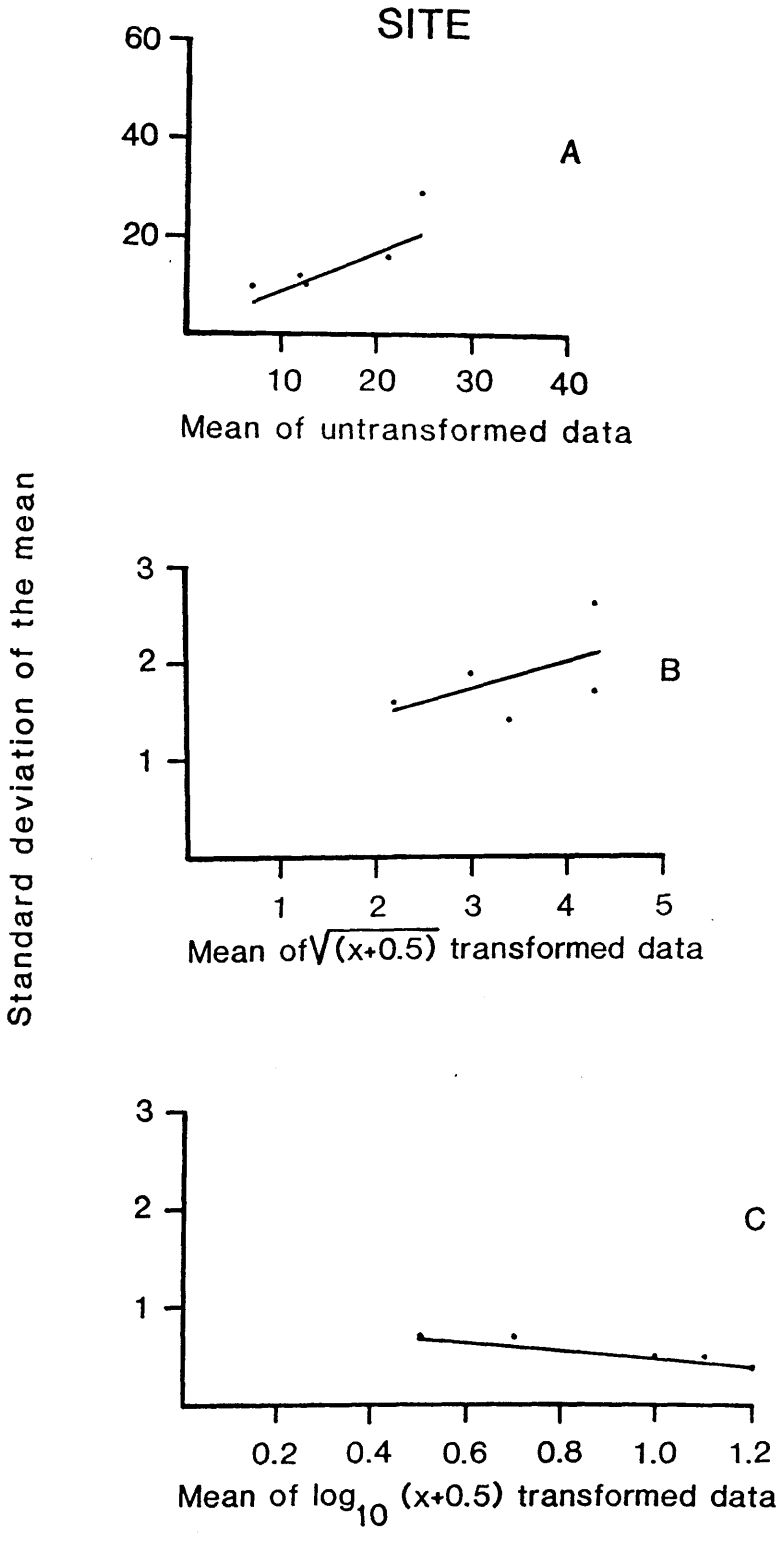

A
99.6
99
98
95
90
80
70
60
50
40
30
20
Cumulative percent (probability scale)
10
20
30
40
50
60
70
Untransformed data of number of isolates/group/depth
15
10
5
0
Arbitrary scale


B
99.6
99
98
95
90
80
70
60
50
40
30
20
1
2
3
4
5
6
7
15
10
5
0
Cumulative percent (probability scale)
Arbitrary scale
Square-root(x+0.5) transformed data of number of isolates/group/depth


C
99.6
99
98
95
90
80
70
60
50
40
30
20
Cumulative percent (probability scale)
0
1
2
3
4
5
6
15
10
5
0
Arbitrary scale
Log10 (x+0.5) transformed data of number of isolates/group/depth

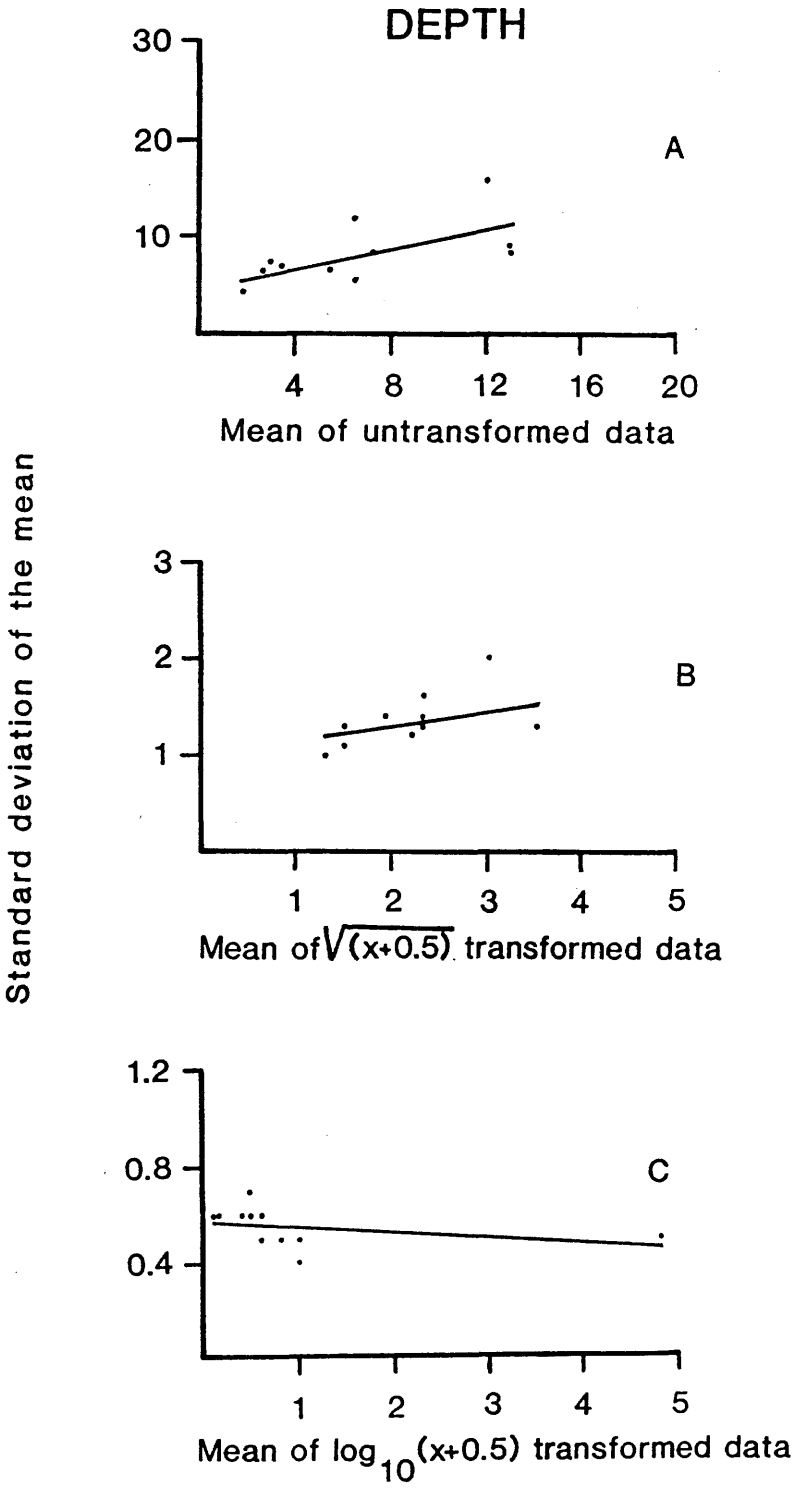
Appendix Figure 4

Number of bacterial isolates/group/site. Relationship between the mean (x-axis) and standard deviation (y-axis) of A: Untransformed data, B: (square-root x+0.5) transformed data. and C: ($\log_{10}$ x+0.5) transformed data. The five points on each graph are the means and standard deviations for the five sites. Cluster analysis at 50% similarity level.


SITE
60
40
20
A
10
20
30
40
Mean of untransformed data
Standard deviation of the mean
3
2
1
B
1
2
3
4
5
Mean of √(x+0.5) transformed data
3
2
1
C
0.2
0.4
0.6
0.8
1.0
1.2
Mean of log10 (x+0.5) transformed data

Appendix figure 5

Number of bacterial isolates/group/depth. Relationship between the mean (x-axis) and standard deviation (y-axis) of A: untransformed data, B: (square-root x+0.5) transformed data, and C: ($\log_{10}$ x+0.5) transformed data. The 11 points on each graph are the means and standard deviations for the 11 depths. Cluster analysis at 50% similarity level.


DEPTH
30
20
10
A
4
8
12
16
20
Mean of untransformed data
Standard deviation of the mean
3
2
1
B
1
2
3
4
5
Mean of √(x+0.5) transformed data
1.2
0.8
0.4
C
1
2
3
4
5
Mean of log10(x+0.5) transformed data

Group	Site 1	2	3	4	5
1	3.240	2.345	5.050	2.121	2.739
2	0.7071	8.093	5.523	5.701	3.674
3	0.7071	3.536	2.345	3.937	2.550
4	3.391	3.082	5.431	3.536	2.739
5	2.739	9.618	4.743	6.595	6.124
6	0.7071	6.042	3.240	5.244	3.808
7	2.550	2.121	0.7071	2.121	4.528
8	0.7071	4.848	0.7071	5.050	2.550
9	5.874	2.550	3.082	2.550	3.674
10	1.871	3.937	1.225	7.106	3.808
11	1.871	1.581	1.225	3.674	0.7071

Appendix Table 16: Number of bacterial isolates/group at each site (e.g. no. isolates/group/site), table 14, Square-root (x + 0.5) transformed data. Cluster analysis at 50% similarity level.

Group	Depth (cm)										
	00.0	00.5	03.0	04.0	05.5	07.5	10.5	11.0	13.0	15.5	20.5
1	4.416	3.240	3.240	0.7071	1.225	2.550	0.7071	1.225	0.7071	1.871	1.225
2	1.871	3.808	4.637	2.345	2.121	5.244	0.7071	7.314	3.391	0.7071	1.581
3	1.581	2.345	1.581	2.345	1.581	1.871	0.7071	3.240	3.082	0.7071	0.7071
4	4.528	2.915	2.121	2.915	1.871	3.240	3.082	1.581	1.225	1.871	0.7071
5	2.550	3.808	4.950	2.915	6.124	4.062	4.848	5.148	3.808	4.301	3.937
6	1.581	3.240	3.391	2.915	2.345	2.915	1.871	4.528	3.808	2.121	1.581
7	0.7071	4.848	2.915	0.7071	1.581	0.7071	0.7071	1.225	0.7071	0.7071	0.7071
8	0.7071	3.937	4.062	0.7071	1.581	0.7071	1.225	3.536	2.915	0.7071	0.7071
9	0.7071	5.612	4.183	1.225	2.550	3.082	0.7071	1.581	0.7071	1.581	0.7071
10	1.225	3.808	5.339	4.416	3.082	0.7071	1.225	1.871	2.739	0.7071	1.225
11	1.225	0.7071	1.871	2.550	0.7071	0.7071	0.7071	1.581	2.739	0.7071	0.7071

Appendix Table 17: Number of bacterial isolates/group at each depth (e.g. no. isolates/group/depth), table 15. Square-root (x + 0.5) transformed data. Cluster analysis at 50% similarity level.

Group	Site				
	1	2	3	4	5
1	1.021	0.7400	1.407	0.6530	0.8750
2	-0.3010	1.816	1.484	1.512	1.130
3	-0.3010	1.097	0.7400	1.190	0.8130
4	1.061	0.9780	1.470	1.097	0.8750
5	0.8750	1.966	1.352	1.638	1.574
6	-0.3010	1.562	1.021	1.439	1.161
7	0.8130	0.6530	-0.3010	0.6530	1.312
8	-0.3010	1.371	-0.3010	1.407	0.8130
9	1.538	0.8130	0.9780	0.8130	1.130
10	0.5440	1.190	0.1760	1.703	1.161
11	0.5440	0.3980	0.1760	1.130	-0.3010

Appendix Table 18: Number of bacterial isolates/group at each site (e.g. no. isolates/group/site), table 14. Log_{10} (x + 0.5) transformed data. Cluster analysis at 50% similarity level.

Group	Depth (cm)										
	00.0	00.5	03.0	04.0	05.5	07.5	10.5	11.0	13.0	15.5	20.5
1	1.290	1.021	1.021	-0.3010	0.1760	0.8130	-0.3010	0.1760	-0.3010	0.5440	0.1760
2	0.5440	1.161	1.332	0.7400	0.653	1.439	-0.3010	1.728	1.061	-0.3010	0.3980
3	0.3980	0.7400	0.3980	0.7400	0.3980	0.5440	-0.3010	1.021	0.9780	-0.3010	-0.3010
4	1.312	0.9290	0.6530	0.9290	0.5440	1.021	0.9780	0.3980	0.1760	0.5440	-0.3010
5	0.8130	1.161	1.389	0.9290	1.574	1.217	1.371	1.423	1.161	1.267	1.190
6	0.3980	1.021	1.061	0.9290	0.7400	0.9290	0.5440	1.312	1.161	0.6530	0.3980
7	-0.3010	1.371	0.9290	-0.3010	0.3980	-0.3010	-0.3010	0.1760	-0.3010	-0.3010	-0.3010
8	-0.3010	1.190	1.217	-0.3010	0.3980	-0.3010	0.1760	1.097	0.9290	-0.3010	-0.3010
9	-0.3010	1.498	1.243	0.1760	0.8130	0.9780	-0.3010	0.3980	-0.3010	0.3980	-0.3010
10	0.1760	1.161	1.455	1.290	0.9780	-0.3010	0.1760	0.5440	0.8750	-0.3010	0.1760
11	0.1760	-0.3010	0.5440	0.8130	-0.3010	-0.3010	-0.3010	0.3980	0.8750	-0.3010	-0.3010

Appendix Table 19: Number of bacterial isolates/group at each depth (e.g. no. isolates/group/depth), table 15 . Log_{10} (x + 0.5) transformed data. Cluster analysis at 50% similarity level.

Site	Untransformed data		Square-root transformed data		$\log_{10}$ transformed data	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
1	6.727	9.911	2.215	1.598	0.472	0.668
2	24.46	29.10	4.341	2.592	1.144	0.496
3	12.00	12.14	3.025	1.919	0.746	0.696
4	21.00	15.75	4.330	1.738	1.203	0.375
5	12.45	9.812	3.355	1.368	0.958	0.478

Appendix Table 20: Number of bacterial isolates/group for each of the five sites. Mean and standard deviation of untransformed, square-root $\sqrt{(x + 0.5)}$ transformed and $\log_{10}$ $(x + 0.5)$ transformed data. Cluster analysis at 50% similarity level.

Depth	Untransformed data		Square-root transformed data		$\log_{10}$ transformed data	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
0.00	4.909	7.422	1.918	1.380	0.382	0.582
00.5	13.091	8.408	3.479	1.279	0.996	0.476
03.0	13.091	8.803	3.481	1.273	1.022	0.356
04.0	5.455	5.592	2.159	1.193	0.513	0.585
05.0	6.455	10.44	2.252	1.440	0.579	0.477
07.5	7.182	8.412	2.345	1.550	0.522	0.689
10.5	3.364	7.047	1.499	1.333	0.131	0.596
11.0	12.00	16.03	2.985	1.988	0.788	0.546
13.0	6.455	5.466	2.348	1.259	0.574	0.621
15.5	2.727	5.293	1.453	1.107	0.145	0.556
20.5	1.909	4.415	1.254	0.960	4.836	0.481

Appendix Table 21: Number of bacterial isolates/group for each of the 11 depths. Mean and standard deviation of untransformed, square-root $(x + 0.5)$ transformed and $\log_{10}$ $(x + 0.5)$ transformed data. Cluster analysis at 50% similarity level.

	Regression equation	r	Student's s.t	d.f.	P
Untransformed data	$y = 0.2115x + 4.624$	0.9518	24.63	63	P<0.001
Square-root (x + 0.5) transformed data	$y = 2.113\sqrt{x} - 0.0784$	0.9871	48.94	63	P<0.001
Log_{10} (x + 0.5) transformed data	$y = 9.451x - 1.723$	0.9692	31.24	63	P<0.001

Appendix Table 22: No. bacterial isolates)/group/site. Regression analysis applied to untransformed, square-root (x + 0.5) transformed, and log_{10} (x + 0.5) transformed data (Appendix Figure 2). The regression analysis was performed on values of y measured on a cm linear scale (see Appendix Figure 2, legend p. 259). r = correlation coefficient. Cluster analysis at 50% similarity level.

	Regression equation	r	Student's t	d.f.	P
Untransformed data	$y = 0.3942x + 3.274$	0.9878	34.16	29	$P < 0.001$
Square-root $(x + 0.5)$ transformed data	$y = 2.719\ x - 0.8977$	0.9963	62.43	29	$P < 0.001$
Log_{10} $(x + 0.5)$ transformed data	$y = 9.501x - 0.2755$	0.9223	12.85	29	$P < 0.001$

Appendix Table 23: No. bacterial isolates/group/depth. Regression analysis applied to untransformed, square-root $(x + 0.5)$ transformed, and log_{10} $(x + 0.5)$ transformed data (Appendix Figure 3). The regression analysis was performed on values of y measured on a cm linear scale (see appendix figure 3 legend p. 262). r = correlation coefficient. Cluster analysis at 50% similarity level.

	Regression equation	r	Student's.t	d.f.	P
Untransformed data	y = 0.9634x + 0.5775	0.8638	2.970	3	0.10>P>0.05
Square-root (x + 0.5) transformed data	y = 0.2712x + 0.9065	0.5288	1.079	3	0.40>P>0.30
Log_{10} (x + 0.5) transformed data	y =-0.3961x + 0.0909	-0.8773	3.166	3	0.10>P>0.05

Appendix Table 24: Number of bacterial isolates/group/site. Regression analyses applied to the five values (one/site) of the mean (x) and standard deviation (y). Three regression analyses were conducted, on the untransformed, the square-root transformed and log_{10} transformed data. Cluster analysis at 50% similarity level. r = correlation coefficient.

	Regression equation	r	Student's.t	d.f.	P
Untransformed data	y = 0.5234x + 4.293	0.6541	2.594	9	0.05>P>0.02
Square-root (x + 0.5) transformed data	y = 0.1531x + 0.9917	0.4402	1.471	9	0.20>P>0.10
Log_{10} (x + 0.5) transformed data	y =-0.0241x + 0.5662	-0.3525	1.130	9	0.30>P>0.20

Appendix Table 25: Number of bacterial isolates/group/depth. Regression analyses applied to the 11 values (one/depth) of the mean (x) and standard deviation (y). Three regression analyses were conducted, on the untransformed, the square-root transformed and the log_{10} transformed data. Cluster analysis at 50% similarity level. r = correlation coefficient.

APPENDIX C

Parametric and Non-Parametric Statistics and the use of Spearman's Rank Order Correlation Coefficient

Statistical tests are divided into two parts, parametric and non-parametric. Parametric tests include Pearson's product moment correlation coefficient and linear regression. Non-parametric tests include Spearman's rank correlation and Kendall's tau correlation, 2 x n X^2 tests. The difference between parametric and non-parametric statistics is that parametric techniques involve assumptions of normality about the distribution of a sample population, while non-parametric techniques require far fewer assumptions about the data. Cohen & Holliday (1984) state the advantage of using non-parametric statistics is they are simple and quick to apply under a wide range of conditions. However, their disadvantage is that they are less powerful. The greater the ability of a test to reject a false hypothesis, the greater its power.

In general, various correlation coefficients are used to test whether there is a significant relationship between two variables. These correlation coefficients can be either parametric or non-parametric, depending on whether the data is normal or not. The parametric correlation coefficient is Pearson's product moment correlation coefficient and the non-parametric correlation coefficients are Kendall's coefficient of rank correlation and Spearman's coefficient of rank correlation (Snedecor and Cochran, 1967, p. 172-188, 193-195; Sokal and Rholf, 1969, p. 495-515, 532-537). The range of all three correlation coefficients varies from +1 to -1. Values close to +1 indicate a strong direct relationship between the two variables, and values close to -1 indicate a strong inverse relationship. Values close to 0 indicate that there is no

statistical relationship between the two variables. Appendix figure 6 illustrates different degrees of correlation between two variables. Kendall's and Spearman's coefficients are equally powerful but both are less powerful than Pearson's coefficient (c. 90%). Kendall's coefficient is more difficult to compute than Spearman's.

The non-parametric correlations that I have investigated in my thesis have all been statistically analysed using Spearman's rank order correlation coefficient. This test uses the differences in the ranks of the two variables and applies the following equation:

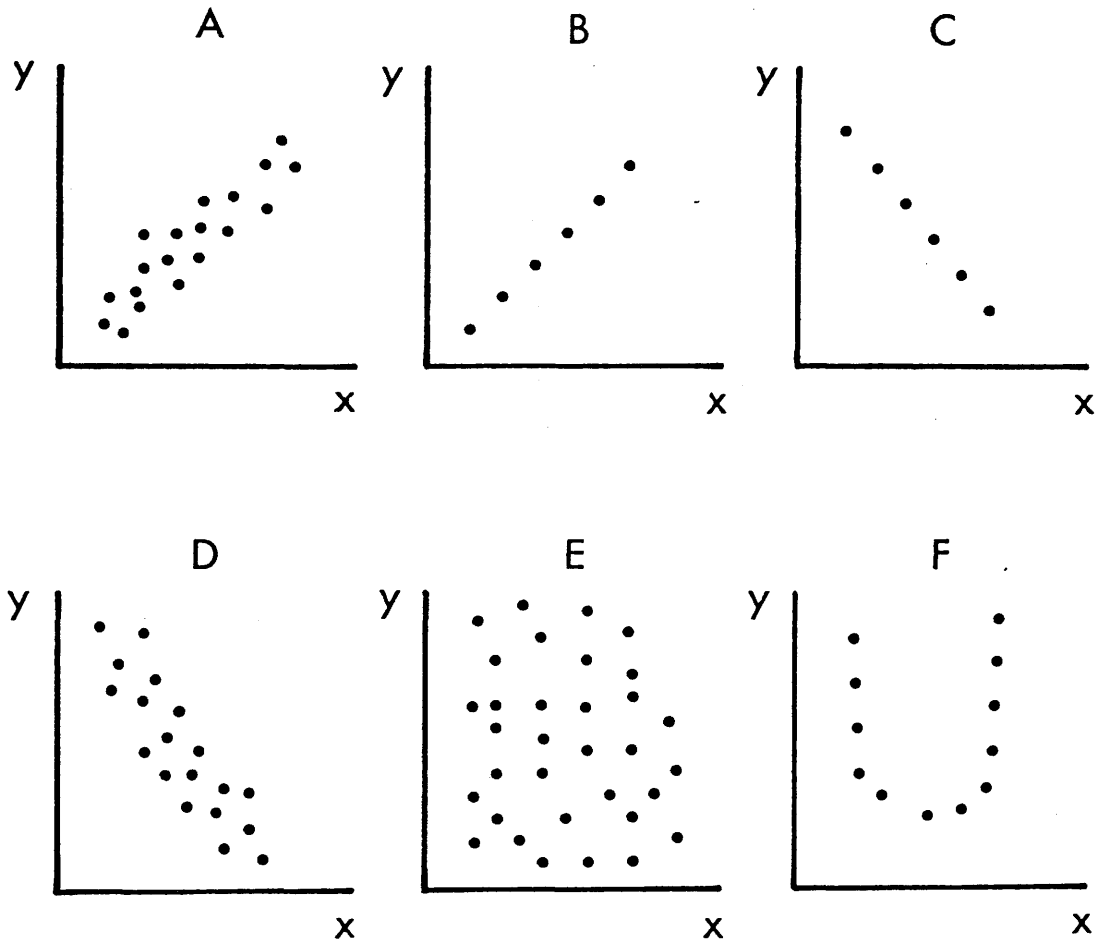
$$r_s = \frac{1 - 6\,d^2}{n\,(n^2-1)}$$

where d^2 is the sum of the squares of the differences between ranks of corresponding values x and y, and n is the number of pairs of x and y values.

There are two methods for testing the signficance of Spearman's rank order correlation coefficient depending on the size of the sample. Shaw & Wheeler (1985) state that for small samples ($n<10$), it is best to use prepared tables (Cohn and Holliday, 1984. Appendix 9, p. 335 or Shaw & Wheeler, 1985. Appendix IX p. 352). For large samples ($n\geqslant10$) r_s is converted to a t value using the formula:

$$t = r_s \sqrt{\frac{n - 2}{1 - r^2_s}}$$

Degrees of freedom are calculated by d.f. = n-2 where n is the number of ranks being compared.



Appendix Figure 6

Illustrates the way in which values of correlation (r) indicate the degree of relationship between two variables (x and y). (A) r is +ve and fairly large; (B) r = +1; (C) r = -1; (D) r is -ve and fairly large; (E) and (F) r is approximately 0.

APPENDIX D

Homogeneity of variance

There are several methods available for testing the homogeneity of variance. Snedecor and Cochran (1980, p. 252-254) and Sokal and Rohlf (1981, Box 13.1 p. 404-407) explain in detail the procedure of the following methods:

1- Bartlett's Test

Sokal & Rohlf (1981) used an F-test in order to test the homogeneity of variances of two samples prior to applying other statistics. Bartlett's test for the homogeneity of variance is often suggested for comparing more than two samples. The final stage of the statistics is a Chi-square test. The disadvantage of this test as well as all the other tests is that it is very sensitive to departures from normality.

2- Hartley's F-max test

This variance ratio or F-test is a quick, simple test that works on the ratio of the largest to the smallest of several sample variances. The F-value is found from the F-distribution table for the significance level corresponding to the numerator and denominator degrees of freedom.

3- Log-anova or Scheffe'-Box test

This test requires some data preparation before it can be used. It is based on randomly dividing the (n_i) observations in each group into (m_i) subsamples. The number of subsamples, m_i, should be approximately equal to the square-root of n_i, and the number of observations (i.e. readings) in each subsample of each group should be about the same. The sample variance S^2ij is calculated separately for each of the subsamples and then its natural log (ln = $\log_e$) is taken.

An analysis of variance is then conducted on the ln(variance)/d.f., of each variance, where d.f. = degrees of freedom (i.e. n-1) of that variance. The final stage of this test is to find the variance ratio from the analysis of variance and then the significance level.

4- Levene's Test

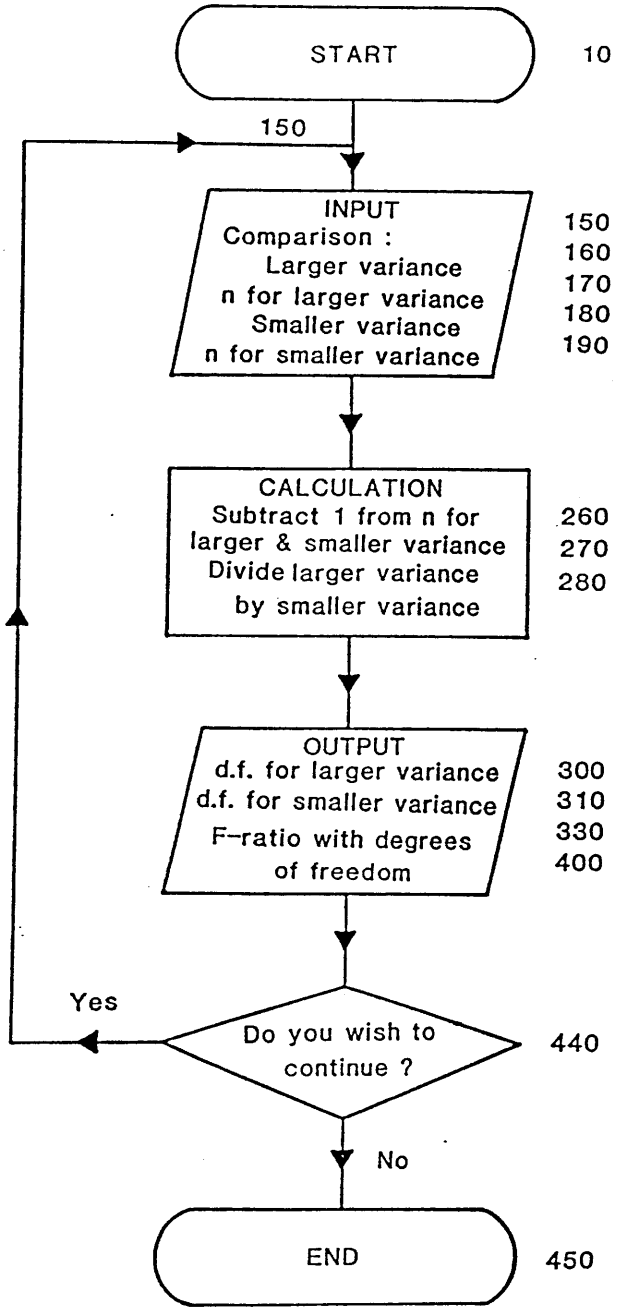
Levene's test of homogeneity of variance is a test based on dividing the mean of the observations by each individual observation for several classes and then applying an analysis of variance to the mean deviations. Snedecor & Cochran (1980, p. 253) state that Levene's test is much less sensitive to non-normality of data than is Bartlett's test.

Appendix Table 26

Computer program "F-ratio" for calculations of the variance ratio test (Sokal and Rohlf, 1981, Box 8.1, p. 190 and table 16 in Rohlf and Sokal, 1969).

```
10 REM *** F-ratio, F. EDDEB, DEC.1985 ***
20 LPRINT CHR$(27);"1";CHR$(10)
30 CS$=CHR$(126)+CHR$(28)
40 PRINT CS$
50 PRINT "THIS PROGRAMME CALCULATES THE VARIANCE RATIO TEST"
60 PRINT "(SOKAL & ROHLF, 1981. BOX 8.1, P.190"
70 PRINT "AND TABLE 16 IN ROHLF & SOKAL, 1969)"
80 LPRINT "THIS PROGRAMME CALCULATES THE VARIANCE RATIO TEST"
90 LPRINT "(SOKAL & ROHLF, 1981. BOX 8.1, P.190"
100 LPRINT "AND TABLE 16 IN ROHLF & SOKAL, 1969)"
110 PRINT:PRINT
120 LPRINT:LPRINT
130 LPRINT "------------------------------------------------------------
-----------"
140 PRINT
150 INPUT "COMPARISON :";T$
160 INPUT "LARGER VARIANCE ";L
170 INPUT "n FOR LARGER VARIANCE ";A
180 INPUT "SMALLER VARIANCE ";S
190 INPUT "n FOR SMALLER VARIANCE ";B
200 LPRINT "COMPARISON :";T$
210 LPRINT
220 LPRINT "LARGER VARIANCE = ";L

230 LPRINT "n FOR LARGER VARIANCE = ";A
240 LPRINT "SMALLER VARIANCE = ";S
250 LPRINT "n FOR SMALLER VARIANCE = ";B
260 C = A-1
270 D = B-1
280 F = L/S
290 PRINT
300 PRINT "d.f FOR LARGER VARIANCE ";C
310 PRINT "d.f FOR SMALLER VARIANCE ";D
320 PRINT
330 PRINT "F-ratio ";F
340 LPRINT
350 LPRINT "d.f FOR LARGER VARIANCE ";C
360 LPRINT "d.f FOR SMALLER VARIANCE ";D
370 LPRINT
380 LPRINT "F-ratio = ";F
390 A$ = "WITH d.f"
400 PRINT A$ "= "; "("C ","D")"
410 PRINT "------------------------------------------------------------
-----------"
420 LPRINT A$ "= "; "("C ","D")"
430 LPRINT "------------------------------------------------------------
-----------"
440 PRINT "DO YOU WISH TO CONTINUE, Y/N":INPUT B$:IF B$="Y" THEN GOTO
150
450 END
```



APPENDIX FIGURE 7

A flow diagram of the programme calculating the variance ratio test (Sokal & Rohlf, 1981. Box 8.1, p.190 and Table 16 in Rohlf & Sokal, 1969).

Appendix Table 27

Example of calculating the variance ratio using "F-ratio" computer program.

```
THIS PROGRAMME CALCULATES THE VARIANCE RATIO TEST
(SOKAL & ROHLF, 1981. BOX 8.1, P.190
AND TABLE 16 IN ROHLF & SOKAL, 1969)


-----------------------------------------------------------------
COMPARISON :DEEP-SEA BACTERIA

LARGER VARIANCE =  927
n FOR LARGER VARIANCE =  9
SMALLER VARIANCE =  456
n FOR SMALLER VARIANCE =  6

d.f FOR LARGER VARIANCE  8
d.f FOR SMALLER VARIANCE  5

F-ratio =  2.0329
WITH d.f= ( 8 , 5 )
-----------------------------------------------------------------
COMPARISON :DEEP-SEA BACTERIA

LARGER VARIANCE =  82
n FOR LARGER VARIANCE =  8
SMALLER VARIANCE =  65
n FOR SMALLER VARIANCE =  6

d.f FOR LARGER VARIANCE  7
d.f FOR SMALLER VARIANCE  5

F-ratio =  1.26154
WITH d.f= ( 7 , 5 )
-----------------------------------------------------------------
COMPARISON :DEEP-SEA BACTERIA

LARGER VARIANCE =  64
n FOR LARGER VARIANCE =  5
SMALLER VARIANCE =  35
n FOR SMALLER VARIANCE =  4

d.f FOR LARGER VARIANCE  4
d.f FOR SMALLER VARIANCE  3

F-ratio =  1.82857
WITH d.f= ( 4 , 3 )
-----------------------------------------------------------------
```

Appendix Table 28

A breakdown of the contents of each of the 21 clusters formed at 72% similarity level. Column 1= isolate number, column 2= sampling site (1-5), column 3= sediment depth (cm), column 4=incubation temperatures (4 or 10°C) and column 5= source of sample (1= sediment, 2= burrow linings, 3= faecal pellets).

CLUSTER 1

Isolate No.	Site	Depth	Incub. temp.	Source										
1	2	0.5	10	1	7	2	0.5	10	1	8	2	0.5	10	1
470	4	15.5	10	1	803	4	15.5	10	1	804	4	15.5	10	1
829	1	3.0	10	1	34	2	20.5	10	1	38	2	11.0	10	3
825	1	3.0	10	1	222	5	0.5	10	1	819	1	3.0	10	1
826	1	3.0	10	1	827	1	3.0	10	1	653	1	7.5	4	3
818	1	3.0	10	1	812	1	3.0	10	1	835	3	7.5	10	3
813	1	3.0	10	1	823	1	3.0	10	1	831	3	7.5	10	3
832	3	7.5	10	3	96	4	3.0	10	1	483	1	5.5	10	1
217	5	0.5	10	1	223	5	0.5	10	1	225	5	0.5	10	1
226	5	0.5	10	1	227	5	0.5	10	1	247	5	0.5	10	1
647	3	7.5	4	3	654	3	7.5	4	3					

CLUSTER 2

329	3	0.0	4	1	330	3	0.0	4	1	335	3	0.0	10	1
340	3	0.0	4	1	336	3	0.0	4	1	331	3	0.0	4	1
348	3	0.0	4	1	332	3	0.0	4	1	333	3	0.0	4	1
334	3	0.0	4	1	350	3	0.0	4	1	341	3	0.0	4	1
337	3	0.0	4	1	342	3	0.0	4	1	344	3	0.0	4	1
343	3	0.0	4	1	345	3	0.0	4	1	347	3	0.0	4	1
346	3	0.0	4	1										

CLUSTER 3

6	2	0.5	10	1	357	4	4.0	10	1	176	2	11.0	10	2
485	3	7.5	10	3	752	4	13.0	10	2	86	4	3.0	10	1
88	4	3.0	10	1	109	2	3.0	10	1	349	3	0.0	4	1
651	3	7.5	4	3	228	5	0.5	10	1	645	3	7.5	4	3
646	3	7.5	4	3	166	2	11.0	10	2	308	3	0.0	10	1
387	4	4.0	10	1	204	2	5.5	10	1	643	3	7.5	10	3
644	3	7.5	10	3	220	5	0.5	10	1	486	3	7.5	10	3
487	3	7.5	10	3	490	3	7.5	10	3	491	3	7.5	10	3
492	3	7.5	10	3	496	3	7.5	10	3	493	3	7.5	10	3
494	3	7.5	10	3	499	3	7.5	10	3	503	3	7.5	10	3
489	3	7.5	10	3	500	3	7.5	10	3	498	3	7.5	10	3
504	3	7.5	10	3	797	3	7.5	4	3	495	3	7.5	10	3
502	3	7.5	10	3	507	3	7.5	10	3	510	3	7.5	10	3
509	3	7.5	10	3	508	3	7.5	10	3					

CLUSTER 4

33	2	20.5	10	1	636	5	5.5	10	1	163	2	11.0	10	2
170	2	11.0	10	2	168	2	11.0	10	2	552	5	3.0	10	1
167	2	11.0	10	2	309	3	0.0	10	1	174	2	11.0	10	2
760	4	13.0	10	2	363	4	4.0	10	2	425	4	13.0	10	1
229	5	0.5	10	1	433	2	0.5	10	1	689	2	11.0	4	2
698	2	11.0	4	2	699	2	11.0	4	2	744	2	11.0	4	3
108	2	3.0	10	1	690	2	11.0	4	2	737	2	11.0	4	3
702	2	11.0	4	2	703	2	11.0	4	2	707	2	11.0	4	2
154	2	11.0	10	3	157	2	11.0	10	1	156	2	11.0	10	2
182	2	11.0	10	3	186	2	11.0	10	3	158	2	11.0	10	3
159	2	11.0	10	3	183	2	11.0	10	3	160	2	11.0	10	3
184	2	11.0	10	3	187	2	11.0	10	3	188	2	11.0	10	3
682	2	11.0	4	2	683	2	11.0	4	2	704	2	11.0	4	2

cluster 4 cont'd.

739	2	11.0	4	3	745	2	11.0	4	3	177	2	11.0	10	3
543	5	5.5	10	1	265	5	3.0	10	1	548	5	5.5	10	1
526	4	0.5	10	1	563	4	13.0	10	1	738	2	11.0	4	3
572	4	13.0	10	1	245	5	0.5	10	1	257	5	3.0	10	1
268	5	3.0	10	1										

CLUSTER 5

9	2	0.5	10	1	39	2	11.0	10	3	32	2	20.5	10	1
47	2	11.5	10	3	527	4	0.5	10	1	528	4	0.5	10	1
517	4	0.5	10	1	522	4	0.5	10	1	564	4	13.0	10	1
570	4	13.0	10	1	565	4	13.0	10	1	581	4	3.0	10	1
580	4	3.0	10	1	582	4	3.0	10	1	583	4	3.0	10	1
584	4	3.0	10	1	585	4	3.0	10	1	413	4	13.0	10	1
416	4	13.0	10	1	734	2	11.0	4	3	515	4	0.5	10	1
716	2	11.0	4	3	718	2	11.0	4	3	726	2	11.0	4	3
730	2	11.0	4	3	731	2	11.0	4	3	717	2	11.0	4	3
719	2	11.0	4	3	724	2	11.0	4	3	733	2	11.0	4	3
747	2	11.0	4	3	721	2	11.0	4	3	732	2	11.0	4	3
411	4	13.0	10	1	587	4	3.0	10	1	445	2	3.0	10	1
451	2	3.0	10	1	452	2	3.0	10	1	111	2	3.0	10	1
696	2	11.0	4	2	736	2	11.0	4	3	551	5	3.0	10	1
375	4	4.0	10	2	385	4	4.0	10	2	697	2	11.0	4	2
586	4	3.0	10	1	230	5	0.5	10	1					

CLUSTER 6

63	5	0.5	10	1	784	4	13.0	10	2	768	4	13.0	10	2
102	2	3.0	10	1	110	2	3.0	10	1	301	3	0.0	10	1
537	5	5.5	10	1	64	5	0.5	10	1	151	2	11.0	10	3
155	2	11.0	10	3	152	2	11.0	10	3	179	2	11.0	10	3
178	2	11.0	10	3	180	2	11.0	10	3	181	2	11.0	10	3

cluster 6 cont'd.

388	4	4.0	10	1	420	4	13.0	10	1	218	5	0.5	10	1
384	4	4.0	10	2	511	4	0.5	10	1	532	5	5.5	10	1
224	5	0.5	10	1	360	4	4.0	10	2	648	3	7.5	4	3

CLUSTER 7

172	2	11.0	10	2	173	2	11.0	10	2	759	4	13.0	10	2
175	2	11.0	10	2	320	3	7.5	10	2	339	3	0.0	4	1
650	3	7.5	4	3	772	4	13.0	10	2	353	4	4.0	10	2
770	4	13.0	10	2	356	4	4.0	10	2	751	4	13.0	10	2
771	4	13.0	10	2	778	4	13.0	10	2					

CLUSTER 8

2	2	0.5	10	1	35	2	11.0	10	3	37	2	11.0	10	3
94	4	3.0	10	1	432	2	0.5	10	1	464	4	0.5	10	1
52	5	0.5	10	1	615	5	10.5	10	1	840	1	3.0	10	1
95	4	3.0	10	1	137	1	0.5	10	1	624	5	5.5	10	1
260	5	3.0	10	1	232	5	0.5	10	1	326	3	7.5	10	2
68	5	0.5	10	1	321	3	7.5	10	2	479	1	5.5	10	1
194	2	5.5	10	1	802	1	15.5	10	1	219	5	0.5	10	1
294	3	0.0	10	1	298	3	0.0	10	1					

CLUSTER 9

22	2	10.5	10	1	501	3	7.5	10	3	391	4	4.0	10	1
403	4	4.0	10	1	338	3	0.0	4	1	808	3	7.5	10	2
471	1	10.5	10	1	472	1	10.5	10	1	474	1	10.5	10	1
789	4	13.0	10	2	28	2	15.5	10	1	799	3	7.5	4	3
800	3	7.5	4	3	302	3	0.0	10	1	305	3	0.0	10	1
304	3	0.0	10	1	303	3	0.0	10	1	29	2	15.5	10	1
300	3	0.0	10	1	327	3	0.0	10	1	476	1	10.5	10	1
792	3	7.5	4	3	404	4	4.0	10	1	405	4	4.0	10	1

cluster 9 cont'd.

410	4	4.0	10	1	288	3	0.0	10	1	406	4	4.0	10	1
389	4	4.0	10	1	291	3	0.0	10	1	793	3	7.5	4	3
402	4	4.0	10	1	795	3	7.5	4	3	796	3	7.5	4	3
475	1	10.5	10	1	477	1	10.5	10	1	478	1	10.5	10	1
290	3	0.0	10	1	292	2	0.0	10	1	295	3	0.0	10	1
293	3	0.0	10	1	296	3	0.0	10	1	297	3	0.0	10	1
299	3	0.0	10	1	307	3	0.0	10	1	306	3	0.0	10	1

CLUSTER 10

10	2	3.0	10	1	14	2	3.0	10	1	214	2	5.5	10	1
779	4	13.0	10	2	562	4	13.0	10	1	277	2	20.5	10	1
622	4	10.5	10	1	153	2	11.0	10	3	801	1	15.5	10	1
15	2	5.5	10	1	195	2	5.5	10	1	198	2	5.5	10	1
208	2	5.5	10	1	199	2	5.5	10	1	769	4	13.0	10	2
200	2	5.5	10	1	121	2	15.5	10	1	271	2	20.5	10	1
126	2	15.5	10	1	278	2	20.5	10	1	279	2	20.5	10	1
282	2	20.5	10	1	31	2	20.5	10	1	791	3	7.5	4	3
191	2	5.5	10	1	203	2	5.5	10	1	757	4	13.0	10	2
419	4	13.0	10	1	612	5	10.5	10	1	629	5	5.5	10	1
25	2	15.5	10	1	27	2	15.5	10	1	122	2	15.5	10	1
26	2	15.5	10	1	118	2	15.5	10	1	392	4	4.0	10	1
393	4	4.0	10	1	400	4	4.0	10	1	764	4	13.0	10	2
311	3	0.0	10	1	312	3	7.5	10	2	319	3	7.5	10	2
313	3	7.5	10	2	45	2	11.0	10	3	49	2	11.0	10	3
50	2	11.0	10	3	560	5	3.0	10	1	48	2	11.0	10	3
443	2	3.0	10	1	462	4	0.5	10	1	435	2	0.5	10	1
617	5	10.5	10	1	619	5	10.5	10	1	618	5	10.5	10	1
437	2	0.5	10	1	439	2	0.5	10	1	626	5	5.5	10	1
742	2	11.0	4	3	743	2	11.0	4	3					

CLUSTER 11

205	2	5.5	10	1	461	4	0.5	10	1	289	3	0.0	10	1
794	3	7.5	4	3	805	3	7.5	10	3	641	3	0.0	4	1
655	3	7.5	4	3	656	3	7.5	4	3	642	3	0.0	4	1
664	1	0.5	10	1	666	1	0.5	10	1					

CLUSTER 12

16	2	5.5	10	1	212	2	5.5	10	1	213	2	5.5	10	1
575	2	10.5	10	1	286	2	20.5	10	1	637	5	5.5	10	1
781	4	13.0	10	2	192	2	5.5	10	1	201	2	5.5	10	1
272	2	20.5	10	1	274	2	20.5	10	1	287	3	0.0	10	1
408	4	4.0	10	1	595	4	3.0	10	1	606	4	3.0	10	1
596	4	3.0	10	1	602	4	3.0	10	1	614	5	10.5	10	1
613	5	10.5	10	1	609	5	10.5	10	1	610	5	10.5	10	1
628	5	5.5	10	1	24	2	10.5	10	1	632	4	10.5	10	1
755	4	13.0	10	2	750	5	15.5	10	1	725	2	11.0	4	3
746	2	11.0	4	3	130	2	15.5	10	1	273	2	20.5	10	1
780	4	13.0	10	2	215	2	5.5	10	1	756	4	13.0	10	2
131	2	15.5	10	1	281	2	20.5	10	1	280	2	20.5	10	1
285	2	20.5	10	1	44	2	11.0	10	3	60	5	0.5	10	1
556	5	3.0	10	1	649	1	20.5	10	1	383	4	4.0	10	2
536	5	5.5	10	1	193	2	5.5	10	1	196	2	5.5	10	1
197	2	5.5	10	1	631	4	10.5	10	1	473	1	10.5	10	1
207	2	5.5	10	1	621	4	10.5	10	1	603	4	3.0	10	1
611	5	10.5	10	1	210	2	5.5	10	1	633	4	10.5	10	1
634	4	10.5	10	1	283	2	20.5	10	1	211	2	5.5	10	1
216	2	5.5	10	1	19	2	5.5	10	1	729	2	11.0	4	3
124	2	15.5	10	1	129	2	15.5	10	1	762	4	13.0	10	2
396	4	4.0	10	1	775	4	13.0	10	2	116	2	15.5	10	1

cluster 12 cont'd.

117	2	15.5	10	1	120	2	15.5	10	1	123	2	15.5	10	1
206	2	5.5	10	1	323	3	7.5	10	2	722	2	11.0	4	3
723	2	11.0	4	3	788	4	13.0	10	2	728	2	11.0	4	3
18	2	5.5	10	1	161	2	11.0	10	3	382	4	4.0	10	2
390	4	4.0	10	1	267	5	3.0	10	1	314	3	7.5	10	2
315	3	7.5	10	2	523	4	0.5	10	1	538	5	5.5	10	1
237	5	0.5	10	1	264	5	3.0	10	1	688	2	11.0	4	2
269	5	3.0	10	1	430	4	13.0	10	1	741	2	11.0	4	3
695	2	11.0	4	2	709	2	11.0	4	2	700	2	11.0	4	2
705	2	11.0	4	2	701	2	11.0	4	2	708	2	11.0	4	2
107	2	3.0	10	1	549	5	5.5	10	1	810	3	7.5	10	2
807	3	7.5	10	2	809	3	7.5	10	2	811	3	7.5	10	2
557	5	3.0	10	1	558	5	3.0	10	1	608	5	10.5	10	1
669	1	00.5	10	1	670	1	0.5	10	1					

CLUSTER 13

17	2	5.5	10	1	591	4	3.0	10	1	598	4	3.0	10	1
605	4	3.0	10	1	597	4	3.0	10	1	599	4	3.0	10	1
604	4	3.0	10	1	630	5	5.5	10	1	749	5	15.5	10	1
727	2	11.0	4	3	21	2	10.5	10	1	59	5	0.5	10	1
318	3	7.5	10	2	51	5	0.5	10	1	748	2	11.0	4	3
735	2	11.0	4	3	559	5	3.0	10	1	623	5	5.5	10	1
592	4	3.0	10	1	607	5	10.5	10	1	640	3	0.0	4	1
616	5	10.5	10	1	620	5	10.5	10	1	625	5	5.5	10	1

CLUSTER 14

5	2	0.5	10	1	185	2	11.0	10	3	639	3	0.0	4	1
753	4	13.0	10	2	783	4	13.0	10	2	773	4	13.0	10	2
231	5	0.5	10	1	262	5	3.0	10	1	364	4	4.0	10	2
652	3	7.5	4	3	806	3	7.5	10	3	23	2	10.5	10	1

cluster 14 cont'd.

576	2	10.5	10	1	30	2	20.5	10	1	164	2	11.0	10	2
169	2	11.0	10	2	40	2	11.0	10	3	429	4	13.0	10	1
354	4	4.0	10	2	401	4	4.0	10	1	409	4	4.0	10	1
202	2	5.5	10	1	710	4	5.5	10	1	284	2	20.5	10	1
568	4	13.0	10	1	422	4	13.0	10	1	423	4	13.0	10	1
127	2	15.5	10	1	209	2	5.5	10	1	540	5	5.5	10	1
310	3	0.0	10	1	578	2	10.5	10	1	424	4	13.0	10	1
427	4	13.0	10	1	785	4	13.0	10	2	440	2	0.5	10	1
453	2	3.0	10	1	436	2	0.5	10	1	671	2	11.0	4	2
119	2	15.5	10	1	128	2	15.5	10	1	125	2	15.5	10	1
782	4	13.0	10	2	165	2	11.0	10	2	171	2	11.0	10	2
366	4	4.0	10	2	371	4	4.0	10	2	488	3	7.5	10	3
42	4	11.0	10	3	412	4	13.0	10	1	679	2	11.0	4	2
421	4	13.0	10	1	754	4	13.0	10	2	266	5	3.0	10	1
675	2	11.0	4	2	676	2	11.0	4	2	677	2	11.0	4	2
678	2	11.0	4	2	680	2	11.0	4	2	99	4	3.0	10	1
359	4	4.0	10	2	395	4	4.0	10	1	235	5	0.5	10	1
236	5	0.5	10	1	672	2	11.0	4	2	512	4	0.5	10	1
681	2	11.0	4	2	691	2	11.0	4	2	692	2	11.0	4	2
673	2	11.0	4	2	674	2	11.0	4	2	513	4	0.5	10	1
546	5	5.5	10	1	71	5	0.5	10	1	324	3	7.5	10	2
316	3	7.5	10	2	317	3	7.5	10	2	322	3	7.5	10	2
325	3	7.5	10	2	253	5	3.0	10	1	254	5	3.0	10	1
221	5	0.5	10	1	442	2	3.0	10	1	448	2	3.0	10	1
256	5	3.0	10	1	270	5	3.0	10	1	259	5	3.0	10	1

CLUSTER 15

36	2	0.5	10	1	67	5	0.5	10	1	454	2	3.0	10	1
466	4	0.5	10	1	484	1	5.5	10	1	41	2	11.0	10	3

cluster 15 cont'd.

114	2	3.0	10	1	661	1	0.5	10	1	553	5	3.0	10	1
658	1	0.5	10	1	58	5	0.5	10	1	246	5	0.5	10	1
249	5	0.5	10	1	251	5	3.0	10	1	482	1	5.5	10	1
241	5	0.5	10	1	243	5	0.5	10	1	662	1	0.5	10	1
146	1	0.5	10	1	263	5	3.0	10	1	255	5	3.0	10	1
238	5	0.5	10	1	244	5	0.5	10	1	233	5	0.5	10	1
239	5	0.5	10	1	242	5	0.5	10	1	234	5	0.5	10	1
100	4	3.0	10	1	514	4	0.5	10	1	520	4	0.5	10	1
240	5	0.5	10	1	248	5	0.5	10	1	250	5	0.5	10	1
261	5	3.0	10	1										

CLUSTER 16

12	2	3.0	10	1	766	4	13.0	10	2	456	2	0.5	10	1
458	4	0.5	10	1	468	4	0.5	10	1	758	4	13.0	10	2
763	4	13.0	10	2	103	2	3.0	10	1	444	2	3.0	10	1
457	4	0.5	10	1	469	4	0.5	10	1	463	4	0.5	10	1
465	4	0.5	10	1	449	2	3.0	10	1	455	2	0.5	10	1
20	2	10.5	10	1	112	2	3.0	10	1	113	2	3.0	10	1
77	4	3.0	10	1	447	2	3.0	10	1	415	4	13.0	10	1
428	4	13.0	10	1	450	2	3.0	10	1	459	4	0.5	10	1
460	4	0.5	10	1	555	5	3.0	10	1	569	4	13.0	10	1
593	4	3.0	10	1	600	4	3.0	10	1	62	5	0.5	10	1
66	5	0.5	10	1	594	4	3.0	10	1	601	4	3.0	10	1
75	4	3.0	10	1	414	4	13.0	10	1	693	2	11.0	4	2
518	4	0.5	10	1	519	4	0.5	10	1	544	5	5.5	10	1
530	5	5.5	10	1	521	4	0.5	10	1	711	2	11.0	4	3
720	2	11.0	4	3	712	2	11.0	4	3	713	2	11.0	4	3
714	2	11.0	4	3	554	5	3.0	10	1	567	4	13.0	10	1
684	2	11.0	4	2	686	2	11.0	4	2	687	2	11.0	4	2

cluster 16 cont'd.

694	2	11.0	4	2	685	2	11.0	4	2	715	2	11.0	4	3

CLUSTER 17

3	2	0.5	10	1	4	2	0.5	10	1	10	2	0.5	10	1
11	2	3.0	10	1	843	3	7.5	10	3	815	1	3.0	10	1
824	1	3.0	10	1	135	1	0.5	10	1	150	1	0.5	10	1
136	1	0.5	10	1	144	1	0.5	10	1	149	1	0.5	10	1
142	1	0.5	10	1	147	1	0.5	10	1	57	5	0.5	10	1
65	5	0.5	10	1	140	1	0.5	10	1	92	4	3.0	10	1
93	4	3.0	10	1	143	1	0.5	10	1	839	1	3.0	10	1
816	1	3.0	10	1	822	1	3.0	10	1	830	1	3.0	10	1
820	1	3.0	10	1	821	1	3.0	10	1	838	3	7.5	10	3

CLUSTER 18

53	5	0.5	10	1	55	5	0.5	10	1	139	1	0.5	10	1
524	4	0.5	10	1	525	4	0.5	10	1	69	5	0.5	10	1
657	5	3.0	10	1	141	1	0.5	10	1	56	5	0.5	10	1
638	5	5.5	10	1	134	1	0.5	10	1	659	1	0.5	10	1
145	1	0.5	10	1	542	5	5.5	10	1	189	2	11.0	10	3
190	2	11.0	10	3	842	1	15.5	10	1	668	1	0.5	10	1
541	5	5.5	10	1	547	5	5.5	10	1	841	1	3.0	10	1
843	1	15.5	10	1	87	4	3.0	10	1	386	4	4.0	10	1
252	5	3.0	10	1	497	3	7.5	10	3	505	3	7.5	10	3
506	3	7.5	10	3	258	5	3.0	10	1	480	1	5.5	10	1
481	1	5.5	10	1	833	3	7.5	10	3	836	3	7.5	10	3
837	3	7.5	10	3	138	1	0.5	10	1	665	1	0.5	10	1
667	1	0.5	10	1	660	1	0.5	10	1	817	1	3.0	10	1
663	1	0.5	10	1	798	3	7.5	4	3					

CLUSTER 19

45	5	0.5	10	1	516	4	0.5	10	1	590	4	3.0	10	1
417	4	13.0	10	1	706	2	11.0	4	2	573	4	3.0	10	1
574	4	3.0	10	1	588	4	3.0	10	1	589	4	3.0	10	1
101	2	3.0	10	1	115	2	3.0	10	1	418	4	13.0	10	1
529	4	0.5	10	1	446	2	3.0	10	1	76	4	3.0	10	1
83	4	3.0	10	1	82	4	3.0	10	1	89	4	3.0	10	1
84	4	3.0	10	1	90	4	3.0	10	1	98	4	3.0	10	1
78	4	3.0	10	1	380	4	4.0	10	2	79	4	3.0	10	1
351	4	4.0	10	2	441	2	3.0	10	1	467	4	0.5	10	1
104	2	3.0	10	1	105	2	3.0	10	1	106	2	3.0	10	1

CLUSTER 20

61	5	0.5	10	1	133	1	0.5	10	1	132	1	0.5	10	1
81	4	3.0	10	1	85	4	3.0	10	1	97	4	3.0	10	1
80	4	3.0	10	1	566	4	13.0	10	1	579	4	3.0	10	1
162	2	11.0	10	3	577	2	10.5	10	1	531	5	5.5	10	1
533	5	5.5	10	1	627	5	5.5	10	1	535	5	5.5	10	1
635	5	5.5	10	1	545	5	5.5	10	1	550	5	5.5	10	1
70	5	0.5	10	1	72	5	0.5	10	1	74	4	3.0	10	1
539	5	5.5	10	1	367	4	4.0	10	2	434	2	0.5	10	1
91	4	3.0	10	1	534	5	5.5	10	1	352	4	4.0	10	2
787	4	13.0	10	2	376	4	4.0	10	2	398	4	4.0	10	1
394	4	4.0	10	1	397	4	4.0	10	1	399	4	4.0	10	1
407	4	4.0	10	1	73	5	0.5	10	1	148	1	0.5	10	1
373	4	4.0	10	2	275	2	20.5	10	1	374	4	4.0	10	2
378	4	4.0	10	2	276	3	0.0	10	1	361	4	4.0	10	2
377	4	4.0	10	2	370	4	4.0	10	2	368	4	4.0	10	2
369	4	4.0	10	2	365	4	4.0	10	2	431	2	0.5	10	1
740	2	11.0	4	3	438	2	0.5	10	1	426	4	13.0	10	1

cluster 20 cont'd.

561	4	13.0	10	1	571	4	13.0	10	1					

CLUSTER 21

43	2	11.0	10	3	46	2	11.0	10	1	328	3	0.0	4	1
790	1	3.0	10	1	828	1	3.0	10	1	814	1	3.0	10	1
355	4	4.0	10	2	362	4	4.0	10	2	358	4	4.0	10	1
372	4	4.0	10	2	767	4	13.0	10	2	774	4	13.0	10	2
379	4	4.0	10	2	381	4	4.0	10	2	761	4	13.0	10	2
777	4	13.0	10	2	765	4	13.0	10	2	786	4	13.0	10	2
776	4	13.0	10	2										

APPENDIX E

Clustering of variables

It is usual to apply cluster analyses to cases (rows) not to variables (columns). However, I was able to find a program that would cluster using the variables. This was the BMDP (P1M) program. I applied the program three times: to the antibiotics alone (7 variables), to the heavy metals alone (6 variables), and to the antibiotics and heavy metals together (13 variables).

The program PIM procedures illustrating the clustering process were shown in appendix table 30.

Appendix Table 29

An example of calculating the correlation coefficient matrix for the clustering process of heavy metals based on the single linkage method. d = distance, max = maximum distance.

	Cd	Cr	Hg	Cu	Mn	Pb
Cd	0					
Cr	0.1423	0				
Hg	0.2005	-0.2757	0			
Cu	0.2958	-0.0756	0.4152	0		
Mn	0.2954	0.0857	0.2232	0.3331	0	
Pb	0.3073	-0.0213	0.3566	0.4023	0.2812	0

d (Hg, Cu) Mn = max {d HgMn, d CuMn} = d CuMn = 0.3331

d (Hg, Cu) Pb = max {d HgPb, d CuPb} = d CuPb = 0.4023

d (Hg, Cu) Cd = max {d HgCd, d CuCd} = d CuCd = 0.2958

d (Hg, Cu) Cr = max {d HgCr, d CuCr} = d HgCr =-0.2757

	Cd	Cr	(HgCu)	Mn	Pb
Cd	0				
Cr	0.1423	0			
(HgCu)	0.2958	-0.2757	0		
Mn	0.2954	0.0857	0.3331	0	
Pb	0.3073	-0.0213	0.4023	0.2812	0

d (HgCu, Pb) Mn = max {d HgCu-Mn, d PbMn} = d HgCu-Mn = 0.3331

d (HgCu, Pb) Cd = max {d HgCu-Cd, d PbCd} = d PbCd = 0.3073

d (HgCu, Pb) Cr = max {d HgCu-Cr, d PbCr} = d HgCu-Cr =-0.2757

	Cd	Cr	(HgCuPb)	Mn
Cd	0			
Cr	0.1423	0		
(HgCuPb)	0.3073	-0.2757	0	
Mn	0.2954	0.0857	0.3331	0

d (HgCuPb, Mn) Cd = max {d HgCuPb-Cd, d MnCd} = d HgCuPb-Cd = 0.3073

d (HgCuPb, Mn) Cr = max {d HgCuPb-Cr, d MnCr} = d HgCuPb-Cr =-0.2757

	Cd	Cr	(HgCuPbMn)
Cd	0		
Cr	0.1423	0	
(HgCuPbMn)	0.3073	-0.2757	0

d (HgCuPbMn, Cd) Cr=max {d HgCuPbMn-Cr, d CdCr}=d HgCuPbMn-Cr =-0.2757

	Cr	(HgCuPbMnCd)
Cr	0	
(HgCuPbMnCd)	-0.2757	0

Finally, Cr joined the metals Hg, Cu, Pb, Mn and Cd at a correlation measure of -0.2757.

Appendix Table 30

The program "BMDP1M-cluster analysis of variables" procedures illustrating the clustering process of heavy metals based on the single linkage method.

```
1PAGE   1  BMDP1M

 BMDP1M - CLUSTER ANALYSIS OF VARIABLES
 BMDP STATISTICAL SOFTWARE, INC.
 1964 WESTWOOD BLVD. SUITE 202
 (213) 475-5700
 PROGRAM REVISED OCTOBER 1983
 MANUAL REVISED -- 1983
 COPYRIGHT (C) 1983 REGENTS OF UNIVERSITY OF CALIFORNIA

 TO SEE REMARKS AND A SUMMARY OF NEW FEATURES FOR
 THIS PROGRAM, STATE NEWS. IN THE PRINT PARAGRAPH.
CTHIS VERSION OF BMDP HAS BEEN CONVERTED FOR USE ON
 ICL 2900 COMPUTERS BY THE PROGRAM LIBRARY UNIT,
 UNIVERSITY OF EDINBURGH.          TEL 031-667 1081.
 PROGRAM IS PLU VERSION 7.0.

     APRIL 14, 1986  AT 14:24:06

 PROGRAM CONTROL INFORMATION

 /PROBLEM TITLE IS 'DSBTAX'.
 /INPUT VARIABLES ARE    13.
 FORMAT IS '(17x,7F4,F6,3F4,F5,F4)'.
 /VARIABLE NAMES ARE AMPIC,CHLOR,POLYB,STREP,NALAC,TETRA,METBL,CADM,CHRO,
                     MERC,COPP,MANG,LEAD.
 USE=CADM,CHRO,MERC,COPP,MANG,LEAD.
 /PRINT     CORRELATION.
            SHADE.
 /END.

 PROBLEM TITLE IS
 DSBTAX

 NUMBER OF VARIABLES TO READ IN. . . . . .      13
 NUMBER OF VARIABLES ADDED BY TRANSFORMATIONS. .       0
 TOTAL NUMBER OF VARIABLES . . . . . .      13
 NUMBER OF CASES TO READ IN. . . . . . TO END
 CASE LABELING VARIABLES . . . . . .
 MISSING VALUES CHECKED BEFORE OR AFTER TRANS. . NEITHER
 BLANKS ARE. . . . . . MISSING
 INPUT FILE. . . . . UNIT    5 . . . . . .
 REWIND INPUT UNIT PRIOR TO READING. . DATA. . .     NO
 NUMBER OF WORDS OF DYNAMIC STORAGE. . . . . .   14998
 NUMBER OF CASES DESCRIBED BY INPUT FORMAT . . .       1

 VARIABLES TO BE USED
      8 CADM       9 CHRO       10 MERC       11 COPP       12 MANG
     13 LEAD
1PAGE   2  BMDP1M DSBTAX

0INPUT FORMAT IS
 (17x,7F4,F6,3F4,F5,F4)

 MAXIMUM LENGTH DATA RECORD IS   72 CHARACTERS.
1PAGE   3  BMDP1M DSBTAX

CI N P U T   V A R I A B L E S . . . . .
    VARIABLE      RECORD    COLUMNS     FIELD  TYPE              VARIABLE      RECORD    COLUMNS     FIELD  TYPE
  INDEX   NAME      NO.  BEGIN  END    WIDTH                   INDEX   NAME      NO.  BEGIN  END    WIDTH
```

Appendix Table 30 (con'd.)

1	AMPIC	1	19	21	4	F	8	CADM	1	46	51	6	F
2	CHLOR	1	22	25	4	F	9	CHRO	1	52	55	4	F
3	POLYB	1	26	29	4	F	10	MERC	1	56	59	4	F
4	STREP	1	30	33	4	F	11	COPP	1	60	63	4	F
5	NALAC	1	34	37	4	F	12	MANG	1	64	68	5	F
6	TETRA	1	38	41	4	F	13	LEAD	1	69	72	4	F
7	METBL	1	42	45	4	F							

PROCEDURE MEASURE . . . . . .ABSC
PROCEDURE AMALGAMATION RULE IS MINIMUM DISTANCE (SINGLE LINKAGE)
0BASED ON INPUT FORMAT SUPPLIED 1 RECORDS READ PER CASE.

NUMBER OF CASES READ. 843
1PAGE 4 BMDP1M DSBTAX

STATISTICS FOR EACH VARIABLE

VARIABLE NO.	NAME	MEAN	STANDARD DEVIATION	SMALLEST VALUE	SMALLEST Z-SC	SMALLEST CASE	LARGEST VALUE	LARGEST Z-SC	LARGEST CASE
8	CADM	29.4525	31.5623	8.0000	-0.68	3	256.0000	7.18	394
9	CHRO	188.7446	188.7275	8.0000	-0.96	65	512.0000	1.71	23
10	MERC	24.1312	31.2869	2.0000	-0.71	3	128.0000	3.32	35
11	COPP	81.7978	52.2468	4.0000	-1.49	824	256.0000	3.33	102
12	MANG	2041.3979	1963.7456	128.0000	-0.97	55	8192.0000	3.13	103
13	LEAD	321.6335	169.8589	64.0000	-1.52	41	512.0000	1.12	1

1PAGE 5 BMDP1M DSBTAX

CORRELATIONS

		CADM 8	CHRO 9	MERC 10	COPP 11	MANG 12	LEAD 13
CADM	8	1.0000					
CHRO	9	0.1423	1.0000				
MERC	10	0.2005	-0.2757	1.0000			
COPP	11	0.2958	-0.0756	0.4152	1.0000		
MANG	12	0.2954	0.0857	0.2232	0.3331	1.0000	
LEAD	13	0.3073	-0.0213	0.3566	0.4023	0.2812	1.0000

1PAGE 6 BMDP1M DSBTAX

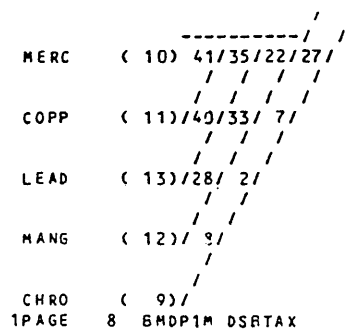
NAME	VARIABLE NO.	OTHER BOUNDARY OF CLUSTER	NUMBER OF ITEMS IN CLUSTER	DISTANCE OR SIMILARITY WHEN CLUSTER FORMED
CADM	8	2	6	27.57
MERC	10	5	4	33.31
COPP	11	3	2	41.52
LEAD	13	3	3	40.23
MANG	12	1	5	30.73
CHRO	9	1	6	27.57

1PAGE 7 BMDP1M DSBTAX

TREE PRINTED OVER ABSOLUTE CORRELATION MATRIX.
CLUSTERING BY MINIMUM DISTANCE METHOD.

VARIABLE NAME	NO.	
		----------/
CADM	(8)	2) 29 30 29/14/

Appendix Table 30 (con'd.)



1PAGE 8 BMDP1M DSBTAX

THE VALUES IN THIS TREE HAVE BEEN SCALED 0 TO 100
ACCORDING TO THE FOLLOWING TABLE

VALUE ABOVE	CORRELATION	VALUE ABOVE	CORRELATION
0	0.000	50	0.500
5	0.050	55	0.550
10	0.100	60	0.600
15	0.150	65	0.650
20	0.200	70	0.700
25	0.250	75	0.750
30	0.300	80	0.800
35	0.350	85	0.850
40	0.400	90	0.900
45	0.450	95	0.950

1PAGE 9 BMDP1M DSBTAX

ABSOLUTE VALUES OF CORRELATIONS IN SORTED AND SHADED FORM

```
+    8 CADM      X
+                0
+                *

+   10 MERC      +X
+                 0
+                 *

+   11 COPP      XXX
+                NOO
+                 **

+   13 LEAD      XXXX
+                NOOO
+                  **

+   12 MANG      XXXXX
+                N ONO
+                    *

+    9 CHRO      -X. .X
+                 N   O
```

Appendix Table 30 (con'd.)

```
 THE ABSOLUTE VALUES OF
 THE MATRIX ENTRIES HAVE BEEN PRINTED ABOVE IN SHADED FORM
 ACCORDING TO THE FOLLOWING SCHEME

+
+
+                  LESS THAN OR EQUAL TO         0.071
+      .         0.071  TO AND INCLUDING         0.120
+
+
+      -         0.120  TO AND INCLUDING         0.169
+
+
+      +         0.169  TO AND INCLUDING         0.218
+
+
+      X         0.218  TO AND INCLUDING         0.268
+
+
+      X         0.268  TO AND INCLUDING         0.317
+      N
+
+      X         0.317  TO AND INCLUDING         0.366
+      O
+
+      X                     GREATER THAN         0.366
+      O
+      *

 NUMBER OF INTEGER WORDS OF STORAGE USED IN PRECEDING    PROBLEM     834
 CPU TIME USED      7.055 SECONDS

1PAGE  10  BMDP1M

 BMDP1M - CLUSTER ANALYSIS OF VARIABLES

     APRIL 14, 1986  AT 14:24:20

 NO MORE CONTROL LANGUAGE.

 PROGRAM TERMINATED

END OF LISTING OF FILE :GBZV28.CLUMETAL(1,*,1) FOR USER :GBZV28 AT 1986/04/14__15:14:27
```

Appendix Table 31

Data on the responces (MIC) of 843 deep-sea isolates and 27 reference cultures to antibiotics and heavy metals. Site number, depth (cm), source of sample and incubation temperatures are also shown.

Isolate No.	Site	Depth	Incub.temp.	Source	Minimum inhibitory concentration (MIC)											
					Antibiotics							Heavy metals				
					Am	Ch	Po	St	Na	Te	Me	Cd	Cr	Hg	Cu	Mn	Pb
001	2	00.5	10	1	512	008	004	128	512	128	512	032	256	004	064	4096	512
002	2	00.5	10	1	512	002	256	256	128	128	512	016	256	004	048	0512	512
003	2	00.5	10	1	512	002	004	064	512	128	512	032	512	004	064	2048	512
004	2	00.5	10	1	512	008	004	128	512	128	512	048	512	004	064	2048	512
005	2	00.5	10	1	008	002	128	004	512	128	002	053	203	004	064	1024	512
006	2	00.5	10	1	008	004	032	128	256	032	008	128	256	004	128	4096	512
007	2	00.5	10	1	512	002	043	128	512	128	512	032	256	004	064	4096	512
008	2	00.5	10	1	512	002	004	128	256	128	512	032	512	004	064	4096	512
009	2	00.5	10	1	032	004	004	128	032	256	512	064	008	032	128	4096	512
010	2	00.5	10	1	512	032	008	256	512	128	512	032	512	004	064	2048	512
011	2	03.0	10	1	512	002	004	256	512	128	512	032	512	004	128	2048	512
012	2	03.0	10	1	098	002	049	222	016	141	390	050	173	047	140	1024	229
013	2	03.0	10	1	002	002	032	016	256	032	002	008	256	002	048	0512	128
014	2	03.0	10	1	098	002	064	016	256	032	032	008	256	047	064	0512	128
015	2	05.5	10	1	035	032	076	055	137	078	002	008	097	005	061	0483	160
016	2	05.5	10	1	002	004	076	004	256	032	002	008	097	002	032	0256	128
017	2	05.5	10	1	002	032	046	055	256	078	512	008	097	002	032	0256	128
018	2	05.5	10	1	002	002	128	032	128	128	042	016	256	002	048	0256	512
019	2	05.0	10	1	002	002	076	055	128	078	042	008	256	005	061	0128	160
020	2	10.5	10	1	002	004	130	013	032	008	512	015	134	005	064	1024	267
021	2	10.5	10	1	002	003	004	004	128	128	512	016	256	004	128	0512	512
022	2	10.5	10	1	002	003	004	004	128	128	002	016	256	004	128	0512	512
023	2	10.5	10	1	002	002	130	004	103	057	002	008	134	002	032	0786	267
024	2	10.5	10	1	002	004	130	016	016	032	002	032	134	016	048	0128	192
025	2	15.5	10	1	003	004	028	004	064	064	002	008	207	002	048	0472	128
026	2	15.5	10	1	003	002	028	051	090	046	002	008	207	002	039	0472	064
027	2	15.5	10	1	003	002	028	051	090	046	002	008	207	002	064	0472	128
028	2	15.5	10	1	002	002	004	064	064	256	002	048	512	004	064	0512	192
029	2	15.5	10	1	002	002	008	128	128	256	002	048	512	004	064	0512	192
030	2	20.5	10	1	042	002	021	052	081	004	063	024	040	006	041	0932	135
031	2	20.5	10	1	032	002	004	032	032	128	016	032	040	004	032	0512	256
032	2	20.5	10	1	128	002	004	064	064	256	512	128	064	032	128	4096	512
033	2	20.5	10	1	032	002	004	256	256	064	128	048	256	016	128	4096	256
034	2	20.5	10	1	512	004	004	128	064	256	512	128	128	032	128	4096	512
035	2	11.0	10	3	512	002	256	128	128	256	512	048	256	004	128	0512	256
036	2	11.0	10	3	512	004	004	032	128	256	512	128	128	032	128	1024	512
037	2	11.0	10	3	512	002	256	128	128	256	512	016	256	004	128	0256	256
038	2	11.0	10	3	512	002	128	128	128	256	512	128	256	032	128	4096	512
039	2	11.0	10	3	064	002	008	032	032	256	512	064	016	032	128	4096	512
040	2	11.0	10	3	008	002	004	064	064	016	008	016	095	016	032	1024	256
041	2	11.0	10	3	512	002	128	256	256	256	512	032	256	002	128	1024	192
042	2	11.0	10	3	008	005	004	064	064	032	008	016	064	016	048	1024	512
043	2	11.0	10	3	002	002	064	128	064	109	187	008	095	042	107	3446	128
044	2	11.0	10	3	002	005	064	128	064	008	187	008	095	002	064	0128	192
045	2	11.0	10	3	002	002	064	256	256	064	008	008	512	004	048	0512	192
046	2	11.0	10	1	002	008	064	256	256	008	187	040	095	042	016	3446	192
047	2	11.0	10	3	064	004	008	064	064	128	512	048	032	032	128	4096	512
048	2	11.0	10	3	093	032	064	128	128	064	187	008	095	002	032	0512	128
049	2	11.0	10	3	002	002	064	256	256	032	187	008	256	002	032	0512	128
050	2	11.0	10	3	002	005	064	256	256	004	187	040	256	002	032	0512	128
051	5	00.5	10	1	008	008	004	256	016	256	512	016	032	002	128	0256	512
052	5	00.5	10	1	512	002	004	128	256	064	128	048	256	004	048	0256	512
053	5	00.5	10	1	512	002	004	128	128	064	128	048	256	004	048	2048	512
054	5	00.5	10	1	064	008	004	064	064	128	512	016	032	002	128	2048	512
055	5	00.5	10	1	512	002	004	128	256	128	128	048	256	023	064	2048	512
056	5	00.5	10	1	512	002	004	128	512	064	128	048	256	023	064	2048	128
057	5	00.5	10	1	319	002	024	064	512	256	512	016	256	023	032	2282	256

058	5	00.5	10	1	319	002	004	032	256	194	128	048	256	004	032	1024	256
059	5	00.5	10	1	008	004	004	064	032	128	512	032	064	064	256	0256	512
060	5	00.5	10	1	008	032	004	032	064	128	064	032	032	016	128	0128	256
061	5	00.5	10	1	032	002	004	128	256	064	128	064	256	023	064	2048	512
062	5	00.5	10	1	008	002	004	128	032	128	512	032	032	023	256	1024	512
063	5	00.5	10	1	032	002	004	128	064	128	512	064	016	064	128	8192	512
064	5	00.5	10	1	064	002	004	256	032	128	128	064	064	016	128	8192	512
065	5	00.5	10	1	319	032	016	199	216	256	512	032	256	032	128	2282	512
066	5	00.5	10	1	002	004	004	128	016	256	512	032	064	023	128	1024	512
067	5	00.5	10	1	256	032	016	004	064	128	512	064	256	004	128	1024	512
068	5	00.5	10	1	512	002	004	032	256	128	128	064	512	004	128	0256	512
069	5	00.5	10	1	256	002	004	128	256	128	128	064	032	004	128	2048	512
070	5	00.5	10	1	319	004	004	128	064	256	166	064	032	032	256	2282	512
071	5	00.5	10	1	064	005	024	199	216	194	166	032	264	023	106	1024	371
072	5	00.5	10	1	002	005	004	199	004	194	166	033	256	023	064	2282	371
073	5	00.5	10	1	319	002	064	016	216	194	166	008	256	023	106	2282	064
074	4	03.0	10	1	032	004	256	032	016	064	128	032	128	016	128	2048	512
075	4	03.0	10	1	128	004	004	064	016	032	512	016	032	004	128	1024	512
076	4	03.0	10	1	032	002	004	064	032	128	512	008	032	002	128	1810	512
077	4	03.0	10	1	032	002	004	032	064	128	512	016	032	002	128	1024	256
078	4	03.0	10	1	032	002	004	032	064	128	512	016	032	004	128	2048	256
079	4	03.0	10	1	032	011	004	064	032	032	512	016	032	004	128	2048	128
080	4	03.0	10	1	008	011	004	064	064	064	032	008	128	002	128	2048	385
081	4	03.0	10	1	008	064	004	032	177	128	032	016	128	004	093	1810	512
082	4	03.0	10	1	008	016	004	064	177	128	512	016	128	004	128	1810	512
083	4	03.0	10	1	008	002	004	064	016	128	512	016	128	004	128	1810	512
084	4	03.0	10	1	002	011	032	083	177	004	338	017	256	013	128	1810	385
085	4	03.0	10	1	077	011	032	083	177	090	004	017	256	013	128	1810	385
086	4	03.0	10	1	002	002	256	016	512	008	004	008	512	002	128	4096	385
087	4	03.0	10	1	002	002	256	032	512	008	008	008	512	002	128	1810	385
088	4	03.0	10	1	002	002	256	016	512	090	004	008	512	002	064	4096	385
089	4	03.0	10	1	002	064	004	032	177	128	512	016	128	008	128	1810	512
090	4	03.0	10	1	077	011	032	083	177	090	338	017	256	002	093	1810	385
091	4	03.0	10	1	032	002	256	004	256	256	002	008	256	002	008	1810	256
092	4	03.0	10	1	512	004	004	032	177	128	512	032	256	016	128	1810	512
093	4	03.0	10	1	512	008	004	032	128	064	512	032	256	032	128	1810	256
094	4	03.0	10	1	512	008	004	064	064	008	512	032	256	016	008	0512	256
095	4	03.0	10	1	512	011	004	064	016	064	004	048	008	064	128	0512	512
096	4	03.0	10	1	512	008	004	128	128	128	016	048	512	032	128	4096	385
097	4	03.0	10	1	032	008	004	032	004	016	004	048	256	032	128	1810	512
098	4	03.0	10	1	002	032	032	032	177	128	338	032	128	032	128	1810	256
099	4	03.0	10	1	128	002	004	032	177	016	032	032	256	064	128	1024	512
100	4	03.0	10	1	512	002	004	032	064	016	032	032	256	064	128	1024	512
101	2	03.0	10	1	032	002	004	256	016	256	512	128	128	128	256	2048	512
102	2	03.0	10	1	032	002	004	256	032	256	512	064	128	128	256	8192	512
103	2	03.0	10	1	098	002	004	128	032	256	512	016	256	016	128	1024	128
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432 2 00.5 10 1   512 002 128 032 064 128 512   008 064 004 032 0512 160
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525 4 00.5 10 1   512 004 032 016 128 064 256   048 064 004 128 2048 512
526 4 00.5 10 1   128 008 008 064 128 128 128   008 008 064 128 4096 512
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532 5 05.5 10 1   512 004 128 512 128 128 512   048 032 032 128 8192 512
533 5 05.5 10 1   135 007 028 148 128 085 157   008 055 030 066 1879 348
534 5 05.5 10 1   002 016 256 148 240 085 157   008 064 032 066 1879 128
535 5 05.5 10 1   002 007 028 148 240 032 002   025 016 002 066 1879 348
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548 5 05.5 10 1   064 004 004 128 128 256 032   016 032 064 128 4096 512
549 5 05.5 10 1   064 002 016 064 512 064 032   016 128 004 032 0256 512
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551 5 03.0 10 1   256 008 008 256 128 128 512   032 008 008 032 4096 160
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755	4	13.0	10	2	008	002	091	032	032	064	002	008	008	002	016	0128	160
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762	4	13.0	10	2	008	002	091	064	032	128	111	008	256	002	064	0128	128
763	4	13.0	10	2	008	004	032	256	032	128	512	008	032	016	064	1024	192
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765	4	13.0	10	2	015	003	091	072	197	045	111	016	163	007	054	2752	064
766	4	13.0	10	2	128	008	128	256	032	064	512	017	128	016	128	1024	256
767	4	13.0	10	2	015	003	004	072	004	045	111	008	163	007	054	2752	143
768	4	13.0	10	2	008	008	004	064	032	256	512	016	008	016	064	8192	192
769	4	13.0	10	2	002	002	128	016	064	008	002	008	008	002	048	0512	128
770	4	13.0	10	2	015	002	091	016	128	064	002	017	163	016	032	8192	128
771	4	13.0	10	2	008	002	256	032	256	032	008	016	512	004	016	8192	064
772	4	13.0	10	2	008	002	256	256	512	064	512	016	512	032	128	8192	256
773	4	13.0	10	2	008	004	256	256	512	064	064	016	128	004	128	1024	256
774	4	13.0	10	2	015	002	004	016	064	004	111	008	128	004	008	2752	160
775	4	13.0	10	2	002	002	016	016	256	064	002	017	256	002	128	0128	128
776	4	13.0	10	2	015	003	091	072	197	045	111	017	008	007	054	2752	143
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842	1	15.5	10	1	032	002	016	032	512	256	002	008	008	002	016	2048	288
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*854	9	99.9	99	9	299	016	088	512	256	064	002	008	016	002	058	0128	256
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*856	9	99.9	99	9	299	026	088	004	263	102	125	012	166	002	058	1616	299
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*858	9	99.9	99	9	512	026	004	107	263	032	125	008	016	004	058	1616	299
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*862	9	99.9	99	9	299	026	088	107	263	102	125	012	166	004	058	1616	299
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*865	9	99.9	99	9	512	004	016	004	004	064	256	008	256	004	008	2048	256
*866	9	99.9	99	9	299	008	016	004	128	102	008	008	128	004	008	2048	512
*867	9	99.9	99	9	299	026	088	004	263	102	125	012	166	004	058	1616	299
*868	9	99.9	99	9	299	026	008	004	512	102	125	008	166	004	008	1616	299
*869	9	99.9	99	9	064	128	004	107	256	102	008	008	016	002	008	1616	299
*870	9	99.9	99	9	064	002	004	004	032	102	002	048	064	004	032	1024	512

* = Reference cultures.

9, 99, 99.9 = Codes for the computer.

SECTION 2

Annual survey of microorganisms in intertidal sediments and seawater at Ardmore, Clyde Estuary

SECTION 2

INTRODUCTION

Marine bacteria

Bacteria living in the sea are different from those in fresh water and bacteria of the rivers are different from those in lakes (Rheinheimer, 1985). Most of the aquatic bacteria are heterotrophic (i.e. they live on organic substances). Morphologically, the majority of aquatic bacteria have their equivalent amongst the basic types of terrestrial bacteria (i.e. rods, cocci, commas or spirals).

The majority of marine bacteria are Gram-negative. Moriarty and Hayward (1982) studied an Australian coast sediment and found that 90% of Gram-negative bacteria in the aerobic surface zone (0-1cm) and 70% in the underlying anaerobic zone (20-21cm). Most marine bacteria are motile. ZoBell (1946a) found that 75-85% of pure cultures examined for this feature possess flagella. The author also studied the growth of bacteria and found that marine bacteria generally grow more slowly than soil bacteria. For example, the maximum number of visible colonies of marine bacteria incubated at optimal temperature was reached after 14-18 days, while soil bacteria was only after 2-7 days. Most of aquatic bacteria are facultative anarobes, but they prefer the presence of oxygen (Rheinheimer, 1985).

There seems to be a large proportion of proteolytic (protein-decomposing) bacteria in marine habitats. ZoBell (1938; 1946a) carried out a study on the bacterial flora of marine sediments and found that a large number of bacteria were proteolytic as indicated by their ability to liquefy gelatin and to release ammonia or hydrogen sulphide from proteinaceous substances. On the other hand, saccharolytic (sugar-decomposing) bacteria play an important role, but it is rather smaller in the sea than in the other habitats. Other organisms

decompose materials of high molecular weight like cellulose, agar, chitin or hydrocarbons and phenols. ZoBell (1938) found that lipoclastic bacteria which liberate the fatty acids from various lipids and utilize the glycerol are widely distributed in bottom deposits. He suggested that may they play an important role in the genesis of petroleum. The process of denitrification (reduction of nitrate via nitrite to free nitrogen) in the sea is carried out by the denitrifying bacteria. Sulphate reduction also is a process following nitrate reduction in sediment. This process is carried out by the genus Desulphovibrio. Few bacteria able to produce methane (Toerien and Hattingh, 1969) as the result of stepwise degradation of simple organic compounds such as ethanol to H_2 and acetic acid followed by the reduction of CO_2. The process of methane production is carried out by methanogenic bacteria and become in significant quantities only after the sulphate reduction process is complete.

Bacteria may live in the water free or attached to some solid substances. Therefore, the microbiology of intertidal sediments has been given considerable attention and several investigators have studied the attachment of microorganisms onto the surface of sand grains (Anderson and Meadows, 1965; 1969; 1978; Anderson et al. 1981; Dale, 1974; Deans et al. 1982; Fukami et al. 1983; Goulder, 1977; Meadows, 1965; Meadows and Anderson, 1966; 1968).

Bacteria and fungi in the sea play a very important function in the food cycle by synthesising cell substances and converting waste or dissolved organic matter into a particular form which can be used as food for the fauna of the sea bed (Rheinheimer, 1985; Wood, 1965; ZoBell, 1938; 1946). The importance of microorganisms in feeding most of the marine animals such as invertebrates has been reported in extensive literature (Christian and Welzel, 1978; Gray, 1966; Meadows and Williams, 1963; Meadows, 1964a; Meadows and Campbell, 1972;

Wilson, 1955). Some animals in the sea may live almost entirely on bacteria and fungi which contain high protein value. For example, sponges take up bacteria and digest them. In addition, bacteria and fungi affect the formation of sediments. For example, colonization by microorganisms destroy partly if not completely the suspended particles if they are used for food. The growth of microorganisms however may modify the marine sediment properties. Webb (1969) showed that the presence of bacterial films change the size, shape and adhesion of particles as well as their geometrical arrangement. Fungi are also able to hold together a number of particles and unite them using rhizoids or hyphae (Rheinheimer, 1985).

Yeasts

Yeasts have served humansfor many centuries by fermenting fruit juices, bread and many other food. The important of the yeasts today has become greater than in earlier eras because of the development of fermentation processes and also for synthesising certain vitamins, fats and proteins from simple sugars. Other species however, cause diseases for many plants and animals. Kreger-van Rij (1973) defined yeasts as "those fungi which in a stage of their life cycle , occur as single cells, reproducing by budding or fission". Kohlmeyer and Kohlmeyer (1979) isolated 177 species from seawater, sediments, plants animals and organic matter in the marine habitats. They classified their species into obligate and facultative groups. The authors defined obligate marine yeasts as "those yeasts that thus far, have never been collected anywhere but in the marine environment. Whereas facultative marine yeasts are also known from terrestrial habitats".

During the past twenty years, many investigators have studied the occurrence and distribution of yeasts in marine environments

(Ahearn et al. 1968; Buck, 1975; Combs et al. 1971; Roth et al. 1962; Taysi and Van Uden, 1964; Van Uden and Castelo-Granco, 1963). Ahearn et al. (1968) studied the ecology of 1000 yeasts from aquatic regions of South Florida. They found the highest yeast densities in freshwater. The recovery of yeasts per sample decreased from 100% to approximately 70% with lowered organic content, increasing salinity and distance from land. Taysi and Van Uden (1964) also found the numbers of yeasts and yeast species decreased with increasing distance from estuaries. Roth et al. (1962) investigated the ecology of yeasts obtained from various marine substrates. They found that most of the yeasts present in all the environments they studied, were oxidative and asporogenous forms which require one or two vitamins for their growth. Roth and his co-workers also found the number of yeast flora on marine vegetation was low and consistent with the species obtained from surrounding waters and sediments.

Fungi

Generally, living organisms have been divided into two Kingdoms: the plant Kingdom and the animal Kingdom. However, biologists found that it might be more important to recognise at least four major divisions: the animal Kingdom, the Kingdom of green plants, the fungal Kingdom and the Kingdom of bacteria (Ingold, 1961). The fungal Kingdom which I am concerned with here is a large one, forming 50,000 to 100,000 known species. It includes the moulds which grow on dumped organic materials.

Fungi differ fundamentally from green plants in their lack of chlorophyll (i.e. they are unable to photosynthesise their own organic food from carbon dioxide and water). According to Moor-Landecker (1982) fungi are heterotrophic organisms which must consume organic matter. They live either as saprophytes (i.e. digest and consume dead

organic matter as plants or animals) or as parasites (i.e. assimilate tissues of living plants and animals).

Fungi are divided into two divisions; marine and terrestrial species. The ecological definition of marine fungi is as follows. Obligate marine fungi are those that grow and sporulate exclusively in marine or estuarine habitats. Facultative marine fungi are those from fresh water or terrestrial milieux able to grow (and possibly also to sporulate) in marine environments (Kohlmeyer and Kohlmeyer, 1979). The marine fungi occur as parasites on plants and animals in all marine environments. Although the distribution of fungi is mainly limited by dissolved oxygen and the temperature of the water, organic substances such as algae, marsh plants and plant litter accumulated along the shores also play an important role in providing nutrients for fungi.

Microorganism counts

There are various techniques available for the enumeration of microorganisms in aquatic environments. These techniques include direct counts of stained cells (e.g. epifluorescence and immunofluorescence), indirect counts by dilution and growth of bacteria on solid media (e.g. plate counts and most probable number determinations), and other chemical methods. These latter can include measuring a unique bacterial component, such as lipopolysaccharide (LPS) or muramic acid in a mixed population (Costerton and Geesey, 1979). The method used in this study was the plate count technique. This technique has been used to estimate the viable counts of microorganisms. Several advantages and disadvantages were listed by Buck (1979). This author described the advantages of plate counts as convenient, simple, economic, a good source of viable organisms and a semiquantitative method for both horizontal and vertical environmental profiles. The disadvantages of this technique are that it gives viable

counts only and that it may select particular physiological types of bacteria. Other problems that can occur with plate counts are clumping and aggregation, and the effects of dilution pipetting and counting.

Clyde Estuary

The Clyde sea area can be divided into three parts, the Firth, the sea Lochs and the Estuary. The hydrography, geology and biology of the Clyde Estuary have been studied by several investigators such as Collar (1974), Deegan (1974) and Smyth (1974) respectively. The estuary of the River Clyde comprises two distinct parts; an upper shallow drowned estuary, and the lower Firth of Clyde. This represents a total area of over 2,500 Km^2 contained in a series of deep glaciated basins separated by shallow sills (Anon, 1974). Generally it can be described as partially or well mixed in terms of water circulation. As well as the River Clyde, three major tributaries discharge freshwater to the upper estuary (collar, 1974). The River Kelvin enters at 3.2 Km, the Carts at 10.4 Km and the Leven at 22.2 Km below Glasgow Bridge.

In comparison with other estuaries in the United Kingdom, the Clyde has a relatively small tidal range. The admiralty tide tables quote mean ranges at the entrance to the dredged channel (mouth of the estuary) of 3.08m Springs and 1.89m Neaps, rising to 4.11m Springs and 2.40m Neaps at Glasgow Bridge.

Input of sediment to the estuary occur from many sources. 87.5% of the annual inflow of sediment is riverborne, 6.25% comes as dredged spillage, and another 6.25% from solids discharged at sewage works (collar, 1974).

Pollution in the Clyde Estuary

The Clyde estuary receives many pollutants at various places along its length. Most of these discharges are organic materials, heavy metals and some other substances. These discharges of pollutants have three main sources; (1) domestic sewage and industrial effluent from urban places carried by the River Clyde and its tributaries; (2) dumping of sludge and sewage off Garroch Head; and (3) the enrichment of the Irvine Bay area through the discharge from industrial plants (Heath, 1974). In addition to these sources power stations also release some radioactive materials into the estuary.

The upper estuary receives an average input of 0.41×10^6 m^3/day of treated sewage and 0.59×10^6 m^3/day of untreated and partially treated sewage (Anon. 1974). These flows may be compared with a daily input of 22.3×10^6 m^3 of freshwater, of which 9.2×10^6 m^3 is discharged via the Clyde, Cart, Kelvin and Leven. The lower estuary receives untreated and partially treated sewage from a population of 150,000 people estimated at 0.045×10^6 m^3/day. During periods of low flow in the summer, the estuary can become totally deoxygenated for a distance of over 20 Km downstream of the tidal weir (Glasgow city centre). Reports conducted by the Clyde River Purification Board reported other problems of pollution in the Clyde area (Collar, 1974).

Distribution of marine and estuarine microorganisms in the Clyde sea area

Environmental variables have a dominant effect on the qualitative and quantitative abundance of marine microorganisms in estuaries and the intertidal zone. Physical and chemical factors such as tides, concentrations of suspended solids, pollution, sunlight, and fluctuations in salinity and temperature, may influence vertical and seasonal variations in the population density of microorganisms in the

open seas on coasts, and in estuaries (ZoBell, 1946; Brock, 1966; Campbell, 1982; Rheinheimer, 1985).

Many investigators have studied seasonal variations of microorganisms and factors related to these differences (Lloyd, 1930; Meadows and Anderson, 1966; 1968; Stevenson et al., 1973; Dale, 1974; Ezura et al., 1974; Anderson and Meadows, 1978; Bent and Goulder, 1981). Bent and Goulder (1981) studied seasonal variation in the population density and activity of heterotrophic bacteria. They found that density and activity of attached bacteria showed seasonal variations according to the concentration of suspended solids (low in summer and high from autumn to spring). However, the same authors state that free bacteria showed no seasonal variation and were not dependent on suspended solids. During an investigation of the factors related to the distribution of bacteria in intertidal sediments, Dale (1974) studied the bacterial numbers in intertidal sediments and the factors related to their distribution. He found that numbers of bacteria ranged from 1.17×10^8 to 9.97×10^9 g^{-1} dry sediment. He also found that there was a strong relationship (high correlation) between bacterial numbers and particular sediment properties such as, grain size, organic carbon content and total nitrogen. Hagler and Mendonca-Hagler (1981) studied quantitatively and qualitatively the yeast populations of marine and estuarine sites around Rio de Janeiro (Brazil). They found that the mean total yeast counts were 2880 C.F.U./100ml for very polluted sites, 202 C.F.U./100ml for moderately polluted sites, and 3 C.F.U./100ml for lightly polluted and unpolluted sites. The authors concluded that the total yeast counts were positively correlated with increased pollution levels. For example, Candida krusei and other phenotypically similar yeasts were prevalent as a group in polluted estuarine water but rare in unpolluted seawater. Hagler and his co-worker found that the most frequently

isolated genera in polluted estuary water were Candida, Rhodotorula, Torulopsis, Hanseniaspora, Debaryomyces and Trichosporon.

A number of microbiological studies have been carried out in the Clyde sea area. Most of these studies have been done by the former Royal technical College, University of Strathclyde, by the University of Glasgow, and by the Millport marine station (Ellis, 1925; 1926; 1929; Lloyd, 1929; 1930; Ellis, 1932; Ross and Morris, 1965; Meadows and Anderson, 1966; 1968; Morris, 1968; Anderson and Meadows, 1969; Lloyd and Morris, 1971; Lloyd et al., 1971; Anderson and Meadows, 1978; Anderson et al., 1981).

Lloyd (1930) studied vertical, seasonal and diurnal variations in bacterial populations in water samples obtained from Loch Striven, Loch Long, and Greenock. Water samples were also taken from Cumbrae Deep, a place used for the dumping of Glasgow sewage (activated sludge). She concluded that bacterial numbers were greater in surface waters and decreased with increasing depths. Lloyd also found that Loch Long water contained more bacteria than Loch Striven, and that there was little pollution in both lochs. However, due to the presence of pollution in the sewage sludge at Cumbrae Deep and in the river off Greenock, bacterial numbers were higher at these two sites than in the lochs. Lloyd (1931) examined marine mud samples from Loch Striven Head, Clapochlar, Carroch-Corrie and Kames Bay in the Clyde sea area. She found that bacterial numbers decreased with increasing sediment depth, and that these numbers fluctuated near the surface of the mud but were more constant in the deeper layers of mud. The predominant microorganisms found were water bacteria of the Achromobacter and Chromobacterium types, and large spore forming bacilli similar to common soil bacteria. Factors affecting the bacterial content of muds were also discussed by this author.

Anderson and Meadows have studied microorganisms in Clyde sediments in a series of papers. Meadows and Anderson (1968) studied bacteria on sand grains from littoral (i.e. intertidal) and sublittoral environments. They found microbial colonies in hollows and on flat surfaces of sand grains, but in general, microorganisms did not colonize surfaces subject to abrasion. Anderson and Meadows (1969) continued this work by studing the number of bacteria attached to the surfaces of sand grains from intertidal beaches between Wemyss Bay and Troon on the Ayrshire coast (Scotland). They found that total and viable numbers of bacteria on the surfaces of sand grains showed high variation, but viable counts varied more. Estimated counts varied from 2 to 241 x 10^3 g^{-1} dry sand. The authors also suggested that tides, rain water and run-off from the land will decrease the number of bacteria on the surfaces of intertidal sand grains. Anderson and Meadows (1978) reported that shallow water and intertidal marine sediments are heterogeneous and consist of several microenvironments. Sediment properties such as Eh, total carbon, organic carbon, total nitrogen, heterotrophic bacterial content and chlorophyll content can change notably over short distances in the sediment. Bioturbation structures such as animal burrows were shown to affect these properties. Meadows and Anderson (1966) found that a wide range of microorganisms occur on marine and fresh water samples in conjunction with organic material. In general in marine sediments, microbial numbers are higher at mid-tide level and at low-tide level than at high-tide level. These workers correlated their results with an increase in the surface area of sand grains down the shore. They also found a similar pattern with an increase in the surface area of fresh water sand grains.

Ross and Morris (1965) investigated the yeast flora of marine fish in the Clyde Estuary. They isolated a total of 189 yeasts from 16

fish species. The isolates from marine fish were found to comprise six genera, Debaryomyces, Torulopsis, Candida, Rhodotorula, Pichia and Cryptococcus, and 17 species within these genera. They also found that D. kloeckeri was the most abundant species in all geographical sampling sites, whereas the occurrence and proportion of isolates of other species varied in each area. The highest numbers of bacteria and fungi are almost always found in the top few centimetres of the sediments and mostly at the sediment surface. In the uppermost zone (0-1cm) of mud sediments of the middle Kiel Bay, total numbers of bacteria found were 46.7 - 77.7 x 10^9 g^{-1} dry sediment (Rheinheimer 1985).

The objectives of the work reported in this section were to estimate the seasonal viable counts of heterotrophic bacteria, yeasts and fungi in overlying, interstitial waters and sediments (vertical profiles) at Ardmore point in the Clyde Estuary.

SECTION 2

MATERIALS AND METHODS

Sampling site and sampling collection

Samples were collected from intertidal sediments (vertical profiles), interstitial and overlying water at Ardmore Point in the Clyde Estuary (National grid reference 55° 58' 23" N , 04° 41' 7"W). Ardmore Point is situated approximately four miles East of Helensburgh on the North bank of the Clyde Estuary. It is shown in figure 1 and plates 1 and 2.

Samples were taken monthly from February 1984 to February 1985 in order to study the seasonal abundance of heterotrophic bacteria, fungi and yeasts. Interstitial water samples were taken after digging a hole in the sediment and overlying water was collected from about one metre water depth using sterile plastic universal containers. Undisturbed sediment samples were obtained using the technique of coring. The core was pushed into the sediment to the required depth and then removed after digging away the surrounding sediment to free it. The core was then split at the site and exterior surfaces of its sub-fractions were removed using a sterile spatula (in order to reduce the possibility of extraneous contamination). Sediment was sampled from 0, 5, 10, 20 and 35cm and transferred into sterile universal containers. Duplicate samples were kept overnight at 4°C and subsequently processed.

Enumeration of microorganisms

Heterotrophic bacteria

The enumeration of heterotrophic bacteria present in a specific quantity of sediment and water was carried out using the following dilution technique and surface plate counts on solid agar media.

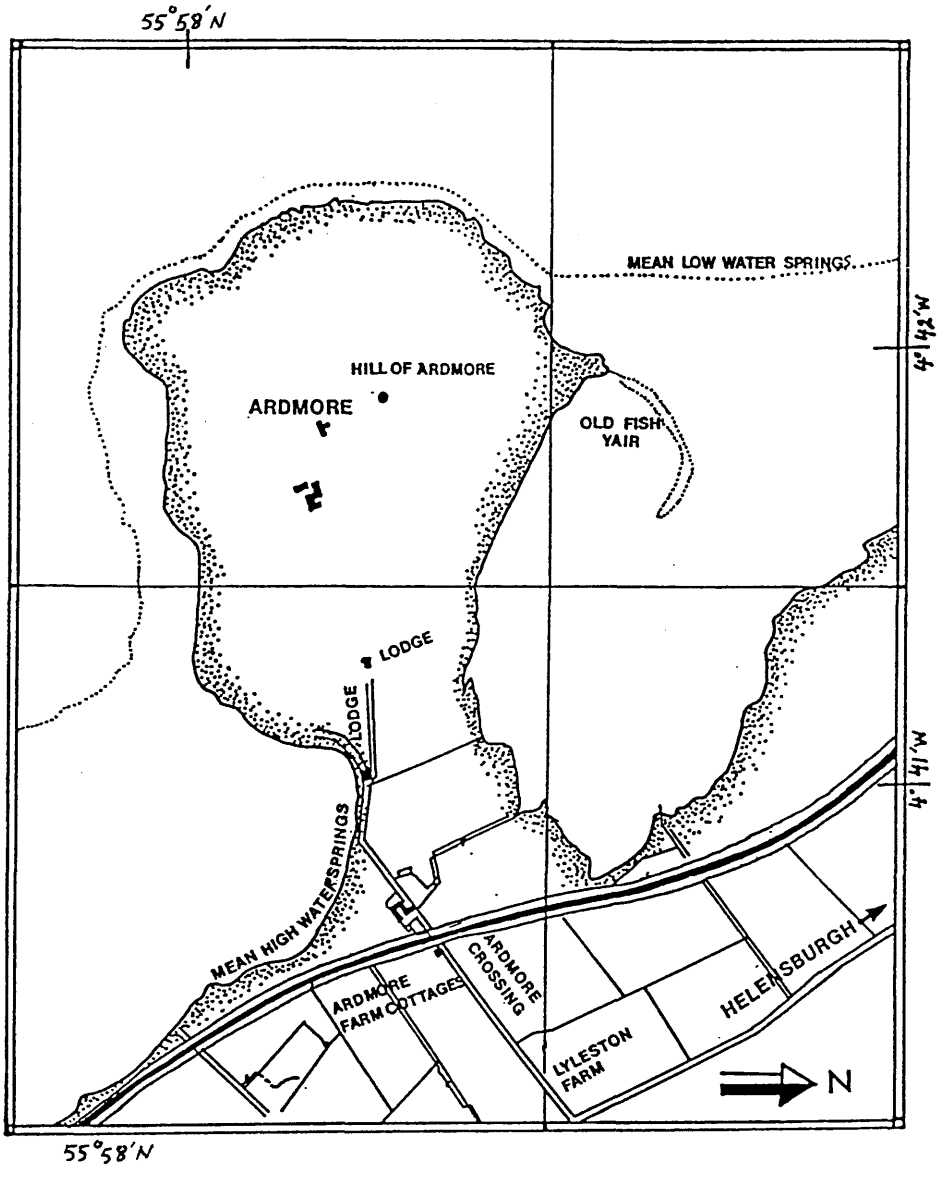


Figure 1

Diagram showing the location of Ardmore Point (Clyde Estuary).

Plates 1 and 2:

Study area at Ardmore Point in the Clyde Estuary.

Plate 1

Plate 2

A 1g sample of sediment or 1 ml sample of water was diluted with 9 ml of sterile 75% seawater in a sterile universal container. Sediment samples were mixed for 10 seconds using a vortex mixer. Shaking served both to break up clumps of bacteria in the sediment and to separate bacteria attached to sediment particles. Serial dilutions to a concentration of 10^{-3} were made from the first container. Surface plate spreading using 0.1 ml aliquots of each dilution were made on culture plates of ZoBell marine agar medium. Plates were previously dried at 37°C for 60 minutes to allow better adsorption of the 0.1 ml aliquot and to prevent confluent growth of bacteria. Replicate plates were made for each dilution and plates incubated at 10°C. Colony forming units on plates were recorded after 3, 5, 7, 10, 15, 20 and 25 days incubation.

Numbers of bacteria were calculated per gram wet weight sediment and converted to per gram dry weight using the water content values obtained separately for the same sediment samples. For water samples, numbers of bacteria were expressed as colony forming units/ml (C.F.U.). A computer program "Bact" was developed to calculate the numbers of heterotrophic bacteria per gram dry weight sediment or per ml water (Appendix table 1). The flow diagram used to develop the program is shown in appendix figure 1. An example of the calculation performed by the computer is given in appendix table 2.

Yeasts and Fungi

Yeasts and fungal abundance were determined using the method described for bacterial counts, except that Sabouraud dextrose agar was used to culture fungi and Potato dextrose agar for yeasts. These two media were supplied by Oxoid Ltd. Their composition is as follows:

Sabouraud dextrose agar		Potato dextrose agar	
Mycological peptone	10g	Potato Extract	4g
Dextrose	40g	Dextrose	20g
Agar No. 1	15g	Agar No. 1	15g
pH 5.6 (approx.)		pH 5.6 (approx.)	

The computer program "Bact" developed to calculate the numbers of heterotrophic bacteria was also used to calculate the numbers of yeasts and fungal colony forming units per gram dry weight sediment or per ml water.

SECTION 2

RESULTS

BACTERIA

The mean and standard deviation of the bacterial colony forming units (C.F.U.) per gram dry weight sediment or per ml water calculated by the computer program "Bact" is given in table 1.

Figure 2 shows the number of bacteria in overlying and interstitial waters at Ardmore Point (Clyde Estuary) from February 1984 to February 1985. In general, interstitial water showed higher numbers of bacteria than overlying water. In overlying water, the number of bacteria was in the range 5.250-58.75 $x10^3$ ml^{-1}. while in interstitial water the range was 6.525-131.5 $x10^3$ ml^{-1}.

Overlying water showed a peak in bacterial numbers in February 1984 followed by a decrease in the number of bacteria until May after which fluctuation occurred. Interstitial water showed a marked number of peaks of high bacterial numbers early in March and between April-November 1984. This was followed by a decrease in bacterial numbers from November 1984 to February 1985.

The results in figure 3 show the variation in the number of bacteria at different sediment depths at Ardmore Point during the year from February 1984 to February 1985. In general, the number of bacteria was greatest at the surface sediment and tended to decrease with depth. The number of bacteria (g^{-1}dry sediment) at the depths 0, 5, 10, 20 and 35cm was in the range 13.48-325.9 $x10^3$, 4.984-21.23 $x10^3$, 2.449-28.52 x 10^3, 0.3722-7.882 x 10^3 and 0.4910-8.160 $x10^3$ respectively.

At the surface sediment, high levels of bacteria occurred in February 1984 followed by a sharp decrease to a very low level in March. This decrease did not occur at the other depths for the same

	Feb 1984	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1985	Feb
O.W.	58.75 ± 21.39	17.68 ± 2.990	11.68 ± 1.814	5.250 ± 0.9469	9.275 ± 1.595	9.300 ± 2.486	11.00 ± 3.559	6.775 ± 0.1477	11.10 ± 0.5477	13.88 ± 3.711	16.15 ± 2.661	11.70 ± 1.175	13.33 ± 2.185
I.W.	65.00 ± 36.67	75.50 ± 11.68	10.45 ± 1.237	95.25 ± 19.82	71.25 ± 13.50	79.00 ± 20.07	131.5 ± 19.60	63.00 ± 14.51	106.0 ± 16.25	37.75 ± 23.51	22.08 ± 5.537	8.850 ± 1.277	6.525 ± 1.443
S.S.	195.6 ± 34.56	26.91 ± 7.229	325.9 ± 61.22	18.29 ± 5.232	17.29 ± 1.540	24.10 ± 2.437	115.0 ± 23.84	13.48 ± 2.217	15.85 ± 3.435	88.47 ± 13.98	25.72 ± 1.417	129.7 ± 7.206	26.06 ± 2.013
5cm	21.23 ± 22.77	20.77 ± 9.674	9.491 ± 1.867	4.984 ± 1.762	7.898 ± 0.447	9.970 ± 4.466	16.12 ± 1.330	8.126 ± 2.652	5.835 ± 0.4637	11.14 ± 0.3577	7.659 ± 0.7345	8.136 ± 1.258	18.97 ± 1.954
10cm	2.449 ± 3.463	28.52 ± 5.063	7.485 ± 0.1739	3.383 ± 1.653	25.18 ± 14.39	7.696 ± 1.404	5.916 ± 3.191	4.827 ± 0.5250	3.725 ± 0.08925	3.640 ± 1.658	5.437 ± 1.048	6.040 ± 0.2643	3.214 ± 0.6993
20cm	3.621 ± 0.08695	7.882 ± 3.716	6.276 ± 5.120	0.4275 ± 0.08636	0.3722 ± 0.5263	7.260 ± 2.675	5.647 ± 1.042	0.9835 ± 0.6955	1.870 ± 0.8816	3.083 ± 0.5232	4.434 ± 0.9715	6.651 ± 0.9669	2.389 ± 1.299
35cm	0.4910 ± 0	2.802 ± 1.551	0.9741 ± 0.6888	2.461 ± 2.088	0.7885 ± 0.08578	5.316 ± 2.679	8.160 ± 1.722	0.8704 ± 0.1759	0.5012 ± 0.1772	0.5451 ± 0.08566	1.604 ± 0.1745	2.223 ± 1.347	0.6231 ± 0.1762

TABLE 1 : Bacterial colony forming units ($x10^3$) per ml water or per gram dry weight of sediment (mean ± standard deviation) for the annual survey at Ardmore Point (Clyde estuary).
O.W. and I.W. are overlying and interstitial waters respectively. S.S. is surface sediment. 5,10,20 and 35cm are vertical sediment depths.

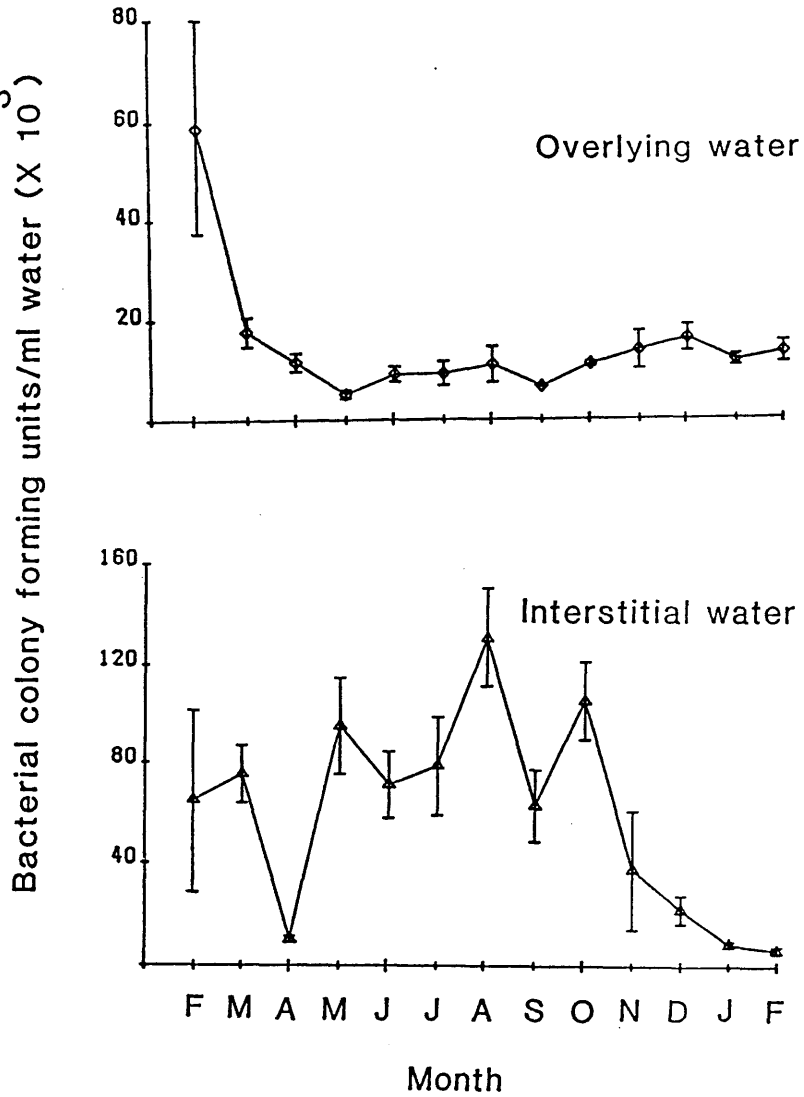
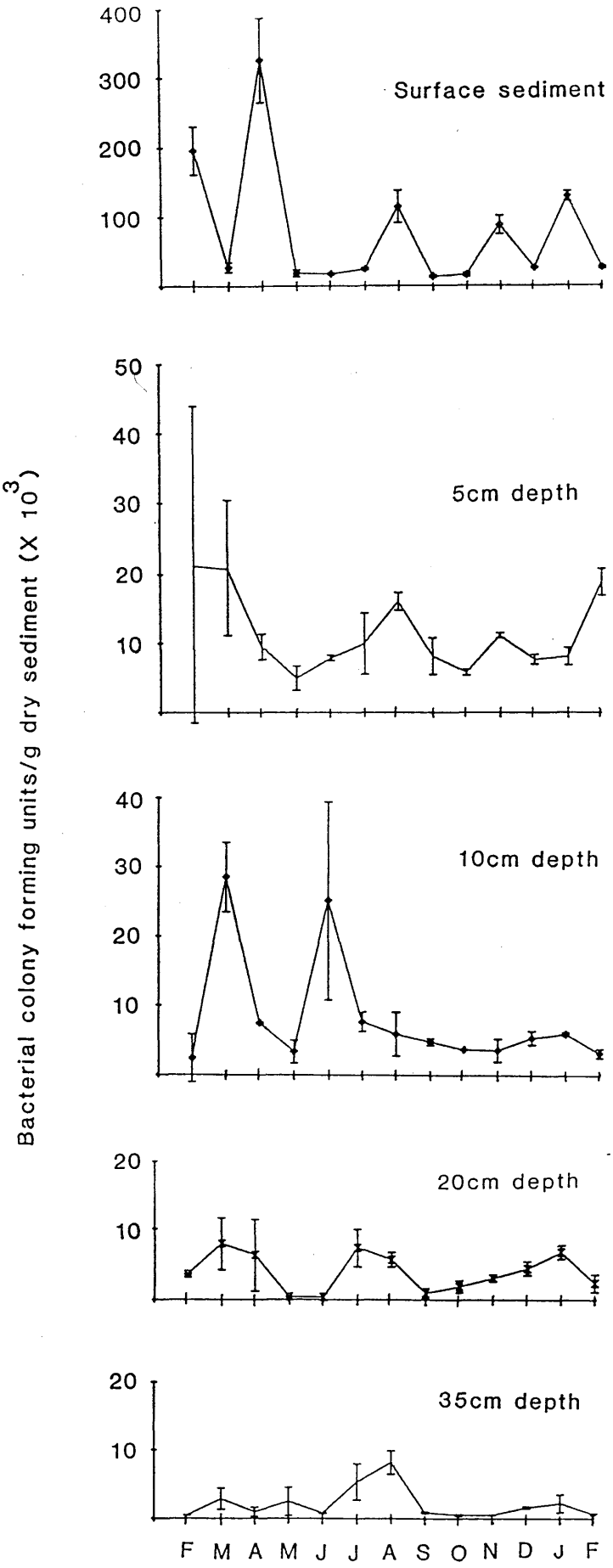


Figure 2

Bacterial colony forming units /ml of overlying and interstitial waters from February 1984 to February 1985 at Ardmore Point (Clyde Estuary).

Figure 3

Bacterial colony forming units/g dry weight of sediment at different depths (0, 5, 10, 20 and 35cm) from February 1984 to February 1985 at Ardmore Point (Clyde Estuary).


Surface sediment
5cm depth
10cm depth
20cm depth
35cm depth
Bacterial colony forming units/g dry sediment (X 10^3)
Month
F M A M J J A S O N D J F

period. Separate peaks also occurred in April, August, and November 1984, and in January 1985.

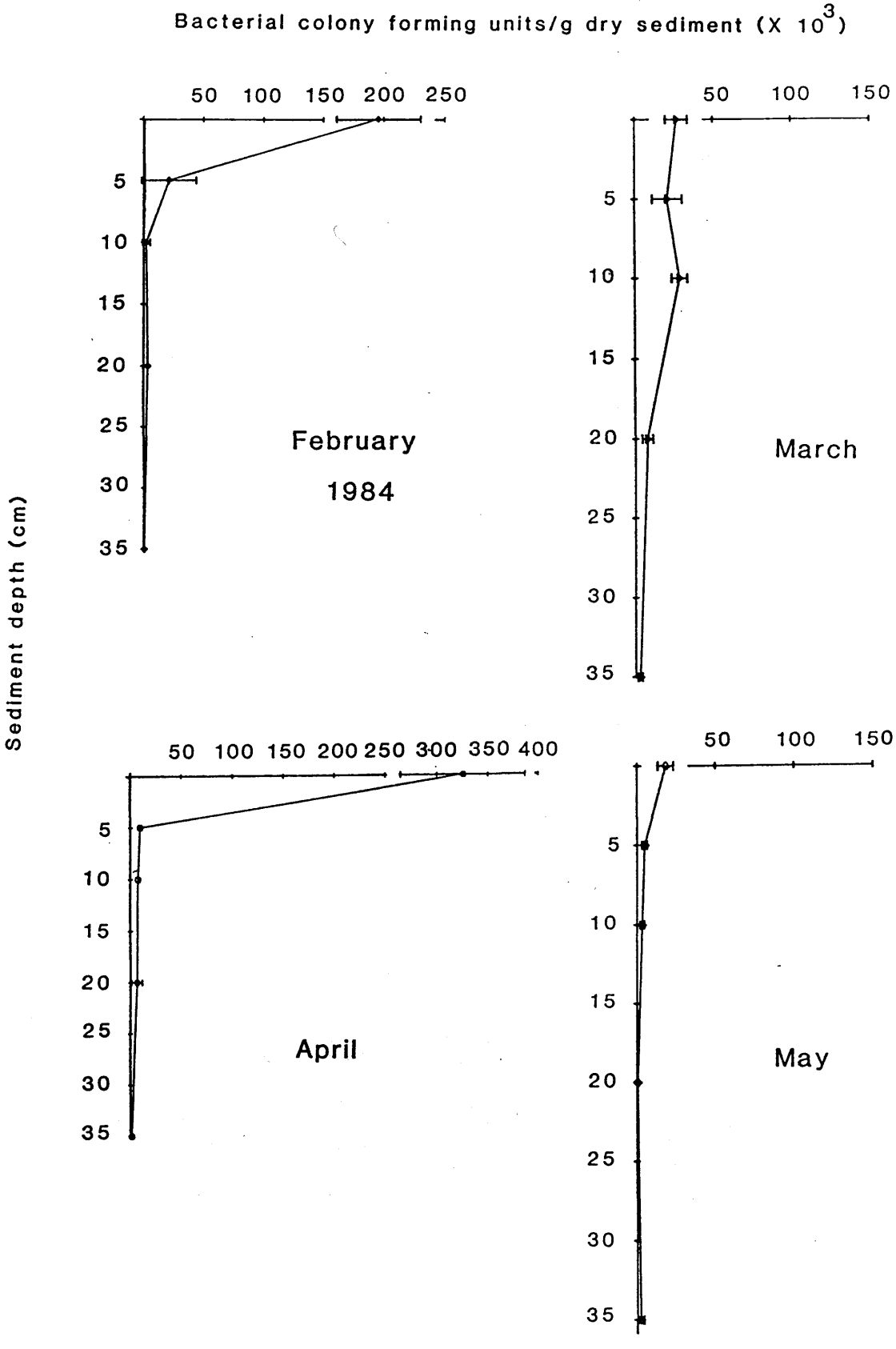
At 5 and 10cm depth, similar ranges of bacterial numbers occurred although peaks were found in different months at the two depths. At 5cm depth, three major peaks occurred in February-March, and August 1984 and in January 1985. A minor peak also occurred in November 1985. At 10cm depth, only two major peaks were found, in March and June 1984. At 20 and 35cm depth, much lower ranges of bacterial numbers were found than at 5 and 10cm depth. At 20cm depth, three minor peaks occurred in March-April 1984, July-August 1984, and in January 1985. In general, at 35cm depth, only one peak occurred, and this was in August 1984.

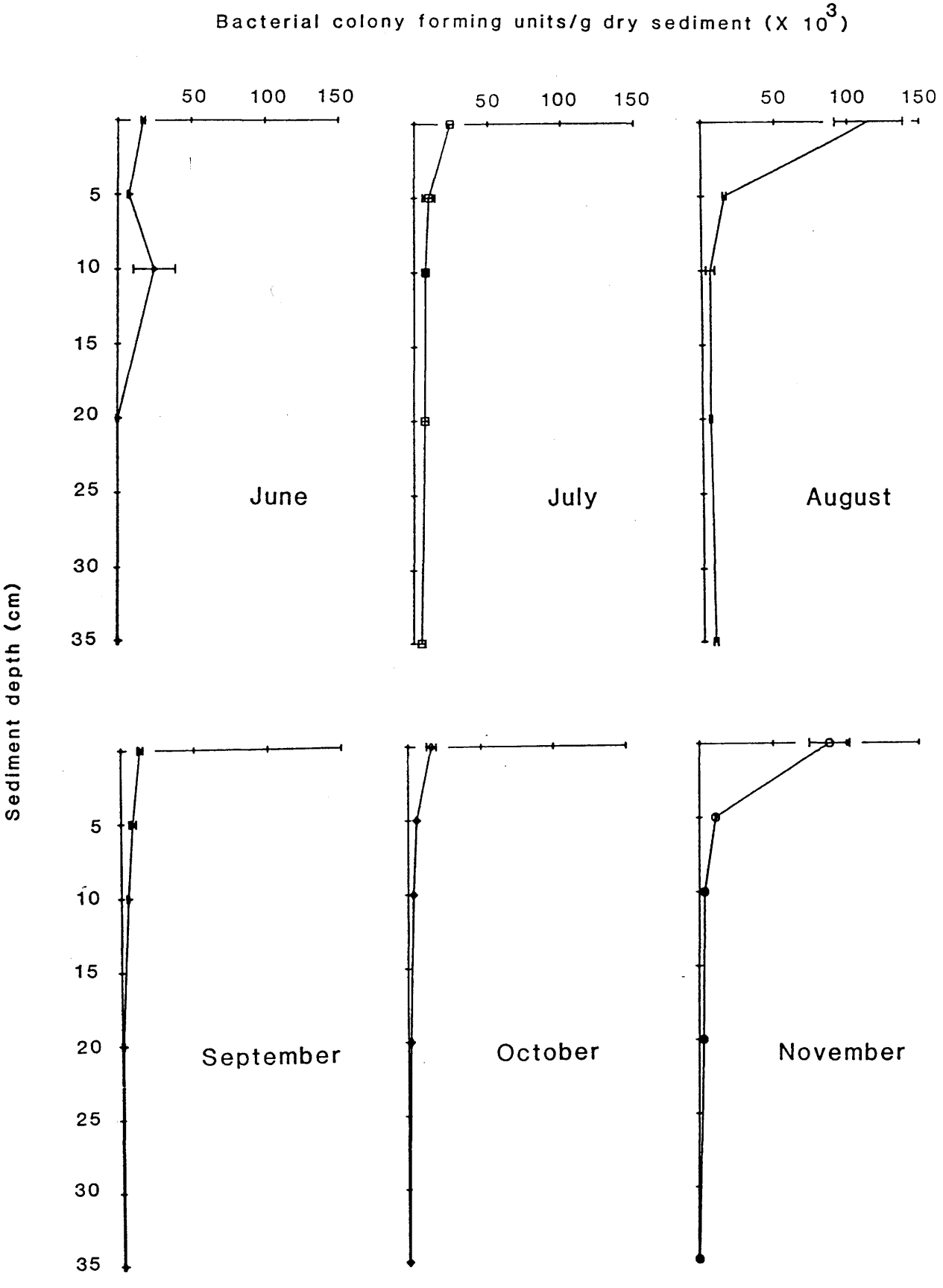
The variation in the number of bacterial colony forming units with depth for each month from February 1984 to February 1985 is shown in figure 4. In general, the pattern of decreasing numbers of bacteria with depths was similar for all months with the exception of the surface sediment which showed high levels of bacteria in February, April, August, November 1984 and in January 1985. These levels fell dramatically to 5cm depth. In March and June, there was a slight increase in the number of bacteria at the 10cm depth.

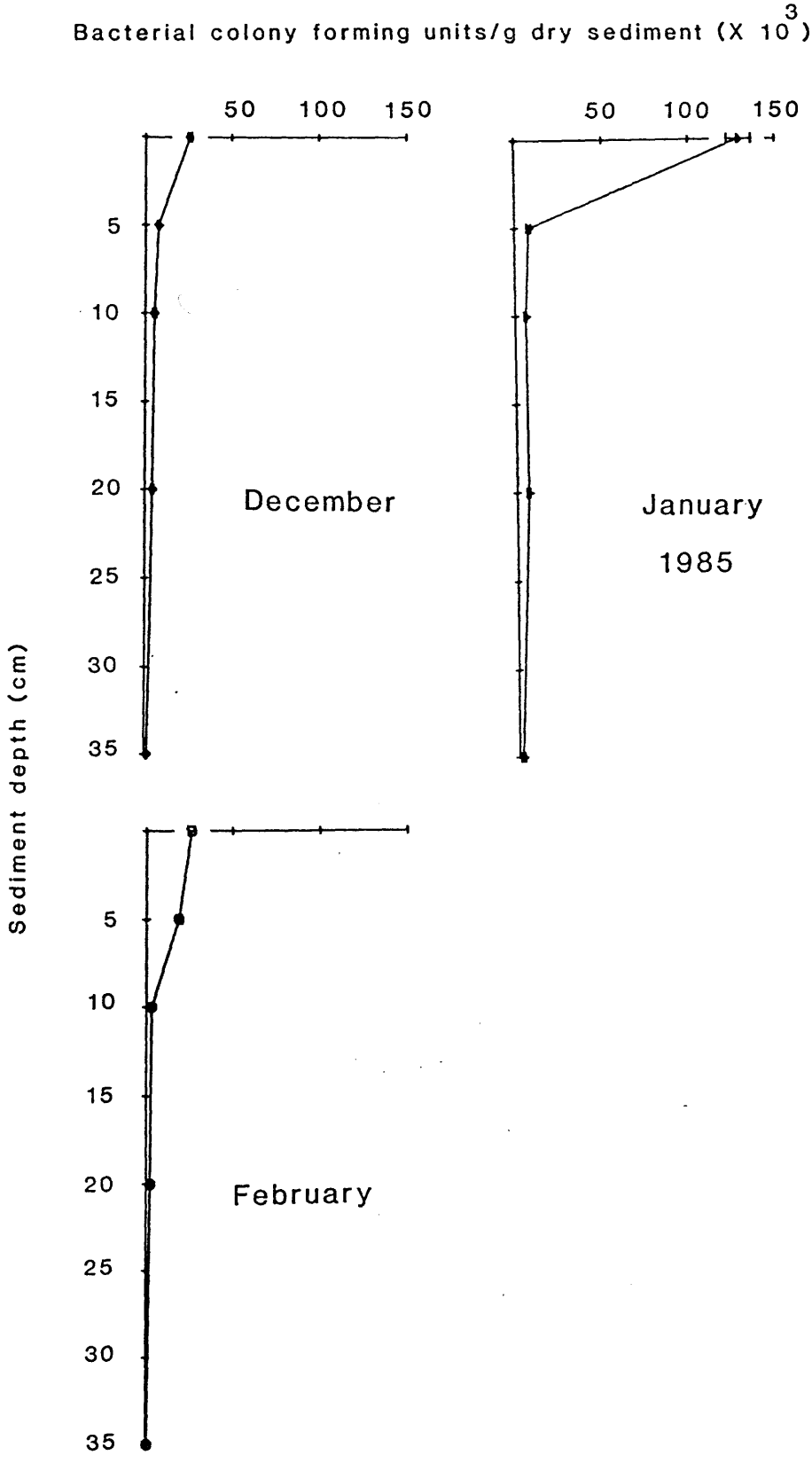
The bacterial numbers at each sampling depth were compared using all data collected over the thirteen months. This was done using regression analyses and student's t-tests. Figures 5 and 6 and table 2 show the results of these comparisons. In figure 5, the bacterial numbers found at the sediment surface over the thirteen months were plotted against the bacterial numbers found at the other depths over the same period. Figure 6 shows the same plots but comparing all the other depths. A direct relationship in bacterial numbers occurred in all cases with only a few exceptions. In the comparisons surface-10cm and surface-35cm, an inverse relationship was found. No significant

Figure 4

Relationships between bacterial colony forming units/g dry weight of sediment and sediment depth from February 1984 to February 1985 at Ardmore Point (Clyde Esuary). Graphs are on 3 pages.


Bacterial colony forming units/g dry sediment (X 10^3)
Sediment depth (cm)
February 1984
March
April
May


Bacterial colony forming units/g dry sediment (X 10³)
Sediment depth (cm)
50
100
150
5
10
15
20
25
30
35
June
July
August
September
October
November


Bacterial colony forming units/g dry sediment (X 10³)
50
100
150
Sediment depth (cm)
5
10
15
20
25
30
35
December
January
1985
February

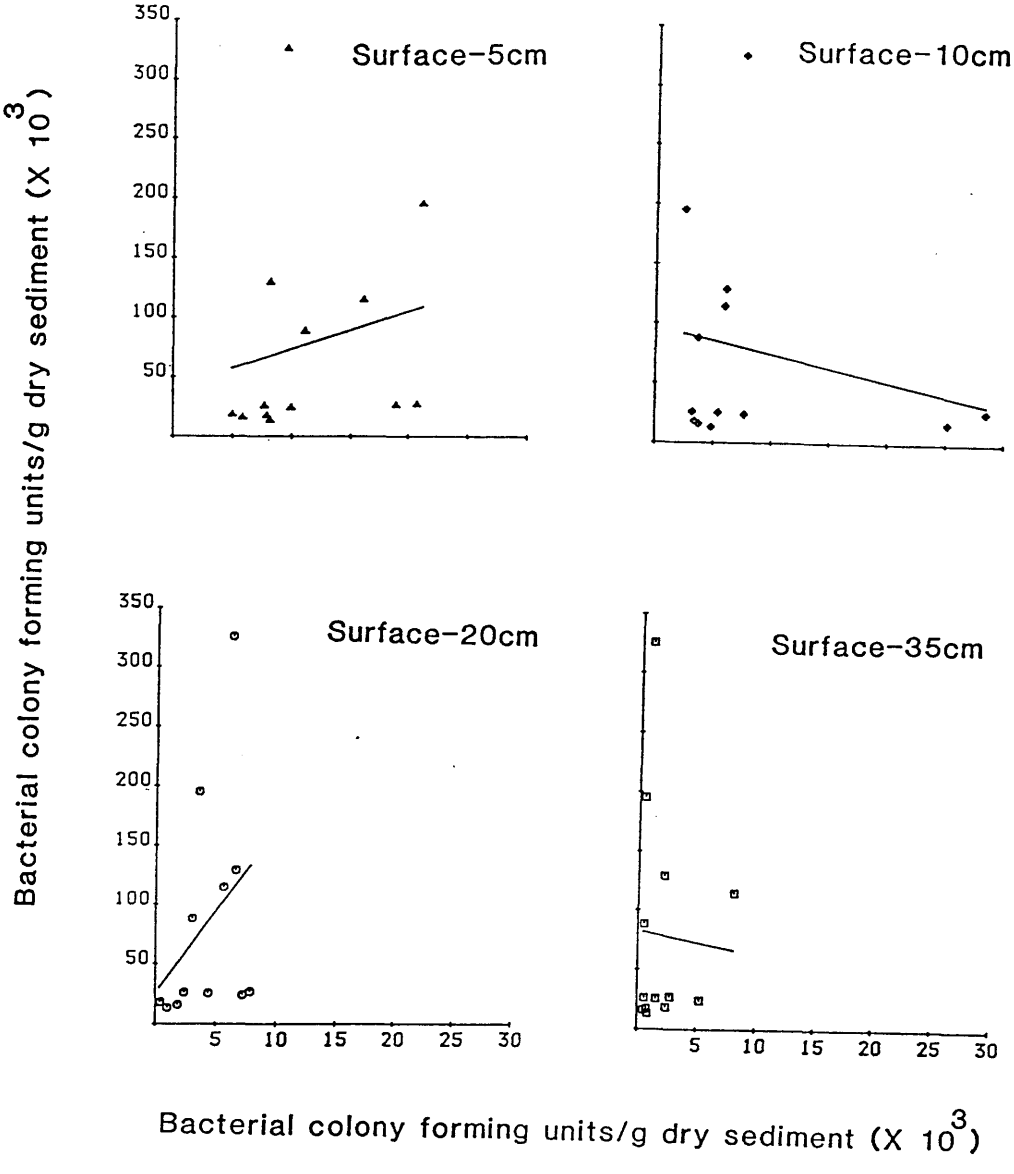


Figure 5

Relationship between bacterial colony forming units/g dry weight of surface sediment and colony forming units/g dry weight of sediment at 5, 10, 20 and 35cm depth. y-axis is surface sediment, x-axis is the other depths. Each point represents data for one month.

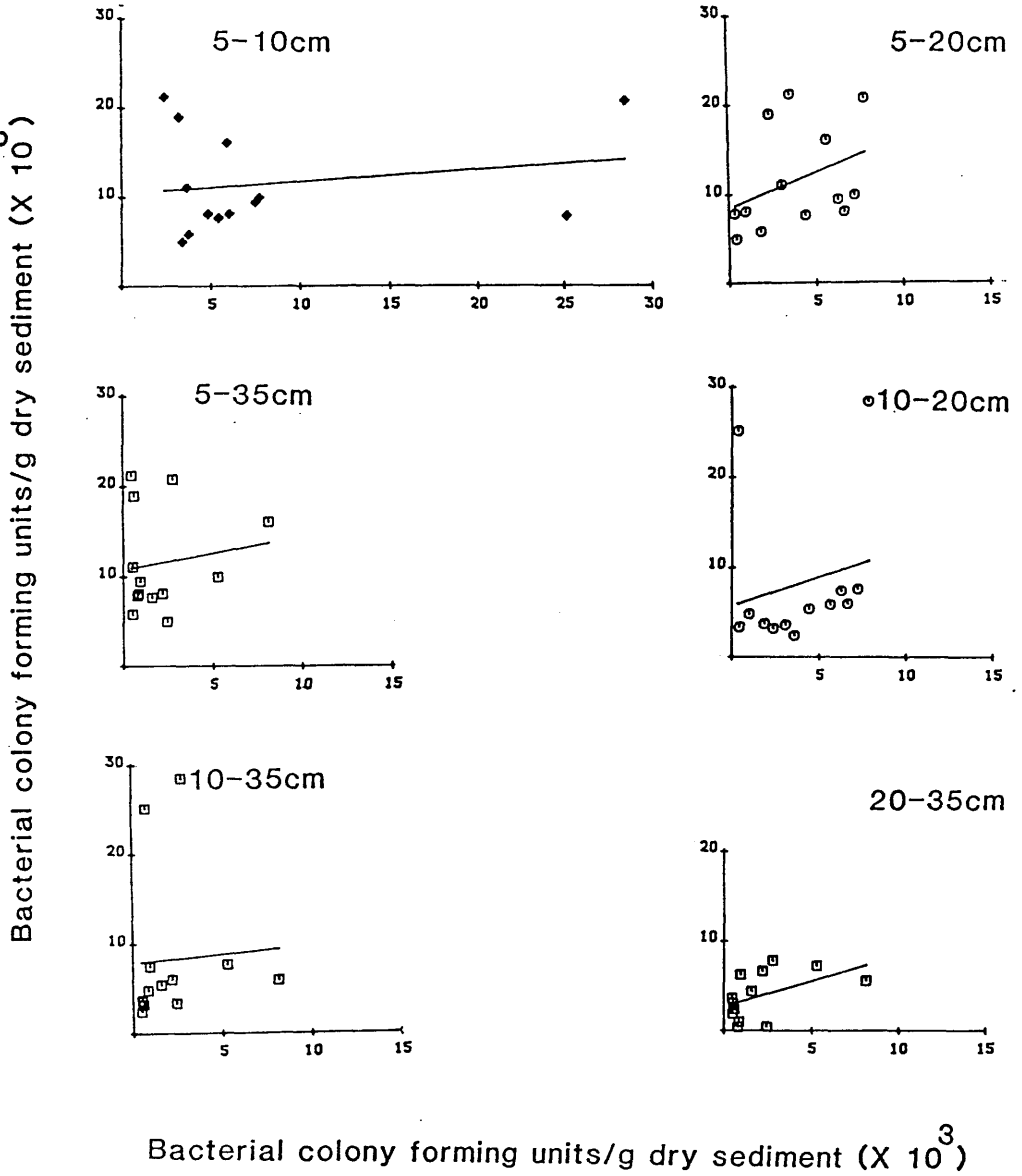


Figure 6

Relationship between bacterial colony forming units/g dry weight of sediment at each depth and the other depths. y-axis (5, 10, 20cm), x-axis (10, 20, 35cm). The figures at the top of each graph are the depths compared. For example, 5-10cm means 5cm data on y-axis and 10cm data on x-axis. Each point represents data for one month.

Data	Regression equation	t-test	d.f.	P
S.S-5cm	Y = 3.183x + 41.81	2.603	12	0.05>P>0.02
S.S-10cm	Y =-2.290x + 97.59	2.645	12	0.05>P>0.02
S.S-20cm	Y = 13.79x + 24.66	2.902	12	0.02>P>0.01
S.S-35cm	Y =-1.992x + 82.84	2.937	12	0.02>P>0.01
5-10cm	Y = 0.1328x + 10.47	1.291	12	0.30>P>0.20
5-20cm	Y = 0.7957x + 8.449	5.184	12	P<0.001
5-35cm	Y = 0.3663x + 10.79	5.865	12	P<0.001
10-20cm	Y = 0.6525x + 5.716	1.891	12	0.1>P>0.05
10-35cm	Y = 0.1883x + 7.874	2.579	12	0.05>P>0.02
20-35cm	Y = 0.5747x + 2.706	2.619	12	0.05>P>0.02

Table 2

Results of regression analyses and student's t-tests applied to data to compare numbers of bacteria present at the different sampling depths. S.S = surface sediment. The regression plots are shown in figures 5 and 6.

relationship was found in the 5-10cm comparison. The t-tests showed a significant difference in bacterial numbers between all depths with the exception 5-10cm, where no significant difference in bacterial numbers was shown ($0.30>P>0.20$).

Figure 7 shows the relationship between the numbers of bacteria (C.F.U.) in overlying and interstitial waters and incubation time (days) for the month of July 1984. Interstitial water contained much higher numbers of bacteria than overlying water (Table 3). It was also found that bacteria in overlying water reached a maximum growth after 20 days incubation, while in interstitial water, the maximum growth occurred after only 7 days. This suggested that prolonged incubation time was very important to ensure the maximum phase of bacterial growth.

Figure 8 shows the relationship between the numbers of bacteria (C.F.U.) in sediment from different depths, and incubation time (days). Surface sediment contained more bacteria than the other depths (twice to four times as much approximately). In general, there was a decrease in bacterial numbers with sediment depth at each incubation time (Table 3). In addition, at greater sediment depths there was a less pronounced increase in bacterial growth with increasing incubation time. However, the maximum growth was always reached approximately after 15 days incubation.

YEASTS

The mean and standard deviation of the yeast colony forming units (C.F.U.) per gram dry weight sediment or per ml water calculated by the computer program "Bact" is given in table 4.

Figure 9 shows the relationship between numbers of yeasts in overlying and interstitial water at Ardmore Point from February 1984 to February 1985. Interstitial water contained significantly higher

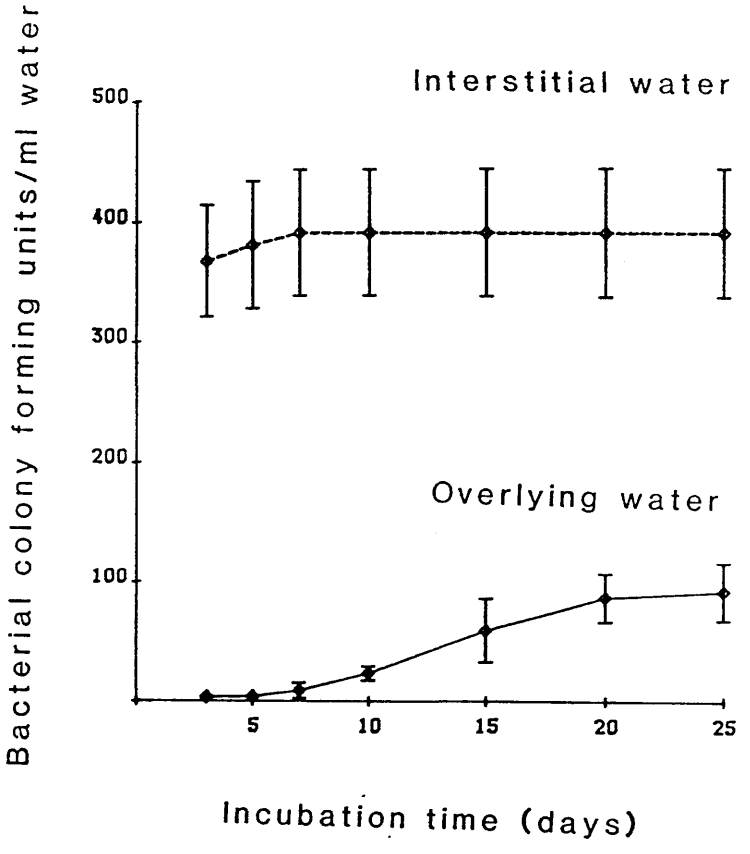


Figure 7

Relationship between bacterial colony forming units/ml of overlying and interstitial waters and the incubation time (days) for July 1984.

Table 3

The numbers of bacteria (mean ± s.d.) for July 1984 obtained from each sampling depth after incubation times of 3 to 25 days. This data is plotted in figures 7 and 8.

Sample	Incubation time (days)	No. bacteria (C.F.U.) (mean ± s.d.)
Overlying water	3	3.000 ± 0.8160
	5	3.750 ± 0.9570
	7	8.500 ± 6.856
	10	23.25 ± 6.397
	15	59.50 ± 27.26
	20	87.50 ± 20.50
	25	93.00 ± 24.86
Interstitial water	3	368.0 ± 47.46
	5	381.8 ± 53.93
	7	392.3 ± 53.45
	10	392.8 ± 53.45
	15	393.0 ± 53.81
	20	393.5 ± 54.09
	25	394.3 ± 54.30
Surface sediment (0cm depth)	3	107.3 ± 36.75
	5	172.4 ± 24.94
	7	184.8 ± 30.53
	10	202.0 ± 30.25
	15	212.2 ± 28.20
	20	217.8 ± 25.91
	25	224.0 ± 22.65

Table 3 cont'd.

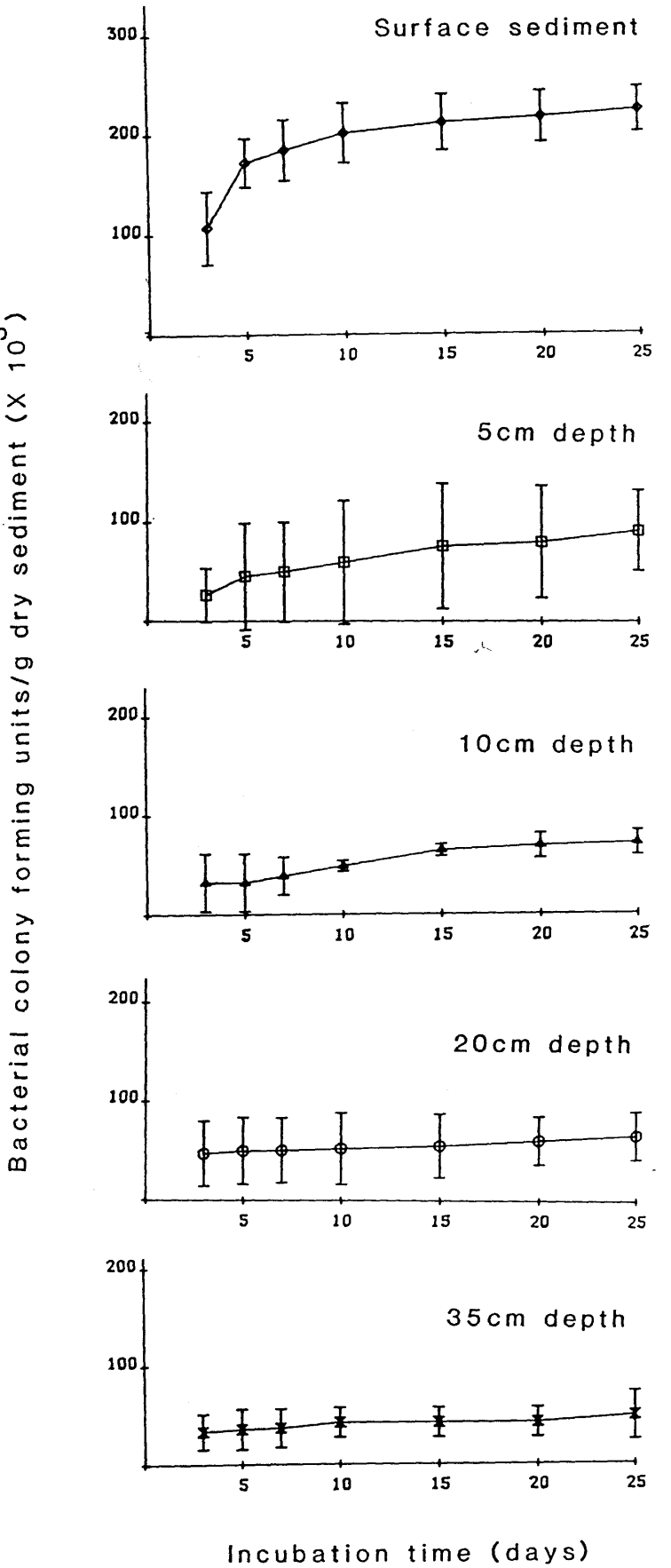
Sample	Incubation time (days)	No. bacteria (C.F.U.) (mean ± s.d.)
	3	26.26 ± 27.45
	5	45.09 ± 54.09
	7	49.66 ± 50.86
5cm depth	10	59.37 ± 62.96
	15	75.92 ± 63.77
	20	80.49 ± 57.31
	25	91.90 ± 41.17
	3	32.09 ± 29.18
	5	32.09 ± 29.18
	7	38.97 ± 19.45
10cm depth	10	48.71 ± 5.673
	15	64.19 ± 6.483
	20	68.77 ± 12.96
	25	71.06 ± 12.96
	3	47.26 ± 33.42
	5	50.07 ± 34.21
	7	50.63 ± 33.42
20cm depth	10	52.86 ± 36.60
	15	55.69 ± 32.62
	20	61.32 ± 24.66
	25	66.94 ± 24.66

Table 3 cont'd.

Sample	Incubation time (days)	No. bacteria (C.F.U.) (mean ± s.d.)
	3	33.16 ± 18.47
	5	35.97 ± 20.66
	7	36.53 ± 19.87
35cm depth	10	42.15 ± 15.11
	15	42.15 ± 15.11
	20	42.15 ± 15.11
	25	48.89 ± 24.64

Figure 8

Relationship between bacterial colony forming units/g dry weight of sediment (0, 5, 10, 20 and 35cm) and incubation time (days) for July 1984.


Surface sediment
5cm depth
10cm depth
20cm depth
35cm depth
300
200
100
5
10
15
20
25
Bacterial colony forming units/g dry sediment (X 10^3)
Incubation time (days)

	Feb 1984	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1985	Feb
O.W.	3.250 ± 0.2121	0.7000 ± 0.5657	3.250 ± 3.182	0.1500 ± 0.07071	0.3500 ± 0.2121	0 ± 0	1.550 ± 0.3535	0.4500 ± 0.3536	0.4000 ± 0.1414	1.200 ± 0.2828	1.750 ± 0.3536	4.350 ± 0.2122	1.950 ± 0.364
I.W.	9.800 ± 4.384	7.150 ± 3.606	2.500 ± 0.9899	57.00 ± 7.072	18.75 ± 2.051	17.10 ± 4.384	119.0 ± 4.247	54.50 ± 13.43	84.50 ± 6.364	8.250 ± 0.06986	11.15 ± 1.202	3.450 ± 0.9192	2.200 ± 0.8485
S.S.	18.24 ± 9.022	19.84 ± 1.032	18.19 ± 2.858	15.30 ± 5.852	6.836 ± 0.5687	10.98 ± 7.953	87.59 ± 21.29	19.92 ± 0.03200	12.79 ± 3.655	46.74 ± 5.666	10.24 ± 4.952	25.14 ± 7.110	12.76 ± 2.090
5cm	0.5774 ± 0.09072	2.157 ± 1.482	0.3143 ± 0.2667	0.1869 ± 0.08811	1.516 ± 0.8936	3.344 ± 4.029	1.599 ± 1.862	2.313 ± 2.210	3.147 ± 2.596	1.899 ± 0	2.467 ± 1.285	2.288 ± 0.7192	10.49 ± 0.4440
10cm	4.019 ± 2.842	3.086 ± 0.5238	1.104 ± 0.6941	0 0	17.19 ± 3.684	3.352 ± 4.564	2.745 ± 1.984	1.423 ± 0.9628	0.6944 ± 0.08928	0.5553 ± 0.2618	0.8649 ± 0.5242	2.304 ± 1.145	1.360 ± 0.1748
20cm	2.700 ± 2.778	2.383 ± 1.987	0.4827 ± 0.5120	0 0	0.1861 ± 0.08772	9.090 ± 6.125	7.488 ± 6.771	0.8606 ± 0.3477	1.309 ± 0.4408	1.233 ± 0.1744	1.311 ± 0.7948	1.865 ± 0.7032	1.103 ± 1.213
35cm	0.6138 ± 0.3472	0.3046 ± 0.08615	0.1826 ± 0.08610	0 0	0.1820 ± 0.2573	5.805 ± 0.9505	1.096 ± 0.1722	1.181 ± 0.08792	0.6265 ± 0.7088	0.3028 ± 0.2570	0.3701 ± 0	2.350 ± 2.066	0.2493 ± 0.1762

TABLE 4 : Yeast colony forming units ($x10^3$) per ml water or per gram dry weight of sediment (mean ± standard deviation) for the annual survey at Ardmore Point (Clyde estuary).
O.W. and I.W. are overlying and interstitial waters respectively. S.S. is surface sediment. 5,10,20 and 35cm are vertical sediment depths.

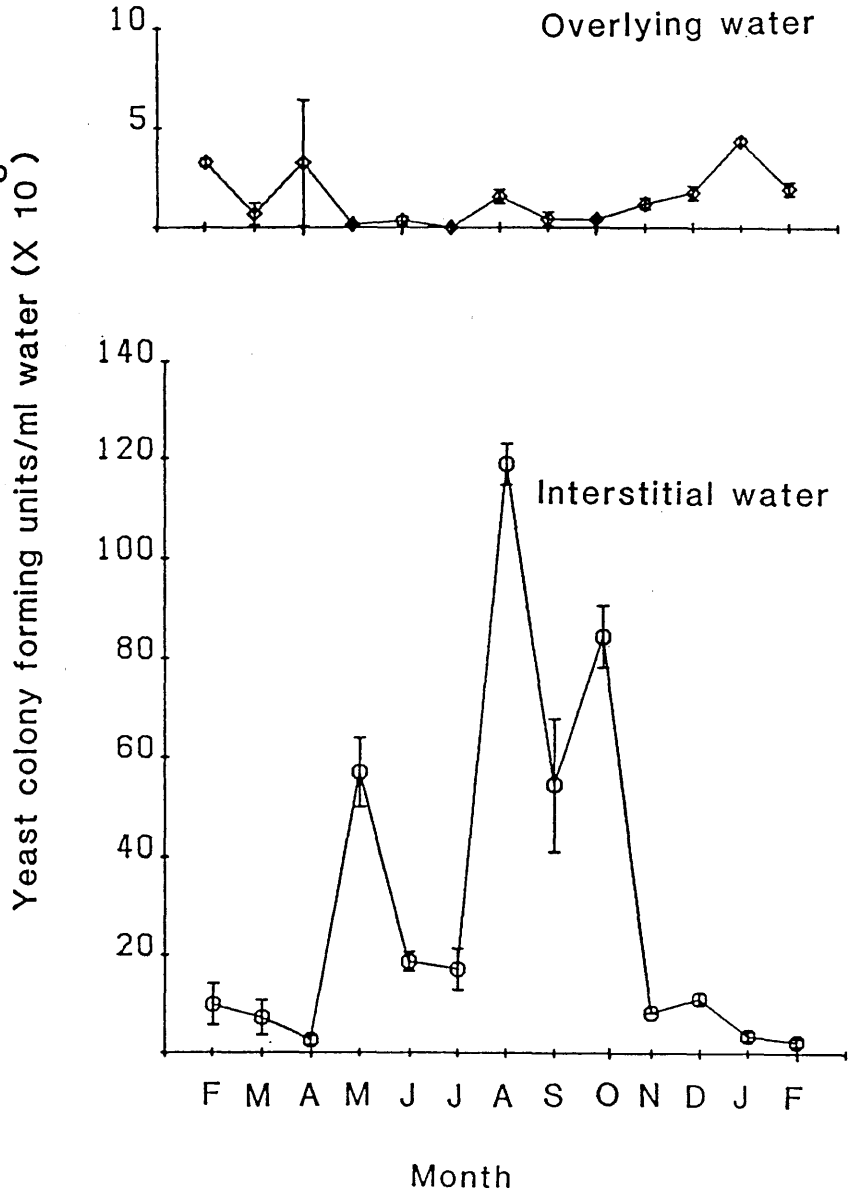


Figure 9

Yeast colony forming units/ml of overlying and interstitial waters from February 1984 to February 1985 at Ardmore Point (Clyde Estuary)

numbers of yeasts than overlying water. The number of yeasts in overlying water was in the range 0-4.350 $x10^3$ ml^{-1} while interstitial water showed a range of 2.200-119.0 $x10^3$ ml^{-1}.

Overlying water showed 4 peaks in the numbers of yeasts. These occurred in February, April, and August 1984 and in January 1985.

Interstitial water showed 3 peaks. These occurred in May, August and October 1984.

Figure 10 shows the variation in the numbers of yeasts in sediment at different depths during the year. The numbers of yeasts were greatest at the surface sediment and in general tended to decrease with depth although some peaks were found at different depths. The numbers of yeasts (g^{-1}dry sediment) at the depths 0, 5, 10, 20 and 35cm were in the range 6.836-46.74 $x10^3$, 0.1869-10.49 $x10^3$, 0-17.99 $x10^3$, 0-9.090 $x10^3$ and 0-5.805 $x10^3$ respectively.

Maximum yeast numbers (peaks) occurred at different months of the year at different sediment depths. In the surface sediment, maximum numbers were found in November with lesser peaks in March, and September 1984 and in January 1985. At 5cm depth, maximum numbers occurred in February 1985. At 10cm depth, maximum numbers were found in June 1984. At 20 and 35cm depth, similar curves were found although peaks occurred at slightly different times of the year (August at 20cm depth, and July at 35cm depth).

The variation in the numbers of yeasts with depth for each month from February 1984 to February 1985 is shown in figure 11. In general, the pattern of decreasing numbers of yeasts with depths was similar for all months with the exception of February, March, June, July and August which showed peaks of maximum numbers of yeasts at 10cm and 20cm depth.

Figure 10

Yeast colony forming units/g dry weight of sediment at differnt depths (0, 5, 10, 20 and 35cm) from February 1984 to February 1985 at Ardmore Point (Clyde Estuary).

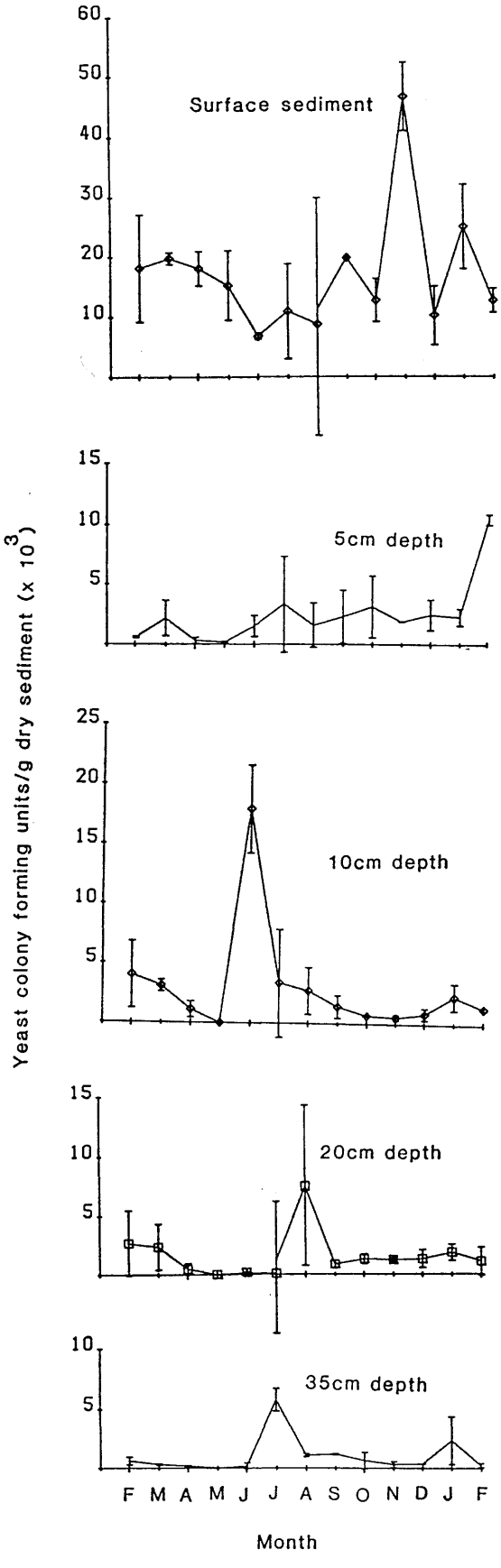
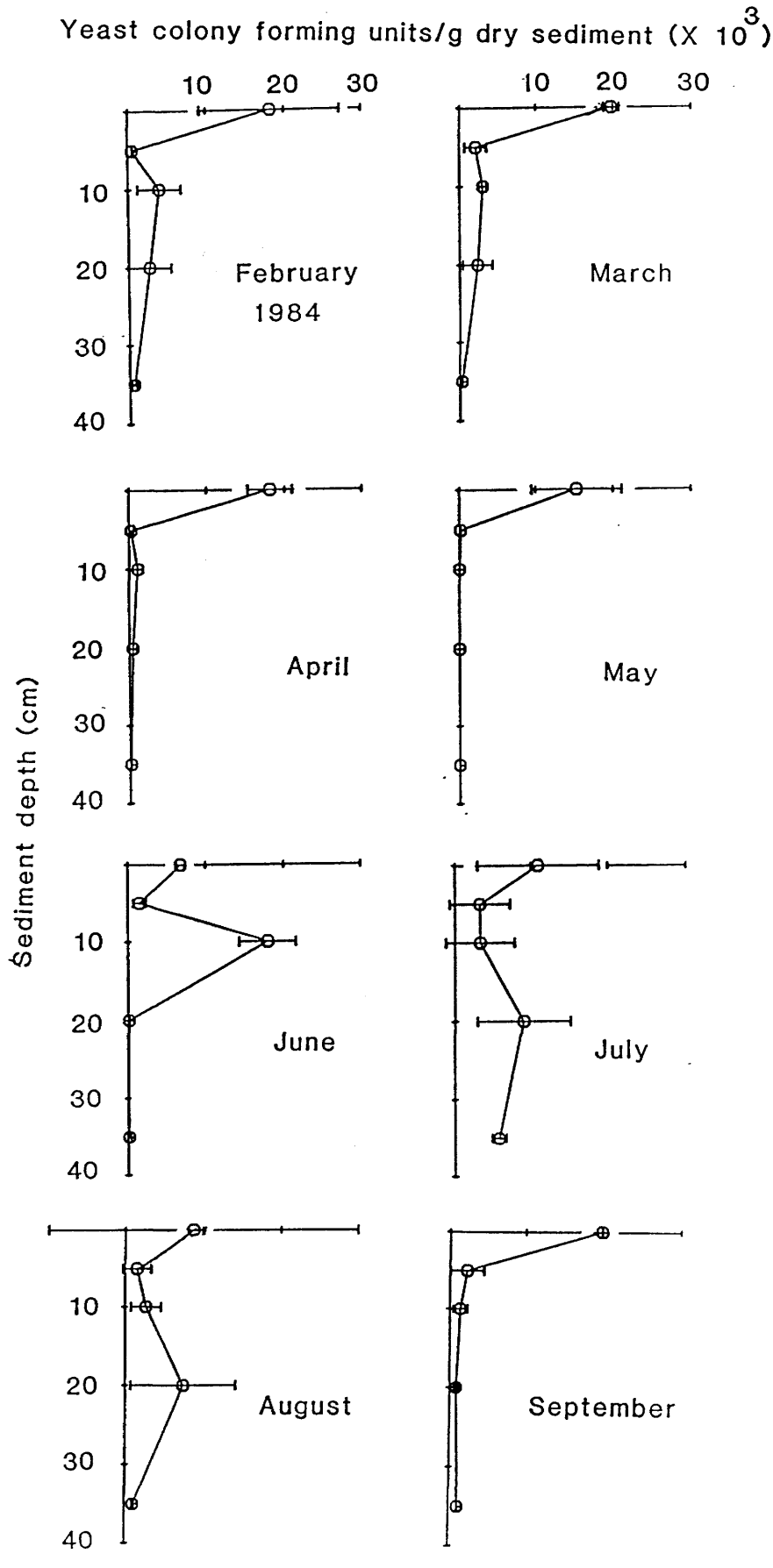
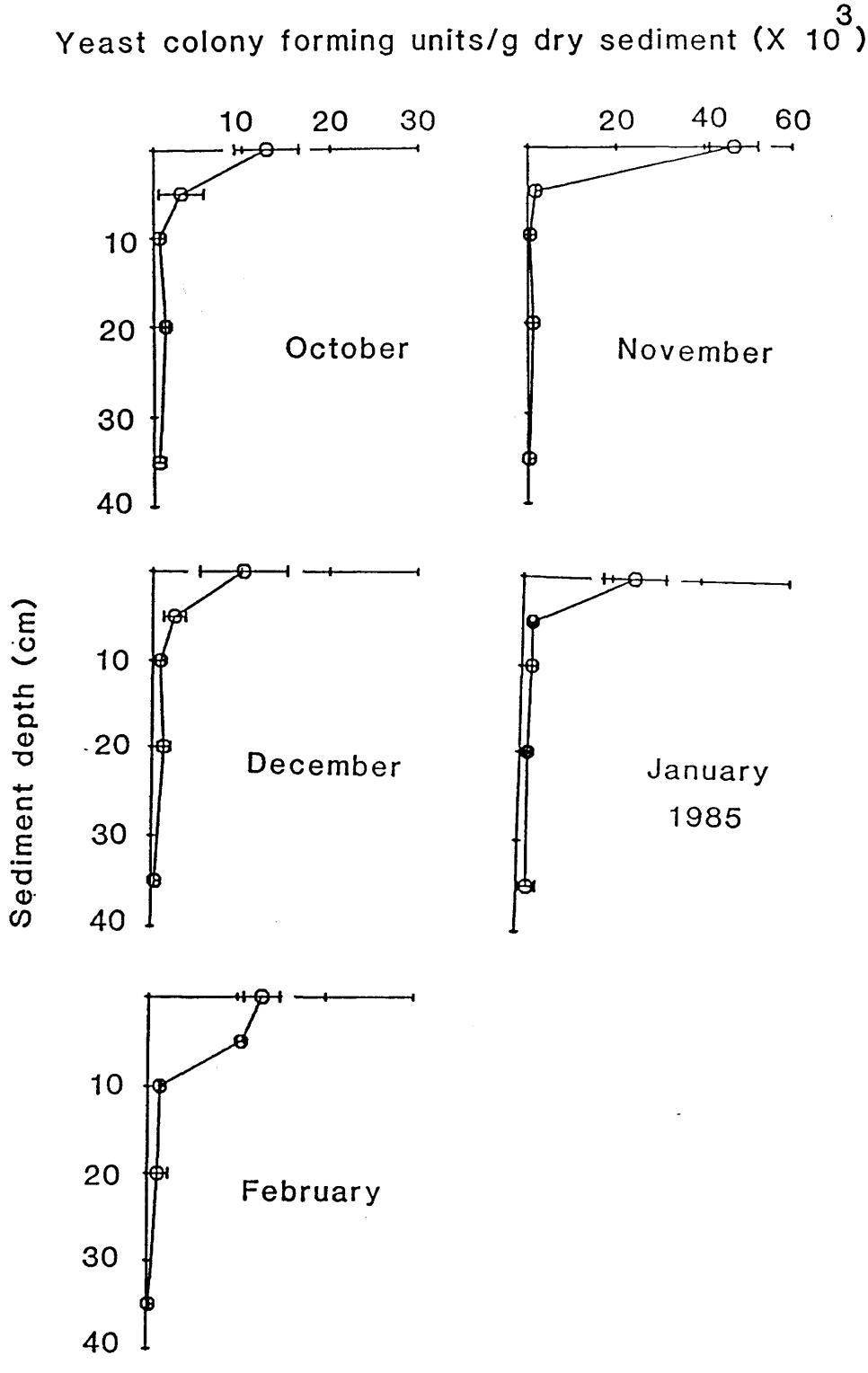

Surface sediment
5cm depth
10cm depth
20cm depth
35cm depth
Yeast colony forming units/g dry sediment (x 10^3)
F M A M J J A S O N D J F
Month

Figure 11

Relationship between yeast colony forming units/g dry weight of sediment and sediment depths from February 1984 to February 1985 at Ardmore Point (Clyde Estuary). Graphs are on 2 pages.


Yeast colony forming units/g dry sediment (X 10^3)
10
20
30
Sediment depth (cm)
10
20
30
40
February
1984
March
April
May
June
July
August
September


Yeast colony forming units/g dry sediment (X 10³)
10
20
30
20
40
60
Sediment depth (cm)
10
20
30
40
October
November
December
January
1985
February

Comparisons in the numbers of yeasts present (C.F.U.) at each sampling depth were carried out using regression analysis and student's t-tests in the same manner as that conducted with bacteria (see page 340). The results of these comparisons are shown in figures 12 and 13 and table 5. Highly significant inverse relationships were found with all the surface comparisons (all $P<0.001$). A significant direct relationship was found in the 20-35cm comparison ($0.05>P>0.02$). The other comparisons (5-10cm, 5-20cm, 5-35cm, 10-20cm and 10-35cm) were not significant ($0.80>P>0.70$, $0.90>P>0.80$, $0.20>P>0.10$, $0.70>P>0.60$, and $0.20>P>0.10$ respectively).

FUNGI

The mean and standard deviation of the fungal colony forming units (C.F.U.) per gram dry weight sediment or per ml water calculated by the computer program "Bact" is given in table 6.

Figure 14 shows the numbers of fungal colony forming units in overlying and interstitial water at Ardmore Point from February 1984 to February 1985. The highest numbers of fungi in overlying water were found in February 1984, while in interstitial water the highest numbers occurred in March 1984. Apart from these differences, similar seasonal abundance curves were found with overlying and interstitial water, and the ranges in fungal abundance were close (overlying water, 0-4.250 $x10^3$, interstitial water, 0-2.500 $x10^3$ C.F.U. ml^{-1}). In general, there were 3 peaks of fungal growth for both overlying and interstitial waters. With overlying water, peaks occurred in February, July and November 1984. With interstitial water, peaks occurred in February-March and November 1984.

Figure 15 shows the variation in the numbers of fungi in sediment at different depths. No clear decrease in the numbers of fungi with depth was found. In general, at all depths, there were two

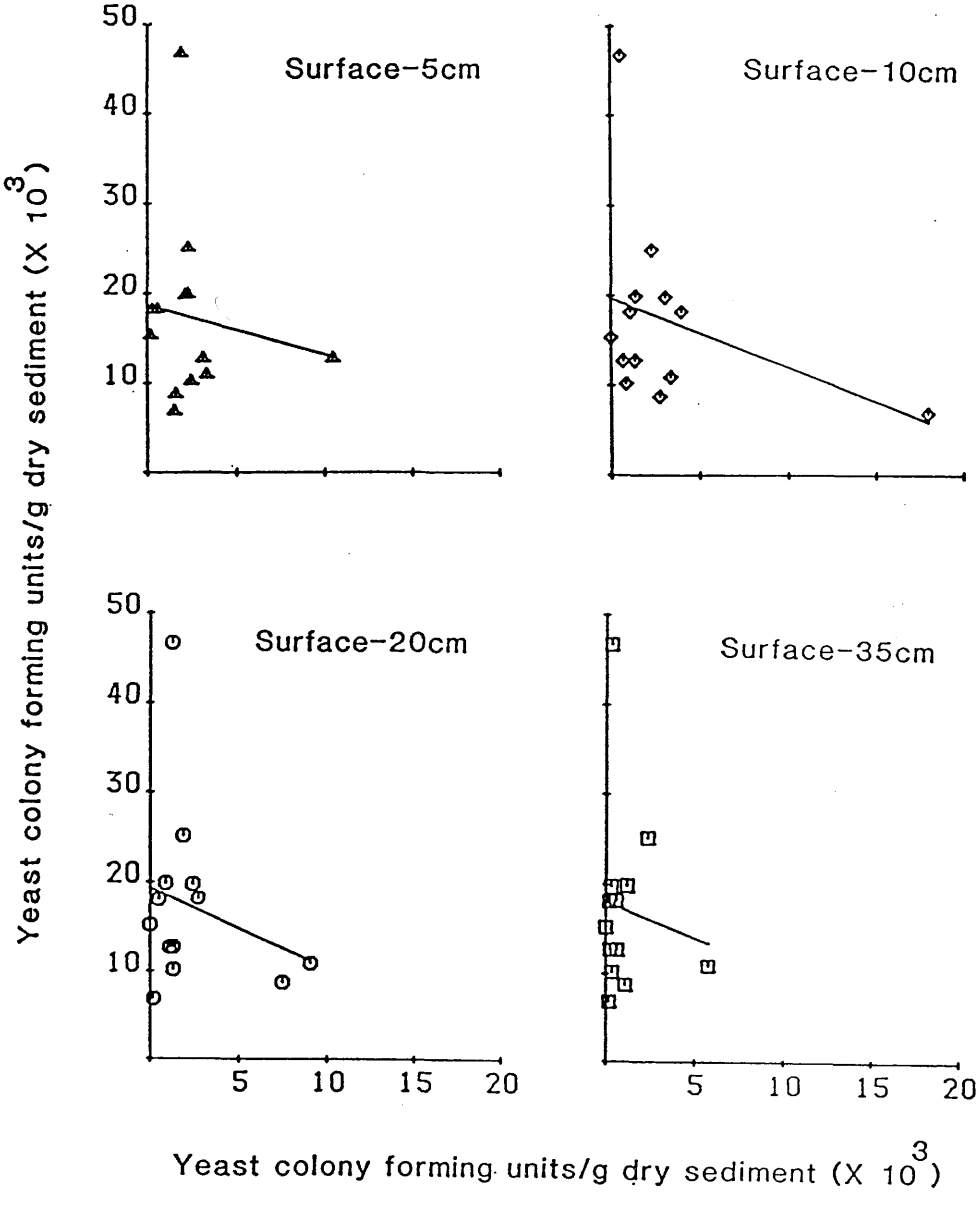
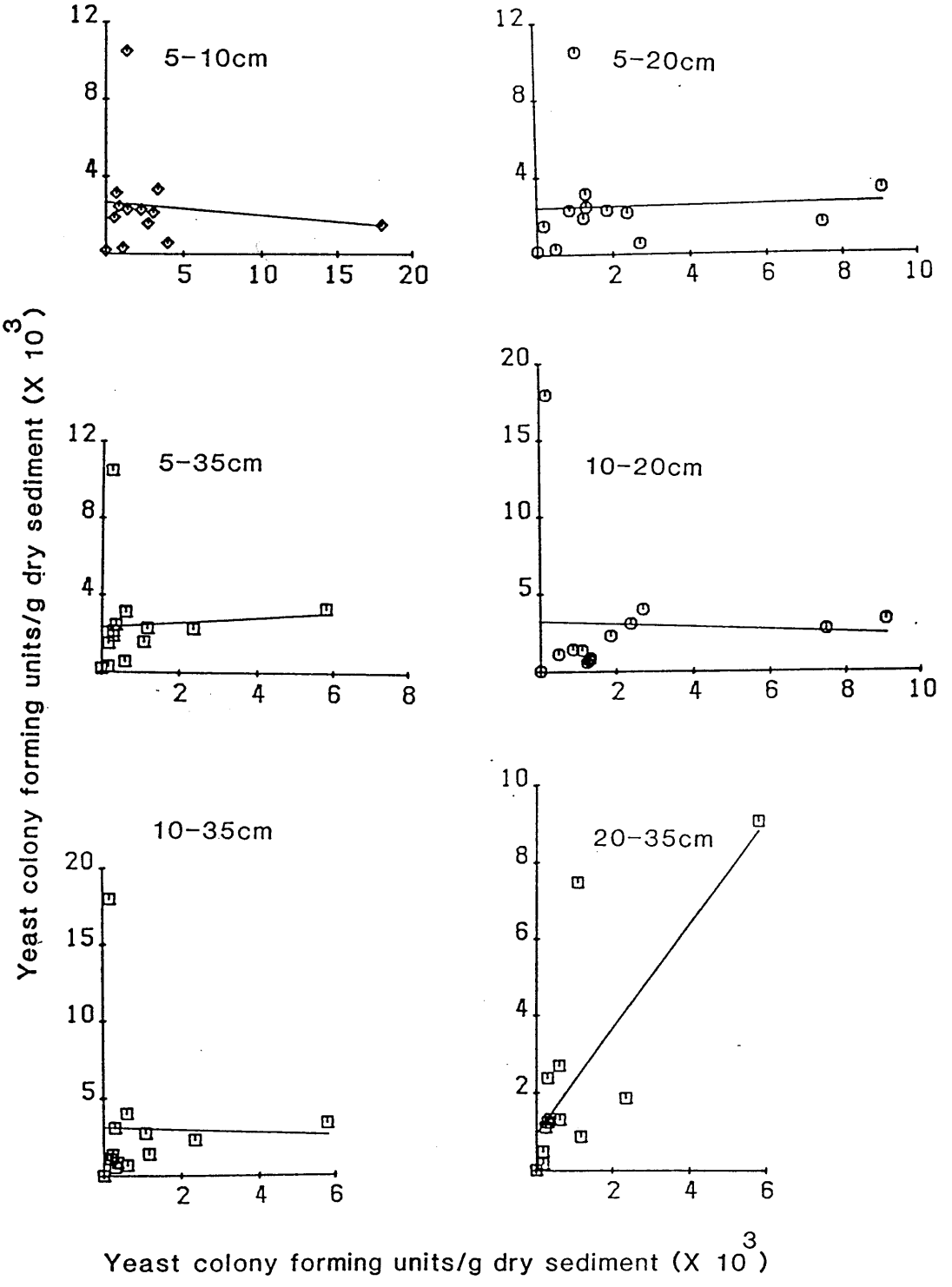


Figure 12

Relationship between yeast colony forming units/g dry weight of surface sediment and colony forming units/g dry weight of sediment at 5, 10, 20 and 35cm depth. y-axis is surface sediment, x-axis is the other depths. Each point represents data for one month.

Figure 13

Relationship between yeast colony forming units/g dry weight of sediment at each depth and the other depths. y-axis (5, 10, 20cm), x-axis (10, 20, 35cm). The figures at the top of each graph are the depths compared. For example, 5-10cm means 5cm data on y-axis and 10cm data on x-axis. Each point represents data for one month.


5-10cm
5-20cm
5-35cm
10-20cm
10-35cm
20-35cm
Yeast colony forming units/g dry sediment (X 10 3)
Yeast colony forming units/g dry sediment (X 10 3)

Data	Regression equation	t-test	d.f.	P
S.S-5cm	Y =-0.5686x + 18.78	4.908	12	P<0.001
S.S-10cm	Y =-0.3413x + 14.95	4.480	12	P<0.001
S.S-20cm	Y =-0.8948x + 19.43	4.824	12	P<0.001
S.S-35cm	Y =-0.7941x + 18.17	5.584	12	P<0.001
5-10cm	Y =-0.0660x + 2.671	0.3658	12	0.80>P>0.70
5-20cm	Y = 0.0265x + 2.423	0.1455	12	0.90>P>0.80
5-35cm	Y = 0.1332x + 2.334	1.779	12	0.20>P>0.10
10-20cm	Y =-0.0895x + 3.245	0.4740	12	0.70>P>0.60
10-35cm	Y =-0.0879x + 3.128	1.468	12	0.20>P>0.10
20-35cm	Y = 1.363x + 0.9179	2.479	12	0.05>P>0.02

Table 5

Results of regression analyses and student's t-tests applied to data to compare numbers of yeasts present at the different sampling depths. S.S = surface sediment. The regression plots are shown in figures 12 and 13.

	Feb 1984	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan 1985	Feb
O.W.	4.250 ± 2.051	0.2500 ± 0.07071	0	0	0.3000 ± 0	0.5500 ± 0.07071	0.3500 ± 0.07071	0.1000 ± 0.1414	0.1500 ± 0.07071	0.3500 ± 0.07071	0.1000 ± 0	0	0
			0	0								0	0
I.W.	1.400 ± 1.414	2.500 ± 0.9899	0	0	0.1000 ± 0	0.2000 ± 0.1414	0	0.2500 ± 0.07071	0	0.5500 ± 0.6364	0.1500 ± 0.07071	0	0
			0	0			0		0			0	0
S.S.	2.384 ± 0.2734	1.858 ± 0.5631	0	0	0.3351 ± 0.2844	3.548 ± 4.640	0	0.5419 ± 0	0	0	0.1347 ± 0	0.2011 ± 0.09480	0
			0	0			0		0	0			0
5cm	6.287 ± 7.439	0.3698 ± 0.1743	0	0	0.2527 ± 0.1787	0.8051 ± 0.4379	0.1881 ± 0.08869	0	0	0	0	0	0
			0	0				0	0	0	0	0	0
10cm	4.395 ± 5.328	1.049 ± 1.309	0	0	0.2481 ± 0.1754	0.2483 ± 0.3511	0	0	0	0	0	0	0
			0	0			0	0	0	0	0	0	0
20cm	2.823 ± 2.256	1.222 ± 1.210	0	0	0.8684 ± 0.1754	0.2440 ± 0.1725	0.4297 ± 0.2604	0	0.1247	0	0	0	0
			0	0				0	0	0	0	0	0
35cm	7.243 ± 1.215	0.5482 ± 0.4307	0	0	0.3033 ± 0.08577	0.7943 ± 0.6049	0	0.3109 ± 0.2638	0.1253 ± 0	0	0	0	0
			0	0			0		0	0	0	0	0

TABLE 6 : Fungal colony forming units ($x10^3$) per ml water or per gram dry weight of sediment (mean ± standard deviation) for the annual survey at Ardmore Point (Clyde estuary).
O.W. and I.W. are overlying and interstitial waters respectively. S.S. is surface sediment. 5,10,20 and 35cm are vertical sediment depths.

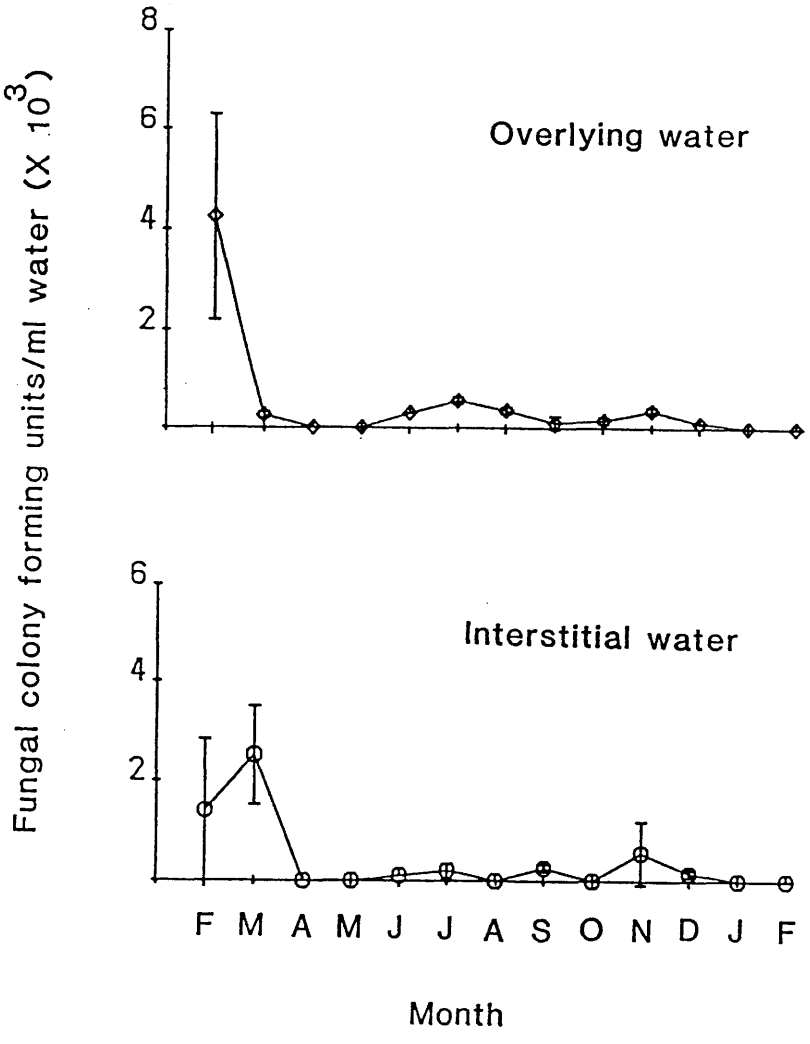


Figure 14

Fungal colony forming units/ml of overlying and interstitial waters from February 1984 to February 1985 at Ardmore Point (Clyde Estuary).

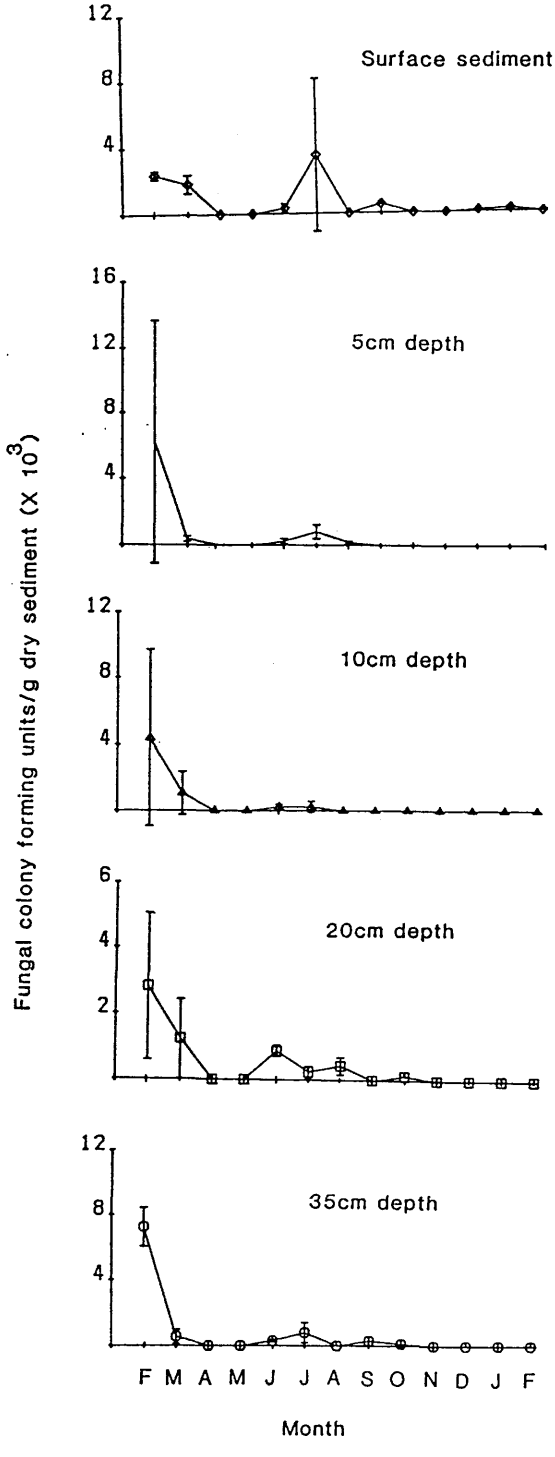


Figure 15

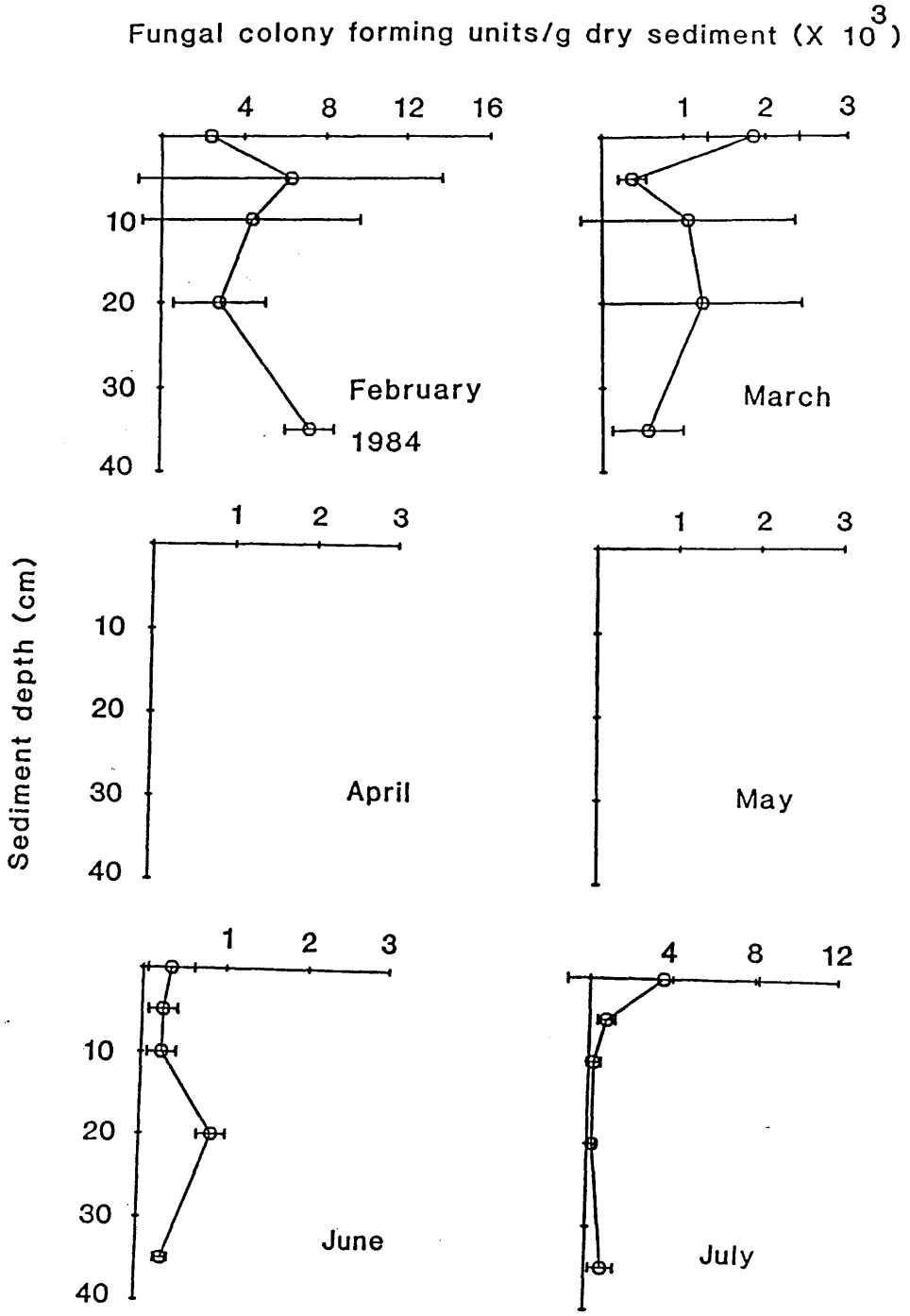
Fungal colony forming units/g dry weight of sediment at different depths (0, 5, 10, 20 and 35cm) from February 1984 to February 1985 at Ardmore Point (Clyde Estuary).

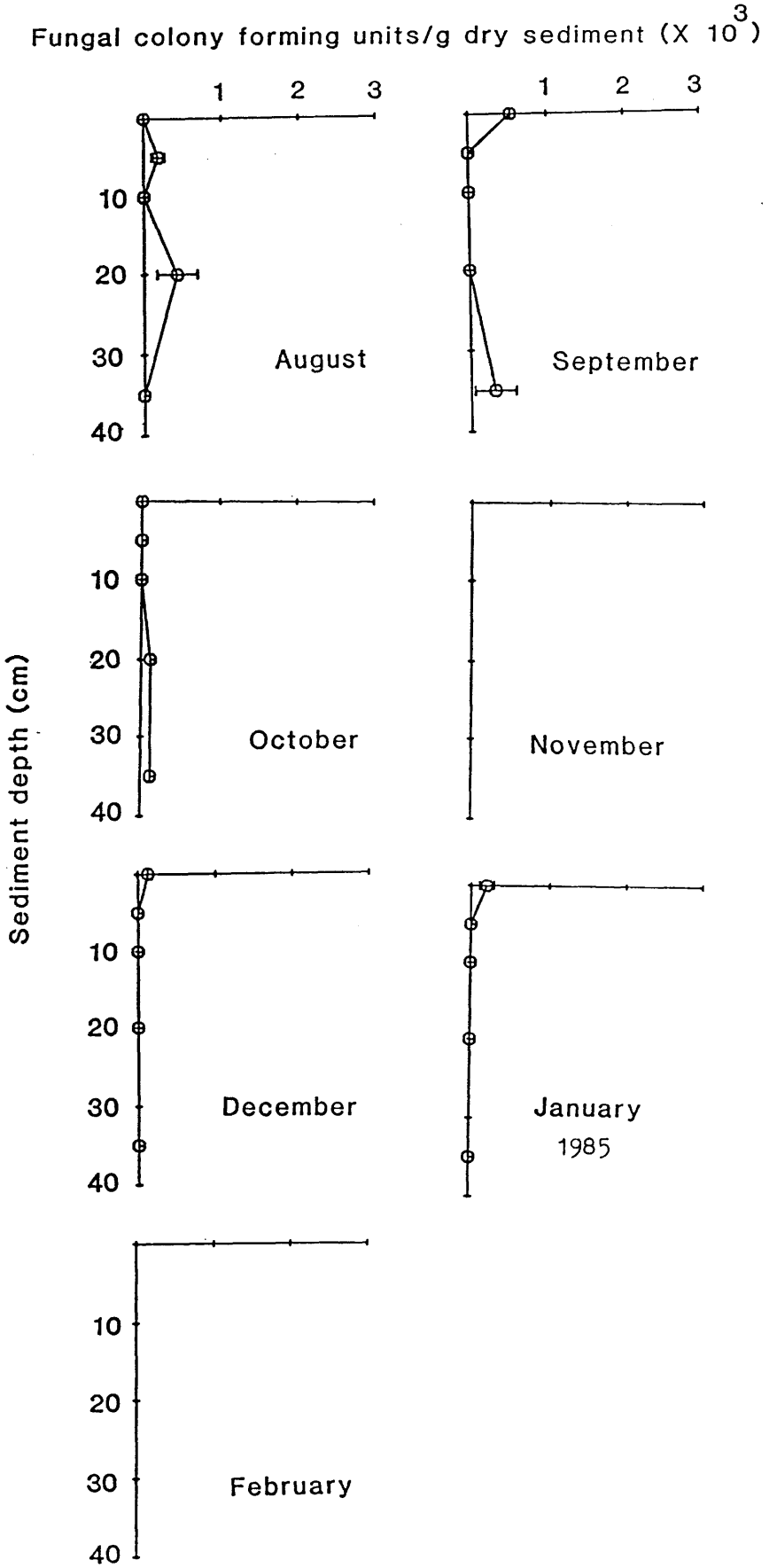
peaks of high numbers of fungi, early in the year 1984, in February, and at the middle of the year, in May-August.

The fluctuation in the numbers of fungi with depth, for each month from February 1984 to February 1985 is shown in figure 16. Unlike the results of the bacterial and yeast numbers, there was not a clear pattern of decreasing numbers of fungi with depth. In some months (February, March, June, July, August, September and October 1984) both increases and decreases were observed with increasing sediment depth. In the other months, very few or no fungi observed.

Figure 16

Relationship between fungal colony forming units/g dry weight of sediment and sediment depth from February 1984 to February 1985 at Ardmore Point (Clyde Estuary). Graphs are on 2 pages.


Fungal colony forming units/g dry sediment (X 10³)
Sediment depth (cm)
4 8 12 16
1 2 3
10
20
30
40
February
1984
March
April
May
June
July


Fungal colony forming units/g dry sediment (X 10³)
1
2
3
Sediment depth (cm)
10
20
30
40
August
September
October
November
December
January
1985
February

SECTION 2

DISCUSSION

The intertidal zone is an extremely complex environment. Wave action and tidal activity cause movement of the upper-most sediment layer and this results in resuspension of fine sediment particles and other sedimented material into the water column. Water and sediments (especially muddy ones) are relatively rich in organic material. Although organic carbon exists in both interstitial water and overlying water, the concentration of particulate organic carbon is generally at least three orders of magnitude greater in interstitial than in overlying water (Meyer-Reil et al., 1978). This situation is often reflected by the quantity and variety of microorganisms present in each habitat (Fukami et al., 1983). It is generally accepted that only about 20% of bacteria in the water column are cells that are usually attached to solid particles, in contrast to more than 99% attached cells in sediment (Meyer-Riel et al., 1978). The attached bacteria in sediments are usually found in depressions and crevices on the surface of the sediment particles (Meadows and Anderson, 1966).

The work in this section was concerned with investigating two main subjects related to microbial numbers in the Clyde Estuary.

(i) Whether there are seasonal patterns in the abundance of bacteria, yeasts and fungi in the estuary and whether there are significant differences between the overlying water and sediment pore water (interstitial water) in terms of microbial abundance. The effect of incubation time of cultures on the results of bacterial counts was also studied.

(ii) Whether microbial abundance in the sediment varies with depth into the sediment. Seasonal variation in microbial growth at specific depths was also studied.

The above two subjects were studied separately with respect to bacteria, yeasts and fungi, and will be discussed separately below, in that order.

1- BACTERIA

(i) Seasonal periodicity and abundance in overlying and interstitial water

My results showed that significantly higher bacterial numbers occurred in the interstitial or pore water than in the overlying water. This is a fairly widely accepted phenomenon (Hayes, 1964; Novitsky, 1983) and has been noted in earlier studies carried out in other areas. ZoBell and Feltham (1942) working in Mission Bay, California, found that there were thousands of bacteria per ml of overlying water, in contrast to millions of bacteria in the same volume of mud. Meyer-Riel et al. (1978) found that bacterial numbers in Baltic sea sediment were three orders of magnitude higher than those found in the water above. High bacterial numbers in sediment are thought to result from high levels of organic matter in the sediment (Hayes, 1964; Gerba and McLead, 1976). This organic matter is replenished to a great extent by sedimentation of plant and animal materials from the water column. Other factors in estuaries such as dilution by incoming water, tend to decrease the amount of bacteria present in overlying water even more, relative to sediment (Ketchum et al., 1952).

My studies showed that there were seasonal variations in the numbers of bacteria present in both the overlying and interstitial waters at Ardmore Point. In overlying water a peak occurred in February 1984, while in interstitial water peaks occurred in March 1984 and April-November 1984. Many workers have studied seasonal variations in bacterial numbers in other areas. Ezura et al. (1974)

found that viable bacterial counts in water and mud samples from Akkeshi Bay, Japan, attained a maximum in summer and decreased in the cold water season. Bent and Goulder (1981) found no seasonal variation in free bacteria in the Humber Estuary although attached bacteria had a clear seasonal abundance pattern. Kenyon et al. (1984) working off the Californian Coast found that Vibrio cholerae showed a 5 to 56-fold increase in numbers over the summer months when water temperatures rose by approximately 2 to 5°C. Rheinheimer (1985) quotes seasonal variations in bacterial numbers in the Western Baltic, where two peaks occur. The first peak occurs in Spring (April/May), the other in Autumn (October/November). Laanbroek and Verplanke (1986) studied the seasonal changes in percentage of attached bacteria in the tidal Oosterschelde basin and in the stagnant but saline lake Grevelingen, delta area of the rivers Rhine. They found that the percentage of attached bacteria enumerated in subsurface water (1m below the water surface) and deep-water (1m above the sediment) appeared to be highly dependent upon the season.

Many suggestions have been given to account for seasonal periodicity in bacterial numbers. Goulder (1976), Goulder (1977), Bent and Goulder (1981) and Laanbroek and Verplanke (1986) consider that attached bacterial numbers vary with the concentration of suspended solids in the estuary. ZoBell and Feltham (1942) state that bacterial numbers are affected by depth, tidal cycles, temperature and amount of organic matter present. Fukami et al. (1983) found that bacterial numbers were correlated with the concentration of particulate organic carbon present in the water. Dale (1974) found a strong relationship between the number of bacteria present and sediment grain size, organic carbon content and total nitrogen content. Ezura et al. (1974) found a correlation between bacterial numbers and ammonia , seston-

carbon and chlorophyll a levels in seawater. Sieburth (1967) found that bacterial numbers were related to water temperature. Kenyon et al. (1984) also found that the number of Vibrio cholerae in seawater was significantly related to water temperature. Erkenbrecher and Stevenson (1977) found that bacterial numbers in Salt-March Creeks were influenced by the tidal cycle and the particulate organic carbon content of the water.

My results showed that maximum bacterial growth was attained quicker on agar plates with interstitial water as the inoculant than with overlying water (7 days for interstitial water compared with 20 days for overlying water). This is almost certainly due to the much higher concentration of bacterial cells in the interstitial water sample initially, compared to the overlying water.

I also studied the effect of increasing incubation time on the growth of bacteria from different depths in the sediment to ensure that counting was carried out when the bacteria had reached a phase of maximum growth. I found that, in general, bacteria from surface sediment did not reach their maximum growth phase as quickly as bacteria from underneath the sediment surface. In general, the time taken to reach maximum growth decreased with increasing depth into the sediment, although the 20cm and 35cm curves were similar to each other. A number of factors may be responsible for these findings. Firstly, there may be a change in the composition of the bacterial population with increasing depth into the sediment with only specialised fast growing species occurring deep in the sediment. Secondly, the level of nutrients in the media may favour the growth of bacteria from specific depths in the sediment and delay the growth of bacteria from other levels.

(ii) **Variation in bacterial abundance with sediment depth and seasonal variation between depths**

My results showed that in general, at the sediment surface, high bacterial counts in the approximate range 14-326 $x10^3$ g^{-1} dry sediment occurred in most months. These counts fell dramatically to 5-21 $x10^3$ g^{-1} dry sediment at 5cm depth. This count remained approximately the same to a depth of 10cm after which a further, though less dramatic decrease in numbers occurred (0.5-8 $x10^3$ g^{-1} dry sediment at 35cm). Seasonal variation in bacterial abundance occurred at all depths and there were usually 2 or 3 peaks in abundance. However, there were differences in the timing of the peaks between different months.

Decrease in bacterial abundance with depth in marine sediments was first recognised in the Clyde Sea Area by Lloyd (1931). The author found levels of 70-300 $x10^3$ g^{-1} dry sediment at the sediment surface falling to 58 $x10^3$ g^{-1} dry sediment at 30cm depth. More recent studies in the Clyde Estuary have been conducted by Anderson et al. (1981). They found levels of approximately 40±24 $x10^4$ g^{-1} dry sediment at the sediment surface decreasing to 11±12 $x10^4$ g^{-1} dry sediment at a depth of 10cm. This phenomenon has been noted in other intertidal and nearshore areas (Hayes, 1964, Collins, 1977). ZoBell and Feltham (1942) working in Mission Bay, USA, found levels of 172-400 $x10^3$ g^{-1} dry sediment at the sediment surface decreasing to 8.2-62 $x10^3$ g^{-1} dry sediment at 85-90cm depth. Meadows and Tait (1985) reported a similar exponential decrease in bacteria abundance with sediment depth in deep-sea sediment from the North East Atlantic (c. 10^2-10^5 g^{-1} dry sediment at the sediment surface falling to c. 1 g^{-1} dry sediment at 20cm depth).

Several reasons have been put forward for the exponential

decrease in bacterial numbers commonly observed in marine sediments. Rheinheimer (1985) states that it is caused by a decrease in nutrient concentration and faunal activity with depth into the sediment. The decrease in bacterial numbers with depth may also be caused by high levels of hydrogen sulphide produced at depth by specialised sulphate reducing bacteria (Fenchel and Riedl, 1970). High levels of bacteria at the sediment surface at particular times of the year may result from fallout from phytoplankton blooms which temporarily increase the organic nutrient level of the surface sediment and facilitate bacterial growth (Hayes, 1964; Wolter, 1982; Linley et al., 1983; Lancelot and Billen, 1984; Rheinheimer, 1985). Peaks in bacterial numbers at depths in the sediment may be due to the presence of animal burrows in the sediment which contain high levels of bacteria in their linings. Anderson and Meadows (1978) found high levels of bacteria in burrows of Nereis diversicolor in the Clyde Estuary (c. 15×10^7 g^{-1} dry sediment). These levels were close to levels found at the sediment surface (c. 18×10^7 g^{-1} dry sediment) and were much higher than that found in sediment close to the burrow (c. 1×10^7 g^{-1} dry sediment). Meadows and Tait (1985) found that deep-sea burrows contained high levels of bacteria relative to surrounding sediment at the same depth. ZoBell (1938) suggests that burrowing animals may also be important for transferring aerobic bacteria from the sediment surfaces to depths in the sediment. The activities of burrowing animals may have been responsible for producing peaks in my data at certain depths in the sediment. Bacteria may also be carried from the surface to depths in the sediment by water draining through the sediment during low tide. Anderson and Meadows (1969) suggest that run-off from the land and rain water may wash bacteria from the surface of sand grains.

When I applied regression analysis and t-tests to my data to

assess whether specific sediment depths were related in terms of bacterial abundance, I found the following. Surface-5cm and surface-20cm had a direct relationship, i.e. as bacterial abundance at the first depth increased or decreased, bacterial abundance at the other depth varied in the same way. Surface-10cm and surface-35cm had an indirect relationship, i.e. as bacterial abundance increased or decreased at one depth, it changed in the opposite way at the other depth. Comparisons between all the other depths showed a direct relationship. The reason for the above findings are not clear but may result from several factors such as changes in particle size with sediment depth and the rate of transfer of organic material and other nutrients between sediment depths in the interstitial water. Bioturbation may be important in influencing particle size and nutrient transfer between sediment depths. Burrowing animals are known to rework sediments and alter their particle size distribution (Fager, 1964; Rhoads, 1974). Burrows also play an important role in sediment pore-water chemistry and can alter fluxes of ions between sediment depths and across the sediment-water interface (Aller, 1980).

2- YEASTS

(i) Seasonal periodicity and abundance in overlying and interstitial water

My results showed that significantly greater numbers of yeasts were present in interstitial than in overlying water (2.2-119 $x10^3 ml^{-1}$, 0-4.350 $x10^3$ ml^{-1} respectively). I can find no references in the literature to work in which the number of yeasts in overlying water is compared to interstitial water. However, it is generally accepted that yeast abundance in seawater is influenced to a great extent by organic content (Fell and Van Uden, 1963; Van Uden and

Castelo-Branco, 1963; Ahearn et al. 1968; Ahearn, 1973; Fell, 1974). Since sediments generally contain levels of particulate organic matter much in excess of that found in overlying water (Meyer-Reil et al. 1978) it would be expected that the above results would be obtained.

I found that peaks of yeast abundance occurred in overlying water, in February, April and August 1984, and in January 1985. In interstitial water, peaks occurred in May, August and October 1984, and were more dramatic than those found with overlying water. Peaks in yeast abundance may be related to several factors. Increases in input of organic material to the environment in terms of pollution and phytoplankton blooms may increase yeast numbers. Spencer et al. (1970) found that seasonal variation in yeast abundance in the Saskatchewan river was directly related to the input of sewage to the river. Hagler and Mendonca-Hagler (1981), working in Brazilian estuaries and coastal sites, found 2880 C.F.U./100ml water in heavily polluted areas, 202 C.F.U./100ml water in moderately polluted areas and 3 C.F.U./100ml water in lightly polluted areas. These workers also found that high yeast numbers coincided with algal blooms. Morris (1968) stated that high yeast levels are possibly related to high levels of phytoplankton waste products present in the water after blooms. Meyers et al. (1967) found that seasonal peaks in yeast numbers in the North sea coincided with the end of dinoflagellate blooms.

Increases in temperature may affect yeast numbers at particular times of the year. Van Uden and Costelo Branco (1963) found that temperature affected the numbers of yeasts in water samples taken along the Californian coast. Seshadri and Sieburth (1971) detected maximum yeast numbers on seaweed samples from Narrangansett Bay, USA. during the warmest months of the year. Fell (1974) related the distribution and abundance of yeasts in the South Indian, South Pacific and South Atlantic oceans to several factors, including

temperature.

Changes in salinity can affect yeast numbers in marine environments, and may cause variations in numbers throughout the year. Seasonal changes in input of fresh water to estuaries from rivers and from run-off can alter salinity significantly. Ahearn et al. (1968) compared yeast abundance in water samples taken from rivers, estuaries and coastal sites around Florida. They found highest yeast numbers in fresh water, than in estuaries or coastal water. Combs et al. (1971) had similar results when they compared yeast abundance in long Island Sound and Housatonic River Estuary, USA. Higher levels occurred in the estuary than in the coastal samples.

(ii) **Variation in yeast abundance with sediment depth, and seasonal variation between depths**

My results showed that in general, for each month there was a decrease in yeast numbers with depth. The decrease was most dramatic between the surface and 5cm depth (6.836-46.74 $x10^3$ and 0.1869-10.49 $x10^3$ g^{-1} dry sediment respectively). After 5cm, the decrease in numbers was more gradual. However, in three months, June , July and August, peaks were found at depths in the sediment, but these months were exceptions to the pattern described above. The greatest variation in yeast numbers between months occurred at the sediment surface. Similar variations in yeast numbers occurred below 10cm depth.

Most of the literature concerned with yeasts from the marine environment, describes yeasts isolated from water samples. Very few workers have looked at yeast populations in marine sediments. ZoBell (1938) isolated bacteria and actinomycete-like organisms from deep-sea sediment collected along the Californian coast, but could not find any yeasts or mould fungi. Fell and Van Uden (1963) found that yeast numbers decreased with sediment depth in estuaries, coastal and deep-

sea sediments although they found a few yeast abundance peaks at some sediment depths. They noted that yeasts occurred at greater sediment depths in estuarine sediments than in coastal sediments. The authors suggested that the decrease in yeast numbers with depth was due to decreasing O_2 levels, and that higher wave agitation and sedimentation in estuaries promoted yeast numbers. Rheinheimer (1985) states that yeasts generally occur in the topmost few centimeters of marine sediments. The decrease in yeast numbers with depth that I found at Ardmore Point is probably caused by a decrease in organic matter with sediment depth. This trend may be altered by localised phenomena such as the presence of animal burrows containing faeces where yeast levels are high (Fell and Van Uden, 1963).

As with the bacterial data, sediment depths were compared in terms of C.F.U. g^{-1} dry sediment using regression analyses and t-tests. Direct relationships were found between the surface and all the other depths. When the depths were compared with each other, no significant relationship was found between them with the exception of the 20-35cm comparison where a direct relationship occurred. These findings probably result from a combination of several factors such as changes in particle size with sediment depth, exchange of organic nutrients between depths and across the sediment-water interface, and bioturbation effects.

FUNGI

(i) **Seasonal periodicity and abundance in overlying and interstitial water**

My results showed that, in general, unlike bacteria and yeasts, fungal abundance in overlying and interstitial water were similar. Seasonal variations in fungal abundance in overlying and

interstitial water were also similar apart from only small differences in time between some peaks. For example, the highest numbers of fungi in overlying water occurred in February 1984 while the highest numbers in interstitial water occurred in March.

I can find no previous reference in the literature to studies in which fungal abundance in interstitial and overlying water are compared. Indeed very little is known about factors responsible for establishing distribution patterns of fungi in marine environments (Johnson, 1968). Salinity and temperature have been tentatively explored as factors determining the distribution and occurrence of saprobic fungi in salt water (Sparrow and Johnson, 1961; Ritchie and Jacobson, 1963; Johnson, 1968; Sparrow, 1968).

It is not clear why similar fungal numbers were found in interstitial and overlying water. One would expect to find the highest numbers in interstitial water due to the correspondingly greater organic matter levels. Elliott (1930) found higher fungal numbers in organic rich sediments than in organic poor sediments. My results may have been influenced by the presence of spores or conidia of terrestrial fungi in the overlying water. It is stated by Elliott (1930) that terrestrial spores or conidia that are washed into salt-marshes can retain their viability for years.

(ii) **Variation in fungal abundance with sediment depth, and seasonal variation between depths**

No clear variation in the numbers of fungi with sediment depth was found. The depth profiles showed large fluctuations between months. However, fungal abundance peaked at similar times of the year at all depths (February and May-August).

Varying accounts have been given of seasonal periodicity in marine fungi. Borut and Johnson (1962) found no seasonal variation in

fungal abundance in a North Carolina estuary. However, Elliott (1930) found higher numbers of Dovey salt marsh fungi in June than in other months.

Little information is given in the literature on the variation in fungal abundance with sediment depth. Elliott (1930) looked at variations in the species present at particular depths but did not study changes in abundance. Litchfield and Floodgate (1975) carried out a study on the bacteriology of Irish sea sediments, and mentioned finding higher numbers of fungi in deeper sediment layers than at the surface. They suggested that the fungi had come from soil drainage and laid dormant as spores. It is difficult to put forward suggestions for the erratic depth profiles that were found in my study.

SECTION 2

SUMMARY

The aims of my work in this section were to estimate the seasonal viable counts of hetrotrophic bacteria, yeasts and fungi in overlying, and interstitial waters and sediments (vertical profiles) at Ardmore, Clyde Estuary.

BACTERIA

1- The relationship between numbers of bacteria in overlying and interstitial water at Ardmore Point over a period of thirteen months was studied. The results showed that the numbers of bacteria in interstitial water were higher than that of overlying water.

2- In overlying water, a peak in bacterial numbers was shown in February 1984, while in interstitial water, a number of marked peaks of bacterial numbers occurred in March and between April-November 1984.

3- The variation in the numbers of bacteria at different sediment depths was studied. The following conclusions were made.

(a) The numbers of bacteria were greatest at the surface sediment and tended to decrease with depth.

(b) At the surface sediment, peaks of high levels of bacteria occurred in February, April, August and November 1984 and in January 1985.

(c) At 5 and 10cm sediment depth, similar ranges of bacterial numbers occurred.

(i)- At 5cm depth, three peaks occurred in February-March, and August 1984 and in January 1985.

(ii)- At 10cm depth, two peaks occurred in March and June 1984.

(d) At 20 and 35cm depth, much lower numbers of bacteria were found than at 5 and 10cm depth.

(i)- At 20cm depth, three minor peaks occurred in March-

April, July-August and in January 1985.

(ii)- At 35cm depth, only one peak of bacterial numbers occurred in August 1984.

4- The variation in the numbers of bacteria with depth for each month from February 1984 to February 1985 was studied. The results showed that in general, the patterns of decreasing numbers of bacteria with depth was similar for all months except for surface sediment which showed high levels of bacteria in February, April, August, November 1984 and in January 1985.

5- The bacterial numbers at each sampling depth were compared using all data collected over thirteen months. This was carried out using regression analyses and student's t-tests. The bacterial numbers at each depth were also plotted against the other depths (e.g. surface against the other depths). The results showed the following.

a) A direct relationship in bacterial numbers occurred in all cases with only a few exceptions as follows.

(i) An inverse relationship was found in the comparisons of surface-10cm and surface-35cm depth.

(ii) No significant relationship was found in the 5-10cm comparison.

(iii) The t-tests showed significant differences in bacterial numbers between all depths with the exception of 5-10cm, where no significant difference was found.

6- The relationship between the numbers of bacteria (C.F.U.) and incubation time (days) was investigated for overlying and interstitial waters collected in July 1984. The results showed that bacteria in overlying water reached a maximum growth after 20 days, while in interstitial water, the maximum growth occurred after only 7 days.

7- The relationship between the numbers of bacteria (C.F.U.) in sediment from different depths, and incubation time (days) was also

studied. The results showed that in general, there was a decrease in bacterial numbers with sediment depth at each incubation time. In addition at greater sediment depths there was a less pronounced increase in bacterial growth with increasing incubation time. The maximum growth was always reached approximatley after 15 days incubation.

YEASTS

1- The relationship between numbers of yeasts in overlying and interstitial waters at Ardmore Point over a period of thirteen months was studied. The results showed that interstitial water contained significantly higher numbers of yeasts than overlying water.

2- In overlying water, 4 peaks of yeast numbers were shown in February, April and August 1984, and in January 1985, while in interstitial water, 3 peaks of yeasts were shown in May, August and October 1984.

3- The variation in numbers of yeasts in sediment at different depths was studied. The results showed the following conclusions.

(a) The numbers of yeasts were greatest at the surface sediment and in general, tended to decrease with depth.

(b) At the surface sediment, peaks of high levels of yeasts occurred in November with lesser peaks in March and September 1984 and in January 1985.

(c) At 5cm depth, maximum numbers of yeasts were found in February 1985.

(d) At 10cm depth, maximum numbers were found in June 1984.

(e) At 20cm and 35cm depth, similar curves were found although peaks occurred at slightly different times of the year (August at 20cm and July at 35cm).

4- The variation in the numbers of yeasts with depth for each month from February 1984 to February 1985 was studied. The results

showed that in general, the patterns of decreasing numbers of yeasts with depths was similar for all months with the exception of February, March, June, July and August which showed peaks of numbers of yeasts at 10cm and 20cm depth.

5- The yeast numbers at each sampling depth were compared using all data collected over thirteen months. This was carried out using regression analysis and student's t-tests. the yeast numbers at each depth were also plotted against the other depths (e.g. surface against the other depths). The results showed the following.

(a) Highly inversely significant relationships were found with all the surface comparisons.

(b) There were no significant relationships between all the other depths with exception of 20cm-35cm depth which showed a direct significant relationship.

FUNGI

1- The relationship between numbers of fungi in overlying and interstitial waters at Ardmore Point over a period of thirteen months was studied. The results showed similar seasonal abundance with overlying and interstitial waters and the ranges in fungal abundance were close in both samples.

2- In general, there were 3 peaks of fungal growth for both overlying water (February, July and November 1984) and interstitial water (February-March, and November 1984).

3- The variation in the numbers of fungi at different sediment depths was studied. There was no clear decrease in the numbers of fungi with deptn. The results also showed at all depths, there were 2 peaks of high numbers of fungi (February 1984 and May-August).

4- The variation in the numbers of fungi with depth for each month from February 1984 to February 1985 was studied. The results showed no clear pattern of decreasing numbers of fungi with depth.

SECTION 3

The use of API test-kits and their function on marine and non-marine isolates and on sediments

SECTION 3

INTRODUCTION

Biochemical tests

Many methods have been used to identify bacteria from different sources, including, conventional biochemical tests and test-kits.

Identifying bacteria using conventional tests is often time consuming. These tests usually involve detecting one or more bacterial enzymes by colour changes or gas formation according to enzyme activity (Shewan and Hodgkiss, 1954; Kovacs, 1956; Gaby and Hadly, 1957; Shewan et al., 1960; Leifson, 1963). However, identification of bacteria has been greatly simplified by the developement of new test-kits for routine bacteriological work.

Many investigators have used the test-kits systems. The systems used to#date are API 20E (Holmes et al., 1978; Edberg et al., 1979; Aldridge and Hodges, 1981; Rüger, 1981; Holmes et al., 1982; MacDonell et al., 1982; O'Reilly et al., 1984). API 20B (Rüger, 1981), API ZYM (Smith et al., 1972; Humble et al., 1977; Tharagonnet et al., 1977; Hofstad, 1980; Waitkins et al., 1980; Kelley, 1982; O'Reilly et al., 1984), Minitek (Back and Oberhofer, 1978; Retter and Bannatyne, 1981; Rüger, 1981), MICRO-ID (Aldridge et al., 1978; Edberg et al., 1979), R/B tubes (Nord et al. 1974), and Entero-set 20 (Aldridge and Hodges, 1981). Appendix p. 476 gives further particulars about the API ZYM, 20E, 20NE, 20B, 50CH, and Minitek systems.

The API ZYM system is in increasing use at the moment to detect bacterial enzymes and to identify bacteria and I shall review some of the studies that have been conducted using it. Humble et al. (1977) used the API ZYM system to detect enzymes for 18 clinical bacterial isolates from several species. They reported in this

preliminary study that the API ZYM system produced results that may be useful in the identification of a variety of bacteria. Tharagonnet et al. (1977) studied the use of API ZYM system in the identification of Gram-negative anaerobes from human sources. The method has also been used by Waitkins et al. (1980) who identified Streptococci by the same technique. The API ZYM system however, can not differentiate between certain groups of bacteria. Hofstad (1980) used the technique to identify many species of Bacteroides and Fusobacterium. He found that the system only discriminated between certain Bacteroides species but did not discriminate between Fusobacterium species.

Over the past few years, other biochemical test-kits have been widley used in clinical laboratories. Aldridge and Hodges (1981) studied the degree of accuracy and reproducibility of Entero-set 20, API 20E and conventional media systems for identification of members of the family Enterobacteriaceae collected from clinical sources. They showed that both systems performed with a high degree of accuracy and reproducibility when compared with conventional tube media. Holmes et al. (1982) used the API 20E for biotyping the clinical strains of Haemophilus influenzae. They concluded that API 20E provided 100% correlation with the conventional methods. Edberg et al. (1979) evaluated the ability of the MICRO-ID, API 20E, and the conventional media system to identify members of the Enterobacteriaceae. Edberg and his associates found that MICRO-ID compared quite favorably with conventional methods. They also found that the MICRO-ID and the API 20E agreed on the genus and species names in 85.7% of cases. Nord et al. (1974) studied the accuracy of five test-kits; API, Auxotab, Enterotube, Patho-Tec and R/B tubes in trials to identify members of Enterobacteriaceae. They found that the API system was the most reliable technique followed by Enterotube, R/B, Patho-Tec and Auxo-Tab in that order. Although these systems showed reasonable

reproducibility in this study some questionable results and problems have been shown by the API system. For example, O'Reilly et al. (1984) tried to evaluate the systems API 20E and API ZYM for clinical purposes. They succeeded in characterising and differentiating 30 Haemophilus and 6 Actinobacillus strains, but they reported that identification and nomenclature proved difficult in some cases. This may be due to defects in some of the test media in the available kits. Holmes et al. (1978) examined 206 strains of Enterobacteriaceae using the API 20E system and conventional media. They suggested that slight modification of either the kit or the reagent might further improve its diagnostic performance. Holmes and his co-workers determined nitrate reduction in 86 strains using the API 20E system and the reagents of Crosby (1967). Only 10 strains gave a negative result. When these 10 strains were re-tested with the same technique but using the reagents recommended by the manufacturer, 6 strains gave a positive result and 4 were negative. The authors therefore recommended that the manufacturer of the API system should consider recommending the reagents of Crosby (1967) to future users of the API 20E system. Earlier studies by Leifson (1963) showed that the indicator bromthymol blue, used in the API 20E system to detect acid production from carbohydrates, is toxic to many marine bacteria and this point should be taken into consideration as well.

There is very little literature and some critisism on the applicability of the commercially available test-kits to marine bacteria (Gauthier and Clement, 1978; Pyle and Shotts, 1980; Rüger, 1981; MacDonell et al., 1982). Rüger (1981) compared the API 20B, API 20E, API 50E and Minitek with conventional methods for differentiating marine and terrestrial strains of Alcaligenes, Bacillus and Vibrio. With the strain of Alcaligenes, only the Minitek system agreed with the

conventional method while with the other two strains, all test systems showed reliable results. The author reported that the media of the commercially available kits are not suitable for marine bacteria. He therefore substituted Leifson's (1963) MOF media for the media of API 20B, API 50E and Minitek. With the API 20E, sterile seawater (17 ‰) was used as the test medium instead of the distilled water recommended by the manufacturer. MacDonell et al. (1982) evaluated the influence of nine chemical inoculation diluents on the biochemical profiles of 30 marine and estuarine cultures including Vibrio, Aeromonas, Allomonas and Photobacterium using the API 20E system. The authors showed that the API 20E system , modified by the use of marine salts diluent prepared with the salinity adjusted to 20 ‰ and incubated at 22°C, can provide a rapid characterisation of marine and estuarine bacteria.

The manufacturer of the API test-kits state that API ZYM kits are applicable to specimens such as body fluids and soil. There appears, however, to be no published work on the applicability of the commercially available API ZYM test-kits to intertidal sediments, or to overlying and interstitial seawater.

The aim of the work reported in this section was to apply the API ZYM, API 20E and API 20NE micromethod systems to marine and non-marine samples in a number of experiments. The API 20E and API 20NE systems were applied to marine and non-marine bacterial strains in order to study the accuracy and reproducibility of these kits in identifying marine bacteria. The API ZYM was used in an attempt to classify sediments only, and was not used with bacterial strains.

SECTION 3

API ZYM

EXPERIMENT No. 1

Aim of experiment

The aim of this experiment was to determine the degree of variation in the quantity of sediment dispensed by (a) successive drops from a given pasteur pipette following one filling, and (b) drops from different pipettes.

Materials and methods

Sediment samples were taken from the surface of a core collected from Ardmore Point and carefully washed into 5ml bijou bottles with sterile artificial seawater. Four drops were then dispensed from a pasteur pipette into each of four weighed metal foil cups. This process was repeated twice using the same pipette. The whole procedure was also repeated twice using two separate pasteur pipettes. Twelve drops were therefore dispensed from each pasteur pipette (Table 1). Samples were dried in the oven at 60^{o}C for 24 hours. After drying, sediment was kept in a desiccator until cooled and then weighed in order to calculate the dry weight of sediment obtained from each drop. This value was then used to determine the amount of inoculum dispensed from the pasteur pipette used in the inoculation.

EXPERIMENT No. 2

Aim of experiment

The aim of this experiment was to determine the optimum incubation time of inoculated API ZYM strips using surface sediment samples.

	Pasteur pipette (A)			Pasteur pipette (B)			Pasteur pipette (C)		
	I	II	III	I	II	III	I	II	III
First drop	1	1	1	1	1	1	1	1	1
Second drop	2	2	2	2	2	2	2	2	2
Third drop	3	3	3	3	3	3	3	3	3
Fourth drop	4	4	4	4	4	4	4	4	4

Table 1: Experimental procedure used to determine the degree of variation in the quantity of sediment dispensed by (a) successive drops from a given pasteur pipette following one filling, and (b) drops from different pipettes. For each pipette, three replicates were used.

Materials and methods

Sediment samples were collected at Ardmore Point and transported to the laboratory for analysis. Approximately 3 g of sediment were suspended in 3ml of sterile artificial membrane filtered seawater. Two drops of sediment suspension were dispensed into each cupule using a pasteur pipette. Two replica strips and one control, containing only sterile filtered seawater, were used for each incubation time. Incubation times of 2.5, 5, 10 and 20 hours were tested both before and after addition of ZYM A and B reagents. Figure 1 shows the procedure adopted to the API ZYM experiments.

EXPERIMENT No. 3

Aim of experiment

The aim of this experiment was to determine how soon colour developed after addition of reagents and how soon it reached a maximum intensity. Sediment from a range of sediment depths was used in this experiment.

Materials and methods

Sediment was collected from Ardmore Point using a sediment corer. The sediment core was kept at $4^{o}C$ overnight then subdivided. 3g samples of sediment were taken from 0, 5, 10, 20 and 35cm and washed separately into 5ml bijou bottles using 3ml of membrane filtered sterile seawater (0.45um). After shaking to resuspend sediment, each cupule of the API strip was inoculated with two drops of the suspension using a pasteur pipette. After 12 hours incubation at $10^{o}C$, one drop from each reagent of ZYM A and B was dispensed into each cupule. Colour development for the duplicate tests were recorded 5, 15, 30, 60, 120, 300 and 720 minutes after the addition of reagents. The strips were set up at staggered times to allow readings to be taken at the correct time intervals.

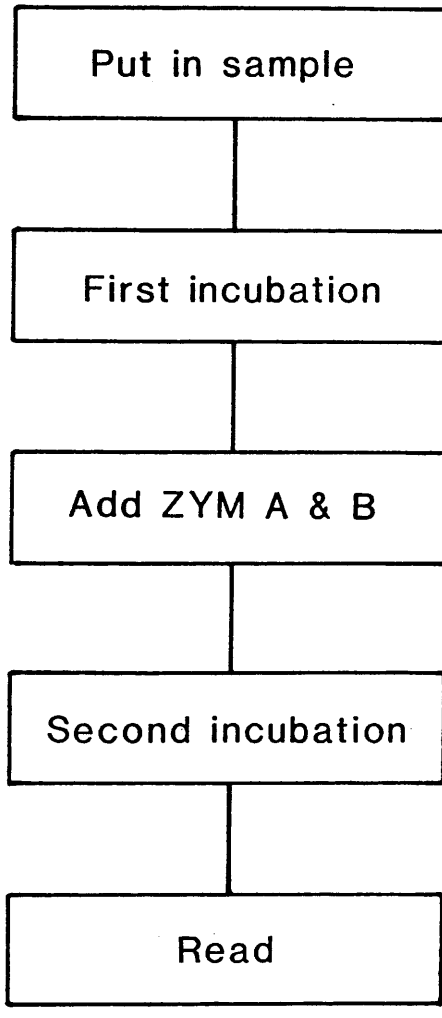


Figure 1

Flow diagram showing procedure adopted in the API ZYM experiments.

EXPERIMENT No. 4

Aim of experiment

The aim of this experiment was to test whether colour present in API cupules was produced by membrane filterable products and if so, whether these substances were heat stable or heat labile.

Materials and methods

Samples of overlying and interstitial water were collected and brought to the laboratory for immediate processing. Each sample was subdivided into four parts (Figure 2) and treated as follows:

1- Control sample (untreated).

2- Filtered through a 0.22um membrane filter.

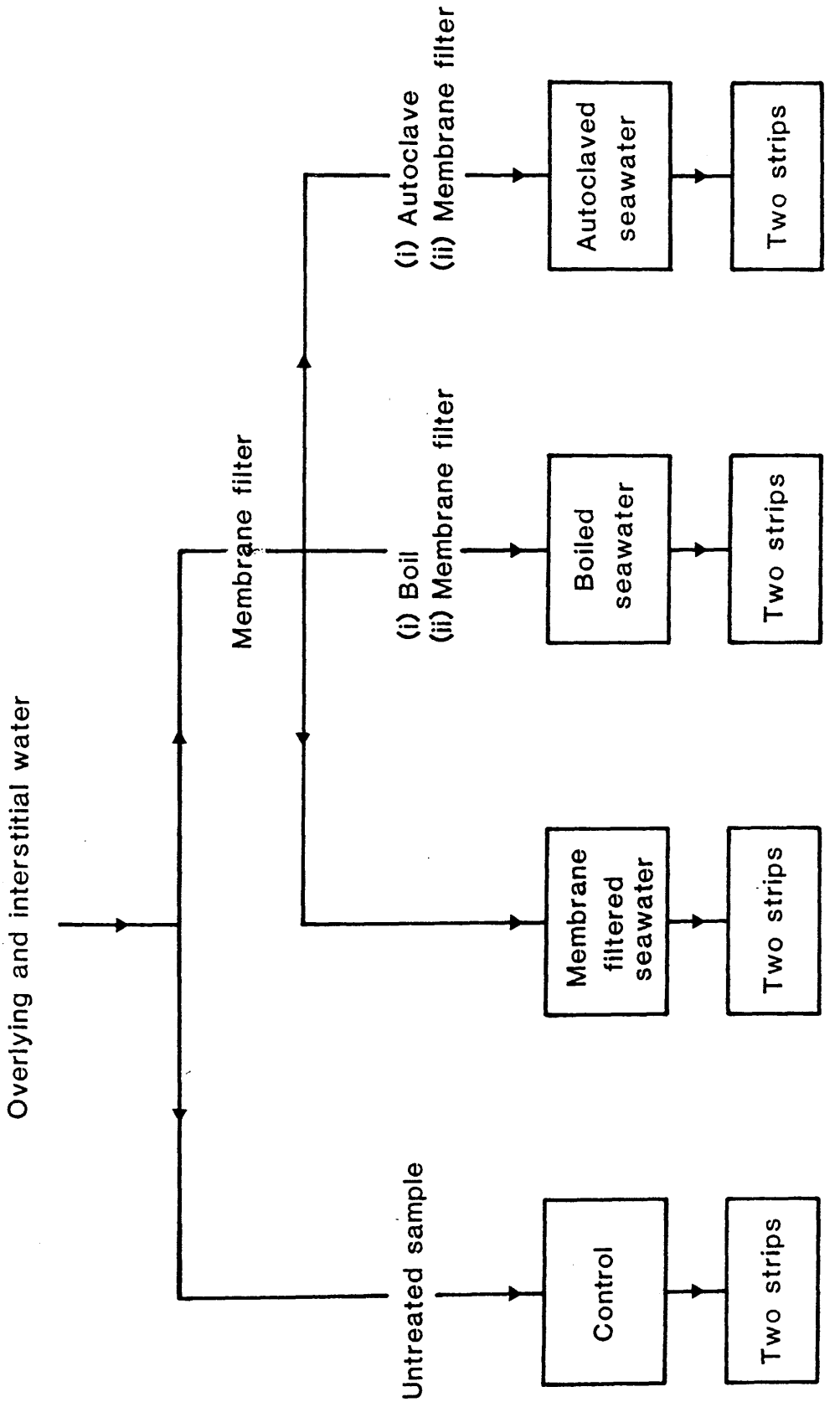
3- Membrane filtered, boiled for 15 minutes, cooled and re-filtered.

4- Membrane filtered, water was autoclaved at 121^{o}C for 15 minutes cooled and re-filtered using the same size of filter as described above.

Two drops of treated samples were dispensed into each API ZYM cupule using a pasteur pipette. About 5ml of tap water was dropped into the incubation trays to provide a humid atmosphere. Following inoculation of all the strips, plastic lids were placed on the trays and then they were incubated for 12 hours at 10^{o}C. Following incubation, one drop of reagent ZYM A and one drop of reagent ZYM B were added into each cupule. Trays were then re-incubated for 6 hours at 10^{o}C. All strips were then read against a reading scale and results were recorded.

Figure 2

Flow diagram showing how overlying and interstitial water samples were subdivided and treated before being tested using the API ZYM system.


Overlying and interstitial water
Untreated sample
Control
Two strips
Membrane filter
(i) Boil
(ii) Membrane filter
Boiled seawater
Two strips
Membrane filtered seawater
Two strips
(i) Autoclave
(ii) Membrane filter
Autoclaved seawater
Two strips

API 20E and 20NE

EXPERIMENT A

Aim of experiment

The aim of this experiment was to test the effect of bacterial cell concentrations on the API system. It is important to obtain a growth curve showing the phase at which the culture must be inoculated into the API system.

Materials and methods

Preparation of broth medium

The cultural broth medium used in this experiment was seawater yeast peptone medium. The composition of this medium was as follows:

Yeast extract ..... 0.3% (w/v)

Peptone 0.5%

Filtered, aged seawater ..... 75% (v/v)

Distilled water 25%

The ingredients of this medium were dissolved by boiling 3-5 minutes than filtered using Millex-HA 0.45um and the pH was adjusted to 7.5. Sterilization was achieved by autoclaving at 121^{o}C for 15 minutes.

Culture method

The marine strains, Vibrio fischeri and Micrococcus sp. and the non-marine strains, Aeromonas hydrophila and Escherichia coli were used. These cultures were resubcultured on ZoBell agar and incubated at 20^{o}C for 7 days. Good-sized distinct colonies was picked up from each culture and inoculated into 5 tubes of 10ml seawater yeast peptone medium. Two uninoculated tubes containing medium were used as controls to obtain "blank" readings. Following inoculation, the tubes were incubated at 20^{o}C and turbidity readings were taken every 6

hours. These readings were compared with MacFarland barium sulphate tubes until peak growth had clearly been reached. Figure 3 shows the procedures adopted in this experiment.

EXPERIMENT B

Aim of experiment

The aim of this experiment was to test how the API 20E and API 20NE systems work with marine and non-marine bacteria in relation to the oxidase reaction. This experiment also tested the effect of bacterial cell concentration on the kit-system reaction.

Materials and methods

The marine strains, Vibrio fischeri (oxidase positive) and Micrococcus sp. (oxidase negative) and the non-marine strains, Aeromonas hydrophila (oxidase positive) and Escherichia coli (oxidase negative) were resubcultured on ZoBell agar and incubated at 20°C for 48 hours. For the API 20E, each isolate was inoculated into sterile universal bottles containing 9ml sterile artificial seawater adjusted to 20 ‰ salinity. The concentration of the bacterial suspension was adjusted so that it's turbidity was equivalent to that of the No. 5 MacFarland tubes. A series of dilutions were made and API 20E strips were inoculated from each dilution. For the API 20NE, each isolate was inoculated into 3ml of 8.5 ‰ sterile NaCl as the initial suspension and a series of dilutions were made after the cell concentration was adjusted to 5 on the MacFarland turbidity scale. Using sterile pipettes, the first 8 tests of the API 20NE strip were inoculated with the saline suspension. 200µl of the remaining saline suspension was transferred by micropipette into the assimilation medium provided. The 12 assimilation tests were inoculated with the medium filling both the

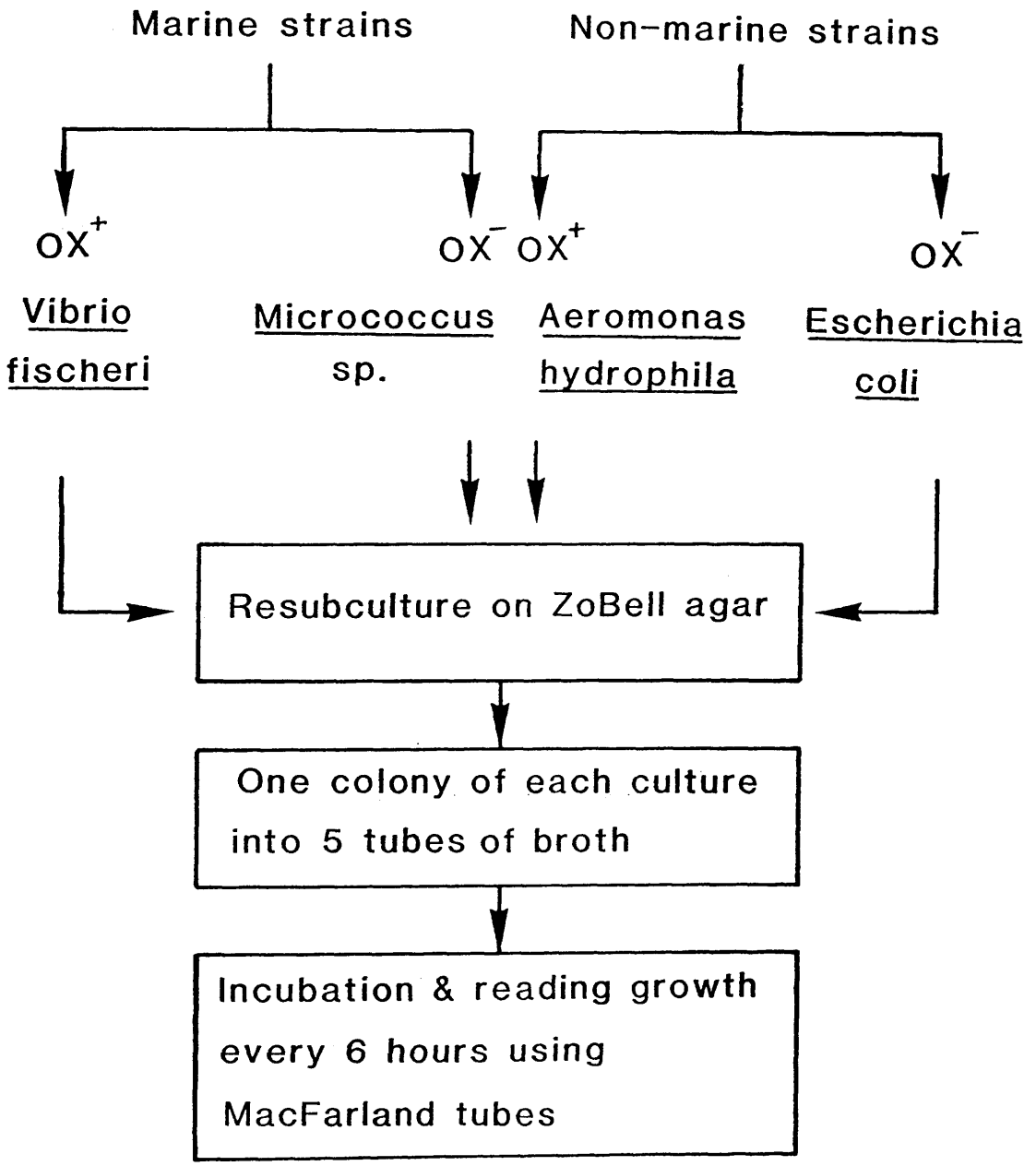


Figure 3

Flow diagram showing procedure adopted in experiment to test the effect of bacterial cell concentrations on the API system and obtaining a growth curve. OX = Oxidase test.

tube and cupule sections of the microtubes so as to leave a flat or slightly convex surface. Following inoculation, some tests were covered with mineral oil and strips were incubated at 20°C. After 24, 48, 72 and 96 hours, the strips were read and the results recorded.

EXPERIMENT C

Aim of experiment

The aim of this experiment was to test the effect of the number of Escherichia coli cells and incubation time on the API 20E system.

Materials and methods

This study was divided into two experiments, preliminary and definitive experiments.

Preliminary experiment

Tubes containing Escherichia coli used in the growth curve experiment (see above, experiment A) were used in the preliminary study to test the effect of bacterial cell numbers and incubation time on the API 20E tests. The procedure was carried out with centrifuging tubes containing culture. The cultures were centrifuged at 1250 'g' for 15 minutes. The supernatant was then removed and 10ml of sterile artificial seawater was added and the tubes shaken to re-suspend the bacterial cells. Two dilutions, 1:10 and 1:100, were used to inoculate 2 API strips. Following inoculation, strips were incubated at 20°C for 24 hours before being read. The cupules containing no added reagents were cut off and reincubated prior to reading after 48 and 96 hours.

Definitive experiment

One colony of Escherichia coli was inoculated into 10ml of seawater yeast peptone broth and incubated at 20°C for 12 hours. The

inoculated medium was centrifuged for 15 minutes at 1250 'g' to spin down the supernatant and the sedimented culture was then washed into 10ml sterile artificial seawater (20 ‰). A series of dilutions were made from this culture (10^{0} to 10^{-4}) and duplicate strips of API 20E were inoculated from each dilution. Following inoculation, strips were incubated at 20^{o} and readings were taken after 24 hours. Tests containing no added reagents were cut off and reincubated at 20^{o}C for further readings at 32, 48 and 72 hours.

EXPERIMENT D

Aim of experiment

The aim of the experiment was to test whether the API 20NE system produced replicable results with the non marine isolate Aeromonas hydrophila when used by two separate workers.

Materials and methods

Four strips of API 20NE were inoculated with Aeromonas hydrophila. Bacterial cell concentrations equivalent to 0.5 and 5 on the MacFarland barium sulphate scale tubes were prepared and used to inoculate strips. This preliminary study was carried out by 2 workers and personal error was avoided by alternating tasks. Following inoculation, strips were incubated at 20^{o}C and readings were taken after 24, 48 and 120 hours.

Note:

MacFarland barium sulphate standard set. Difco Laboratory, Detroit, Michigan, U.S.A. Set of 10 tubes. Code no. 0691-32-6.

SECTION 3

RESULTS

Experiment 1

The results of the two replicate experiments are presented in table 2, and their statistical analysis is shown in table 3. The original results are given in appendix tables 1 and 2.

The variation in the amount of sediment dispensed in metal foil from the three pasteur pipettes was tested using two two-way analyses of variance.

In the two-way analysis comparing drops against pipettes (Table 3), no significant variation was found in the first replica experiment between drops and pipettes (both, $0.50>P>0.25$).

In the second replica experiment, there was also no significant variation between drops (Factor A; $0.75>P>0.50$) and pipettes (Factor B; $0.50>P>0.25$).

Experiment 2

The results of this experiment suggested that the highest amount of hydrolysed substrate occured after 10-20 hours in the first incubation time and 5-20 hours in the second incubation time at $10^{o}C$ as shown in figure 4 and appendix table 3.

Student's t-tests comparing the quantity of hydrolysed substrate in each API cupule at the first and second incubation times were carried out. The results are given in tables 4 and 5.

In general, differences were found between the first incubation times in all the cupules except between 10 x 20 in cupule 2; 2.5 x 5 in cupule 7; 2.5 x 5, 2.5 x 20 and 5 x 20 in cupule 8; 2.5 x 5, 2.5 x 20 and 5 x 20 in cupule 9; 2.5 x 5 in cupule 11 and 2.5 x 5 and 10 x 20 in cupule 12.

In the second incubation time, no significant variation was

Replicate Experiment 1

Drop	Pipette I		Pipette II		Pipette III	
	$\bar{X}$	s.d.	$\bar{X}$	s.d.	$\bar{X}$	s.d.
1	0.01695	± 0.002148	0.01631	± 0.003881	0.01871	± 0.002420
2	0.01620	± 0.003340	0.02013	± 0.001362	0.01666	± 0.01289
3	0.02267	± 0.008604	0.008843	± 0.001914	0.0878	± 0.1215
4	0.01183	± 0.007896	0.005583	± 0.001636	0.02076	± 0.006639

Replicate Experiment 2

Drop	Pipette I		Pipette II		Pipette III	
	$\bar{X}$	s.d.	$\bar{X}$	s.d.	$\bar{X}$	s.d.
1	0.01873	± 0.003435	0.01904	± 0.001314	0.01750	± 0.004908
2	0.02449	± 0.01113	0.02474	± 0.01341	0.01645	± 0.0007550
3	0.02056	± 0.002565	0.01508	± 0.002690	0.02101	± 0.004699
4	0.02094	± 0.0007353	0.01524	± 0.004695	0.01768	± 0.005575

Table 2: Weights (g) of intertidal sediment obtained from disposable glass Pasteur pipettes in the first, second, third and fourth drops from one filling. Means ± s.d. of 3 replicate readings.

Replicate Experiment 1

	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	3.982×10^{-3}	1.327×10^{-3}	3	1.051	0.50 > P > 0.25
Factor B	3.690×10^{-3}	1.845×10^{-3}	2	1.461	0.50 > P > 0.25
A/B interaction	7.363×10^{-3}	1.227×10^{-3}	6	-0.9716	0.50 > P > 0.25
Error	3.031×10^{-2}	1.263×10^{-3}	24		
Total	0.04535		35		

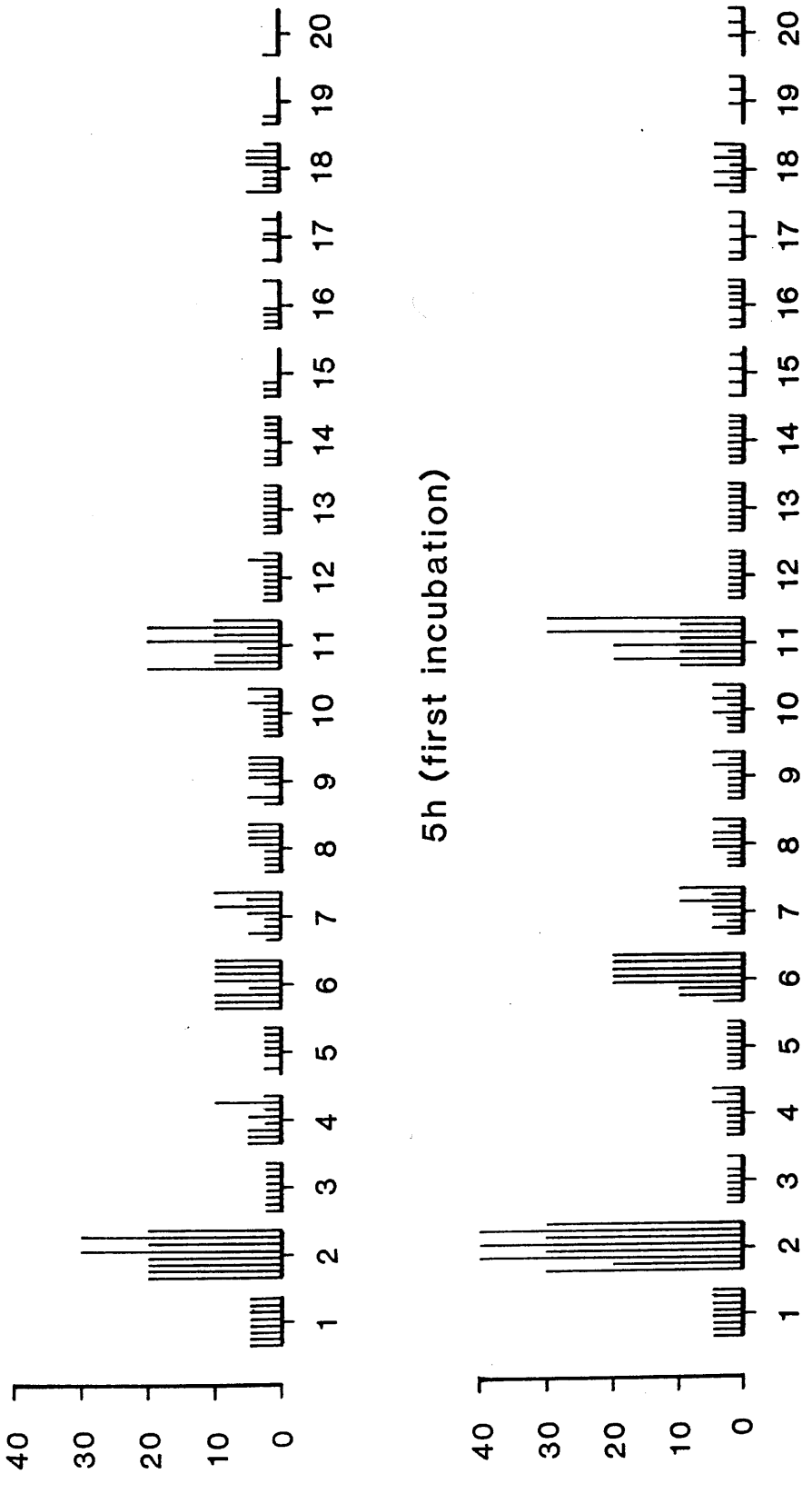
Replicate Experiment 2

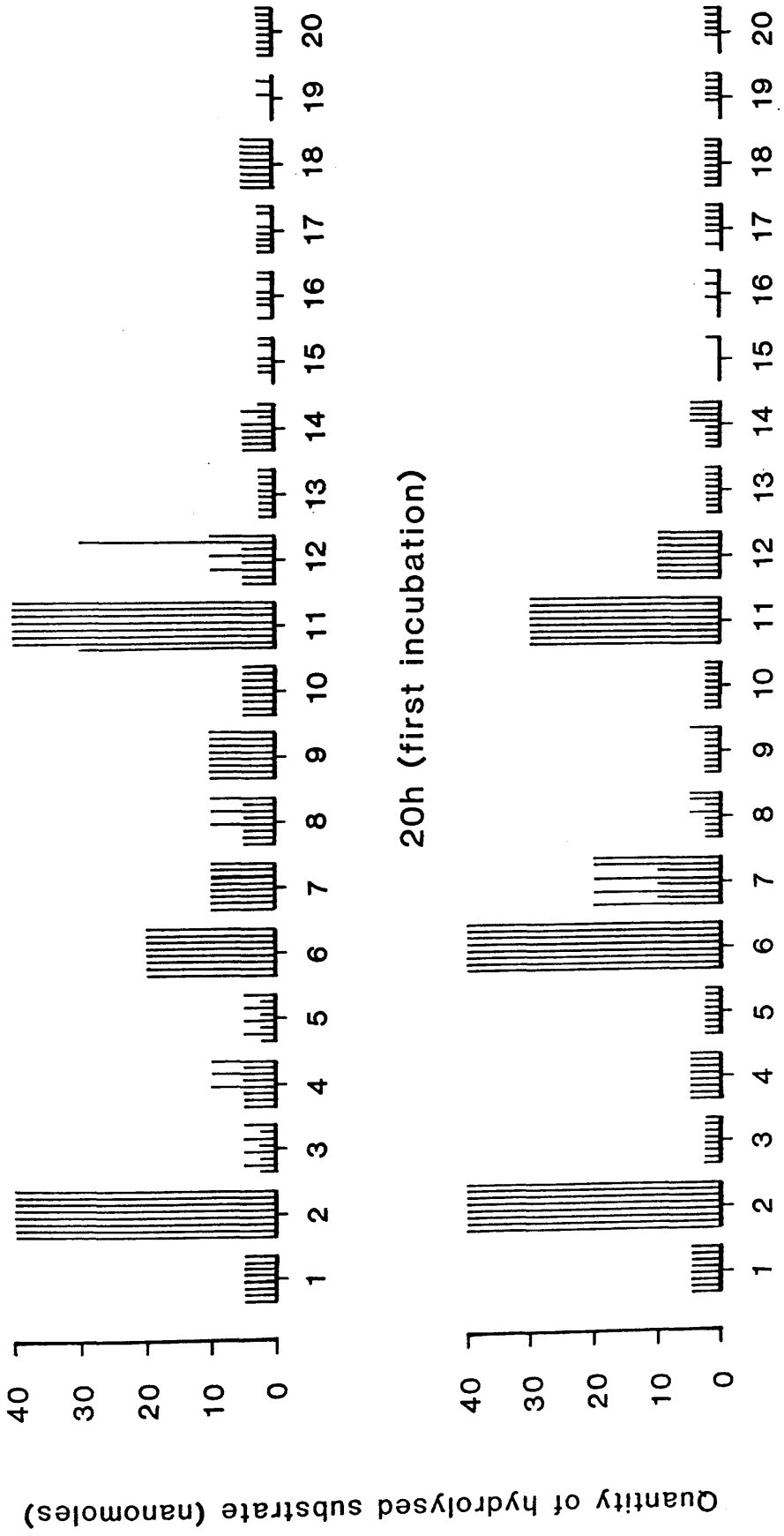
	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	8.535×10^{-5}	2.845×10^{-5}	3	0.7915	0.75 > P > 0.50
Factor B	6.522×10^{-5}	3.261×10^{-5}	2	0.9072	0.50 > P > 0.25
A/B interaction	1.868×10^{-4}	3.113×10^{-5}	6	0.8660	0.75 > P > 0.50
Error	8.626×10^{-4}	3.594×10^{-5}	24		
Total	1.200×10^{-3}		35		

Table 3: Pasteur pipette experiments 1 and 2. Two way analyses of variance testing differences between weights of sediment in drops 1-4 (Factor A) from 3 pipettes (Factor B).

Figure 4

Amount of hydrolysed substrate (nanomoles) in API ZYM cupules at each first and second incubation times (2.5, 5, 10 and 20 hours). First incubation time = before addition of reagents, second incubation time = after addition of reagents. Vertical lines represent the amount of hydrolysed substrate at the second incubation time. At each cupule, the 1st and 2nd lines represent 2.5 hours of second incubation time (replicates a and b); the 3rd and 4th lines represent 5 hours (replicates a and b); the 5th and 6th lines represent 10 hours (replicates a and b) and the 7th and 8th lines represent 20 hours of second incubation (replicates a and b).


2.5h (first incubation)
5h (first incubation)
Quantity of hydrolysed substrate (nanomoles)
0
10
20
30
40
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Cupule number (substrate number)


10h (first incubation)
20h (first incubation)
Quantity of hydrolysed substrate (nanomoles)
Cupule number (substrate number)
0
10
20
30
40
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20

Cupule Number	First incubation Time Comparison (hour)	Student t	d.f.	Probability (P)
	2.5 x 5	3.347	14	0.01 > P > 0.001
	2.5 x 10	10.69 *	7	P < 0.001
	2.5 x 20	10.69 *	7	P < 0.001
2	5 x 10	3.000 *	7	0.02 > P > 0.01
	5 x 20	3.000 *	7	0.02 > P > 0.01
	10 x 20	0	14	P > 0.9
	2.5 x 5	2.731 *	8	0.05 > P > 0.02
	2.5 x 10	16.99 *	7	P < 0.001
	2.5 x 20	48.97 *	7	P < 0.001
6	5 x 10	1.983 *	7	0.1 > P > 0.05
	5 x 20	11.06 *	7	P < 0.001
	10 x 20	748.4	14	P > 0.001
	2.5 x 5	0.2069	14	0.9 > P > 0.8
	2.5 x 10	4.254 *	7	0.01 > P > 0.001
	2.5 x 20	5.121	14	P < 0.001
7	5 x 10	4.249 *	7	0.01 > P > 0.001
	5 x 20	5.061	14	P < 0.001
	10 x 20	3.416 *	7	0.02 > P > 0.01
	2.5 x 5	0	14	P > 0.9
	2.5 x 10	3.035	14	0.01 > P > 0.001
	2.5 x 20	0.4745	14	0.7 > P > 0.6
8	5 x 10	3.035	14	0.01 > P > 0.001
	5 x 20	0.4745	14	0.7 > P > 0.6
	10 x 20	3.360 *	11	0.01 > P > 0.001
	2.5 x 5	0.7977	14	0.5 > P > 0.4
	2.5 x 10	9.350 *	7	P < 0.001
	2.5 x 20	1.270 *	11	0.3 > P > 0.2
9	5 x 10	16.79	7	P < 0.001
	5 x 20	0.6061	14	0.6 > P > 0.5
	10 x 20	22.96	7	P > 0.001
	2.5 x 5	1.158	14	0.3 > P > 0.2
	2.5 x 10	10.48	14	P < 0.001
	2.5 x 20	8.034 *	7	P < 0.001
11	5 x 10	6.298 *	10	P < 0.001
	5 x 20	3.981	7	0.01 > P > 0.001
	10 x 20	7.000 *	7	P > 0.001
	2.5 x 5	1.002 *	7	0.4 > P > 0.3
	2.5 x 10	3.199 *	8	0.02 > P > 0.01
	2.5 x 20	22.96 *	7	P < 0.001
12	5 x 10	3.416 *	7	0.02 > P > 0.01
	5 x 20	394.9 *	7	P > 0.001
	10 x 20	0.6832*	7	0.6 > P > 0.5

Table 4: Students t-tests comparing the amount of hydrolysed substrate in each API ZYM cupule at each first incubation time. First incubation time = before addition of developing reagents. * = unequal variance t-test.

Cupule Number	Second incubation Time Comparison (hour)	Student t	d.f.	Probability (P)
	2.5 x 5	0.5239	14	0.7 > P > 0.6
	2.5 x 10	0.8509	14	0.5 > P > 0.4
	2.5 x 20	0.8509	14	0.5 > P > 0.4
2	5 x 10	0.2977	14	0.8 > P > 0.7
	5 x 20	0.2977	14	0.8 > P > 0.7
	10 x 20	0	14	P > 0.9
	2.5 x 5	0.1855	14	0.9 > P > 0.8
	2.5 x 10	0.4906	14	0.7 > P > 0.6
	2.5 x 20	0.4906	14	0.7 > P > 0.6
6	5 x 10	0.3011	14	0.8 > P > 0.7
	5 x 20	0.3011	14	0.8 > P > 0.7
	10 x 20	0	14	P > 0.9
	2.5 x 5	0.1057	14	P > 0.9
	2.5 x 10	0.7157	14	0.5 > P > 0.4
	2.5 x 20	1.077	14	0.3 > P > 0.2
7	5 x 10	0.8129	14	0.5 > P > 0.4
	5 x 20	1.159	14	0.3 > P > 0.2
	10 x 20	0.4752	14	0.7 > P > 0.6
	2.5 x 5	0.9165*	10	0.4 > P > 0.3
	2.5 x 10	2.593	14	0.05 > P > 0.02
	2.5 x 20	2.593	14	0.05 > P > 0.02
8	5 x 10	1.048	14	0.4 > P > 0.3
	5 x 20	1.048	14	0.4 > P > 0.3
	10 x 20	0	14	P > 0.9
	2.5 x 5	0.3489	14	0.8 > P > 0.7
	2.5 x 10	0.3839	14	0.8 > P > 0.7
	2.5 x 20	0.5932	14	0.6 > P > 0.5
9	5 x 10	0.7234	14	0.5 > P > 0.4
	5 x 20	0.9285	14	0.4 > P > 0.3
	10 x 20	0.2072	14	0.9 > P > 0.8
	2.5 x 5	0.1012	14	P > 0.9
	2.5 x 10	0.4441	14	0.7 > P > 0.6
	2.5 x 20	0.4441	14	0.7 > P > 0.6
11	5 x 10	0.4840	14	0.7 > P > 0.6
	5 x 20	0.4840	14	0.7 > P > 0.6
	10 x 20	0	14	P > 0.9
	2.5 x 5	0.3567	14	0.8 > P > 0.7
	2.5 x 10	0.3567	14	0.8 > P > 0.7
	2.5 x 20	1.158	14	0.3 > P > 0.2
12	5 x 10	0	14	P > 0.9
	5 x 20	0.8721	14	0.4 > P > 0.3
	10 x 20	0.8721	14	0.4 > P > 0.3

Table 5: Students t-tests comparing the amount of hydrolysed substrate in each API ZYM cupule at each second incubation time. Second incubation time = after addition of developing reagents. * = unequal variance t-test.

found except in cupule 8 (2.5 x 10 and 2.5 x 20). The original results are shown in appendix table 3.

Experiment 3

The results of this experiment showed that some colour development of surface sediment was noticed immediately after reagents were added. Colour developed more slowly and to a lesser degree in deep than in surface sediment as shown in figure 5 and plate 1. Even after 12 hours of incubation, the amount of substrate hydrolysed by surface sediment was generally greater than for deep sediment.

The variation in nanomole scores between sampling depths and between number of cupules (Table 6) was tested statistically using a two-way analysis of variance. The results of this analysis are shown in table 7. Possible statistical interaction was found ($0.10>P>0.05$); therefore breakdown one-way analyses of variance were carried out. The results of these analyses are shown in table 8. A highly significant variation between cupules was found at a depth of 5 cm ($P<0.001$). At depths of 10cm, 20cm and 35cm, no significant variation between cupules was found (all, $0.50>P>0.25$). With the surface sediment, only possible variation between cupules was found ($0.10>P>0.05$).

A significant variation between depths was found at cupule 2 ($0.01>P>0.005$) and cupule 6 ($0.005>P>0.001$). At cupule 7 and 11, no significant variation between depths was found (both $0.25>P>0.10$).

The variation in time at which maximum scores were reached (Table 9) was analysed statistically using a two-way analysis of variance. The results are shown in table 10. A significant variation was found with depth (Factor A; $0.05>P>0.025$). No significant variation between cupules was found (Factor B; $0.75>P>0.50$).

Figure 5

Relationship between the quantity of hydrolysed substrate in API ZYM cupules (2, 6, 7 and 11) and time.

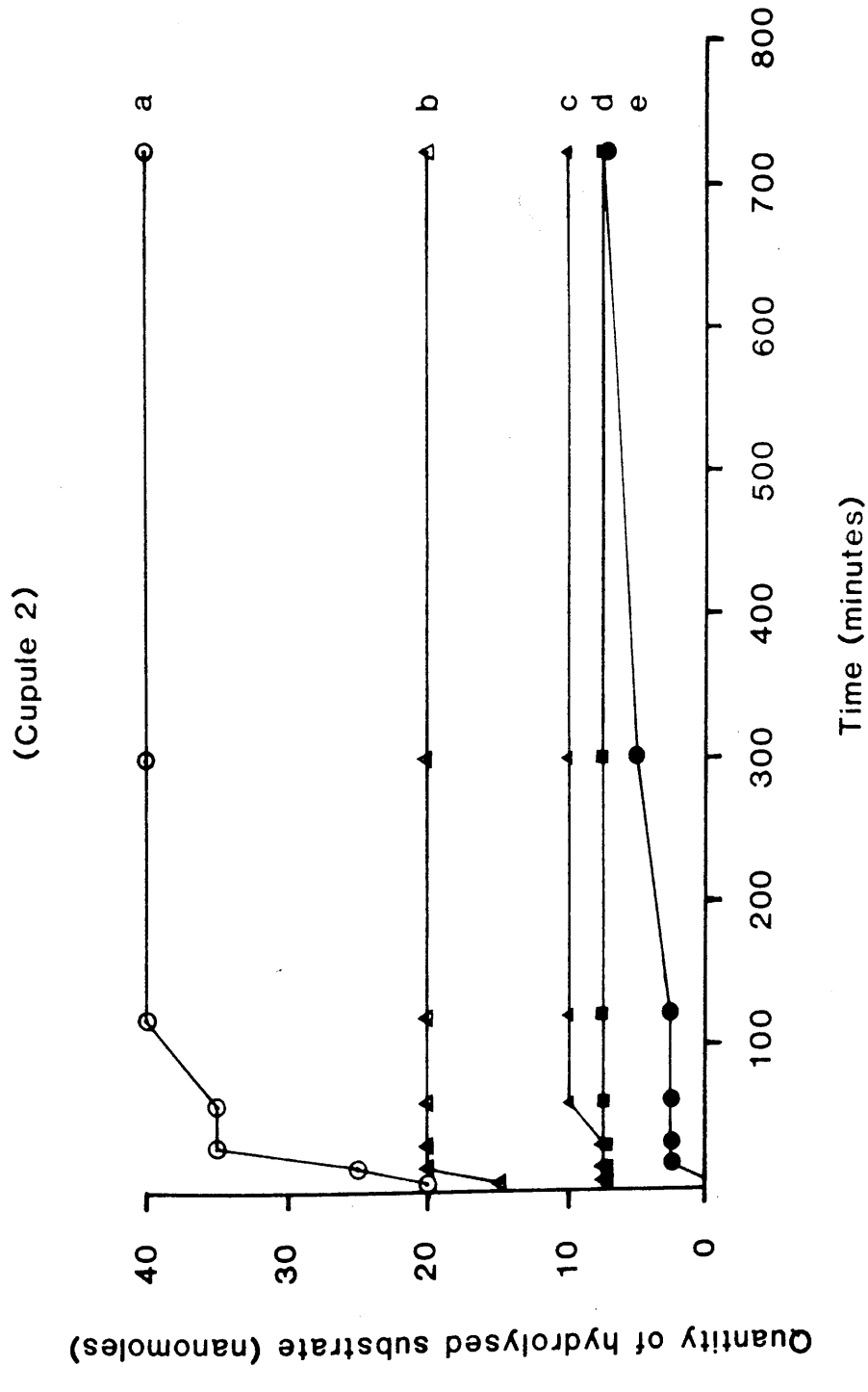
a- (o) surface sediment.

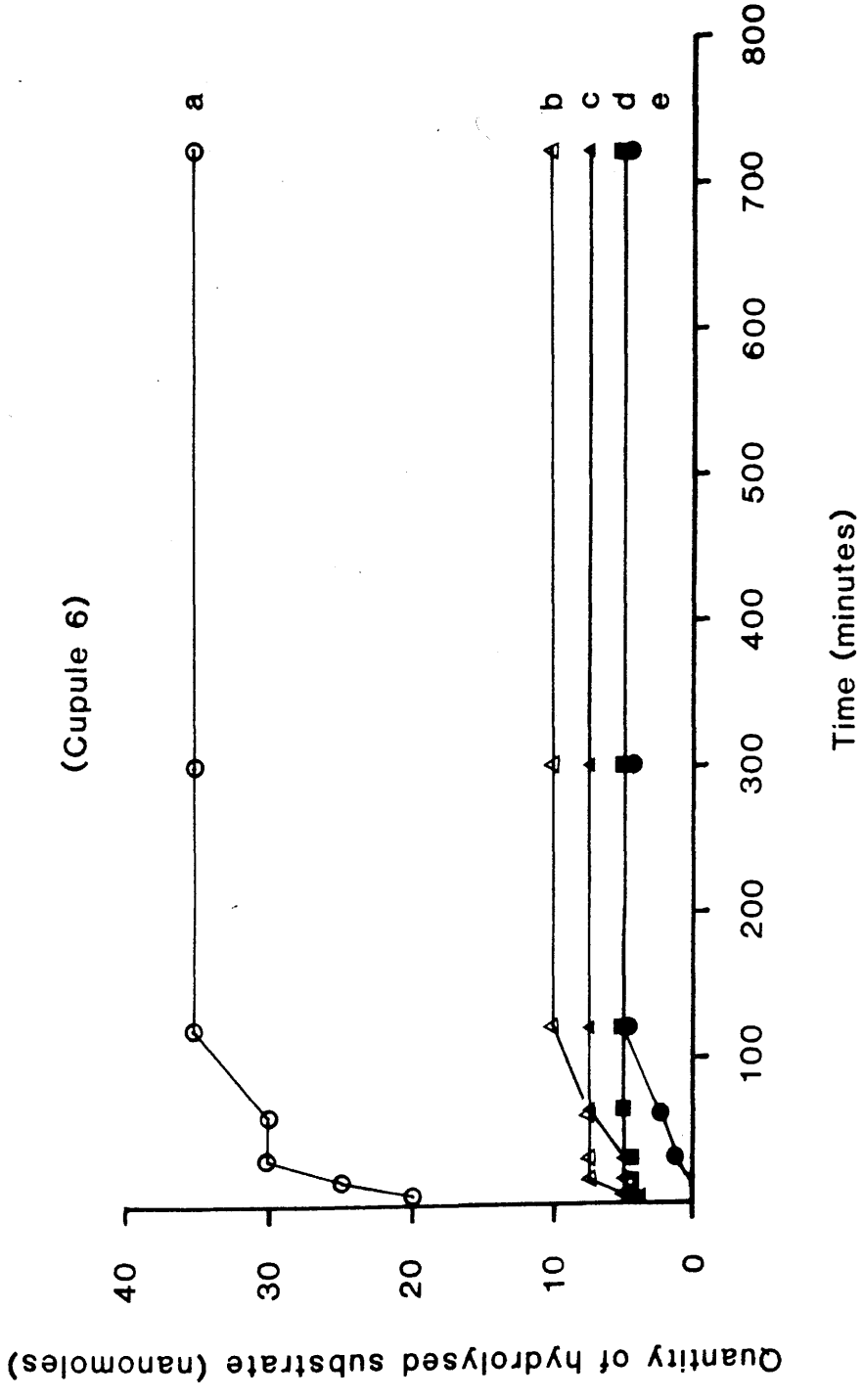
b- (Δ) sediment from 5cm depth.

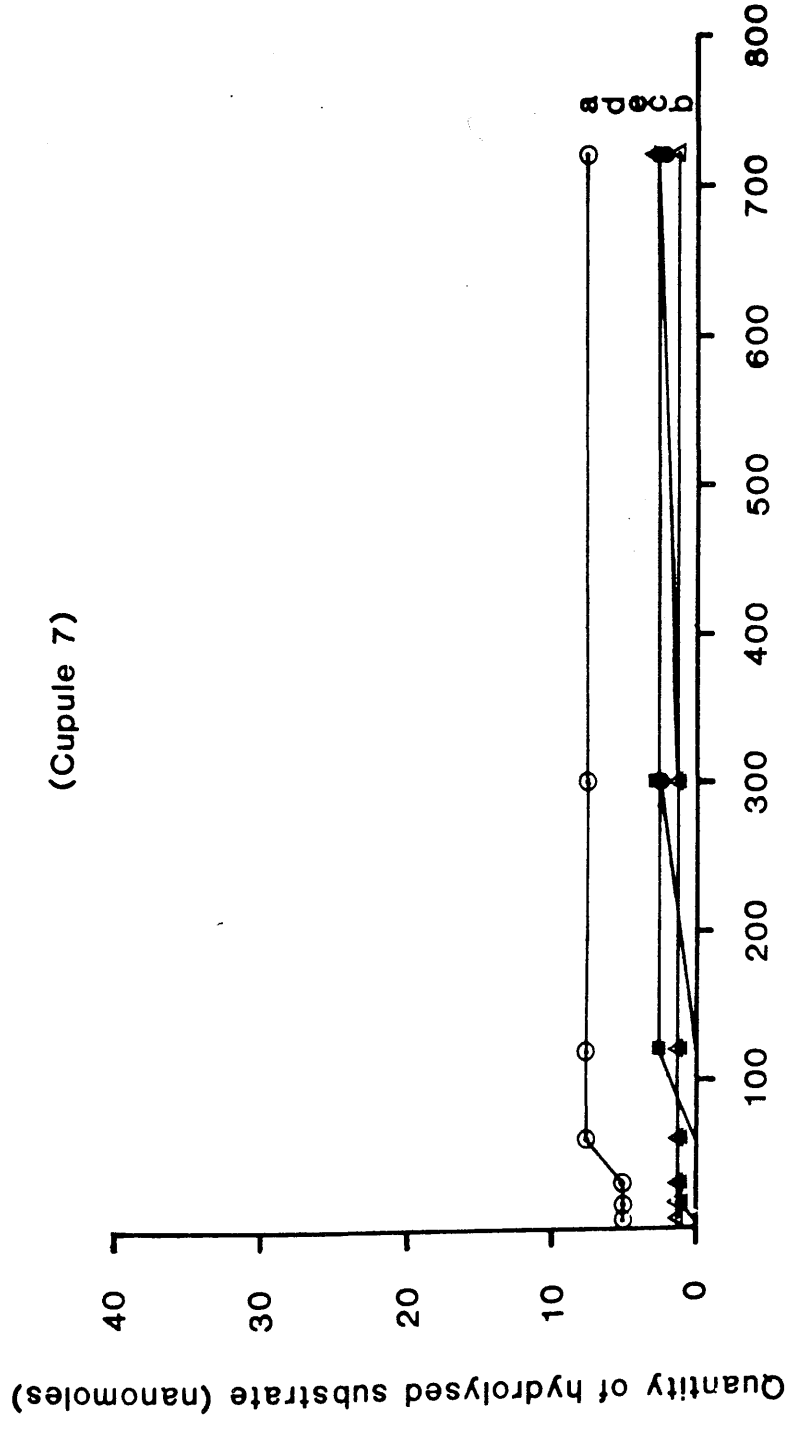
c- (▲) sediment from 10cm depth.

d- (■) sediment from 20cm depth.

e- (●) sediment from 35cm depth.


(Cupule 2)
Quantity of hydrolysed substrate (nanomoles)
40
30
20
10
0
100
200
300
400
500
600
700
800
Time (minutes)
a
b
c
d
e


(Cupule 6)
Quantity of hydrolysed substrate (nanomoles)
40
30
20
10
0
100
200
300
400
500
600
700
800
Time (minutes)
a
b
c
d
e


(Cupule 7)
Quantity of hydrolysed substrate (nanomoles)
40
30
20
10
0
100
200
300
400
500
600
700
800
Time (minutes)
a
d
e
c
b

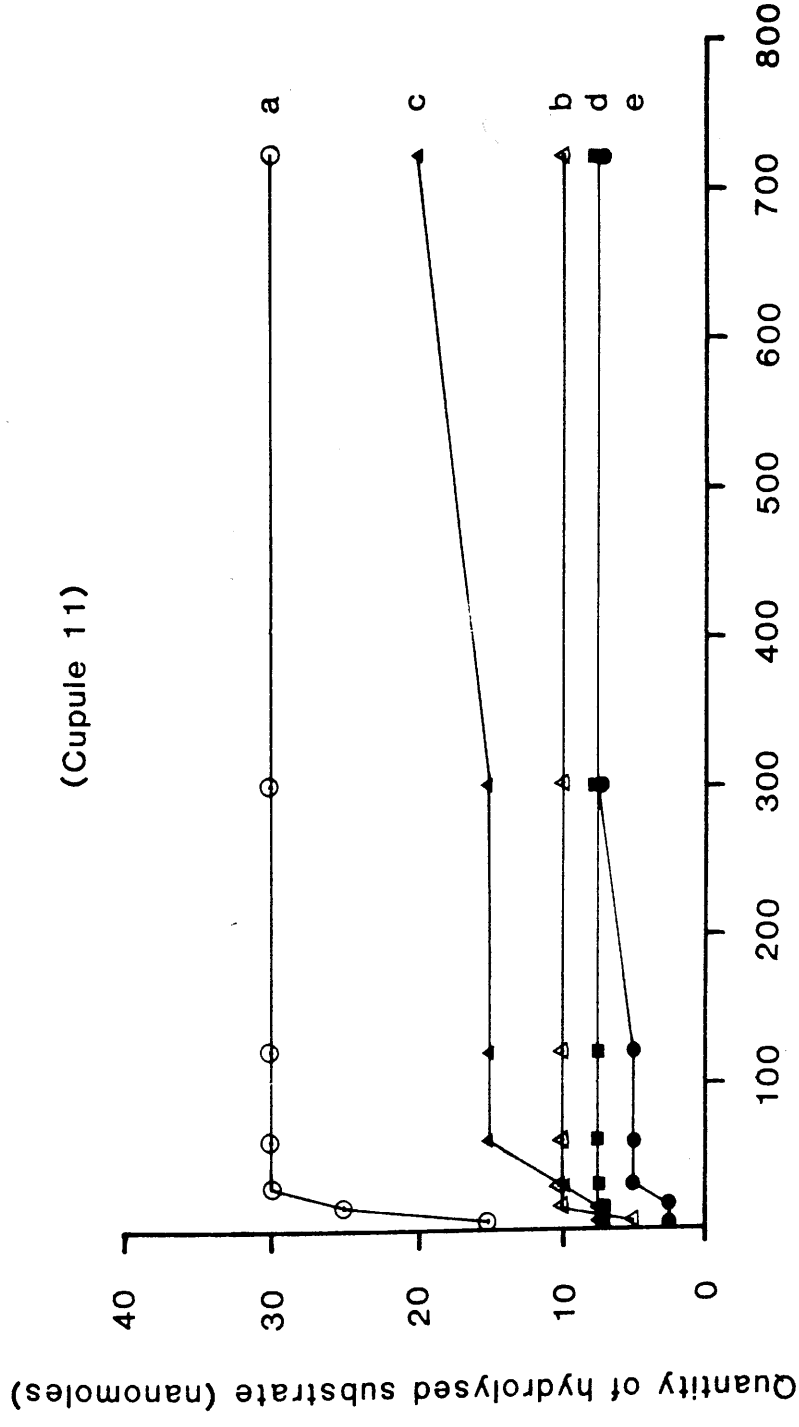

(Cupule 11)
Quantity of hydrolysed substrate (nanomoles)
40
30
20
10
0
100
200
300
400
500
600
700
800
Time (minutes)
a
c
b
d
e

Plate 1

API ZYM strips containing sediment from a range of depths (A = surface sediment, B = 5cm, C = 10cm, D = 20cm and E = 35cm).

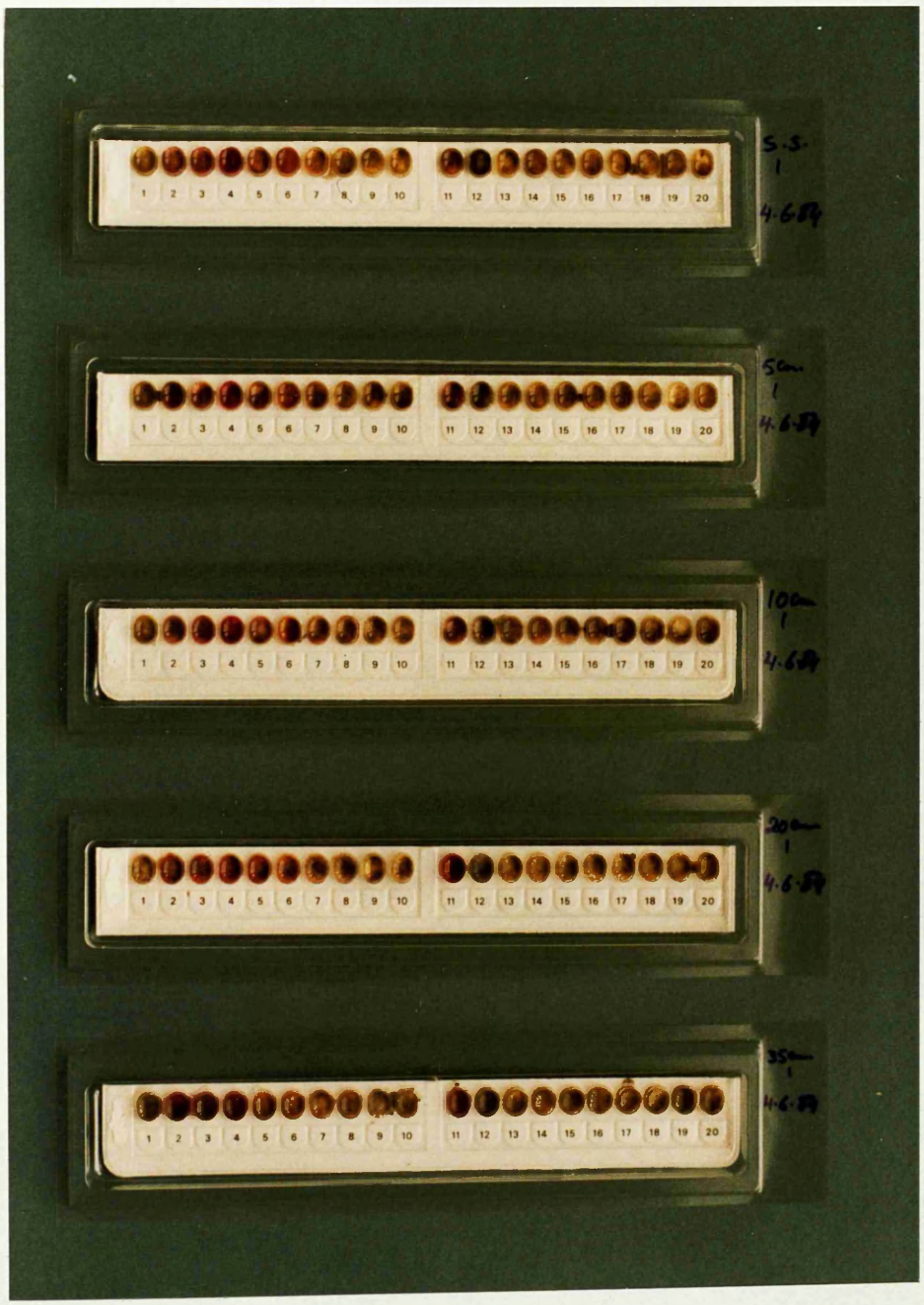
A

B

C

D

E



Sample depth (cm)	Replica	Cupule number			
		2	6	7	11
0	1	40	40	10	40
	2	40	30	5	20
5	1	20	10	-	10
	2	20	10	2.5	10
10	1	5	5	-	10
	2	20	10	5	30
20	1	10	5	2.5	10
	2	5	5	2.5	5
35	1	5	5	2.5	5
	2	10	5	2.5	10

Table 6: Shows the maximum nanomole scores for each strip (replicas 1 and 2) for cupules 2, 6, 7 and 11. - = zero score.

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	2768	692.0	4	21.19	P<0.001
Factor B	1160	386.8	3	11.85	P<0.001
A/B interaction	829.4	69.11	12	2.116	0.10>P>0.05
Error	653.1	32.66	20	-	-
Total	5411	-	39	-	-

Table 7: Two-way analysis of variance on maximum nanomole scores for each strip (replicas 1 and 2) for cupules 2, 6, 7 and 11. Factor A = sediment depths (surface sediment, 5cm, 10cm, 20cm and 35 cm), Factor B = cupules (2, 6, 7 and 11).

		Factor	Sum of squares (SS)	Mean squares (MS)	d.f.	F- Ratio	Probability (P)
Depth (cm)	0	Main Error Total	1234 262.5 1497	411.5 65.63	3 4 7	6.270	0.10>P>0.05
	5	Main Error Total	352.3 3.125 355.5	117.4 0.781	3 4 7	150.3	P<0.001
	10	Main Error Total	334.4 337.5 671.9	111.5 84.38	3 4 7	1.321	0.50>P>0.25
	20	Main Error Total	34.38 25 59.38	11.46 6.25	3 4 7	1.833	0.50>P>0.25
	35	Main Error Total	34.38 25 59.38	11.46 6.25	3 4 7	1.833	0.50>P>0.25

Table 8: One-way analysis of variance on maximum nanomole scores for each strip (replicas 1 and 2) for cupules 2, 6, 7 and 11.

Sample depth (cm)	Replica	Cupule number			
		2	6	7	11
0	1	30	120	60	30
	2	120	30	5	15
5	1	15	120	-	15
	2	5	15	5	15
10	1	5	15	-	60
	2	60	60	720	720
20	1	5	5	120	5
	2	5	5	120	5
35	1	300	120	300	30
	2	720	120	300	300

Table 9: Time in minutes at which maximum score was reached for each strip separately (replicas 1 and 2) for cupules 2, 6, 7 and 11. - = no reading because zero score throughout experiment.

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	418041	104510	4	3.394	0.05>P>0.025
Factor B	54392	18130	3	0.589	0.75>P>0.50
A/B interaction	392738	32728	12	1.063	0.50>P>0.25
Error	615875	30793	20	-	-
Total	1481047	-	39	-	-

Table 10: Two-way analysis of variance on time at which maximum score was reached (replicas 1 and 2) for cupules 2, 6, 7 and 11. Factor A = sediment depths (surface sediment, 5cm, 10cm, 20cm and 35cm), Factor B = cupules (2, 6, 7 and 11).

Experiment 4

The results of this experiment showed higher intensity colour in cupules containing untreated and membrane filtered samples than in samples exposed to heat (boiled or autoclaved), especially in interstitial water samples (Figure 6).

In general, there was little difference in the amount of hydrolysed substrate (colour) between the cupules containing untreated and membrane filtered overlying water (Plate 2), while there was a variation in the intensity of colour with untreated and membrane filtered interstitial water (Plate 3). For example, most cupules containing overlying water contained 5 nanomoles of hydrolysed substrate (Table 11) but in interstitial water samples, the range was from 5 to 30 nanomoles as shown in table 12.

The significance of the results were tested by a series of two-way and one-way analyses of variance, as follows.

Firstly, four two-way analyses of variance were conducted, two on the overlying water results and two on the interstitial water results. The first of each pair compared control with membrane filtered water (Factor A) and cupules 2, 4, 6, 7, and 11 (Factor B). The second of each pair compared boiled with autoclaved water (Factor A) and cupules 2, 4, 6, 7, and 11 (Factor B).

In the two-way analysis comparing control and membrane filtered overlying water against cupule number (Table 13A), significant variation was found between cupules (Factor B; $0.01>P>0.005$) but not between treatments (control vs filtered water) (Factor A; $0.50>P>0.25$).

Similarly, in the two-way analysis comparing boiled and autoclaved overlying water against cupule number (Table 13B), highly significant variation was found between cupules (Factor B; $P<0.001$) but not between treatments (boiled vs autoclaved water) (Factor A;

Figure 6

Effect of membrane filtration and heat on quantity of hydrolysed substrate in cupules 2, 4, 6, 7 and 11 for overlying and interstitial water. ■ = control, □ = membrane filtered, ▨ = 100°C heat, ▩ = 121°C heat (autoclave).

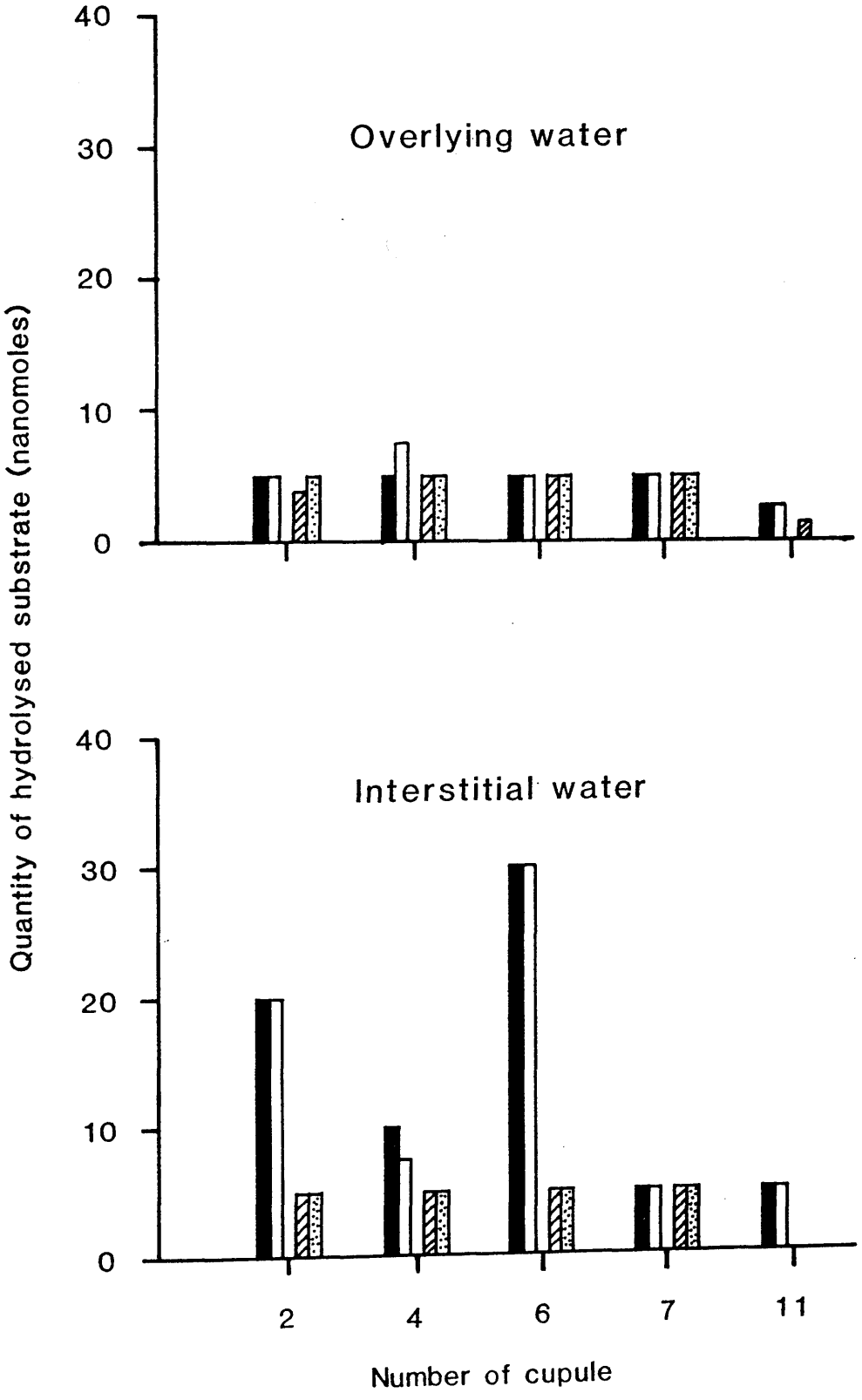

Overlying water
Interstitial water
Quantity of hydrolysed substrate (nanomoles)
40
30
20
10
0
2
4
6
7
11
Number of cupule

Plate 2

API ZYM strips containing overlying water. A = untreated (control), B = membrane filtered, C = boiled, and D = autoclaved.

D

C

B

A

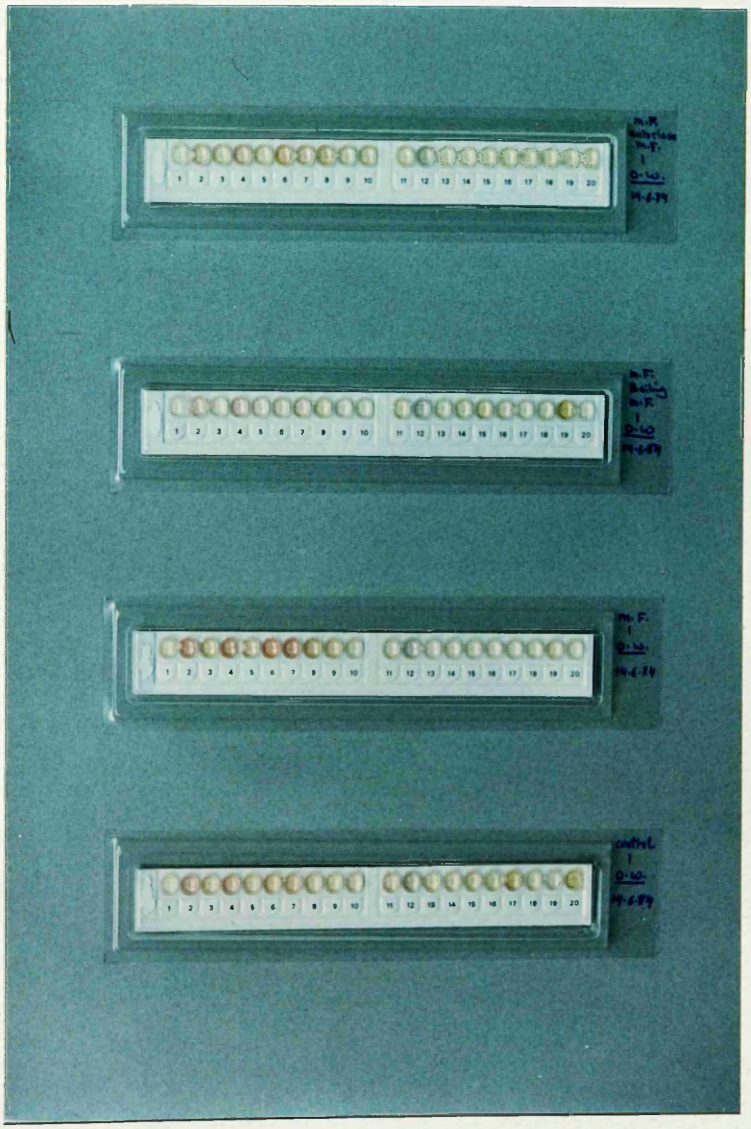


Plate 3

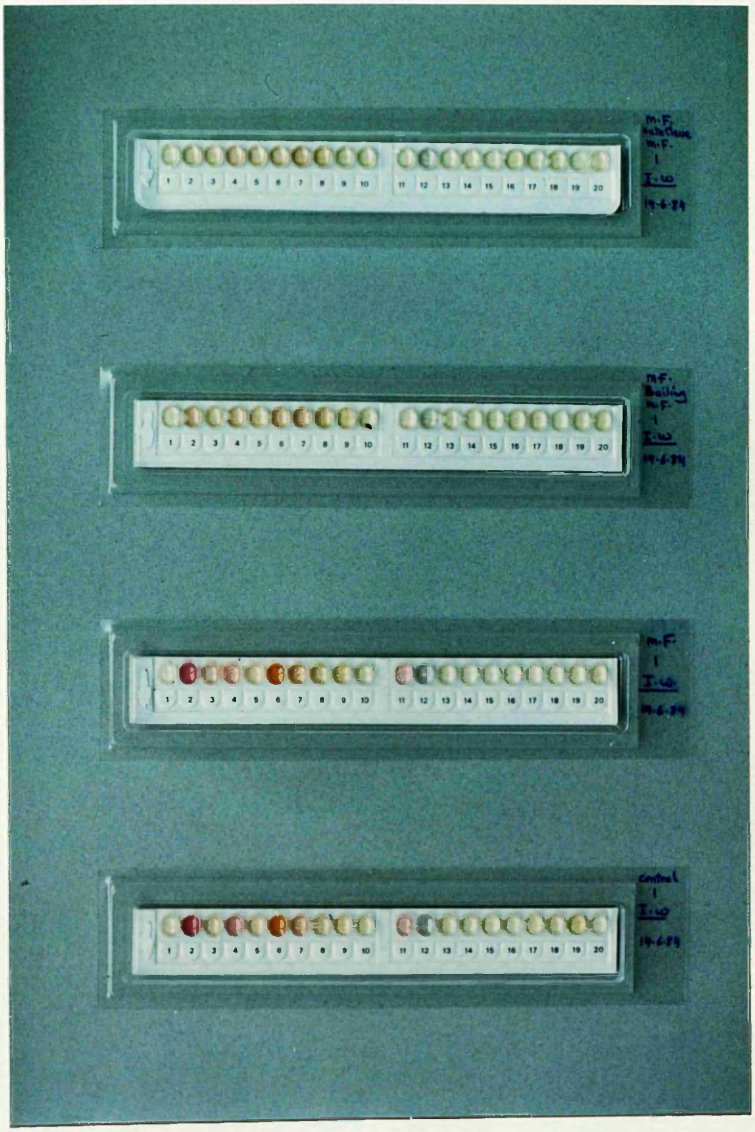
API ZYM strips containing interstitial water. A = untreated (control), B = membrane filtered, C = boiled, and D = autoclaved.

D

C

B

A



A

		Cupule				
		2	4	6	7	11
O.W.	Control	5.001	5.001	5.001	5.001	2.500
		4.999	4.999	4.999	4.999	2.499
	M.F.	5.001	5.000	5.001	5.001	2.500
		4.999	10.000	4.999	4.999	2.499

B

		Cupule				
		2	4	6	7	11
O.W.	Boil.	5.001	5.001	5.001	5.001	2.500
		2.500	4.999	4.999	4.999	0.0
	Auto.	5.001	5.001	5.001	5.001	0.001
		4.999	4.999	4.999	4.999	0.0

Table 11: Amount of hydrolysed substrates (nanomoles) in API ZYM cupules (2,4,6,7 and 11) inoculated with overlying water samples. A = control, membrane filtered. B = boiled, autoclaved.

A

		Cupule				
		2	4	6	7	11
I.W.	Control	20.001	10.001	30.001	5.001	5.001
		19.999	9.999	29.999	4.999	4.999
	M.F.	20.001	10.000	30.000	5.001	5.001
		19.999	5.000	29.999	4.999	4.999

B

		Cupule				
		2	4	6	7	11
I.W.	Boil.	5.001	5.001	5.001	5.001	0.001
		4.999	4.999	4.999	4.999	0.0
	Auto.	5.001	5.001	5.001	5.001	0.001
		4.999	4.999	4.999	4.999	0.0

Table 12: Amount of hydrolysed substrates (nanomoles) in API ZYM cupules (2,4,6,7 and 11) inoculated with interstitial water samples. A = control, membrane filtered. B = boiled, autoclaved.

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	1.25	1.25	1	0.9999	0.50>P>0.25
Factor B	30.01	7.502	4	6.002	0.01>P>0.005
A/B interaction	5	1.25	4	0.9999	0.50>P>0.25
Error	12.50	1.250	10	-	-
Total	48.76	-	19	-	-

Table 13A: Two-way analysis of variance comparing control and membrane filtered overlying water against cupule number. Factor A = treatments (control, membrane filtered), Factor B = cupule number (2,4,6,7 and 11).

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	0	0	1	0	P>0.75
Factor B	58.12	14.53	4	23.24	P<0.001
A/B interaction	3.123	0.7806	4	1.248	0.50>P>0.25
Error	6.253	0.6253	10	-	-
Total	67.49	-	19	-	-

Table 13B: Two-way analysis of variance comparing boiled and autoclaved overlying water against cupule number. Factor A = treatments (boiled, autoclaved), Factor B = cupule number (2,4,6,7 and 11).

$P>0.75$).

In the two-way analysis comparing control and membrane filtered interstitial water against cupule number (Table 14A), highly significant variation was found between cupules (Factor B; $P<0.001$) but not between treatments (control vs filtered water) (Factor A; $0.25>P>0.10$).

Similarly, in the two-way analysis comparing boiled and autoclaved interstitial water against cupule number (Table 14B), highly significant variation was found between cupules (Factor B; $P<0.001$) but not between treatments (boiled vs autoclaved water) (Factor A; $P>0.75$).

These four two-way analyses of variance show that for both overlying water and interstitial water, there is no difference between control and membrane filtered seawater and no difference between boiled and autoclaved seawater. This allowed me to combine the control and membrane filtered data together and to combine the boiled and autoclaved data together for further statistical analyses. These analyses were designed to find out whether there was any difference between the control + membrane filtered results and the boiled + autoclaved results for the different cupules.

A two-way analysis of variance was applied to the overlying water comparing control + membrane filtered with boiled + autoclaved water (Factor A) and cupules (Factor B). Factor A and Factor B were both highly significant (Table 15).

A similar two-way analysis of variance was applied to the interstitial water data, however in this case there was highly significant interaction between the two factors (Table 16A). Five breakdown one-way analyses of variance were therefore applied to each of the five cupules. Each of these compared control + membrane

filtered water with boiled + autoclaved water. Four out of five comparisons were statistically significant (Table 16B) and showed that the cupules containing control or membrane filtered had more activity (deeper colours) than the cupules containing boiled or autoclaved water.

The overall conclusions from these statistical analyses are as follows. There are significant differences between the cupules. The activity is not affected by membrane filtration but is abolished by boiling or autoclaving.

Finally, the variation in colour development found with overlying and interstitial water (control and membrane filtered) (Table 17) was tested statistically using a one-way analysis of variance. The results of this analysis are shown in table 18. A highly significant variation between overlying and interstitial water samples was found at cupules 2, 6 and 11 (all, $P<0.001$). No significant variation between both samples was found at cupule 4 ($0.25>P>0.10$) and cupule 7 ($P>0.75$). This means that the activity tested for by cupules 2, 6, and 11, but not by cupules 4 and 7, is greater in interstitial water than in overlying water.

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	11.25	11.25	1	1.799	0.25>P>0.10
Factor B	1820	455	4	72.78	P<0.001
A/B interaction	20	5	4	0.7997	0.75>P>0.50
Error	62.52	6.252	10	–	–
Total	1913	–	19	–	–

Table 14A: Two-way analysis of variance comparing control and membrane filtered interstitial water against cupule number. Factor A = treatments (control, membrane filtered), Factor B = cupule number (2,4,6,7 and 11).

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	0	0	1	0	P>0.75
Factor B	79.98	20.00	4	1.176×10^{-7}	P<0.001
A/B interaction	0	0	4	0	P>0.75
Error	1.7×10^{-6}	1.7×10^{-6}	10	–	–
Total	79.98	–	19	–	–

Table 14B: Two-way analysis of variance comparing boiled and autoclaved interstitial water against cupule number. Factor A = treatments (boiled, autoclaved), Factor B = cupule number (2,4,6,7 and 11).

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	5.622	5.622	1	5.997	0.025>P>0.01
Factor B	82.82	20.70	4	22.09	P<0.001
A/B interaction	5.309	1.327	4	1.327	0.50>P>0.25
Error	28.13	0.9375	30	-	-
Total	121.9		39	-	-

Table 15: Overlying water. two-way (2 x 5) analysis of variance (4 observations/cell) comparing control + membrane filtered (2 x 2=4 observations/cupule) and boiled + autoclaved (2 x 2=4 observations/cupule) water against cupule number. Factor A = treatments (control + membrane filtered and boiled + autoclaved), Factor B = cupule number (2, 4, 6, 7 and 11).

Source of variation	Sum of squares (SS)	Mean squares (MS)	d.f.	F	Probability (P)
Factor A	950.6	950.6	1	1521.0	-
Factor B	1177.5	294.4	4	471.0	-
A/B interaction	827.5	206.9	4	331.0	P<0.001
Error	18.75	0.6250	30	-	-
Total	2974.3	-	39	-	-

Table 16A: Interstitial water. Two-way (2 x 5) analysis of variance (4 observations/cell) comparing control + membrane filtered (2 + 2=4 observations/cupule) and boiled + autoclaved (2 + 2=4 observations/cupule) water against cupule number. Factor A = treatments (control + membrane filtered and boiled + autoclaved), Factor B = cupule number (2, 4, 6, 7 and 11).

		Factor	Sum of squares (SS)	Mean squares (MS)	d.f.	F-Ratio	Probability
Cupule	2	Main	450	450	1	3.375×10^8	P<0.001
		Error	0	0	6		
		Total	450		7		
	4	Main	28.13	28.13	1	9.00	0.025>P>0.01
		Error	18.75	3.125	6		
		Total	46.88		7		
	6	Main	1250	1250	1	1.111×10^9	P<0.001
		Error	0	0	6		
		Total	1250		7		
	7	Main	0	0	1	0	P>0.75
		Error	0	0	6		
		Total	0		7		
	11	Main	49.99	49.99	1	5.999×10^7	P<0.001
		Error	0	0	6		
		Total	49.99		7		

Table 16B: Interstitial water. Breakdown one-way (1 x 2) analysis of variance (4 observations/cell) comparing control + membrane filtered with boiled + autoclaved water for cupules 2, 4, 6, 7 and 11.

Cupule	O.W. (c + m.F.)		I.W. (c + m.F)	
2	5.001	5.001	20.001	20.001
	4.999	4.999	19.999	19.999
4	5.001	5.000	10.001	10.000
	4.999	10.000	9.999	5.000
6	5.001	5.001	30.001	30.001
	4.999	4.999	29.999	29.999
7	5.001	5.001	5.001	5.001
	4.999	4.999	4.999	4.999
11	2.500	2.500	5.001	5.001
	2.499	2.499	4.999	4.999

Table 17: Amount of hydrolysed substrates (nanomoles) in API ZYM cupules (2,4,6,7 and 11) inoculated with overlying and interstitial water (control, membrane filtered). O.W. = overlying water, I.W. = interstitial water, C = control, and M.F. = membrane filtered.

		Factor	Sum of squares (SS)	Mean squares (MS)	d.f.	F- Ratio	Probability (P)
Cupule	2	Main Error Total	450 8.0×10^{-6} 450	450 1.333×10^{-6}	1 6 9	3.375×10^{8}	P>0.001
	4	Main Error Total	12.5 37.50 50.00	12.5 6.250	1 6 7	2.00	0.25>P>0. 10
	6	Main Error Total	1250 8.0×10^{-6} 1250	1250 1.333×10^{-6}	1 6 7	9.375×10^{8}	P>0.001
	7	Main Error Total	0 8.0×10^{-6} 8.0×10^{-6}	0 1.333×10^{-6}	1 6 7	0	P>0.75
	11	Main Error Total	12.51 5.0×10^{-6} 12.51	12.51 8.333×10^{-7}	1 6 7	1.501×10^{7}	P>0.001

Table 18: One-way analysis of variance comparing colour development between overlying and interstitial water (control, membrane filtered).

Experiment A

The bacterial growth rate of the marine strains, Vibrio fischeri, Micrococcus and the non-marine strains, Aeromonas hydrophila, and Escherichia coli was determined (Figure 7) after 6, 12, 18, 24, 30 and 36 hours. It was found that the number of cells increased with increasing incubation time up to 12 hours, but there was a greater increase in the number of cells with the non-marine strains than with the marine strains. For example, Escherichia coli and Aeromonas hydrophila showed an average number of cells after 12 hours of 54×10^7 and 24×10^7 respectively, whereas Vibrio fischeri and Micrococcus showed only an average number of 15×10^7 (Table 19).

In general, the log phase of all strains started just before 6 hours, with the exception of Escherichia coli which reached this stage slightly earlier.

The stationary phase of Vibrio fischeri, Micrococcus and Escherichia coli was always reached after 12 hours. The strain of Aeromonas hydrophila used in the experiment had a transitional period between it's log and stationary phases. The stationary phase was reached after 18 hours.

Experiment B

The results of this experiment are divided into two parts as follows:

1- API 20E system.

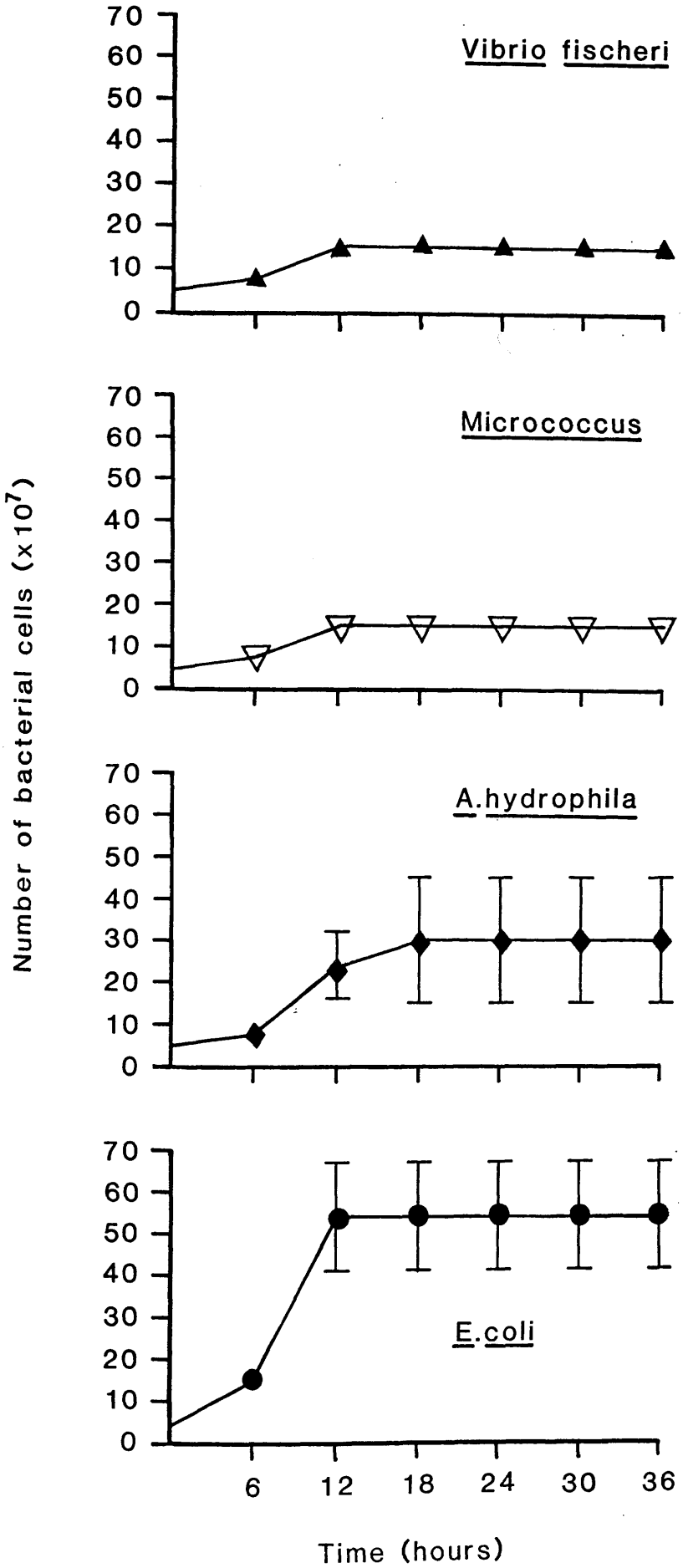
2- API 20NE system.

1- **API 20E system**

The results of the API 20E system showed that after 24 hours of incubation, the strain Vibrio fischeri gave some positive results (Table 20). The glucose test showed positive results after 24 hours, therefore developing reagents were added. It was also found in some

Figure 7

Bacterial growth rate of the marine strains, Vibrio fischeri, Micrococcus sp. and the non-marine strains, Aeromonas hydrophila and Escherichia coli after 6, 12, 18, 24, 30 and 36 hours.


Vibrio fischeri
Micrococcus
A.hydrophila
E.coli
Number of bacterial cells (x10⁷)
Time (hours)
0
10
20
30
40
50
60
70
6
12
18
24
30
36

Time (hour)		Number of cells and equivalent McFarland scale values																				
		Vibrio fischeri					*Micrococcus* sp.					*Aeromonas hydrophila*					*Escherichia coli*					Control
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	
	M.C	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	
0	B.C	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	0 0
		$5 \times 10^7 \pm 0$					$5 \times 10^7 \pm 0$					$5 \times 10^7 \pm 0$					$5 \times 10^7 \pm 0$					
	M.C	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.5	0.5	
6	B.C	75	75	75	75	75	75	75	75	75	75	75	75	75	75	75	150	150	150	150	150	0 0
		$7.5 \times 10^7 \pm 0$					$7.5 \times 10^7 \pm 0$					$7.5 \times 10^7 \pm 0$					$15 \times 10^7 \pm 0$					
	M.C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1	0.5	1	0.5	2	2	2	1	2	
12	B.C	150	150	150	150	150	150	150	150	150	150	300	300	150	300	150	600	600	600	300	600	0 0
		$15 \times 10^7 \pm 0$					$15 \times 10^7 \pm 0$					$24 \times 10^7 \pm 8.216 \times 10^7$					$54 \times 10^7 \pm 13.42 \times 10^7$					
	M.C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5	1	0.5	1.5	0.5	2	2	2	1	2	
18	B.C	150	150	150	150	150	150	150	150	150	150	450	300	150	450	150	600	600	600	300	600	0 0
		$15 \times 10^7 \pm 0$					$15 \times 10^7 \pm 0$					$30 \times 10^7 \pm 15 \times 10^7$					$54 \times 10^7 \pm 13.42 \times 10^7$					
	M.C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5	1	0.5	1.5	0.5	2	2	2	1	2	
24	B.C	150	150	150	150	150	150	150	150	150	150	450	300	150	450	150	600	600	600	300	600	0 0
		$15 \times 10^7 \pm 0$					$15 \times 10^7 \pm 0$					$30 \times 10^7 \pm 15 \times 10^7$					$54 \times 10^7 \pm 13.42 \times 10^7$					
	M.C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5	1	0.5	1.5	0.5	2	2	2	1	2	
30	B.C	150	150	150	150	150	150	150	150	150	150	450	300	150	450	150	600	600	600	300	600	0 0
		$15 \times 10^7 \pm 0$					$15 \times 10^7 \pm 0$					$30 \times 10^7 \pm 15 \times 10^7$					$54 \times 10^7 \pm 13.42 \times 10^7$					
	M.C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1.5	1	0.5	1.5	0.5	2	2	2	1	2	
36	B.C	150	150	150	150	150	150	150	150	150	150	450	300	150	450	150	600	600	600	300	600	0 0
		$15 \times 10^7 \pm 0$					$15 \times 10^7 \pm 0$					$30 \times 10^7 \pm 15 \times 10^7$					$54 \times 10^7 \pm 13.42 \times 10^7$					

Table 19

Number of bacterial cells (equivalent to specific McFarland scale values) of *Vibrio fischeri*, *Micrococcus* sp., *Aeromonas hydrophila*, and *Escherichia coli*, obtained after 0 to 36 hours incubation. Five replicate tubes were set up for each species, and the mean and standard deviation are shown with the original values for each tube.

M.C = McFarland scale value. B.C. = bacterial cell concentration (millions).

Conc.	Incub. time(hr)	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NIT
	24	+	-	+	+	-	-	+w	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+
5	48		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	72		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	96		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	24	+	-	+	+	-	-	+w	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+
0.5	48		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	72		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	96		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	24	+	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+
0.05	48		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	72		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	96		-	+	+	-	-	-				-		+	+	+	+	+	+	+	+		
	24	+	-	+	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	+
0.005	48		-	+	+	-	-	-				-		+	+	+	+	-	+	+	+		
	72		-	+	+	-	-	-				-		+	+	+	+	-	+	+	+		
	96		-	+	+	-	-	-				-		+	+	+	+	-	+	+	+		

Table 20

API 20 E profiles of the marine species Vibrio fischeri obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +w =weak positive, - =negative reaction.

tests that there was a decrease in the colour intensity with increasing dilutions of cell suspension. This was obvious after 24 hours with the tests ONPG, lysine decarboxylase (LDC), ornithine decarboxylase (ODC) and urease (URE) as shown in Plate 4.

The strain Micrococcus sp. gave no positive results in all the tests as shown in table 21 and Plate 5.

With the strain Aeromonas hydrophila, there were some positive results (Table 22). In some tests, the colour intensity decreased with increasing dilutions of cell suspension (e.g. saccharose (SAC), glucose (GLU), and gelatinase (GEL) as shown in Plate 6.

The strain Escherichia coli, showed positive reactions in some tests (Table 23). In several tests, the colour intensity decreased with increasing dilutions of cell suspension. This was very clear in Plate 7 with the arabinose (ARA), melibiose (MEL), rhamnose (RHA), lysine decarboxylase (LDC) and ONPG tests.

After 48, 72 and 96 hours incubation, the strain Vibrio fischeri, Aeromonas hydrophila and Escherichia coli showed little change in the results except for the carbohydrate tests. This can be interpreted as an effect from the reagents added after 24 hours.

The strain Micrococcus sp. showed no reaction except for the carbohydrate tests which showed positive reactions only after 96 hours. This change proved that the recommended reagents added after 72 hours affected the carbohydrate tests.

In general, after 24 hours, the difference between API 20E profiles (Plate 8) of the four strains inoculated with concentrations equivalent to 5 on the MacFarland scale was as follows.

Plate 4

Decrease in colour intensity with increasing dilutions of cell suspensions of Vibrio fischeri using the API 20E system. Arrows (left to right) show the tests ONPG, lysin decarboxylase (LDC), ornithine decarboxylase (ODC) and urease (URE) respectively. 24 hours reading. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

Plate 5

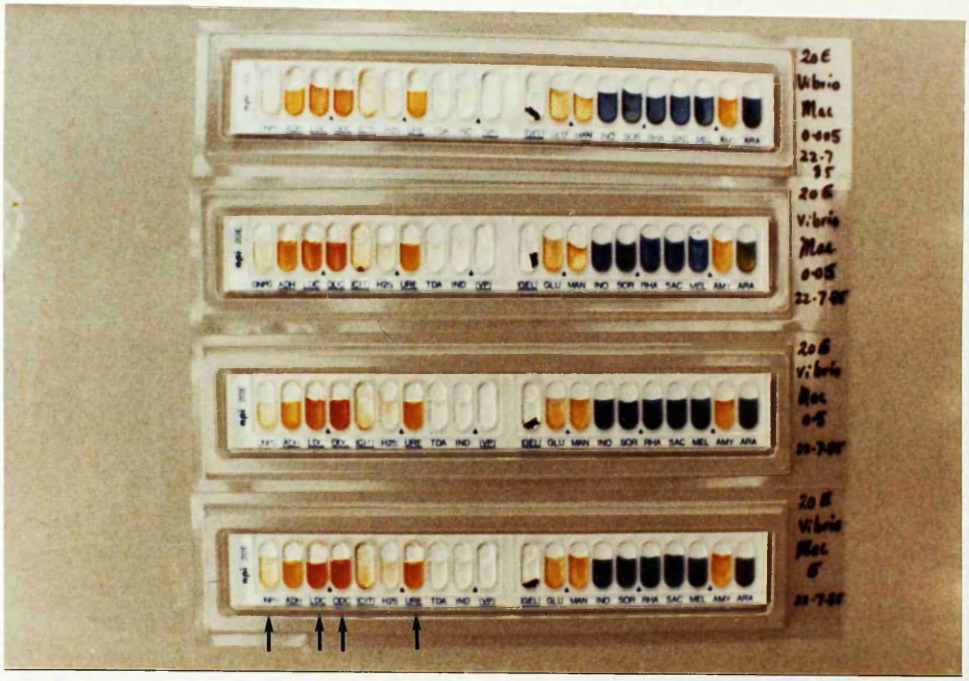
API 20E strips inoculated with different dilutions of Micrococcus sp., showing no positive results after 24 hours. The neutral, yellow and blue coloured cupules are negative in these strips. Compare plate 5 with plates 4, 6, 7, and 8. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

D

C

B

A

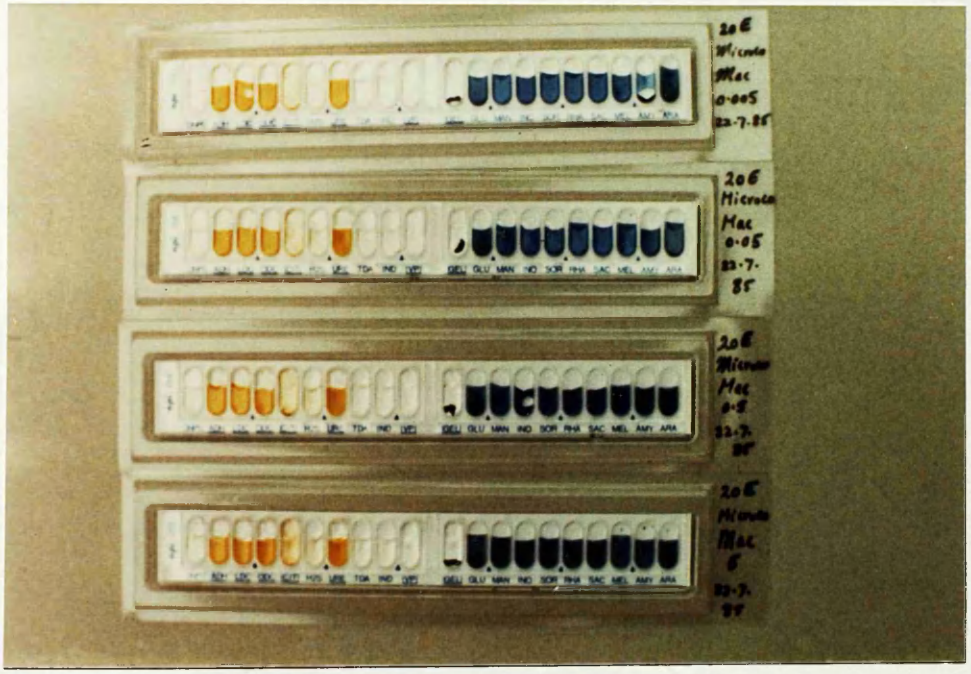

20E
Vibrio
Mac
0-005
22-7
85
20E
Vibrio
Mac
0-05
22-7-85
20E
Vibrio
Mac
0-5
20E
Vibrio
Mac
5

D

C

B

A


20E
Microto
Mac
0-005
22-7-85
20E
Microto
Mac
0-05
22-7-
85
20E
Microto
Mac
0-5
20E
Microto
Mac
5

Conc.	Incub. time(hr)	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NIT
5	24	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	48	+	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	72	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96		-	-	-	-	-	-				-		+	+	+	+	+	+	+	+		
0.5	24	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	48	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96		-	-	-	-	-	-				-		+	+	+	+	+	+	+	+		
0.05	24	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	48	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96		-	-	-	-	-	-				-		+	+	+	+	+	+	+	+		
0.005	24	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	48	-	-	-	-	-	-	-				-	-	-	-	-	-	-	-	-	-		
	72	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	96		-	-	-	-	-	-				-		+	+	+	+	-	+	+	+		

Table 21

API 20 E profiles of the marine species Micrococcus obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, - =negative reaction.

Conc.	Incub. time(hr)	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NIT
5	24	+	+	-	-	-	-	-	-	+	+	+	+	+w	-	-	-	+	-	-	+	+	+
	48		+	+	-	-	-	-				+		+	+	+	+	+	+	+	+		
	72		+	+	-	-	-	-				+		+	+	+	+	+	+	+	+		
	96		+	+	-	-	-	-				+		+	+	+	+	+	+	+	+		
0.5	24	-	+	-	-	-	-	-	-	+	+	+	+	+w	-	-	-	+	-	-	+	+	+
	48		+	+	-	-	-	-				+		+	-	-	-	+	-	-	+		
	72		+	+	-	-	-	-				+		+	-	-	-	+	-	-	+		
	96		+	+	-	-	-	-				+		+	+	+	+	+	+	+	+		
0.05	24	-	+	-	-	-	-	-	-	+	-	+w	+	+w	-	-	-	+	-	-	-	-	+
	48		+	+	-	-	-	-				+		+	-	-	-	+	-	-	-		
	72		+	+	-	-	-	-				+		+	-	-	-	+	-	-	-		
	96		+	+	-	-	-	-				+		+	+	+	+	+	+	+	+		
0.005	24	-	-	-	-	-	-	-	-	+	-	-	+	+w	-	-	-	+	-	-	-	-	+
	48		+	+	-	-	-	-				-		+	-	-	-	+	-	-	-		
	72		+	+	-	-	-	-				+		+	-	-	-	+	-	-	-		
	96		+	+	-	-	-	-				+		+	+	+	+	-	+	+	+		

Table 22

API 20 E profiles of the non-marine species Aeromonas hydrophila obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +w =weak positive, - =negative reaction.

Plate 6

Decrease in colour intensity with increasing dilutions of cell suspensions of Aeromonas hydrophila using the API 20E system. Arrows (left to right) show the tests gelatinase (GEL), glucose (GLU), and saccharose (SAC) respectively. 24 hours reading. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

Plate 7

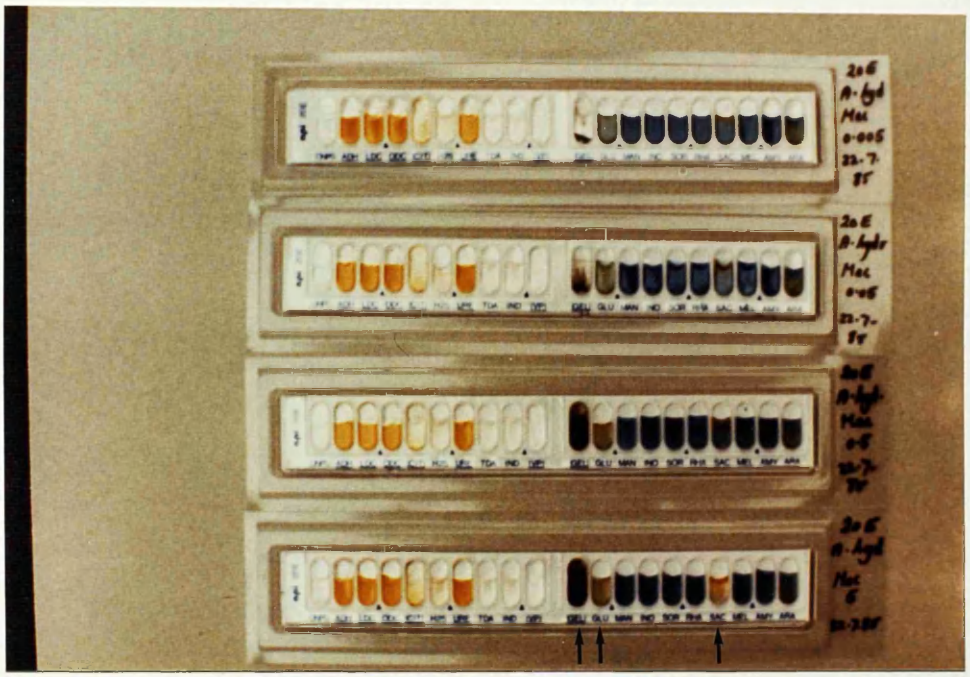
Decrease in colour intensity with increasing dilutions of cell suspensions of Escherichia coli using the API 20E system. Arrows (left to right) show the tests ONPG, lysine decarboxylase (LDC), rhamnose (RHA), melibiose (MEL), and arabinose (ARA) respectively. 24 hours reading. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

D

C

B

A

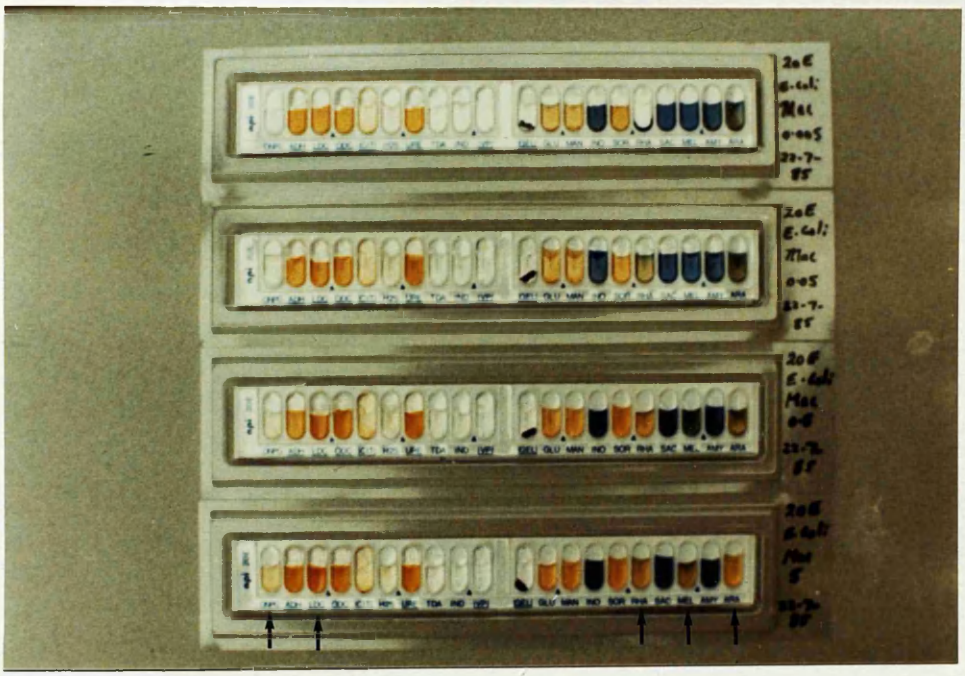


D

C

B

A



Conc.	Incub. time(hr)	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NIT
	24	+	-	+	-	-	-	-	-	+	-	-	+	+	-	+	+	-	+	-	+	-	+
5	48		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	72		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	96		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	24	+	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	+
0.5	48		-	+	-	-	-	-				-		+	+	+	+	-	+	-	+		
	72		-	+	-	-	-	-				-		+	+	+	+	-	+	-	+		
	96		-	+	-	-	-	-				-		+	+	+	+	-	+	-	+		
	24	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	+
0.05	48		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	72		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	96		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	24	-	-	-	-	-	-	-	-	+	-	-	+	+	-	+	+	-	-	-	-	-	+
0.005	48		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	72		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		
	96		-	+	-	-	-	-				-		+	+	+	+	+	+	-	+		

Table 23

API 20 E profiles of the non-marine species Escherichia coli obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, - =negative reaction.

Strain tested	test result	
	+ve (+)	-ve (-)
Vibrio fischeri	9	13
Micrococcus	0	20
Aeromonas hydrophila	11	11
Escherichia coli	10	12

2-**API 20NE system**

The results of the API 20NE system showed that after 24 hours of incubation, the strain Vibrio fischeri gave only two positive tests (urease and PNPG) as shown in table 24. The urease test was only positive at the cell suspension concentration equivalent to 5 and 0.5 on the MacFarland scale.

The strain Micrococcus sp. showed only one positive result in the PNPG test (Table 25 and Plate 9). In this test, the colour intensity decreased with increasing dilution of cell suspension (i.e. decreasing the cell concentration).

The non-marine strains, Aeromonas hydrophila and Escherichia coli showed numerous positive tests after 24 hours (Tables 26 and 27 and Plates 10 and 11 respectively). In general, Aeromonas hydrophila showed more positive tests than Escherichia coli. Almost all the assimilation tests with the two strains were positive (+), weak positive (+/-) or very weak positive (-/+) results. The glucose test with Aeromonas hydrophila was negative. However, with Escherichia coli, the test showed positive results at the high cell concentration. With the aesculin (ESC), it was vice versa.

After 48, 72 and 96 hours of incubation, the strain Vibrio fischeri showed few positive tests at the high cell concentration.

Plate 8

The difference in API 20E profiles of the strains Vibrio fischeri (A), Micrococcus sp. (B), Aeromonas hydrophila (C), and Escherichia coli (D) after 24 hours. Bacterial cell concentrations equivalent to 5 on the MacFarland scale.

Plate 9

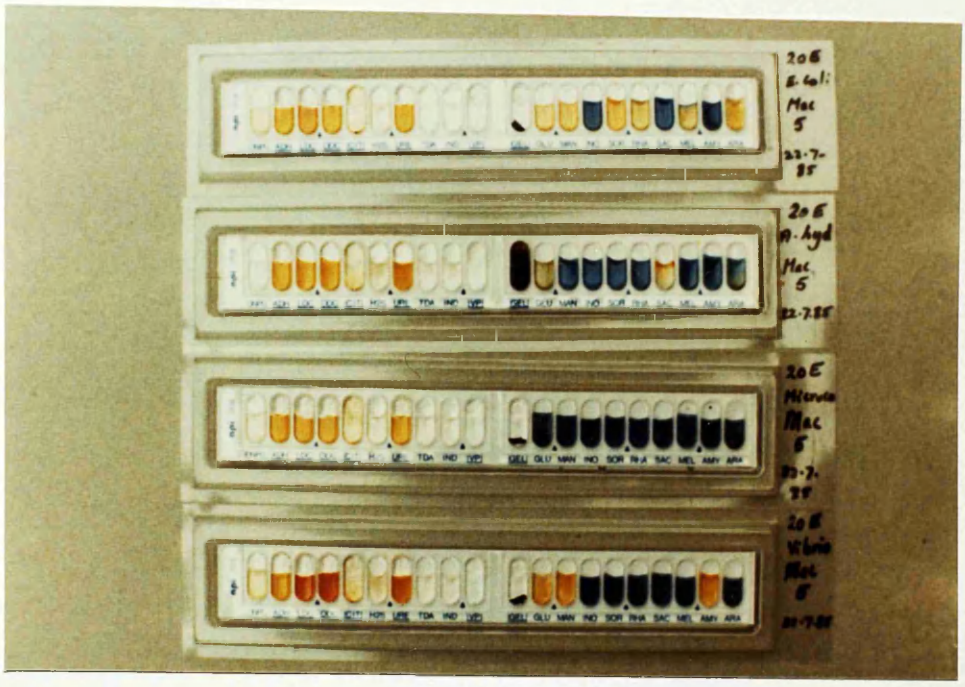
API 20NE strips inoculated with different dilutions of Micrococcus sp., showing only one positive result in the PNPG test. Arrow shows the PNPG test. 24 hours reading. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

D

C

B

A

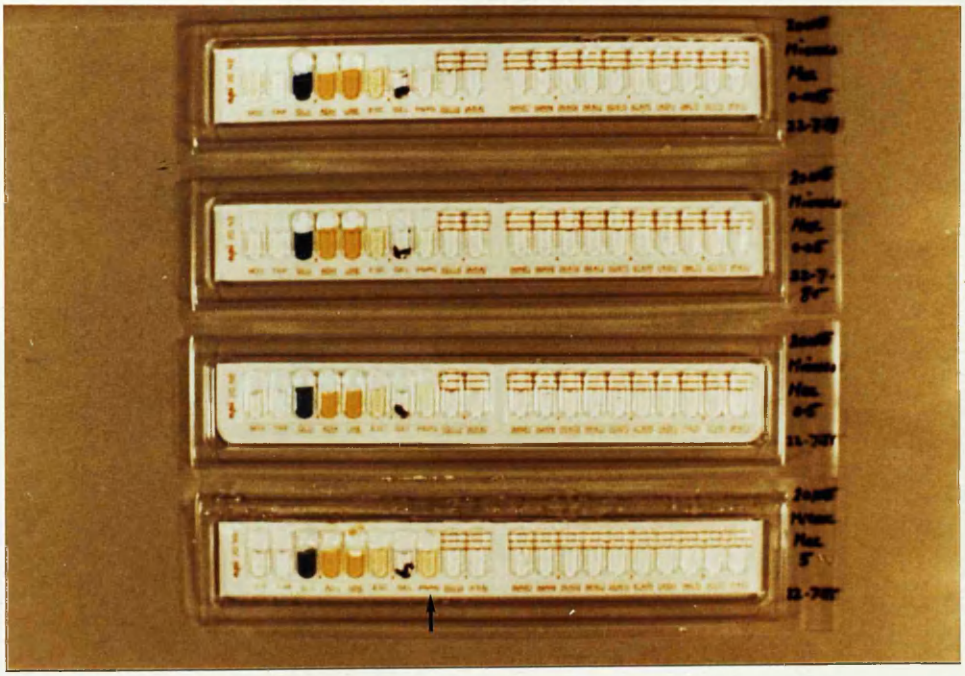


D

C

B

A



Conc.	Incub. time(hr)	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
	24			-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
5	48	+	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	72			+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	96			+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	24			-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.5	48	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	72			-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	96			-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	24			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.05	48	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	72			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	96			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	24			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
0.005	48	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	72			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	96			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

Table 24

API 20 NE profiles of the marine species Vibrio fischeri obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after.

Conc.	Incub. time(hr)	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
	24			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
5	48			-	-	-	-	-	+	-	-	-	-/+	-	-/+	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	+	+/-	-	-/+	+	-	+/-	+/-	-	-	-/+	-	-
	96			-	-	-	-	+	+	+/-	-/+	+/-	+	-	+	+	-	-	-/+	-	-
	24			-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-
0.5	48			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	+	-/+	-	-/+	-/+	-	-/+	-/+	-	-	-	-	-
	96			-	-	-	-	-	+	+/-	-/+	+/-	+/-	-	+	+/-	-	-	-	-	-
	24			-	-	-	-	-	-/+	-	-	-	-	-	-	-	-	-	-	-	-
0.05	48			-	-	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	96			-	-	-	-	-	+	-	-	-/+	-/+	-	-/+	-	-	-	-	-	-
	24			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.005	48			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	72	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	96			-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

Table 25

API 20 NE profiles of the marine species Micrococcus obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +/- =weak positive, -/+ =very weak positive, - =negative reaction.

Conc.	Incub. time(hr)	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
	24			-	+	-	+	-	+	+	+/-	+	+	+	+	+	+/-	-	+	-/+	-
5	48	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+/-	-
	72			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
	96			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
	24			-	+	-	+	-	+	+/-	+/-	+	+	+	+	+	+/-	-	+/-	-/+	-
0.5	48	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+/-	-
	72			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
	96			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-
	24			-	+	-	+	-	+	-/+	-/+	+/-	+/-	+/-	+/-	+/-	-/+	-	+/-	-	-
0.05	48	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-/+	-
	72			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+/-	-
	96			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+/-	-
	24			-	+	-	+	-	+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-	-/+	-	-
0.005	48	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	-/+	-
	72			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+/-	-
	96			+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+/-	-

Table 26

API 20 NE profiles of the non-marine species Aeromonas hydrophila obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +/- =weak positive, -/+ =very weak positive, - =negative reaction.

Conc.	Incub. time(hr)	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC
5	24	+	+	+	−	−	−	−	+	+	+	+/−	+	+	+	+	−	−	+	−	−
	48			+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	72			+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	96			+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
0.5	24	+	+	−	−	−	−	−	+	+	+	+/−	+	+	+	+	−	−	+	−	−
	48			+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	72			+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	96			+	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
0.05	24	+	+	−	−	−	−	−	+	+	+	+/−	+	+	+	+	−	−	+/−	−	−
	48			−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	72			−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	96			−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
0.005	24	+	+	−	−	−	−	−	+	+	+/−	+/−	+/−	+/−	+/−	+/−	−	−	−	−	−
	48			−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	72			−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−
	96			−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	−	−

Table 27

API 20 NE profiles of the non-marine species Escherichia coli obtained after 24, 48, 72 and 96 hours at bacterial cell concentrations equivalent to 0.005, 0.05, 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +/- =weak positive, - = negative reaction.

Plate 10

API 20NE strips inoculated with different dilutions of Aeromonas hydrophila, showing many positive results in the assimilation tests (these positive results appear as turbidity and cannot be seen in the photograph). Arrow shows the aesculine (ESC) test. 24 hours reading. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

Plate 11

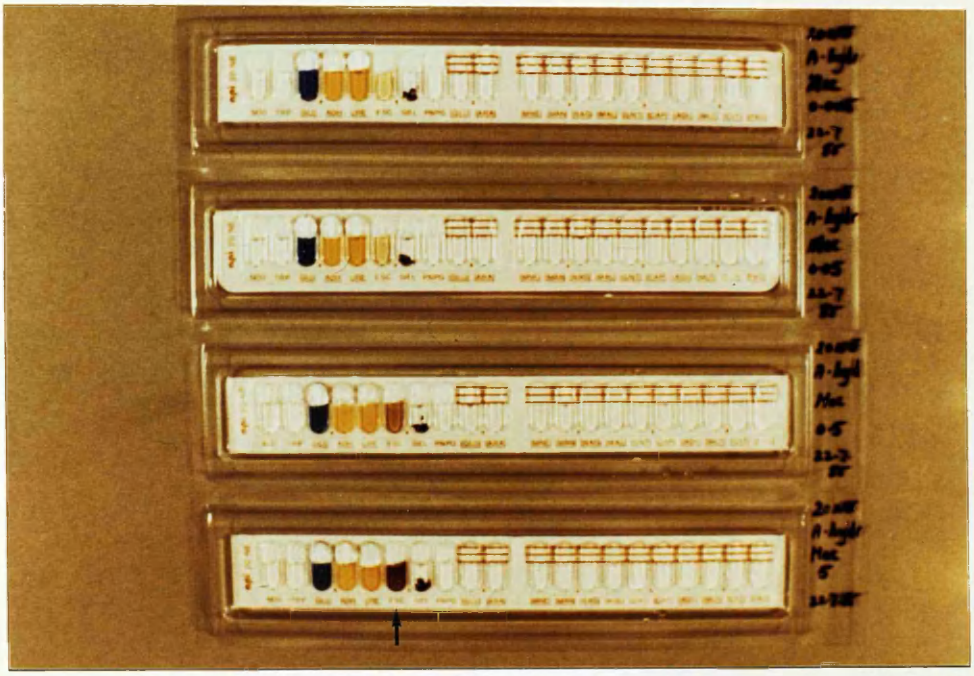
API 20NE strips inoculated with different dilutions of Escherichia coli, showing many positive results in the assimilation tests (these positive results appear as turbidity and cannot be seen in the photograph). Arrow shows the glucose (GLU) test. 24 hours reading. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

D

C

B

A

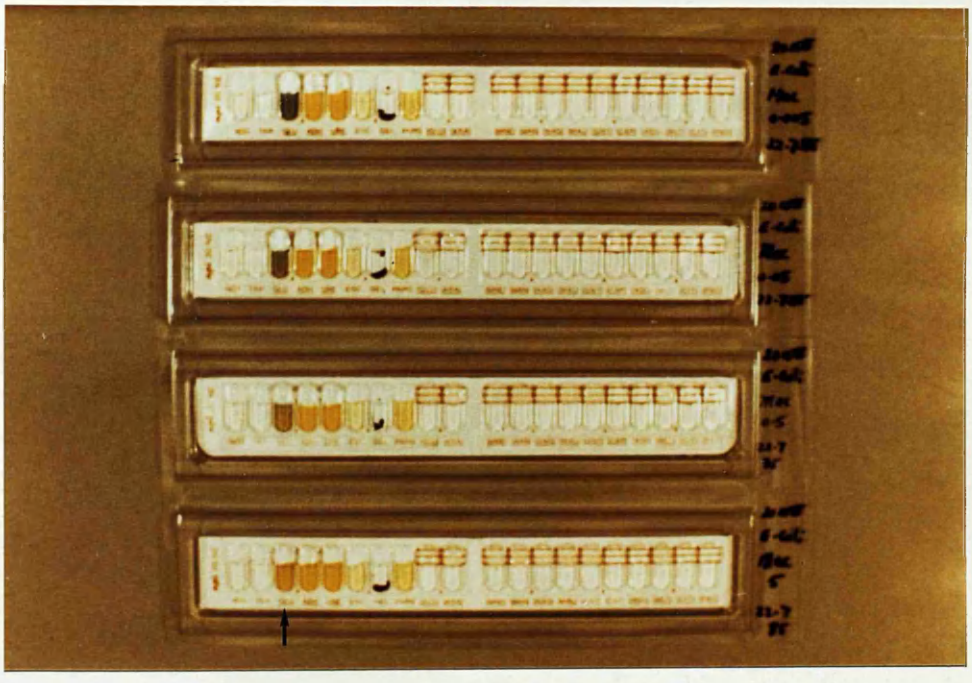


D

C

B

A



With the strain Micrococcus sp., there were very few positive reactions in the conventional test. However, after 72 and 96 hours , there were many positive reactions (growth) in the assimilation tests at the high cell concentration (i.e. 5 and 0.5 on the MacFarland scale).

With the strain Aeromonas hydrophila and Escherichia coli, there were more positive reactions than at 24 hours. The positive results were almost all from the assimilation tests. These were very clear-cut after 48 hours (i.e. there was no difference in the test results between 48, 72 and 96 hours).

In general, after 24 hours, the difference between the API 20NE profiles (Plate 12) for the four strains inoculated with concentration equivalent to 5 on the MacFarland scale was as follows.

strain tested	test result			
	+ve (+)	weak +ve (+/-)	very weak +ve (-/+)	-ve (-)
Vibrio fischeri	2	0	0	18
Micrococcus sp.	1	0	0	19
Aeromonas hydrophila	10	2	1	7
Escherichia coli	9	1	0	10

Experiment C

The results of this experiment was divided into two parts: preliminary and definitive.

Preliminary experiment

Only two dilutions (10^{-1}, 10^{-2}) were used in the preliminary experiment. The results showed that there was very little difference in colour intensity between dilutions after 24 hours. For example, the

ONPG test showed positive results at the first dilution (10^{-1}), while at 10^{-2} it showed a weak positive result (Table 28). This phenomenon also occurred after 48 and 96 hours with the melibiose and arabinose. It was observed from the results that the gelatinase test was positive at 10^{-1} after 24 hours. However, it was negative at 10^{-2} dilution. In general, in this preliminary study, there was an indication of the effect of bacterial cell concentration on the biochemical tests in the cupules. In other words, the colour intensity decreased with increasing dilutions.

Definitive experiment

In the definitive experiment, a series of dilutions were made from 10^{0} to 10^{-4} and duplicate strips of API 20E were inoculated from each dilution. In this experiment, the developing reagents were added after 24 hours and results demonstrated in general, that colour intensity decreased with increasing dilutions of cell suspension. Prolonged incubation time to 48 hours improved some tests at low cell concentrations and caused a change to positive (Table 29). The differences in colour intensity between some tests of API 20E are shown in Plate 13. The difference in colour intensity between 10^{-1} and 10^{-4} dilutions is shown in Plate 14.

The definitive experiment results agreed with the API profiles with the exception of the oxidase test which showed a very weak positive result.

Experiment D

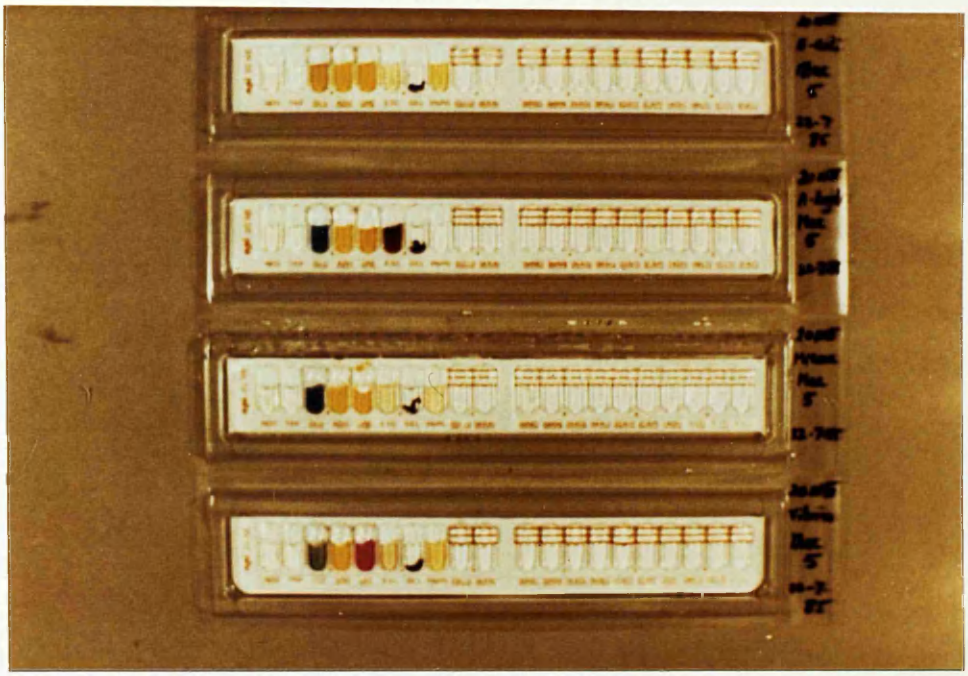
The bacterial cell concentrations used in this experiment were equivalent to 0.5 and 5 on the MacFarland scale. The results of this experiment (Table 30) showed that cell concentrations of 5 on the MacFarland scale was much better than 0.5. This can be observed in Plate 15 with the gelatin (GEL) and aesculin (ESC) which showed a

D

C

B

A



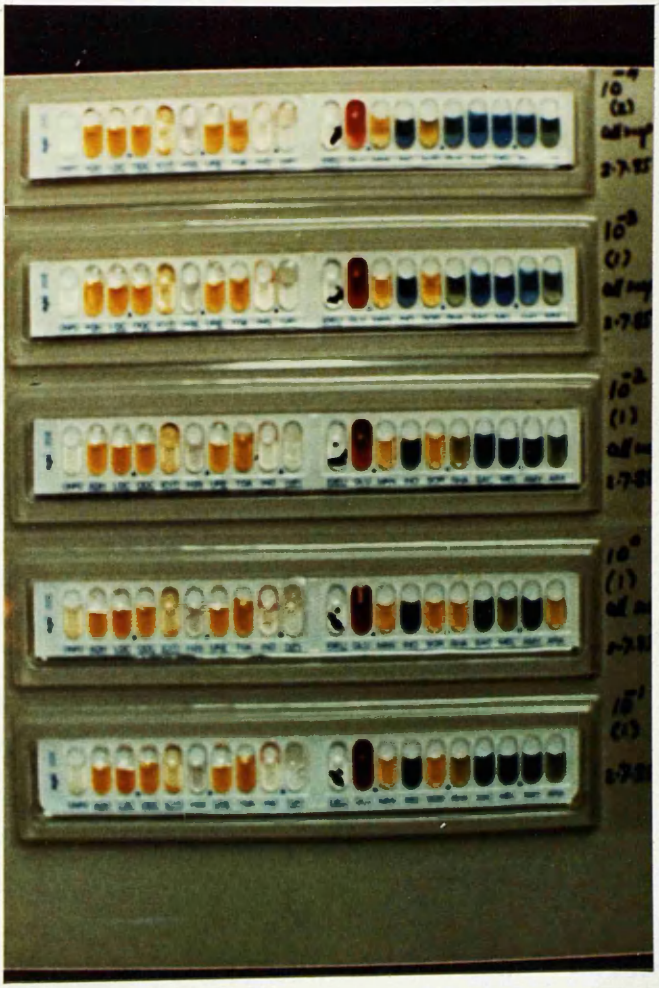
E

D

C

A

B



Conc.	Incub. time(hr)	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NIT
10^{-1}	24	+	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	-	+	+	+
	48		-	+	-	-	-	-				+		+	-	+	+	+	+	-	+		
	96		-	+	-	+	-	-				+		+	-	+	+	+	+	-	+		
10^{-2}	24	+w	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	+	-	-	+	+	+
	48		-	+	-	-	-	-				+		+	-	+	+	+	+w	-	+w		
	96		-	+	-	+	-	-				+		+	-	+	+	+	+w	-	+w		

Table 28

API 20 E profiles of Escherichia coli species obtained in the preliminary experiment after 24, 48 and 96 hours at 10^{-1} and 10^{-2} dilutions. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +w =weak positive, - =negative reaction.

Conc.	Incub. time(hr)	ONPG	ADH	LDC	ODC	CIT	H_2S	URE	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	OX	NIT
10^0	24	++	—	++w	—	—	—	—	—	++	—	—	++	++	—	++	++	—	++w	—	++	++w	++
	32		—	++w	—	—	—	—				—		++	—	++	++	—	++w	—	++		
	48		—	++	—	—	—	—				—		++	—	++	++	—	++w	—	++		
	72		—	++	—	—	—	—				—		++	—	++	++	—	++w	—	++		
10^{-1}	24	++w	—	++w	—	—	—	—	—	++	—	—	++	++	—	++	++	—	—	—	++w	++w	++
	32		—	++w	—	—	—	—				—		++	—	++	++	—	—	—	++w		
	48		—	++	—	—	—	—				—		++	—	++	++	—	—	—	++w		
	72		—	++	—	—	—	—				—		++	—	++	++	—	—	—	++		
10^{-2}	24	—	—	—	—	—	—	—	—	++	—	—	++	++	—	++	++w	—	—	—	++w	++w	++
	32		—	++w	—	—	—	—				—		++	—	++	++w	—	—	—	++w		
	48		—	++w	—	—	—	—				—		++	—	++	++w	—	—	—	++w		
	72		—	++	—	—	—	—				—		++	—	++	++w	—	—	—	++		
10^{-3}	24	—	—	—	—	—	—	—	—	++w	—	—	++	++	—	++	—	—	—	—	++w	++w	++
	32		—	—	—	—	—	—				—		++	—	++	++w	—	—	—	++w		
	48		—	++w	—	—	—	—				—		++	—	++	++w	—	—	—	++w		
	72		—	++	—	—	—	—				—		++	—	++	++w	—	—	—	++		
10^{-4}	24	—	—	—	—	—	—	—	—	++w	—	—	++w	++w	—	++w	—	—	—	—	—	++w	++
	32		—	—	—	—	—	—				—		++	—	++	++w	—	—	—	++w		
	48		—	++w	—	—	—	—				—		++	—	++	++w	—	—	—	++w		
	72		—	++	—	—	—	—				—		++	—	++	++w	—	—	—	++		

Table 29

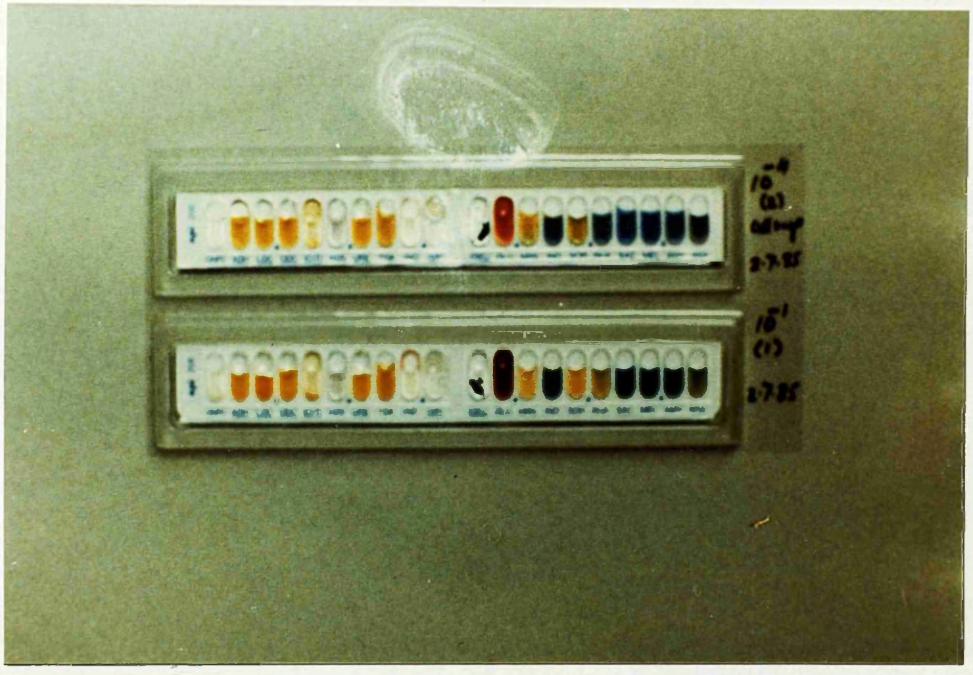
API 20 E profiles of Escherichia coli species obtained in the definitive experiment after 24, 32, 48 and 72 hours at a series of dilutions (10^0-10^{-4}). Duplicate strips were used. w=weak reaction. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. ++w =weak positive reaction in the two strips, — =negative reaction.

Plate 14

The difference in colour intensity between 10^{-1} (A) and 10^{-4} (B) dilutions of Escherichia coli suspensions.

B

A



Incub. time(hr)	Conc.	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	Ox
24	0.5			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	5			-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
48	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-/+	-	-	+
	5	-	-	-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	-/+	-	-	+
120	0.5			-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	-	+	-	-	+
	5			-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	+	+	-	+
24	0.5			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	5			-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+
48	0.5	-	-	-	-	-	+	+/-	-	+	-	-	-	-	-/+	-	-	-	-	-	-	+
	5	-	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	+	-	-	+
120	0.5			-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	+/-	-	-	+
	5			-	-	-	+	+	-	+	-	-	-	-	+	-	-	-	+	+	-	+

Table 30

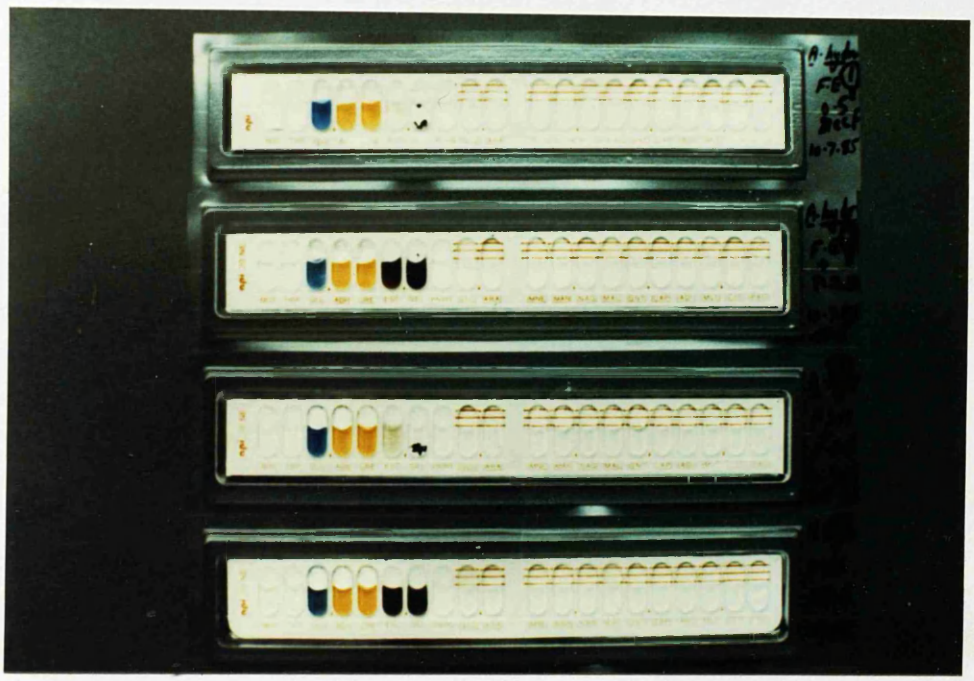
API 20 NE profiles of the Aeromonas hydrophila species to test whether the system produced replicable results when used by two separate workers. Bacterial concentrations used were equivalent to 0.5 and 5 on the MacFarland scale. No readings were taken where blanks occur in the table. Readings were only taken immediately after the addition of reagents, and not before or after. + =positive reaction, +/- =weak positive, -/+ =very weak positive, - =negative reaction.

A

B

C

D



diffusion of black pigments. Some of the assimilation tests also showed positive growth, e.g. glucose (GLU), maltose (MAL) and malate (MLT). The results of these tests can only be noticed as turbidity (bacterial growth). 48 hours of incubation showed more positive tests than that of 24 hours. According to the API quality control profiles, the results of this experiment were not completely identical.

SECTION 3

DISCUSSION

API ZYM is a technique has been used by several investigators to identify clinical microorganisms (Washington et al., 1971; Smith et al., 1972; Tharagonnet et al., 1977; Hofstad, 1980; Waitkins et al., 1980). However, I can find no previous published application of this technique to seawater and sediment although it has been used for several years by Mr Meadows's research group.

The accuracy of the technique has been studied by several investigators using different groups of bacteria. Smith et al. (1972) used the API system for identification of Enterobacteriaceae. They found that the accuracy of identification of the system was 96.4%. These results agreed with the studies made on Enterobacteriaceae by Washington et al. (1971) who found the accuracy of identification on initial testing was nearly 90% and 93% in the repeat experiment. Tharagonnet et al. (1977) used the API ZYM system to identify anaerobic Gram-negative organisms from human sources. They found that the technique distinquished clearly between the different genera and species and seemed to be reliable for identification of that particular group of organisms.

The results of this section can be summarized and discussed as follow:

I- Experiment 1

The variation in the amount of sediment dispensed in metal foil from 3 pasteur pipettes was tested. It was found that there was no significant variation between drops and pipettes in the two replica experiments. These results show that replicable amounts of sediment can be dispensed using the pipette technique.

II- **Experiment** 2

The results of this experiment showed that the highest amount of hydrolysed substrate in most cupules occured in the time between 10-20 hours of the first incubation and between 5-20 hours of the second incubation time. In general, there were differences between the first incubation times in most of the API cupules. However, between the second incubation times, the variation was not significant except in one cupule.

The amount of hydrolysed substrate may vary depending on many factors such as the sample to be tested, the amount of sample dispensed in the cupules and incubation time and temperature. In this study, sediment was used to determine the optimum incubation time of the API ZYM, so the highest reaction between the sediment enzymes and substrates was recorded within the time mentioned above. There appeared to be a reduction in colour intensity in some cupules after 10 hours during the first incubation time (e.g. cupule 9 and 11). This may be due to some enzymes losing their activity after a particular time and under certain conditions. Reichardt et al. (1967) pointed out that the enzymes they examined lost half of their activity after exposure for 3.2 days at 18^{o}C.

III- **Experiment** 3

The results of this experiment showed that in general, colour developed more slowly and to a lesser degree in deep than in surface sediment.

The occurence of various cell-free enzymes in aquatic environments has been tested by several investigators (Harvey, 1925; Berman, 1970; Hanson and Kim, 1970). Berman (1970) determined alkaline phosphatase activities in lake waters. He found that there were seasonal fluctuations in enzyme activity. Hanson and Kim (1970)

determined the activities of some cell-free enzymes dissolved in oceanic and saline lake environments. They found that some enzymes e.g. alkaline phosphatase, and amylase had significant activity levels. The authors also found that some of the enzymes exhibited a diverse activity related to pH and salt concentration. In my experiment the amount of hydrolysed substrate varied from one cupule to another, also from one sample to another (e.g. colour intensity with surface sediment higher than with deep sediment). This phenomenon may be due to the effect of many factors on the activity of sediment enzymes reacting with the cupule substrates. These factors include the pH of the sediment and the substrates. The higher numbers of microorganisms present in surface sediment may also affect the activity of enzymes. Reichardt et al. (1967) found that there was a correlation between the concentration of free enzymes and the numbers of phytoplankton and bacteria.

IV- Experiment 4

This experiment showed that a higher intensity of colour was reached in the cupules containing untreated (control) and membrane filtered (0.22um) seawater than in the cupules containing boiled (100°C) or autoclaved (121°C) seawater for 15 minutes. This phenomenon was clearer with interstitial water than with overlying water.

Some workers have studied the cell-free enzymes released from living or dead organisms in aquatic environments (Harvey, 1925; Reichardt et al., 1967; Hanson and Kim, 1970; Kim and ZoBell, 1974; Thompson and Eribo, 1984). Many microorganisms have the ability to produce extracellular enzymes. Thompson and Eribo (1984) studied the ability of the fungi Rhizopus and Mucor to release extracellular enzymes into the culture medium. They found that phosphatase was released into the medium by all the fungi examined. However, some

species produced more phosphatase enzyme than others. They also found that most of the fungi they studied were able to produce many other enzymes. Hanson and Kim (1970) studied the activity of a range of cell-free enzymes including alkaline phosphatase and amylase in the marine environments, and demonstrated the ecological importance of cell-free enzymes. Kim and ZoBell (1974) discussed the effect of pressure, temperature, pH and salinity of the oceanic environment on the activities of cell-free enzymes including some recovered from saline lakes, seawater and marine sediments and some prepared from microbial cells. They found that types and concentrations of the cell-free enzymes were influenced by the sampling sites, the dominant types of life in that environment, and the density of the life forms. For example, alkaline phosphatase showed varying optimum pH depending on the sampling sites.

Overlying water showed less intensity of colour than interstitial water. This may due to the enzymes dissolved in overlying water being more diluted than those in interstitial water. Kim and ZoBell (1974) pointed out that one of the problems in preparing cell-free enzymes from seawater samples was that enzymes dissolved in seawater are highly diluted. The higher intensity of colour in the cupules containing untreated and membrane filtered samples may indicates that the concentration of the enzymes in these samples is high. It also demonstrates that enzymes are present in solution in seawater and can pass through a membrane filter (0.22um). The lower intensity of colour presented in the cupules containing boiled ($100^{o}C$) and autoclaved ($120^{o}C$) seawater, is evidence for the inhibition of enzyme activity by heat. For example, Reichardt et al. (1967) found that optimum activity of alkaline phosphomonoesterases occurred at a temperature of $27^{o}C$. They also found that about 75% of the activity of

this enzyme was inhibited after 10 minutes at 100°C.

V- **Experiment** A

Bacterial reproduction occurs by binary fission (i.e. one cell divides, producing two cells). The increase in bacterial numbers is by geometric progression, i.e. 1, 2, 4, 8, 16. The time interval required for the cell to divide or for the population to double, is known as the generation time. Not all bacteria have the same generation time. For example with Escherichia coli, it may be 15 to 20 minutes, while Mycobacterium tuberculosis is several hours (Pelczar and Reid, 1972). Similarly the generation time is not the same for a particular bacterium under all conditions. It is strongly dependent on many factors such as nutrients in the medium, temperature, gases required, and pH.

After bacterial cells were inoculated into the medium, there was an initial period of what appears to be no growth (lag phase), followed by rapid growth (log phase), which started just before 6 hours. This stage was followed by a leveling off (stationary phase) which started after 12 hours, except for Aeromonas hydrophila which started after 18 hours. As a final stage, a decline in the viable population must occur after a particular time. This stage had not appeared after 36 hours, as shown in figure 7. The reason for this was probably because cells were dying at the same rate as new cells were being produced. This may be due to the presence of high nutrient levels in the media or because this bacterial species dies slowly.

The strain Aeromonas hydrophila showed a transitional period (curved portion) between the log and stationary phases. This represents the time required before all the cells enter the new phase. This may mean that not all the cells of Aeromonas hydrophila were in the same physiological condition towards the end of log phase.

From the results, the following conclusions can be made with regard to future experiments of this kind. Firstly, API strips should be inoculated with cell suspension while the bacteria are in the log phase (i.e. between 6 and 12 hours). Secondly, bacteria of the species tested should be inoculated before 36 hours (i.e. stationary phase) because bacteria can resume reproduction, i.e. re-enter log phase when fresh media and optimal physical conditions are supplied.

VI- **Experiment** B

The API 20E system is designed for rapid differentiation and identification of bacteria belonging to the Enterobacteriaceae family. The API 20NE system is designed for the identification of Gram-negative rods not belonging to the Enterobacteriaceae family. Both of these systems have identification codes based on how fast positive results occur in the test-kits. Because marine bacteria generally require low growth temperatures and prolonged incubation times, the identification codes are not very suitable for use with marine environmental isolates. The API 20NE system is a relatively new system and there is little evidence so far to indicate that marine bacteria can be handled in the same way as clinical isolates. I have therefore tried to test both systems with marine and non-marine strains in relation to the oxidase test, which is necessary for the the API 20NE system. In addition, I have tested the effect of cell concentration on these two systems.

There are rare reports of the application of commercially available test-kits to marine bacteria. Gauthier and Clement (1978) studied 10 marine bacterial strains through several API systems. The authors compared their results with the conventional tests and found these systems allowed to define to a certtain extent, the limits of applicability of such micromethods to the taxonomic study of

hetrotrophic bacteria isolated from seawater. Pyle and Shotts (1980) reported that the API 20E system gave reasonably differentiation with pathogenic flexibacteria from non-marine cold- and warmwater fish. The authors found only two dubious results. These occurred with glucose-fermentation and gelatin liquification.

When bacterial strains are to be characterised by the API 20E system, the manufacturer's recommended procedure is to prepare a cell suspension in sterilised distilled water and to inoculate the various biochemical test cupules. I substituted the distilled water with sterile artificial seawater, adjusted to 20 ‰ salinity. Rüger (1981) used sterile seawater adjusted to a salinity of 17 ‰ as the test medium instead of distilled water. MacDonell et al. (1982) tested the applicability of the API 20E system in conjunction with various diluents for use in identifying marine and estuarine bacteria. The authors concluded that marine salt diluent prepared with the salinity adjusted to 20 ‰, yielded the largest fraction of positive tests, while the 0.85% NaCl diluent consistently yielded the smallest proportion of positive tests. Therefore, the authors recommended the use of marine salt adjusted to a salinity of 20 ‰ for characterising both marine and estuarine isolates. Because of the optimal growth temperature of marine bacteria, I have chosen an incubation temperature of 18°C and incubation periods of 24, 48, 72 and 96 hours. The API 20E system contains bromthymol blue as an indicator to detect acid production from carbohydrates. This indicator is known to be toxic to a number of marine bacteria (Leifson, 1963), therefore the system is not suitable for differentiating marine bacteria unless this indicator is replaced with another indicator such as phenol red. Leifson (1963) compared several indicators and found that phenol red was the most satisfactory and at a concentration of 0.001%, has no toxic effect on marine bacteria. Some of the API tests need additional

developing reagents prior to reading (indole, nitrate reduction, Voges Proskauer). These tests must be performed last since these reactions release gaseous products which interfere with other tests such as the carbohydrate tests. This can be seen in the series of photographs shown in Plate 16, where the colour of carbohydrate tests (negative) changes to positive with increasing the incubation time.

My results with Aeromonas hydrophila using the API 20E system agree with the results of MacDonell et al. (1982), with the exception of citrate and Voges Proskauer tests. With API 20E galleries, the marine and non-marine isolates tested gave negative results for citrate utilization, H_2S, urease and tryptophan deaminase. All the marine and non-marine strains gave negative reactions with the gelatinase test, with the exception of Aeromonas hydrophila which gave positive results. MacDonell et al. (1982) explained the divergent results in some tests as an interaction between the diluent and the biochemical tests in the cupules.

VII- **Experiment** C

The results of the preliminary experiment showed that there was very little difference in colour intensity between the 10^{-1} and 10^{-2} dilutions after 24 hours. The variation in colour was not very pronounced but there was an indication that colour intensity is affected by the cell concentration of cell suspension. This would have to be tested using more than two dilutions. In the definitive experiment, where a series of dilutions were made, the decrease in colour intensity with increasing dilution of cell suspension was very clear (e.g. arabinose, rhamnose, glucose, indole and ONPG as shown in Plate 13.

Prolonged incubation time affected the results of some tests, especially at low concentrations of cell suspension. This was because

Plate 16

Series of photographs of API 20E strips inoculated with different dilutions of a Micrococcus suspension. These show false positive results (yellow colour) for the carbohydrate tests (bracketed) due to interference by the gases produced by the developing reagents added to the indol, nitrate reduction, and Voges Proskauer test cupules. Reagents added after 48 hours, and photographs were then, taken after 0, 2.30, 5 and 24 hours respectively as shown on next two pages. A = 5, B = 0.5, C = 0.05, and D = 0.005 on the MacFarland scale.

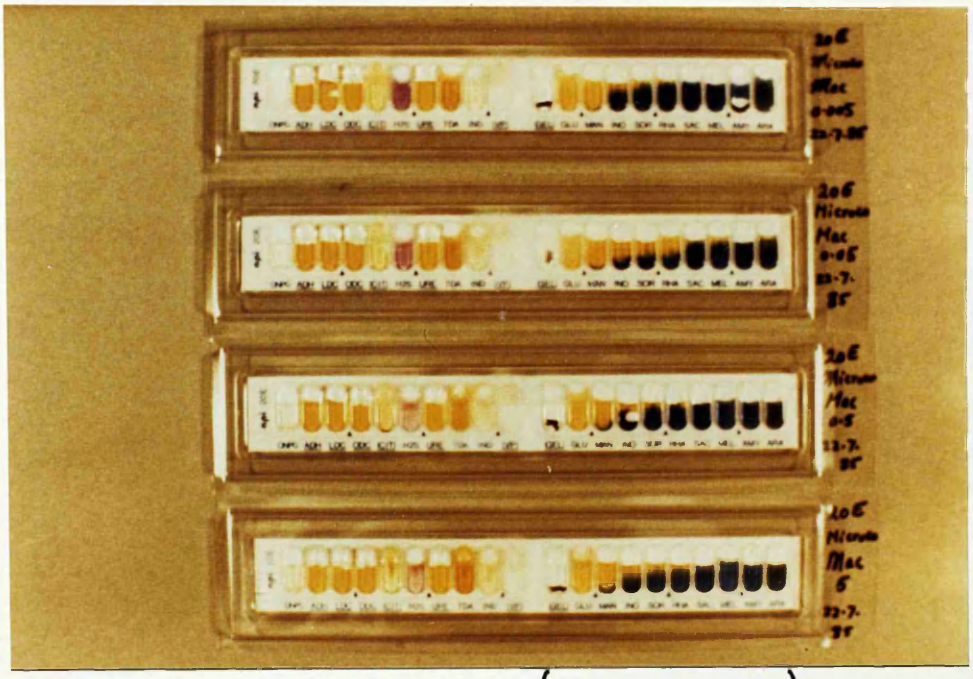
0 hr

D

C

B

A



(————————)

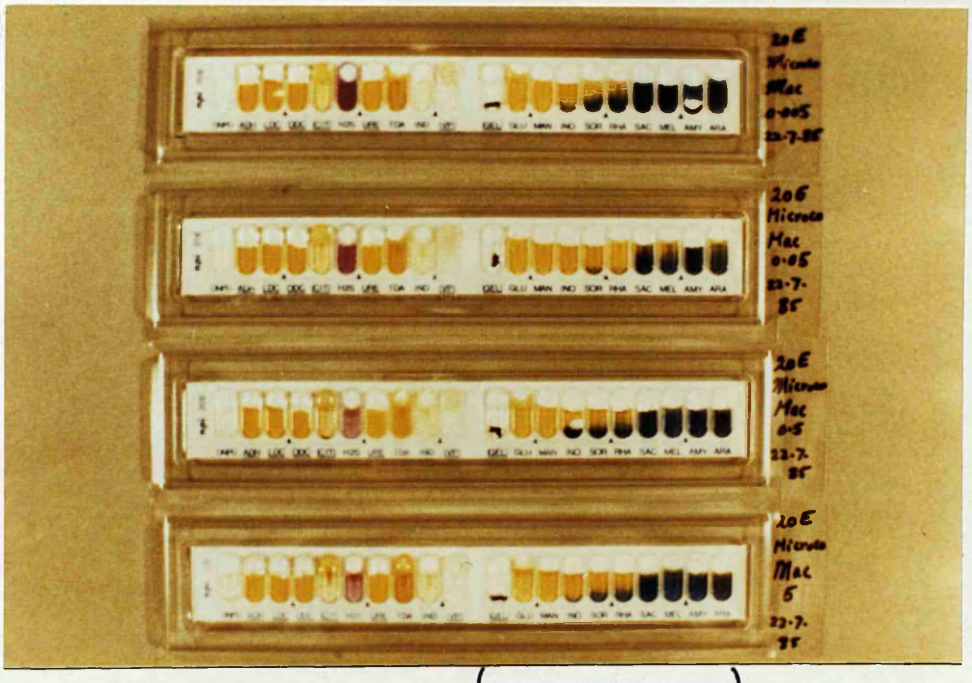
2·30 hr

D

C

B

A



(————————)

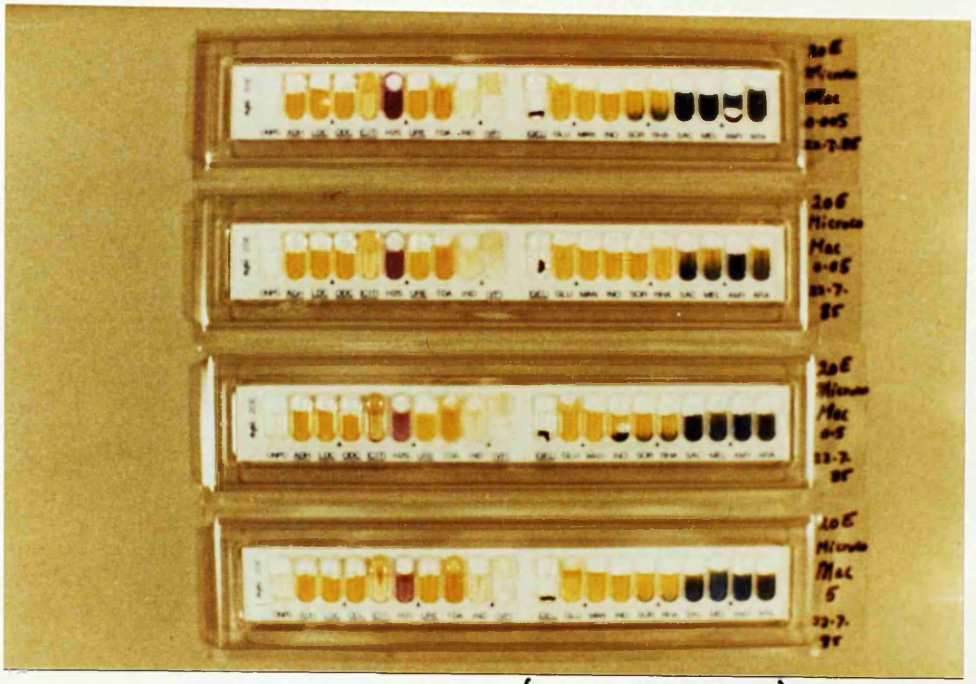
5 hr

D

C

B

A



(————————)

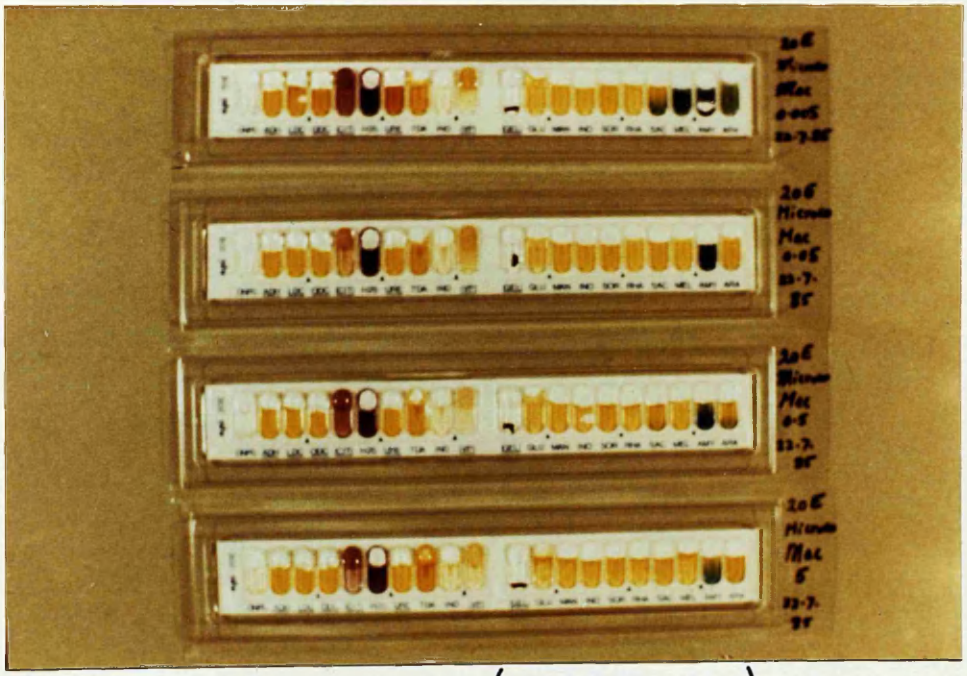
24 hr

D

C

B

A



(————————)

there were not enough bacterial cells to utilize the test substrates very quickly.

Since some reactions release gaseous products which interfere with other tests, precautions had to be taken during the experimental procedure. Those cupules containing added reagents were therefore cut from the API strips before the other tests were reincubated. This gave results that agreed with the API profiles.

VIII- **Experiment** D

The cell concentration of Aeromonas hydrophila equivalent to 5 on the MacFarland scale gave better results than those given at 0.5 on the scale. This may be due to the differences in the number of cells able to hydrolyse the substrates in the API 20NE cupules. Plate 15 shows the number of cells at the concentration of 5 that were able to hydrolyse the gelatin (GEL) and aesculin (ESC) using the protease and β-glucosidase enzymes, thereby producing black pigments. It was noticed that fungal colonies grew in the cupule containing arabinose (ARA). Contamination may have occurred during addition of reagents or when the galleries were being photographed. The reasons for the differences obtained between these test results and the API profiles are not clear.

SECTION 3

SUMMARY

1- The aim of my work in this section was to apply the API ZYM, API 20E and API 20NE systems to marine and non-marine samples in a number of experiments. The samples consisted of intertidal sediment and marine and non-marine named bacterial isolates.

2- An experiment was conducted to determine the quantity of sediment dispensed from a pasteur pipette into each API ZYM cupule. Statistical analysis using two-way analyses of variance showed that in both the first and second replicate experiments there was no significant variation between drops or pipettes.

3- An experiment was carried out to determine the optimum incubation time of inoculated API ZYM strips using intertidal surface sediment. The results of this experiment suggested that the highest amount of hydrolysed substrate in most cupules occurred in the time between 10-20 hours of the first incubation and between 5-20 hours of the second incubation time.

Student's t-tests were applied to the data and showed that there were differences between the first incubation times in most cupules. However, in the second incubation times, there was no significant variation.

4- An experiment was conducted to determine how soon after addition of API ZYM reagents colour developed and reached a maximum intensity. A two-way analysis of variance applied to the data showed possible statistical interaction. Subsequent one-way analyses showed a highly significant variation between cupules at a depth of 5cm. However, there was no significant variation between cupules at 10cm, 20cm and 35cm depth. At the surface, there was only possible variation between cupules. One-way analyses of variance also showed a significant variation between depths with cupules 2 and 6. However,

with cupules 7 and 11, there was no significant variation between depths.

The variation in time at the which maximum score was reached was studied. Two-way analysis of variance showed that there was a significant variation between depths, but that there was no significant variation between cupules.

5- An experiment was conducted to test whether the colour noticed in API ZYM cupules was produced by membrane filterable products and if so, whether these products were heat stable or heat labile. The results showed that for both overlying and interstitial water, there is no difference between control and membrane filtered seawater and no difference between boiled and autoclaved seawater. The results also showed that there are significant differences between the cupules, and the activity is not affected by membrane filtration but is abolished by boiling and autoclaving.

Statistical analysis comparing overlying water with interstitial water (control and membrane filtered) for each cupule, showed a highly significant variation between overlying and interstitial water samples in 3 out of 5 cupules. In these 3 cupules, the activity in the interstitial water was greater than that in the overlying water.

6- An experiment was conducted to determine the growth rate of marine and non-marine bacteria. The results showed the following conclusions:

(a) The number of cells increased with increasing incubation time up to 12 hours and the non-marine strains showed a greater increase in the number of cells than marine strains.

(b) The log phase of all strains started just before 6 hours, with the exception of Escherichia coli which reached slightly

earlier.

(c) The stationary phase of all strains examined was always reached after 12 hours, with the exception of Aeromonas hydrophila which reached the stationary phase after 18 hours.

7- An experiment was conducted to test how well the API 20E and API 20NE systems work with marine and non-marine bacteria. The effect of bacterial cell concentration on the kit-system reaction was also tested. The results showed the following conclusions.

(a) With the API 20E system and after 24 hours, all the strains gave some positive results and in general, the colour intensity decreased with increasing dilutions of cell suspension. The exception to this was Micrococcus sp. which gave negative results with all the tests.

(b) The results with Vibrio fischeri, Aeromonas hydrophila and Escherichia coli showed little change after 48, 72 and 96 hours except in the carbohydrate tests which were affected by the added reagents.

(c) With the API 20NE system and after 24 hours, the marine strains Vibrio fischeri and Micrococcus sp. gave two and one positive result respectively. The non-marine strains Aeromonas hydrophila and Esherichia coli gave numerous positive results.

(d) After 48, 72 and 96 hours of incubation, the strains Vibrio fischeri and Micrococcus sp. showed only a few positive results in the conventional tests. However, after 72 and 96 hours, there were many positive results (growth) in the assimilation tests at the high cell concentrations.

(e) With the strains Aeromonas hydrophila and Esherichia coli, there were more positive results after 48, 72 and 96 hours than at 24 hours. The majority of these positive results were

from the assimilation tests.

8- Preliminary and definitive experiments were conducted to test the effect of the number of Escherichia coli cells and incubation time on the API 20E system. The results demonstrated that colour intensity decreased with increasing dillutions after 24 hours. Prolonged incubation time to 48 hours improved the results of some tests at low cell concentrations and caused a change to positive.

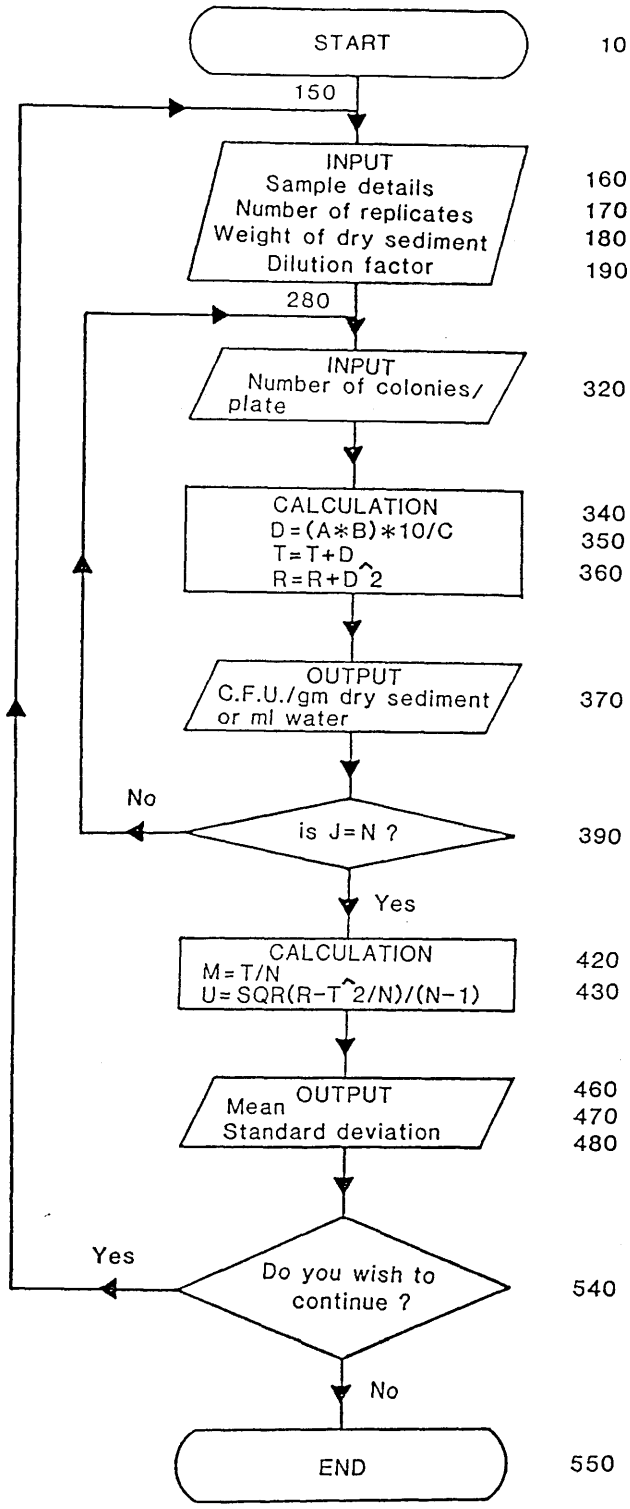
9- An experiment was carried out to test whether the API 20NE system produced replicable results with the non-marine strain Aeromonas hydrophila when used by two separate workers. The results showed that cell concentrations of 5 on the MacFarland scale were much better than 0.5. The results also showed that 48 hours of incubation produced more positive tests than 24 hours.

Section 2 appendix

Appendix Table 1

Computer program "BACT" for calculations of the numbers of heterotrophic bacteria per gram dry weight sediment or per ml water.

```
10 REM *** BACT, F. EDDEB, OCT.1984 ***
20 PRINT:PRINT
30 LPRINT CHR$(27);"1";CHR$(10)
40 CS$=CHR$(126)+CHR$(28)
50 PRINT CS$
60 PRINT"THIS PROGRAMME CALCULATES NUMBERS OF HETEROTROPHIC BACTERIA"
70 PRINT"PER GRAM DRY SEDIMENT OR PER ml WATER(C.F.U.=COLONY FORMING"
80 PRINT"UNITS)"
90 PRINT:PRINT
100 LPRINT"THIS PROGRAMME CALCULATES NUMBERS OF HETEROTROPHIC BACTERIA
"
110 LPRINT"PER GRAM DRY SEDIMENT OR PER ml WATER(C.F.U.=COLONY FORMING
"
120 LPRINT"UNITS)"
130 LPRINT:LPRINT
140 LPRINT"------------------------------------------------------------
-----------"
150 A=0:B=0:C=0:D=0:N=0:T=0:R=0:U=0
160 INPUT"SAMPLE DETAILS(REMEMBER TO SPECIFY SEDIMENT OR WATER)= ";A$
170 INPUT"NUMBER OF REPLICATE PLATES= ";N
180 INPUT"ENTER WEIGHT OF DRY SEDIMENT(IF WATER COUNTS ENTER 1)= ";C
190 INPUT"DILUTION FACTOR= ";B
200 PRINT
210 LPRINT"SAMPLE DETAILS(REMEMBER TO SPECIFY SEDIMENT OR WATER)= ";A$
220 LPRINT"NUMBER OF REPLICATE PLATES= ";N
230 LPRINT"ENTER WEIGHT OF DRY SEDIMENT(IF WATER COUNTS ENTER 1)= ";C
240 LPRINT"DILUTION FACTOR= ";B
250 LPRINT:LPRINT
260 :
270 :
280 REM"LINES 280-390 ARE A LOOP"
290 FOR J=1 TO N
300 LPRINT
310 PRINT
320 INPUT"NUMBER OF COLONIES PER PLATE= ";A
330 LPRINT"NUMBER OF COLONIES PER PLATE= ";A
340 D=(A*B)*10/C
350 T=T+D
360 R=R+D^2
370 PRINT"C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER= ";D
380 LPRINT"C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER= ";D
390 NEXT J
400 :
410 :
420 M=T/N
430 U=SQR((R-T^2/N)/(N-1))
440 LPRINT:LPRINT
450 PRINT:PRINT
460 PRINT"C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER"
470 PRINT"MEAN= ";M
480 PRINT"STANDARD DEVIATION= ";U
490 PRINT"------------------------------------------------------------
-----------"
500 LPRINT"C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER"
510 LPRINT"MEAN= ";M
520 LPRINT"STANDARD DEVIATION= ";U
530 LPRINT"------------------------------------------------------------
-----------"
540 PRINT"DO YOU WISH TO CONTINUE,Y/N":INPUT A$:IF A$="Y" THEN GOTO 15
0
550 END
```



Appendix Figure 1

A flow diagram of the programme calculating the numbers of heterotrophic bacteria per gram dry weight sediment or per ml water (if water counts enter 1 as dry weight input). C.F.U. = colony forming units.

Appendix Table 2

Example of calculating the numbers of heterotrophic bacteria per gram dry weight sediment or per ml water using "BACT" computer program.

```
THIS PROGRAMME CALCULATES NUMBERS OF HETEROTROPHIC BACTERIA
PER GRAM DRY SEDIMENT OR PER ml WATER(C.F.U.=COLONY FORMING
UNITS)

-----------------------------------------------------------------
SAMPLE DETAILS(REMEMBER TO SPECIFY SEDIMENT OR WATER)= FEB.BACT.O.W
NUMBER OF REPLICATE PLATES=  3
ENTER WEIGHT OF DRY SEDIMENT(IF WATER COUNTS ENTER 1)=  1
DILUTION FACTOR=  100


NUMBER OF COLONIES PER PLATE=  65
C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER=  65000

NUMBER OF COLONIES PER PLATE=  87
C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER=  87000

NUMBER OF COLONIES PER PLATE=  97
C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER=  97000


C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER
MEAN=  83000
STANDARD DEVIATION=  16370.3
-----------------------------------------------------------------
SAMPLE DETAILS(REMEMBER TO SPECIFY SEDIMENT OR WATER)= FEB.FUN.S.S
NUMBER OF REPLICATE PLATES=  3
ENTER WEIGHT OF DRY SEDIMENT(IF WATER COUNTS ENTER 1)=  .7542
DILUTION FACTOR=  10


NUMBER OF COLONIES PER PLATE=  154
C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER=  20419

NUMBER OF COLONIES PER PLATE=  164
C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER=  21744.9

NUMBER OF COLONIES PER PLATE=  137
C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER=  18164.9


C.F.U.PER GRAM DRY SEDIMENT OR PER ml WATER
MEAN=  20109.6
STANDARD DEVIATION=  1809.88
-----------------------------------------------------------------
```

Section 3 appendix

ENZYMATIC REACTIONS

API ZYM

The API ZYM system is a semiquantitative micromethod which was originally designed to detect enzymatic activities in a variety of specimens, such as tissues, cells, bacterial fluids, microorganisms and soil. It allows the systematic and rapid study of 19 enzymatic reactions using very small quantities of sample. The API ZYM gallery has 20 microtubes. The bottom of 19 of the microtubes is specially made to contain enzymatic substrate and buffer impregnated into inert supportive fabric, while cupule No.1 contains no substrate and is used as a control. Enzymatic activity is revealed after addition of suitable developing indicators.

API 20E

The API 20E system is a standardized, miniaturized version of conventional procedures for the identification of Enterobacteriaceae and other Gram-negative bacteria. It is a microtube system designed for the performance of 23 standard biochemical tests from a single colony of bacteria on plating medium.

Studies made with cultures isolated from clinical samples have considered API 20E as the most complete commercially available system for identification of Enterobacteriaceae (full details in section three introduction). This system combined with clear-cut results, ease of reading and interpretation.

API 20NE

The API 20NE system is a standardized micromethod combining 8 conventional tests and 12 assimilation tests for the identification of Gram-negative rods not belonging to the family of Enterobacteriaceae. For example, Acinetobacter, Aeromonas, Flavobacterium, Moraxella, Pseudomonas and Vibrio.

The API 20NE strip consists of 20 tubes and cupules containing dehydrated media and substrates. The principle of the technique is to inoculate the conventional tests with a bacterial suspension in saline which reconstitutes the media. During the incubation period metabolism produces colour changes that are spontaneous or develop after addition of suitable developing reagents. The assimilation tests are inoculated with a minimal saline suspension added to the assimilation medium provided and the bacteria only grow if they are capable of utilizing the corresponding substrate. Turbidity acts as an indicator of bacterial growth.

API 20B

The API 20B is a system has been designed to study heterotrophic, aerobic bacteria and of their distribution in the environment (water, air, and soil). The API gallery consists of 20 microtubes containing dehydrated substrates which enable 22 biochemical tests to be obtained. In addition, other microscopic observation tests (morphology, motility) are recommended to be carried out. The results of these test-kits are obtained as a characteristic numerical profile of the strain studied.

API 50CH

The API 50CH is a system can be used to study assimilation, oxidation or fermentation of substrates by a wide range of micro-organisms. The principle of the technique is to prepare a standardized bacterial suspension in the medium recommended for a particular strain and then use it to inoculate the API 50CH strips.

Minitek

The Minitek system utilizes paper discs impregnated with appropriate biochemicals. The discs are dispensed into the Minitek

plate and a suspension of the test organism in the appropriate Minitek broth is added. The Minitek plates are then incubated and after the addition of the appropriate reagents, the discs are examined for specific colour reactions. Colour chart cards are supplied by the manufacturer as an aid to read the organism reactions. The Minitek qualitative procedure is based upon establised biochemical methods for the differentiation of microorganisms according to their metabolism of certain substrates by bacteria. The Minitek set provide consumable products suffecient to differentiate up to 50 isolates in the Minitek system.

Drop	Pipette I			Pipette II			Pipette III		
1	0.01716	0.01898	0.01470	0.01249	0.01620	0.02025	0.2149	0.01753	0.01710
2	0.01260	0.01679	0.01920	0.01920	0.02169	0.01949	0.01837	0.02862	0.0030
3	0.01755	0.01785	0.03260	0.00678	0.01056	0.00919	0.01889	0.2281	0.01641
4	0.00309	0.01845	0.01395	0.00710	0.00385	0.00580	0.01850	0.02823	0.01554

Appendix Table 1: Replicate experiment 1. Four drops of sediment dispensed from different pasteur pipettes following one filling into each of four weighed metal foil cups.

Drop	Pipette I			Pipette II			Pipette III		
1	0.02067	0.01476	0.02075	0.02045	0.01882	0.01785	0.01183	0.02026	0.02040
2	0.03725	0.01680	0.01942	0.01960	0.03996	0.01467	0.01575	0.01635	0.01725
3	0.02093	0.02292	0.01783	0.01807	0.01286	0.01430	0.02610	0.01684	0.02008
4	0.02128	0.02010	0.02145	0.01995	0.01522	0.01056	0.01770	0.01210	0.02325

Appendix Table 2: Replicate experiment 2. Four drops of sediment dispensed from different pasteur pipettes following one filling into each of four weighed metal foil cups.

Incubation time (hour) 1st	2nd		Cupules 2	6	7	8	9	11	12
2.5	2.5	a	20	10	2.5	2.5	2.5	20	2.5
		b	20	10	5	2.5	5	10	2.5
	5	a	20	10	2.5	2.5	0	10	2.5
		b	20	5	2.5	2.5	2.5	5	2.5
	10	a	30	10	5	5	5	20	2.5
		b	20	10	10	5	5	10	2.5
	20	a	30	10	5	5	5	20	5
		b	20	10	10	5	5	10	2.5
5.0	2.5	a	30	5	2.5	2.5	2.5	10	2.51
		b	20	10	5	2.5	2.5	20	2.49
	5	a	40	10	2.5	2.5	2.5	10	2.5
		b	30	20	5	5	2.5	20	2.5
	10	a	40	20	5	5	2.5	10	2.5
		b	30	20	10	5	5	30	2.5
	20	a	40	20	5	2.5	2.5	10	2.5
		b	30	20	10	5	5	30	2.5
10	2.5	a	40.1	20.1	10.1	5	10.1	30	5
		b	39.9	19.9	9.9	5	9.9	40	5
	5	a	40	20	10	5	10	40	10
		b	40	20	10	10	10	40	5
	10	a	40	20	10	5	10	40	10
		b	40	20	10	10	10	40	5
	20	a	40	20	10	5	10	40	20
		b	40	20	10	10	10	40	10
20	2.5	a	40.1	40.1	20	2.5	2.5	30.1	10.1
		b	39.9	39.9	10	2.5	2.5	29.9	9.9
	5	a	40	40	20	2.5	2.5	30	10
		b	40	40	10	2.5	2.5	30	10
	10	a	40	40	20	5	2.5	30	10
		b	40	40	10	2.5	2.5	30	10
	20	a	40	40	20	5	2.5	30	10
		b	40	40	20	5	5	30	10

Appendix Table 3: Amount of hydralysed substrates (nanomoles) in API ZYM cupules (2,6,7,8,9,11 and 12) at each first and second incubation times. First incubation time = before addition of developing reagents, Second incubation time = after addition of developing reagents.

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