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Bacteriology of Larval Turbot Development

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in the Faculty of Science, University of Glasgow

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SUMMARY

The gut of newly-hatched turbot larvae contained very few bacteria but was rapidly colonised once feeding commenced and was generally dominated by *Vibrio* species. The source of the larval gut flora was the rotifers used as food, rather than the ambient water. In extensive-rearing systems the larval turbot gut was colonised more slowly than in intensive-rearing systems and this was correlated with better growth and survival rates. A wide variety of bacteria were represented in the gut flora of turbot larvae but recognised pathogens were rarely found and their presence was not correlated with low survival rates.

Scanning electron microscopy revealed that the bacterial flora of rotifers was almost entirely associated with the surface of the rotifers; approximately 65% of bacteria were removed by rinsing rotifers in sterile seawater and only 5% remained after treatment with benzalkonium chloride.

Attempts to reduce the viable bacterial load of rotifers with detergents and other antibacterial compounds were unsuccessful, as all the substances tested were either toxic to rotifers, or were ineffective at reducing the number of rotifer-associated bacteria. Exposure to lysozyme at 0.2% salinity resulted in >70% reduction in the bacterial load of rotifers and lysozyme-treated rotifers were palatable to turbot larvae. However, the osmotic shock due to the low salinity caused the rotifers to stop swimming and sink to the bottom of the larval-rearing tanks. Although the rotifers recovered overnight, their guts were empty, thus, they were probably of little nutritional value to the turbot larvae.

Ultra-violet irradiation of rotifers was more successful. Large numbers of rotifers were treated by passing the culture through a water jacket surrounding a U.V. tube lamp. A >99% reduction of the bacterial load of unenriched rotifers was obtained at a flow rate of 1.5 litres per min and a concentration of 200 rotifers per ml. This decreased to a >96% reduction if the rotifers were enriched with algae prior to treatment. U.V.-treated rotifers were palatable to turbot larvae and in field trials at Golden Sea Produce, Hunterston, the gut of larvae fed with U.V.-treated rotifers was colonised more slowly than in controls fed untreated rotifers. In two field trials with

extensively-reared turbot larvae, the survival rates of the larvae fed with U.V.-treated rotifers were higher than those of larvae fed untreated rotifers. The gut microflora of intensively-reared turbot larvae fed with U.V.-treated rotifers was diverse and resembled that of unfed larvae, whereas the gut microflora of larvae fed untreated rotifers was dominated by a few *Vibrio* species.

The use of rotifers as carriers was considered a potentially effective means of introducing a probiotic bacterial flora into the gut of turbot larvae. Axenically cultured rotifers were rapidly colonised when the culture water was inoculated with defined bacteria. Within two hours, approximately 250 bacteria adhered per rotifer, or were ingested by rotifers, with each of the five bacterial species tested. Attempts were made to colonise rotifers subsequently fed to larval turbot, with a strain of *Vibrio alginolyticus* isolated from healthy, intensively-reared larvae. However, the bacteria either lost viability or did not stably colonise the gut of turbot larvae.

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ABBREVIATIONS

BKD	Bacterial kidney disease
c.f.u.	Colony forming units
CLED	Cysteine lactose electrolyte deficient
cm	Centimetre
C.P.D.	Critical point dryer
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
g	Gravity
gm	Gram
G.S.P.	Golden Sea Produce
ICL	International Computers Limited
INFL	Intestinal necrosis of flounder larvae
i.u.	International unit
kg	Kilogram
M	Molar
m	Metre
mA	Milliamperes
M.A.F.F.	Ministry of Agriculture, Fisheries and Food
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
MOF	Marine oxidative/fermentative
NCMB	National collection of marine bacteria
ng	Nanogram
nm	Nanometre
O/129	2,4-diamino-6,7-diisopropyl pteridine

O.D.	Optical density
ONPG	O-nitrophenyl-ß-D-galactopyranoside
ppm	Parts per million
PUFA	Polyunsaturated fatty acid
rpm	Revolutions per minute
sp	Species
SEM	Scanning electron microscopy
TCBS	Thiosulphate-citrate bile-salt sucrose
µg	Microgram
µm	Micrometre
U.V.	Ultra-violet
$\mu W.s/cm^2$	Microwatt seconds per square centimetre
v	Volume
w	Weight

INTRODUCTION

1.1 Development of Turbot Culture

Flatfish such as plaice, sole, flounder, halibut and turbot are among the most commercially important fish of northern temperate waters. Overfishing and environmental deterioration led to attempts at artificial propagation and restocking of flatfish, particularly plaice (*Pleuronectes platessa*), at the end of the nineteenth century. The efficacy of this practice was doubtful and it was discontinued by 1920 (Blaxter, 1987).

Most of the early research into flatfish culture was carried out in the U.K. by the White Fish Authority (W.F.A.) and the Ministry of Agriculture, Fisheries and Food (M.A.F.F.). The first species chosen was plaice but its production was uneconomical. Attention was switched to Dover sole (*Solea solea*) and turbot (*Scophthalmus maximus*) (Bardach *et al.*, 1972).

Trials of the suitability of juvenile turbot for cultivation were made by Purdom *et al.* (1972). Fish were caught in push nets from beaches since O-group turbot live in the surf zone. The turbot were fed on mashed trash fish and reached a marketable size (30cm) within 14 months, starting from a mean length of 5cm. They proved to be easy to manage at high density and food conversion rates were good, averaging 32.4%. The authors concluded that post-metamorphosed turbot were suitable for cultivation.

Rearing of turbot, and also lemon sole (*Microstomus kitt*), was hindered by the lack of a suitable food for the early larval stages since the larvae were too small to ingest brine shrimp (*Artemia salina*) used to rear plaice. Howell (1972) tested five easily cultured organisms which might support the growth of lemon sole. The organisms used were the marine flagellate *Dunaliella tertiolecta*, an unidentified hypotrichid ciliate, the "vinegar eelworm" *Tubatrix*, the trochophore larvae of the mussel *Mytilus edulis*, and the marine rotifer *Brachionus plicatilis*. Only mussel larvae and rotifers supported growth until *Artemia* nauplii could be fed. Turbot larvae were reared beyond metamorphosis for the first time in 1972 at the M.A.F.F. laboratory, Lowestoft, using rotifers followed by *Artemia* nauplii and subsequently

larger stages of *Artemia* (Jones, 1973; Jones *et al.*, 1974).

In 1976, survival rates of turbot larvae between different batches of eggs at the W.F.A. hatchery at Ardtoe, Scotland, fluctuated between 0% and 20.7%. However, the gross survival of 7.2% was below the level of 10% estimated to be necessary for commercial viability (Kingwell *et al.*, 1977).

The technique used by the W.F.A. employed circular black fibre-glass tanks with flat bases housed in a heated building and supplied with ambient seawater filtered through sand. Howell (1979) found that black tanks were more suitable than white tanks for rearing larval turbot since slightly higher survival rates and significantly increased lengths of larvae were recorded in black tanks.

The tanks were continuously illuminated and aeration was supplied using airstones and a compressor. The tank temperatures were thermostatically controlled using sheathed heaters. Purdom *et al.* (1972) showed that the growth of turbot was temperature dependent and was maximal in the range 18 to 22°C.

In turbot, as in most fish, spawning is seasonal, occurring usually in mid-summer. The development of techniques for inducing spawning by controlling the light and temperature conditions allowed the production of viable embryos throughout the year (Bye and Htun-Han, 1979). Devauchelle *et al.* (1988) investigated the optimum conditions for embryonic development of turbot. The temperature required for maximal spawning was 13 to 15°C, however, the optimal temperature for incubation was 15 to 17°C. No differences in hatching performance were recorded in salinities between 2.5% and 3.5%.

The fertilised eggs were incubated in seawater treated with antibiotics. Shelbourne (1964) discovered that by adding a mixture of penicillin and streptomycin to the hatching tanks up to 80% of turbot larvae survived to metamorphosis.

Due to the high cost of live food production, turbot larvae have been weaned at an early age onto cheaper diets such as trash fish and artificial dry foodstuffs. Deniel (1976) fed captured wild juvenile turbot on moist pellets with a high protein content for 13 months at 15°C. After nine months the fish fed on moist pellets were slightly

larger than the fish fed natural food (molluscs or fish). After this period, fish fed on natural food grew better than those fed the artificial diet.

Bromley (1978) successfully weaned hatchery-reared turbot larvae onto a commercial, high-fat, salmon starter diet. The duration of the weaning period was influenced by the initial size of the larvae, with larger larvae being weaned more quickly than smaller ones. The complete absence of live food during weaning resulted in poor growth and survival of the larvae. Kuhlmann *et al.* (1981) found that the growth of post-metamorphosed turbot was greatest when fed herring meat plus living mysids compared to combinations of pelleted trout feed and mysids or trout feed alone. The trout feed alone gave the poorest growth rates.

The quality of the live food influenced the subsequent weaning of turbot larvae onto an inert diet (Bromley and Howell, 1983). Larvae fed newly-hatched *Artemia* nauplii before weaning did not survive as well as larvae fed metanauplii which had previously been given algae for two days. The water content of the artificial diet was unimportant in weaning late-stage larvae.

1.2 Current Status of Turbot Culture

Turbot is considered to be one of the most promising species for marine aquaculture since it has a high value at £20-30 per kg and, as culture techniques have improved, it appears to be relatively easy to rear. The commercial production of turbot has developed most rapidly along the Atlantic coast of northern Spain and southern France. In northern Europe attention has now focused on rearing juveniles for ongrowing in southern Europe due to the lack of seawater at suitable temperatures for rearing turbot. In the U.K. and Scandinavia, cooling water from power plants has been utilised in fish farms (Paulsen, 1989).

Such a system is employed by Golden Sea Produce (G.S.P.), based at Hunterston power station in Scotland. The hatchery was built in 1984 after G.S.P. took over the site of an eel farm. Currently G.S.P. export juvenile turbot to northern Spain for ongrowing.

Survival rates of turbot larvae at G.S.P. have varied widely from 0% to approximately 30%. Intensive rearing (10-15 larvae per litre), although potentially efficient, is particularly prone to low survival rates. The reasons for this are unclear but have generally been attributed to poor "water quality" (A. Barbour, personal communication). The factors influencing water quality are numerous. They include the level of particulate matter, the concentration of heavy metals or other pollutants, the level of dissolved gases, the organic content and the concentration of living organisms such as phytoplankton and bacteria. An investigation of the water quality in the area revealed no obvious answer (Perkins, 1981). This thesis reports a study of the role of bacteria in the early stages of development of turbot larvae which was carried out in collaboration with G.S.P., Hunterston.

1.3 Nutrition of Turbot Larvae

Newly Hatched Turbot Larvae

Newly hatched turbot larvae are typically 2.3-2.8mm long and possess a large yolk sac containing a single oil globule (Al-Maghazachi and Gibson, 1984). During the first few days after hatching, the energy requirement of the larvae is fulfilled by this endogenous yolk supply. Blaxter (1966) found that, in herring (*Clupea harengus*) larvae, the rate and efficiency of yolk utilization can influence the size and condition of the larvae. This affects their survival since their size and condition determines their ability to swim and catch prey and their ability to survive a short period of malnutrition such as occurs during the period of first feeding (May, 1973).

Quantz (1985) examined the effect of temperature and salinity on yolk absorption by larvae of Baltic Sea turbot in brackish water (1.8%) at 15, 18 and 21°C, and North Sea turbot in seawater (3.2%) at 15°C. The lengths of the larvae at yolk absorption did not differ significantly between the groups, however, yolk sac utilization increased with temperature.

The larvae in brackish water showed a slightly lower efficiency of yolk

conversion into tissue than the larvae in seawater (60.2% compared to 72.1%). The possibility that this was due to genetic differences between the two stocks of turbot was mentioned by Quantz but he believed the difference was due to the different larval behaviours. The larvae in seawater were buoyant and floated passively with only occassional movements whereas the larvae in brackish water were below their point of neutral buoyancy (2.8%; Kuhlmann and Quantz, 1980) and had to swim up to the surface regularly to compensate for their sinking.

During the process of yolk conversion into tissue, the dry weight of tissue did not increase constantly but reached a maximum at about 48 hours after hatching and then decreased by approximately 11% (15°C) until the end of the yolk sac period. The resorption of larval tissue indicated the onset of the "critical period". A similar event was observed in the larvae of the Pacific sardine (*Sardinops caerulea*) by Lasker (1962). However, Quantz pointed out that at this stage the larvae are morphologically capable of uptake of prey organisms and a problem only occurs if food is unavailable. He recommended that turbot larvae reared at 15, 18 or 21°C should be supplied with an adequate concentration of suitable prey within 90, 75 and 60 hours post-hatch, respectively, and larvae reared in seawater at 15°C, within 100 hours post-hatch.

Gadomski and Petersen (1988) found that first-feeding halibut (*Paralichthys californicus*) and diamond turbot (*Hypsopsetta guttulata*) also required food by the day of total yolk absorption. The growth rate of halibut larvae which survived starvation for one or two days after yolk absorption was significantly less than that of larvae fed earlier. The length of time to "irreversible starvation" was examined in three-week-old larvae and survival was reduced with longer periods of starvation. One day of food deprivation reduced survival of halibut larvae from 73% to 64% and deprivation for three and four days resulted in less than 25% survival. Three-week-old turbot larvae survived more prolonged periods of starvation than halibut although mortality was also pro-

portional to time. The turbot larvae were able to resume feeding and survive, after about a week of starvation. In contrast, halibut larvae rarely resumed feeding and even when they did they frequently failed to survive, seemingly being incapable of digestion.

Cousin *et al.* (1986) made histological observations of turbot larvae from intensive cultures in which high mortalities occurred. The larvae dying between days 6 and 8 after first-feeding were usually thin and presented a regression of the digestive tract with atrophy and/or desquamation of the mucosa of the digestive tube. Later mortalities, between days 9 and 15, appeared in larvae which had ingested food. Again, atrophy and desquamation of the digestive tract were observed. Muscular degeneration, dystrophy of the swim bladder and glomerular atrophy were noted. These alterations were attributed to inadequate quality of the food organisms, either in size or nutritional value, essentially causing starvation, since no infectious, parasitic, or dysplasic factors were detected.

Rosenberg and Haugen (1982) demonstrated that starvation was size-selective in larval turbot. Samples of larvae were taken during the first 12 days post-hatch and individual body growth was estimated based on back calculations from daily growth rings on their otoliths. Starvation occurred on the seventh day. The survivors beyond this period were larger by 0.18mm on average.

Dietary Enzymes in Turbot Larvae

Differences in the enzymic activities of fed and starved turbot larvae have been investigated by several authors. Munilla-Moran and Stark (1989) measured the proteolytic activity of fed and starved 3-, 6- and 9-day-old larvae by the hydrolysis of casein at a range of pH values. The results were compared with those from digestive tracts of 30-day-old juveniles and the guts of 24-hour-starved adults.

The pH profile of starved specimens revealed three peaks of maximum

activity at pH 2-3, 5.5 and 9.0. The highest specific activity was noted at pH 5.5 for larvae, but in adults proteolytic levels were highest at pH 2.5 and 9.0. The level of proteolytic activity detected in adult guts, after the removal of the gut contents, was found to be similar to that of the larvae with the exception of the acid protease which was higher in adults.

The proteolytic activity of fed larvae and juveniles was very different to that of the starved specimens. In fed specimens the highest activity was detected at pH 9.0. At acid pH values the specific activities in fed and starved larvae and juveniles were very similar but at pH 9.0 the activity of fed specimens was two or three times higher than that of starved specimens. Hjelmeland *et al.* (1988), using a very sensitive radioimmunoassay, discovered that ingestion of polystyrene spheres by herring larvae induced trypsin secretion to a higher level than in non-feeding fish.

The possibility that exogenous proteases were responsible for the difference between fed and starved specimens was investigated by Munilla-Moran and Stark (1989) but their results indicated that this was very unlikely.

The protease activity at acid pH values of whole 30-day-old juveniles, with the gut removed was similar to that of larvae. Thus, the authors concluded that the acid protease activity in early larval stages was not located in the digestive tissues.

In a later paper, Munilla-Moran *et al.* (1990) measured the levels of nine enzymes in turbot larvae and the most common live diets (rotifers, copepods and *Artemia*). Turbot larvae possessed the complete range of digestive enzymes tested at three days post-hatch. Adult copepods showed very high enzymic levels as did *Artemia* metanauplii but in *Artemia* the enzymic levels varied with the developmental stage and the nutritional state of the animal. The lowest enzymic levels, expressed as units per individual, were found in rotifers. This was in agreement with their earlier suggestion that rotifers were unlikely to contribute to the digestive enzymes of turbot larvae.

Cousin *et al.* (1987) used histoenzymological techniques to investigate the activity of seven enzymes in fed and starved turbot up to 40 and 10 days old, respectively. Alkaline phosphatase and aminopeptidase activities were detected in the distal region of the digestive tube in one-day-old larvae. Non-specific esterase and protease activities became apparent in the digestive tract at days 2 and 3, respectively. Amylase occurred in the exocrine pancreas at day 3 and in the lumen of the intestine at day 4. Acid phosphatase activity was detected in the cells surrounding the yolk sac and the lipid droplets at day one and in the epithelium of the intestine at day 3. Lipase was only detected at day 15 in the muscular layers of the oesophagus. All the enzymes investigated were found in starving larvae with the exception of lipase, however, the enzymic activities were weaker in the starved larvae compared to the fed larvae between days 7 and 10.

Natural Food of Turbot Larvae

Few studies have been published on the diet of larval turbot in the wild. Lebour (1919) found the cladoceran, *Podon*, the copepods *Pseudocalanus* and *Calanus*, *Calanus* nauplii and euphasiid and gastropod larvae in young turbot (*Rhombus maximus*) caught in Plymouth Sound. Last (1979) examined the stomach contents of pre-metamorphosed larval turbot (*S. maximus*) between 3 and 18mm in length from the west central North Sea. The food items consisted of a wide variety of planktonic crustaceans, the most important of which were the cladocerans *Podon leukarti* and *Evadne normanni*, and the copepodites of 5 species of calanoid copepods. Phytoplankton were found in the stomachs of some of the smaller larvae. It was estimated that the larvae ate 28% of their body weight daily.

Spectorova *et al.* (1974) fed Black Sea turbot (*S. maeoticus*) larvae on wild microzooplankton, rotifers and dinoflagellates. The plankton represented were bivalve and gastropod larvae, copepod nauplii, rotifers and polychaete larvae. Experiments involving different concentrations of rotifers fed to turbot larvae

showed that a concentration of one organism per ml was suitable and this concentration was maintained in the experiments with wild zooplankton. The turbot larvae had a definite preference for bivalve mollusc larvae and when these were present gastropod and copepod nauplii were not consumed. However, if bivalve mollusc larvae were absent, gastropod and copepod nauplii were taken. Rotifers were taken even when bivalve mollusc larvae were present. Among the organisms found in the stomach were larval Lamellibranchia, gastropod and polychaete larvae, copepod nauplii, rotifers, unidentified cysts, eggs and dinoflagellates.

Nutrition of Hatchery-Reared Turbot Larvae

The use of zooplankton as living feeds for larval marine fishes is practiced world-wide since suitable artificial diets are so far unavailable for most species. The types of zooplankton used include rotifers, copepods of the marine species *Tigriopus*, *Acartia*, *Oithona* and *Paracalanus* or the freshwater species *Moina* and *Daphnia* and the brine shrimp, *Artemia salina*. The most extensively used is the rotifer *Brachionus plicatilis*.

The use of rotifers was pioneered by Ito (1960). The first successful attempts to rear turbot larvae from eggs obtained at sea from wild fish used rotifers at the initial stages of feeding, followed by *Artemia* nauplii (Jones, 1973). Metamorphosis of the larvae occurred when they reached 23-30mm in length. At this stage they were weaned onto a diet of trash fish. The overall survival from hatching to metamorphosis was very low at approximately 1%.

Alderson and Howell (1973) found that the presence of unicellular algae in the rearing water of juvenile sole improved the growth of the fish. The authors surmised that this was due to the removal of dissolved ammonia by the algae. The use of algae in the rearing water of larval fish was adopted by most workers thereafter.

Spectorova and Doroshev (1976) reared Black Sea turbot larvae on rotifers

fed either a combination of algal species or a monoculture. The unicellular algae chosen were *Dunaliella tertiolecta*, *D. viridis*, *Tetraselmis salina* and *Phaeodactylum tricornatum*. The algae were cultured in $2m^3$ tanks for 3-4 days until, at the peak of algal development, the rotifers were added. The peak densities of the algae varied from 1.2×10^6 cells per ml for *Ph. tricornatum* and 0.7×10^6 cells per ml for *D. tertiolecta* to $0.05\text{-}0.1 \times 10^6$ cells per ml for the other algae. When the density of rotifers had increased to 5-10 per ml the turbot larvae were introduced. None of the larvae survived beyond metamorphosis, however, the authors deduced that a mixture of algae was preferable to a monoculture.

Howell (1979) compared growth of larval turbot (*S. maximus*) in tanks containing either a mixture of 6 species of unicellular algae (*D. tertiolecta*, *Rhodamonas* sp., *Isochrysis galbana*, *Ph. tricornatum*, *Tetraselmis suecica* and *Prorocentrum micans*) or rotifers given *Isochrysis* or *Dunaliella* daily. The value of oyster (*Crassostrea gigas*) veligers as food for early turbot larvae was also assessed by comparing larvae fed rotifers, veligers or a mixture of the two. Algae alone did not sustain early growth of turbot larvae and after five days feeding all the larvae died. After nine days feeding, larvae fed on rotifers given *Isochrysis* were significantly larger and survived better than larvae fed on rotifers given *Dunaliella*. Oyster veligers alone, or with rotifers, did not produce significantly higher larval growth or survival than rotifers alone.

Scott and Middleton (1979) also found that the growth and survival rate of turbot larvae varied depending on which algal species was added to the tanks during the rotifer feeding stage. They found that *Dunaliella* resulted in poor growth and survival of larvae compared to *Isochrysis*, *Phaeodactylum* or *Pavlova*. They demonstrated that *Dunaliella* was not toxic but probably caused a dietary deficiency in the turbot larvae. Fatty acid analysis of the algae, rotifers and turbot revealed that *Dunaliella* was noticeably deficient in the long-chain (20 and 22 carbon) polyunsaturated fatty acids (PUFA's) of the n-3 series. Rotifers

grown at 18°C showed the same pattern of fatty acids as the algae on which they were fed but rotifers fed at 23 and 28°C showed little difference in composition.

Scott and Baynes (1978) found the type of algae used had little influence on the growth rate and yield of rotifers in culture, however, at low temperatures (18°C) rotifers consumed their food slowly and maintained a relatively high level of lipid and carbohydrate for a long period of starvation. At high temperatures (28°C), food was consumed rapidly and the nutritional value of the rotifers declined markedly when they were starved. The authors stressed the importance of keeping rotifers well-fed in the turbot-rearing tanks considering the significant loss of weight and nutritional value that occurred with starvation. Diet had a marked effect on the fatty acid composition of turbot larvae. Fish from tanks with added *Dunaliella* had much lower levels of long-chain PUFA's than other fish. Scott and Baynes suggested that larval turbot were probably unable to synthesize their own long-chain PUFA's.

Cowey *et al.* (1976) showed that the growth rate and food conversion of post-larval turbot given a diet containing preformed PUFA's of the n-6 series was inferior to that of turbot given a diet containing n-3 series PUFA's. In addition, turbot were unable to desaturate 18-carbon fatty acids.

The gill epithelium was found to be a sensitive indicator of deficiency of 22:6(n-3) PUFA in turbot by Bell *et al.* (1985). Three groups of turbot were fed a diet containing 10% of the dry weight as natural fish oil, (n-3 and n-6 series PUFA's), palmitic acid (no PUFA's) or an n-6 PUFA-deficient diet with a ratio of 20:5(n-3)/22:6(n-3) of 13.8. The diet incorporating natural fish oil was the only one which produced significant growth over a 15-week period. There were high mortalities in the groups fed the other diets, however, mortalities were reduced in the group fed the n-6 PUFA- deficient diet by changing the ratio of 20:5(n-3)/22:6(n-3) from 13.8 to 2.2. Fish fed the PUFA-deficient diets developed gross changes in the gill structure with the tissue ultimately disintegrating to leave a skeleton of connective tissue.

Watanabe *et al.* (1983) demonstrated that rotifers can take up lipid and fat-soluble vitamins directly when fed an emulsion of lipid, raw egg yolk and water together with baker's yeast (*Saccharomyces cerevisiae*). The concentration of n-3 PUFA in the rotifers was proportional to the content of n-3 PUFA in the emulsion and reached a maximum between 6 and 12 hours after feeding. Soybean lecithin, casein-Na and raw egg yolk were equally suitable as emulsifiers. Kissil and Koven (1990) developed a method of increasing the PUFA content of oils used in emulsions by low temperature crystallization separation at -70°C for 8 hours.

The value of various enrichments for increasing the PUFA content of rotifers, including baker's yeast with capelin or cod liver oil, herring roe meal, squid meal, shrimp meal and fish meal or combinations of these, was investigated by Rainuzzo *et al.* (1989). Baker's yeast contained no n-3 PUFA and herring roe meal contained the largest amount, however, rotifers fed on squid meal showed the highest n-3 PUFA level. This was thought to have been due to the difference in suitability of the particles for ingestion by the rotifer.

Perez-Benavente and Gatesoupe (1988a) examined the n-3 PUFA content of turbot larvae fed rotifers once a day or semicontinuously throughout the day. The rotifers were enriched with cod liver oil for 24 hours before feeding. Although the survival rates of the two groups of larvae did not differ significantly after 10 days, the larvae fed semicontinuously had higher levels of 22:6(n-3) PUFA than the larvae which were fed once a day. The authors concluded that the continuous distribution of rotifers in larval turbot rearing tanks was probably beneficial.

Whyte and Nagata (1990) identified the sugars and fatty acids present in rotifers fed baker's yeast or phytoplankton (*Thalassiosira pseudona, Isochrysis galbana, Tetraselmis suecica* or *Chlorella saccharophila*). Glucose, as glycogen, formed 61-80% of the rotifer carbohydrate; ribose was also present at a relatively high percentage (9-18%) and the content of sugars in the rotifers did

not vary with diet as much as did the fatty acid levels.

1.4 Bacteria Associated with Fish and Their Culture

Bacterial Flora of Seawater

One of the possible sources of bacteria associated with fish and other marine organisms is seawater. The number of bacteria in seawater has been determined to be in the range 10^3 to 10^7 per ml depending on the season, location and detection technique (Jannasch and Jones 1959; Patrick 1978; Watson 1977). Direct count techniques (Bowden, 1977; Jones and Simon, 1975) yield the highest values and it is possible that many bacteria are present in aquatic systems in a dormant form (Stevenson, 1978).

Gilmour *et al.* (1976) carried out quantitative and qualitative analyses of warmed seawater, from power station effluent used for fish culture, before and after the addition of starved plaice. The bacterial counts from the stocked tanks were significantly higher than those from tanks with no fish. The bacteria identified from both sets of tanks were similar, the predominant type being the *Pseudomonas* GroupIV/ *Achromobacter*/ *Alcaligenes*/ *Agrobacterium* group. Other bacteria present were *Vibrio*/anaerogenic *Aeromonas, Flavobacterium*/ *Cytophaga, Moraxella, Pseudomonas* Groups I and II/ *Achromobacter*/ *Alcaligenes*/*Agrobacterium, Acinetobacter*, Gram-positive cocci, coryneforms and unidentified Gram-negative rods.

Austin (1982; 1983) studied the bacterial flora associated with a coastal, marine fish-rearing unit and found a seasonal distribution with maximum numbers of bacteria isolated in summer. Isolates from seawater were identified as *Acinetobacter, Bacillus, Cytophaga*/ *Flavobacterium*/ *Flexibacter, Hyphomicrobium*, Gram-positive cocci, *Photobacterium, Pseudomonas, Vibrio* and unidentified isolates. In addition, water from tanks stocked with turbot contained *Alteromonas*, Enterobacteriaceae, *Asticcacaulis, Caulobacter*,

coryneforms, *Janthinobacterium*, *Lucibacterium* and *Prosthecomicrobium*. The numbers of bacteria in the tank water were consistently higher than those of the seawater but the highest counts were always obtained from the effluent.

Bolinches and Egidius (1987) found a ten-fold increase in bacteria in the water of halibut-rearing tanks compared to the inlet water and the bacterial flora also differed. The inlet water had high percentages of *Vibrio* and *Pseudomonas/Moraxella* species whereas the water in the spawning tank contained more *Photobacterium* and *Flavobacterium* with water from a tank containing halibut being dominated by Actinomycetes. Vibrio counts were estimated using thiosulphate-citrate-bile salt-sucrose (TCBS) agar and these were very low (0.1-100 c.f.u. per ml) except in the tank of halibut larvae where levels reached 1,000 c.f.u. per ml.

The bacteria in the rearing tank water may be very important in the development of the gut flora of early fish larvae. Drinking by yolk-sac stage larvae of the halibut was demonstrated by Tytler and Blaxter (1988). They immersed the larvae in a fluorescein isothiocynate dextran solution and examined them by epifluorescent microscopy. Drinking was observed in yolk-sac larvae three days post-hatch, long before first feeding normally occurs.

The ingestion of rhodamine-labelled bacteria by cod larvae was shown by Olafsen (1984) using fluorescent microscopy. He suggested that some mechanism other than the passive uptake of bacteria with water during osmoregulation by the larvae must be responsible for the large numbers of bacteria ingested.

Bacterial Flora of Zooplankton and Algae

The alternative source of bacteria associated with fish is their food. In the case of marine fish larvae in captivity this refers to live food organisms such as copepods, rotifers, *Artemia* and, perhaps indirectly, algae.

Sochard *et al.* (1979) studied the bacteria associated with healthy and moribund laboratory-reared copepods of the species *Acartia tonsa*, and from

several species of wild copepods collected from a marine or estuarine environment. They found greater numbers of bacteria in the surface samples (copepod washings) than in the "gut-surface" samples (washed, homogenised copepods). The estuarine copepods had the highest number of *Vibrio* species whereas the marine copepods possessed more *Pseudomonas* species in the gut-surface flora than either estuarine or laboratory-reared copepods. These differences were attributed to differences in salinity and feeding habits, *A. tonsa* being herbivorous, the others being omnivorous.

Aseptic removal of the gut of laboratory-reared copepods revealed only *Vibrio* species present. In all the copepods studied, *Vibrio* species were dominant, being three times more abundant than *Pseudomonas* species. The authors concluded that a commensal flora was established in the gut of copepods.

The numbers of bacteria associated with the cysts and nauplii of *Artemia* were estimated by Austin and Allen (1981/1982). The dried cysts contained 2.5 x 10^5 bacteria per gram which approximated to less than one bacterium per cyst. There was a rapid increase in total bacterial numbers upon the onset of hatching. The number of bacteria in the seawater-based growth medium reached 1.2 x 10^7 per ml after 24 hours but gentle washing revealed that few bacteria were closely associated with the *Artemia*. Representative isolates were identified as *Bacillus, Erwinia, Micrococcus, Staphylococcus* and *Vibrio* species.

Hayashi *et al.* (1975) found 10^8 to 10^{10} bacteria per gram of dried *B. plicatilis* and *Moina macrocopa* (used to produce larvae of ayu, *Plecoglossus altivelis*) on fresh and salt water media, and 10^7 to 10^8 bacteria per gram on TCBS agar. Miyakawa and Muroga (1988) counted 10^7 to 10^8 c.f.u. per gram of rotifers on marine agar, *Pseudomonas* and *Moraxella* being the dominant types.

Nicolas *et al.* (1989) examined the bacterial flora associated with two similar trophic chains consisting of unicellular algae (*Pavlova lutheri* or *Platymonas suecica*), rotifers and turbot larvae. Direct counts revealed about 10^7 bacteria per ml in the culture water of the algae and rotifers. The ratio of direct

counts/plate counts was very low and the number of bacteria was not affected by the type of algae present. In the algal cultures the bacteria were identified as *Pseudomonas, Vibrio, Aeromonas, Oceanospirillum, Alteromonas, Acinetobacter* and *Flavobacterium*. Previously, Berland *et al.* (1969) found similar bacteria associated with six different algal cultures, the dominant genera being *Pseudomonas, Flavobacterium* and *Achromobacter*. In the studies by Nicolas *et al.* (1989) the number of bacteria associated with the rotifers fed *Pavlova* was 2 and 25 times greater than that of rotifers fed *Platymonas* at the beginning and end of the experiment, respectively. This was attributed to the fact that the rotifers fed *Pavlova* grazed almost ten times more than the rotifers fed *Platymonas*. The bacterial flora of the *Pavlova*-fed rotifers resembled that of the culture water but *Platymonas*-fed rotifers selected a specific microflora.

Turbot larvae given the *Platymonas*-fed rotifers fed normally and 40-60% were alive on day 10. In contrast, larvae given *Pavlova*-fed rotifers did not ingest the prey and none survived beyond day 8. However, the number of bacteria per larval turbot did not differ significantly between the two groups.

No bacteria isolated from the algal cultures were isolated from the rotifers and, except for some *Vibrio* isolates, most bacteria associated with the rotifers were not found in the larvae. The authors suggested that the gut of larval turbot selected for certain bacteria since only certain *Vibrio* strains were isolated from larvae.

Normal Bacterial Flora of Marine Fish

Liston (1956) studied the bacterial flora of skate (*Raja* species) and lemon sole (*Pleuronectes microcephalus*) caught in the North Sea over a period of 27 months. A seasonal variation in the size of the bacterial populations on the gills was observed. In skate the greatest numbers occurred in early summer and early winter, and in lemon sole the peaks were in mid-winter and possibly again in summer. Seasonal variation was not observed in the gut bacteria and the

microflora of the skin was thought to be influenced by the trawling procedure used to catch the fish and was therefore not a reasonable indicator of seasonal variation.

Raised temperatures did not appear to be responsible for the increases in the bacterial flora, instead plankton blooms were suspected. The peak of bacterial development occurred some two months after a plankton bloom and the sinking of the decaying remains of plankton, providing an additional source of organic nutrients, was thought to be responsible.

Identification of the bacteria isolated from skate and lemon sole (Liston, 1957) revealed a very similar flora in the two flatfish. The most common strains isolated were *Pseudomonas, Achromobacter, Alcaligenes, Flavobacterium*, corynebacteria, cocci and *Vibrio*. The flora of the gut was distinct from that of the skin or gills and consisted mainly of "gut group vibrios". The bacteria on the skin and gills varied seasonally, reflecting the flora of the environment.

The flora of the skin and gills of Atlantic salmon (*Salmo salar*) was also shown to be influenced by that of the environment (Horsley, 1973). In contrast, Mudarris and Austin (1988) found that the microflora of turbot gills was quite distinct from that of the surrounding water and the fish skin. Scanning electron microscopy (S.E.M.) of the gill surface revealed that the bacteria were found in protected niches, such as the clefts between the lamellae, and not on exposed parts of the gills.

Austin (1983) examined the skin of healthy turbot by S.E.M. and observed few bacteria although spread plates prepared from similar samples showed abundant bacterial growth. Austin assumed that the bacteria were either loosely associated with fish skin, and therefore removed by the fixation procedure, or they were masked by debris.

Gilmour *et al.* (1976) examined the flora of the skin and intestines of farmed plaice. Five generic groups were isolated from the skin, namely, *Pseudomonas* Group IV/*Achromobacter/Alcaligenes/Agrobacterium*, *Pseudomonas* Groups I

and II/ *Achromobacter/ Alcaligenes/ Agrobacterium, Vibrio/* anaerogenic *Aeromonas*, Enterobacteriaceae and *Moraxella*. These isolates were also recovered from the intestines and, in addition, *Aeromonas, Acinetobacter*, unidentified Gram-negative rods, Gram-positive cocci and coryneforms were found.

Cousin and Baudin-Laurencin (1987) found that bacteria were associated with areas of desquamation of the intestinal epithelium in moribund juvenile (15 to 40-day-old) turbot. Few bacteria were attached to the wall of the intestine of healthy turbot although the gut lumen contained many bacteria.

Aiso *et al.* (1968) found that the aerobic bacterial flora of the intestine of the jackmackerel (*Trachurus japonicus*) was composed almost exclusively of *Vibrio* species whereas the flora of the stomach was more varied. The microflora of the stomach and intestine of red sea bream (*Pagrus major*) was compared to that of file-fish (*Stephonolepsis cirrhifer*), a fish with a relatively undeveloped stomach, by Sera and Ishida (1972). Two *Vibrio* strains, which were resistant to bile and low pH, were always dominant in the stomach and intestines of the red sea bream, irrespective of the diet. The intestines of file-fish contained mostly *Vibrio* and *Achromobacter* and the components of the flora reflected those of the diet. The authors suggested that the environmental conditions within the gut of fish with a developed stomach selected for a few specific types of bacteria.

Sakata *et al.* (1978) found vibrios to be the dominant group in the intestinal microflora of yellowtail. A possible role of these vibrios in the nutrition of the host fish was suggested since most were able to hydrolyse chitin although none hydrolysed casein, gelatin or starch.

MacDonald *et al.* (1986) dissected the digestive tract of Dover sole into three sections, foregut, midgut and hindgut/rectum and found a progressive increase in the numbers of aerobic heterotrophic bacteria along the digestive tract. On a w/w basis, juveniles had 10 to 20 times more bacteria in their intestines than adult fish. The bacteria, identified as *Acinetobacter, Alcaligenes*,

Enterobacteriaceae, *Photobacterium*, Gram-positive cocci and *Vibrio*, with a predominance of *Vibrio*, were capable of degrading *p*-nitrophenyl-ß-N-acetyl-glucosaminide, chitin and collagen. The authors concluded that these bacteria were probably involved in the digestion of food in Dover sole.

The intestinal microflora of farmed Dover sole at different stages of development, and in relation to different diets, was investigated by Campbell and Buswell (1983). The dominant generic group in the fish larvae was *Pseudomonas* Group IV/*Alcaligenes* which was also the dominant group in the tank water. An increase in the percentage incidence of *Vibrio*/anaerogenic *Aeromonas* in the intestines was observed with increasing age of the fish and change of diet. This increase coincided with the period when the fish were fed *Lumbricillus rivalis*, the dominant flora of which was identified as *Vibrio*/anaerogenic *Aeromonas*. However, the composite pellet diet, used for ongrowing fish, had a high percentage of Gram-positive bacteria but there was no relationship between the pellet microflora and that of the fish intestines.

A similar study was carried out by Muroga *et al.* (1987) on the intestinal microflora of farmed red seabream and black seabream (*Acanthropagrus schlegeli*). Few bacteria were found in four-day-old larvae. Juvenile fish contained 7.4×10^4 c.f.u. per fish and 3.4×10^4 c.f.u. per fish for red seabream and black seabream, respectively. Vibrios were the dominant gut bacteria in both types of fish. Pseudomonads were also common. The dominant genera in the fish intestines were present in the tank waters and live diets but constituted a much lower percentage of the total flora. The authors concluded that the indigenous microflora of fish intestines arose from the diet and became established by selection due to specific conditions in the gut.

Investigation of the gut microflora of larval and juvenile Japanese flounder (*Paralichthys olivaceous*) by Tanasomwang and Muroga (1988) revealed a similar picture. Pseudomonads predominated in newly hatched larvae and in the rearing water but after feeding, the gut flora of larval and juvenile flounder was

influenced quantitatively and qualitatively by the microflora of the foods ingested, with vibrios predominating. A decrease in the number of bacteria was observed when the fish went onto an artificial diet which contained less bacteria than the live diets. The incidence of *Vibrio* also decreased but the dominant bacteria in the artificial diet, namely *Acinetobacter* and Gram-positive cocci, were not found in the fish intestine. It was suggested that they failed to colonise due to unsuitable conditions within the gut.

Bacterial Pathogens of Fish

Horne (1982) stated that whenever the natural gut and skin microflora of healthy fish have been described, large numbers of different strains of bacteria known to be the cause of fish diseases have been isolated. He suggested that the normal or commensal bacterial flora of fish was one possible source of pathogens and that disease might arise from a change in the balance between the host and the indigenous microflora.

In a review of the bacterial pathogens of fish, Austin and Allen-Austin (1985) stated that representatives of 25 bacterial genera had been implicated in diseases of fish. The majority were Gram-negative although there were important Gram-positive fish pathogens such as *Mycobacterium* species, *Streptococcus* species, *Nocardia asteroides* and *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD). Pseudotuberculosis, streptococcicosis, nocardiosis and BKD were listed among the most important bacterial infections in mariculture in Japan by Sano and Fukuda (1987).

The Gram-negative pathogens included by Austin and Allen-Austin (1985) were: *Aeromonas hydrophila; A. salmonicida* which causes furunculosis, an important disease of salmonids; *Flexibacter; Flavobacterium; Cytophaga psychrophila; Edwardsiella ictaluri; Pasteurella piscida*; the pseudomonads *P. anguiseptica, P. chlororaphis* and *P. fluorescens; Sporocytophaga; Yersinia ruckeri* and several *Vibrio* species.

Vibriosis

One of the most important diseases of fish and shellfish, known as vibriosis, is caused by members of the Gram-negative genus *Vibrio*. This disease was also listed by Sano and Fukuda (1987) who reported economic losses due to vibriosis in Japan of red seabream, yellowtail, horsemackerel and kuruma shrimp totalling 1,092 million yen in 1984.

In their review on vibriosis, Anderson and Conroy (1970) listed over 40 fish species from which vibrio-associated diseases had been reported.The geographical distribution of the disease was worldwide. They described the disease as an ulcerative, haemorrhagic septicaemia, the clinical symptoms being loss of appetite, inactivity and discolouration of the skin followed by congestion of the fins and haemorrhages of the body surface. The haemorrhages later become ulcerative. The gut is distended and filled with a clear, viscous fluid and anaemia is usually evident.

The pathogenic *Vibrio* species described by Austin and Allen-Austin (1985) were *V.alginolyticus, V. damsela, V. cholerae* (non O1), *V. vulnificus, V. anguillarum and V. ordalii*. The authors stated that *V. alginolyticus* was mainly a shellfish pathogen but it had been found in diseased red seabream. *V. damsela* had been isolated from skin ulcers on damsel fish (*Chromis punctipinnis), V. cholerae*, from wild and cultured ayu in Japan and, *V. vulnificus*, from eels, also in Japan. *V. ordalii* was noted as the cause of red pest in eels. The name *V. ordalii* was proposed by Schiewe *et al.* (1981) for *V. anguillarum* biotype 2.

Egidius (1987) described an additional two *Vibrio* species as fish pathogens, namely, *V. carchariae*, isolated fron a brown shark which died in captivity, and *V. salmonicida*, the cause of "cold-water vibriosis" (Hitra disease) which was first recorded in Norwegian salmonid farms.

Serology of Pathogenic Vibrios

Pacha and Kiehn (1969) studied pathogenic marine vibrios isolated from fish

in the Pacific Northwest and compared them with *V. anguillarum*. Based on the morphological and physiological characteristics the isolates closely resembled this organism. Three serotypes were distinguished by their thermostable antigens. Serotype 1 contained strains isolated from Northwest salmonids, serotype 2, strains from European waters, and serotype 3 consisted of strains from Pacific herring.

Over 200 bacterial isolates from eleven species of fish in Japan were compared to 29 reference strains by Ezura *et al.* (1980). The majority were identified as *V. anguillarum* or were closely related. 21 strains which varied widely couldn't be identified as any *Vibrio* species and 3 strains were recognised as *Aeromonas* species. Most of the *V. anguillarum* and the closely related strains could be separated into 3 serological groups. The largest group, J-O-1, was comprised of 110 strains from ayu, eel, rainbow trout (*Oncorhyncus mykiss*), amago (*Oncorhynchus rhodurus*) and yamabe (*Oncorhynchus masou*) reared in fresh water. Serotype J-O-2 contained 11 strains from ayu and eel reared in fresh or salt water and serotype J-O-3 contained 56 strains from ayu, coho salmon (*Oncorhynchus kisutch*), yellowtail and amberjack, mostly reared in salt water. Further studies on the serology of these isolates revealed the existence of 8 serological groups (J-O-1 to J-O-8), (Tajima *et al.*, 1985), however, more than 90% belonged to serotypes J-O-1 to J-O-3. Serotypes J-O-4 to J-O-7 each consisted of only one strain.

Sorensen and Larsen (1986) distinguished Danish *Vibrio* strains isolated from diseased wild and cultured fish, from invertebrates, and from the environment into 10 serotypes (O1 to O10) based on detection of O antigens by slide agglutination. Not all the strains were typable but over 90% of the fish-pathogenic strains and 40% of the environmental strains could be distinguished. The dominant serotype among strains from cultured rainbow trout was type O1 whereas most of the strains from wild fish were serotype O2. No dominating serotype was found in the environmental isolates.

Toranzo *et al.* (1987) found that the majority of *V. anguillarum* strains isolated from diseased turbot, salmon and trout reared on the Atlantic coast of Northwest Spain belonged to serotype O1. One strain responsible for a turbot epizootic in 1985 belonged to serotype O2. The isolates belonging to serotype O1 shared one plasmid of 47 megadaltons, whereas no plasmids were detected in the pathogenic vibrios assigned to serotype O2, hence, their virulence properties were chromosome coded. The extracellular products from all the *Vibrio* strains displayed strong cytotoxic activity against five fish cell lines, but a non-pathogenic reference strain gave similar results, indicating that no relationship could be established between virulence and cytotoxicity. In addition, the isolates exhibited similar drug resistance patterns, strong protease, amylase and phospholipase activities and all produced haemolysins against human, sheep and trout erythrocytes.

Pathogenesis of Vibriosis

Crosa *et al.* (1977) reported a relationship between the presence of a 50-megadalton plasmid in *V. anguillarum* and enhanced virulence of this organism. It was later shown that the plasmid specified an efficient iron-sequestering system (Crosa, 1980). The ability of a pathogen to multiply in the host is known to be influenced by the availability of iron. In animals the amount of iron freely available to bacteria is extremely small since it is held in complexes with high-affinity binding proteins such as transferrin and lactoferrin, therefore, the pathogenicity of an organism is greatly influenced by its ability to obtain iron (Bullen and Griffiths, 1987). Crosa and Hodges (1981) demonstrated that when grown in conditions of iron limitation, *V. anguillarum* cells had at least two novel outer-membrane proteins, one of which, OM2, was only inducible in cells with the virulence plasmid, pJM1. OM2 was shown to be a receptor for complexes of iron and a diffusible siderophore which was also specified by the plasmid (Walter *et al.*, 1983). Andrus *et al.* (1983) assayed six pathogenic *Vibrio* species

for the production and utilization of siderophores; all the bacteria tested produced compounds which stimulated their growth in iron-limited media but the compounds varied between the species.

Munn (1978) reported that a virulent strain of *V. anguillarum* produced a haemolytic toxin and suggested that the toxin played a role in the pathogenicity of the organism. Toranzo *et al.* (1983) found that both pathogenic and non-pathogenic strains of *V. anguillarum* produced haemolysin. They also found no relationship between virulence and haemagglutination of fish erythrocytes with *V. anguillarum* strains isolated from the Pacific Northwest although a positive correlation was shown in strains isolated from striped bass (*Morone saxitilis*) on the Atlantic coast. Trust *et al.* (1981) found no relationship between virulence and the ability of *V. anguillarum* or *V. ordalii* to agglutinate fish erythrocytes but virulence was associated with resistance to the bactericidal activity of normal (non-immune) rainbow trout serum.

V. anguillarum produces an extracellular, heat-stable toxin which is lethal to goldfish (*Carassius auratus*) (Umbriet and Tripp, 1975). A heat-labile exotoxin produced by *V. anguillarum* was also lethal when injected into goldfish, Japanese eel(*Anguilla japonica*), ayu and mouse. Proteolytic activity coexisted with lethal toxicity and the exotoxin and protease were correlated with virulence (Inamura *et al.*, 1984).

DiSalvo *et al.* (1978) isolated a pathogenic strain of *V. anguillarum* from a shellfish hatchery in California. A water-soluble, heat-stable exotoxin produced by the vibrio inhibited swimming and contributed to mortality of larvae of oysters (*Crassostrea gigas*). Brown and Roland (1984) purified a heat-labile exotoxin produced by a *Vibrio* species isolated from diseased oyster (*Crassostrea virginica*) larvae. The exotoxin showed no proteolytic or amylase activity and the molecular weight was estimated to be 68,000. Nottage and Birkbeck (1986) found two factors in the culture supernate of a bivalve-pathogenic *Vibrio* species which they suggested could be involved in the pathogenesis of vibriosis in oysters since

both were lethal to oyster spat. The first was a heat-labile proteinase of molecular weight 30,000, while the second factor was a heat-stable, ciliostatic toxin of molecular weight less than 5,000.

The adhesion of *V. anguillarum* to host tissues is thought to be essential in initiating infection. *V. anguillarum* cells adhered to excised sections of rainbow trout gut (Horne and Blaxendale, 1983) and gut sections of vaccinated trout had less adherent bacteria than sections from non-vaccinated fish. Horne and Blaxendale postulated that localised immune mechanisms, most likely antibodies, existed in the gut wall.

Chart (1983) found a correlation between the ability of *V. anguillarum* to cause disease in eels and the possession of more than one flagellum. He suggested that additional flagella may be involved in chemotaxis and/or penetration of mucosal surfaces.

Myxobacteriosis

Diseases associated with myxobacteria are common in fish but not in higher vertebrates. Anderson and Conroy (1969) reviewed the fish-pathogenic bacteria. Columnaris is perhaps the best known of the fish myxobacterioses, so called because of the column-like masses of bacterial cells which can be seen on tissue collected from infected sites. Its distribution is worldwide and it occurs in many species of warm- and cold-water fish. It is caused by *Flexibacter columnaris* and characteristically gives rise to greyish-white spots on the head, fins and body. The lesions are the site of a progressive necrosis involving the epidermis and the connective tissue and the sites of infection are frequently small injuries on the skin surface with the gills often being attacked.

The genus *Flexibacter* belongs to the family Cytophagaceae. Members of the family are long, thin, Gram-negative rods which form carotenoid pigments and exhibit gliding motility. The family also includes the genera *Flavobacterium* and *Cytophaga* (Sanders and Fryer, 1989).

Mudarris and Austin (1989) described a disease in turbot (*S. maximus*), caused by a previously unrecognised *Cytophaga*-like bacterium. Infected fish showed gill hyperplasia and a systemic haemorrhagic septicaemia. The disease was reproduced by intraperitoneal injection with the *Cytophaga*-like bacterium. Based on their earlier work on the identification of bacteria from the gills of turbot (Mudarris and Austin, 1988), the authors considered that the isolate was probably part of the normal gill microflora of turbot. The factors involved in the change of status from a harmless organism to a pathogen were not elucidated.

Diseases of Larval and Juvenile Stage Fish

Several bacterial pathogens cause mortalities in larval and juvenile fish. The cause of massive losses of black sea bream fry in Japan was investigated by Kusuda *et al.* (1986) and three dominant species were isolated in the bacterial flora of moribund fish., *V. alginolyticus, V. neresis* and *Alcaligenes cupidis*. Waterborne infection and oral infection by feeding rotifers artificially inoculated with bacteria were used in an attempt to identify the causative agent. *V. alginolyticus* and *A. cupidis*, but not *V.neresis*, gave high mortalities of fish.

Masumura *et al.* (1989) isolated a *Vibrio* species from the intestine of diseased Japanese flounder larvae. The disease was characterised by an opaque intestine or intestinal necrosis and the *Vibrio* species was tentatively named *Vibrio* species INFL (Intestinal Necrosis of Flounder Larvae). The disease was reproduced by oral administration of the isolate incorporated into rotifers and brine shrimp.

An outbreak of acute vibriosis in juvenile turbot in Scotland was described by Horne *et al.* (1977). Affected fish were very dark in colour, immobile and displayed distinctive eye changes. The orbits were swollen and a white ring appeared below the cornea. The lens was more opaque than normal although the fish did not appear to be blind. The abdomen was grossly distended but there was no food in the gut, only clear fluid. Most of the internal organs showed necrosis, oedema or other abnormalities. There were no obvious skin lesions,

however, a pure culture of *V. anguillarum* was readily obtained from the heart, kidney and blood of all the specimens examined. Temperature reduction to less than 10°C was the most successful method of treatment, reducing losses from 40% to 5%. The rapid onset of the disease, with fish dying within hours of the appearance of clinical abnormalities, was thought to explain the lack of skin lesions. Stress and skin trauma resulting from handling were suggested as the stimulus to development of the disease since lack of detectable effect on the gut wall was thought to rule out the intestine as the route of infection.

Vibriosis in juvenile turbot was also reported by Devesa *et al.* (1985) in Spain. The symptoms were similar to those described by Horne *et al.* except that the disease developed more slowly and skin lesions occurred. The outbreak was attributed to stress of handling and to a reduction in water salinity since when the fish were transferred to another rearing facility with appropriate salinity the mortalities ceased.

McVicar (1987) suggested that stress was almost certainly the most important factor responsible for the occurrence of outbreaks of a disease in juvenile farmed Dover sole in Scotland. The disease was described by McVicar and White (1979) as a darkening and necrosis of the fish skin and fins. The term black patch necrosis (BPN) was used to describe the condition although the authors pointed out that these reactions were a normal response of fish skin to damage from various causes. The disease was generally observed in juvenile fish 60 to 100 days old and outbreaks were more frequent in summer than in winter. Within about five days of the first signs appearing the mortality rate rose to as high as 10.2% per day until most of the fish succumbed. The addition of sand to the rearing tanks markedly reduced mortalities. This was attributed to a possible reduction of stress in fish which were able to bury themselves.

The examination of the skin of Dover sole by electron microscopy revealed that the fish reared in the absence of sand had a layer of dead cells on the surface of the epidermis which was not seen in the fish which had sand added to

their rearing tanks (McVicar, 1987). In addition, the fish reared without sand showed an increased number of macrophage-like cells in the epidermis. McVicar suggested that they were responding to a local immunological stimulus such as bacteria present in the layer of dead cells.

Campbell and Buswell (1982) proposed that BPN was due to a *Flexibacter columnaris*-like organism after they isolated a long, thin, filamentous, Gram-negative bacterium repeatedly from diseased tissue of Dover sole. The disease was reproduced by injecting fish subdermally with cultures of the isolate and the bacterium was reisolated from the necrotic lesions which developed.

Further identification of the bacterium responsible for BPN revealed that it was in fact *Flexibacter maritimus* (Bernardet *et al.*, 1990) an organism responsible for an infection which caused high mortalities (20 to 30%) in red and black seabream fry in Japan (Wakabayashi *et al.*, 1984; 1986).

1.5 Treatment and Prevention of Disease in Aquaculture

The use of Antimicrobial Compounds

Austin (1984) reviewed the antimicrobial compounds used to control bacterial fish diseases and the methods of administration. Four possible methods of treatment were described. These included mixing drugs with the food of fish, bath and flush treatments and injection. The medicated food method is advantageous in that the quantities of drug the fish receive can be carefully controlled but the drug must be palatable and be absorbed intact through the gut. In some infections fish refuse to eat, in which case bath or flush treatments are necessary. In bath treatments the fish are placed in a solution of the drug for a specific time after which they are returned to the holding facility. Insoluble drugs can be dispersed evenly using surfactants. Flush treatments involve the addition of a high concentration of a drug to the holding tank and the subsequent removal of the drug from the system by the normal water flow. In both

these treatments disposal of the spent drug can pose problems. The technique of injecting fish is only feasible for valuable fish, such as brood stock, since it is time consuming and usually requires anaesthesia of the fish.

The range of antimicrobial compounds listed by Austin includes substances such as iodophors, used to treat Acinetobacter disease, furunculosis, BKD and flavobacteriosis; benzalkonium chloride, used in fin rot and gill disease; malachite green, used for columnaris; and numerous antibiotics such as oxytetracycline, chloramphenicol and oxolinic acid. Austin listed 13 antibiotics used to treat vibriosis, namely: aureomycin, chloramphenicol, 5,7-dichloro-8-hydroxyquinoline, halquinol, furanace, kanamycin, nifurprazine, nitrofurantoin, oxytetracycline, sulfisoxazole, sulphamerazine, sulphamethazine-sodium salt and oxolinic acid.

Drugs may be used for chemoprophylaxis as well as chemotherapy. Oppenheimer (1955) found that the control of bacteria which developed on the eggs of the Pacific sardine, the Norwegian codfish (*Gadus callarias*), and turbot (*Pleuronichthys ritteri*), using antibiotics, increased the percentage of eggs which hatched. The quantity of drug used was important as too little gave decreased bactericidal activity and too much was toxic to the fish eggs.

Hayashi *et al.* (1975) attempted unsuccessfully to eliminate the bacteria associated with *B. plicatilis* and *M. macrocopa* by washing with sterilized or running water. Addition of the antibiotic compound "Aivet" to the culture water, although it decreased the counts on TCBS agar, did not affect the total number of heterotrophic bacteria. Gatesoupe (1982) found that rotifers treated with the antibiotic Tribrissen and fed to turbot larvae gave increased survival of larvae compared to untreated rotifers. A similar effect was observed with the antibiotic disinfection of *Artemia* during the second step of the food sequence (Perez-Benavente and Gatesoupe, 1988). Miyakawa and Muroga (1988) obtained a 10- to 100-fold decrease in the bacteria associated with rotifers when treatment with sodium-nifurstyrenate for one hour was carried out after harvest. The composition of the bacterial flora remained relatively

stable. Tanasomwang and Muroga (1989) used sodium nifurstyrenate and tetracycline to treat rotifers. Sodium nifurstyrenate reduced the total number of heterotrophic bacteria slightly and the number of vibrios decreased from 10^7 to 10^5 c.f.u. per gram wet weight of rotifers. Tetracycline did not affect the viable counts of bacteria.

The use of drugs to prevent disease in fish is effective when the occurrence of disease is predictable. Austin *et al.* (1983) used oxolinic acid to prevent outbreaks of furunculosis in rainbow trout. The disease appeared annually, usually in July or August, when the water temperature reached 18°C. When the fish received a medicated diet, commencing when the water temperature reached 17.5°C, mortalities were negligible.

Resistance of Bacteria to Antibiotics

A major problem associated with the use of antibiotics is the occurrence of drug-resistant bacteria. Aoki *et al.* (1974) detected transferable drug resistance plasmids, called R factors, in *V. anguillarum* strains from diseased ayu in Japan. The most common type specified resistance to sulphonamides, streptomycin, chloramphenicol and tetracycline. The high incidence of R factors in bacteria from cultured ayu was assumed to be due to selective pressure exerted by chemotherapeutic agents used in fish culturing. Aoki *et al.* (1980) isolated chloramphenicol- and tetracycline-resistant bacteria from the intestinal tracts of cultured ayu and the water of the culturing ponds. Drug resistant *Aeromonas hydrophila, V. anguillarum, Pseudomonas* species and Enterobacteriaceae were found but resistant bacteria were not isolated from wild ayu. R factors were detected in all the *V. anguillarum* strains and some of the *Hafnia, Enterobacter cloacae, E. aerogenes, A. hydrophila* and other unidentified Enterobacteriaceae. The drug resistance markers present in the R factor-bearing strains were in most cases sulphonamides, streptomycin, chloramphenicol and tetracycline. Aoki *et al.* (1981) examined the sensitivity of 259 strains of *V. anguillarum* isolated from diseased ayu to 12 different chemotherapeutic agents. Only 9

strains were sensitive to all the drugs tested. All the strains were sensitive to streptomycin, kanamycin, aminobenzyl penicillin and colistin. Strains resistant to chloramphenicol, nalidixic acid, furazolidone, sulphamonomethoxine, trimethoprim and tetracycline were frequently isolated. The frequency of nalidixic acid- and furazolidone-resistant strains increased from 1974 to 1977, the period of the study, and trimethoprim-resistant strains appeared after 1976.

Antibiotic Pollution from Aquaculture

Austin (1985) investigated the effect of antimicrobial substances on the aquatic microflora at four rainbow trout rearing sites. One site, which received water from bore holes, did not have a history of disease and, consequently, did not use any drugs during the period of the investigation. The other sites were located on river systems and suffered from outbreaks of furunculosis and enteric redmouth. The infected fish were given food containing oxolinic acid, potentiated sulphonamide or oxytetracycline. The bacterial numbers were very low in the bore hole water (300 per ml) and increased ten fold in the effluent. The river water contained on average 3,000 bacteria per ml and did not increase more than two fold in the effluent. During the periods of chemotherapy the bacterial numbers in the effluent were lower than in the inflow water and the range of bacterial taxa isolated was considerably reduced. After chemotherapy the microflora in the effluent quickly increased in diversity. Antibiotic resistance was a common feature in bacteria from both inflow and effluent waters including the bore hole water. The use of antimicrobial compounds led to an increase in resistant bacteria in the effluent, however, this increased level of resistance was reduced after the conclusion of chemotherapy.

Jeanthon *et al.* (1988) carried out a bacteriological survey of chloramphenicol-treated seawater in a scallop (*Pecten maximus*) hatchery. The total microflora estimated by epifluorescence microscopy remained constant before and after treatment, however, the viable heterotrophic bacteria increased

while the *Vibrio*-like bacteria decreased. The increase in heterotrophic bacteria was explained by the proliferation of chloramphenicol-resistant bacteria. Twenty one *Vibrio*-like strains were isolated and tested for resistance to chloramphenicol. Only one strain was resistant. The authors postulated that repeated use of this antibiotic could eventually promote the spread of potentially pathogenic *Vibrio* strains.

The residues and persistence of oxytetracycline in wild fish and sediments was studied by Bjorklund *et al.* (1990) at two fish farms after chemotherapy of the farmed fish. In wild fish, residues of oxytetracycline were detected up to 13 days after medication. The thickness of the organic sediments was 5-10cm on farm A and 10-30cm on farm B. The half-life of oxytetracycline in the fish farm sediments was 9 days and 419 days on farms A and B, respectively. The authors suggested that under stagnant anoxic conditions, oxytetracycline was very persistent in fish farm sediments and that leakage, rather than decomposition, was the main factor reducing oxytetracycline levels in the sediments.

Vaccination of Fish

Ellis (1988a) stated that vaccination had several advantages over chemotherapy since the effects were long lasting, no toxic residues were produced and resistant pathogens were not derived. He described several methods of vaccinating fish. The most effective method is by intraperitoneal injection, however, this method is labour intensive and only possible for large (over 15g) fish as the handling and anaesthesia required is stressful for the fish.

Oral vaccination is possible and is the only feasible method for pond-reared fish since catching the fish is impractical. No handling is required so stress is avoided but an important limitation of oral vaccines is their poor potency and the inconsistent levels of protection which are produced, thought to be due to the destruction of the antigens in the stomach and foregut before they reach the immune-sensitive areas of the fish gut.

Bath, flush and spray vaccination are varieties of immersion vaccination. Only a few seconds exposure time is necessary for the uptake of antigens, the main route of entry being probably through the gills. A major advantage of immersion vaccination over injection vaccination is that small fish can be immunised.

The most successful fish vaccines developed to date are against enteric redmouth and vibriosis. Enteric redmouth, caused by the Gram-negative, motile bacterium *Yersinia ruckeri*, affects rainbow trout and other salmonids. It produces a systemic infection with subcutaneous haemorrhaging and erosion of the jaws and palate. The vaccine is based on formalin- inactivated whole cells and immersion vaccination is highly effective, however, significant levels of protection do not develop in fish below about 1g in weight. The duration of protection is also size dependent (Ellis, 1988b).

Vibriosis vaccines contain mixtures of inactivated whole cells and extracellular products of *V. anguillarum* and *V. ordalii*. Injection is thought to be the best method although immersion vaccination is effective. Oral vaccination using formalin-killed lyophilized whole cells also provides some degree of protection. The minimum size at which protective immunity develops is 1.0-2.5g and the duration of protection is size dependent (Smith, 1988).

Elimination of Bacteria by Ultra-Violet Radiation

Aboul-Ela (1958) suggested that the development of a control method using ultra-violet (U.V.) radiation to sterilize water for culturing oysters would be beneficial to the oyster farming industry and Waugh (1958) reported considerable success in rearing larvae of *Ostrea edulis* in U.V. radiation-sterilized water.

Kimura *et al.* (1976) examined the effect of U.V. radiation on cell suspensions (10^5 -10^7 cells per ml) of five species of fish-pathogenic bacteria, one strain of *Escherichia coli*, and also on fish-rearing pond waters. A 99.99% or more

reduction in *Aeromonas punctata, A. hydrophila, A. salmonicida, Pseudomonas fluorescens, V. anguillarum* and *E. coli* was achieved with a dose of 22,100 microwatt seconds per square centimeter (uW.s/cm^2). The experiments on four samples of fish-rearing pond waters containing 10^4 -10^6 cells per ml indicated that U.V. radiation was an effective means of disinfection of hatchery water; however, a tendency towards U.V. resistance was shown by some groups in the microflora such as coryneforms and yeast-like organisms.

Bullock and Stuckey (1977) found that filtration and U.V. radiation at doses of 13,100-29,400 uW.s/cm^2 effected a 99.98-100% reduction in *A. salmonicida, A. hydrophila, P. fluorescens* and *Y. ruckeri* when a cell suspension was added to give 10^4 -10^5 cells per ml in clear spring water or spring water containing particulate matter. Filtration and 13,100 uW.s/cm^2 irradiation of water containing *A. salmonicida* prevented transmission of furunculosis.

Brown and Russo (1979) inoculated seawater with five species of oyster-pathogenic bacteria to a concentration of 10^5 cells per ml. U.V. radiation at a dosage of 93,312-155,520 uW.s/cm^2 rendered the seawater safe for rearing fertilised eggs of *Crassostrea virginica.*

Brown (1981) reported the isolation of two highly virulent strains of *Vibrio*, closely related to *V. anguillarum*, implicated in an outbreak of disease in oyster larvae. The pathogens represented an extremely small proportion of the total bacterial population in the seawater system of the hatchery. The disease could be initiated in the laboratory when only two cells per ml of the pathogen were added to each daily change of larval culture water. Brown suggested that the carryover of even extremely low numbers of pathogenic bacteria in a seawater system could eventually lead to disease. U.V. radiation was an effective method of eliminating one of the *Vibrio* strains but the other partially recovered from exposure within 24 hours.

The effect of U.V.-irradiated water on the bacterial levels of cultured *B. plicatilis* and *M. macrocopa* was investigated by Hayashi *et al.* (1976). The effect

of U.V. irradiation of the circulating water of an ayu fish hatchery was also studied. No harmful effect on ayu, *B. plicatilis* or *M. macrocopa* was observed. U.V. irradiation reduced the level of bacteria in the inlet waters from 10^4 -10^5 cells per ml to 10-10^2 cells per ml. Despite the supply of U.V.-treated water the viable counts of bacteria from the pond water outlets were almost the same as those of untreated water. No more than a 10-fold reduction in the bacterial levels of suspensions of *B. plicatilis* or *M. macrocopa* was achieved. Similar results were obtained by Yamanoi and Sugiyama (1987).

Axenic Cultures

The use of gnotobiotic animals in the study of host microbial relationships was reviewed by Gordon and Pesti (1971) but most of the reported work concentrated on the possible contributions to man in the field of medicine. The production of fish in an environment completely free of microorganisms, although possible (Baker and Ferguson, 1942; Trust, 1974; Battalora *et al.*, 1985), is unrealistic in terms of aquaculture with today's technology. However, Droop (1975) demonstrated that the production of axenic algae on a scale suitable for supplying an oyster hatchery was possible using a continuous culture system. The growth of germ-free rotifers was also demonstrated. Droop suggested that it was conceivable that a scaled-up model would be suitable for use in aquaculture. The rotifer chemostat was used in studies of the steady-state energetics and nutrition of rotifers (Droop and Scott, 1978; Scott, 1980; 1983) and a requirement for vitamin B12 by rotifers in axenic conditions was shown by Scott (1981).

Nicolas and Besse (1987) reported that in preliminary experiments germ-free turbot larvae survived better than conventional larvae. The authors concluded that there was a need to develop research in gnotobiology for nutritional and pathological studies in aquaculture.

Probiotics

The value of probiotics has been recognised in land-farmed animals, notably poultry, for some time. Schleifer (1985) reviewed the efficacy and mechanism of probiotics for the control of *Salmonella* in poultry. The term "competitive exclusion" was used to describe the inoculation of chicks with harmless bacteria which occupy the attachment sites in the gut and prevent infection by species of *Salmonella*, an organism capable of causing food poisoning in man.

Bacteria may also be beneficial to the host by producing growth factors or compounds which inhibit the growth of other microorganisms. Thus, Austin (1988) described several antibiotic compounds and enzymes produced by marine bacteria which could be valuable to the host in the digestion of food and Kashiwada *et al.* (1971) demonstrated the production of folic acid by the intestinal bacteria of carp.

Gatesoupe *et al.* (1989) compared two food additives containing live bacteria, given to rotifers and subsequently fed to Japanese flounder. One of the additives, Adjulact, a spray-dried powder containing *Streptococcus thermophilus* and *Lactobacillus helveticus*, increased the production rate of rotifers. The number of bacteria per rotifer increased dramatically after enrichment for 17 hours. In an untreated batch the numbers rose from 3.0×10^3 to 9.9×10^4 bacteria per rotifer. Acosil, a spray-dried extract from sprouting cereal grains fermented with strains of lactic bacteria, reduced the number of bacteria after enrichment to 5.4×10^4 bacteria per rotifer. Although the survival rates of the flounder at day 18 were not significantly different, the mean length of flounder fed Acosil-treated rotifers was significantly greater than that of flounder fed control rotifers. The authors considered that the explanation for this could be the reduction in the number of bacteria per rotifer after enrichment.

Gatesoupe (1989) also investigated the effect of Adjulact and Acosil-enriched rotifers on turbot larvae. Antibiotic treatment of the rotifers was also tested and, in addition, one batch was enriched with spores of *Bacillus toyoi*. Rotifers

without bacterial additives had the lowest production rates and gave the poorest survival rates of turbot larvae when not disinfected but the best survival rate was obtained with the same rotifers when antibiotic treated. *B. toyoi* spores improved the growth rate of turbot when given to rotifers before antibiotic treatment. Whatever their diet, rotifers which were treated with antibiotics for 24 hours, then rinsed, gave improved survival and growth rates of turbot compared to untreated controls.

Gatesoupe (1990) compared the growth and survival rates of turbot larvae fed continuously on rotifers which were either untreated or given the food additive Acosil. The rotifers were reared at pH 7-8 and 19°C or pH 5.25-6 and 26°C to determine whether the latter conditions would enhance the possible effect of the lactic bacteria in Acosil. The mean survival rates were not significantly different due to the high degree of variability in the survival of turbot fed Acosil-treated rotifers reared at low pH and high temperature. The number of bacteria per rotifer was especially variable within this group and Gatesoupe postulated that this explained the survival rates since batches with a high number of bacteria at day 9 presented a high mortality at day 10.

A positive correlation between the proportion of *Vibrio alginolyticus* in the flora and the larval survival rate was found. However, when an *Aeromonas* species dominated, correspondingly high mortalities occurred and this species was thought to be opportunistic since its proliferation occurred in tanks of larvae on the verge of high mortality. Gatesoupe concluded that, while selected bacteria might improve the microflora of rotifers, a high level of bacteria appeared to be detrimental to turbot larvae. This effect remains to be explained.

OBJECT OF RESEARCH

Although turbot larvae can be reared successfully in captivity, survival rates remain highly variable. To date most research has concentrated on the nutritional aspects of this problem. However, bacteria have been implicated as a major cause of high mortalities since improved survival rates have often been obtained when larvae are reared in the presence of antibiotics.

The primary object of the current research was to determine the relationship between the bacterial flora of the gut of turbot larvae during the early stages of fish development and larval growth and survival rates.

A second object was to determine the origin of the gut flora of turbot larvae and whether it was possible to influence the bacterial flora of the gut to improve larval growth and survival rates.

MATERIALS AND METHODS

2.1 Supply of Turbot Larvae and Rotifers

The turbot (*Scophthalmus maximus*) larvae and their live food organisms were provided by Golden Sea Produce, Hunterston, Ayrshire. Rotifers (*Brachionus plicatilis*) were the main food for larval turbot but in one experiment the copepod *Eurytemora hirundoides* was used. The Norwegian larval turbot samples from Mowi, and processed by the method described below Bergen, Norway, were collected by A. Barbour. These fish were fed the copepods *Calanus, Acartia, Temora* and *Centropages*.

2.2 Bacterial Flora of the Gut of Turbot Larvae

Since the small size of larval turbot rendered dissection impractical, the aerobic bacterial flora of the intestine was sampled by a method similar to that of Muroga *et al.* (1987). Turbot larvae (10 fish per sample) were collected from the rearing tanks with a Pasteur pipette and placed into sterile glass tubes, of internal diameter 3.8cm, with a 10μm nylon mesh (Plastok Plastics Ltd., 79 Market Street, Birkenhead) fixed to the bottom. The larvae were anaesthetised by immersion in 0.1% benzocaine (ethyl p-aminobenzoate) (BDH Ltd.) for 30 seconds. This solution was prepared by dissolving the benzocaine in a small volume of acetone and diluting with seawater. The larvae were rinsed with 0.1% benzalkonium chloride (Sigma Chemical Company) in sterile seawater for 60 seconds, followed by sterile seawater alone for 60 seconds, to remove the surface bacteria. The larvae were aseptically transferred to a sterile glass homogeniser (Jencons) and homogenised in 1 or 2ml sterile seawater. Dilutions were prepared in sterile seawater and 0.1ml volumes were spread onto marine agar 2216 (Difco), and thiosulphate-citrate-bile-salt-sucrose (TCBS) agar (Difco) when necessary. The marine agar and TCBS agar plates were incubated at 20°C for 5 days and 48 hours, respectively. After enumeration, a representative selection of colonies was subcultured on marine agar and retained for identification.

2.3 Bacterial Flora of Rotifers

The aerobic bacterial flora associated with rotifers was sampled by filtering 5 to

20ml of rotifer suspension through a 10μm nylon mesh disc, of 2.5cm diameter, mounted in a Swinnex filter holder. When required the rotifers were rinsed with 0.1% benzalkonium chloride in seawater for 30 seconds, followed by sterile seawater alone for 60 seconds, or sterile seawater only, by filling a sterile plastic syringe and slowly passing the liquid through the filter holder. The nylon mesh disc was aseptically transferred to a glass homogeniser with a close-fitting teflon plunger and homogenised in 5ml sterile seawater. Dilutions were prepared in sterile seawater and 0.1ml volumes were spread in duplicate onto marine agar and TCBS agar when required. The conditions for incubation of the plates were identical to those for samples from turbot larvae. After enumeration, a representative selection of colonies were subcultured onto marine agar and retained for identification. The number of rotifers was determined by placing 1ml of suspension dropwise into a petri dish and examining the drops under a low power microscope (x20 magnification) (Vickers Instruments). In this way the number of bacteria per rotifer could be calculated.

2.4 Storage of Bacteria

Bacteria were subcultured 2 or 3 times on marine agar to obtain pure cultures then stab inoculated into 2ml of marine agar in a 5ml screw-capped vial. After 48 hours incubation at 20°C, 1ml of sterile liquid paraffin was added and the cultures were stored at room temperature in the dark.

2.5 Identification of Bacteria

Identification of the bacterial isolates to genus level was based on the taxonomic schemes of Oliver (1982) and Muroga *et al.* (1987). *Vibrio* and related genera were further identified by the computer program BACTERIAL IDENTIFIER, the revised edition of BACTID (Bryant *et al.*, 1986), run on an Amstrad pc1512.

2.6 Cluster Analysis

Cluster analysis of bacterial isolates was performed using BMDP P2M (BMDP

Statistical Software Inc., 1964 Westwood Blvd., Suite 202, Los Angeles, California 90025) run on the University of Glasgow ICL3980 computer using the VME operating system.

2.7 Identification Tests

A total of 42 physiological and biochemical properties were recorded for each bacterial isolate. A list of the tests is given in Table 1. Unless otherwise stated, the incubation temperature was 20°C.

The following tests were performed as described by Furniss *et al.* (1979): arginine, lysine and ornithine decarboxylase, haemolysis of sheep red blood cells, lecithinase and the Voges-Proskauer test. The ability to utilise compounds as the sole carbon source was determined by the replica plating method of Baumann *et al.* (1971) and aesculin hydrolysis was tested as described by Lee and Donovan (1985). Nitrate reduction was tested by the method of Lee *et al.*, (1979). The following tests were performed as described by Cowan (1965) with the addition of 1% NaCl to the media: acid production from arbutin, salicin and sucrose with phenol red as the indicator, amylase, gelatinase (plate method), indole, ONPG (O-nitrophenyl-ß-D-galacto-pyranoside) and oxidase. A description of the methods is given in Appendix I.

The mode of metabolism of glucose was tested by the method of Hugh and Leifson (1953) using MOF (marine oxidative/fermentative) medium (Difco). In addition, pigmentation, swarming and growth at 4°C and 37°C were determined after 5 days incubation on marine agar. A Gram stain was performed and the colonies were examined for luminescence in the dark after incubation for 24 hours on marine agar. Motility was determined by examination of an inverted drop of culture using the X40 objective of a light microscope (Vickers Instruments) after 24 hours incubation in 1% tryptone with 2% NaCl. Growth on CLED (cysteine lactose electrolyte deficient medium) (Oxoid) was determined after 24 hours incubation at 20°C.

Resistance to 10µg and 150µg O/129 (2,4-diamino-6,7-diisopropyl-pteridine) was determined after 48 hours incubation on marine agar using discs (Oxoid). Resistance

TABLE 1

List of Identification Tests

Gram stain	
Oxidation/Fermentation of glucose	
Pigment	
Motility	
Swarming	
Luminescence	
Growth at 4°C	
Growth at 37°C	
Growth on CLED	
Arginine Decarboxylase	
Lysine	"
Ornithine	"
Nitrate Reduction	
Oxidase	
Indole	
ONPG	
Voges-Praskauer	
Resistance to:	O/129 10µg
	O/129 150µg
	ampicillin 10µg
	polymyxin B 50i.u.
Aesculin Hydrolysis	
Elastinase	
Gelatinase	
Lecithinase	
Amylase	
Xanthine Decomposition	
Haemolysis of Sheep red blood cells	
Acid from:	arbutin
	salicin
	sucrose
Utilisation of:	L-arabinose
	D-cellobiose
	D-galactose
	D-melibiose
	D-gluconate
	D-glucuronate
	L-citrulline
	L-leucine
	D-glucosamine
	DL-3-hydroxybutyrate
	succinate

to 10μg ampicillin and 50i.u. polymyxin B was also determined on marine agar using filter paper discs impregnated with 10μl antibiotic solution.

Decomposition of elastin and xanthine was determined by spot inoculation of marine agar plates incorporating 2% elastin or xanthine and incubation for 28 days. A clear zone around the colony indicated a positive result.

2.8 Colonisation of Turbot Larvae with Defined Bacteria

Isolate 3-8, identified as *V. alginolyticus*, was used to inoculate rotifers which were subsequently fed to turbot larvae. This strain was originally isolated from a batch of intensively-reared larvae which had a relatively high survival rate and was therefore presumed to be beneficial, or at least non-pathogenic, to turbot larvae. A streptomycin-resistant derivative, 3-8sr, was produced by plating 3-8 on marine agar containing 50μg streptomycin per ml. Viability of 3-8sr over a 48 hour period was examined in 3 different diluents. Viability decreased in 1.8% and 3.0% NaCl solution but the concentration of bacteria increased in autoclaved seawater therefore, seawater was used as the diluent in all experiments.

Marine broth (Difco) and marine broth containing 50μg per ml streptomycin (50ml in a 250ml flask) were inoculated with 3-8 and 3-8sr, respectively, and incubated at 20°C in an orbital shaker (Gallenkamp) overnight. The cells were harvested by centrifugation at 7,800 g for 10 minutes in a Sorvall RC-5 centrifuge, washed and resuspended in sterile seawater to an O.D. 600nm of 1.0 (approximately 1 x 10^9 c.f.u. per ml). A 50 litre culture of rotifers was inoculated with 50ml of bacterial suspension to give a final concentration of 1.0 x 10^6 c.f.u. per ml one day prior to harvesting the rotifers. The turbot larvae were sampled one hour after feeding. Samples from larvae fed rotifers inoculated with 3-8 and 3-8sr were plated onto marine agar and marine agar containing 50μg per ml streptomycin, respectively.

2.9 Culture of Bacteria-Free Algae

The initial inoculum of bacteria-free algae (*Pavlova lutheri*) was kindly provided by Mr. J. M. Scott, Dunstaffnage Marine Laboratory, Oban. The algae were maintained in a continuous culture system, or chemostat (Figure 1), the construction of which was based on that of Droop (1975). Spherical glass flasks of 2 litres volume were used for the medium reservoir, growth vessel and output vessel. Glass tubing (3.2mm bore, 1.0mm wall) was used inside the flasks and as connectors at the ends of the input and output tubes. The ends of the tubes were clamped and stored in small bottles of dilute iodine solution (Appendix II). Silicon bungs and tubing with a 3.2mm bore and 1.0mm wall thickness (Altec, Mill Lane Estate, Alton, Hampshire) were used throughout. Fresh growth medium (Appendix III) was sterilised by autoclaving at 121°C for 15 minutes in 2 litre glass flasks fitted with cotton wool bungs and silicon tubes to enable connection to the input tube of the growth-medium reservoir.

An Atlantis B1500 airpump was used to aerate and mix the culture; this also provided a positive air pressure system. Sartofluor II Mini Capsules of 0.2μm pore size (Sartorius Ltd., 18 Avenue Road, Belmont, Surrey) were used to filter the air at the input and vent. Growth medium was drawn from the reservoir at a fixed rate by a Watson-Marlow 101U/R variable speed peristaltic pump (Belmont Instruments, Glasgow). Light was provided by two 6 watt, white, strip lamps (Sylvania). The complete apparatus was contained within a perspex cabinet with the airpump positioned outside the cabinet to prevent air circulation.

The apparatus was autoclaved at 121°C for 15 minutes as a single unit. The open tube ends were wrapped in cotton wool and packed into flat-bottomed glass tubes before autoclaving.

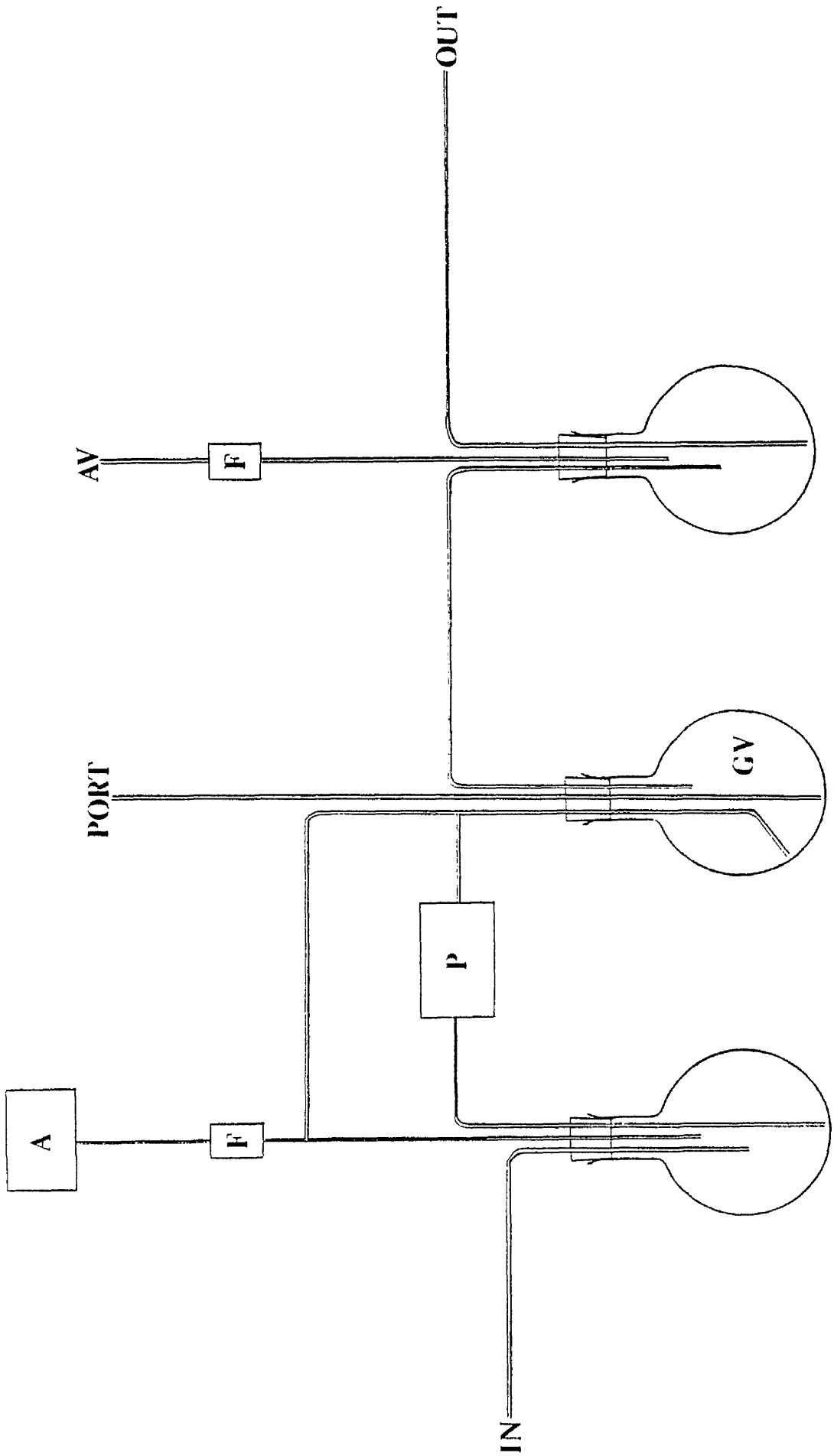
2.10 Culture of Bacteria-Free Rotifers

The initial inoculum of bacteria-free rotifers was kindly provided by Mr. J. M. Scott, Dunstaffnage Marine Laboratory, Oban. The construction of the rotifer

FIGURE 1

Structure of algal chemostat.

A	Air Pump
AV	Air Vent
F	0.2μm Air Filter
GV	Growth Vessel
IN	Medium Input Tube
OUT	Output Tube
P	Peristaltic Pump
PORT	Inoculating/Sampling Port


OUT
AV
F
PORT
GV
P
A
F
IN

chemostat was identical to that of the algal chemostat. An additional peristaltic pump was required to draw algae from the growth vessel of the algal chemostat into that of the rotifer chemostat. The growth medium for the rotifers was seawater diluted to 2.4% salinity with vitamin B12 added to a final concentration of 100ng per litre. The vitamin B12 requirement of *B. plicatilis* was shown by Scott (1981). The medium was autoclaved at 121°C for 15 minutes.

2.11 Colonisation of Bacteria-Free Rotifers with Known Bacteria

The bacterial isolates selected for experiments involving the colonisation of bacteria-free rotifers were mainly isolated from batches of healthy turbot larvae. They included two strains identified as *Vibrio campbellii* (NT9 and NT31), *Vibrio* phenon 36 (NT7) and *Vibrio* phenon 21 (NT29) from Norwegian, copepod-fed larvae and *Vibrio alginolyticus* (3-8) from intensively-reared larvae from Hunterston. *Pseudomonas* 1.1.1. was originally isolated from seawater in Kames Bay, Isle of Cumbrae by Wardlaw and Unkles (1978). This organism is non-pathogenic and forms characteristic black pigmented colonies.

Bacteria were cultured for 24 hours in 50ml marine broth in a 250ml flask at 20°C in an orbital shaker. The cells were harvested by centrifugation at 7,800 g for 10 minutes, washed and resuspended in sterile seawater to give a final concentration of 1.0×10^9 c.f.u. per ml. Bacteria-free rotifer culture (500ml in a 1 litre conical flask) was harvested from the chemostat and inoculated with bacteria to give a final concentration of 1.0×10^6 c.f.u. per ml. The culture was incubated at 20°C and aerated with filter-sterilised air (0.2µm Gelman filter). Rotifer culture water, filtered (40µm) to remove the rotifers, was also inoculated as a control to check the viability of the bacteria with time.

At two-hour intervals between 0 and 6 hours, and in one experiment at 24 hours after inoculation, two samples of rotifers were collected. One sample was rinsed with 0.1% benzalkonium chloride solution followed by sterile seawater to remove surface bacteria while the other was rinsed with sterile seawater only prior to processing in the manner previously described under section 2.3 "Bacterial flora of rotifers". The

rotifer culture water and the culture water with bacteria only were sampled by plating 0.1ml of serial 10-fold dilutions in sterile seawater onto marine agar and incubating at 20°C.

2.12 Scanning Electron Microscopy of Rotifers

The rotifers were narcotized by adding "Analar" methanol dropwise to a final concentration of 10%. After 10 minutes the rotifer suspension was filtered through a 10μm nylon mesh disc of 2.5cm diameter, mounted in a Swinnex filter holder. The rotifer samples were rinsed with 0.1% benzalkonium chloride in seawater followed by sterile seawater alone, rinsed with seawater only or untreated.

The following steps involving highly toxic chemicals were performed in a fume cupboard and plastic gloves were worn. The rotifers were washed off the nylon disc with fixative (Appendix IV) into a plastic universal bottle. After one hour the fixative was removed using a Pasteur pipette and replaced with buffer (Appendix IV). The rotifers were stored in buffer at 4°C until required for further processing. This involved washing with fresh buffer then resuspending in a few drops of buffer and an equal volume of 0.2% osmium tetroxide. The mixture was left at room temperature for one hour then the rotifers were rinsed twice, 10 minutes each time, with distilled water. The distilled water was decanted and replaced with 5ml freshly prepared 0.5% uranyl acetate. This solution is toxic and slightly radioactive. The specimens were left at room temperature in the dark for one hour then rinsed twice with distilled water.

A dense suspension of rotifers, treated as described above, was prepared in distilled water and one drop was transferred to the drying apparatus. The rotifer suspension was held in a teflon ring sandwiched between two Nuclepore polycarbonate filters of 5.0μm pore size and 10mm diameter supported by two metal mesh discs. The "sandwich" was held in a brass filter holder.

The specimens were dehydrated with a series of acetone solutions (30%, 50%, 70%, 90%, absolute and dried absolute acetone). Each acetone solution was applied for 10 minutes. The brass filter holder was quickly transferred to the critical point

dryer (C.P.D.) and the door was carefully sealed. The C.P.D. was filled with liquid carbon dioxide, flushed twice then refilled. This step was repeated every 15 minutes for one hour. Approximately one third of the volume of liquid carbon dioxide was drained then the C.P.D. was heated using two hairdryers until the pressure reached 96 bar. The pressure was reduced slowly over a period of approximately 15 minutes while continuing to heat the C.P.D. with one hairdryer.

The polycarbonate filters were removed and mounted on double-sided sticky tape on a metal stub. The edges were carefully cut away and silver paint applied to improve conductivity between the filter and the stub. The specimens were gold coated in a sputter coater (Polaron Equipment Ltd.) at 15mA for 8 minutes then examined with a Philips 500 scanning electron microscope.

2.13 Decontamination of Rotifers

Cooling and Starvation of Rotifers

Three experiments were carried out, two at G.S.P. and one at Glasgow University. At G.S.P., one batch of rotifers in a round, conical tank was cooled to 7°C in a chilled room. A second batch in a polythene bag was cooled to 14°C in a water bath with a heating element in the chilled room. Both batches were aerated.

At Glasgow one batch of rotifers in a polythene bag was cooled to 14°C in a temperature controlled room and aerated with filtered air (Gelman 0.2μm filter). Rotifers which settled on the bottom were removed using a pipette.

The rotifers were sampled daily and processed as described previously under section 2.3 "Bacterial flora of rotifers".

Chemical Disinfection of Rotifers

A range of concentrations of the following compounds in seawater (2.4% salinity) were investigated for their effect on rotifers and their bactericidal action; benzalkonium chloride, Tween 20 (polyoxyethylene-sorbitan monolaurate), Tween 80 (polyoxyethylene-sorbitan monooleate), Triton X-100 (octyl phenoxy polyethoxy-

ethanol) (Sigma Chemical Co.), Phylatol (di-2-hydroxyethoxy methane) and Panacide (dichlorophen) (BDH Ltd.).

Rotifer culture was filtered through a 10μm nylon mesh fixed to the bottom of a glass tube of internal diameter 3.8cm and immersed in 50ml of the test solution in a 100ml volume beaker. The final concentration of rotifers ranged from 100-300 per ml. After the specified exposure time the rotifers were rinsed twice by immersion in 50ml sterile seawater (2.4% salinity) and the bacterial load was determined as described previously under section 2.3 "Bacterial load of rotifers". In addition, a few rotifers were removed with a Pasteur pipette, resuspended in fresh seawater (2.4% salinity) containing algae and examined under a low power microscope (x20 magnification) to determine whether the swimming and feeding activities of the rotifers were affected.

Bactericidal Activity of Phylatol and Panacide

The susceptibility of two strains of bacteria, 3-8 (*V. alginolyticus*) and 3-15 (*v. anguillarum*) from turbot larvae, to a range of concentrations of Phylatol and Panacide was examined. A 24 hour culture of bacteria in marine broth was harvested by centrifugation at 7,800 g for 10 minutes, washed and resuspended in sterile seawater to an O.D.600nm of 1.0 (approximately 1×10^9 c.f.u. per ml). The test solution was inoculated to give a final concentration of 1.0×10^6 c.f.u. per ml. Bacterial cell viability was measured by plating 0.1ml of serial 10-fold dilutions onto marine agar at regular intervals and incubating at 20°C for 48 hours.

Lysozyme Assay

The enzymic activity of hen-egg-white lysozyme (Sigma) was measured under standard conditions. Freeze-dried *Micrococcus lysodeikticus* (3mg) (Sigma) was suspended in 10ml 0.06M sodium phosphate buffer, pH 6.4 (Appendix V). Aliquots (2.9ml) were dispensed into cuvettes (1cm light path) and 0.1ml of test solution was added. A concentration of lysozyme of 0.5μg per ml in sodium phosphate buffer was

used. Buffer was added to the control in place of the test solution. Distilled water was used as the blank. The O.D. at 600nm was measured at 30 second intervals at 37°C. One enzyme unit was defined as that which produced a reduction in the O.D. 600nm of 0.001 per minute using *M. lysodeikticus* as the substrate.

The activity of lysozyme in seawater, seawater diluted with distilled water and distilled water was tested by substituting these for the sodium phosphate buffer.

The effect of Tween 80 and ethylenediamine-tetra acetic acid (EDTA) (BDH Ltd.), separately and together, on the activity of lysozyme was examined under the standard assay conditions.

Activity of Lysozyme Against Gram-Negative Bacteria

The bactericidal activity of lysozyme, alone or in conjunction with Tween 80 and/or EDTA at various concentrations and different salinities, was examined against isolate 3-8 (*V. alginolyticus*) and *Pseudomonas* 1.1.1. An overnight broth culture of bacteria was harvested by centrifugation at 7,800 g for 10 minutes, rinsed and standardised to 1.0×10^9 c.f.u. per ml in sterile seawater. The cells were diluted to give 1.2×10^4 c.f.u. per ml then 0.2ml of cell suspension was added to 1.0ml of test solution. After 0, 5, 15 and 30 minutes, 0.1ml aliquots were plated onto marine agar and incubated at 20°C for 48 hours before enumeration.

Effect of Lysozyme on the Bacterial Load of Rotifers

The activity of lysozyme at a concentration of 1.0mg per ml, against the bacterial load of rotifers was examined by the method previously described under "Chemical disinfection of rotifers". The effect of a range of salinities from 0.2% to 0.8% and a range of rotifer concentrations from 300 to 1,600 was investigated. In addition, the ability of EDTA (0.1mM) to enhance the activity of lysozyme in low salinity seawater and calcium-free seawater (Appendix VI) was also examined.

Elimination of Bacteria by Ultra-Violet Radiation

A 15 watt U.V. strip lamp (Philips) was switched on 10 minutes prior to the start of the experiment and allowed to stabilise. Overnight broth cultures of *Pseudomonas* 1.1.1., *V. anguillarum* (3-15) and *V. tubiashi* NCMB 1336, were harvested by centrifugation at 7,800 g for 10 minutes, washed and resuspended in sterile seawater to 1.0×10^9 c.f.u. per ml. The cell suspension was diluted to give 1.0×10^6 c.f.u. per ml and 6ml aliquots pipetted into plastic petri dishes giving a depth of approximately 1.0mm. The cells were exposed to U.V. radiation for 0, 15, 30, 60 and 120 seconds after which 0.1ml was plated onto marine agar and incubated at 20°C for 48 hours. Control samples were taken from cell suspensions which were not exposed to U.V. radiation.

Effect of Exposure Time and Distance From the U.V. Source on the Bacterial Load of Rotifers

The U.V. strip lamp was switched on and allowed to stabilise 10 minutes prior to the start of the experiment. Rotifer cultures from G.S.P. were diluted with seawater to give approximately 200 rotifers per ml then 6ml aliquots were pipetted into plastic petri dishes giving a depth of approximately 1.0mm. In one experiment, rotifers were exposed to U.V.radiation for 0, 30, 60, 120 or 300 seconds at a distance of 0.5m from the U.V. source. In a second experiment, rotifers were exposed to U.V. radiation for 120 seconds at a distance of 0.13, 0.21, 0,28, 0.36, 0.44 or 0.5m from the U.V. source. Control samples were not exposed to U.V. radiation. The rotifer suspension (3.0ml) was pipetted into a glass homogeniser, diluted with an equal volume of sterile seawater and homogenised. A series of 10-fold dilutions were prepared and 0.1ml volumes were plated onto marine agar and incubated at 20°C for 5 days.

U.V. Radiation Treatment of Large Volumes of Rotifer Culture

A glass water jacket with an anulus of 4.0mm was fitted over a U.V. strip lamp connected to a transformer. The distance between the U.V. lamp and the inside of the

water jacket was approximately 1.0mm. The water jacket and U.V. lamp were covered to prevent eye and skin exposure to U.V. radiation.

Rotifer culture was held in a 10 litre glass carboy fitted with a tap and a glass tube through the rubber stopper which provided a constant head. Silicone tubing was used to connect the tap and the inlet of the water jacket. A Y-piece was used to connect a second glass carboy containing seawater to the inlet. The U.V. lamp was switched on and allowed to stabilise for 10 minutes prior to the start of all the experiments. During this period seawater was passed through the water jacket to keep it cool. The length of the silicone tubing connected to the outlet of the water jacket determined the operating pressure or flow rate.

U.V.-treated rotifers were sampled by the method previously described under section 2.3 "Bacterial flora of rotifers". They were not rinsed before sampling.

2.14 Feeding Ability of U.V.-Treated Rotifers

Rotifers were enriched with algae, rinsed and starved for 48 hours at G.S.P.. The rotifer culture was U.V.-treated at a flow rate of 1.5 litres per minute with a concentration of 200 rotifers per ml. A control batch was not exposed to U.V. radiation. *Isochrysis* (100ml) was added to 1.0 litre volumes of U.V.-treated and untreated rotifer culture and to seawater containing no rotifers. The cultures were maintained at 20°C with aeration and light. At hourly intervals for 8 hours and at 24 hours, the clearance of algae was measured by filtering (10μm) 5ml of the cultures to remove the rotifers and reading the optical density at 600nm of the filtrate.

RESULTS

3.1 Bacterial Flora of Larval Turbot

One of the main objectives of this project was to isolate and identify the "normal" intestinal microflora of turbot larvae and to determine whether this microflora differed from that of moribund larvae. By comparing these strains with bacteria isolated from various sources associated with the early feeding stages of larval turbot, the origin of the larval gut microflora could be established. Gut bacteria were isolated by rinsing the larvae with benzalkonium chloride solution followed by sterile seawater. The lowest bacterial counts (18%) were obtained after a 60 second rinse with benzalkonium chloride at which point the surface bacteria were presumed to be killed or removed.

Intensively-Reared Turbot Larvae Fed Intensively-Cultured Rotifers

The changes in the bacterial numbers and flora of the gut of intensively-reared turbot larvae from one to nine days posthatch were investigated. Newly-hatched larvae were introduced into a tank containing 1,800 litres of 0.1μm filtered seawater to a density of approximately 16 larvae per litre. Intensively-cultured rotifers were added daily from day 3 onwards. The following samples were analysed for total heterotrophic bacteria and presumptive vibrios, as determined by growth on TCBS agar:

(i) water in which the larvae were introduced into the tank (day one only)

(ii) water from the rearing tank (daily)

(iii) turbot larvae (daily)

(iv) water from the rotifer culture (day 3 to day 9)

(v) rotifers (day 3 to day 9).

The gut of newly-hatched larval turbot contained very few bacteria, however, the level increased rapidly after feeding commenced with $>10^4$ c.f.u. per fish (20 fish per sample) recovered on day 4 (Figure 2a). The number of bacteria per fish did not change significantly after about day 5. The bacterial levels in the rotifers and the rotifer culture water remained relatively constant throughout the experiment (Figures

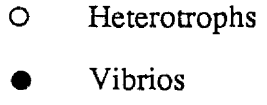
FIGURE 2

a) **Bacteria associated with intensively-reared turbot larvae.**

b) **Bacteria associated with rotifers.**

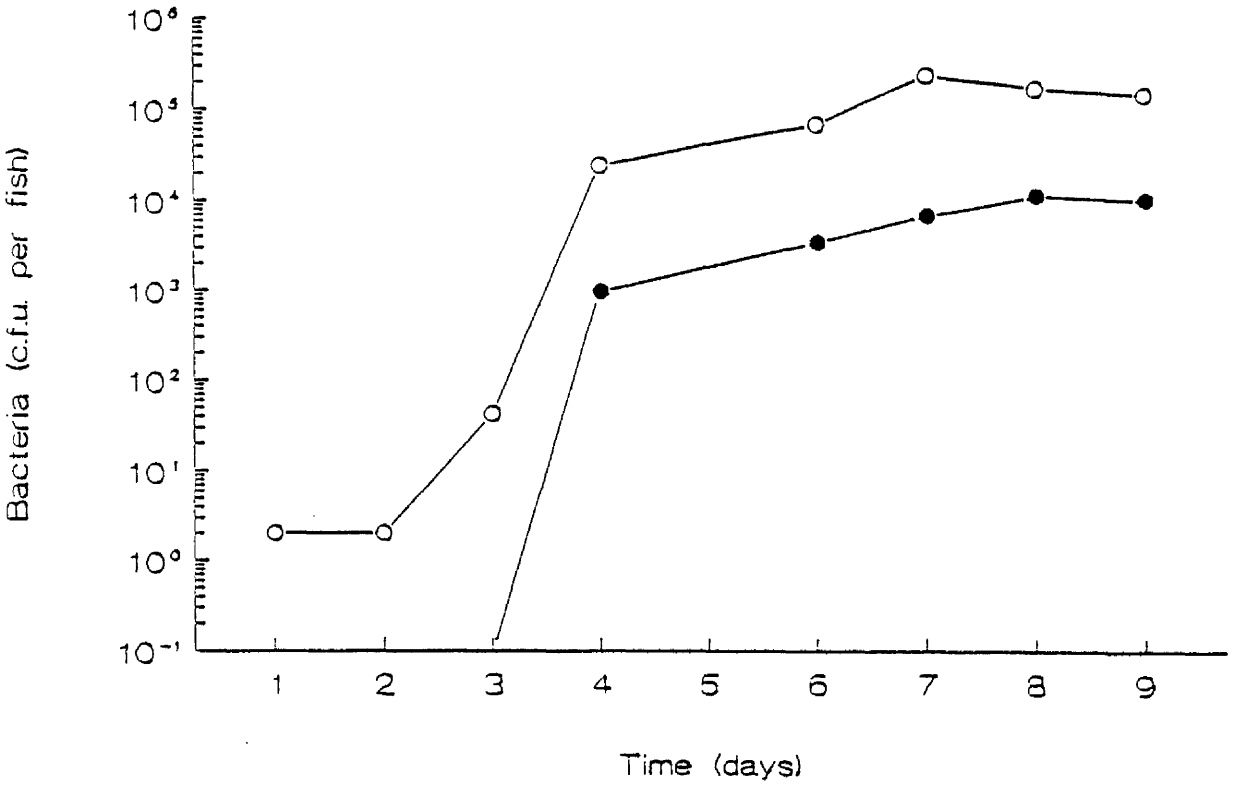
c) **Bacteria associated with rotifer culture water.**

d) **Bacteria associated with tank water.**

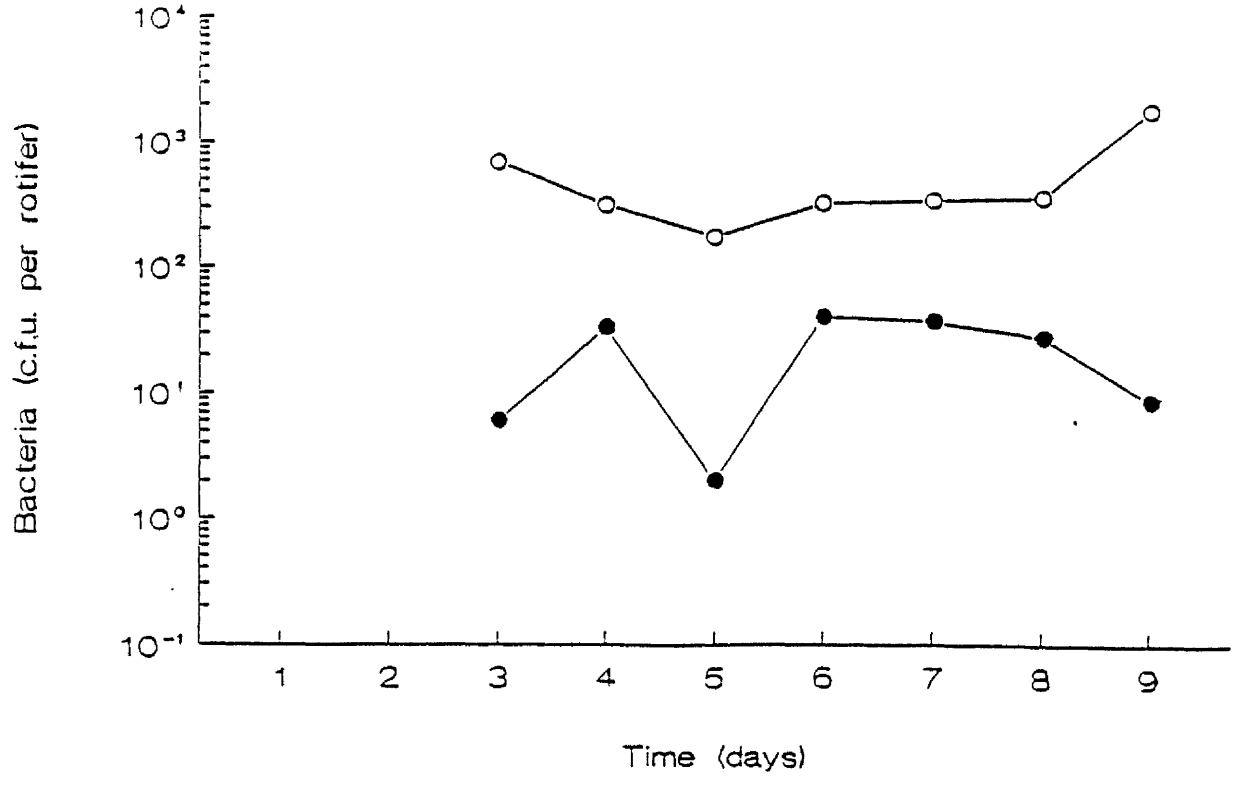


Points represent means of three 10-fold dilutions performed in duplicate. This applies to all graphs.

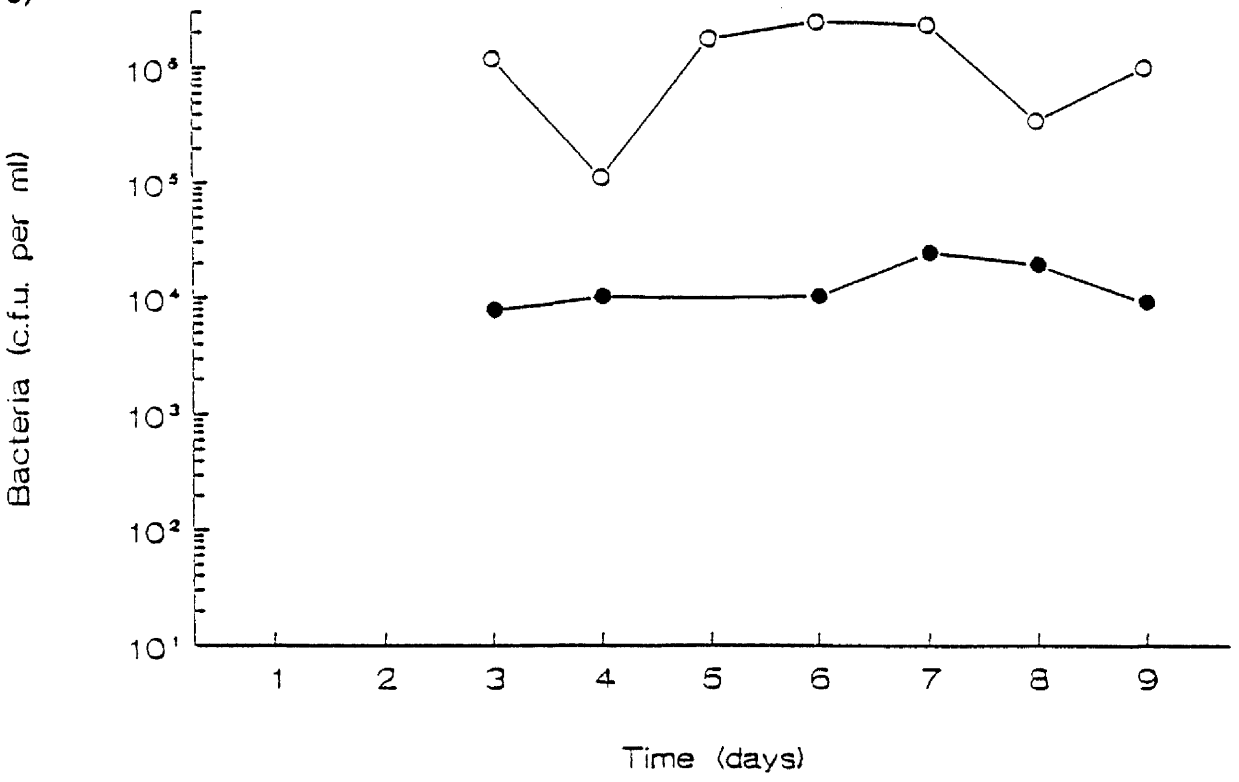
a)


Bacteria (c.f.u. per fish)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
10⁻¹
1
2
3
4
5
6
7
8
9
Time (days)

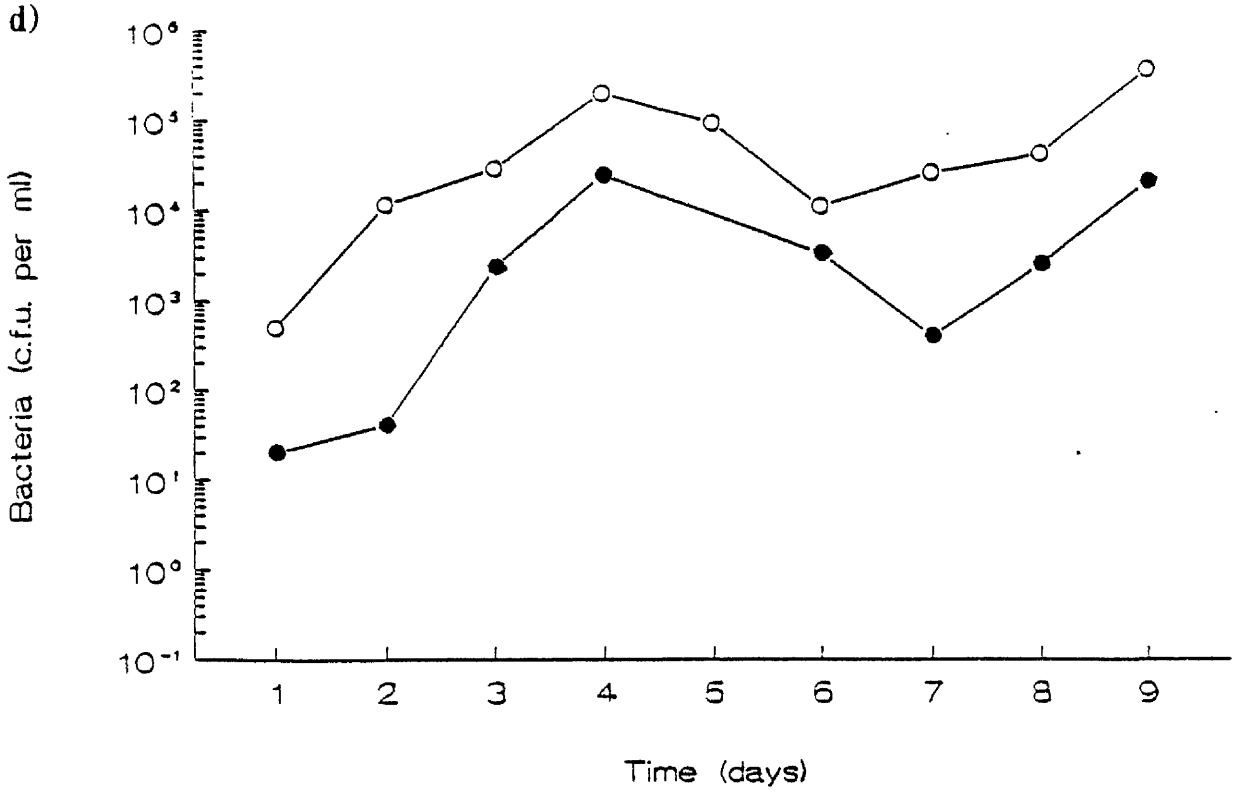
b)


Bacteria (c.f.u. per rotifer)
10⁴
10³
10²
10¹
10⁰
10⁻¹
1
2
3
4
5
6
7
8
9
Time (days)

c)


Bacteria (c.f.u. per ml)
10⁶
10⁵
10⁴
10³
10²
10¹
1
2
3
4
5
6
7
8
9
Time (days)

d)


Bacteria (c.f.u. per ml)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
10⁻¹
1
2
3
4
5
6
7
8
9
Time (days)

2b and 2c), whereas the level of bacteria in the turbot rearing tank water increased from day 1 to day 4 (Figure 2d). The proportion of presumptive vibrios remained fairly constant and rarely exceeded 10% of the total population. Good survival of turbot larvae occurred during the 9-day period of this experiment, as judged by visual examination of the numbers remaining.

Identification of 110 isolates selected from the dominant colony types on marine agar from the samples (i) to (v) above, revealed that the most varied flora occurred in the tank water (Table 2), and that vibrios represented a much higher proportion of the total population than was indicated by the growth on TCBS agar. In fact, the *Vibrio/Aeromonas* group was dominant. This group of isolates from the gut of larval turbot was identified to species level using a probabilistic identification matrix for vibrios (Bryant *et al.*, 1986) (Table 3). If the identification score reached was <0.6 it was considered insignificant and the isolate was designated an "unidentified vibrio". *V. alginolyticus* was the dominant species. A total of 33 isolates from samples (i) to (v), on TCBS agar, were identified to species level where possible (Table 4).

3.2 Source of the Gut Flora of Larval Turbot

Cluster analysis of the isolates from the gut of larval turbot and the various sources associated with their early feeding stages (samples (i) to (v)) was carried out to determine the source of the gut flora and also, to determine any pattern of similarity between the isolates over the 9-day period. There was a high degree of similarity between the isolates from the gut of larval turbot and the rotifer culture (Figure 3) indicating that the rotifer culture, and not the water from the rearing tank or the original suspension of larvae, was the source of the gut flora. Figure 4 shows that identical isolates, identified as *V. alginolyticus*, were detected in turbot larvae on days 4,5,7,8 and 9 and in the rotifers on day 5. A closely related group of identical isolates, also identified as *V. alginolyticus*, were detected in turbot larvae on days 4,6 and 8, in rotifers on days 3,7 and 8, and in the rotifer culture water on days 7 and 9.

TABLE 2

Heterotrophic Bacteria Isolated From the Gut of Turbot Larvae and Various Sources Associated with Their Early Feeding Stages

Source	Identification	Number of Isolates
Gut of turbot larvae	*Vibrio/Aeromonas*	20
	Acinetobacter	2
	Unidentified	1
Rotifers	*Vibrio/Aeromonas*	16
	Unidentified	3
Rotifer Culture Water	*Vibrio/Aeromonas*	19
	Cytophaga/Flexibacter	1
	Pseudomonas/Alcaligenes	1
	Unidentified	3
Turbot Tank Water	*Vibrio/Aeromonas*	17
	Pseudomonas/Alcaligenes	4
	Cytophaga/Flexibacter	2
	Acinetobacter	1
	Moraxella	1
	Chromobacterium	1
	"Coryneform"	1
	Unidentified	7
Water From Suspension of Larvae	*Vibrio/Aeromonas*	6
	Pseudomonas/Alcaligenes	2
	Cytophaga/Flexibacter	1
	Unidentified	1

TABLE 3

Vibrios From the Gut of Intensively-Reared Turbot Larvae

Identification*	Number of Isolates
V. alginolyticus	9
V. natrigens	4
V. anguillarum	1
V. fluvialis	1
V. pelagius	1
Aeromonas caviae	2
Unidentified vibrio	2

*Using the probability matrix of Bryant *et al.* (1986)

TABLE 4

Vibrios Isolated From the Gut of Turbot Larvae and Various Sources Associated With Their Early Feeding Stages

Source	Identification*	Number of Isolates
Gut of Turbot Larvae	*V. alginolyticus*	1
	V. tubiashii	1
	V. anguillarum	1
	V. campbellii	1
	Vibrio phenon 36	1
Rotifers	*V. alginolyticus*	1
	V. fluvialis	4
	V. natrigens	1
	V. pelagius	1
	V. anguillarum	1
	Aeromonas caviae	1
	Unidentified vibrio	2
Rotifer Culture Water	*V. campbellii*	2
	V. alginolyticus	1
Turbot Tank Water	*V. fluvialis*	3
	V. alginolyticus	3
	V. harveyii	1
	V. pelagius	1
	Vibrio phenon 21	1
	Unidentified vibrio	2
Water From Suspension of Larvae	*V. campbellii*	1
	V. splendidus I	1
	Unidentified vibrio	1

*Using the probability matrix of Bryant *et al.* (1986)

FIGURE 3

Cluster analysis of bacterial isolates from turbot larvae, rotifer culture water, rotifers, water from the larval turbot suspension and the tank water.

○	Turbot Larvae
▲	Rotifer Culture Water
▽	Rotifers
□	Water from the Larval Turbot Suspension
Unmarked	Tank Water

Isolates grouped within an amalgamation distance of 0.000 were identical.

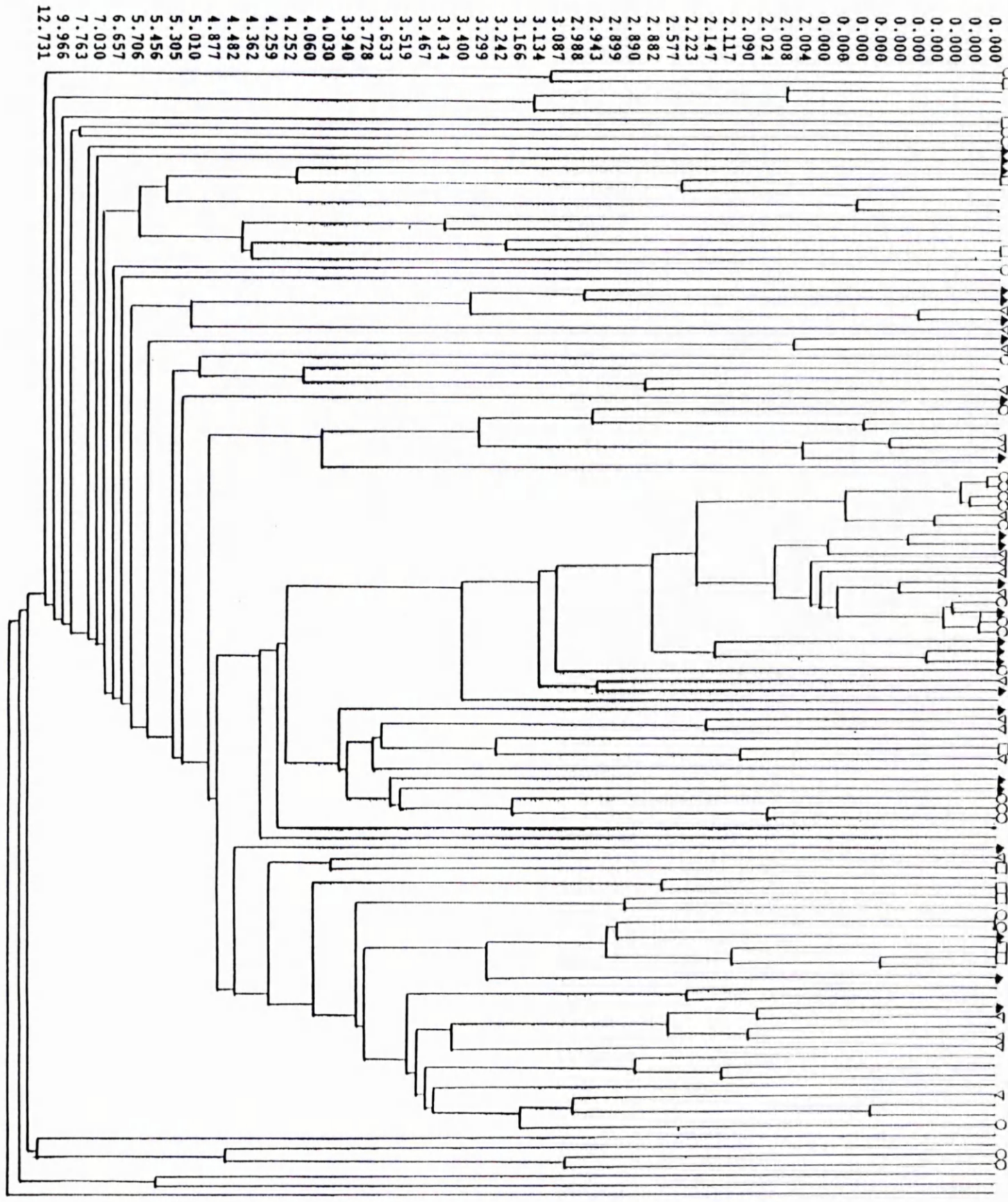
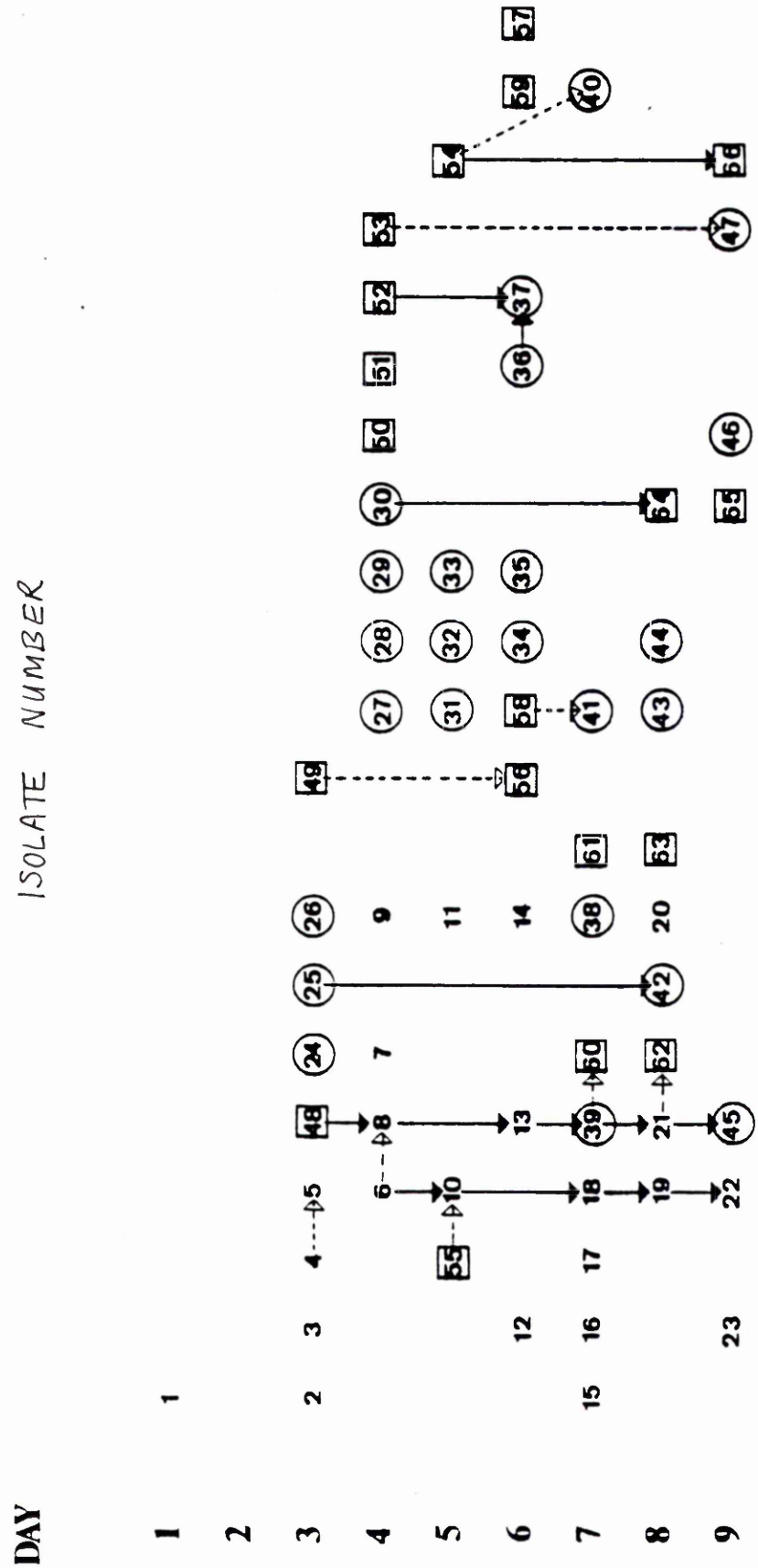

AMALGAMATION DISTANCE
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
2.004
2.008
2.024
2.090
2.117
2.147
2.223
2.577
2.882
2.890
2.899
2.943
2.988
3.087
3.134
3.166
3.242
3.299
3.400
3.434
3.467
3.519
3.633
3.728
3.940
4.030
4.060
4.252
4.259
4.362
4.482
4.877
5.010
5.305
5.456
5.706
6.657
7.030
7.763
9.966
12.731

FIGURE 4

Relationship between bacterial isolates obtained from intensively-reared turbot larvae, rotifer culture water and rotifers, on successive days.

A solid arrow indicates that the isolates were identical, a broken arrow indicates that the isolates differed in one biochemical test and could be the same strain. Isolates marked ○ were isolated from rotifer culture water, those marked □ were isolated from rotifers, the remainder were from turbot larvae.


ISOLATE NUMBER
DAY
1
2
3
4
5
6
7
8
9

3.3 Bacterial Flora of Rotifer Culture Water

Intensive rotifer cultures were produced at Hunterston in 400 litre, white, flat-bottomed tanks. The rotifers were fed on algae (*Pavlova lutheri*) and aerated. Production cycles of seven days were employed, a new cycle being set up by inoculating a tank containing 110 litres of artificial seawater at 2.0% salinity with 5.5 x 10^6 rotifers from the harvest of the previous cycle, and adding approximately 30 litres of artificial seawater daily. Over a period of 14 days, samples of rotifer culture water were taken from 7 tanks (each tank was sampled 10 times) and the mean level of bacteria for each day of the cycle was recorded (Figure 5).

The number of heterotrophic bacteria increased rapidly to give a peak of $>10^6$ c.f.u. per ml on day 2 then declined, almost to the original level, by day 7. The level of presumptive vibrios increased 10 fold to reach a peak of approximately 10^5 c.f.u. per ml by day 3, then decreased to the original level.

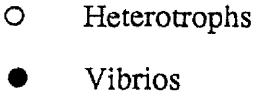
A total of 126 isolates, 66 from marine agar and 60 from TCBS agar, were identified (Table 5). *Vibrio/Aeromonas* species were dominant with *Vibrio* phenon 36 being the most common species isolated from TCBS agar.

3.4 Comparison of the Gut Flora of Apparently Healthy and Unhealthy Turbot Larvae

During the development of turbot larvae, it is possible to distinguish between larvae which are healthy and those which are unhealthy, and likely to die, since the latter appear more darkly pigmented and may not be feeding well. To determine whether the flora of apparently healthy larvae differed from that of unhealthy larvae, 20 bacterial isolates from each group, taken from intensively-reared 8- and 9-day-old larval turbot, were identified (Table 6). The floras of the 2 groups were similar (Figure 6), with *Aeromonas caviae* being the dominant species in both groups. Survival of the turbot larvae was low during this experiment.

FIGURE 5

Bacterial levels in rotifer culture water during one production cycle of rotifers.



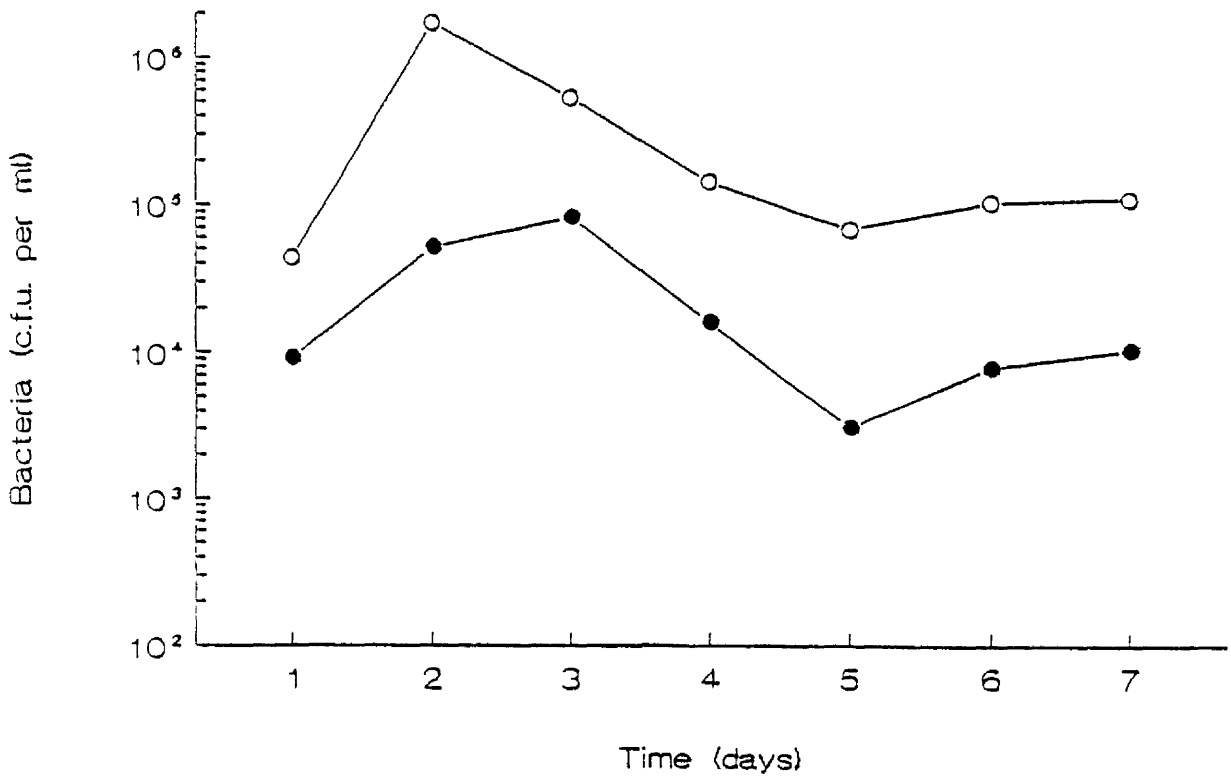

Bacteria (c.f.u. per ml)
10⁶
10⁵
10⁴
10³
10²
1
2
3
4
5
6
7
Time (days)

TABLE 5

Bacteria Isolated From Rotifer Culture Water

Marine Agar

Identification	Number of Isolates
Vibrio/Aeromonas	29
Moraxella	13
Enterobacteriaceae	6
Pseudomonas/Alcaligenes	3
Unidentified	15
Total	66

TCBS Agar

Identification	Number of Isolates
Vibrio phenon 36	41
V. alginolyticus	4
V. pelagius	4
V. campbellii	4
V. harveyii	1
V. fluvialis	1
Unidentified vibrio	5
Total	60

TABLE 6

Bacteria Isolated From Healthy and Unhealthy Turbot Larvae

	Healthy Larvae		Unhealthy Larvae	
	Number of Isolates		Number of Isolates	
	Marine Agar	TCBS Agar	Marine Agar	TCBS Agar
Aeromonas caviae	7	3	7	5
Vibrio phenon 36	2		1	
V. anguillarum		3		1
V. pelagius		1	1	
V. alginolyticus			1	
V. neresis			1	
Unidentified vibrio				1
Acinetobacter	2		2	
Pseudomonas/Alcaligenes	1			
Unidentified	1			
Total	13	7	13	7

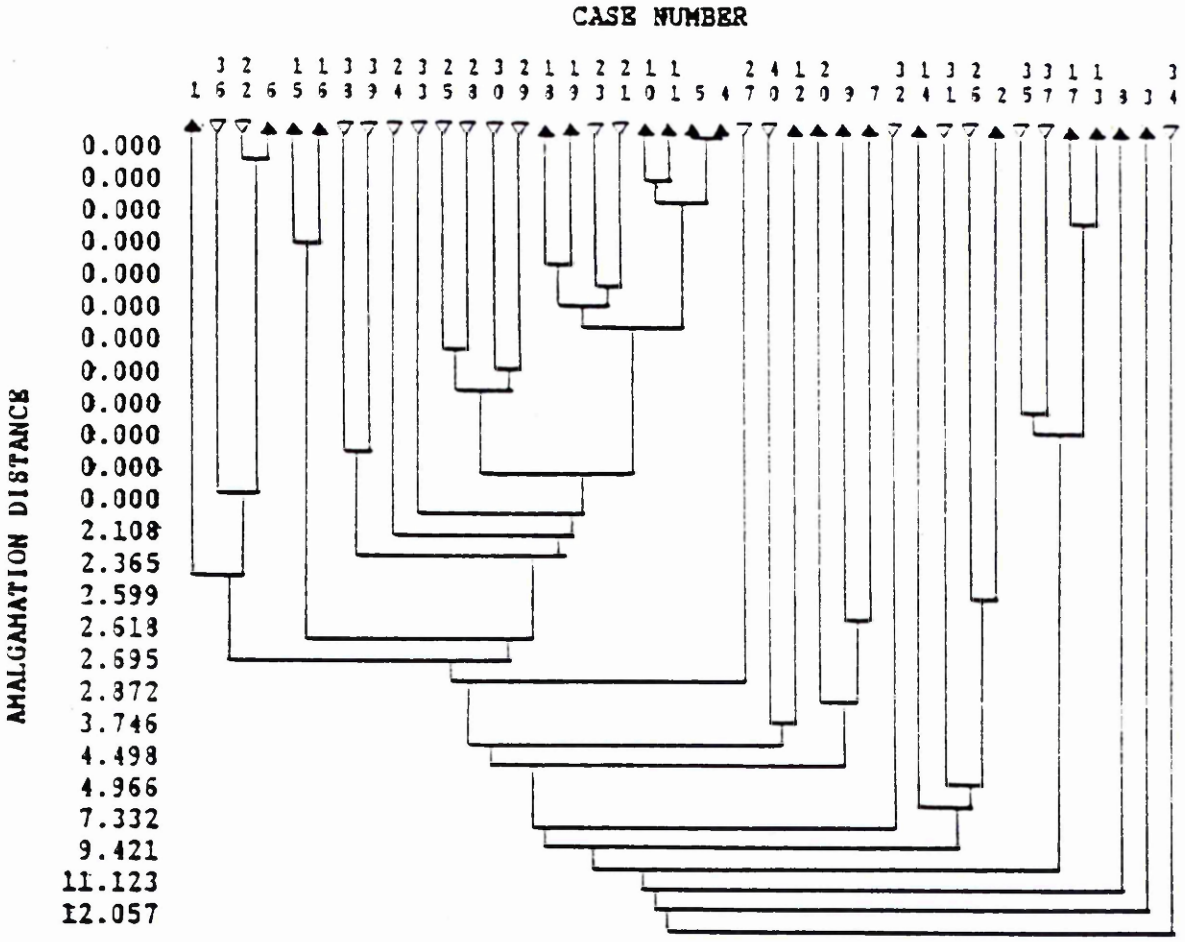
FIGURE 6

Cluster analysis of bacterial isolates from healthy and unhealthy, intensively-reared turbot larvae.

▲ Healthy Larvae

▽ Unhealthy Larvae

Isolates grouped within an amalgamation distance of 0.000 were identical.


CASE NUMBER
1 3 6 2 2 6 1 5 1 6 3 3 3 9 2 4 3 3 2 5 2 8 3 0 2 9 1 8 1 9 2 3 2 1 1 0 1 1 1 5 2 4 7 2 4 0 1 2 2 0 9 7 3 2 3 1 4 3 1 2 6 2 3 3 5 3 7 1 7 1 3 3 3 4
AMALGAMATION DISTANCE
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
2.108
2.365
2.599
2.618
2.695
2.872
3.746
4.498
4.966
7.332
9.421
11.123
12.057

3.5 Comparison of the Gut Flora of Intensively- and Extensively-Reared Turbot Larvae

In a preliminary experiment, isolates from the gut microflora of 10-day-old turbot larvae, which were reared in extensive (low density) conditions and fed rotifers from an extensive, natural source, were identified and found to represent a larger variety of genera than previously detected in intensively-reared larvae (Table 7).

To determine whether the rate of bacterial colonisation of the gut of larval turbot and the nature of the gut flora varied, depending on the rotifer source and the larval rearing conditions, three 1,800 litre tanks (B1, B2 and B10) were set up to mimic intensive (high density) or extensive (low density) rearing conditions. Tank B1 contained 25,000 larvae (intensive conditions) which were fed daily on rotifers from intensive laboratory cultures. Tank B2 contained 3,000 larvae (extensive conditions) fed on rotifers (one addition two days prior to introduction of larvae) from the same intensive laboratory cultures. Tank B10 contained 3,000 larvae (extensive conditions) fed on rotifers (one addition two days prior to introduction of larvae) from a natural, low density source.

The larvae were added to the tanks at 3 days posthatch, and sampled daily thereafter (10 fish per sample). The gut of larval turbot in tanks B1 and B2 was rapidly colonised with $>10^4$ c.f.u. per fish detected in 4-day-old larvae (Figure 7a). The larvae in tank B10 acquired their gut flora at a much slower rate, although all three batches had approximately 10^5 c.f.u. per fish when the larvae were 6 days old. The rate of increase of presumptive vibrios, as shown by growth on TCBS agar, was also slower in larvae from tank B10 (Figure 7b).

The survival rates of the larvae at 20 days posthatch were 4.6%, 11.2% and 32.4% for tanks B1, B2 and B10, respectively, indicating that extensive rearing conditions resulted in higher survival rates than intensive conditions. In addition, increased growth rates of extensively-reared larvae were noted (Table 8).

In another experiment, turbot larvae were reared intensively (25,000 larvae per 1,800 litre tank, fed rotifers daily) or extensively (3,000 larvae per 1,800 litre tank,

TABLE 7

Bacteria Isolated From Extensively-Reared Turbot Larvae

Identification	Number of Isolates
Flavobacterium/Cytophaga	1
Pseudomonas/Alcaligenes	2
Vibrio/Aeromonas	2
Moraxella	2
Gram-positive cocci	1
Unidentified	1
Total	9

FIGURE 7

a) **Gut bacteria from turbot larvae reared under intensive or extensive conditions.**

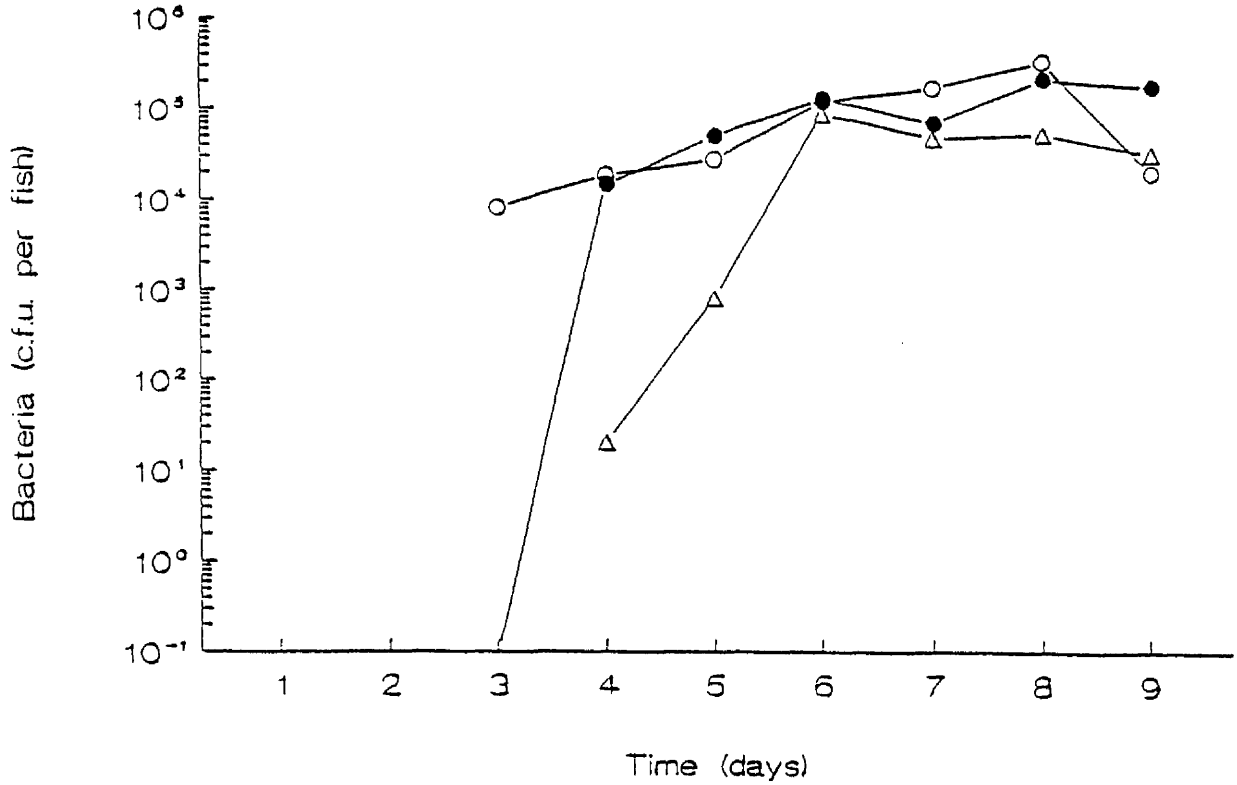
b) **Gut vibrios from turbot larvae reared under intensive or extensive conditions.**

○ Tank B1

● Tank B2

△ Tank B10

a)


Bacteria (c.f.u. per fish)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
1 2 3 4 5 6 7 8 9
Time (days)

b)

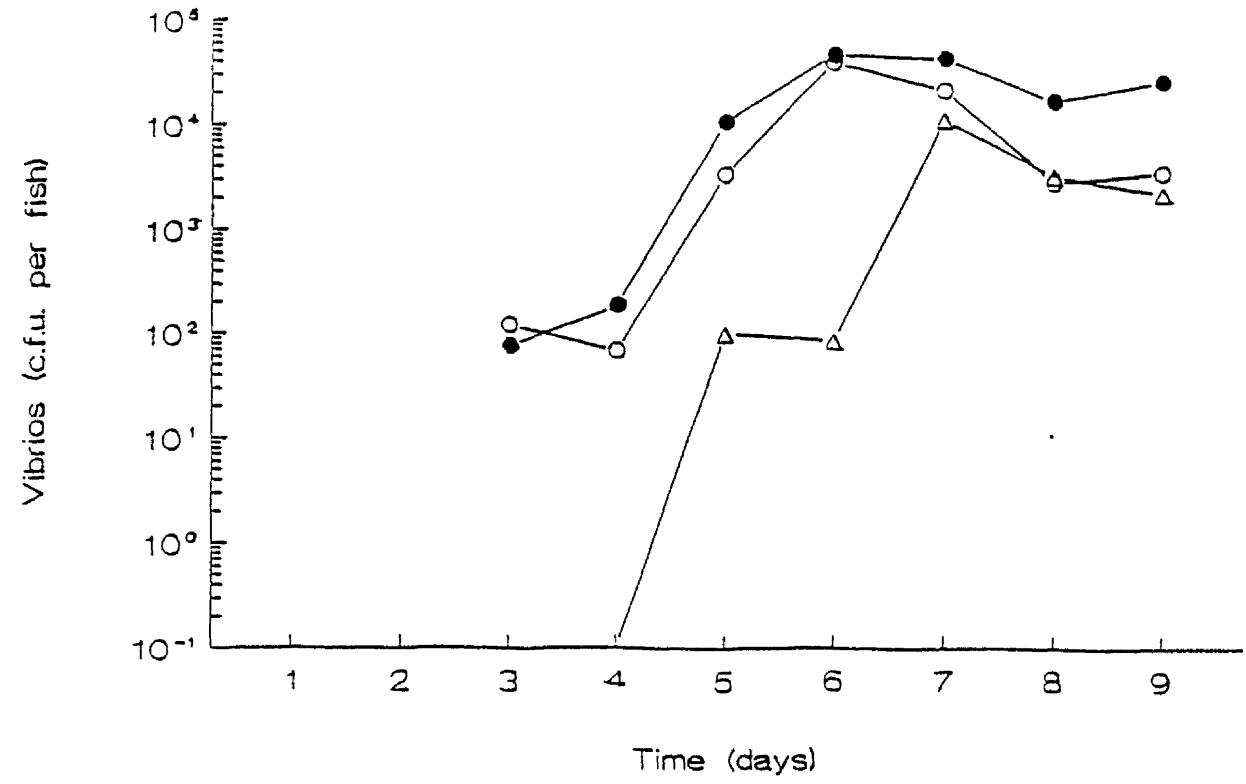

Vibrios (c.f.u. per fish)
10^6
10^4
10^3
10^2
10^1
10^0
10^{-1}
1 2 3 4 5 6 7 8 9
Time (days)

TABLE 8

Comparison of Intensive and Extensive Culture of Turbot Larvae

Tank Number	Number of Larvae in 1,800 litres	Rotifers			Larvae	
		Source	Feeding Regimen	Density (rotifers per litre)	‡Length Range (mm at day 8)	Survival (% at day 20)
Intensive B1	25,000	Laboratory	Daily Additions	3,000 (initially)	5.37-5.80	4.6
Extensive B2	3,000	Laboratory	Single Addition*	300	5.50-6.37	11.2
Extensive B10	3,000	Outdoor	Single Addition*	300	6.30-6.88	32.4

*Rotifers were added to the tanks containing algae two days prior to introduction of turbot larvae.

‡Ten larvae measured.

rotifers added 2 days prior to introduction of larvae), these tanks being equivalent to tanks B1 and B2 above. A slower rate of colonisation of the gut of larvae reared under extensive conditions was found but survival at day 20 was low in both groups (1.5% for intensive and 2.5% for extensive).

Isolates were selected from the dominant colony types on marine agar and TCBS agar from each daily sample of turbot larvae from tanks B1, B2 and B10, to determine whether the composition of the gut flora varied depending on the rearing conditions. *Vibrio* and *Aeromonas* species represented between 90 and 100% of the isolates (Table 9). Figures 8 to 10 show the relationship between the isolates from tanks B1, B2 and B10, respectively, during the 7 consecutive days of sampling. Cluster analysis revealed some similarity between the isolates (Figure 11), with two clusters, identified as *V. alginolyticus* and *A. salmonicida*, being detected in all three groups. One cluster, between isolates from tanks B1 and B10, was identified as *V. parahaemolyticus*, and three clusters, between isolates from tanks B2 and B10, were identified as *V. anguillarum, A. salmonicida* and an unidentified *Vibrio* species. The most similarity, with 6 clusters, was found between isolates from tanks B1 and B2. These isolates were identified as *V. alginolyticus, V. campbellii*, two strains of *V. parahaemolyticus* and two unidentified *Vibrio* species.

In comparison with figure 4, there was no obvious selection of a stable flora in the gut of these larvae.

3.6 Extensively-Reared Turbot Larvae Fed Copepods

Although rotifers are widely used for larval fish rearing, they are not the preferred diet of turbot larvae in the wild. Copepods constitute a significant portion of the natural diet (Last, 1979) and, although less economical to produce than rotifers, they can be cultured in the laboratory. The rate of colonisation and the composition of the gut flora of extensively-reared turbot larvae fed copepods was compared to that of larvae fed rotifers. The gut of copepod-fed larvae was colonised more slowly than that of rotifer-fed larvae. Although similar maximum levels of approximately 10^5 c.f.u. per fish were recorded, this level was not reached until day 8 in copepod-fed larvae, two days later than in extensively-reared, rotifer-fed larvae (Figure 12).

TABLE 9

Bacteria Isolated From Turbot Larvae Reared Under Extensive or Intensive Conditions

Identification	Number of Isolates					
	Marine Agar			TCBS Agar		
	Tank			Tank		
	B1	B2	B10	B1	B2	B10
V. alginolyticus	12	7	2	1		
V. parahaemolyticus	6	10	3	1		2
V. campbellii	3	3	2	4	2	2
V. pelagius	1					
V. splendidus I	1		2	1		1
V. splendidus II			3		2	
V. anguillarum				1		2
V. ordallii			1			
V. furnissi	1					
Vibrio phenon 6	1					1
Vibrio phenon 21	2		1			
Vibrio phenon 36	1					
Aeromonas	3	6	4		4	2
Unknown vibrio	7	7	10	4	9	5
Enterobacteriace			3			
Pseudomonas/Alcaligenes	1		1			
Total	39	33	32	12	17	15

FIGURE 8

Relationship between bacterial isolates from intensively-reared turbot larvae (Tank B1).

A solid arrow indicates that the isolates were identical.

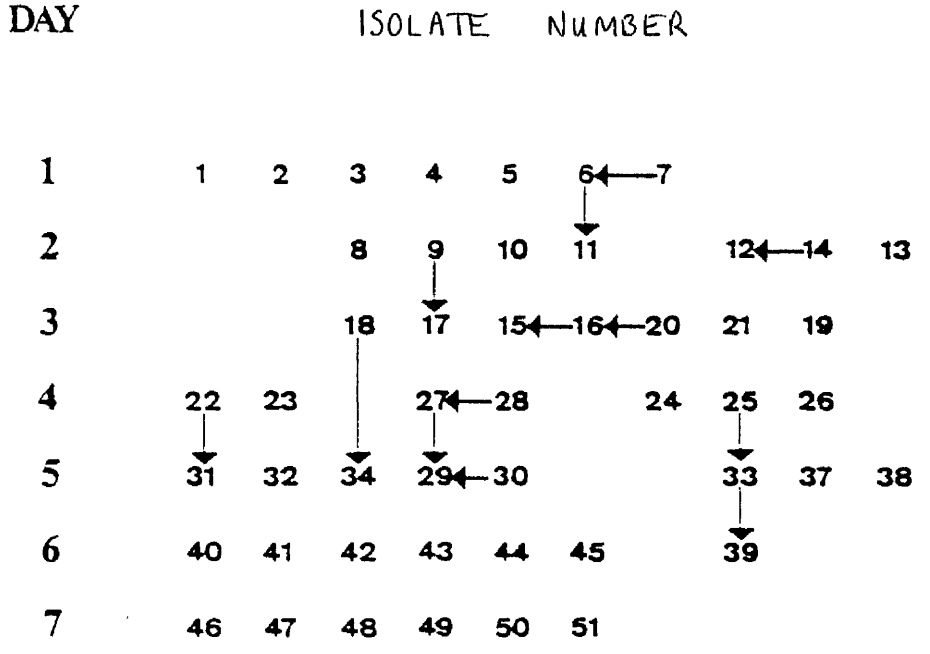

DAY
ISOLATE NUMBER
1
1 2 3 4 5 6 7
2
8 9 10 11 12 14 13
3
18 17 15 16 20 21 19
4
22 23 27 28 24 25 26
5
31 32 34 29 30 33 37 38
6
40 41 42 43 44 45 39
7
46 47 48 49 50 51

FIGURE 9

Relationship between bacterial isolates from extensively-reared turbot larvae fed rotifers from an intensive source (Tank B2).

A solid arrow indicates that the isolates were identical.

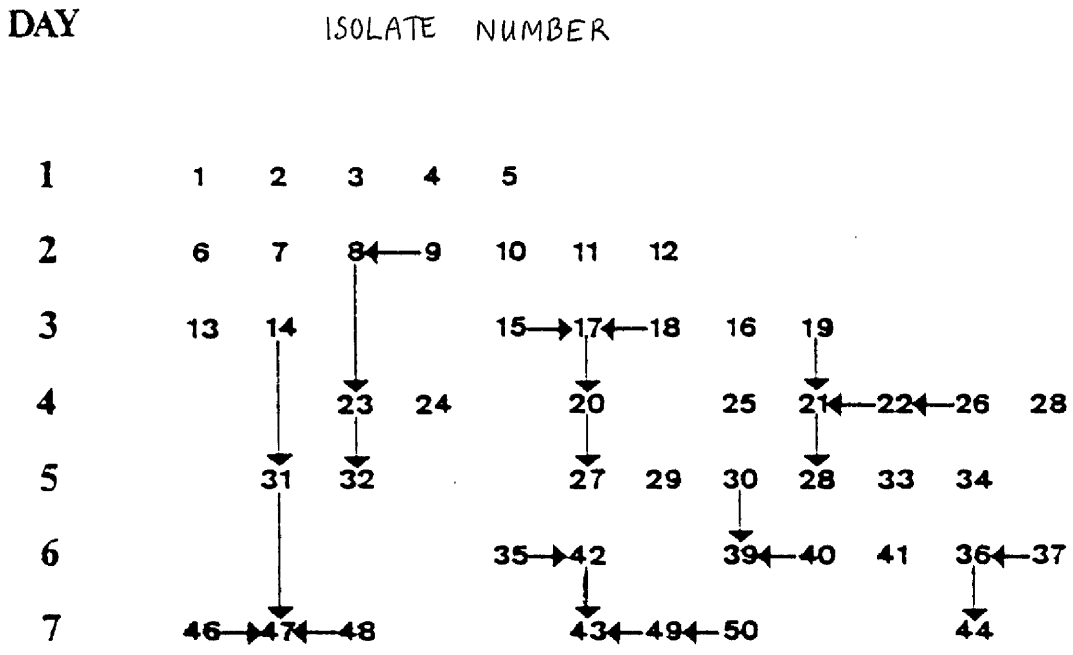

DAY
ISOLATE NUMBER
1
1 2 3 4 5
2
6 7 8 9 10 11 12
3
13 14 15 17 18 16 19
4
23 24 20 25 21 22 26 28
5
31 32 27 29 30 28 33 34
6
35 42 39 40 41 36 37
7
46 47 48 43 49 50 44

FIGURE 10

Relationship between bacterial isolates from extensively-reared turbot larvae (Tank B10).

A solid arrow indicates that the isolates were identical.

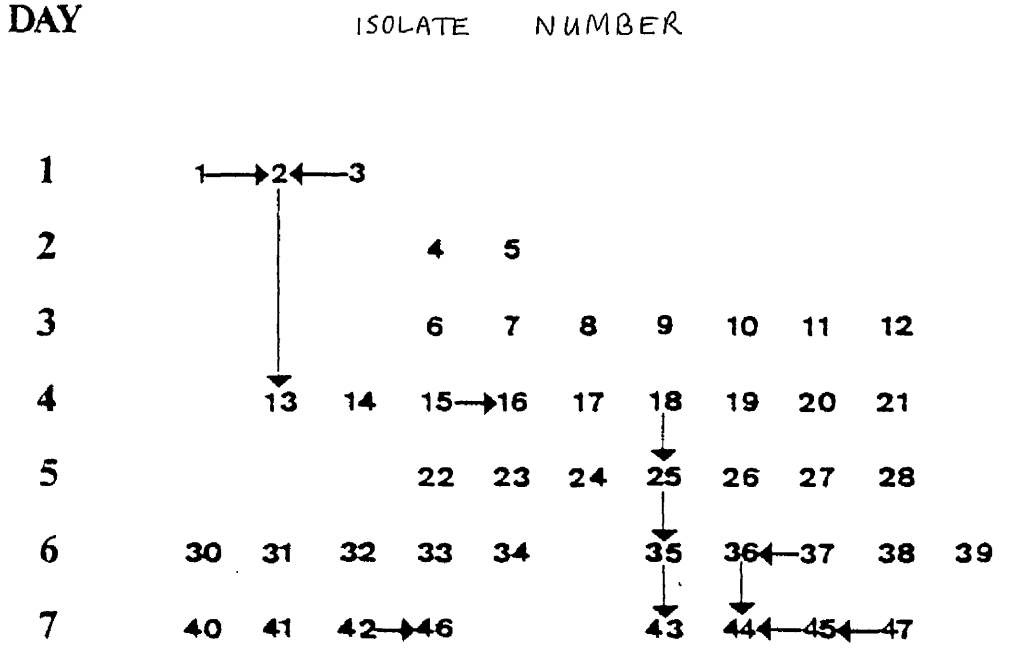
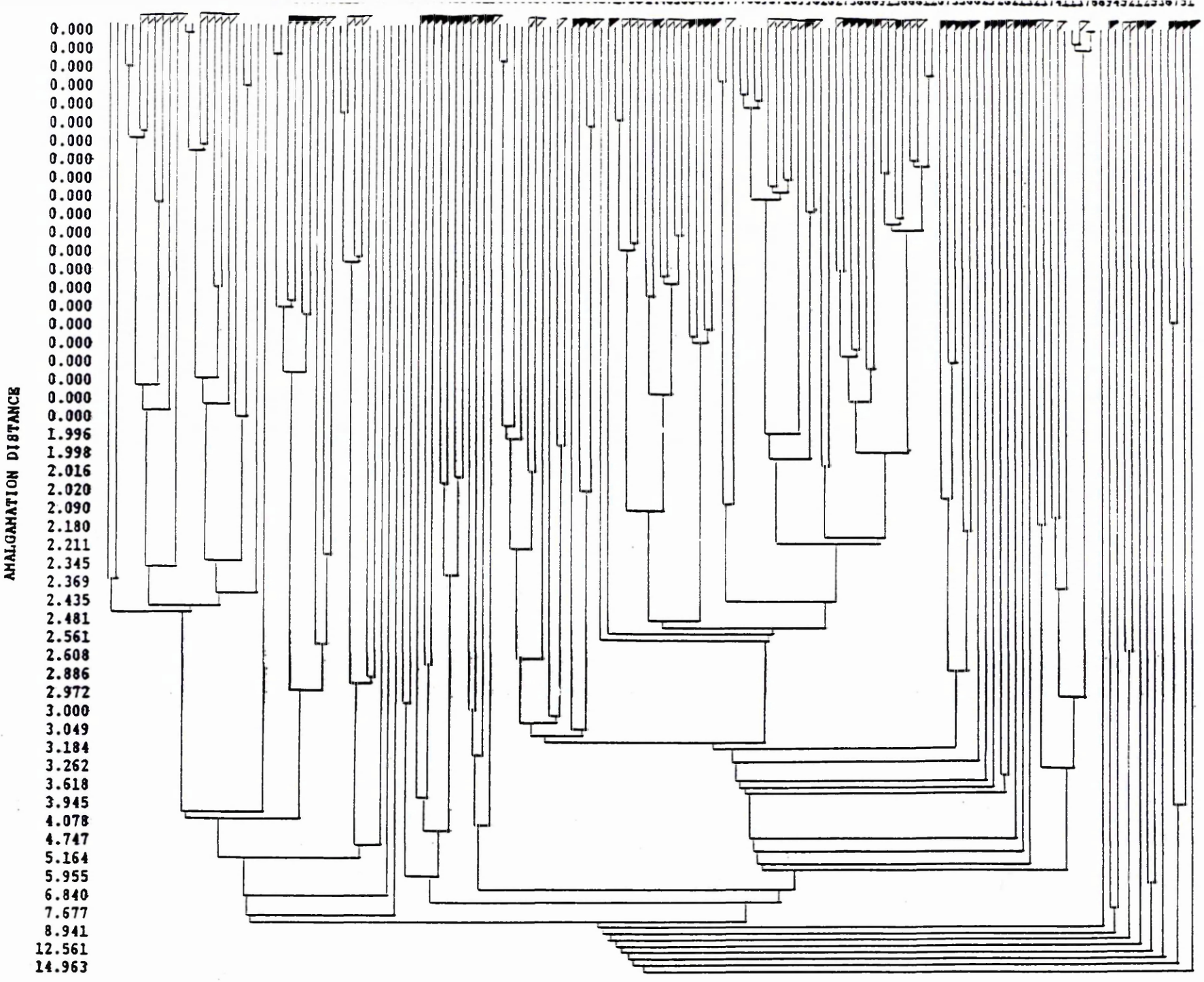

DAY
ISOLATE NUMBER
1
1 2 3
2
4 5
3
6 7 8 9 10 11 12
4
13 14 15 16 17 18 19 20 21
5
22 23 24 25 26 27 28
6
30 31 32 33 34 35 36 37 38 39
7
40 41 42 46 43 44 45 47

FIGURE 11

Cluster analysis of bacterial isolates fron intensively- and extensively-reared turbot larvae.

Unmarked	Intensively-reared Turbot Larvae (Tank B1)
◸	Extensively-reared Turbot Larvae fed Rotifers from an Intensive Source (Tank B2)
◤	Extensively-reared Turbot Larvae

Isolates grouped within an amalgamation distance of 0.000 were identical.


AMALGAMATION DISTANCE
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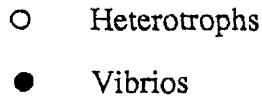
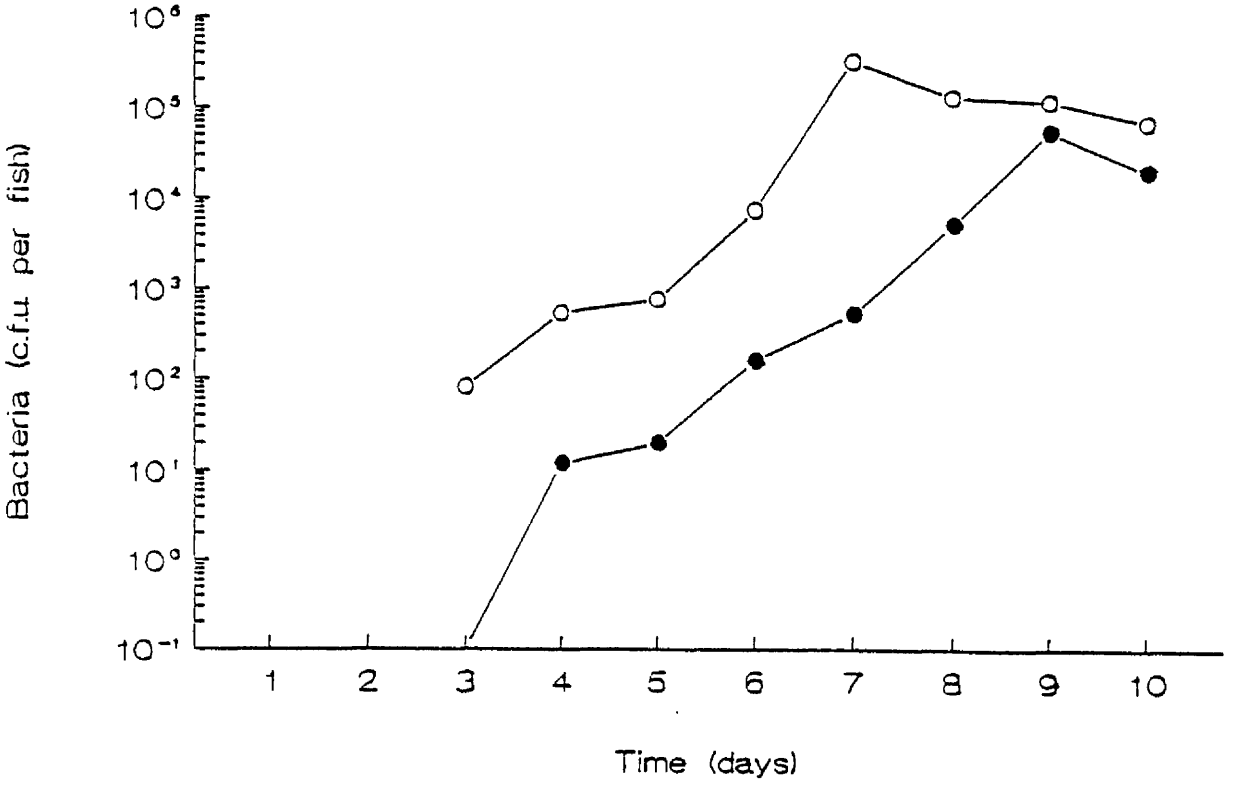
0.000
0.000
0.000
0.000
1.996
1.998
2.016
2.020
2.090
2.180
2.211
2.345
2.369
2.435
2.481
2.561
2.608
2.886
2.972
3.000
3.049
3.184
3.262
3.618
3.945
4.078
4.747
5.164
5.955
6.840
7.677
8.941
12.561
14.963

FIGURE 12

Gut bacteria from turbot larvae fed copepods.




Bacteria (c.f.u. per fish)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
10⁻¹
1
2
3
4
5
6
7
8
9
10
Time (days)

A total of 50 isolates from marine agar and 25 isolates from TCBS agar was selected from the dominant colony types on successive days from samples of turbot larvae fed copepods. As in larvae fed rotifers, *Vibrio* species were dominant in the gut flora (Table 10), however, the microflora was quite distinct from that of turbot larvae fed rotifers, as shown by cluster analysis (Figures 13 and 14). The largest cluster was identified as *V. pelagius*. This strain was detected repeatedly throughout the experiment being isolated on days 3, 4, 6, 7, 8, 9 and 10 (Figure 15).

3.7 Extensively-Reared Turbot Larvae Fed Copepods in Norway

The gut flora of two groups of copepod-fed turbot larvae reared under extensive conditions at Mowi, Norway, was analysed for comparison with the flora of larvae reared at Hunterston. One sample was taken from each group of 9-day-old larvae (10 larvae from group 1 and 15 larvae from group 2). Only 132 and 51 heterotrophic bacteria per fish were recovered from groups 1 and 2, respectively. The low temperature of culture of the Norwegian larvae (9-13°C) compared to that of intensive culture systems (20°C) was thought to be responsible for the extremely low numbers of bacteria from the Norwegian fish.

A total of 32 bacterial isolates from groups 1 and 2 were identified. *Vibrio* and *Aeromonas* species were dominant (94%) with *V. campbellii* and *Vibrio* phenon 21 accounting for 85% of the vibrios present (Table 11). Cluster analysis revealed that the isolates from turbot larvae reared in Norway were distinct from isolates from copepod-fed or rotifer-fed larvae reared at G.S.P. (Figures 16 and 17).

3.8 Colonisation of Larval Turbot with Defined Bacteria

Since their diet appeared to be the source of the intestinal microflora of larval turbot, experiments were carried out in an attempt to "dominate" the flora of rotifer cultures with bacteria isolated from batches of larvae which showed good survival rates. These bacteria were presumed to be potentially beneficial to the larvae by excluding specific or opportunistic pathogens, or by providing some essential nutrient

TABLE 10

Bacteria Isolated From Turbot Larvae fed Copepods

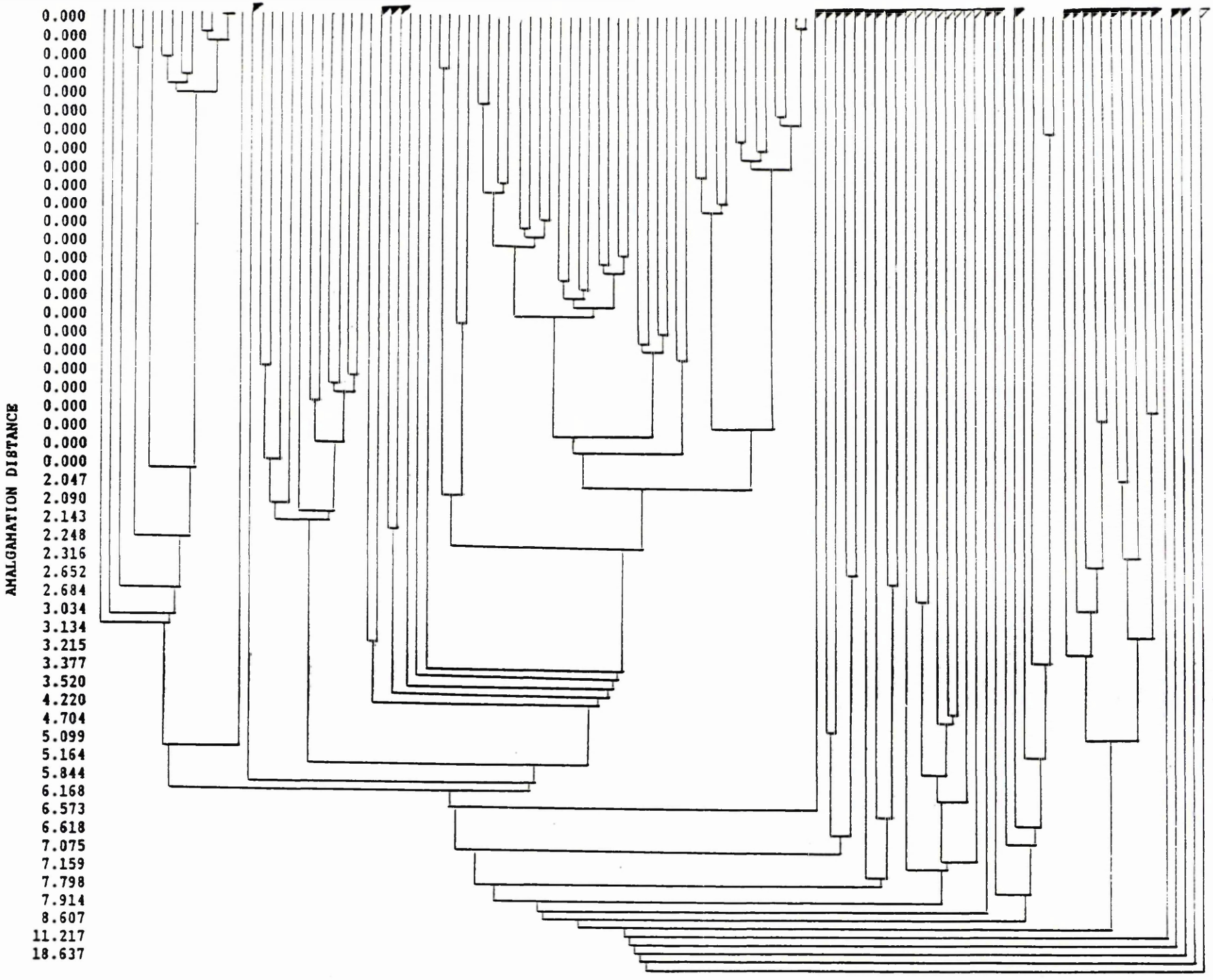
Identification	Number of Isolates	
	Marine Agar	TCBS Agar
Photobacterium	14	
V. pelagius	23	9
V. splendidus II	1	
V. harveyii	1	5
V. campbellii		10
Aeromonas caviae	1	
A. sobria		1
Unidentified vibrio	4	
Moraxella	1	
Enterobacteriaceae	4	
Flavobacterium/Cytophaga	1	
Total	50	25

FIGURE 13

Cluster analysis of bacterial isolates from copepod-fed and intensively-reared, rotifer-fed turbot larvae.

Unmarked	Copepod-fed Turbot Larvae
◤	Intensively-reared, Rotifer-fed Turbot Larvae

Isolates grouped within an amalgamation distance of 0.000 were identical.


AMALGAMATION DISTANCE
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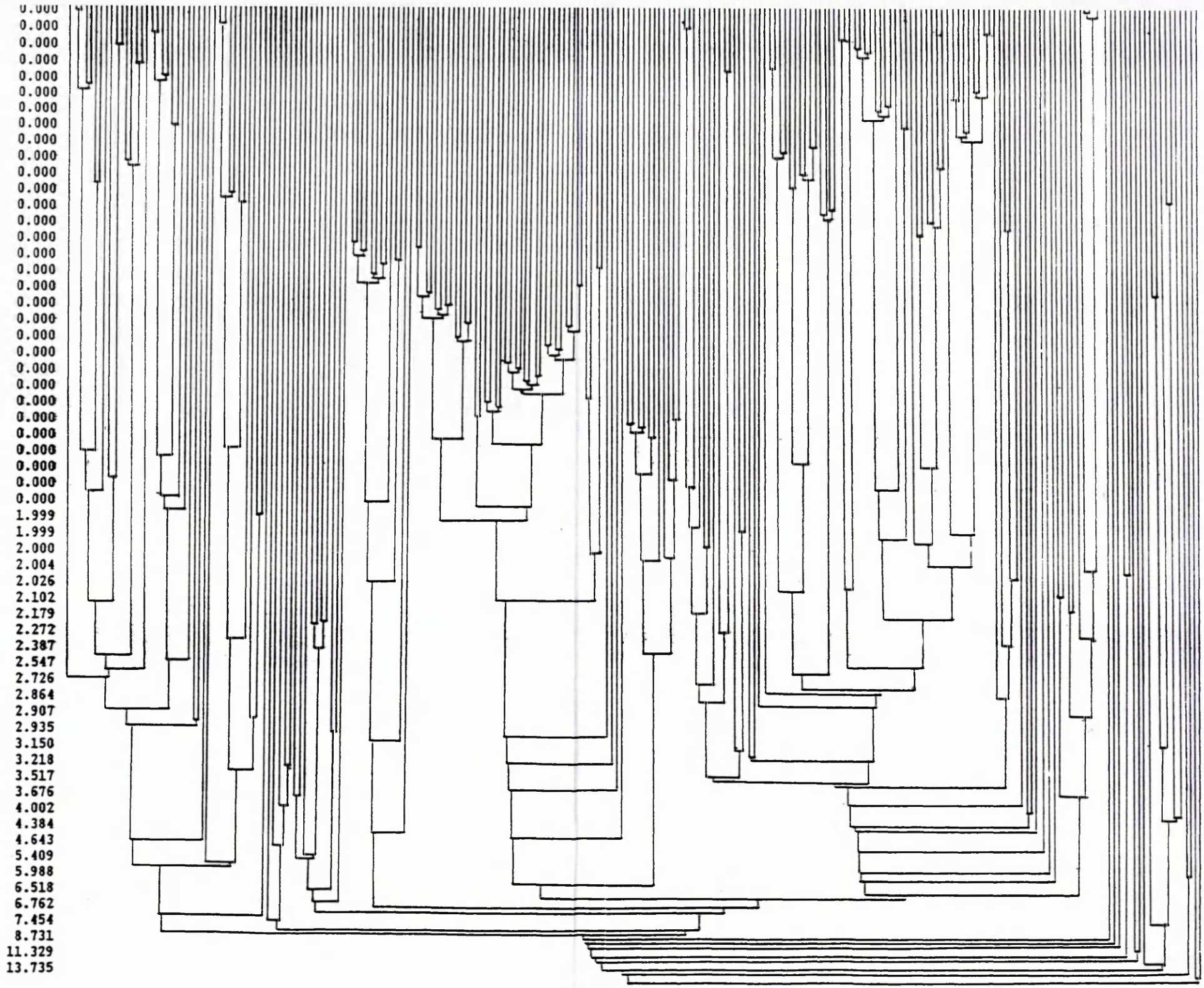
2.047
2.090
2.143
2.248
2.316
2.652
2.684
3.034
3.134
3.215
3.377
3.520
4.220
4.704
5.099
5.164
5.844
6.168
6.573
6.618
7.075
7.159
7.798
7.914
8.607
11.217
18.637

FIGURE 14

Cluster analysis of bacterial isolates from copepod-fed and intensively- or extensively-reared, rotifer-fed turbot larvae.

Unmarked	Copepod-fed Turbot Larvae
◤	Intensively-reared Turbot Larvae (Tank B1)
◸	Extensively-reared Turbot Larvae fed Rotifers from an Intensive Source (Tank B2)
○	Extensively-reared Turbot Larvae (Tank B10)

Isolates grouped within an amalgamation distance of 0.000 were identical.


AMALGAMATION DISTANCE
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0.000
0.000
0.000
0.000
1.999
1.999
2.000
2.004
2.026
2.102
2.179
2.272
2.387
2.547
2.726
2.864
2.907
2.935
3.150
3.218
3.517
3.676
4.002
4.384
4.643
5.409
5.988
6.518
6.762
7.454
8.731
11.329
13.735

FIGURE 15

Relationship between bacteria isolated from extensively-reared turbot larvae fed copepods.

A solid arrow indicates that the isolates were identical.

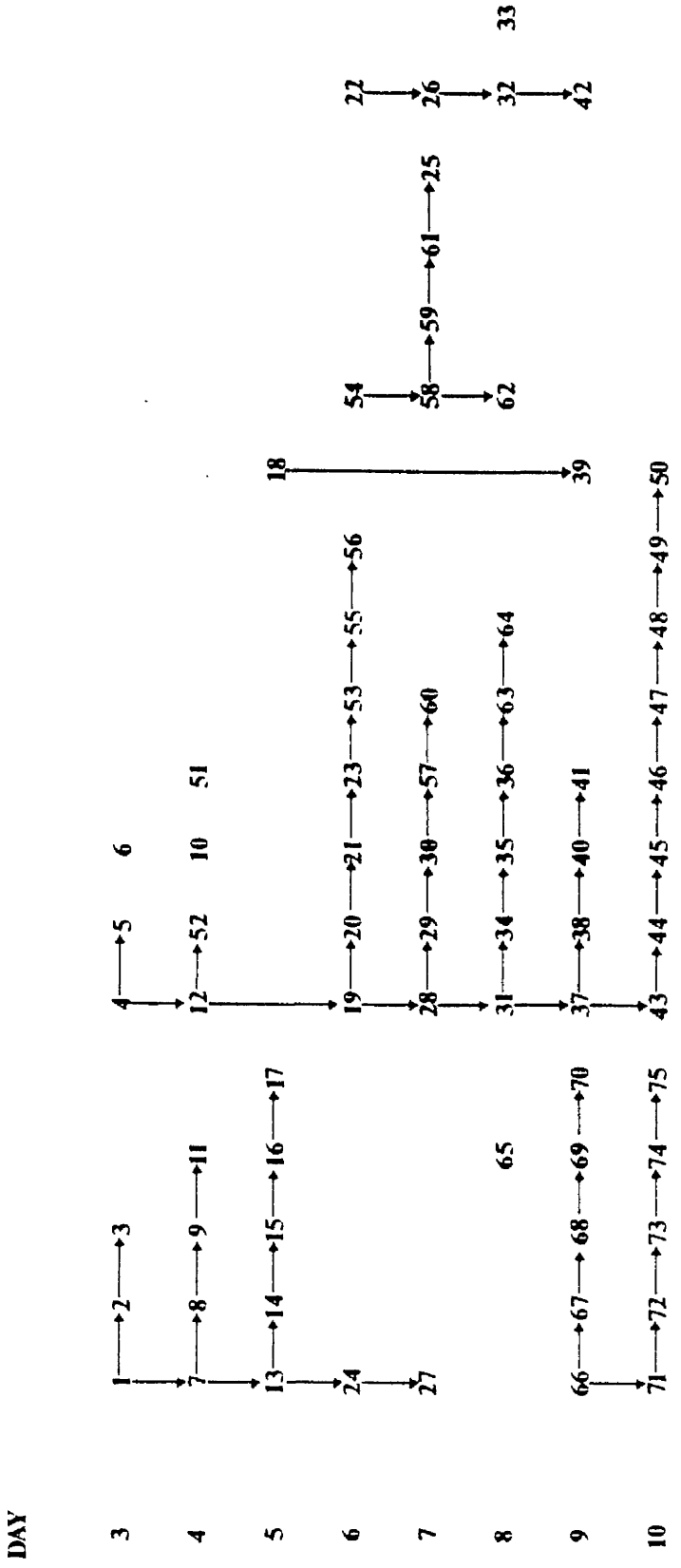

DAY
3
4
5
6
7
8
9
10
1 2 3
4 5 6
7 8 9 11
12 52 10 51
13 14 15 16 17
19 20 21 23 53 55 56
18 39
24
28 29 30 57 60
27
31 34 35 36 63 64
54 58 59 61 25
62
65
37 38 40 41
66 67 68 69 70
22 26 32 33 42
43 44 45 46 47 48 49 50
71 72 73 74 75

TABLE 11

Bacteria Isolated From Norwegian Turbot Larvae fed Copepods

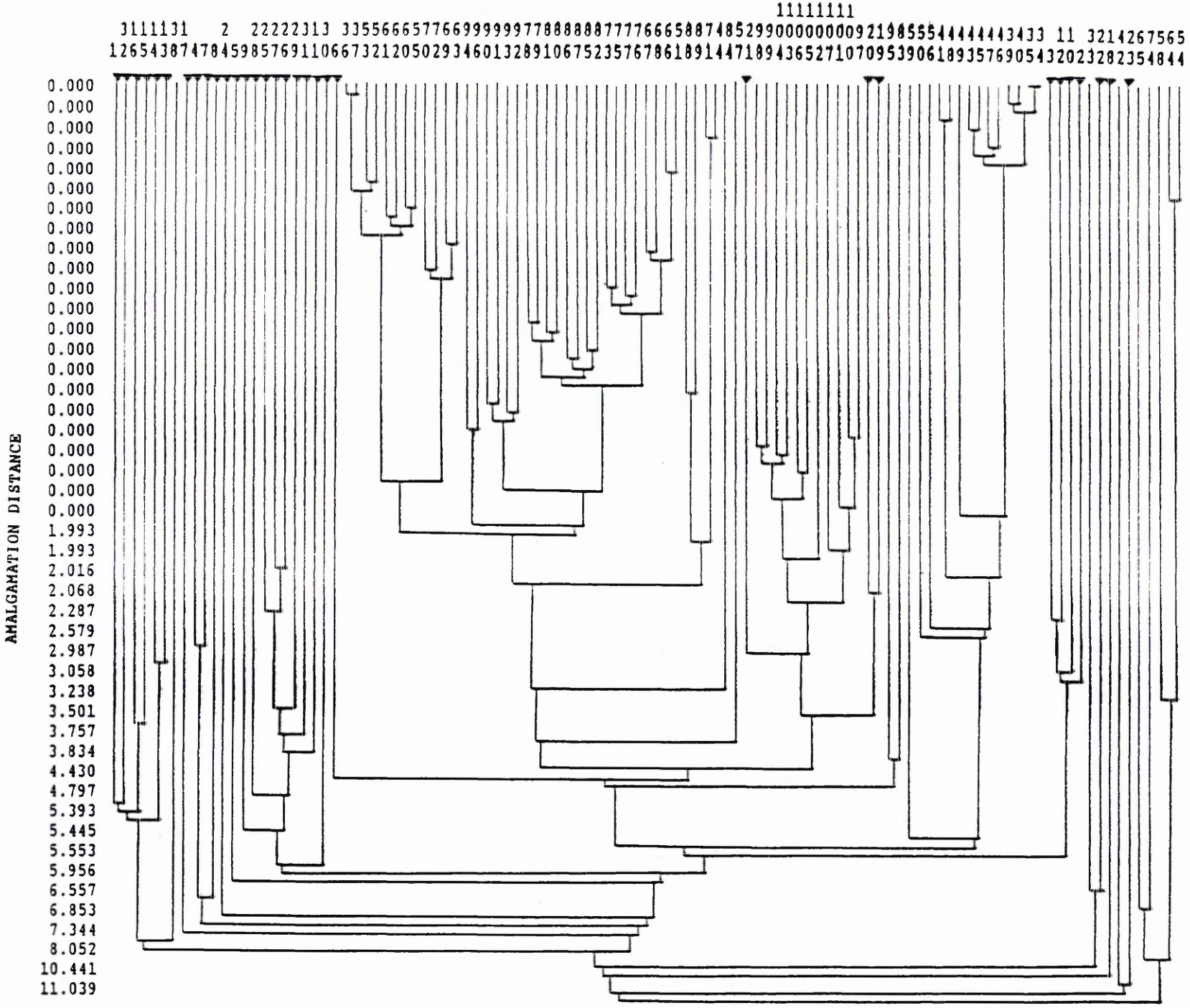
Identification	Number of Isolates
V. campbellii	13
Vibrio phenon 21	8
Vibrio phenon 36	1
Aeromonas sobria	2
Aeromonas species	2
V. pelagius	1
Unidentified vibrio	3
Pseudomonas/Alcaligenes	1
Photobacterium	1
Total	32

FIGURE 16

Cluster analysis of bacterial isolates from copepod-fed turbot larvae reared in Norway or Scotland.

▼	Norway
Unmarked	Scotland

Isolates grouped within an amalgamation distance of 0.000 were identical.


11111111
3111131 2 22222313 3355666577669999997788888877777666588748529900000000921985554444444343311 3214267565
1265438747845985769110667321205029346013289106752357678618914471894365271070953906189357690543202328235484 4
AMALGAMATION DISTANCE
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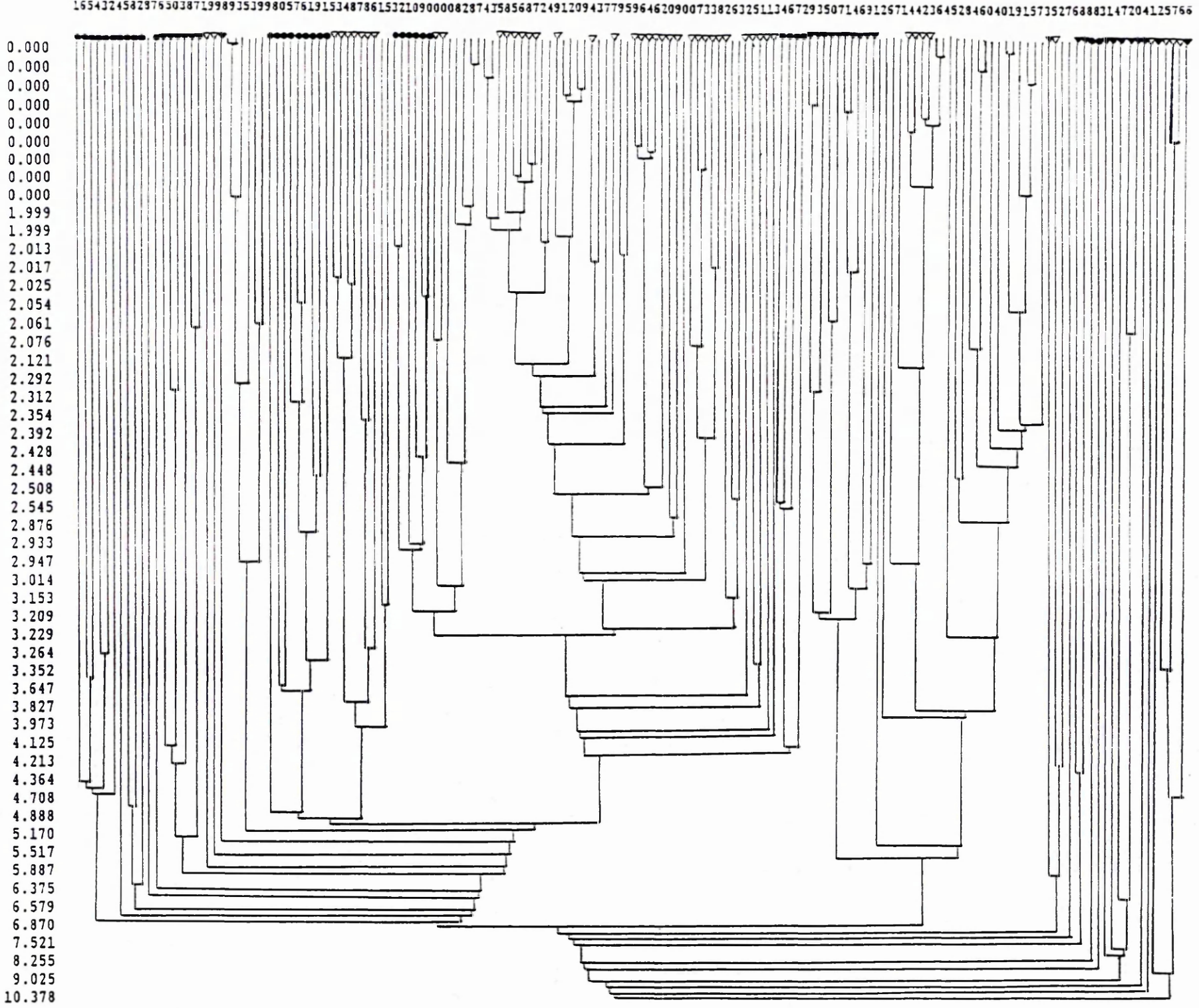
0.000
0.000
1.993
1.993
2.016
2.068
2.287
2.579
2.987
3.058
3.238
3.501
3.757
3.834
4.430
4.797
5.393
5.445
5.553
5.956
6.557
6.853
7.344
8.052
10.441
11.039

FIGURE 17

Cluster analysis of bacterial isolates from Norwegian, copepod-fed larvae and turbot larvae fed rotifers under intensive or extensive rearing conditions.

●	Norwegian, Copepod-fed Turbot Larvae
Unmarked	Intensively-reared, Rotifer-fed Turbot Larvae (Tank B1)
▼	Extensively-reared Turbot Larvae fed Rotifers from an Intensive Source (Tank B2)
▽	Extensively-reared, Rotifer-fed Turbot Larvae (Tank B10)

Isolates grouped within an amalgamation distance of 0.000 were identical.


AMALGAMATION DISTANCE
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
0.000
1.999
1.999
2.013
2.017
2.025
2.054
2.061
2.076
2.121
2.292
2.312
2.354
2.392
2.428
2.448
2.508
2.545
2.876
2.933
2.947
3.014
3.153
3.209
3.229
3.264
3.352
3.647
3.827
3.973
4.125
4.213
4.364
4.708
4.888
5.170
5.517
5.887
6.375
6.579
6.870
7.521
8.255
9.025
10.378

or enzyme.

One of the dominant bacteria isolated, identified as *V. alginolyticus* (3-8), from the gut of healthy intensively-reared larvae fed rotifers, was used to inoculate rotifers (1 x 10^6 c.f.u. per ml) which were subsequently fed to larvae. A streptomycin-resistant derivative of this isolate (3-8sr), which was stable for over 48 hours in seawater, although not in sodium chloride solution, was also used (Figure 18).

The number of bacteria per fish increased 100-fold one hour after feeding. However, this level decreased over the following 24 hours (Figure 19), indicating that the bacteria did not survive or did not stably colonise the gut. The level of streptomycin-resistant bacteria detected in larvae given rotifers inoculated with 3-8sr, was extremely low at approximately 1% of the total bacteria (Figure 19). This level could be accounted for by naturally occurring streptomycin-resistant strains since control samples, from larvae given rotifers inoculated with isolate 3-8, plated onto agar containing streptomycin, gave similar results. This experiment was repeated twice using isolates 3-8 and 3-8sr, and once with a pigmented isolate (NT23) from Norwegian turbot larvae. In each case, few or no bacteria similar to those inoculated were detected in turbot larvae.

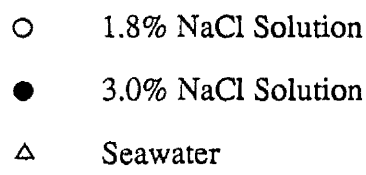
3.9 Colonisation of Axenic Rotifers with Defined Bacteria

A beneficial microflora might be introduced into turbot larvae via rotifers since it was previously shown that their diet was the source of the gut flora of larval turbot. Axenic rotifer cultures were produced in a chemostat in order to determine the rate of uptake of bacteria by rotifers, and also, whether bacteria were accummulated differentially, or taken up at the same rate regardless of species.

In the first colonisation experiments, the rotifers were washed free of algae and concentrated to approximately 100 rotifers per ml. Bacteria, harvested from overnight cultures and rinsed, were added to give a final concentration of 1 x 10^6 c.f.u. per ml. The rotifers were rinsed with benzalkonium chloride solution followed by sterile seawater before homogenisation. No accummulation of bacteria by rotifers was

FIGURE 18

Viability of streptomycin-resistant *Vibrio alginolyticus* (3-8sr).



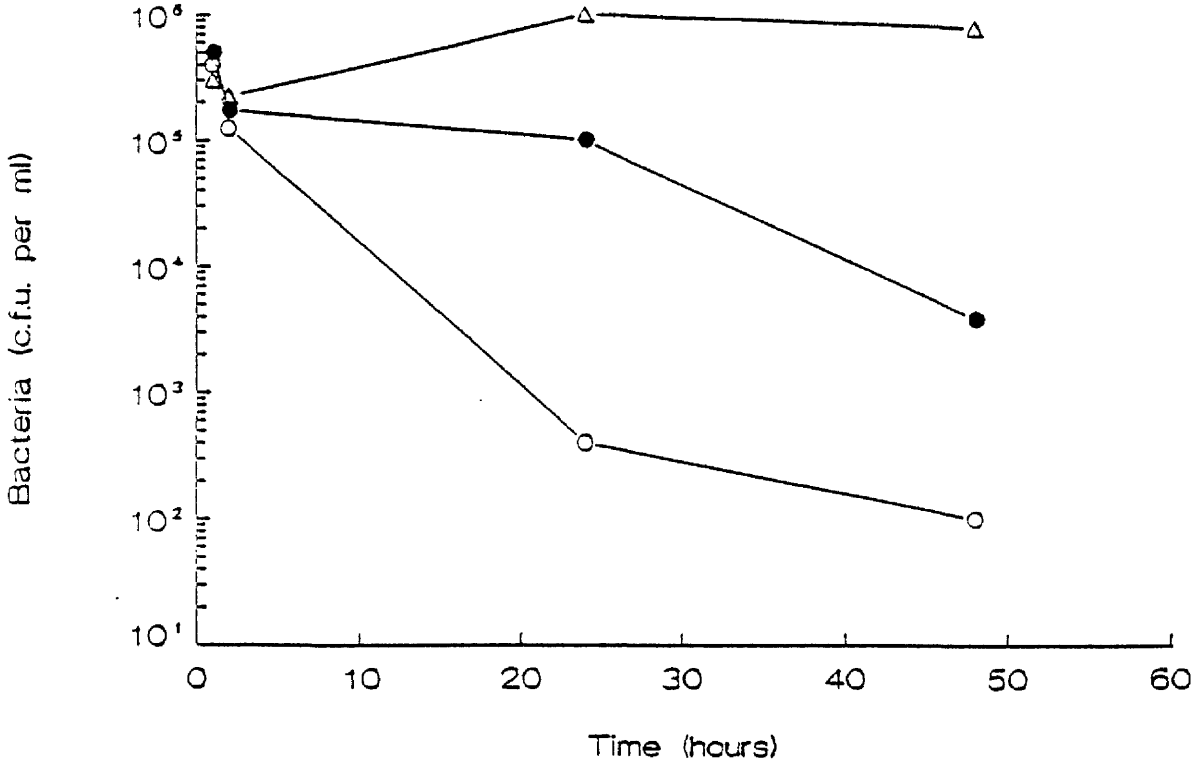
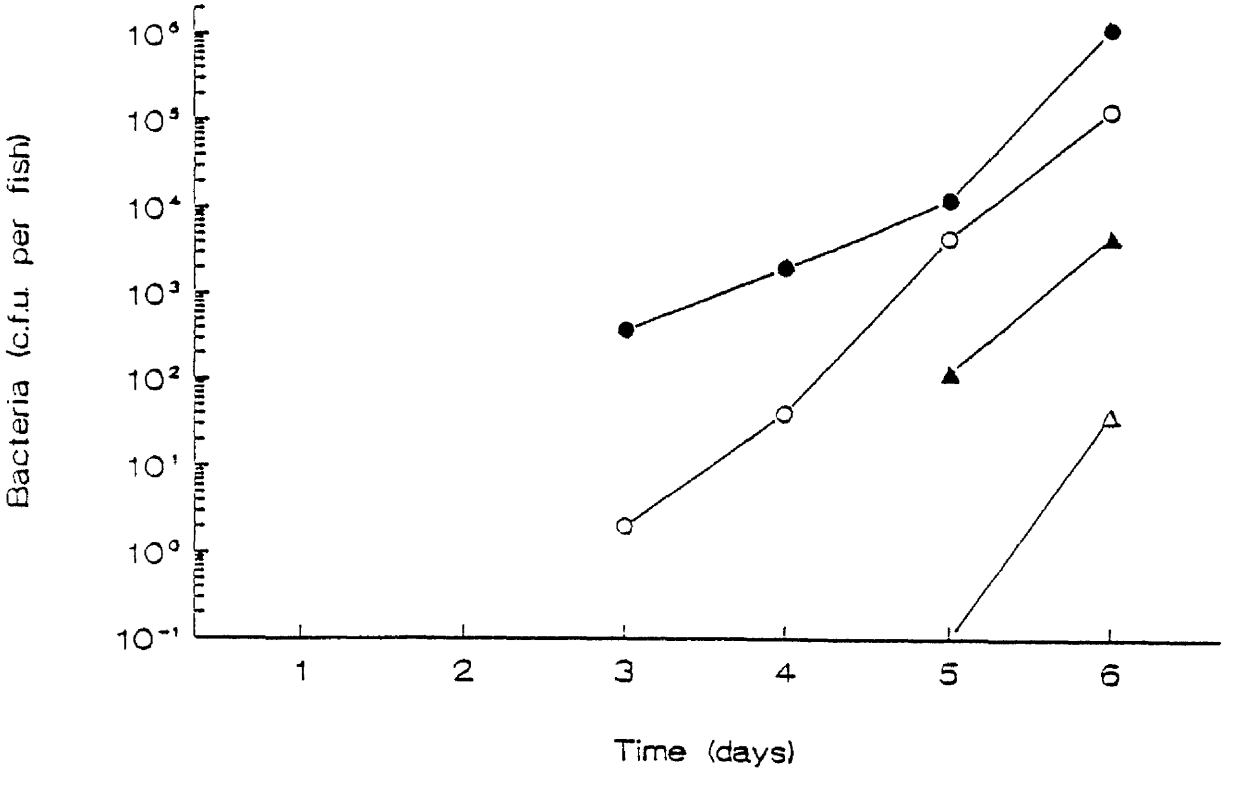

Bacteria (c.f.u. per ml)
10⁶
10⁵
10⁴
10³
10²
10¹
0
10
20
30
40
50
60
Time (hours)

FIGURE 19

Gut bacteria from turbot larvae fed rotifers inoculated with *Vibrio alginolyticus* (3-8) or a streptomycin-resistant derivative (3-8sr).

	Before Feeding	One Hour After Feeding
Larvae fed Rotifers Inoculated with Isolate 3-8	○	●
Larvae fed Rotifers Inoculated with Isolate 3-8sr	△	▲


Bacteria (c.f.u. per fish)
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
10⁻¹
1
2
3
4
5
6
Time (days)

detected within the 6 hour period of these experiments. However, when an axenic culture of algae (*Pavlova lutheri*) was added to the rotifer suspensions and the benzalkonium chloride rinse was omitted, uptake of bacteria was recorded. The uptake of isolate 3-8, from intensively-reared larvae fed rotifers, is shown in figure 20. Although the rotifers were sterile initially, the levels of bacteria measured immediately after addition of the test bacteria were $>10^2$ c.f.u. per rotifer. The rotifers accummulated bacteria rapidly with 700 c.f.u. per rotifer detected after two hours. However, the rate of accummulation decreased thereafter with only 1,000 c.f.u. per rotifer detected after 24 hours. The clearance rate by rotifers was calculated from the rate of uptake over the first four hours, and assuming complete retention of the bacteria during filter feeding, to be 0.044 µl per minute per rotifer. *Cf.* Starkweather and Gilbert, 1977).

A range of bacteria, including NT9 (*V. campbellii*), NT29 (*Vibrio* phenon 21) and NT7 (*Vibrio* phenon 36) from Norwegian turbot larvae, 3-8 (*V. alginolyticus*) from larvae fed rotifers, and *Pseudomonas* 1.1.1., were used to inoculate axenic rotifer cultures. To differentiate between the intestinal bacteria and the bacteria adhering to the surface, the rotifers were rinsed with benzalkonium chloride solution followed by sterile seawater, or sterile seawater alone. The results of inoculation with *Pseudomonas* 1.1.1., NT7 and NT9 are shown in figure 21. An average of 324 bacteria per rotifer were adsorbed/ingested by rotifers two hours after inoculation, whereas the average number of bacteria ingested, i.e. resistant to benzalkonium chloride, was only 24 per rotifer in the same period. This suggested that the bacteria associated with rotifers were mainly adhering to the surface.

3.10 Benzalkonium Chloride Treatment of Rotifers

From the work of Muroga *et al.* (1987), it was considered that benzalkonium chloride removed the surface flora of fish, and this treatment was used in this project for sterile rotifers inoculated with bacteria to determine the number of bacteria ingested over various times. Most of the bacteria associated with these rotifers were removed by rinsing with benzalkonium chloride solution. The effect of benzalkonium

FIGURE 20

Uptake of *Vibrio alginolyticus* (3-8) by rotifers.

- ○ Rotifers
- ● Rotifer Culture Water

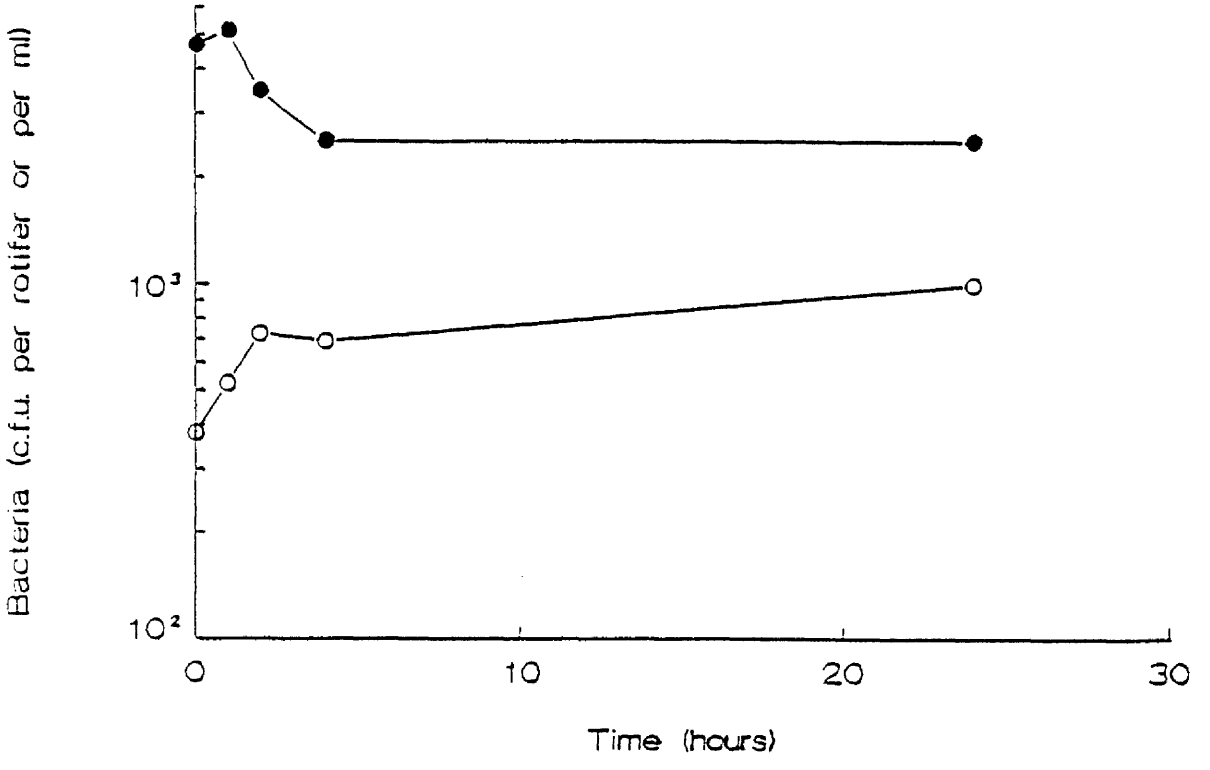
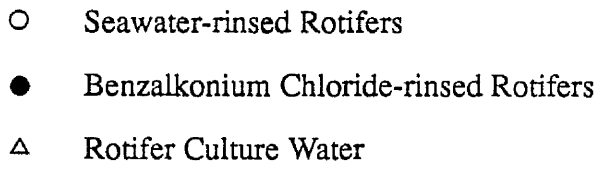

Bacteria (c.f.u. per rotifer or per ml)
10³
10²
0
10
20
30
Time (hours)

FIGURE 21

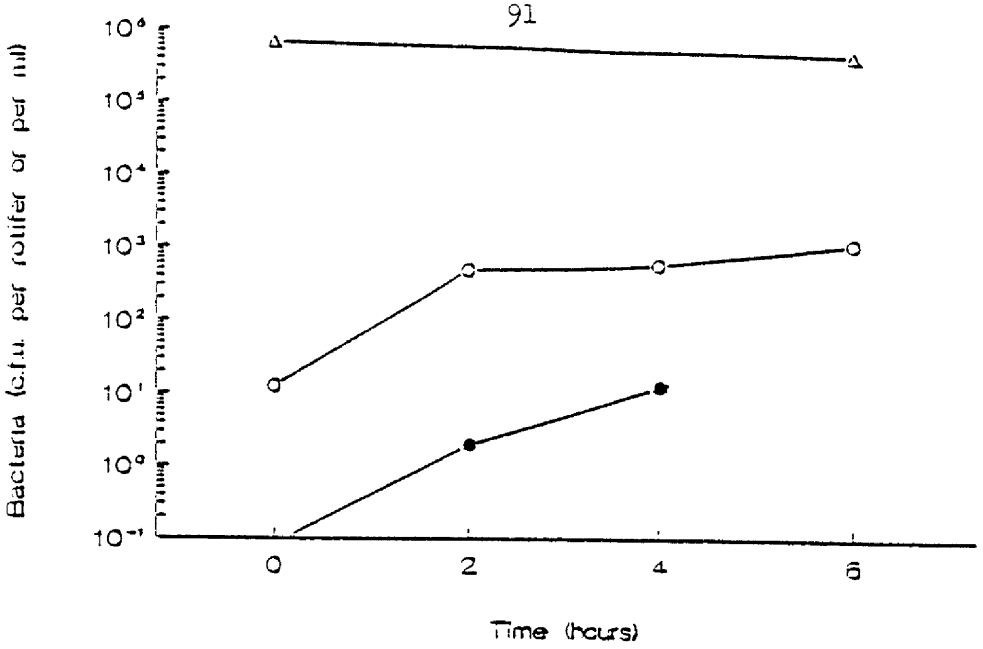
a) **Uptake of *Pseudomonas* 1.1.1. by rotifers.**

b) **Uptake of *Vibrio* phenon 21 by rotifers.**

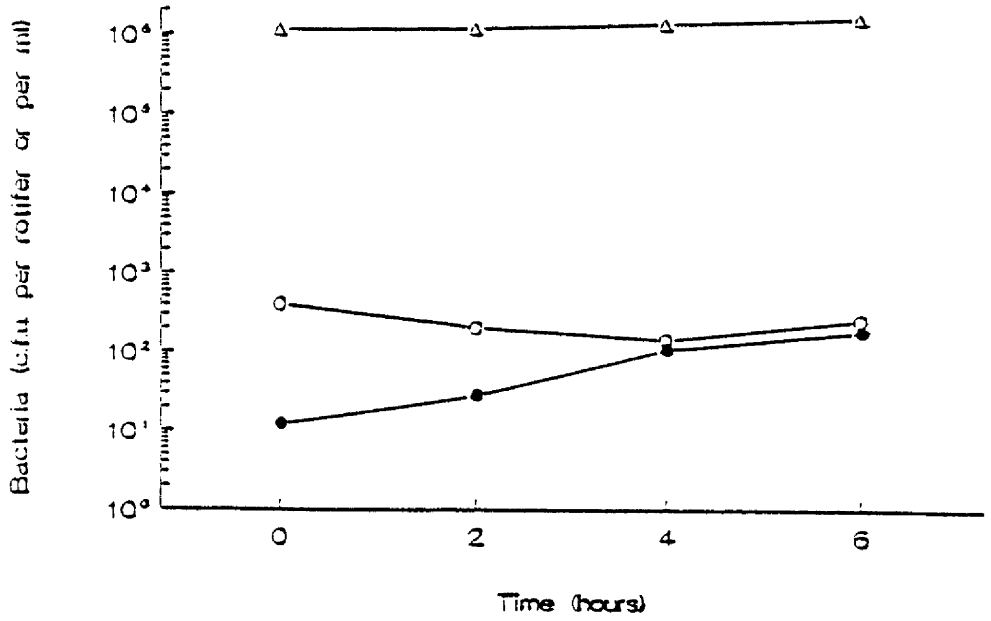
c) **Uptake of *Vibrio campbellii* by rotifers.**



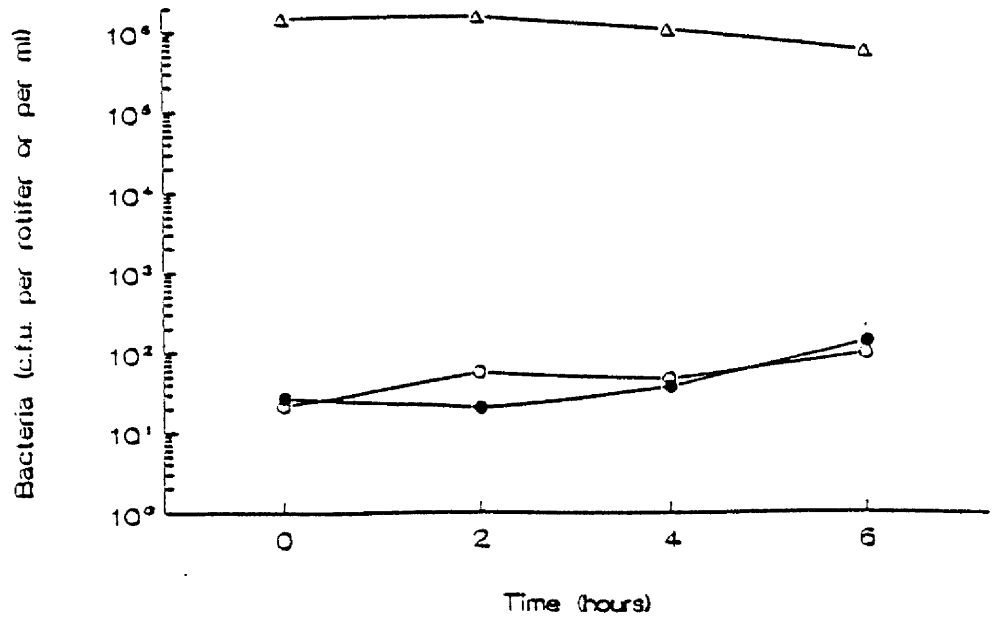
a)


Bacteria (c.f.u. per rotifer or per ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
0
2
4
6
Time (hours)

b)


Bacteria (c.f.u. per rotifer or per ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
Time (hours)

c)


Bacteria (c.f.u. per rotifer or per ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
0
2
4
6
Time (hours)

chloride treatment for various times, on rotifers from intensive, contaminated cultures, is shown in figure 22. Rotifers exposed to benzalkonium chloride solution for 30 seconds before rinsing with sterile seawater had 60% lower bacterial counts than those briefly exposed (time 0).

3.11 Scanning Electron Microscopy of Rotifers

Scanning electron microscopy of rotifers from intensive, contaminated cultures reared at G.S.P., was undertaken to confirm that the microflora was mainly external and was removed by rinsing with benzalkonium chloride solution. Rotifer samples were either rinsed with benzalkonium chloride solution for 30 seconds followed by sterile seawater, rinsed with sterile seawater alone, or untreated before processing for scanning electron microscopy.

Bacteria were clearly visible at medium magnification (x1,600) (Figure 23). Rinsing with sterile seawater alone reduced the bacterial load of rotifers by 65%, and benzalkonium chloride treatment reduced the surface bacteria by 95% compared to untreated rotifers, and by 85% compared to seawater-rinsed rotifers (Table 12). The methanol used to narcotize the rotifers did not appear to affect the number

methanol used to narcotize the rotifers did not appear to affect the number

of bacteria per rotifer viewed by S.E.M.

3.12 Decontamination of Intensively Reared Rotifers

From the results of earlier experiments (see "Comparison of the gut flora of intensively- and extensively-reared turbot larvae"), it was evident that a slow rate of colonisation of the gut of turbot larvae gave increased survival and growth rates and, since the gut microflora was shown to originate from the diet, it was likely that reducing the bacterial load of rotifers would be beneficial to larval turbot.

Cooling and Starvation of Rotifers

The procedure of cooling and starvation for 3-5 days was reputed to substantially decrease the bacterial loading of rotifers (Vadstein O., Skjermo F., Oie G., and Olsen Y., unpublished results). Three experiments were conducted in which rotifers cultured at G.S.P. at 20°C, were cooled to 7°C or 14°C and starved for up to 11 days. The

FIGURE 22

Effect of benzalkonium chloride on the bacterial load of rotifers.

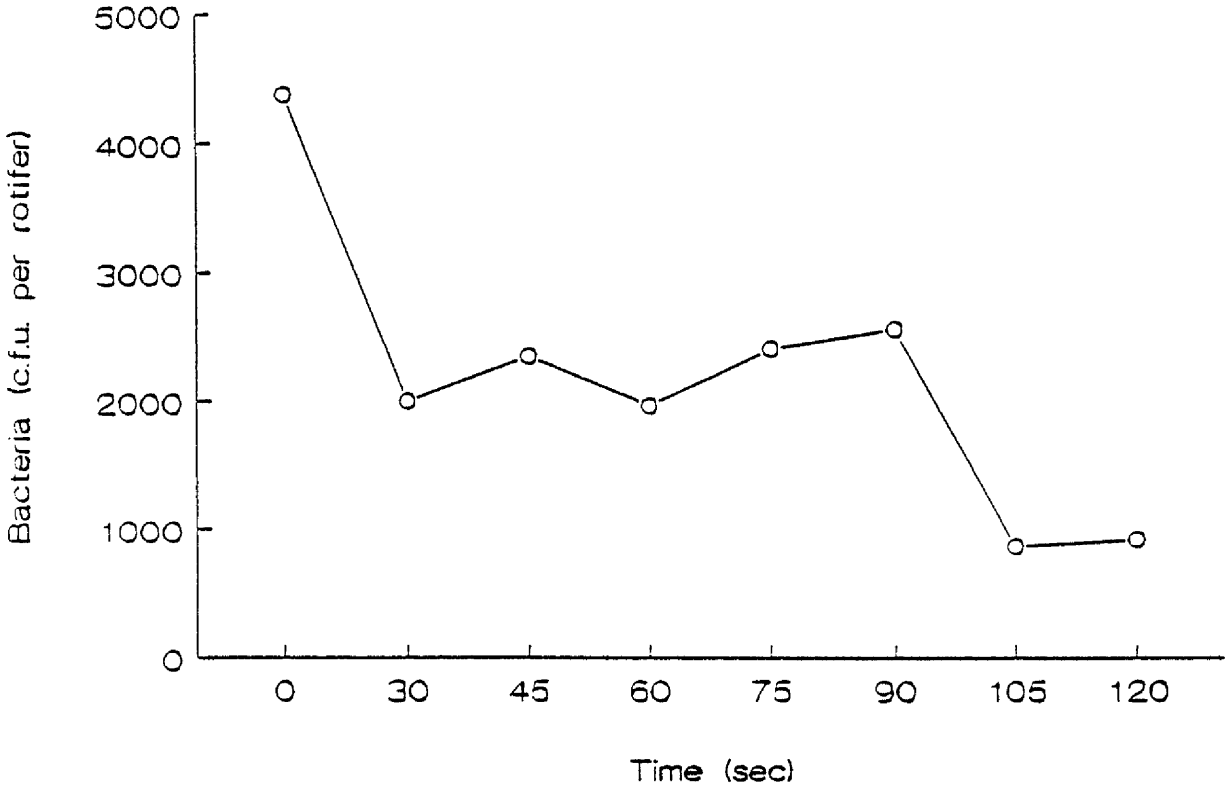

5000
4000
3000
2000
1000
0
Bacteria (c.f.u. per rotifer)
0
30
45
60
75
90
105
120
Time (sec)

FIGURE 23

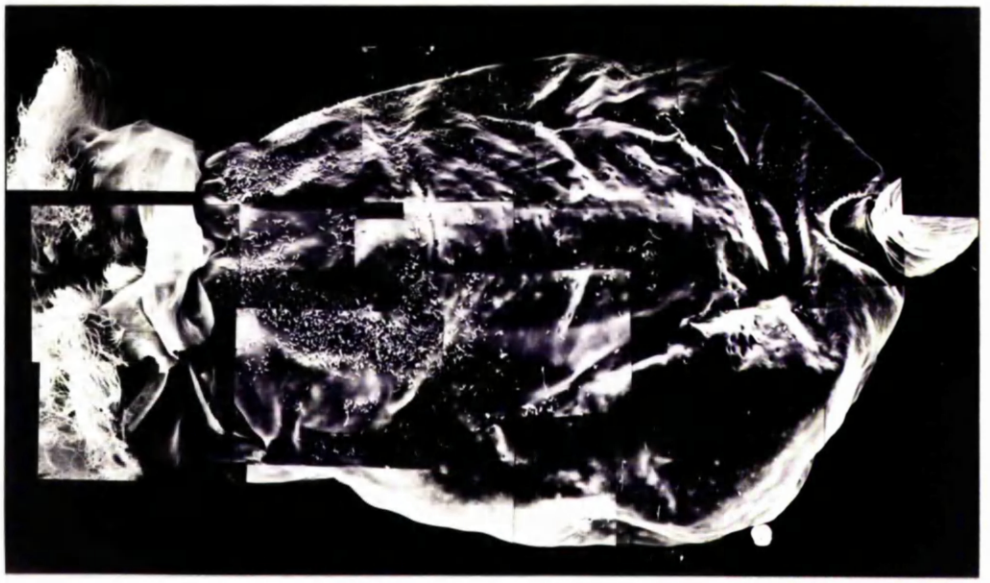
a) **Scanning electron micrograph of an untreated rotifer.**

b) **Scanning electron micrograph of a rotifer rinsed with benzalkonium chloride.**

c) **Scanning electron micrograph of a rotifer rinsed with sterile seawater.**

Magnification x 1,600.

a)



b)

c)

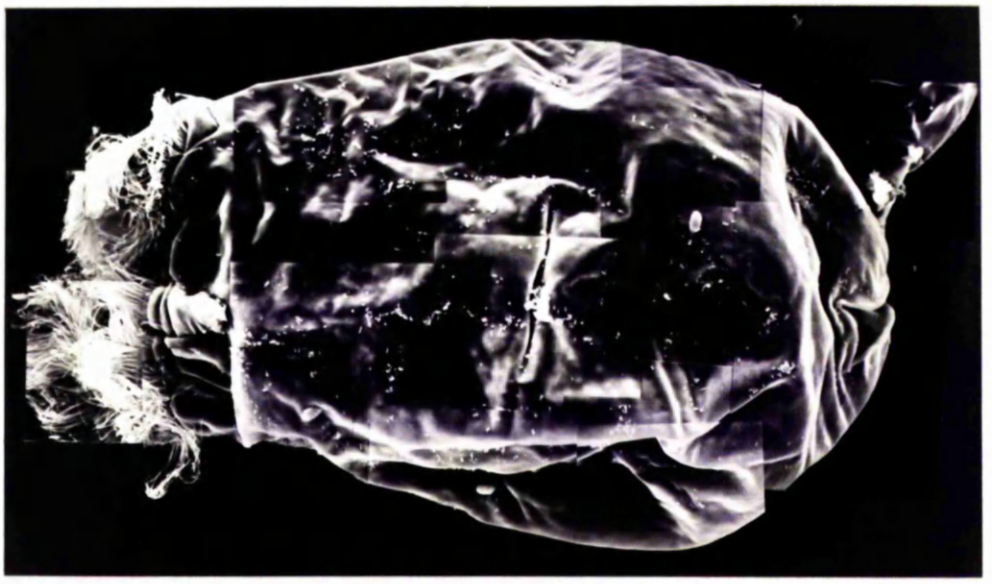


TABLE 12

Surface Bacteria per Rotifer Established by Scanning Electron Microscopy

Treatment	Bacteria per rotifer
Benzalkonium chloride rinsed	400
Seawater rinsed	2,962
Control	8,454

Counts were done by eye and were for single rotifers which were representative of the total population. Actual counts were doubled, assuming that an equal number of bacteria were on the reverse side of each rotifer.

concentration of rotifers decreased dramatically within 24 hours (Figure 24) due to death or settlement.

At 7°C the level of bacteria in both the culture water and the rotifers remained relatively constant. However, the rotifers did not survive and the experiment was concluded after 7 days.

At 14°C the bacterial levels increased in the culture water and also in the rotifers after day 3 or 4 (Figure 25). The rotifers did not survive beyond day 11. It was evident that the strain of rotifers cultured at Hunterston was not as cold-tolerant as those described by Vadstein et al.

Identification of 25 representative bacterial isolates from rotifers before cooling, and 21 isolates from rotifers cooled to 14°C for 11 days, revealed a change in the bacterial flora with a significant decrease in the number of vibrios after cooling ($p < 1$, X^2 test) (Table 13).

3.13 Effect of Potential Rotifer Cleansing Agents

Since attempts to reduce the number of rotifer-associated bacteria by cooling and starvation of rotifers were unsuccessful and the use of antibiotics was considered undesirable due to the possible selection of antibiotic-resistant bacteria, a range of alternative chemical agents were investigated for their cleansing properties.

Benzalkonium Chloride

The response of rotifers to a range of concentrations of benzalkonium chloride, from 0.1-0.001%, was observed, since its effectiveness in removing bacteria adhering to the rotifer surface was demonstrated previously. Less than 50% of the rotifers exposed to 0.001% benzalkonium chloride for one minute were still alive after 30 minutes in fresh seawater (Table 14).

Detergents

The detergents Tween 20, Tween 80 and Triton X-100 were examined for their effect on rotifers and their bactericidal action. Rotifers expelled their gut contents in

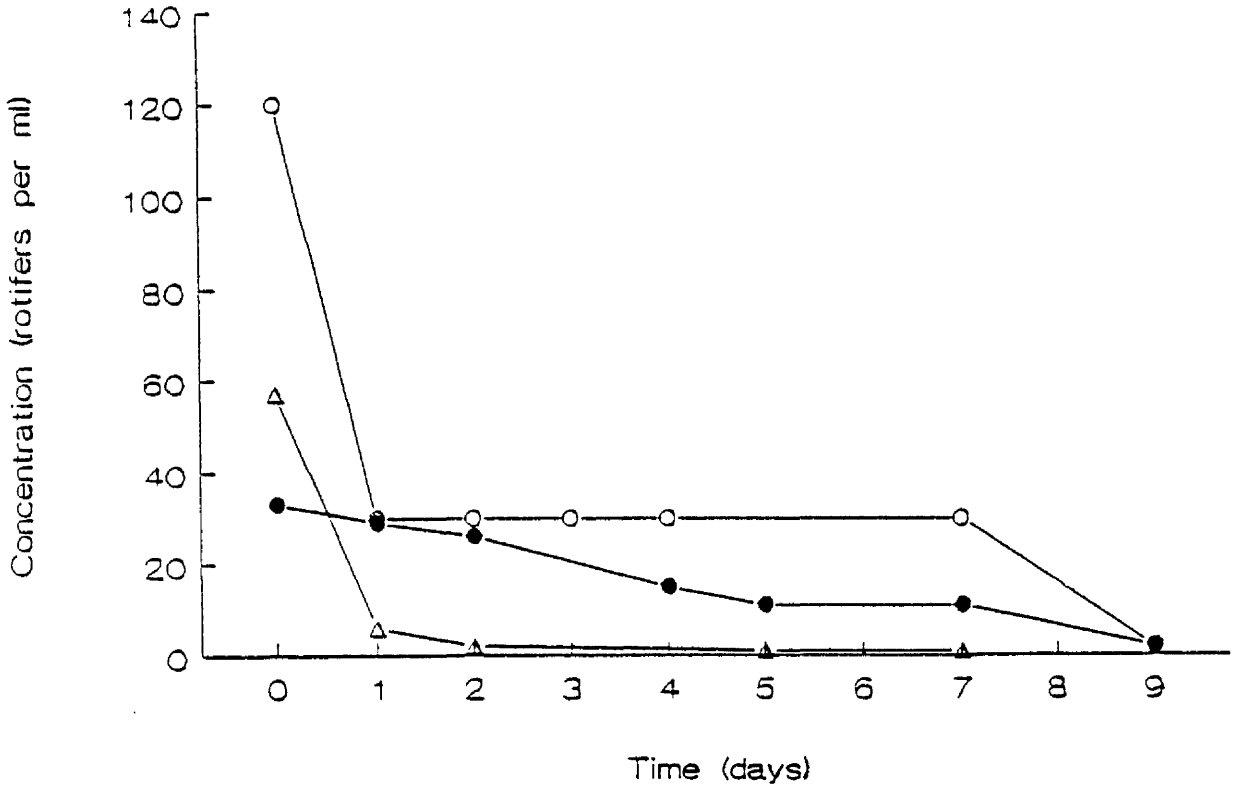

Concentration (rotifers per ml)
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Time (days)

FIGURE 25

Bacteria associated with cooled rotifer cultures.

- ○ Benzalkonium Chloride-rinsed Rotifers
- ● Seawater-rinsed Rotifers
- △ Rotifer Culture Water

The rotifer culture was cooled to 14°C.

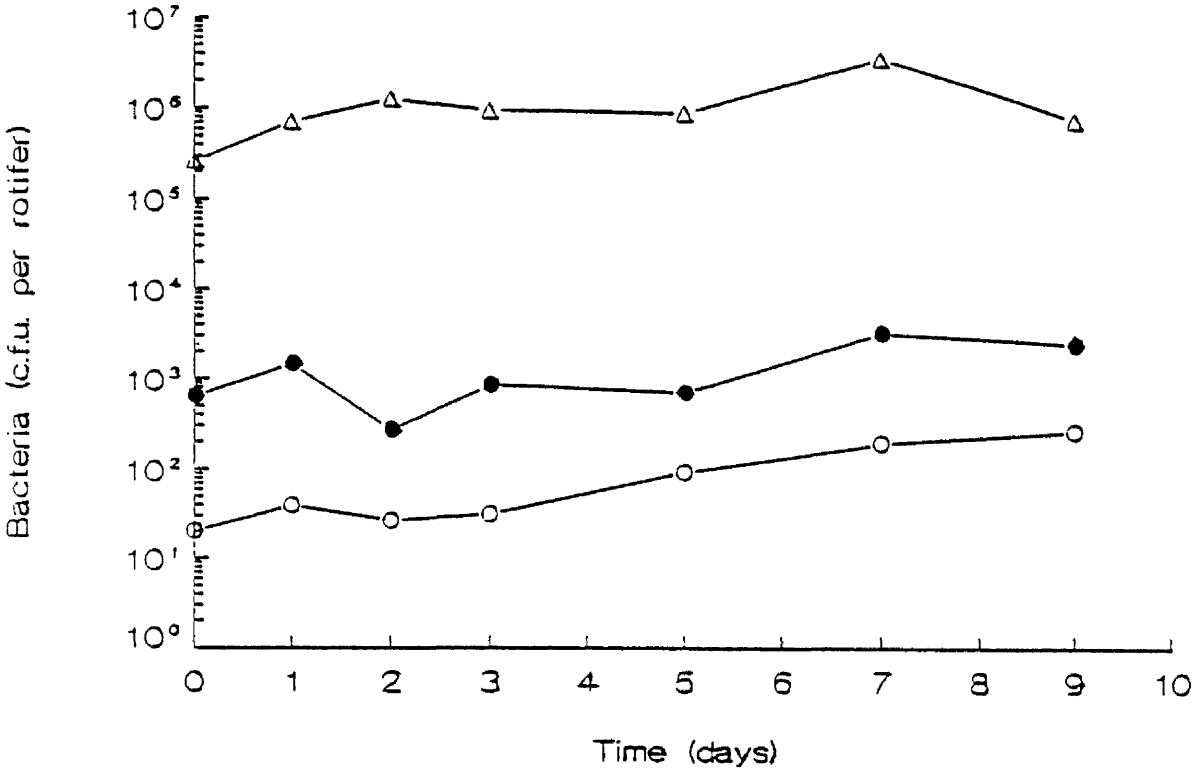

Bacteria (c.f.u. per rotifer)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
0
1
2
3
4
5
6
7
8
9
10
Time (days)

TABLE 13

Bacteria Isolated From Rotifers Before and After Cooling to 14°C for 11 Days

Identification	Number of Isolates	
	Before	After
Cytophaga/Flavobacterium	2	4
Pseudomonas/Alcaligenes	0	5
Pseudomonas/Moraxella	9	5
Aeromonas	3	4
V. campbellii	2	0
V. costicola	0	1
V. natrigens	6	2
V. fluvialis	2	0
Vibrio phenon 21	1	0
Total	25	21
Percentage vibrios	44	14

TABLE 14

Effect of Benzalkonium Chloride on Rotifers

Benzalkonium chloride (%)	Exposure time (min.)	Effect on rotifers	Recovery after 30 min. in fresh seawater
0.1	1	lethal	
0.05	1	lethal	
0.01	1	alive, distressed	died within 30 min.
0.01	5	lethal	
0.005	1	alive, swimming	<50% recovered
0.005	3	alive, not swimming	<50% recovered
0.005	5	alive, not swimming	died within 30 min.
0.005	15	lethal	
0.001	1	alive, swimming	<50% recovered
0.001	5	alive, some swimming	<50% recovered
0.001	20	alive, not swimming	died within 30 min.

5% Tween 20 but were unaffected by 1% Tween 20 for 15 minutes. A similar result was obtained with Tween 80. Triton X-100 was highly toxic to rotifers at concetrations above 0.001%. Effective reduction of the bacterial load of rotifers was not obtained with a 15 minute exposure to 1% Tween 20, 1% Tween 80 or 0.001% Triton X-100 (Figure 26).

Phylatol

The broad spectrum biocide, Phylatol, was chosen as a potential rotifer-cleansing agent since it was recommended as having low toxicity to fish. The effect of a range of concentrations of Phylatol in seawater on rotifers was observed, and their survival in fresh seawater after various exposure times was monitored. A concentration of 0.2% Phylatol was lethal to rotifers in under 2 minutes. Rotifers were distressed and expelled their gut contents, but survived an exposure time of 15 minutes in 0.02% Phylatol. At 0.01%, the lowest concentration tested, the rotifers expelled their gut contents immediately but survived 20 minutes exposure. The bactericidal activity of 0.01% Phylatol on two *Vibrio* species, *V. alginolyticus* (3-8) and *V. anguillarum* (3-15), isolated from turbot larvae, was negligible (Figure 27a).

Panacide

Panacide, another broad spectrum biocide, has a wide range of applications in industry, however, it was extremely toxic to rotifers. A concentration of 0.002% was lethal after only 2 minutes exposure. Some rotifers survived a 5 minute exposure to 0.0016% Panacide but this concentration had little bactericidal effect on isolates 3-8 and 3-15 within 15 minutes (Figure 27b).

Lysozyme

Hen-egg-white lysozyme was chosen as a potential decontaminating agent because of its well-characterised anti-bacterial properties and its synergistic action with other factors such as detergents and EDTA. Several experiments were carried out

FIGURE 26

Effect of detergents on the bacterial loading of rotifers.

- ○ Tween 20
- ● Tween 80
- △ Triton X-100

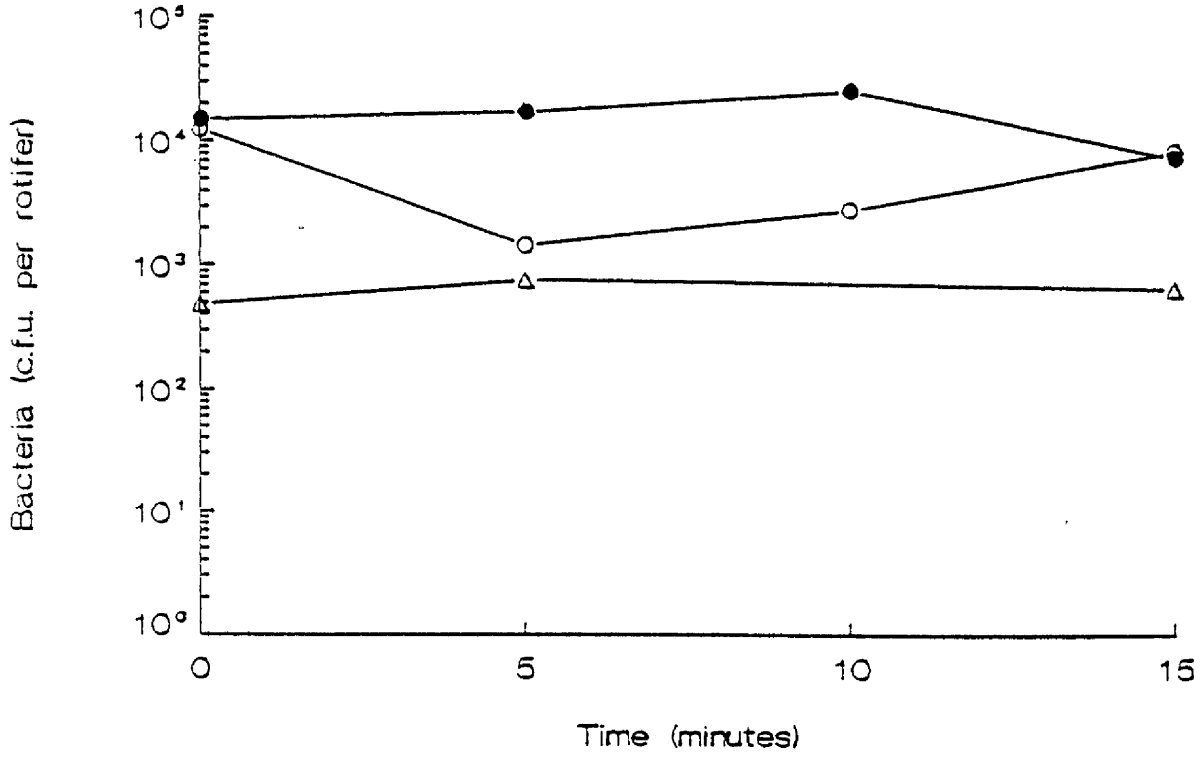

Bacteria (c.f.u. per rotifer)
10⁵
10⁴
10³
10²
10¹
10⁰
0
5
10
15
Time (minutes)

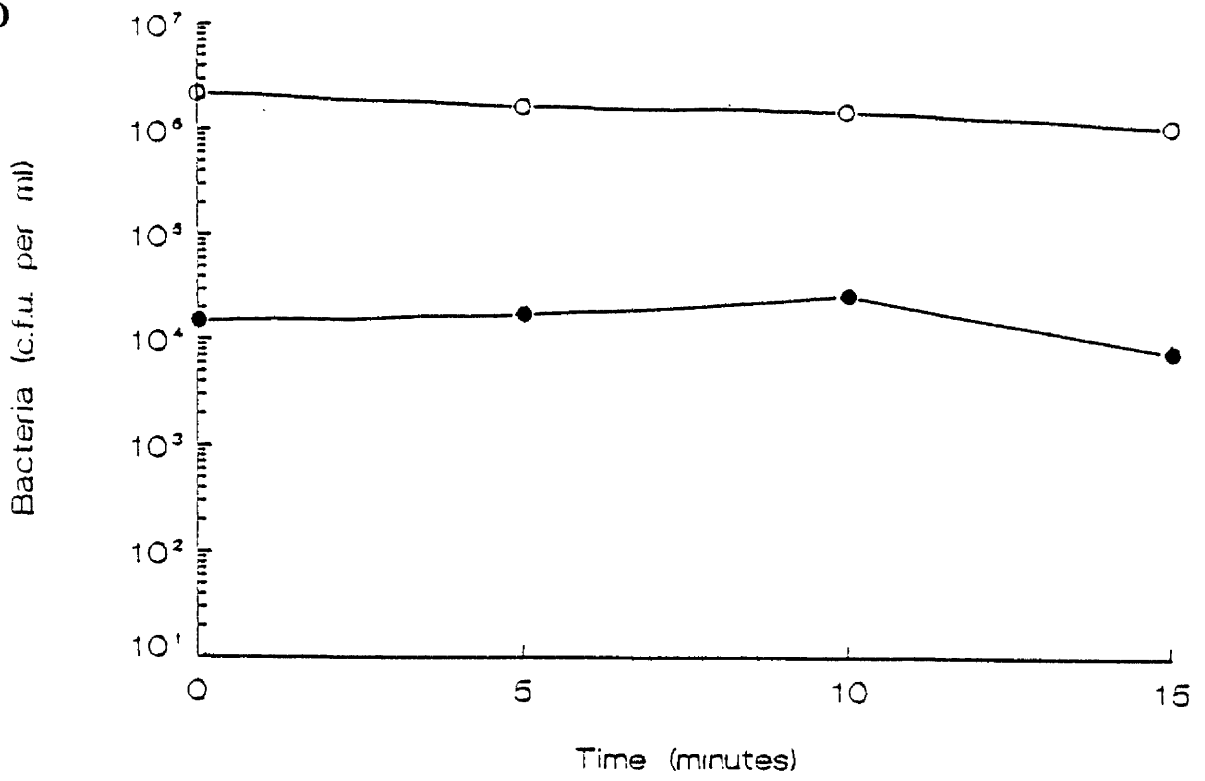
FIGURE 27

a) **Susceptibility of bacteria to Phylatol.**

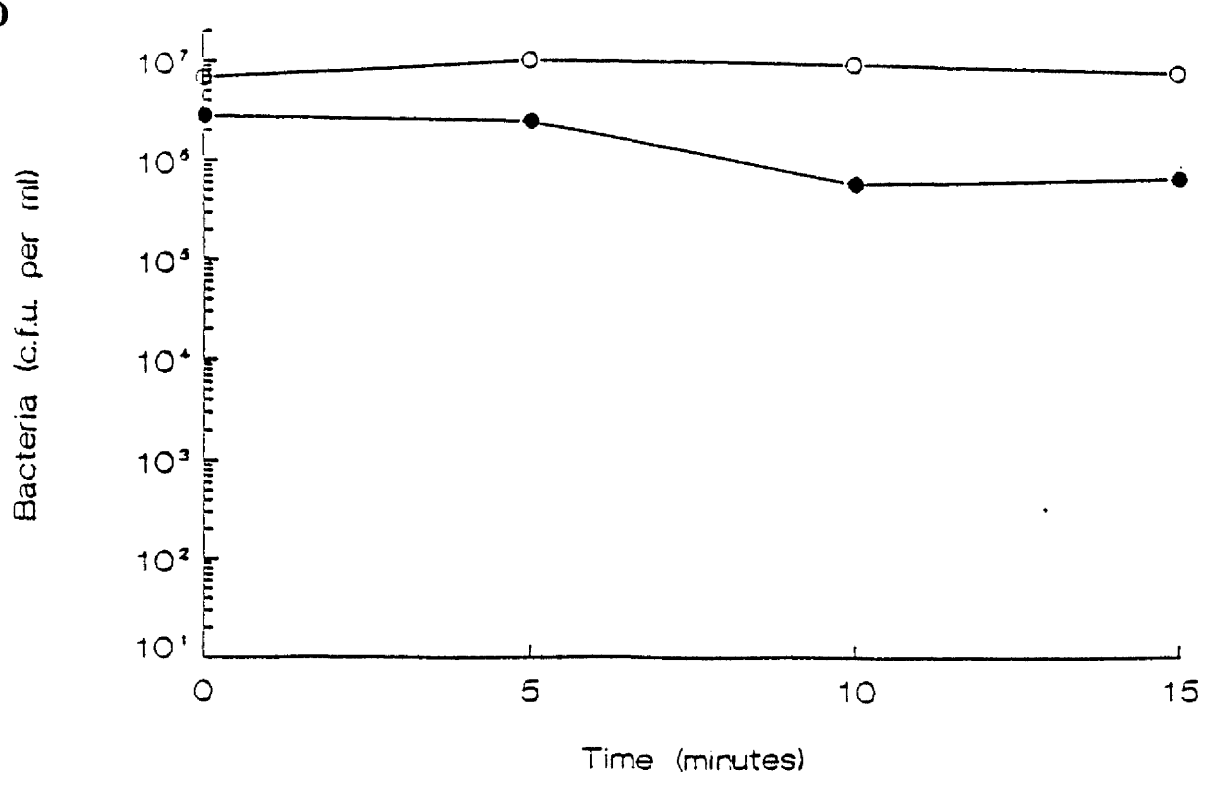
b) **Susceptibility of bacteria to Panacide.**

- ○ *Vibrio alginolyticus* (3-8)
- ● *Vibrio anguillarum* (3-15)

a)


Bacteria (c.f.u. per ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
5
10
15
Time (minutes)

b)


Bacteria (c.f.u. per ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
0
5
10
15
Time (minutes)

using freeze-dried *Micrococcus lysodeikticus* cells under various conditions to check the activity of the lysozyme. A concentration of 0.5mg per ml of lysozyme was used throughout. Seawater and distilled water inactivated lysozyme whereas, seawater diluted to 0.4%, 0.2% and 0.1% salinity gave enzyme activities of 57, 62 and 59 enzyme units compared to 78 enzyme units under standard conditions with 0.06M sodium phosphate buffer, pH 7.2.

M. lysodeikticus cells were also used to investigate the possible potentiating effects of EDTA and the detergent Tween 80 on lysozyme. EDTA at a concentration of 0.01mM, or 1% Tween 80 alone, had no effect on *M. lysodeikticus*. The activities of lysozyme, lysozyme plus 0.01mM EDTA, and lysozyme plus 1% Tween 80, were 86, 92 and 91 enzyme units, respectively. However, the activity of lysozyme plus 0.01mM EDTA and 1% Tween 80 was enhanced with 136 enzyme units detected.

The activity of lysozyme against some Gram-negative, marine bacteria was also investigated. Increasing salinity decreased the effect of lysozyme (1.0mg per ml) against *Pseudomonas* 1.1.1. (Figure 28). The "resistant" fraction of *Pseudomonas* 1.1.1. was cultured and was equally susceptible to lysozyme. Tween 80 alone had no effect on *Pseudomonas* 1.1.1. and it did not enhance the activity of lysozyme (Figure 29). EDTA (0.01mM) appeared to enhance the activity of lysozyme against *V. alginolyticus* (3-8), although in the presence of Tween 80 no increase in activity was observed with EDTA (Figure 30).

Increasing the concentration did not increase the bactericidal activity of lysozyme alone against *Pseudomonas* 1.1.1. (Figure 31a). Concentrations of EDTA above 0.1mM did increase the effect of lysozyme (Figure 31b), however, high concentrations of EDTA alone were lethal to *Pseudomonas* 1.1.1. (Figure 31c).

Effect of Lysozyme on Bacterial Loading of Rotifers

The effect of low salinity and lysozyme on the bacteria load of rotifers is shown in figure 32. A salinity of 0.2% alone reduced the bacterial load by 50% after 15 minutes exposure and even very briefly (time 0), exposure to lysozyme (1.0mg per ml) reduced the bacterial load

FIGURE 28

Effect of salinity on bactericidal action of lysozyme against *Pseudomonas* 1.1.1..

Exposure Time

○ 0 Minutes

● 5 Minutes

△ 10 Minutes

A concentration of lysozyme of 1.0mg per ml was used.

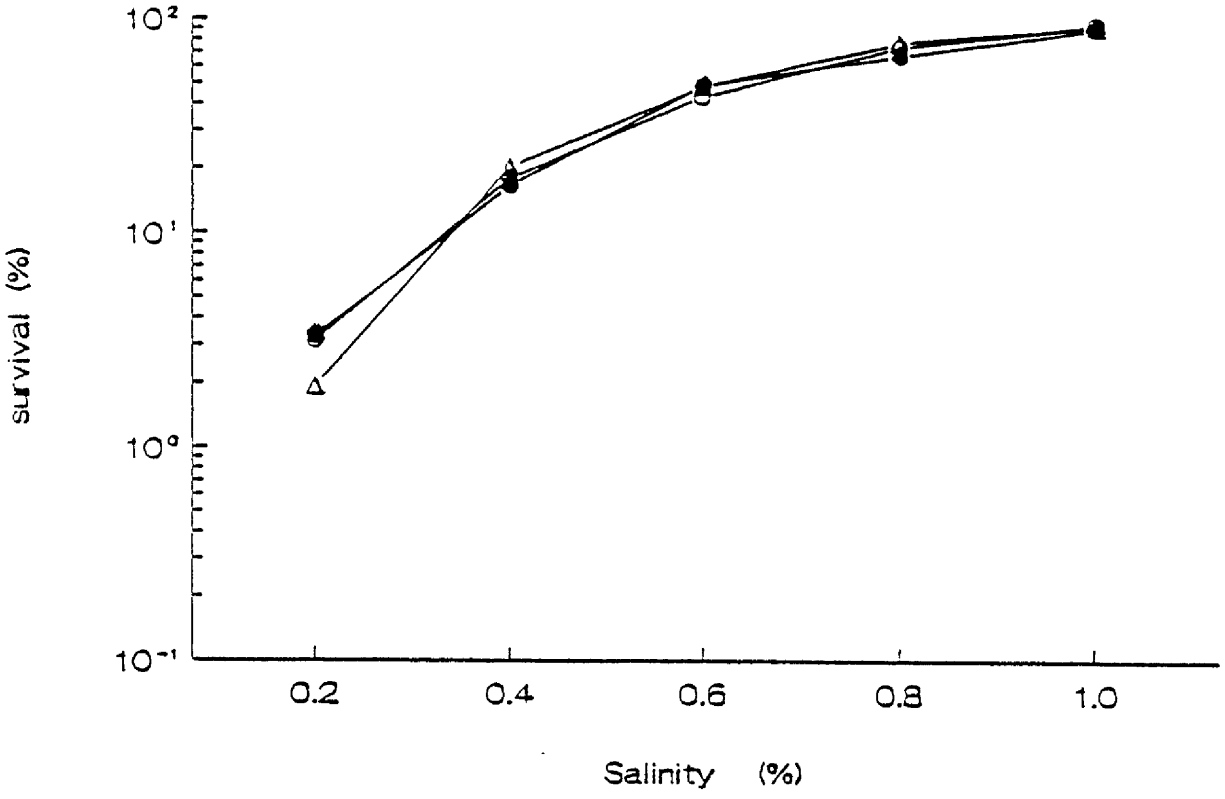

survival (%)
10²
10¹
10⁰
10⁻¹
0.2
0.4
0.6
0.8
1.0
Salinity (%)

FIGURE 29

Effect of Tween 80 on bactericidal action of lysozyme.

- ○ Lysozyme
- ● Lysozyme + Tween 80
- △ Tween 80
- ▲ Seawater at 0.2% Salinity
- □ Seawater

A concentration of lysozyme of 1.0mg per ml was used. The test bacterium was *Pseudomonas* 1.1.1..

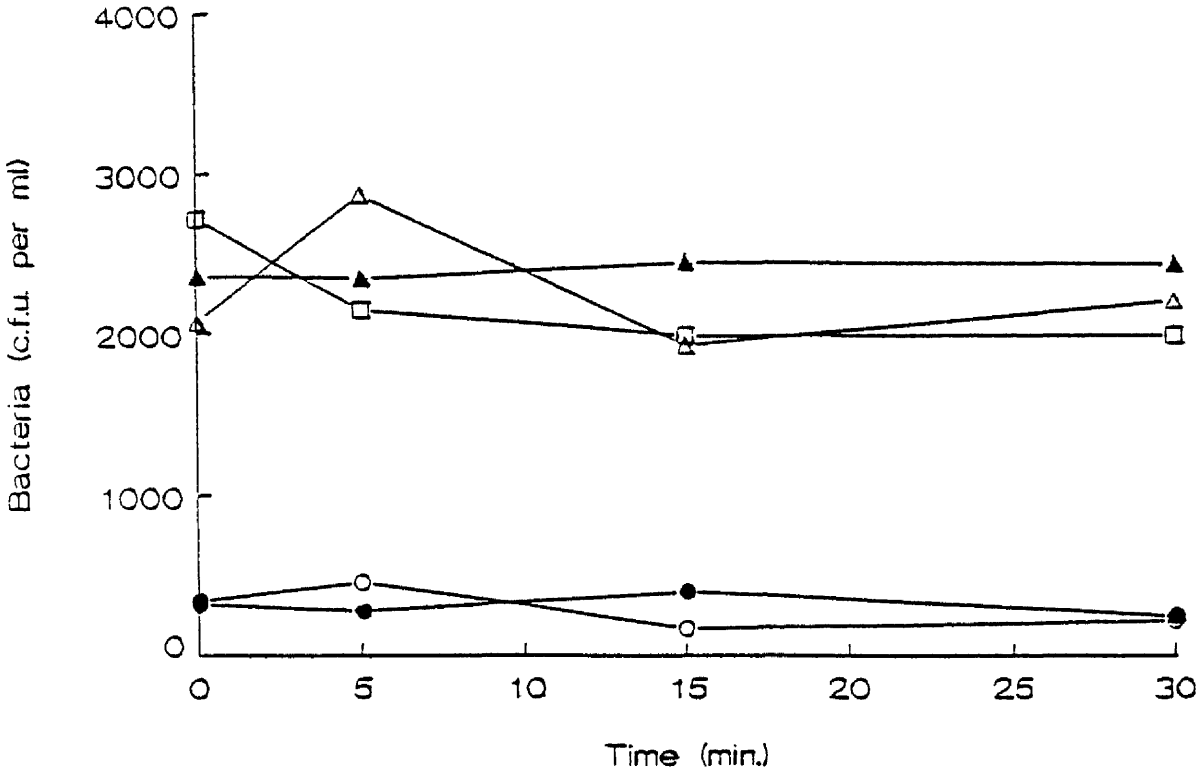

4000
3000
2000
1000
0
Bacteria (c.f.u. per ml)
0
5
10
15
20
25
30
Time (min.)

Figure 30

Effect of Tween 80 and EDTA on the bactericidal action of lysozyme.

- ○ Lysozyme
- ● Lysozyme + Tween 80
- △ Tween 80
- ▲ Seawater at 0.2% Salinity
- □ Seawater
- ■ Lysozyme + EDTA
- ◇ EDTA
- ◆ Lysozyme + EDTA + Tween 80

A concentration of lysozyme of 1.0mg per ml was used. The test bacterium was *Vibrio alginolyticus* (3-8).

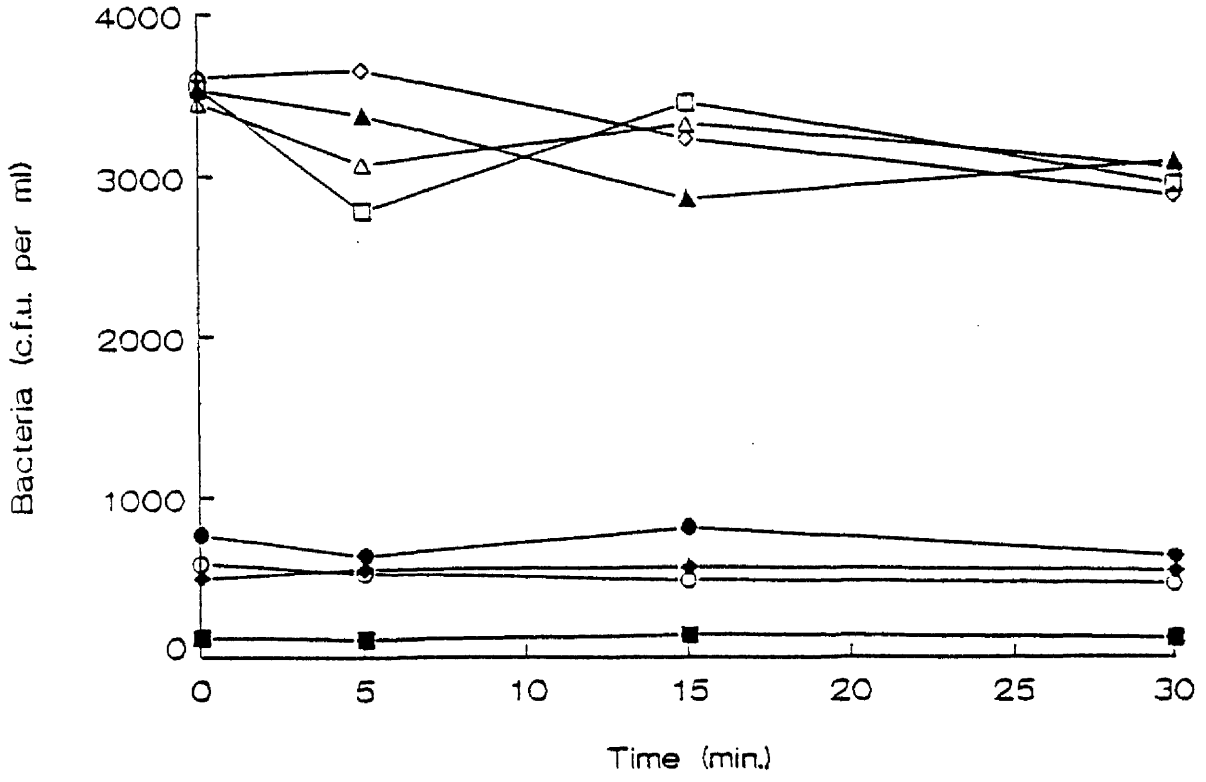

4000
3000
2000
1000
0
Bacteria (c.f.u. per ml)
0
5
10
15
20
25
30
Time (min.)

FIGURE 31

a) **Effect of increasing concentration of lysozyme on *Pseudomonas* 1.1.1..**

b) **Effect of lysozyme plus increasing concentrations of EDTA on *Pseudomonas* 1.1.1..**

A concentration of lysozyme of 0.5mg per ml was used.

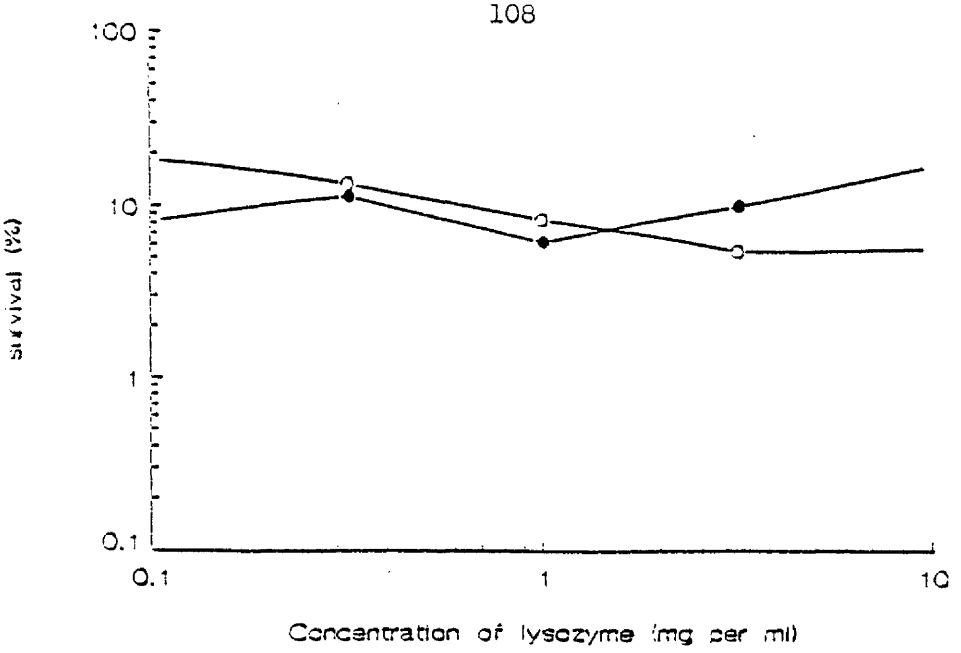
c) **Effect of increasing concentration of EDTA on *Pseudomonas* 1.1.1..**

Exposure Time

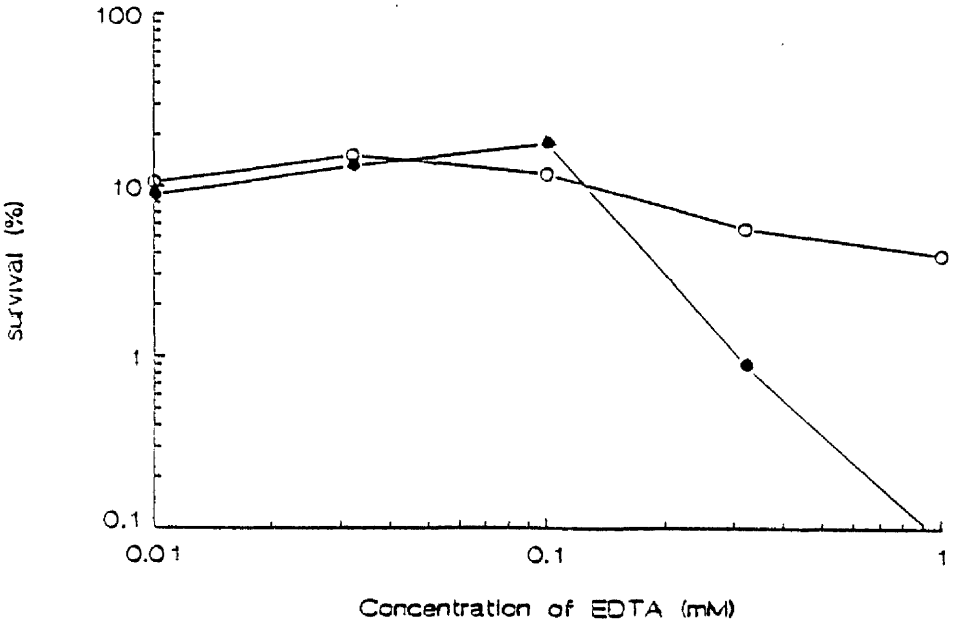
○ 0 Minutes

● 5 Minutes

a)


100
10
1
0.1
Survival (%)
0.1
1
10
Concentration of lysozyme (mg per ml)

b)


100
10
1
0.1
Survival (%)
0.01
0.1
1
Concentration of EDTA (mM)

c)

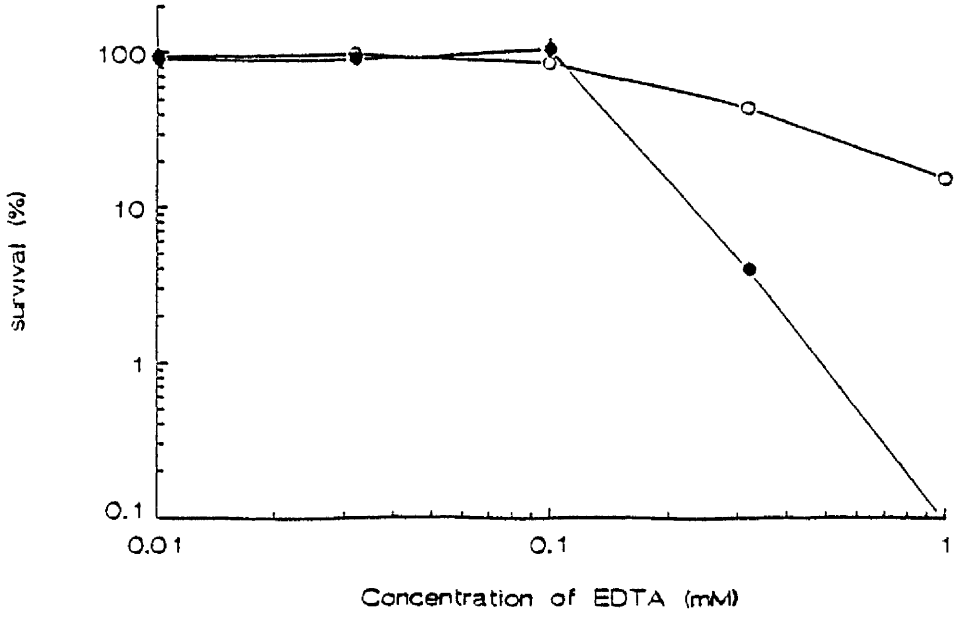
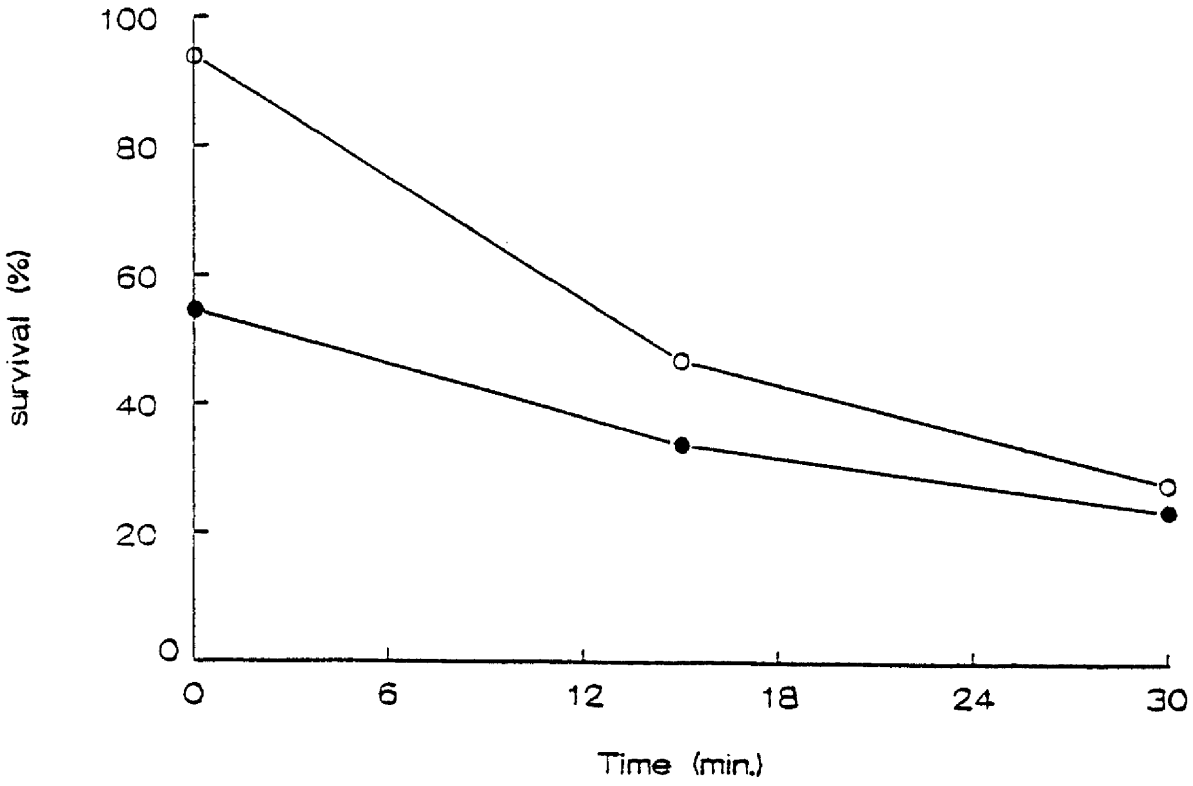

100
10
1
0.1
Survival (%)
0.01
0.1
1
Concentration of EDTA (mM)

FIGURE 32

Effect of low salinity and lysozyme on the bacterial load of rotifers.

- ○ Seawater at 0.2% Salinity
- ● Seawater at 0.2% Salinity + Lysozyme

The action of lysozyme was very rapid and significant bacterial mortality occurred even after the briefest exposure (time 0).


100
80
60
40
20
0
survival (%)
0
6
12
18
24
30
Time (min.)

markedly and by approximately 33% after 15 minutes. The rotifers themselves survived the treatment but stopped swimming and filter-feeding. They appeared to recover after being placed in 2.4% seawater.

The bactericidal activity of lysozyme was again dependent on salinity (Figure 33), and also on rotifer concentration (Figure 34). A concentration of 0.1mM EDTA did not enhance the activity of lysozyme, in fact, it appeared to have the opposite effect (Figure 35). Concentrations of EDTA above 0.1mM were extremely toxic to rotifers. EDTA was possibly being bound by free calcium ions in the 0.2% seawater therefore calcium-free seawater was prepared, however, this did not improve the effect of EDTA on lysozyme (Figure 36).

3.14 Field Trials of Lysozyme-Treated Rotifers

Lysozyme-treated rotifers were given to first-feeding turbot larvae in two trials at G.S.P.. In the first trial both the test group (fed treated rotifers) and the control group (fed untreated rotifers) of larvae were very poor and did not feed properly. The treated rotifers sank to the bottom of the tank. Although they recovered overnight, their guts were empty so they were of little or no nutritional value to the larvae.

In the second trial the lysozyme treatment was preceded by a stepwise reduction in salinity to reduce the osmotic shock to the rotifers. Similarly, after treatment with lysozyme (1.0mg per ml for 10 minutes) the salinity was gradually increased before the rotifers were fed to the larvae. In this trial the bacterial load of the rotifers was reduced by 35-71%. The rotifers sank to the bottom of the tank despite the reduced osmotic shock. Good survival rates were obtained for both groups of larvae but no noticeable benefit was derived from feeding lysozyme-treated rotifers.

Although lysozyme treatment significantly reduced the bacterial load of rotifers, the potential benefit from this was negated by the fact that the rotifers stopped swimming and sank to the bottom of the larval-rearing tanks after treatment. Therefore, an alternative means of disinfecting rotifers was sought.

FIGURE 33

Effect of varying salinity on the activity of lysozyme against the bacterial load of rotifers.

Rotifers were exposed to 1 mg ml^{-1} lysozyme in diluted seawater for 10 minutes.

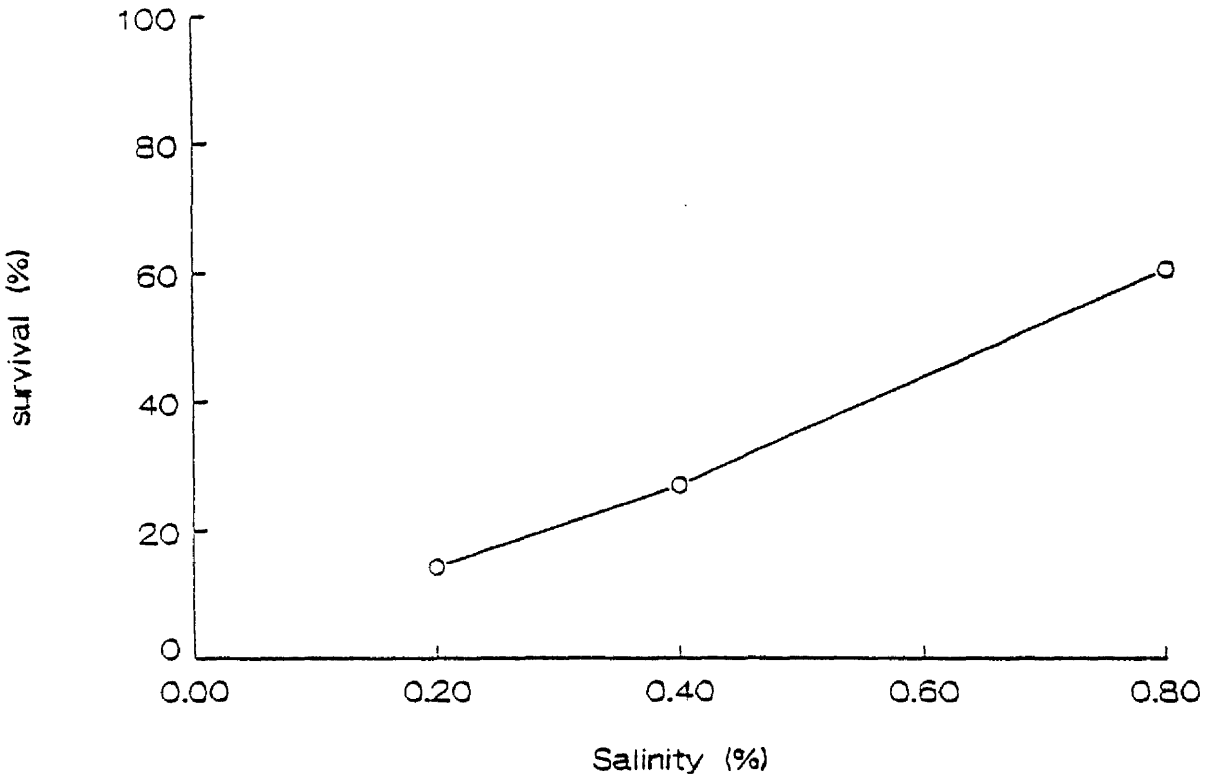

100
80
60
40
20
0
survival (%)
0.00
0.20
0.40
0.60
0.80
Salinity (%)

FIGURE 34

Effect of rotifer concentration on the bactericidal activity of lysozyme.

- ○ 1,400 Rotifers per ml
- ● 600 Rotifers per ml
- △ 300 Rotifers per ml

A concentration of lysozyme of 1.0mg per ml was used.

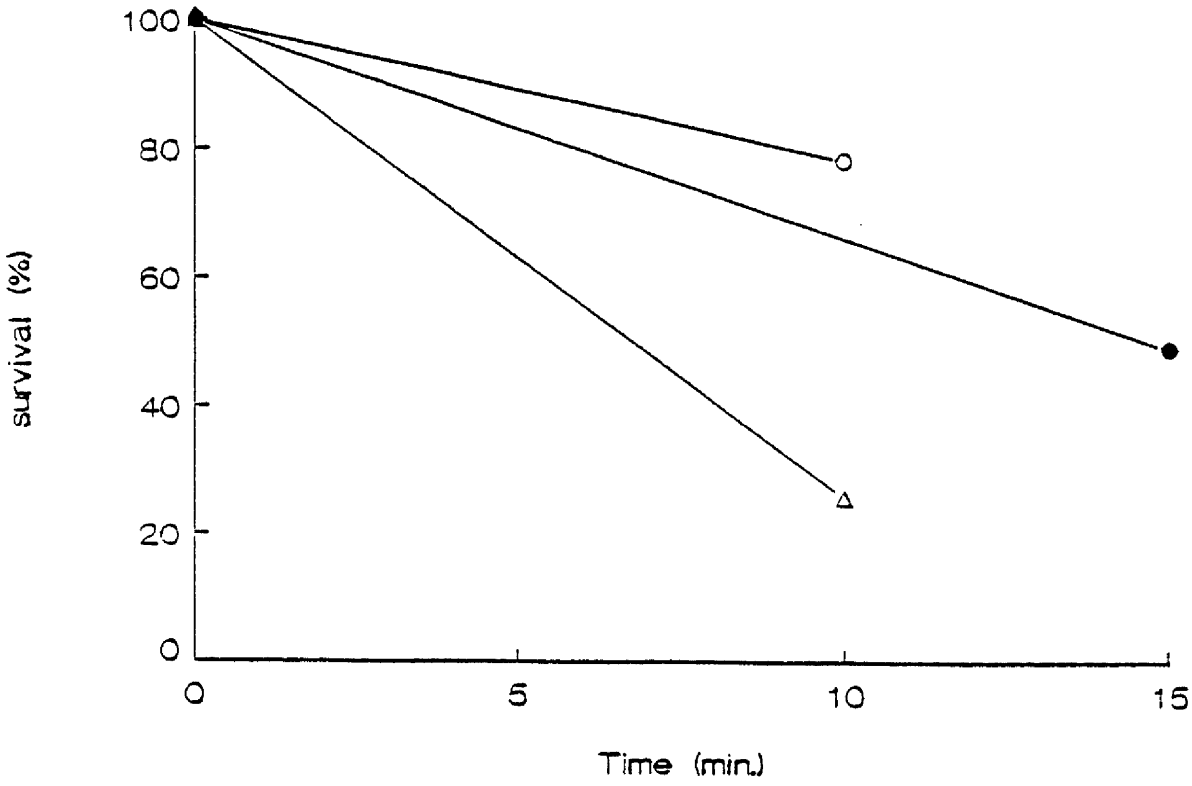

100
80
60
40
20
0
survival (%)
0
5
10
15
Time (min.)

FIGURE 35

Effect of lysozyme plus EDTA on the bacterial load of rotifers.

- ○ Seawater at 0.2% Salinity
- ● Seawater at 0.2% Salinity + Lysozyme
- △ Seawater at 0.2% Salinity + Lysozyme + EDTA

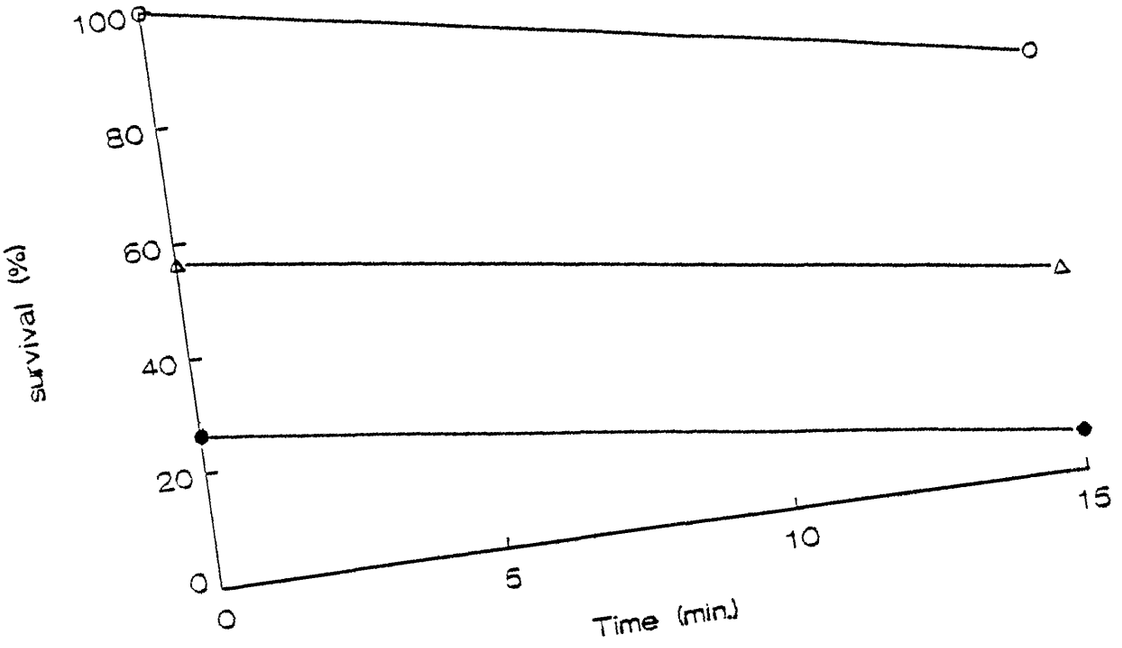
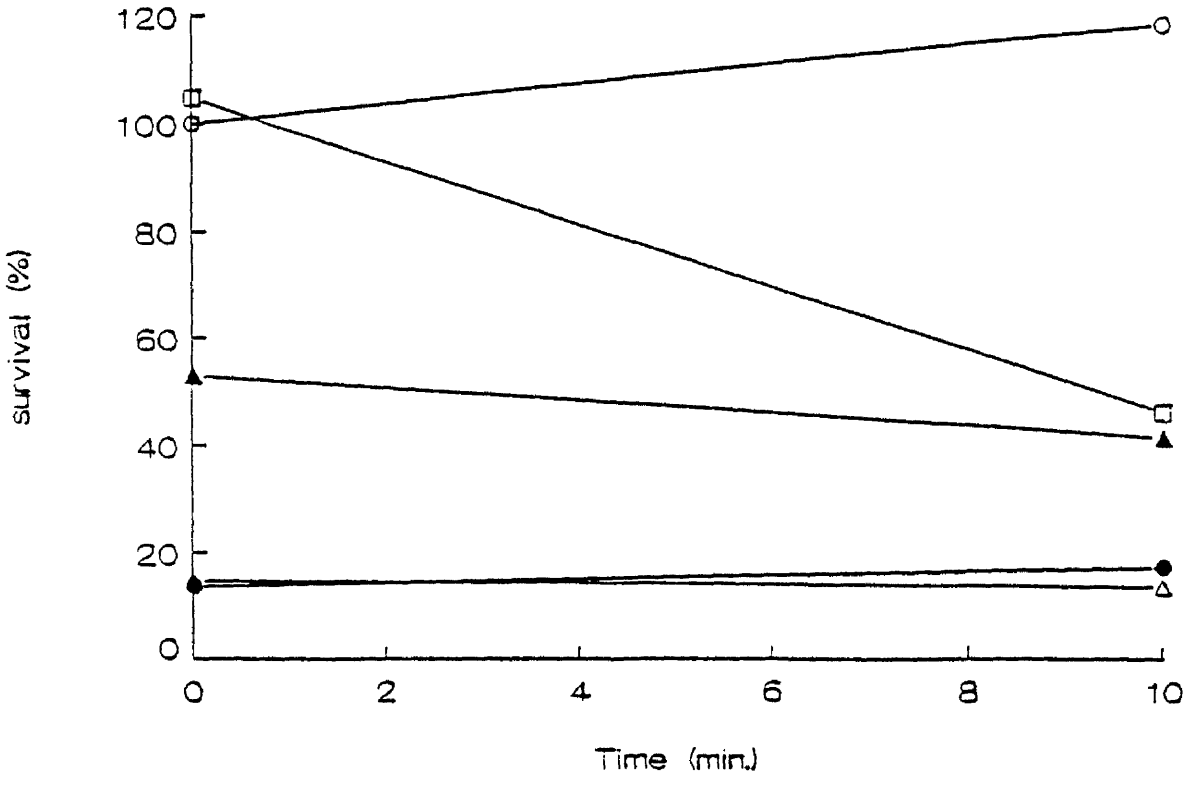

100
80
60
40
20
0
survival (%)
0
5
10
15
Time (min.)

FIGURE 36

Effect of calcium-free seawater on the bactericidal activity of lysozyme and EDTA on the bacterial load of rotifers.

- ○ Seawater
- ● Calcium-free Seawater at 0.2% Salinity
- △ Calcium-free Seawater at 0.2% Salinity + Lysozyme
- ▲ Calcium-free Seawater at 0.2% Salinity + Lysozyme + EDTA
- □ Calcium-free Seawater at 0.2% Salinity + EDTA


120
100
80
60
40
20
0
survival (%)
0
2
4
6
8
10
Time (min.)

3.15Elimination of Bacteria by Ultra-Violet Radiation

The bactericidal activity of a U.V. tube lamp was confirmed by placing suspensions of bacterial cells, in a layer of approximately 1.0mm depth in petri dishes, at a distance of 50cm from the U.V. source. Concentrations of 5.0×10^3 cells per ml of *Pseudomonas* 1.1.1., *V. anguillarum* and *V. tubiashi* were reduced by >99% within 60 seconds (Figure 37).

Effect of U.V. Radiation on the Bacterial Load of Rotifers

Suspensions of rotifers of approximately 120 rotifers per ml were treated in the same manner as the suspensions of bacterial cells above. The rotifers appeared to be unaffected by 5 minutes exposure to U.V. radiation when placed 50cm from the U.V. lamp (Table 15). The effect of U.V. radiation on the bacterial load of rotifers was dependent on time (Figure 38) and distance from the U.V. source (Figure 39). An average reduction of 90% of the bacterial load of rotifers, over 3 experiments, was achieved with an exposure time of 2 minutes, at a distance of 50cm from the U.V. source. This increased to 97% with 5 minutes exposure, and to 98.3% when the distance was reduced to 13.5cm, with an exposure time of 2 minutes.

Such a system is impractical for the U.V. treatment of large numbers of rotifers but this was possible by passing rotifer suspensions (200 rotifers per ml) through a water jacket surrounding a U.V. tube lamp. A reduction in the bacterial load of unenriched rotifers of >99% was possible with a flow rate of 1.5 litres per minute. Increasing the flow rate decreased the bactericidal activity as did enrichment of the rotifers with algae, despite rinsing the enriched rotifers prior to treatment (Table 16).

The ability of the bacterial flora of rotifers to recover after U.V. treatment was investigated by sampling rotifers immediately after treatment then 24 and 48 hours later. There was a rapid increase in the number of bacteria per rotifer and, within 24 hours, the level of bacteria in the culture water exceeded the original level (Figure 40). However, compared to untreated rotifers the bacterial counts at 24 and 48 hours were low (Figure 41).

FIGURE 37

Bactericidal activity of ultra-violet radiation.

- ○ *Pseudomonas* 1.1.1.
- ● *Vibrio anguillarum*
- △ *Vibrio tubiashii*

Distance from the U.V. source was 0.5m.

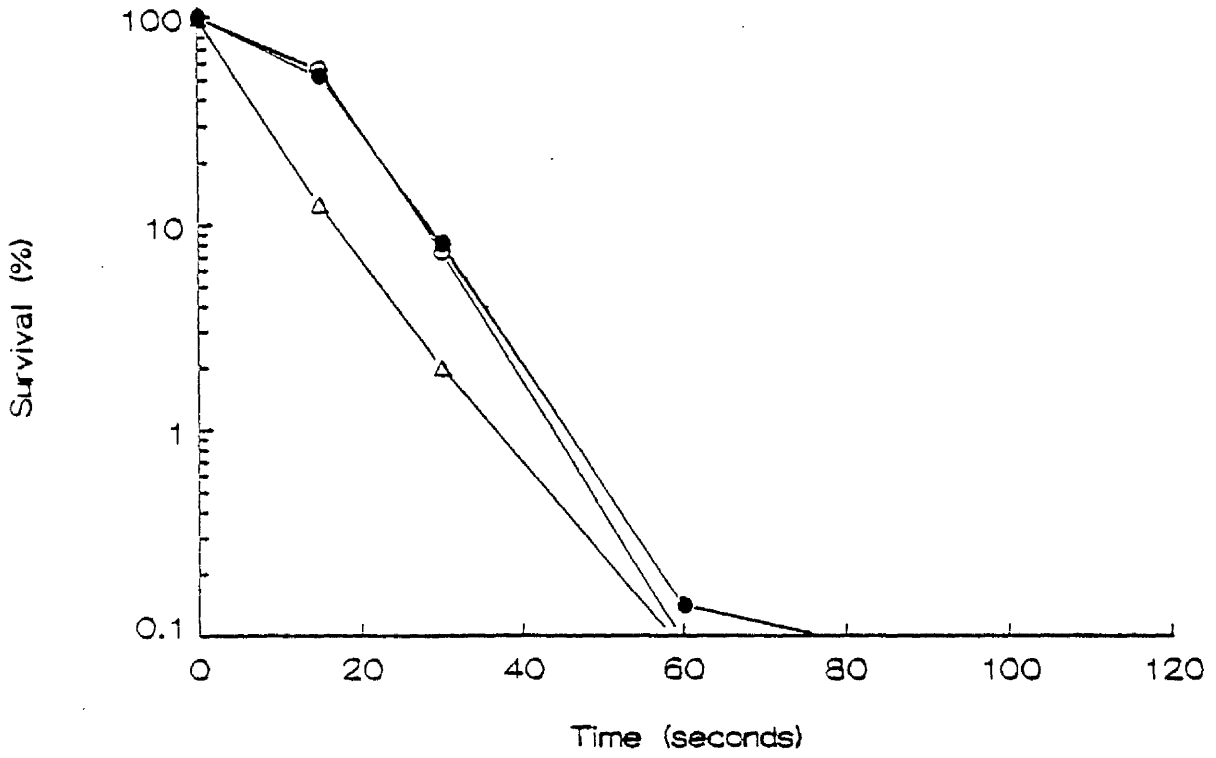

100
10
1
0.1
Survival (%)
0
20
40
60
80
100
120
Time (seconds)

TABLE 15

Effect of Ultra-Violet Radiation on Rotifers

Time of Exposure (min.)	Percent of Rotifers	
	Alive	Swimming
5	100	86.2
10	100	60.6
15	100	44.0
20	100	56.0
25	100	42.7
30	100	36.7

Distance from the U.V. source was 0.5m.

FIGURE 38

Effect of exposure time on the reduction of the bacterial load of rotifers by U.V. radiation.

Distance from the U.V. source was 0.5m.

The values shown are in comparison with unexposed controls.

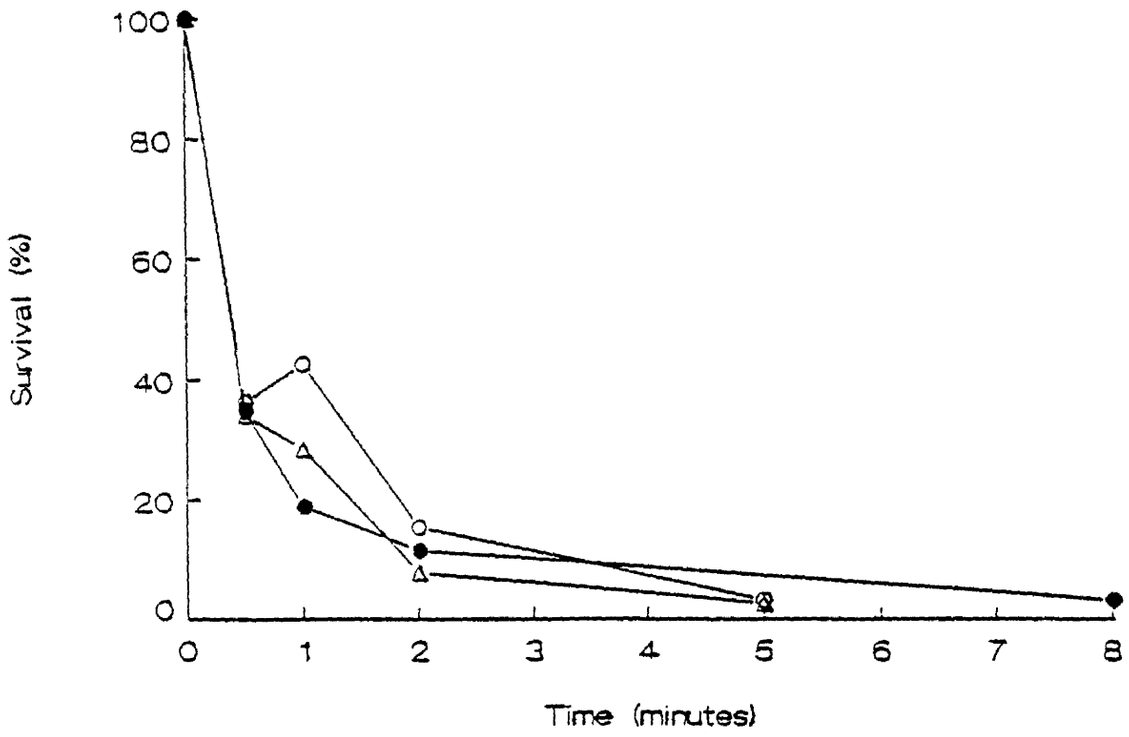

100
80
60
40
20
0
Survival (%)
0
1
2
3
4
5
6
7
8
Time (minutes)

FIGURE 39

Effect of increasing intensity of U.V. radiation by decreasing distance from the U.V. source on the bacterial load of rotifers.

Exposure time was 2.0 minutes.

The values shown are incomparison with unexposed controls.

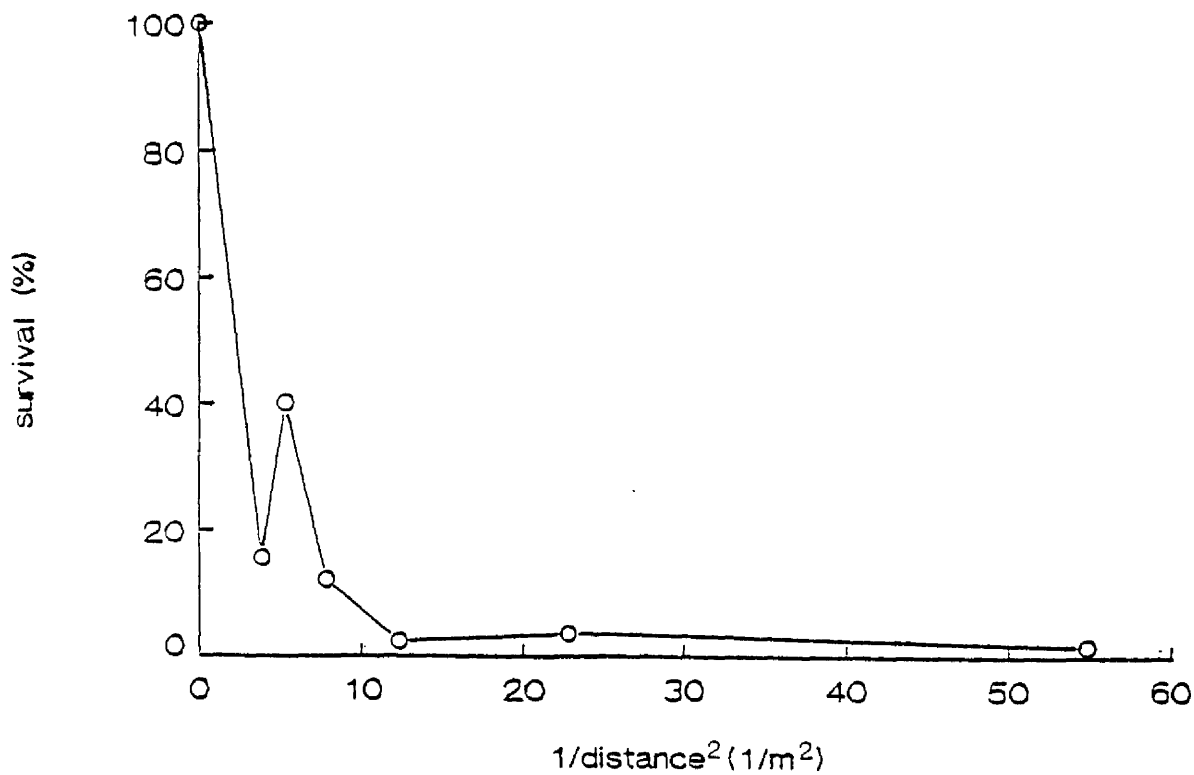

100
80
60
40
20
0
survival (%)
0
10
20
30
40
50
60
1/distance^2 (1/m^2)

TABLE 16

Effect of Flow Rate and Enrichment on the Reduction of the Bacterial Load of Rotifers by Ultra-Violet Radiation

Flow rate (litres per min.)	Mean survival of bacteria per rotifer (%)	
	Unenriched rotifers	Enriched rotifers
0.6	NT	0.32 (4)
1.5	0.06 (6)	1.17 (4)
2.4	3.22 (2)	NT

The number of experiments is shown in parentheses.

NT = not tested.

FIGURE 40

Bacteria associated with rotifers and rotifer culture water after U.V. treatment.

- ○ U.V.-Treated Rotifers
- ● Control Rotifers
- △ U.V.-Treated Rotifer Culture Water
- ▲ Control Rotifer Culture Water

Mean of 6 experiments.

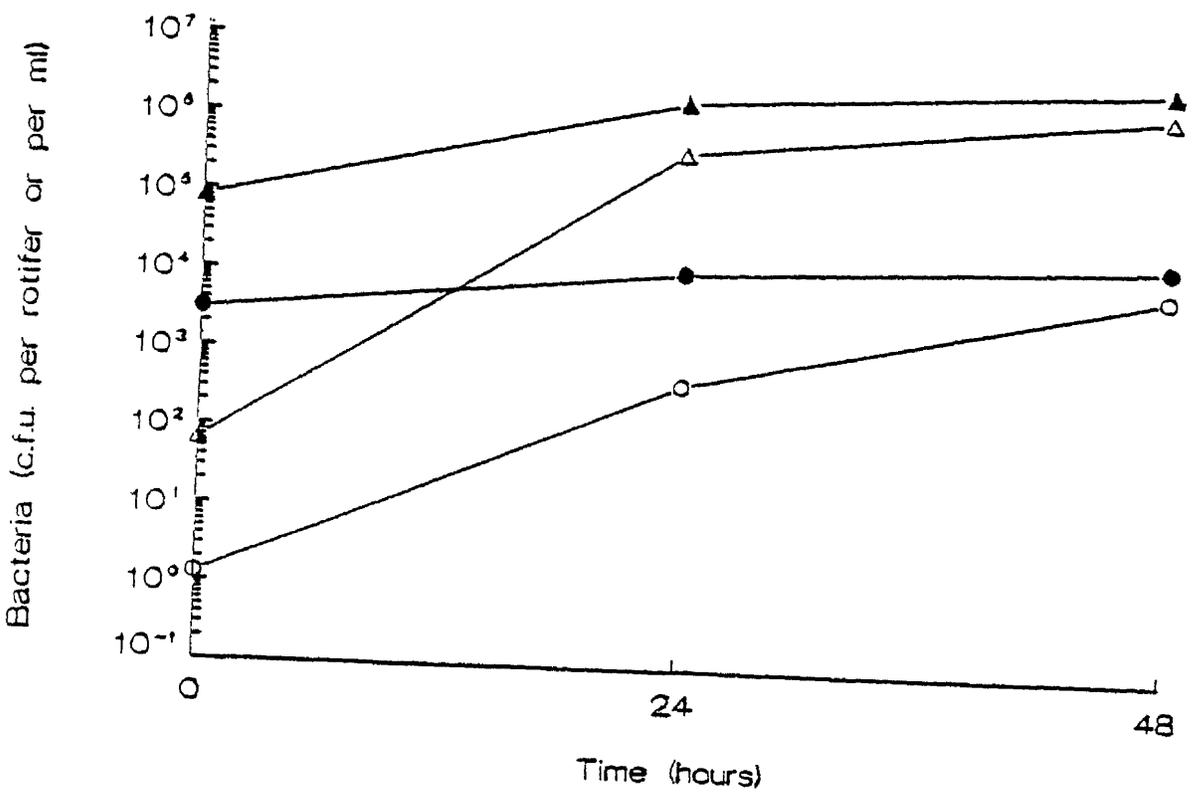
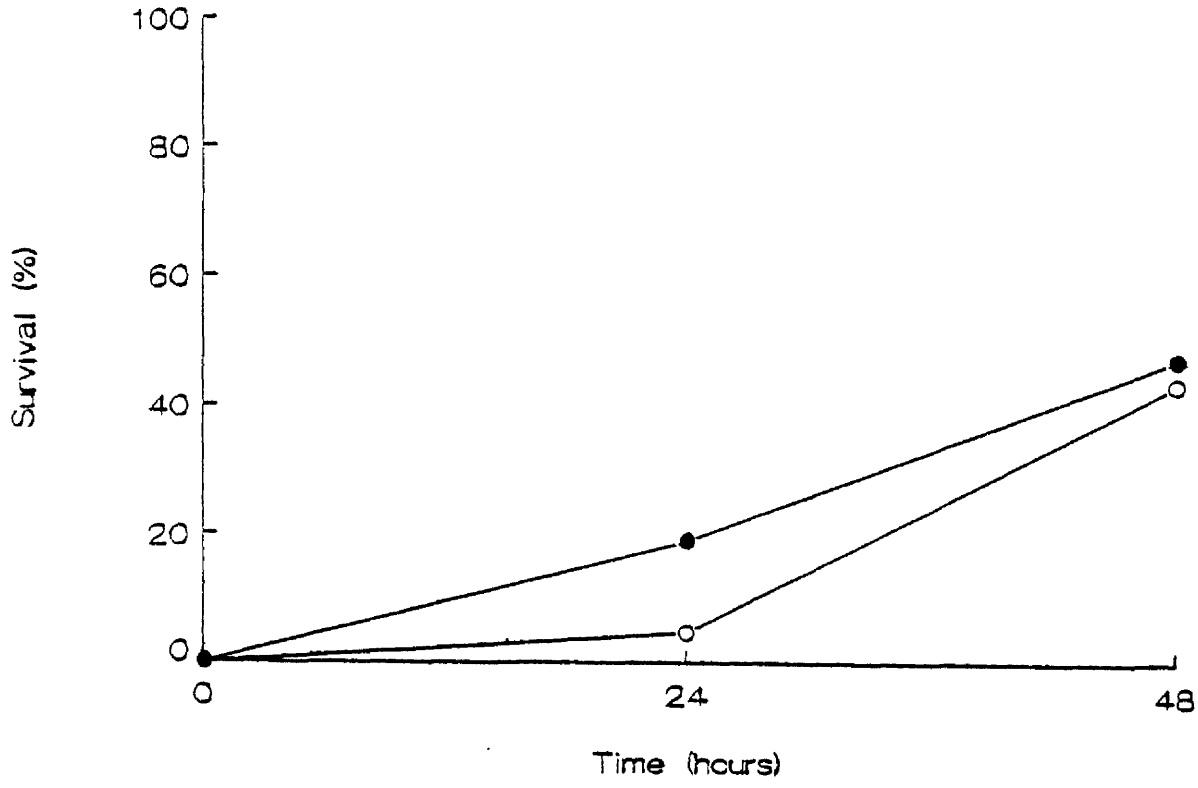

Bacteria (c.f.u. per rotifer or per ml)
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
10⁻¹
0
24
48
Time (hours)

FIGURE 41

Bacteria associated with rotifers and rotifer culture water after U.V. treatment, as a percentage of the controls.

- ○ Rotifers
- ● Rotifer Culture Water

Mean of 6 experiments.


100
80
60
40
20
0
Survival (%)
0
24
48
Time (hours)

To confirm that rotifers were unaffected by U.V.-treatment, algae were added to U.V.-treated rotifers, untreated rotifers, and seawater containing no rotifers as a control. Optical density readings at 600nm of the water (filtered to remove the rotifers) were taken at hourly intervals for 8 hours and at 24 hours after treatment. The optical densities of the treated and untreated rotifer suspensions decreased at similar rates, whereas the control, containing algae only, remained stable (Figure 42), indicating that the rotifers continued to filter feed normally after U.V. radiation treatment. This was confirmed by visual examination of the rotifers.

3.16 Field Trials with U.V.-Treated Rotifers

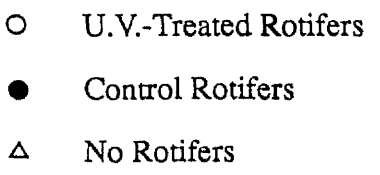
The effect of feeding U.V.-treated rotifers to larval turbot, reared intensively with daily additions of food, was investigated at G.S.P.. Two 2,000 litre tanks, each containing 11,000 turbot larvae, were set up. At 3 days posthatch, and daily thereafter, the test larvae were fed U.V.-treated rotifers and the control larvae were fed untreated rotifers. There was an average reduction of 95% of the bacterial load of the U.V.-treated compared to untreated rotifers (Figure 43a). The number of rotifers available was insufficient (Figure 43b) and the larvae did not survive beyond 8 days posthatch, almost certainly due to starvation.

Daily sampling of the turbot larvae one hour after feeding (10 fish per sample) revealed that the gut of the test larvae was colonised more slowly than that of the control larvae with a 10-fold difference at 4 days posthatch (Figure 44). Length measurements were taken from daily samples of turbot larvae fixed in formalin (10 fish per sample). There was a significant amount of growth from 3-9 days posthatch in both groups ($p<0.1$, Student's t test), however there was no significant difference between the groups (Figure 45).

Similar trials were conducted three times and in each case survival rates of turbot larvae were low because of insufficient numbers of rotifers, but the rate of colonisation of the gut of the larval turbot fed U.V.-treated rotifers was slower than that of the control larvae.

FIGURE 42

Clearance of algae by U.V.-treated and untreated rotifers.



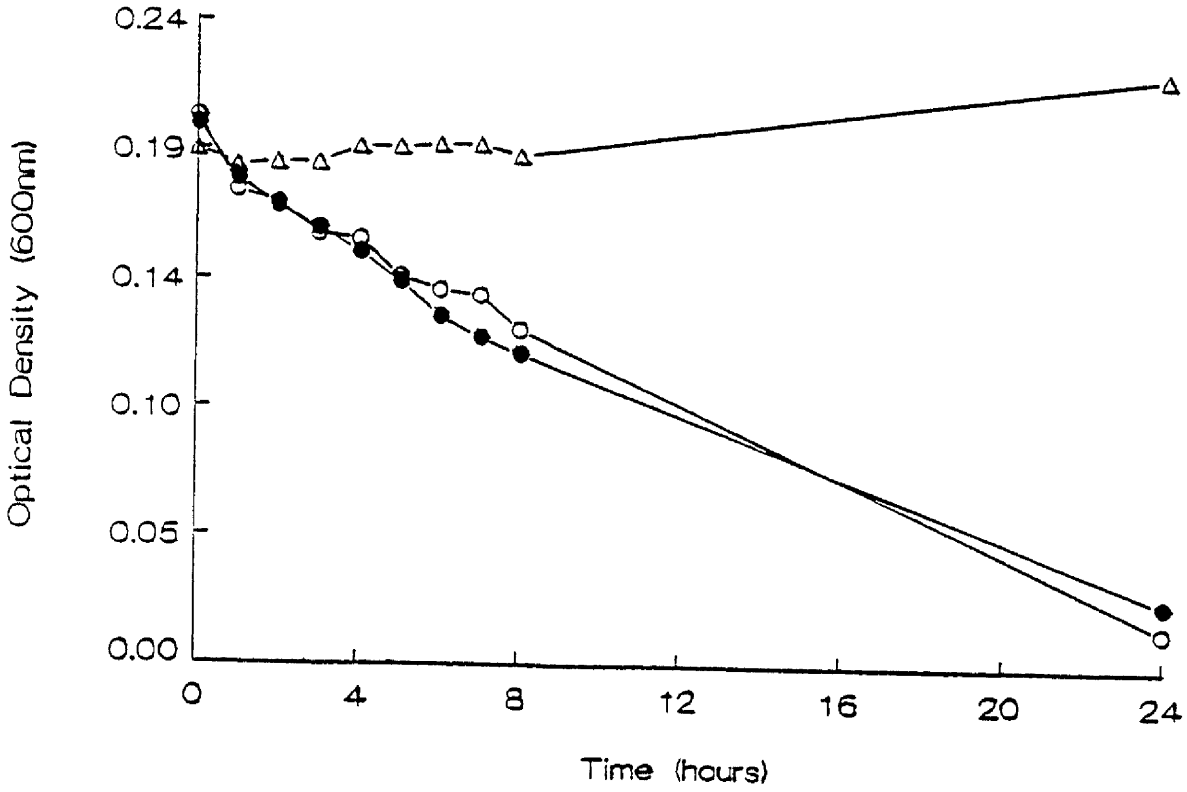

0.24
0.19
0.14
0.10
0.05
0.00
Optical Density (600nm)
0
4
8
12
16
20
24
Time (hours)

FIGURE 43

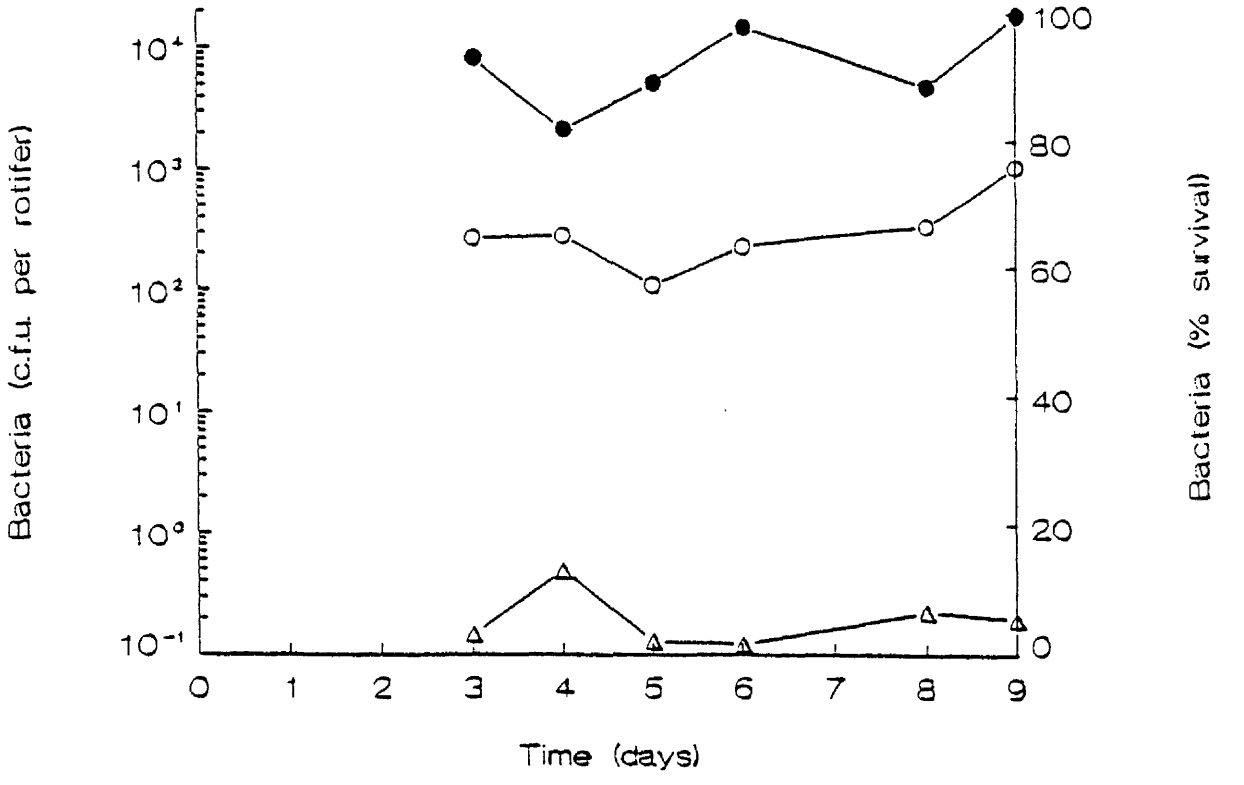
a) **Bacteria associated with U.V.-treated or untreated rotifers fed to turbot larvae.**

- ○ U.V.-Treated Rotifers
- ● Control Rotifers
- △ Bacteria (% Survival)

b) **Rotifers added and uneaten in turbot rearing tanks.**

- ○ Rotifers Added
- ● Uneaten Rotifers in Test Tank
- △ Uneaten Rotifers in Control Tank

a)


Bacteria (c.f.u. per rotifer)
10^4
10^3
10^2
10^1
10^0
10^{-1}
Bacteria (% survival)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
Time (days)

b)

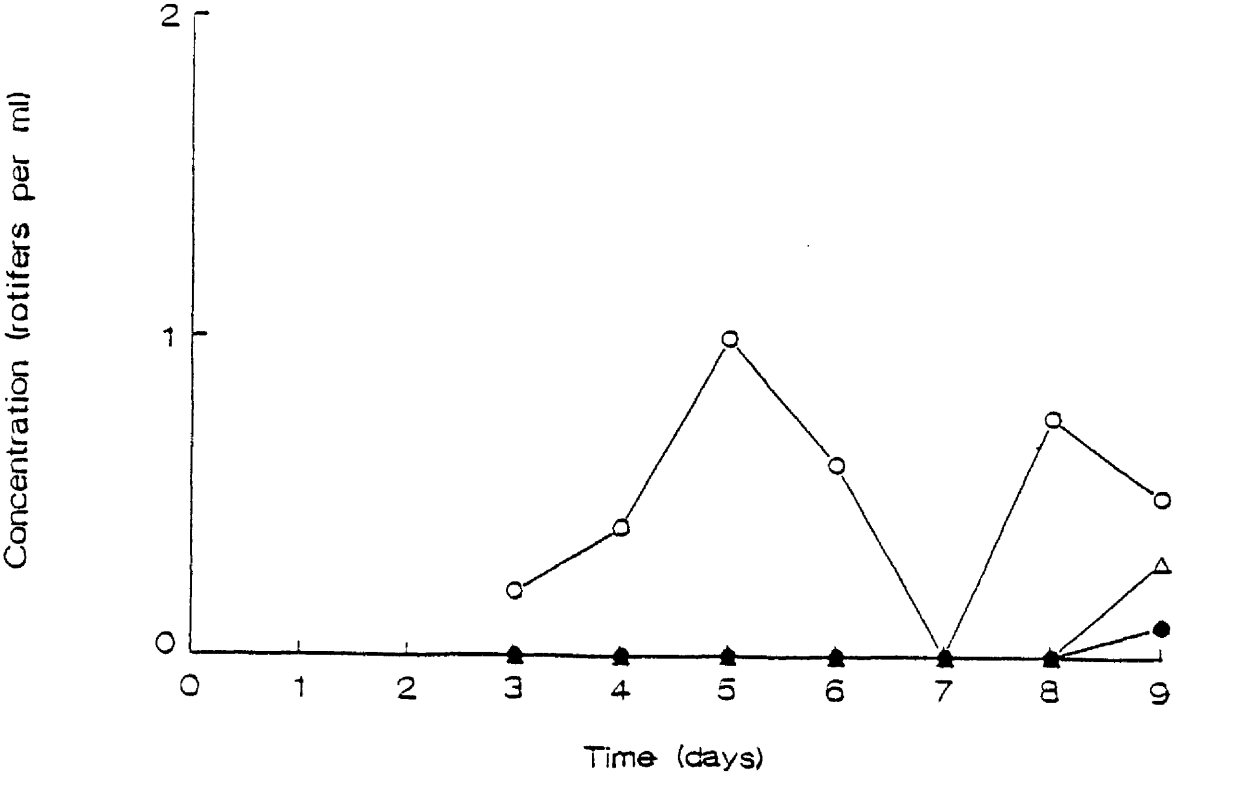

Concentration (rotifers per ml)
2
1
0
0 1 2 3 4 5 6 7 8 9
Time (days)

FIGURE 44

Gut bacteria from intensively-reared turbot larvae fed U.V.-treated or untreated rotifers.

- ○ Turbot Larvae fed U.V.-Treated Rotifers
- ● Turbot Larvae fed Control Rotifers

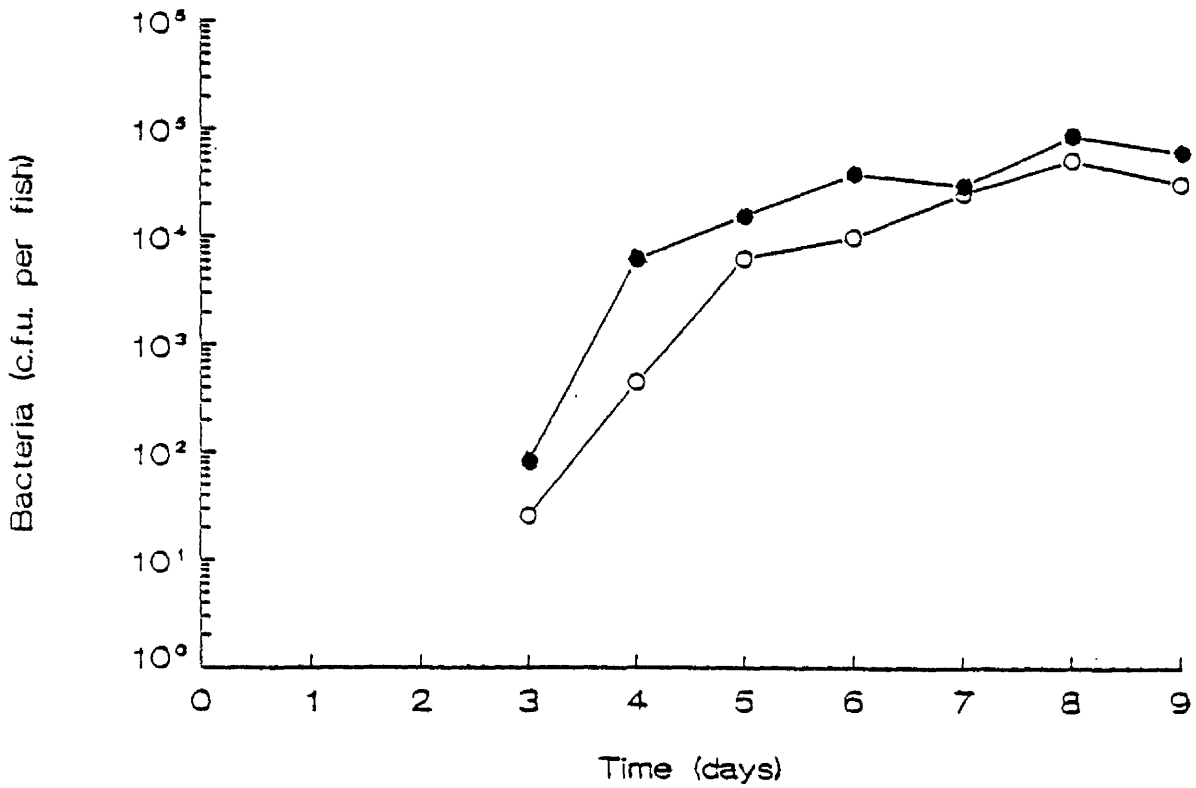
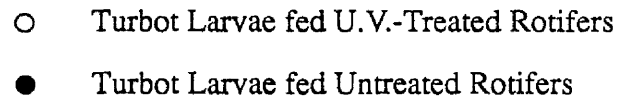
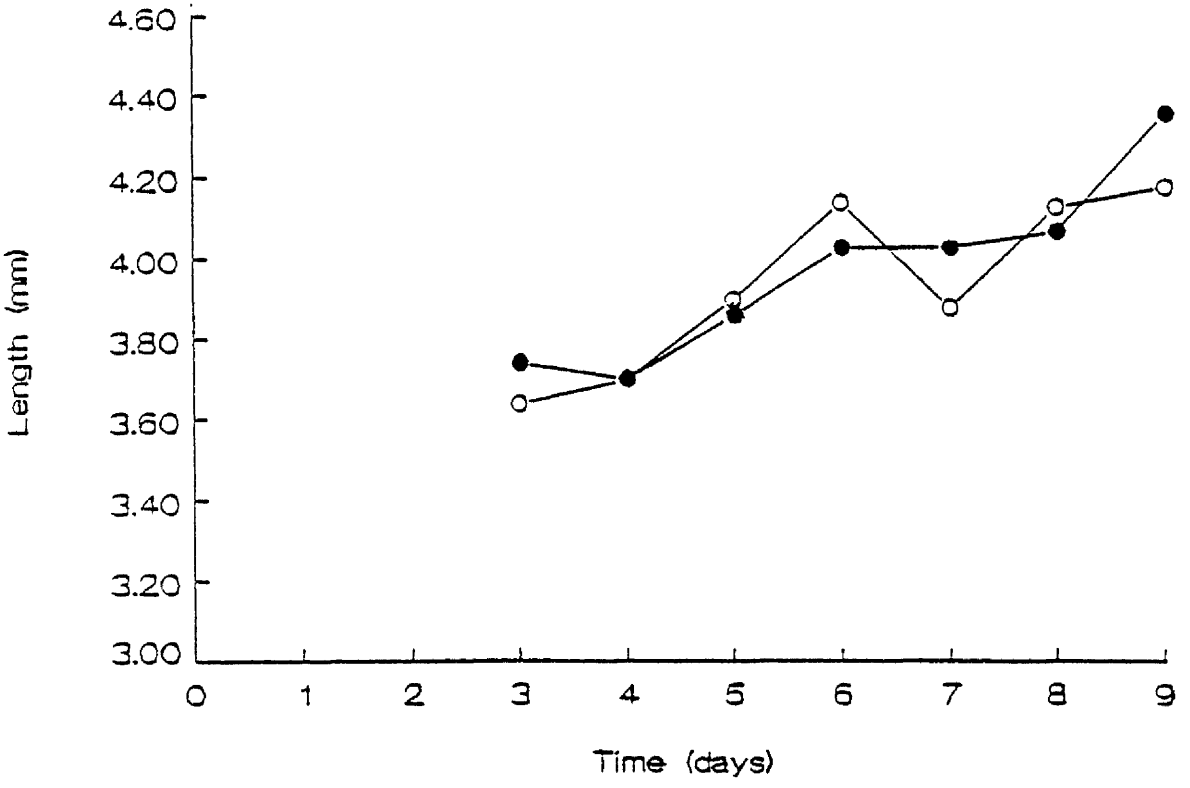

Bacteria (c.f.u. per fish)
10⁵
10⁴
10³
10²
10¹
10⁰
0
1
2
3
4
5
6
7
8
9
Time (days)

FIGURE 45

Growth of intensively reared turbot larvae fed U.V.-treated or untreated rotifers.




4.60
4.40
4.20
4.00
3.80
3.60
3.40
3.20
3.00
Length (mm)
0
1
2
3
4
5
6
7
8
9
Time (days)

In two field trials with extensively-reared turbot larvae, involving 34,000 larvae per tank, the survival rates of larvae fed U.V.-treated rotifers were significantly higher ($p<0.1$, X^2 test), than those of larvae fed untreated rotifers (Table 17). In the first trial larvae were given daily additions of U.V.-treated rotifers after they had established feeding on untreated rotifers for two days. Analysis of the gut flora was not carried out in this trial. In the second trial, U.V.-treated or untreated rotifers were given to turbot larvae at 2, 3, 6, 7, 8 and 9 days posthatch. At 4 days posthatch, the viable counts of bacteria from the gut of larvae given U.V.-treated rotifers were almost 10-fold lower than those of larvae given untreated rotifers (results not shown). There was a mean reduction of 88% of the bacterial load of the U.V.-treated compared to the untreated rotifers with a range from 75 to 97% (results not shown).

3.17 Comparison of the Gut Flora of Turbot Larvae Fed U.V.-Treated or Untreated Rotifers

A total of 58 bacterial isolates were identified from U.V.-treated rotifers, untreated (control) rotifers, and intensively-reared turbot larvae fed either source of rotifers at 3 and 7 days posthatch, i.e. days 1 and 5 of feeding (Table 18). The microflora of the rotifers was very varied, however, based on colony morphology, the dominant groups associated with U.V.-treated rotifers were *Flexibacter/Cytophaga* (19%) and *Pseudomonas/Moraxella* (7%) whereas, the dominant groups associated with the control rotifers were *Pseudomonas/Moraxella* (30%) and *Vibrio/Aeromonas* (19%). The dominant group in both batches of turbot larvae at 3 days posthatch was the *Pseudomonas/Alcaligenes* group (79% and 74% for larvae fed U.V.-treated and control rotifers, respectively). This group was still dominant (70%) in the 7-day-old larvae fed U.V.-treated rotifers, whereas, the *Vibrio/Aeromonas* group was dominant (82%) in the larvae fed control rotifers.

Cluster analysis showed some similarity between bacterial isolates from both batches of 3-day-old larvae, whereas, at 7 days posthatch the floras of the two groups

TABLE 17

Survival of Extensively-Reared Turbot Larvae (%) at 20 Days Posthatch

	Survival (%)	
	Control Larvae	Test Larvae
Field Trial 1.	17.1	27.9
Field Trial 2.	6.4	15.2

TABLE 18

Bacteria Isolated From Rotifers and Turbot Larvae During Field Trial of Ultra-Violet Radiation-Treated Rotifers

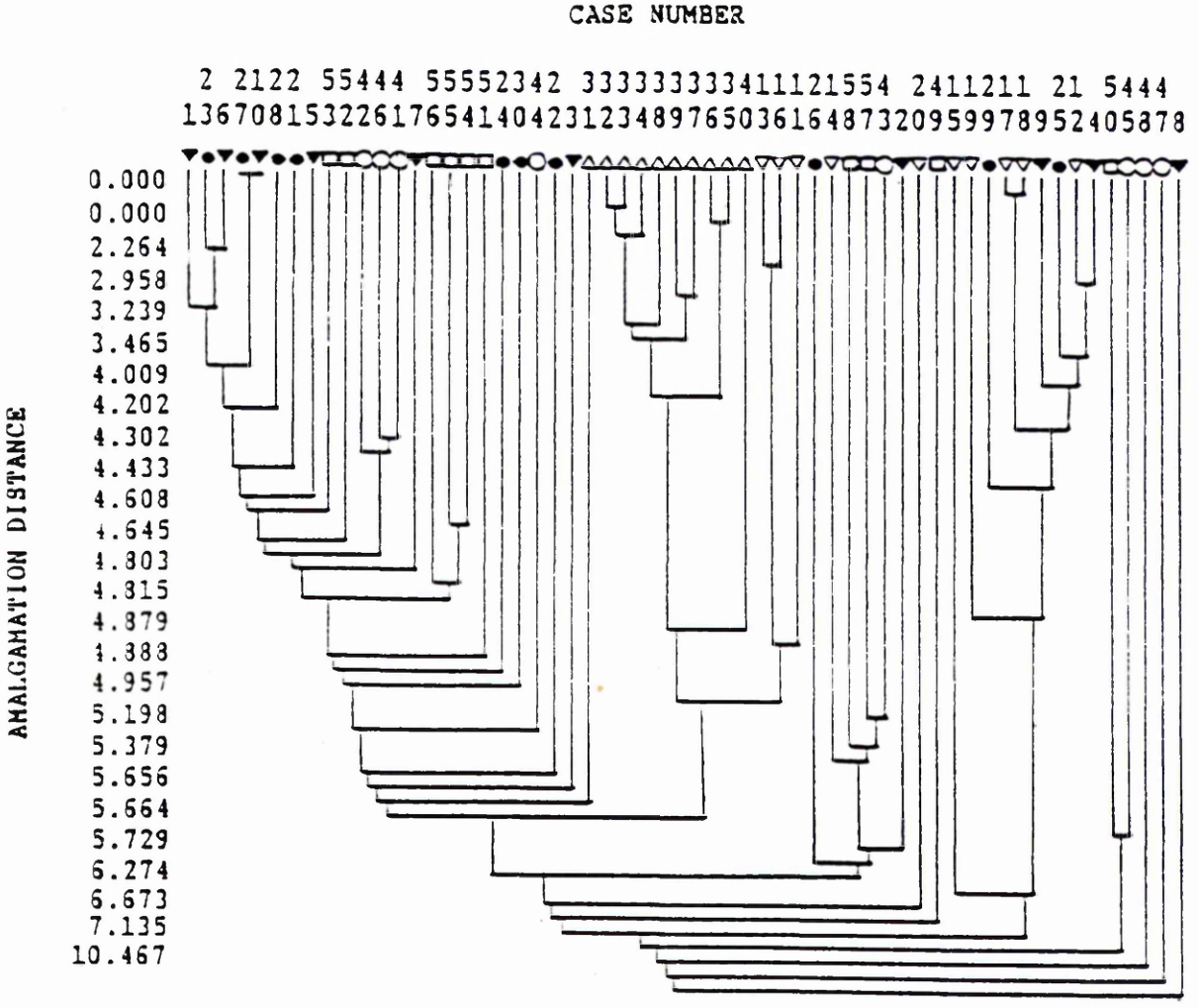
Identification	Source					
	Rotifers		Larvae			
	Test	Control	Test day 3	Control day 3	Test day 7	Control day7
Vibrio/Aeromonas	2	5	4	7	3	9
Flexibacter/Cytophaga	4	1				
Pseudomonas/Moraxella	2	3	1		1	
Pseudomonas/Alcaligenes			3	2	6	
Pseudomonas/Agrobacterium			1			
Flavobacterium		1		1		
Photobacterium						1
Gram positive cocci			1			
Total	8	10	10	10	10	10

were different (Figure 46).

FIGURE 46

Cluster analysis of isolates from a field trial of U.V.-treated rotifers.

- ▼ Turbot Larvae fed U.V.-Treated Rotifers (Day 3)
- ● Turbot Larvae fed Control Rotifers (Day 3)
- ▽ Turbot Larvae fed U.V.-Treated Rotifers (Day 7)
- △ Turbot Larvae fed Control Rotifers (Day 7)
- ○ U.V.-Treated Rotifers
- □ Control Rotifers


CASE NUMBER
2 2122 55444 55552342 333333334111215542411211 21 5444
13670815322617654140423123489765036164873209599789524058 78
AMALGAMATION DISTANCE
0.000
0.000
2.264
2.958
3.239
3.465
4.009
4.202
4.302
4.433
4.608
4.645
4.803
4.815
4.879
4.888
4.957
5.198
5.379
5.656
5.664
5.729
6.274
6.673
7.135
10.467

DISCUSSION

4.1 Bacterial Flora of Turbot Larvae

Since the first attempts to rear larval turbot in captivity in the early 1970s, most research has concentrated on nutritional aspects such as the enrichment of rotifers with long-chain polyunsaturated fatty acids. However, survival rates remain highly variable and the present study is concerned with the development of the bacterial flora of the gut of turbot larvae and whether it is possible to improve survival and growth by influencing the gut flora without the aid of antibiotics.

There are several ways in which the survival and growth of turbot larvae may be dependent on the gut flora. If frank pathogens are present they will obviously have a deleterious effect on the host via virulence factors such as toxins. The entry of opportunistic pathogens may be inhibited by an advantageous bacterial flora, either by competition for binding sites or by the production of antibacterial compounds such as bacteriocins. A probiotic flora might also provide nutritional benefit to the host by the production of cofactors or enzymes important in the breakdown of substrates.

Yolk-sac stage turbot larvae had very few bacteria associated with their gut. This suggests that the surface of the gut does not provide suitable attachment sites for bacteria at this stage or that osmoregulation by drinking may not be as important for these fish as was postulated for yolk-sac stage larvae of the halibut (Tytler and Blaxter, 1988) and cod larvae (Olafsen, 1984).

The gut of larval turbot was rapidly colonised once feeding commenced at 3-4 days posthatch. In intensively-reared larvae the bacterial levels did not alter significantly after about day 4 to 5 (Figure 2a).

The vibrio population was seriously underestimated by using TCBS agar (Figure 2a) as the counts were much lower than the proportion of vibrios identified from isolates on marine agar. Bolinches and Egidius (1987) also found that the vibrio count on TCBS agar was an underestimate when compared to the number of vibrios assessed by isolation from marine agar, but when compared to other media selective for *Vibrio* (data not published) TCBS agar was the best one to assess vibrio levels. Simidu and Tsukamoto (1980) concluded that a noninhibitory medium and

GasPack anaerobic culture system was the best method for the selective enumeration and isolation of vibrios in seawater and marine sediments. The ratio of *Vibrio* counts obtained by this method to the number of vibrios identified from the heterotrophic bacterial population, ranged from 0.5 to 1.3 with a mean of 0.96. However, the use of a selective medium for the enumeration of Enterobacteriaceae, together with the *Vibrio* medium, was recommended if terrigenous contamination was suspected.

In all experiments involving intensively-reared larval turbot, vibrios were the dominant bacteria in the gut (Tables 2, 6 and 9) with *V. alginolyticus* generally being the dominant species (Tables 2 and 7). The *Vibrio/Aeromonas* group was also common among the rotifer culture and the larval rearing water though a much more varied flora existed in the larval rearing water (Table 2). Cluster analysis revealed no marked similarity between bacteria from the rearing water and the gut of larval fish (Figure 3) despite the fact that such bacteria must be ingested when water is taken into the gut during feeding. There were two closely related clusters of isolates from turbot larvae, rotifers and the rotifer culture water, indicating that the diet was the major source of the gut flora. An apparently stable flora developed in the gut since identical isolates, identified as *V. alginolyticus*, were detected in turbot larvae on days 4, 5, 7, 8 and 9 and in rotifers on day 5. A second strain, also identified as *V. alginolyticus*, was detected in larvae on days 4, 6 and 8, in rotifers on days 3, 7 and 8 and in rotifer culture water on days 7 and 9 (Figure 4). This may be due to the selection pressures of the conditions within the gut or the gut flora may merely reflect the flora of the diet. It is probably a combination of these factors which determines the bacterial flora. Many isolates from fish were not closely related to each other and many isolates from rotifers were not detected in fish but this could be due to an insufficient sample size.

The total viable counts of bacteria detected in the gut of turbot larvae in this study are in accordance with the published literature on the bacterial flora of larvae of other fish species. Thus, the studies of Muroga *et al.* (1987) on larvae of red and black seabream and of Tanasomwang and Muroga (1988) on Japanese flounder

indicated concentrations of approximately 10^4 to 10^5 per larva. *Pseudomonas* species were dominant in newly-hatched larvae and the rearing water whereas, after feeding, the gut microflora was influenced, both quantitatively and qualitatively, by the flora of the diet. The bacterial flora of the gut of larval rockfish (*Sebastes schlegeli*), tiger puffer (*Takifugu ribribes*) and red grouper (*Eoinephelus akaara*) was also influenced by the flora of the foods ingested rather than the ambient water (Tanasomwang and Muroga, 1989).

If the gut flora influences the growth and survival rates of turbot larvae, then differences in the flora of healthy and unhealthy fish in a population might be expected. In this study no difference in the intestinal microflora of apparently healthy and unhealthy turbot larvae was detected (Table 6 and Figure 6) however, the overall survival of the batch of larvae examined was low. Although no increased incidence of any recognised potential fish pathogens was apparent, the dominant bacterium, *Aeromonas caviae*, was present in higher proportions than in other batches of larvae with higher survival rates in which *V. alginolyticus* dominated. One of the bacterial isolates, identified as *V. alginolyticus*, from the gut of intensively-reared turbot larvae, was of low toxicity to larvae of *Crassostrea gigas* and did not produce ciliostatic toxins in contrast to most of the fish- and shellfish-pathogenic vibrios surveyed by Nottage *et al.* (1989). Gatesoupe (1990) also noted that *V. alginolyticus* was predominant in healthy turbot larvae and an opportunistic *Aeromonas* species was frequently observed in tanks where the larvae were on the verge of high mortalities. Tanasomwang and Muroga (1988) noted that *V. alginolyticus* was frequently isolated from larval Japanese flounder where no apparent fish mortalities occurred. Conversely, Iwada *et al.* (1978) observed high mortalities of young red seabream when the rearing water was inoculated with *V. alginolyticus* previously isolated from diseased fish. The presence of rotifers appeared to increase mortalities whereas *Chlorella* had a protective effect. *V. alginolyticus* was also associated with mass mortalities of black seabream (Kusuda *et al.*, 1986). Three species, *V. alginolyticus, V. nereis* and *Alcaligenes cupidis*, were dominant in the microflora of moribund fish. *V*

alginolyticus and *A. cupidis*, but not *V. nereis*, gave high mortalities of fish by waterborne infection or oral infection by feeding rotifers inoculated with bacteria. Thus, although *V. alginolyticus* has often been associated with mortalities in other studies, the strain isolated here appeared to be avirulent.

In one experiment where turbot larvae were reared in extensive (low density) conditions and fed rotifers from an extensive source outside the hatchery, the gut microflora was much more varied than that of intensively-reared larvae. This was evident from the high degree of variation in colony morphology on the plate cultures. Although only 9 isolates were identified at least 6 species were present (Table 7).

In a further experiment, turbot larvae were reared intensively on rotifers cultured in the hatchery (Tank B1), extensively with rotifers from the same source (Tank B2) or extensively with rotifers from an extensive source outside the hatchery (Tank B10). The gut of the extensively-reared larvae from tank B10 was colonised more slowly than that of intensively-reared larvae (Figure 7) and the level of presumptive vibrios detected on TCBS agar also increased more slowly in this tank. Thus, there appeared to be a correlation between a slow rate of colonisation and improved survival rates (Table 8).

In all three groups of larvae, vibrios were the dominant bacteria (Table 9) and although most similarity occurred between the isolates from tanks B1 and B2, with 6 clusters, similarities were found between all three groups (Figure 11). This suggested that the slower rate of colonisation, rather than the bacterial flora itself, was responsible for the increased survival and growth rates of the larvae reared under extensive conditions in this experiment.

Further evidence that the rate of colonisation is an important factor in larval survival rates was seen in experiments involving copepod-fed turbot larvae. The rate of colonisation of the gut of larvae fed copepods at G.S.P. was slower than that of either intensively- or extensively-reared larvae fed rotifers (Figure 12) and survival rates of copepod-fed larvae were high. An extremely low bacterial

count was obtained from 9-day-old, extensively-reared larvae fed copepods in Norway where survival rates were high. However, this could be attributed to the lower rearing temperature (9-13°C) compared to that of intensive rearing systems (20°C).

The bacterial flora of copepod-fed larvae was also dominated by *Vibrio* species (Tables 10 and 11) but there was no similarity between the isolates from copepod-fed and rotifer-fed larvae (Figures 13 and 14) or between isolates from Scottish or Norwegian copepod-fed larvae (Figures 16 and 17). Unfortunately, samples of the live food organisms in the larval rearing tanks were not taken so no relationship between the bacterial levels in the larvae and their diet could be determined. Nicolas *et al.* (1989) attributed the failure of turbot larvae given *Pavlova*-fed rotifers to ingest the prey and survive beyond 8 days posthatch, to the high bacterial load of *Pavlova*-fed rotifers, since 40-60% of larvae given *Platymonas*-fed rotifers were still alive at 10 days posthatch and the bacterial load of *Pavlova*-fed rotifer was up to 25 times higher than that of *Platymonas*-fed rotifers. No significant difference between the number of bacteria per fish from the two groups of larvae was detected but the larvae were only sampled twice (at 4 days and 6 or 7 days posthatch). These results are compatible with the proposition that the survival rate is influenced by the rate of colonisation of the gut as a faster colonisation rate is likely to result from more heavily contaminated rotifers.

4.2 **Bacterial Flora of Rotifers**

In the standard rotifer culture system at G.S.P. the level of bacteria associated with rotifers varied from 100 to more than 10^4 c.f.u. per rotifer depending on the age of the culture and whether the rotifers were enriched with algae. The *Vibrio*/*Aeromonas* group was the most common among the bacterial isolates from rotifers (Table 2), not surprisingly since this group was also dominant in seawater samples from Hunterston surveyed over a period of 12 months (results not shown).

The detergent benzalkonium chloride markedly reduced the number of bacteria associated with rotifers (Figure 22) which suggested that the bacteria might be located predominantly on the external surface of the rotifers rather than in the gut. Scanning electron microscopy confirmed this since the numbers of bacteria visible on the rotifer surface equated well with the numbers cultured on marine agar (Table 12). Rinsing with benzalkonium chloride solution for 30 seconds removed 95% of the surface bacteria compared to untreated rotifers and 85% compared to sterile seawater-rinsed rotifers (Table 12). In view of the fact that rotifers almost certainly consume bacteria by passive uptake while feeding on algae, or by active filtration, the period of time required for the bacteria to be degraded or passed through the alimentary canal and excreted, the gut passage time, must be very short. Starkweather and Gilbert (1977) calculated the gut passage time of the rotifer *Brachionus calyciflorus* by radioisotope determination of the feeding rate, to be approximately 20 minutes. It may be even shorter depending on the temperature, food concentration or nutritional state of the rotifers.

Sochard *et al.* (1979) isolated fewer bacteria from washed copepods ("gut-surface" samples) than from the copepod washings (surface samples), indicating that the majority of the bacteria associated with copepods were also external. Again in a scanning electron microscopical study of *Thersitina gasterostei* Pagenstecher, Donoghue (1986) observed large numbers of bacteria on the surface of this parasitic copepod. In studies on the digestive tract and surface of two marine wood-boring isopods and a marine amphipod Boyle and Mitchell (1978) detected no microorganisms by scanning electron microscopy. However, the outer surfaces of these crustaceans supported a dense bacterial flora. In contrast, Austin and Allen (1981) obtained no evidence of surface bacterial colonisation of the brine shrimp *Artemia salina*, either by scanning electron microscopy or by plating techniques. However, many bacteria were loosely associated with the surface of *Artemia* but these were removed by gentle washing with sterile seawater.

The demonstration of a largely surface-associated microflora on rotifers

which could be readily depleted by decontamination was a significant finding as decontaminated rotifers might yield the slow gut bacterial colonisation rate considered desirable for good larval survival rates. Alternatively, they could be recolonised with probiotic bacteria and used as vectors to deliver such bacteria to the larval turbot gut.

4.3 Decontamination of Rotifers

The majority of attempts by others to reduce the number of bacteria associated with rotifers have involved the use of antibiotics (see pages 29-30). The use of antibiotics was considered undesirable in the present study due to the possible selection of antibiotic resistant strains of bacteria which could cause disease problems in the later stages of turbot production. Therefore, other chemical and physical treatments were investigated for their ability to reduce the bacterial load of rotifers. Most of the chemical agents tested, however, were toxic to rotifers or did not significantly reduce their bacterial load (Table 14, Figure 26). The broad spectrum biocides, Phylatol (di(2hydroxyethoxy)-methane) and Panacide (dichlorophen), were also ineffective in killing bacteria under conditions which were not toxic to rotifers (Figure 27). Numerous chemical disinfectants have been described (reviewed by Russell *et al.*, 1982) but a comprehensive study was not conducted since most are likely to be toxic to rotifers.

The bacteriolytic enzyme, lysozyme, was selected as a potential rotifer decontaminating agent because its substrate, peptidoglycan, is unique to the cell walls of bacteria. Since its discovery by Fleming in 1922, the structural and functional properties of lysozyme have been well characterised (reviewed by Jolles and Jolles, 1984). Gram-negative bacteria, generally resistant to the action of lysozyme due to the protection afforded by their outer membrane, are rendered sensitive if the outer membrane is damaged by prior treatment with, for example, EDTA (Repaske, 1956).

A salinity of 0.2% seawater was determined to be optimal for lysozyme activity

against freeze-dried *Micrococcus lysodeikticus* and overnight cultures of *Pseudomonas* 1.1.1 and *V. alginolyticus* (3-8) (Figure 28). Full-strength seawater at a salinity of 3.2% completely inhibited lysozyme. In a comparison of hen-egg-white lysozyme and lysozyme from the mussel, *Mytilus edulis*, McHenery and Birkbeck (1982) described the optimum conditions for hen-egg-white lysozyme as pH 7.5-8.0 at an ionic strength of 0.054 (Figure 47). The *Mytilus* lysozyme was optimally active at an ionic strength of 0.011, pH 7.1, and a secondary optimum was found at an ionic strength of 0.054, pH 4.6. It is not surprising therefore, that full-strength seawater, with an ionic strength of approximately 0.682 (calculation based on the composition of seawater given in Dawson *et al.*, 1986) and a pH of 8.2, inactivated hen-egg-white lysozyme.

Lysozyme in seawater of 0.2% salinity, reduced the bacterial load of rotifers by over 80% (Figure 32) and, as found with the suspensions of bacteria, increasing the salinity decreased the effectiveness of lysozyme (Figure 33). The concentration of rotifers also affected lysozyme activity, a low rotifer concentration giving greater bactericidal activity by lysozyme than a high rotifer concentration (Figure 34). It is conceivable that lysozyme was adsorbed to the surface of the rotifers themselves since they have a chitinous shell or lorica. Chitin is also a substrate for lysozyme (Skujins *et al.*, 1973). The structures of chitin, a ß(1-4) linked polymer of N-acetyl-glucosamine, and peptidoglycan, a ß(1-4) linked polymer of N-acetyl-muramic acid and N-acetyl-glucosamine, are similar (Figure 48).

Addition of EDTA to a concentration of 0.1mM did not enhance the activity of lysozyme, indeed it appeared to have the opposite effect (Figure 35). The use of calcium-free seawater to avoid the possibility of EDTA being bound by free calcium ions in the seawater, did not improve the effect of EDTA and lysozyme (Figure 36). Concentrations of EDTA above 0.1mM were extremely toxic to rotifers.

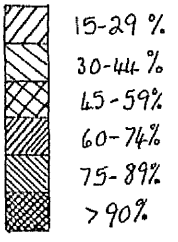
In two field trials at G.S.P. lysozyme-treated rotifers were used as food for turbot larvae but the osmotic shock due to decreasing the salinity from approximately 2.0% to 0.2% caused the rotifers to stop swimming and filter-feeding, even when a step-

FIGURE 47

Effect of pH and ionic strength on activity of hen-egg-white lysozyme.

Reproduced from McHenery and Birkbeck (1982).

The results are expressed as a percentage of the optimum activity of lysozyme.



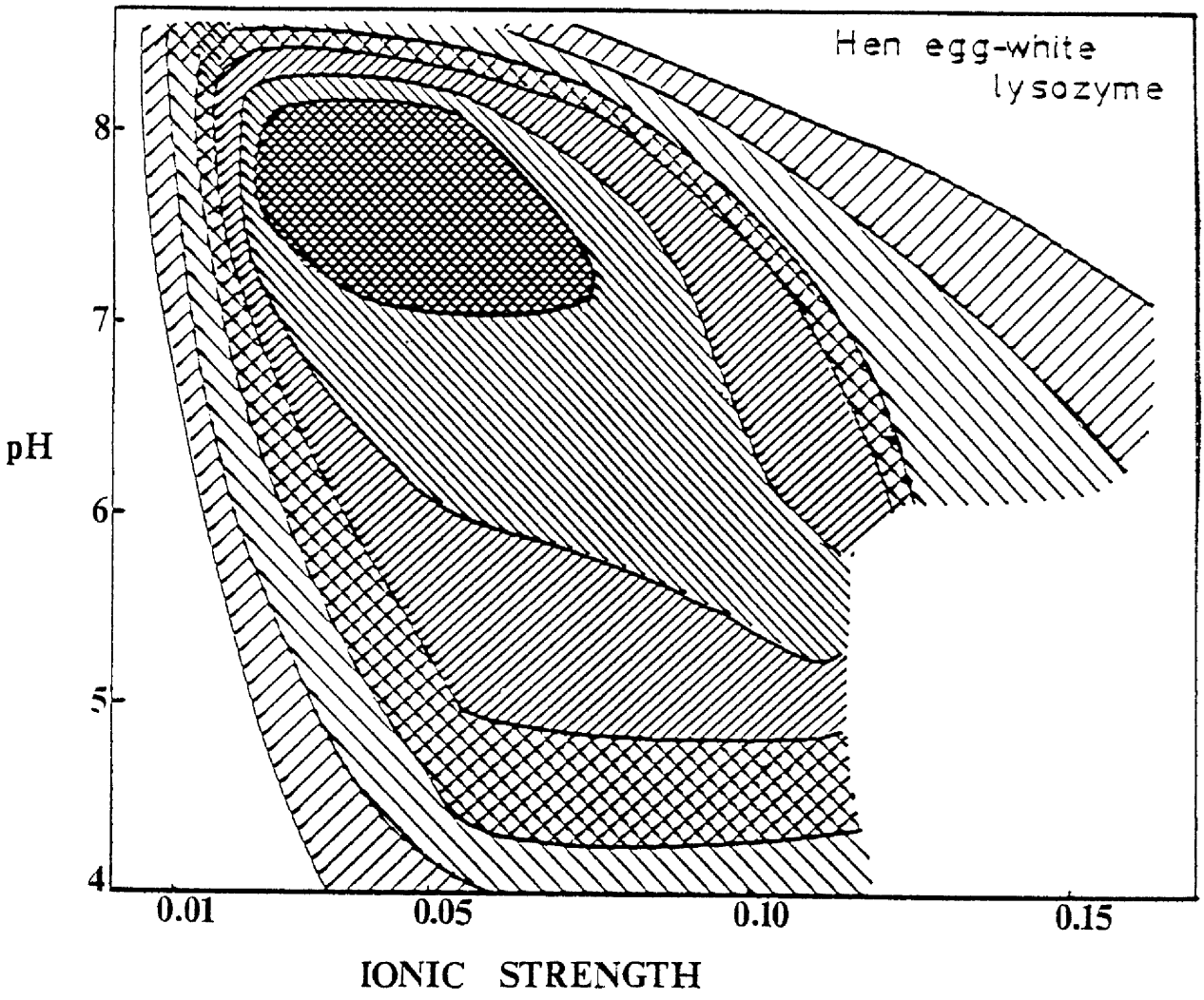

Hen egg-white
lysozyme
8
7
6
5
4
pH
0.01
0.05
0.10
0.15
IONIC STRENGTH

FIGURE 48

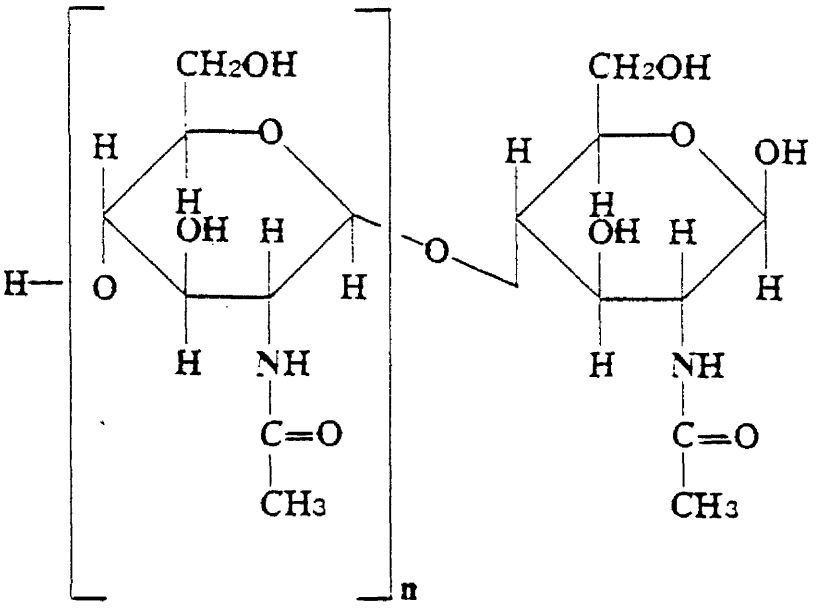
a) **Structure of chitin.**

b) **Structure of peptidoglycan.**

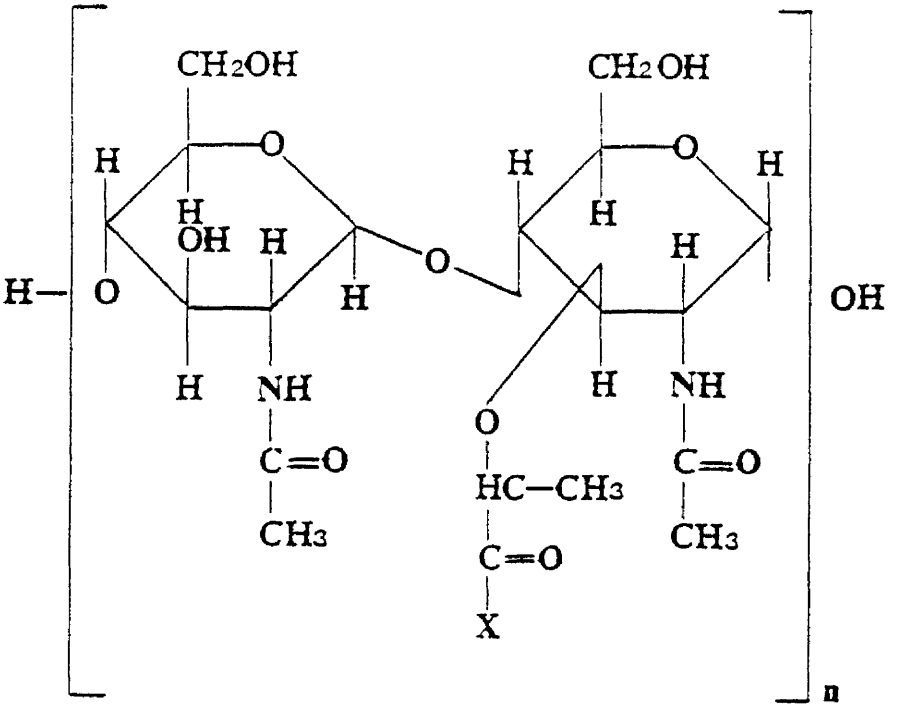
X may be OH or an amino group of a peptide through which the polysaccharide chains are cross-linked.

From Chipman and Sharon (1969).

a)



b)



wise reduction in salinity was introduced. This resulted in the rotifers settling to the bottom of the tanks and although they recovered overnight and were palatable to turbot larvae, no noticeable benefit to the larvae was derived from feeding lysozyme-treated rotifers. The nutritional value of the rotifers was probably low due to their inability to feed immediately after the lysozyme treatment and they may have been recolonised with bacteria from the bottom of the tanks.

The range of detergents and biocides investigated in the present study, in an attempt to disinfect rotifers, were either toxic to the rotifers themselves, or were ineffective at reducing the bacterial load of rotifers. Although lysozyme initially seemed a promising solution to the problem, the osmotic shock of the lysozyme treatment was deleterious. Given these facts and that the use of antibiotics was undesirable, chemical treatments were rejected in favour of ultra-violet radiation.

4.4 Elimination of Bacteria by Ultra-Violet Radiation

Viability of three test bacteria was reduced by >99% within an exposure time of 60 seconds (Figure 37). Further reduction in exposure time may be possible given the recent discovery by Bank *et al.* (1990) that pulsed modulated U.V. radiation was more effective at killing bacteria than conventional, continuous wave U.V. radiation.

The bacterial load of rotifers was dramatically reduced by U.V. radiation but regrowth of the bacterial flora of the rotifers, to the original level, occurred within 48 hours (Figure 40). Nevertheless, the number of bacteria per U.V.-treated rotifer was only approximately 40% of that of untreated rotifers at this time. The rotifers themselves appeared to be unaffected by U.V. radiation at the dose applied and their ability to feed normally was shown by an undiminished clearance of algae given as food (Figure 42). This was confirmed by visual examination of the rotifers since their guts became noticeably darker as they ingested algae.

Ultra-violet treatment of the circulating water of *B. plicatilis* and *M. macrocopa* cultures was investigated by Hayashi *et al.* (1976) in an effort to reduce bacterial contamination, but the rotifers and copepods were trapped by a plankton net and

therefore, were not exposed to U.V. radiation directly. The total viable count of bacteria in the water was reduced from 10^4-10^5 to 10^1-10^2 c.f.u. per ml at the inlet but despite this , the counts at the outlet were similar to those of untreated waters. Yamanoi and Sugiyama (1987) obtained improved results by constant exposure of a 500 litre culture to U.V. radiation by apparently immersing the U.V. lamp in the culture water. This reduced the viable count by 97% and recirculation of the culture, including the rotifers, via a U.V. lamp, reduced the viable count by >99%.

The U.V. treatment of rotifers was tested in field trials at G.S.P. with intensively-reared turbot larvae. Although an average reduction of 95% of the bacterial load of U.V.-treated rotifers was achieved (Figure 43a) and larvae grew from 3-9 days posthatch, the difference in growth rates was not significant (Figure 45). Colonisation of the gut of larvae fed U.V.-treated rotifers was slower than that of larvae fed untreated rotifers with a 10-fold difference between the test and control batches of larvae at 4 days posthatch (Figure 44). However, both batches of larvae failed to survive beyond 8 days posthatch, almost certainly due to starvation since there were insufficient numbers of rotifers available. Similar trials were conducted three times and in each case survival rates of turbot larvae were low due to lack of rotifers, but in all cases the rate of colonisation of the gut of larvae fed U.V.-treated rotifers was slower than that of larvae fed untreated rotifers. The effect of gut colonisation rate on larval survival was not clearly shown in these field trials due to the difficulties inherent in conducting experiments at a production site where some parameters, such as availability of constant supplies of rotifers, cannot be controlled.

In two field trials with extensively-reared larvae, the survival rates of larvae fed U.V.-treated rotifers were higher than those of larvae fed untreated rotifers (Table 17). Bacteriological analysis was only carried out in one of these trials and the rate of bacterial colonisation of the gut of larvae fed U.V.-treated rotifers was reduced compared to that of larvae fed untreated rotifers, again with a 10-fold difference at 4 days posthatch (results not shown). There was an average reduction of 88% of the bacterial load of U.V.-treated

compared to untreated rotifers with a range from 75 to 97%. This compares unfavourably with a mean reduction of >98% of the bacterial load of enriched rotifers under laboratory conditions. This may simply be due to less accurate calculation of the concentration of rotifers during the sampling process under field conditions, or insufficient mixing of the rotifer suspension when treating large volumes of culture. Under laboratory conditions, the concentration of algae in the rotifer suspension did not affect the efficiency of the U.V. treatment (results not shown).

Insufficient data are available concerning the microflora of U.V.-treated and untreated rotifers, to compare the two and draw any conclusions about the susceptibility of any particular group of bacteria to U.V. radiation. However, it is interesting to note that based on colony morphology of the plate cultures, the dominant groups associated with untreated rotifers, *Pseudomonas/Moraxella* (30%) and *Vibrio/Aeromonas* (19%), were much reduced after U.V. treatment, and *Flexibacter/Cytophaga* (19%) became predominant.

The most common group in the gut microflora of both batches of intensively-reared larvae prior to feeding, at 3 days posthatch, was *Pseudomonas/Alcaligenes* (79% and 74% for larvae fed U.V.-treated and untreated rotifers, respectively). After feeding, at 7 days posthatch, the gut flora of larvae fed untreated rotifers was relatively homogeneous (Figure 46), and was dominated by *Vibrio/Aeromonas* (82%). This was consistent with the earlier studies of the gut microflora of larval turbot in this project. In contrast, the gut flora of larvae fed U.V.-treated rotifers was varied and the *Pseudomonas/Alcaligenes* group (70%) was still dominant. This suggests that *Vibrio* species may be more susceptible to U.V. radiation than other bacterial groups.

4.5 Probiotics

The different gut microfloras of turbot larvae fed U.V.-treated or untreated rotifers indicates that the gut microflora of larval turbot can be modified by manipulation of the bacterial flora of their prey. This suggests that rotifers could be used as carriers to

introduce a beneficial, or probiotic, flora into the gut of larval turbot. Such a beneficial flora might function by excluding specific or opportunistic pathogens, or by providing essential nutrients or enzymes.

MacDonald *et al.* (1986) speculated on the role of bacteria in the nutrition of Dover sole; many of the bacterial isolates from the intestinal tract of Dover sole degraded *p*-nitrophenyl-ß-N-acetylglucosaminide, chitin and collagen. Kashiwada *et al.* (1971) demonstrated the production of folic acid by the intestinal bacteria of carp. Dr. E. Ringo (Tromso, unpublished results) found that a strain of *Vibrio pelagius*, isolated during the present study from the gut of turbot larvae fed copepods, contained a relatively high proportion of eicosapentaenoic acid (20:5 n-3). The importance of dietary polyunsaturated fatty acids in the development of turbot larvae has been documented (Cowey *et al.*, 1976; Bell *et al.*, 1985; Perez-Benavente and Gatesoupe, 1988) and it is widely accepted that the biochemical composition of live food organisms, such as rotifers, is affected by their diet (Scott and Baynes, 1978; Watanabe *et al.*, 1983; Ben-Amotz *et al.*, 1987; Rainusso *et al.*, 1989). Thus, the inoculation of rotifer cultures with bacteria rich in polyunsaturated fatty acids might be beneficial to larval turbot.

Bacteria-free rotifer cultures, produced in a chemostat, were used to determine the rate of uptake of bacteria by rotifers; an average of 324 bacteria were isolated per rotifer within two hours of inoculation and benzalkonium chloride treatment indicated that almost all were surface associated (Figures 21a, b and c). These results are compatible with the suggestion that bacteria adhere to the external surface of rotifers and are either not ingested, or are rapidly degraded within the gut. Muroga and Yasunobu (1987) inoculated conventional rotifer suspensions with *V. anguillarum* and calculations based on their results indicate that approximately 800 c.f.u. of *V. anguillarum* were accumulated per rotifer. This is comparable with the result obtained in the present study and the fact that the bacterial level did not decrease on transfer to fresh seawater is consistent with the external surface as the location of bacteria associated with rotifers. However, rotifers can be cultured using bacteria as the sole

food source. Yasuda and Taga (1980) found two strains of *Pseudomonas* capable of supporting growth of *Brachionus plicatilis*. Starkweather *et al.* (1979) noted that *Brachionus calyciflorus* could be cultured on *Aeromonas aerogenes*, but feeding rates of rotifers fed bacteria only were consistently lower than those determined with larger food types, such as the yeast, *Rhodotorula glutinis*.

Hatchery-reared rotifers were inoculated with *V. alginolyticus* (3-8), isolated from healthy, intensively-reared turbot larvae, in an attempt to dominate the bacterial flora of rotifers and subsequently colonise the gut of larval turbot. Although the viable counts of bacteria in the gut of the larval turbot increased 100-fold one hour after feeding, this level decreased over the following 24 hours (Figure 19), indicating that the bacteria failed to survive or did not stably colonise the gut. No noticeable benefit was derived from feeding turbot larvae with rotifers inoculated with *V. alginolyticus* (3-8) indicating that the choice of strain may be extremely important in achieving colonisation or in dominating the microflora. Strain 3-8 was chosen because of its dominance in the flora of intensively-reared turbot larvae with a high survival rate and because of its lack of toxicity towards ciliated cells. However, it may be necessary to consider other properties of potential colonising strains such as their ability to inhibit growth of other microorganisms. Westerdahl *et al.* (1991) recently considered a similar approach to inhibit colonisation of the turbot gut by *V. anguillarum*. Of more than 400 bacteria isolated from adult turbot, 89 produced bacteriocins active against *V. anguillarum*. Several bacteriocin-producing strains were selected and shown to multiply in gut mucus (Olsson *et al.*, 1992), however, colonisation experiments have not been reported for these isolates. In the present work 50 isolates were screened by cross-streaking for their ability to inhibit growth of other bacteria isolated from turbot larvae but only one strain, which comprised a minor component of the larval microflora, had a limited antibacterial effect (results not shown). Other factors, such as the ability to adhere, growth rate and the ability to utilise substrates in the turbot gut, including the chitinous lorica of rotifers, may be just as important for a successful colonising strain.

Other authors have mainly concentrated on the use of "lactic acid" bacteria as potential probiotics. Gatesoupe *et al.* (1989) examined the effect of two food additives containing live bacteria on the production rate of rotifers and their dietary value as food for Japanese flounder. Adjulact, a spray-dried powder containing *Streptococcus thermophilus* and *Lactobacillus helveticus*, improved the production rate of rotifers, whereas, Acosil, a spray-dried extract from sprouting cereal grains fermented with selected lactic acid bacteria, improved their nutritional quality for fish. The improved dietary value of rotifers given Acosil was considered to be due to restriction of the bacterial population of enriched rotifers, although absolute differences were small. In further work, enrichment of rotifers with Adjulact, Acosil, and also spores of *Bacillus toyoi*, improved the survival and growth rates of turbot larvae (Gatesoupe, 1989). Additional improvement was achieved by antibiotic treatment (Gatesoupe, 1990) but due to the highly variable survival rates in these experiments firm conclusions cannot be drawn about the benefits of the probiotics used. Recently, Gatesoupe (1991) showed that three additives, *Lactobacillus plantarum, L. helvetica* and *Streptococcus thermophilus*, improved the nutritional value of rotifers since the mean weight of turbot larvae at day 20 was significantly increased when the rotifers were fed any of the additives. The beneficial effect of *L. plantarum* was considered to be due to its ability to reduce the number of bacteria in the rotifer cultures.

Inoculation of the rearing water of larval turbot with live bacteria, in an attempt to colonise the gut of the larvae directly, is an alternative to the use of rotifers as vectors for probiotics (Ringo E., Vadstein O., Strom E., Hjelmeland K., Ugelstad I., Rosenlund G. and Olafsen J.A. unpublished results) but none of the bacteria tested appeared to have a beneficial effect on larval turbot. Indeed, the increased level of stress caused by high concentrations of bacteria in the water could have a deleterious effect on larvae.

Although the inoculation of the rearing water with high concentrations of bacteria may affect the number of bacteria in the gut of larval turbot, this was not attempted in

the present study since colonisation of rotifers appeared to be a more efficient method of introducing bacteria into the larval gut. Actively feeding turbot larvae consume at least 100 rotifers per day (A. Barbour, personal communication) and since rotifers are associated with 10^3 to 10^4 c.f.u. per individual, the rotifer could contribute 10^5 to 10^6 bacteria per fish per day.

FUTURE WORK

A slow rate of colonisation of the larval turbot gut was correlated with improved growth and survival rates, and U.V. treatment of rotifers certainly appeared to be a practical method of reducing the bacterial load of rotifers. The encouraging results of initial field trials with U.V.-treated rotifers should be repeated in several field trials with high quality larvae and adequate supplies of rotifers. Only in this way can the U.V. treatment method be properly evaluated. In existing literature, too much reliance is placed on the value of single experiments whereas several repetitions of experiments are needed in field trials, given the many variable parameters which are difficult to control.

Although reduction in the bacterial load of rotifers was achieved by U.V. treatment a comprehensive study of the flora of U.V.-treated and untreated rotifers is essential to determine whether certain generic groups of bacteria are more susceptible to U.V. radiation than others. Much of the value of reduction of the bacterial load would be negated if pathogens or deleterious bacteria were more resistant to U.V. radiation than other bacteria.

The possibility that certain bacteria might be advantageous to turbot larvae certainly deserves more attention in the future. Probiotic bacteria could be selected for by their origin, i.e. from healthy fish, their attachment ability, their inhibitory effects against known fish pathogens, their ability to degrade complex substrates such as chitin or their fatty acid profile. The colonisation of rotifers with defined bacteria would certainly be an efficient method of conveying a beneficial microflora to the larval gut. Ideally, bacteria-free turbot larvae fed bacteria-free rotifers inoculated with selected strains of bacteria would be used in laboratory studies to evaluate the potential beneficial effects of a range of bacteria. Conversely, this system would be valuable for screening for pathogenic bacteria. Thus, a profile of the bacteria associated with the current rearing system may be constructed so that the presence of potentially deleterious bacteria could be regularly monitored. The current method of monitoring the bacterial flora of the larval rearing system by the use of routine biochemical tests to identify bacteria is extremely time consuming and more modern

techniques, such as ELISA or probes based on 16s RNA sequences would be needed so that action could be taken before significant numbers of deleterious bacteria accumulate in the rearing system. Such analytical methods will also be valuable in screening juvenile and adult fish for the presence of pathogens as the microbiological problems of rearing turbot are not restricted to the larval stages.

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APPENDICES

APPENDIX I

Acid from carbohydrates (Cowan and Steel, 1965)

Phenol Red Broth:

Peptone water (Oxoid)	10gm
NaCl	10gm
Phenol red	0.018gm
Distilled water	1 litre

The pH was adjusted to 7.1 and the medium autoclaved at 121°C for 15 minutes. Filter-sterilised carbohydrate was added aseptically to a final concentration of 1% for arbutin and sucrose, or 0.5% for salicin. The media were aseptically distributed in 1ml amounts into sterile tubes, inoculated and incubated at 20°C for up to 14 days. Acid production was indicated by a yellow colour.

Aesculin Hydrolysis (Lee and Donovan, 1985)

Aesculin agar:

Tryptone (Oxoid)	10gm
Aesculin	1gm
Ferric citrate	0.5gm
NaCl	10gm
Technical agar No. 3 (Oxoid)	15gm
Distilled water	

The agar, tryptone and NaCl were dissolved by heating then the aesculin and ferric citrate were added. The medium was autoclaved at 115°C for 10 minutes and poured into petri dishes. The plates were spot inoculated with no more than 12 isolates per plate and incubated at 20°C for 5 days. Blackening indicated aesculin hydrolysis.

Amylase (Cowan and Steel, 1965)

Starch agar:

Potato starch	10gm
NaCl	10gm
Distilled water	50ml
Nutrient agar (Oxoid)	1 litre

Lugol's iodine solution

Iodine	5gm
KI	10gm
Distilled water	100ml

The starch was mixed with water to a smooth cream and added to the molten nutrient agar supplemented with NaCl before autoclaving at 115°C for 10 minutes. After cooling, the agar was poured into petri dishes. The plates were spot inoculated and incubated at 20°C for 5 days then flooded with Lugol's iodine solution. A positive result was indicated by a clear zone round the colony.

Decarboxylase tests (Furniss *et al.*, 1979)

Decarboxylase medium (Difco) was prepared with the addition of 1% NaCl, 0.4% $MgCl_2.H_2O$ and 0.4% KCl. For convenience, the medium was dispensed into 4 x 100ml volumes and the amino acids L-lysine, L-arginine and L-ornithine added to a final concentration of 1%. The 3 amino-acid media and the basal medium were distributed into 5ml screw-capped bottles and covered with a layer of liquid paraffin before autoclaving at 121°C for 15 minutes.

The 4 bottles were inoculated through the liquid paraffin and incubated at 20°C for 4 days. Vibrios should show acid (yellow colour) in the blank. A positive result was indicated by an alkaline reaction (purple colour).

Gelatinase (Cowan and Steel, 1965)

Gelatin Agar:

Gelatin	4gm
Distilled water	50ml
NaCl	10gm
Nutrient agar (Oxoid)	1 litre

Acid mercuric chloride

Mercuric chloride	12gm
Distilled water	80ml
Conc. HCl	16ml

The mercuric chloride was mixed with water before addition of acid.

The gelatin was dissolved in distilled water and added to the nutrient agar supplemented with NaCl. The medium was autoclaved at 121°C for 15 minutes then poured into petri dishes. The plates were spot inoculated and incubated at 20°C for 3 days. When the surface was flooded with acid mercuric chloride; clear zones indicated gelatin hydrolysis.

Haemolysis of sheep red blood cells (Furniss *et al.*, 1979)

Brain Heart Thioglycolate Cysteine Agar:

Brain heart infusion broth (Oxoid)	500ml
10% sodium thioglycolate solution	2.5ml
10% L-cysteine in 10% HCl	2.5ml
Technical agar No. 3 (Oxoid)	5gm

The medium was autoclaved at 121°C for 15 minutes, cooled to 51°C and 20ml of a fresh 20% saline suspension of washed sheep red blood cells was added. Plates were spot inoculated (not more than 12 isolates per plate) and incubated at 20°C for 24 hours. A positive result was indicated by a clear zone around the colony.

Indole Production (Cowan and Steel, 1965)

Kovac's reagent

p-dimethylaminobenzaldehyde	5gm
isoamyl alcohol	75ml
Conc. HCl	25ml

The aldehyde was dissolved in isoamyl alcohol by warming to 50°C, cooled and the acid added. The reagent was stored in the dark at 4°C.

A few drops of Kovac's reagent were added to 1ml of a 24-hour culture in 1% tryptone (Oxoid) supplemented with 2% NaCl. A positive result was indicated by a red ring on the surface.

Lecithinase (Furniss *et al.*, 1979)

Lecithin Agar:

egg yolk emulsion	10ml
NaCl	10gm
Nutrient agar (Oxoid)	1 litre

The nutrient agar supplemented with NaCl was autoclaved at 121°C for 15 minutes, cooled to 51°C and the egg yolk emulsion added before pouring into petri dishes. The plates were spot inoculated and incubated at 20°C for 5 days. A positive result was indicated by an opaque zone around the colony.

Nitrate Reduction (Lee *et al.*, 1979)

Nitrate Broth:

KNO	1gm
NaCl	10gm
Nutrient broth (Oxoid)	1 litre

Solution A

Sulphuric acid	0.5gm
Glacial acetic acid	30ml
Distilled water	120ml

Solution B

1-naphthylamine-7 sulphonic acid	0.2gm
Glacial acetic acid	30ml
Distilled water	120ml

Nitrate broth was distributed in 1.0ml amounts into tubes and autoclaved at 121°C for 15 minutes. Inoculated broth was incubated at 20°C for 4 days. Solution A (1ml) was added followed by 1ml of solution B; a red colour indicated nitrate reduction to nitrite. To tubes not showing a red colour, powdered zinc (up to 5mg per ml) was added; a red colour indicated nitrate present in the medium (i.e. no reduction), whereas no red colour indicated reduction of nitrate and nitrite.

ONPG Test (Cowan and Steel, 1965)

ONPG Solution:

ONPG (Koch-Light Laboratories Ltd.)	6gm
0.01 M Na_2HPO_4	1 litre

ONPG (o-Nitrophenyl-ß-D-galactopyranoside) was dissolved in the phosphate solution at pH 7.5 at room temperature, sterilised by filtration and stored in the dark. ONPG solution (250ml) was added to 750ml sterile peptone water (Oxoid) supplemented with 1% NaCl and distributed into 1ml amounts. The inoculated medium was incubated at 20°C for 24 hours. A positive result, i.e. ß-galactosidase activity, was indicated by a deep yellow colour.

Oxidase Test (Furniss *et al.*, 1979)

A few crystals of tetramethyl-*p*-phenylene diamine hydrochloride were dissolved in 10ml distilled water and used to soak filter paper. A small portion of a colony was rubbed onto the paper. A nichrome wire should not be used but platinum, wood or glass are satisfactory. A positive result appeared as a violet colour within a few seconds.

Utilisation of single carbon sources (Baumman *et al.*, 1971)

Artificial seawater

NaCl	0.4M
$MgSO_4.7H_2O$	0.1M
KCl	0.02M
$CaCl_2.2H_2O$	0.02M
Distilled water	1 litre

Basal medium

Tris HCl (pH 7.5)	50mM
NH_4Cl	190mM
$K_2HPO_4.3H_2O$	0.33mM
$FeSO_4.7H_2O$	0.1mM
Half-strength artificial seawater	1 litre

Basal medium agar was prepared by separately sterilising, then mixing, equal volumes of double-strength basal medium and 20gm per litre purified agar (Oxoid), then distributed into 200ml amounts. Each test substance was dissolved in 10ml distilled water, filter-sterilised and added to 200ml of basal medium agar to give a final concentration of 0.1%. The media were poured into petri dishes and the isolates were replica plated (no more than 12 isolates per plate).

Voges-Proskauer Test (Furniss *et al.*, 1979)

Voges-Proskauer (V-P) Medium:

Yeast extract (Oxoid)	1gm
Bacteriological peptone (Oxoid)	12gm
Glucose	10gm
NaCl	10gm
Technical agar (Oxoid)	3gm
Distilled water	1 litre

Solution A

5% Ó-naphthol in absolute alcohol

Protected from light and stored at 4°C

Solution B

KOH	40gm
Creatine	0.3gm
Distilled water	100ml

The pH was adjusted to 7.0 and the V-P medium distributed into 3ml amounts. The medium was inoculated by stabbing and incubated at 20°C for 24 hours then 0.2ml of solution A and 0.1ml of solution B were added. The results were read after 15 minutes and a positive result was indicated by a red ring at the surface of the agar.

APPENDIX II

Iodine Solution

KI	3gm
I_2	2gm
Distilled H_2O	to 50ml

The KI was dissolved in a little of the distilled H_2O then the iodine was addded and the volume made up to 50ml with distilled H_2O. The solution was diluted 1:100 for use.

APPENDIX III

Algal Growth Medium (Turner *et al.*, 1979)

Dry Mix

NaCl	320gm
KCl	8gm
$MgSO_4.7H_2O$	50gm
$CaSO_4.2H_2O$	10gm
Glycine	5gm
KNO_3	2gm
KBr	0.65gm
K_2HPO_4	0.2gm
Na_2 EDTA	1gm
$FeSO_4.7H_2O$	0.05gm

Solution A

KI	0.025gm
$SrCl_2.6H_2O$	6.5gm
$AlCl_3.6H_2O$	0.25gm
$RbCl_2$	0.1gm
LiCl	0.05gm
Distilled H_2O	1 litre

Solution B

$MnSO_4.4H_2O$	0.203gm
$ZnSO_4.7H_2O$	0.022gm
$CuSO_4.5H_2O$	0.0196gm
$CoSO_4.7H_2O$	0.0013gm
$NaMoO_4.2H_2O$	0.00126gm
Distilled H_2O	1 litre

Vitamin B1 Solution

Vitamin B1	0.005gm
Distilled H_2O	100ml

Vitamin B12 Solution

Vitamin B12	0.0001gm
Distilled H_2O	1 litre

Dry mix (19.85gm), 5gm NaCl, 0.5gm $MgSO_4.7H_2O$ and 0.5gm glycylglycine were dissolved in 1 litre distilled H_2O. Aliquots of 1.0ml of solution A, solution B, vitamin B1 solution and vitamin B12 solution were added. The pH was adjusted to 8.0 and the medium was autoclaved at 121°C for 15 minutes.

APPENDIX IV

Fixative for Scanning Electron Microscopy

1.0M sodium cacodylate	10ml
25% glutaraldehyde	8ml
1.0M NaCl	5ml
1.0M $CaCl_2$	0.05ml
Filtered seawater	50ml
Distilled H_2O	make up to 100ml

pH 7.6

Buffer for Scanning Electron Microscopy

1.0M sodium cacodylate	10ml
1.0M NaCl	20ml
1.0M $CaCl_2$	0.05ml
Filtered Seawater	50ml
Distilled H_2O	make up to 100ml

pH 7.8

APPENDIX V

Sodium Phosphate Buffer for Lysozyme Assay

Solution A: 0.2M $NaH_2 PO_4$

Solution B: 0.2M $Na_2 HPO_4$

Solution A (44.1ml) and solution B (15.9ml) were mixed and made up to 200ml with distilled water. NaCl (2.0gm) was added and the pH was adjusted to 7.2.

APPENDIX VI

Calcium-Free Seawater

NaCl	20.758gm
$Na_2SO_4.10H_2O$	7.889gm
KCl	0.587gm
$NaHCO_3$	0.17gm
KBr	0.0845gm
H_3BO_3	0.0225gm
NaF	0.0027gm
Distilled H_2O	1 litre

Calcium-free seawater was diluted to a salinity of 0.2% with distilled water for use in the lysozyme assay.

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