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The Use of Cationic Surfactants in Marine Anti-fouling Applications

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August 1997

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Dedicated to my late Parents.

Abstract

The protection, from fouling, of marine sensors is at present one of the major problems facing those wishing to collect data from the world's oceans. Sensors, such as transmissometers, have their ability to monitor light levels reduced to practically zero within seven days during marine trials in Scottish coastal waters. In this study the use of cationic surface-active agents (surfactants), in particular quaternary ammonium compounds and biguanides, incorporated into hydrogels are investigated as possible anti-fouling protection for such sensors. In this research it has been shown that the dual chemical and physical properties of some quaternary ammonium compounds and biguanides prevent marine fouling, including microfouling, for up to 15 weeks. Due to the ability of these materials to form micelles their release is slowed down and they are able to remain bound to their polymeric substrate, hydrogel. The slow to zero release of these chemicals makes them attractive alternatives to the current toxic anti-fouling materials.

The qualitative and quantitative analysis of the quaternary ammonium compounds and the biguanides are considered and successful, and robust, analytical methods are developed and used within this study. The principles behind the analytical methods are explained, as is the credibility of the chosen technique.

Acknowledgements

My special thanks go to Professor Mike Cowling for his supervision and help throughout this research. I also wish to thank Dr Harry Duncan for his expert comment and consistent encouragement, and Dr Agnes Parr for all her useful advice and helpful discussion.

Thanks also goes to Mr Alan Birkbeck and Mr Alex Torry for their workshop skills in the preparation of acrylic racks, and to the staff of the University Biological Marine Station Millport for allowing me to use their facilities. Thanks also to Miss Isabelle Lawson and Miss Lynn Cullen for their secretarial advice.

I would like to express my thanks to Anne and Susan for inspiring me to embark on this post-graduate course.

Lastly, I would like to just **thank** Norrie.

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Abbreviations

ATAB	-	alkyltrimethylammonium bromide
ATP	-	adenosine triphosphate
AUFS	-	Attenuated Units Full Scale
B-50	-	Arquad B-50
BAC	-	benzalkonium chloride
BP	-	British Pharmacopoeia
CG	-	chlorhexidine gluconate
CMC	-	critical micelle concentration
CTAB	-	cetyltrimethylammonium bromide
di-EDTA	-	ethylenediaminetetraacetic acid, di-sodium salt
DMHTB-75	-	Arquad DMHTB-75
DMMCB-50	-	Arquad DMMCB-50
EDTA	-	ethylenediaminetetraacetic acid
EMR	-	Electron Magnetic Resonance
GMTC	-	Glasgow Marine Technology Centre
HPLC	-	high performance liquid chromatography
ICI	-	Imperial Chemicals Industry
IO	-	Instant Ocean
k'	-	Capacity Factor
m	-	metres
M	-	Molar
MAST	-	Marine Science and Technology
min	-	minutes
ml	-	millilitres
mol ⁻¹	-	moles per litre
N	-	theoretical number of plates
nm	-	nanometres
NMR	-	Nuclear Magnetic Resonance

pKa	-	dissociation constant
PC	-	Personal Computer
prep	-	preparation
QAC	-	quaternary ammonium compounds
RP-HPLC	-	reverse phase high performance liquid chromatography
Surfactant	-	surface active agent
t	-	time
TBT	-	tri-butyl tin
TEA	-	triethylamine
tetra-EDTA	-	ethylenediaminetetraacetic acid, tetra-sodium salt
tri-EDTA	-	ethylenediaminetetraacetic acid, tri-sodium salt
UK	-	United Kingdom
UMBSM	-	University Marine Biological Station Millport
USA	-	United States of America
USP	-	United States Pharmacopoeia
UV	-	ultra-violet
V	-	volume
v/v	-	volume/volume
w/v	-	weight/volume
w/w	-	weight/weight
α	-	Selectivity Factor
λ	-	wavelength
μ	-	micro

Chapter 1

Introduction and Research Aims

- 1.1 Introduction
- 1.2 Sensors
- 1.3 Biofilm Formation and Fouling
- 1.4 Active Substances
- 1.5 Mechanisms of Active Substances
- 1.6 Research Aims
- 1.7 References

1.1 Introduction

The problem of protecting any structure from marine fouling when it is immersed in seawater is age-old and throughout the centuries the quest for a successful anti-fouling surface has continued. The first recorded use of protection of ships' hulls was in the 5th Century B.C. [1] and ever since a great variety of deterrents have been utilised. Some of which have proved to be successful anti-fouling agents, but at what environmental cost? During the last six decades many anti-fouling methods have been proposed. These have varied in their mechanism of action, from isotope introduction and toxic leaching paints to electrical systems, ultrasonics and periodic underwater hull cleaning [1]. At present the great majority of anti-fouling coatings are based on copper compounds, organotins and their derivatives such as tri-butyl tin (TBT) and their combinations. Existing legislation allows the use of cuprous oxide as a bio-active material but the use of organotin is more tightly controlled [2]. The tighter control on organotin products came into force in 1986. This legislation was due to the effects these materials had on development of shellfish such as oysters and mussels [3,4]. Legislation in many parts of the world now prohibits the use of organotin anti-fouling paints on boats less than 25 metres in length [5] and this legislation may eventually extend to other craft.

The task faced in this area of research is to protect underwater sensors from fouling although the solution for sensors may be able to be transferred to protect other submerged structures.

1.2 Sensors

The deployment of underwater sensors for the measurement of light levels, fluorescence, pH, conductivity, oxygen, temperature and turbidity has, due to improved oceanic measurement techniques, become possible. Furthermore, there is a need for non-toxic antifouling agents to protect these instruments if stable measurements have to be maintained over long term exposure trials.

Figure 1.2.1 shows a typical sensor. This instrument is used to measure transmitted light from a source at one end of the instrument, through a fixed path length in the ocean, to a detector at the other end.

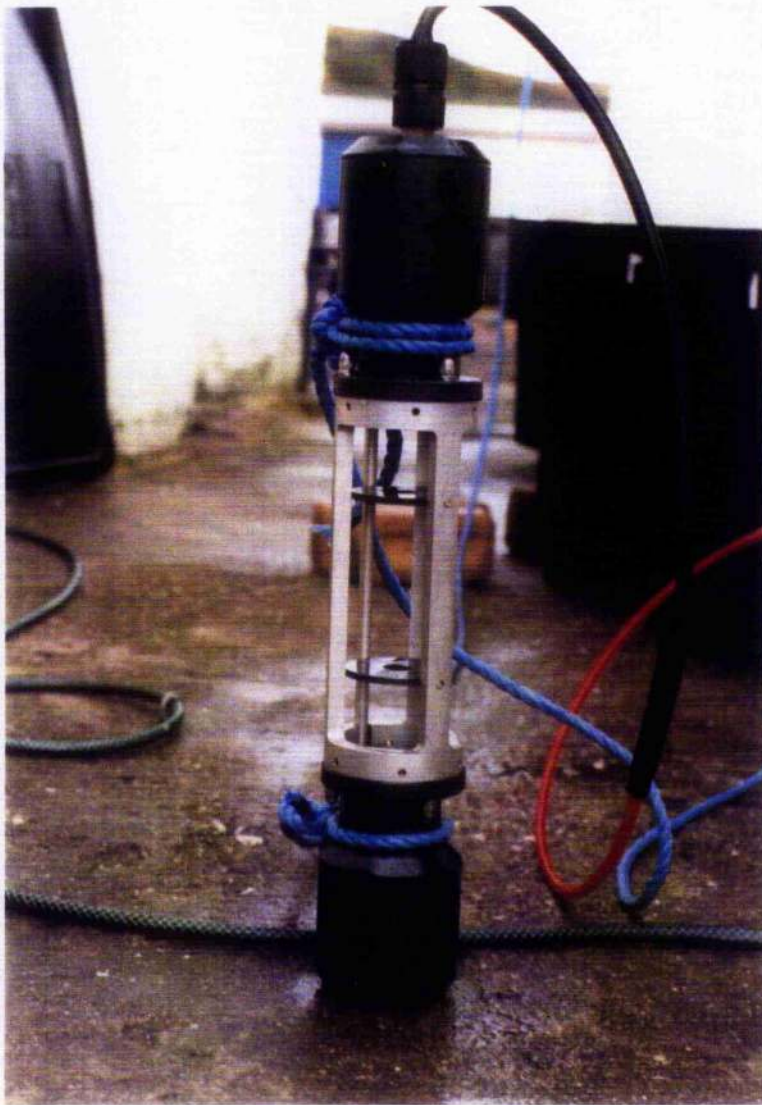


Figure 1.2.1 A transmissometer.

All parts of such an instrument are prone to fouling. Anti-fouling protection must not affect the measurements being taken and so the problem facing the developers of such an anti-fouling agent is complex.

For accurate measurements to be taken over a prolonged period, marine fouling of any sort must be prevented i.e. microfouling as well as macrofouling. Trials at Glasgow Marine Technology Centre [6] showed that typically, after seven days in Scottish coastal waters, a transmissometer was fouled to such an extent that the transmission had been reduced to virtually zero. The complete prevention of microfouling i.e. by bacteria is not possible as the sea is not a sterile environment. However, microfouling such as bacteria and microalgae must be prevented from flourishing and forming a

"slime" layer known as a biofilm. This is a water based matrix and is the accumulation of bacteria and subsequently microalgae, the initial foulers of any surface in the marine environment.

1.3 Biofilms and Fouling Formation

The formation of a biofilm is usually considered to be a prerequisite to fouling by larger organisms such as algae and molluscs [7] i.e. macrofouling. It has been shown that this is not always the case since, although some invertebrates settle preferentially on microbial films, others such as barnacles and bryozoas do not require such films [8]. However, as all fouling, large or small, affects the performance of sensors the task here is to prevent the occurrence of any fouling. Figure 1.3.1 shows a Perspex rack containing trial anti-fouling samples which had been immersed at sea for six months. This rack is very heavily fouled and it is obvious that any sensor with this much fouling would be unable to produce realistic results e.g. a light meter would be unable to measure light. Other structures which are immersed in a marine environment would experience similar growth and any ship would have a larger fuel consumption due to the drag which the fouling would cause. Thus a successful anti-fouling surface would have many applications. It has been noted that fouling communities on remote buoys, i.e. stationary structures are frequently more mature than those found on the hulls of ships [1]. However, merely the growth of a biofilm on a ship's hull caused the hydrodynamic drag on a ship to increase considerably [9].

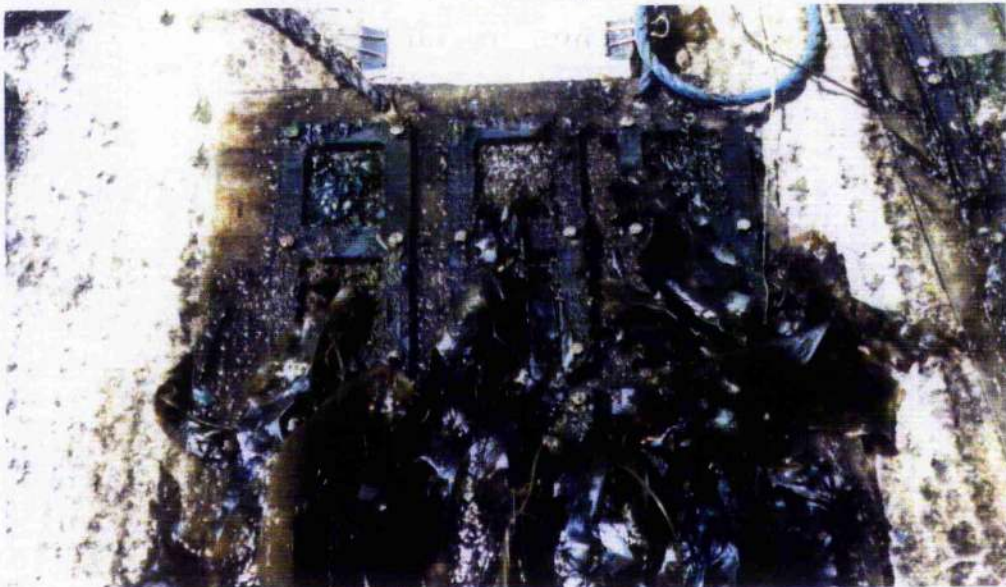


Fig. 1.3.1 Fouling on a perspex rack after 6 months in a marine environment.

The term "biofilm" encompasses a large range of microbial associations which are usually found at phase boundaries. The diverse areas in which biofilms are found are shown in Table 1.3.1 The fouling of all these surfaces originates from the biofilm formation and therefore prevention of this biofilm layer should in turn prevent the majority of macrofouling.

Biofilms form on the following surfaces :
Swimming pool filters
Car wash bottles
Domestic drains
Oral surfaces such as teeth i.e. dental plaque
Prostheses and catheters
Marine surfaces

Table1.3.1 Surfaces on which biofilms form.

This research is predominantly interested in biofilm formation in the marine environment. However, as the mechanism of biofilm formation follows similar mechanisms for all surfaces the only differences are the bacteria flora which colonise these surfaces [10]. There has obviously been much research carried out in all areas where biofilms create problems. A marine biofilm formation at the solid / liquid interface occurs when any surface is immersed in the sea and this biofilm formation occurs due to a sequence of events which is shown below :

1. A clean surface is available for colonisation.
2. A conditioning film of organic molecules forms. (fast formation i.e. a few seconds [9])
3. Colonising bacteria become loosely attached
4. Colonising bacteria become firmly attached.
5. Microcolonies form, extracellular polymer secretion is produced.
6. Colonies spread outwards and upwards to form regular or irregular structures.
7. Biofilm matures, new species enter and grow, organic and inorganic debris is incorporated and solute gradients develop leading to a spatial heterogeneity.

At this point there are three possible ways in which the biofilm can develop. It may either slough off or organisms may begin to graze it [11] allowing the cycle to repeat itself or alternatively, larger organisms such as algae and molluscs may become established on it.

1.3.1 Mechanism of Bacterial Attachment

The means by which bacteria attach to a conditioned surface is by polysaccharide fibre which protrudes from the surface of the bacteria (a similar mechanism is employed by diatoms). These fibres form a felt-like "glycocalyx" which surrounds an individual cell or colony of cells [10]. The production of this polysaccharide matrix by these sessile bacteria i.e. bacteria attached to a surface (free bacteria are known as planktonic) is present in marine systems and when this material is examined using microscopy it was found to be a highly hydrated mass of polyanionic material (a biofilm is known to be 99% water [12]). It has been shown that bacteria form microcolonies within the biofilm [13]. This is followed by microcolonies of diatoms and then larger algae as already described in the sequence of biofilm formation. When these microcolonies form within the biofilm the glycocalyx occurs as a diffuse slime layer surrounding the microbial cells and can be considered as an additional barrier to entry by an anti-fouling agent [12]. Therefore any active substance used to prevent this microbial growth must be able to prevent the formation of a biofilm as well as preventing the fouling of a surface by invertebrates.

1.4 Novel Anti-fouling Agents

In this work the use of quaternary ammonium compounds (QACs), a biguanide and the chelating agent ethylenediaminetetraacetic acid (EDTA) will all be investigated as possible antifouling agents, singularly as well as in combination. These materials have been chosen due to their antimicrobial action and also due to their surface active properties. The hydrophilic hydrogel material used as the substrate in this research was developed for use in anti-fouling applications by Parr et al [14]. This hydrogel was based on hydroxyethylmethacrylate, with a water content of 40%. It is a transparent polymer and has been manufactured with a thickness of 1 mm by precasting it into Perspex moulds and storing it in distilled water until required, thus keeping the material in its hydrated form. Research [15] has shown the hydrogel

material with benzalkonium chloride incorporated to be anti-fouling for up to 12 weeks in the marine environment.

The use of the quaternary ammonium compound benzalkonium chloride (BAC) as an antimicrobial agent and its mode of action will be described in more detail in further chapters. Reference to the anti-algal properties of BAC dates back to 1949 when it was applied in sewerage treatment [16]. Further evidence of its algal control action is shown by its use in outdoor swimming pools.

Other quaternary ammonium compounds (QACs) are known to be antimicrobial. The current study includes an investigation into the anti-fouling action of the material known as cetrimide. Cetrimide is alkyltrimethylammonium bromide where the single chain is usually a mixture of C₁₂ and C₁₄.

The biguanide which is investigated here is chlorhexidine which has been recognised for its antimicrobial properties since it was first synthesised by ICI in the 1940's [17]. From the literature [18] it has been shown that the combination of chlorhexidine gluconate with benzalkonium chloride has improved the antimicrobial properties of both materials and thus hydrogels containing this mix were investigated for their anti-fouling ability.

Another mechanism for improving the action of benzalkonium chloride is by combining it with a chelating agent such as EDTA [19,20]. Marine trials using this partnership were also set-up.

1.4.1 Environmental Impact of Novel Anti-fouling Agents

Quaternary ammonium compounds (QACs) are present in small quantities in the world's oceans due to their extensive pharmaceutical and industrial applications and therefore monitoring of these levels is continuously carried out in order that these quantities do not become too high. The release rates from the hydrogel material of the QACs is slow to zero and thus the impact on the environment is negligible. Previous investigation into the environmental impact [21] has shown that QACs are inactivated by clay minerals and are neutralised by anionic surfactants, which are also waste products in the sea. Another important aspect of the impact of QACs in the sea was the work on oysters by His et al [22]. Oyster boxes were coated with hydrogel

material which had been soaked in 5%w/v benzalkonium chloride and the growth and development of these oysters was monitored over a period of time, all measurements indicated that the benzalkonium chloride had no detrimental effect and was therefore found to be non-toxic to oysters. These results are positive indicators for pursuing the use of QACs in the anti-fouling field as well as the fact that they are not new biocides which are being added to the environment with unknown consequences.

1.5 Mechanisms of Anti-fouling Agents

The quaternary ammonium compounds, benzalkonium chloride and cetrinide, and the biguanide, chlorhexidine are often described as cationic surfactants i.e. surface-active agents that have a positive charge. Such materials have a two pronged attack, antimicrobial and surface-active. It is these two properties that make this class of materials so useful in the control of micro-organisms. The mechanisms by which surfactants operate can be briefly summarised by stating that the positive charge enables them to attach themselves to negative micro-organisms e.g. bacteria, while the alkyl chain gives them their hydrophobic character which is able to alter the surface chemistry by an antistatic action. Both these actions impair the successful growth of micro-organisms.

Antimicrobial and surfactant properties are dealt with in Chapter 2. The antimicrobial actions vary in the way that they attack the cell and a brief description of how they operate is given below.

1.5.1 Antimicrobial Action

Quaternary ammonium compounds (QAC) attack the bacterium cell by targeting its outer membrane in order to make it permeable. They also prevent coagulation by making the cell weak and thus more vulnerable to attack. Chlorhexidine works in a similar manner to the QACs but it also has a severe effect on the adenosine triphosphate (ATP) which is essential to the transport of ions and molecules across cell membranes [19].

1.5.2 Why Antimicrobial Agents Fail

The main problem in the past was assessing the ability of an antimicrobial agent in the laboratory as the results frequently did not concur well with the results obtained in the

"real world". There were various reasons for this. Initially the tests in the laboratory were carried out on one species of one organism and therefore were not very challenging. Scientists soon realised that this was a problem and began introducing tests which included a variety of species and thus the power of the antimicrobial agent could be determined with respect to a more diverse environment. However, the most difficult problem had yet to be overcome as the differences between planktonic and sessile bacteria had not yet been discovered. This difference was first published in 1943 by Zobell [23]. However, little interest was taken until more than three decades later when microbiologists realised the significance of Zobell's discovery. This being that bacteria in the laboratory were planktonic i.e. not attached to a surface while in the "real world" the problems caused by bacteria such as infection, surface degradation and marine fouling were the result of sessile bacteria. These sessile bacteria had an outer membrane, not present in planktonic bacteria, which made them more resistant to antimicrobial attack [10,12]. Thus the failure of some antimicrobial agents such as biocides and antibiotics could now be understood. One of the most obvious examples of an antimicrobial failing is in the treatment of chest and urinary tract infections by antibiotics. This failure can be attributed to the fact that a biofilm has formed and the antibiotic is unable to penetrate the biofilm and thus the infection recurs a short time after treatment finishes [24]. This is comparable to the use of antibiotics in marine trials in that they are very effective over a short time but fail to prevent the bacteria reforming in the long term.

1.5.3 Mechanisms of Cationic Surfactants

In the marine environment the formation of a biofilm has a vast effect on either a sensor or a ship and therefore its formation must be prevented. From the initial work done in this area [14] it appears that the quaternary ammonium compound benzalkonium chloride is able to prevent biofilm formation on the surface of the hydrogel substrate. This action is likely to be a combination of mechanisms i.e. antimicrobial and surface activity. It has been suggested by Cowling et al [25] that a successful anti-fouling surface is likely to consist of a mixture of mechanisms. Therefore the dual chemical and physical actions of the cationic surfactants have been investigated within this research with an aim to understand the different component mechanisms that make cationic surfactants, such as benzalkonium chloride, successful in their anti-fouling application.

1.6 Aims

The main aim of this research is to develop an environmentally acceptable anti-fouling surface which can be used to protect underwater sensors, in order that they can collect data without requiring to be cleaned of biofouling frequently. Such cleaning is costly in both manpower and equipment.

The work described here concentrates on cationic surfactants as anti-fouling agents. Chapter 2 describes the properties of these cationic surfactants and gives the mechanism of their diverse actions and uses. The analytical analysis of such materials is studied in chapter 3. The research included a study of the analytical methods which were robust and cost effective. The theory behind the chosen methods is described and explained with reference to acceptability for routine analysis.

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Chapter 2

The history and experimental work of surface-active agents.

2.1 History of Quaternary Ammonium Compounds

2.2 Benzalkonium Chloride

2.3 Cetrимide

2.4 Chlorhexidine

2.5 Ethylenediaminetetraacetic Acid

2.6 Micelles

2.7 Experimental - Benzalkonium Chloride

2.8 Experimental - Cetrимide

2.9 Experimental - Chlorhexidine

2.10 Experimental - Ethylenediaminetetraacetic Acid

2.11 Experimental - Mixed Surfactant Systems

2.12 Conclusions

2.13 References

Introduction

In this chapter details of each of the potential anti-fouling materials will be discussed. Each material is incorporated in the hydrogel material which is described in Chapter 1. The first four sections deal with the history, development and chemical character of each of the cationic surfactants which were investigated as potential anti-fouling materials. This is followed by Section 2.5 which deals with the chelating agent ethylenediaminetetraacetic acid (EDTA). Previous publications have demonstrated the improved performance of cationic surfactants when EDTA is added and this idea is explored in anti-fouling field trials. Section 2.6 gives a brief explanation of the nature of surface active agents, surfactants and their ability to form micelles. These factors govern the chemistry and mechanisms of these materials. Sections 2.7 to 2.11 are experimental sections which deal with each material from its supplied state up to its reaction when it is introduced into the hydrogel for anti-fouling trials. Section 2.12 gives conclusions from the experimental sections. In the experimental sections there is occasionally a brief mention regarding fouling results; this is only where it is relevant to the understanding of the experiment. Full anti-fouling behaviour is described in Chapter 4.

In each of the experimental sections the suppliers are named but it can be assumed when benzalkonium chloride is discussed without reference to a specific supplier then this material is supplied by Aldrich Chemical. In most of the work with seawater, "real seawater" from University Marine Biological Station Millport (UMBSM) is used in the filtered state i.e. large debris has been removed using Whatman GF/C. However, some work was carried out using artificial seawater if real seawater was not available in the laboratory.

2.1 History of Quaternary Ammonium Compounds

The first published work on quaternary ammonium compounds (QACs) was by Jacobs et al in 1916 [1], where the structure, preparation and antimicrobial activity were examined. During the 1920's some further investigations were carried out, however, it was not until 1935 that Domagk, a German scientist, showed the antimicrobial activity of the long-chain quaternary ammonium salts [2].

The QACs which were examined for their anti-fouling potential in this work were benzalkonium chloride and cetrимide. The advantage of QACs over most other antimicrobial agents is that they are odour free, relatively non-toxic and stable in storage. The character and details of these compounds will now be discussed in Sections 2.2 and 2.3.

2.2 Benzalkonium Chloride

The compound benzalkonium chloride, which is a mixture of various alkyldimethylbenzylammonium chlorides, was initially marketed under the trade name "Zephirin Chloride". In 1949 the XIIIth edition of the United States Pharmacopoeia (USP) [3], recognised the material alkyldimethylbenzylammonium chloride as an antiseptic and renamed it benzalkonium chloride.

Figure 2.2.1 shows the general structure of benzalkonium chloride.

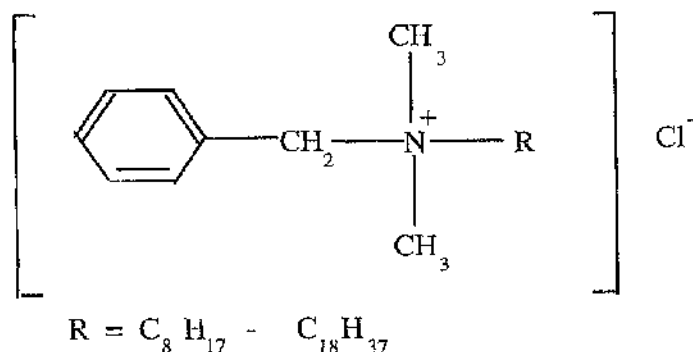


Fig. 2.2.1 Structure of benzalkonium chloride

2.2.1 Production and Chemistry

Benzalkonium chloride is prepared by the alkylation of the tertiary amine with benzyl chloride.

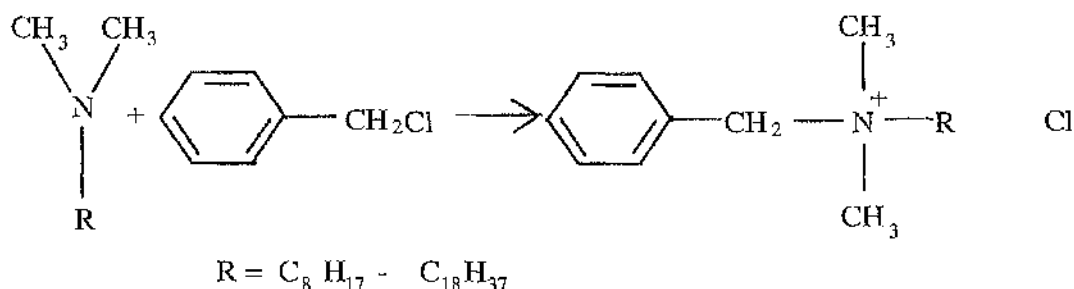


Fig. 2.2.2 Production of benzalkonium chloride.

The quaternization reaction is carried out by heating the tertiary amine with benzyl chloride at 60°C to 80°C. The reaction is carried out in the presence of water and/or iso-propanol which accelerates the reaction. The long chain alkyl group is usually derived from coconut fatty acid which contains the alkyl chains C₁₂, C₁₄ and C₁₆ whereas benzalkonium chloride which is predominantly C₁₈ is derived from tallow fat. The four carbon atoms are linked to the nitrogen through covalent bonds and the anion, in this case chloride, is linked electrovalently.

Benzalkonium chloride (BAC) is very soluble in water, forming a slightly viscous liquid which, in high concentrations, is very sticky. The critical micelle concentration (CMC) is 0.003 mol l⁻¹ although this value is calculated when the material is mainly C₁₂ and thus can vary depending on which alkyl chains are present. It is usually a white/creamy powder or a semi-solid when supplied as the pure material. BAC is practically insoluble in non-polar solvents such as benzene and ether [4]. These properties are generally associated with the most common form of benzalkonium chloride and are less applicable to BAC with higher alkyl chains, which exhibit more hydrophobic characteristics.

2.2.2 Chain Length

The chain length of BAC varies and consequently different chain lengths have different applications and uses. It follows that the molecular weight also varies. The

most common formulation contains mainly C₁₂ and C₁₄ homologues which give the material antimicrobial properties. Listed below are the main properties of each of the homologues from C₈ to C₁₈.

- | | |
|-----------------------------------|--|
| C ₈ - C ₁₀ | Poor detergency. Thus the compound is low foaming, which is useful for washing areas where rinsing is difficult e.g. walls of dairies. These homologues have little antimicrobial power. |
| C ₁₂ - C ₁₄ | Superior antimicrobial action is shown by these homologues compared with others. Surface activity appears at C ₁₂ thus increasing the foaming action. |
| C ₁₆ | Has antimicrobial power although this is reduced in the presence of organic matter. Surface activity is greater than the previous homologues. |
| C ₁₈ | This homologue is practically insoluble in water i.e. is hydrophobic. Has superior surface activity to all lower homologues. |

2.2.3 Applications and Uses of Benzalkonium Chloride

Many different descriptions are attributed to BAC depending on its use. A concise list of these is shown below :

- Algicide/algistat
- Antimicrobial
- Bactericide/Bacteriostat
- Biocide
- Cationic Surfactant
- Detergent
- Disinfectant
- Fungicide/Fungistat

BAC has a broad spectrum of commercial and consumer uses. It is likely that many people will have used BAC at sometime in their lives. The list shown below gives the main applications of this material.

Examples of BAC's antimicrobial applications are :

- Antimicrobial mouth washes
- Burn/wound cleaning
- Cleaning hard contact lenses
- Industrial hygiene

Examples of BAC's applications where surface active properties are useful :

- Fabric softeners
- Corrosion - when pickling steels
- Road building - in bitumen

The variation in the character of benzalkonium chloride is attributed to the different chain lengths from which the compound can be composed. Throughout the world pharmacopoeia differ in how they define it. The British, [4], International [5], and European Pharmacopoeia [6] all state that the alkyl chain length must be between C_8H_{17} - $C_{18}H_{37}$, however the United States Pharmacopoeia [3] states that the alkyl chain "is a mixture of alkyls, including all or some of the group beginning with C_8H_{17} - $C_{18}H_{37}$ and to be pharmaceutically acceptable benzalkonium chloride should contain, not less than 40% of $C_{12}H_{25}$ compound and not less than 20% of the $C_{14}H_{29}$ compound and not less than 70% of these two compounds".

In summary the only pharmacopoeia which dictates the presence in high concentrations of the antimicrobial homologues is the USP. Calls for the BP to state clearly which homologues should be present for acceptable pharmaceutical application have as yet been unheeded [7].

2.2.4 Antimicrobial Mechanism

The benzalkonium ion is positively charged at all pH's, although it is most effective in a neutral to alkaline medium. This positive charge enables the molecule to

be attracted to negatively charged surfaces causing attack on these negative sites. Bacteria, both gram-negative and positive, fungi, yeasts and viruses have surface negative charge, thus BAC is an ideal biocide to prevent the growth of these organisms. The actual mechanism operates by the positively charged hydrophilic moiety of quaternary ammonium compound being strongly adsorbed onto the negatively charged bacterial surface; thereafter damage to the cytoplasmic membrane occurs and an apparently fatal leakage of cytoplasmic material follows [8]. This mechanism describes the bacteriocidal effects of BAC. However, when less concentrated BAC is employed the effect on organisms can be bacteriostatic. This is when enough damage is done to the cytoplasm to prevent growth and development, but the organism is not actually dead.

Many papers have been written on the effect chain length has on the antimicrobial activity of benzalkonium chloride. An antimicrobial agent must be able to prevent the growth of bacteria, both gram-negative and positive as well as fungi, yeasts and viruses. Thus many studies have taken place in order to determine which chain length or lengths are able to prevent the growth of the above mentioned organisms. In 1966 Cutler et al [9] concluded that 14 carbon atoms was the optimum chain length in terms of biocidal activity. This was the result also concluded in 1983 by Daoud et al [10] who stated that "antimicrobial activity is parabolically related to n-alkyl chain length and optimises for bacteria and yeasts at n-alkyl chain lengths of C =14 and 16. Merianos concluded that the major determining factor for biocidal efficacy is the hydrophilic/lipophilic balance of the chain lengths [11]. Various other authors backed this up and a concise summary of these results can be found in Chapter 13 of Disinfection, Sterilisation and Preservation, 4th Edition, edited by Seymour S Block [11]. In 1986 Jono [12] et al stated that the C₁₂ chain was the best length to use as a biocide, although the investigations were limited to bacteria. From the work in this area, one clear picture emerges; namely benzalkonium chlorides containing predominately C₁₂ and C₁₄ homologues have the optimum antimicrobial activity, in that this combination is able to prevent the growth of these organisms.

All the work reviewed so far concentrates on the antimicrobial effects of benzalkonium chloride. Although both the C₁₂ and C₁₄ homologues have surface active properties these properties are much more pronounced in the longer chains i.e. C₁₆ and C₁₈. A study carried out by Linfield [13] stated that the C₁₆ and C₁₈ alkyl chains possess optimum surface activity in terms of lowering the surface tension. It

should be noted that this study did not look at alkyl chains greater than C_{19} . However, the surface active properties alone play an equally important role in the biocidal action of benzalkonium chloride. The successful use of benzalkonium chloride as a deterrent for microfouling on contact lenses is thought to be the result of the action of the hydrophobic alkyl chain of the benzalkonium chloride, which is able to adhere itself to polymers through various possible interactions e.g. ion-ion interaction, van der Waal's interactions and ion-dipole interactions. When it is adhered, the long alkyl chain of the benzalkonium chloride then forms layers through similar slight charges causing a build up of the benzalkonium chloride [14]. Another theory is given by Landa et al [15], who found that microbial detachment by oral rinses is more likely due to a detergent system rather than the antimicrobial component and concluded that "an efficient detergent system to detach adhering bacteria as a means of controlling biofilms in general, may turn out to be preferable to antimicrobials, because the latter only leave a dead biofilm, the surface of which is prone to renewed bacterial adhesion". Results from the marine studies in the current investigation show that benzalkonium chloride containing these longer alkyl chains remained free of any visible fouling for 14-16 weeks. Thus surface activity must play an important role here where antimicrobial action is practically non-existent.

2.3 Cetrimide

Cetrimide was the name given to trimethylalkylammonium bromide which was a mixture of alkyl chains from C_{12} - C_{14} . The British Pharmacopoeia [4] classifies cetrimide as trimethyltetradecylammonium bromide, although the compound may contain smaller amounts of dodecyl and hexadecyltrimethylammonium bromides.

From 1942 onwards it was a principal component of antiseptics and in 1953 the revised form, tetradecyl trimethyl ammonium bromide i.e. the C_{14} form, was introduced as it had the most powerful antimicrobial chain. The general structure of cetrimide is shown in figure 2.3.1.

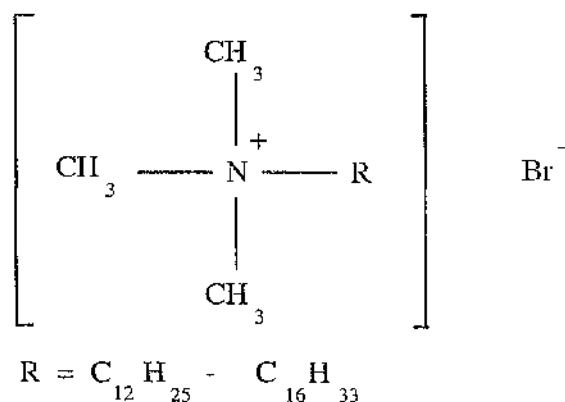


Fig. 2.3.1 Structure of cetrimide

2.3.1 Production and Chemistry

Cetrimide is produced in the same manner as benzalkonium chloride except that trimethylamine is alkylated with an alkyl halide containing the appropriate chain length.

The chemical properties of cetrimide are listed below :

Molecular Weight	:	336.5 (tetradecyl)
Melting Point	:	149-151°C
Solubility	:	Freely Soluble (1g in 10 ml)
Appearance	:	White or almost white, voluminous, free-flowing powder; slight odour.
Category	:	Antimicrobial, preservative
Formula	:	$[\text{RN}(\text{CH}_3)_3]^+ \text{Br}^-$ $\text{R} = \text{C}_{12}\text{H}_{25} - \text{C}_{16}\text{H}_{33}$

2.3.2 Character

Alkyltrimethylammonium bromide which is often described as "cetrimide" has many different forms. The alkyl chain can vary from C₁₂ - C₁₆ and therefore the manner in which it is described depends on the interpretation.

Listed below are the descriptions attributed to the compound :

1. The Penguin Dictionary of Chemistry (D W A Sharp) [16]

Cetrimide, cetyltrimethylammonium bromide, CTAB [C₁₆H₃₃N(CH₃)₃]⁺Br⁻

2. Aldrich Fine Chemicals [17]

a) Cetrimide, alkyl trimethylammonium bromide, predominately C₁₄ but also contains C₁₂ and C₁₆.

b) It details cetyltrimethylammonium bromide as cetrimonium bromide where C₁₆ is the predominant homologue.

3. Sigma Chemical Company Ltd. [18]

a) Cetrimide USP hexadecyltrimethylammonium bromide. Also known as cetyltrimethylammonium bromide and cetrimonium bromide.

Supplier states that this product is soluble in water 10%w/v. Purity is approximately 99%.

b) Cetrimide BP Mixed alkyltrimethylammonium bromide which is predominantly C₁₄ but also contains C₁₂ and C₁₆. Supplier states that this product is soluble in water 10%w/v.

As with all quaternary ammonium compounds which have alkyl chains which can vary in length, the actions and names attributed to these lengths differ. Therefore, it is important to understand what is being implied when a compound is being supplied.

e.g. a document from the USA would describe cetrimide as hexadecyltrimethylammonium bromide, whereas in the UK cetrimide would be thought to be tetradecyltrimethylammonium bromide.

2.3.3 Applications and Uses of Cetrimide

Cetrimide is used as a general antiseptic for cleaning burns, wounds etc. of the skin. It is used as a disinfectant for surfaces in areas that require to be sterile. It is the active ingredient in topical antimicrobial creams and in anti-dandruff shampoos. It is also used in combination with chlorhexidine in many antimicrobial preparations [19].

2.3.4 Antimicrobial Mechanism

Cetrimide, as with other QAC's, is known as a surface-active agent. It operates by being attracted to the negatively charged bacterial cell wall which absorbs the cationic agent into its structure thus disrupting the cell's balance [20].

Cetrimide's properties differ depending on the alkyl chain length. The same actions are attributed to cetrimide which are attributed to benzalkonium chloride, in that the optimum antimicrobial activity occurs between C₁₂-C₁₆.

Cetrimide is known to act differently at different concentrations. At lower concentrations it is attracted towards the negative bacteria causing cell disruption i.e. bacteriostasis. At higher concentrations over a long period, cetrimide causes the cell to leak its constituents resulting in complete cell death i.e. bacteriocidal action [21].

2.4 History of Biguanides

The first reported synthesis of a biguanide was by Rathke in 1879, [22]. However, it was not until 1929 that the biological activity of these compounds was noted by Stotta and Tschesche [23] when they demonstrated the ability of the substituted biguanides to lower blood sugar. The first reported antimicrobial properties of biguanides were reported in 1933 by IG Faben. During the 1950's at ICI England, chlorhexidine was first synthesised by Davies et al [24]. It quickly became widely used as an antimicrobial and is still one of the most commonly used today.

2.4.1 Chlorhexidine

Chlorhexidine is described as a bisbiguanide where N¹ and N⁵ are substituted. Its chemical name is 1, 1'- hexamethylenebis[5-(4-chlorophenyl)biguanide].

Figure 2.4.1 shows the structure of chlorhexidine.

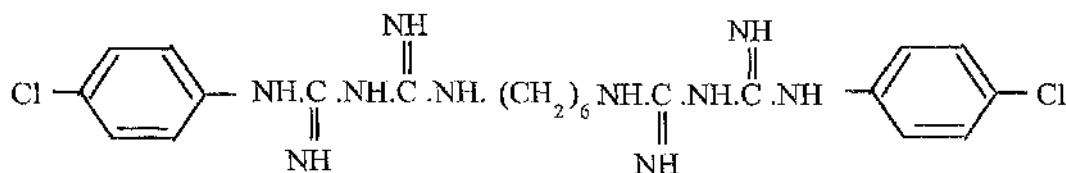


Fig. 2.4.1 Structure of chlorhexidine

2.4.2 Chemistry

Chlorhexidine is a strong base which is practically insoluble in water but when it is formulated with acids to form salts, the solubility increases. The salts formed are shown below, together with their solubility in water :

◇	Chlorhexidine dihydrochloride	1 in 1700
◇	Chlorhexidine diacetate	1 in 55
◇	Chlorhexidine digluconate	miscible

Chlorhexidine diacetate and dihydrochloride are white/pale cream microcrystalline powders while chlorhexidine di-gluconate is a pale straw-coloured solution [4], usually as a 20%w/v solution.

Chlorhexidine is a surface active agent i.e. it is a surfactant and it forms micelles. The critical micelle concentration (CMC) of the acetate is 0.01%w/v at 25°C [21] (details of the chemistry of the surfactants can be found in Section 2.6). It is also termed as "cationic" which enables it to act as a positively charged species. The combined cationic and surface active properties make chlorhexidine extremely useful.

Chlorhexidine is reported by Hugo and Longworth [25] to carry four positive charges over the ten nitrogen atoms, however, only two pKa values of 2.2 and 10.3 have been experimentally confirmed [26].

2.4.3 Applications and Uses of Chlorhexidine

Chlorhexidine is widely used as an antimicrobial agent. Listed below are some of its applications :

- Skin disinfection
- Clean-rooms
- Oral hygiene
- Cleaning burn and wound sites

It is its action in mouth-wash preparations, i.e. preventing the formation of plaque and fungal infections, that has been attributed to its antiseptic and antiadhesive effects. The formation of plaque has been compared to the biofilm formation on marine structures and thus chlorhexidine may be useful in the prevention of the initial bacterial layer which forms prior to macro fouling in the marine environment [27]. Marine trials should be able to determine this.

2.4.4 Mechanism

The biguanides can be classed amongst the so-called membrane active antimicrobial agents. They induce leakage of low molecular weight cytoplasmic components such as potassium from bacteria cells. Due to cytoplasmic component leakage, changes in the permeability are generally attributed to a change in the integrity of the cytoplasmic membrane and thus cell inhibition results [28].

As with benzalkonium chloride and cetrimide, chlorhexidine is bacteriostatic at low concentrations and bactericidal at high concentrations. Cells treated with bacteriostatic levels of chlorhexidine can recover viability despite having lost 50% of their potassium content. However, as the concentration increases cell contents of higher molecular weight, such as nucleotides, begin to leak. It has been shown that if this leakage is more than 15% the cell damage is irreversible and therefore, at this level, chlorhexidine is bactericidal [28]. The levels at which these actions take place are obviously organism dependant.

2.5 Ethylenediaminetetraacetic Acid a Chelating Agent

Ethylenediaminetetraacetic acid (EDTA) has been utilised as a chelating agent since it was synthesised over 60 years ago. Chelating agents of this type were originally

developed to counteract the deleterious effects of water hardness and heavy metal ions on dyestuffs [29].

The word chelate comes from the Greek meaning claw. Other descriptions such as potentiating agent and sequestering agent are also used.

EDTA is classified as a polydentate ligand and has the following chemical structure :

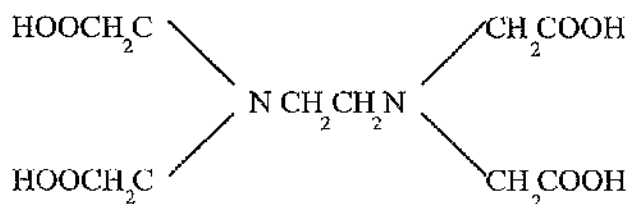


Fig. 2.5.1 Structure of ethylenediaminetetraacetic acid

Many papers have illustrated the synergistic effect, i.e. when one compound increases the accessibility of targets for another, of the addition EDTA to quaternary ammonium compounds [30] and [31]. It is stated that in some cases the improvement to antimicrobial action is 100 fold when only 100 ppm of EDTA is added [32]. Most of the work in this field has been carried out by the ophthalmic industry in order to prevent the formation of biofilms on contact lenses [33]. EDTA itself has never exclusively been antimicrobial on its own, however, Phillips et al [34] did find that addition of EDTA to borate buffered saline did decrease the contamination.

2.5.2 Production and Chemistry

Ethylenediaminetetraacetic acid (EDTA) is produced by the reaction of diamine with sodium cyanide and formaldehyde under alkaline conditions at elevated temperatures. The tetrasodium EDTA salt produced by this reaction is easily converted to the tri-, di-, or monosodium salt, or the free acid, by treatment with sulphuric or hydrochloric acid [35]. EDTA is a white anhydrous crystalline solid with a molecular weight of 292, which melts at 240°C with decomposition.

Although it is practically insoluble in water, it forms a series of mono-, di-, tri- and tetrasodium salts which increase in water solubility as well as in pH. Table 2.5.1 lists the increasing solubility of the sodium salts of EDTA.

Salt	Solubility	pH of a 2% solution
EDTA Na ₂	12.1g/100 ml at 25°C	4.7
EDTA Na ₃	73g/100 ml at 25°C	9
EDTA Na ₄	very soluble at 25°C	12

Table 2.5.1 Solubilities of the sodium salts of EDTA

There are four ionisation constants associated with EDTA and these are listed below :

$$pK_1 = 1.996$$

$$pK_2 = 2.672$$

$$pK_3 = 6.161$$

$$pK_4 = 10.262$$

The pH is important in complex formation with EDTA because the species of the acid present in solution are greatly dependent on pH. The stability of the complexes formed with divalent metal cations can be seen by the substantially negative enthalpy values and the substantially positive entropy values which these complexes exhibit.



2.5.3 Applications and Uses of EDTA

EDTA has many uses as a chelating agent where it is able to attract cations and bind with them thus rendering them neutral. Below are listed some of the varied uses of EDTA :

- ◇ Textile industry - for dyeing
- ◇ Cosmetic industry - many purposes e.g. stabilize colour additives
- ◇ Household detergents - improves performance in hardwater areas
- ◇ Antimicrobial agents - improves performance in hardwater areas

2.5.4 Mechanism

The improved performance of antimicrobial agents has been reported on many occasions when EDTA is added to the antimicrobial agent [35]. This is due to the action of EDTA acting as a chelating agent on the cations in the outer cell membrane of the bacterial cells which causes disruption to the cell's structure, leaving it more porous to attack from the antimicrobial agent.

In hardwater areas EDTA is used to sequester the calcium ions and again the antimicrobial agent is able to perform more efficiently [31]. It should be noted that in order to sequester all the calcium ions a high quantity of EDTA is required [32], which is often not practical due to solubility.

2.6 Chemistry of Surface Active Agents

In this section the properties of the surface active agents, surfactants, will be discussed. A surfactant is classified by its hydrophilic moiety. When it carries a negative charge the material is known as an anionic surfactant; when it carries a positive charge the material is known as a cationic surfactant; when the polyoxyalkylene group is neutral the material is known as a non-ionic surfactant [36]. In the current research all the work is concentrated on the cationic surfactants.

Both the quaternary ammonium compounds, benzalkonium chloride and cetrimide as well as the biguanide, chlorhexidine are included in this group. Surfactants have two, special properties which make them invaluable to their users, these being the ability to form micelles and their amphiphilicity. These properties lead to their interesting behaviour at surfaces and interfaces. When a surfactant is added to a liquid it changes the interfacial properties of that liquid [37] and this explains why when detergent is added to water it feels slippery. Here benzalkonium chloride will be used as an example to demonstrate these properties. The structure of benzalkonium chloride is such that it is composed of two sections. The hydrophilic portion which is polar and comes from the nitrogen moiety and the hydrophobic alkyl chain which is non-polar. In figure 2.6.1 these parts are shown on the molecule.

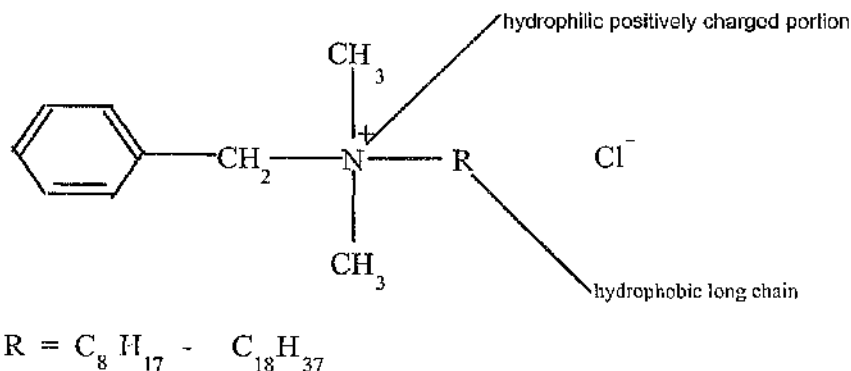


Fig. 2.6.1 Structure of benzalkonium chloride

It is due to this structure that a surfactant is able to lower the interfacial tension between phases [38] and thus make it an extremely useful material. In figure 2.6.2 benzalkonium chloride is added to water and the ions are shown to align themselves between water and air so as to modify the surface of the water.

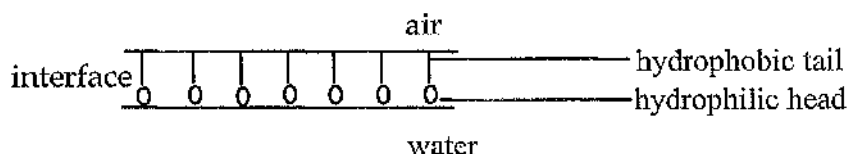
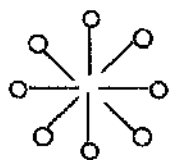
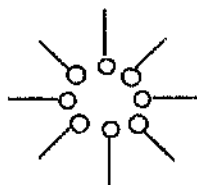


Fig. 2.6.2 Alignment of a surfactant at the interface of water and air.

From the figure it can be seen that the hydrophobic tail has positioned itself as far away as possible from the polar solvent, in this case water. This only occurs at the interface. In the bulk of the solution clusters of the benzalkonium ions form micelles which also align themselves in such a way that their hydrophobic tails are distanced from the water. However, if a surfactant is dissolved or dispersed in a non-aqueous solvent the orientation is reversed and the tail portion aligns itself towards the bulk of the solution. Figure 2.6.3 shows these two effects.



Orientation in water



Orientation in a non-polar solvent

Fig. 2.6.3 Micelle structure.

In figure 2.6.3 the micelle is shown as a sphere. However, as the concentration of the surfactant increases the shape of these micelles change and become cylindrical (rod like), then hexagonal and lastly lamellar, in order to conserve space [39].

Micelle Formation

Micelles have an important role in both industry and biology on account of their solubilising function. In order for these micelles to form the concentration of the solute must reach the critical micelle concentration (CMC) which is achieved only if the Kraft Temperature is reached, i.e. the minimum temperature required for micelle formation. Each compound has a particular temperature when this will occur. CMC is measured by various methods, these being electrical conductance, surface tension and solubilization although, due to recent progress in research techniques, NMR, ESR, neutron scattering and quasielastic light scattering are now more frequently used. [38]. When micelles form, many properties of solutions are drastically changed. One of these properties is the surface tension. Surface tension drops before the CMC is reached, after which it remains constant.

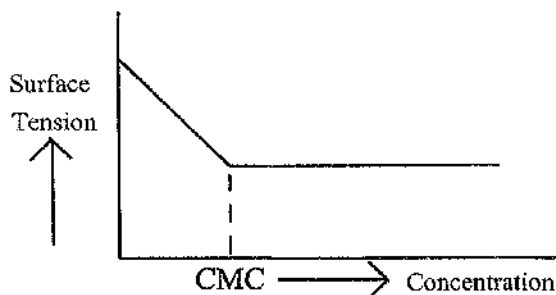


Fig. 2.6.4 Surface Tension as a Function of Concentration.

The reason that surfactants are able to reduce tension at a surface when they are added to water is because they are positively adsorbed. This adsorption can be calculated for dilute solutions using Gibb's Adsorption Isotherm

$$\text{Gibb's Adsorption Isotherm} \quad d\gamma = -\sum \Gamma d\mu$$

Where γ is the surface tension, Γ is the surface adsorption and μ is the chemical potential.

At the water-air interface the excesses of surfactant and water are zero and in a dilute solution the chemical potential (μ) can be substituted by $RT \ln c$. The Gibb's equation can be reduced to the following form:

$$\Gamma = -\frac{c}{RT} \cdot \frac{d\gamma}{dc}$$

In contrast, an ionic compound such as NaCl is negatively adsorbed when it is added to a solvent and thus the surface tension of that solvent is increased.

Many properties of solutions change when the CMC of a surfactant is reached. Figure 2.6.5 gives a diagrammatic representation of these changes [40].

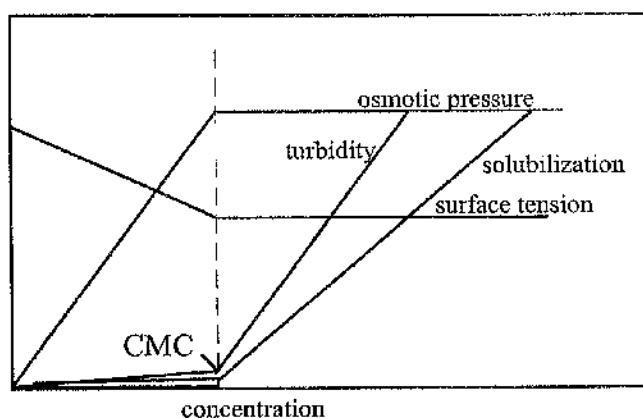


Fig. 2.6.5 Schematic representation of micelle effects.

Surfactant Action of Quaternary Ammonium Compounds

The cationic nature of the quaternary ammonium compounds has given rise to a wide variety of functions in many applications. These applications are derived from four basic functions:

- ◇ Surface activity
- ◇ Substantivity
- ◇ Reactivity
- ◇ Antimicrobial activity

The surface active properties which result from the hydrophilic and hydrophobic parts of the molecule structure gives rise to the orientation at interfaces. This property enables surfactants to act as emulsifiers, wetting agents, thickeners and foaming agents. Substantive action refers to the adsorption properties of surfactants and results from the electrostatic charge on the alkyl chain. This leads to surface modification and thus makes them useful as fabric softeners, anti-static agents, lubricators, as corrosion inhibitors, in adhesion and in general hydrophobic actions. The reactive properties of surfactants lead to the formation of complexes which exhibit low water solubility, making them useful in flocculation. Lastly, these materials exhibit antimicrobial properties for which they are most commonly known, e.g bactericidal [36].

The application of surfactants in this work is as antifouling agents in a hydrogel substrate and therefore the physical characteristics play an important role. When hydrogel material is soaked in a solution of benzalkonium chloride it becomes slippery to touch, however, it should be noted that when a hydrogel is immersed in seawater this slippery feeling is drastically reduced. Surface tension is an obvious area to monitor these changes and further research is necessary for this application.

As the hydrogels used within the current study had a 40% water content all the properties of surfactants should operate in this portion. The 60% non-polar portion will also have surfactant content. However, it is suspected that the surfactant will be held more tightly in this section and this could account for the portion of surfactant still detected after long periods of marine exposure. The release values in Chapter 3

show that not all of the quaternary ammonium compound is released even after very long exposure trials and thus interaction with the non-polar portion must be occurring.

2.7 Experimental Work - Benzalkonium Chloride

2.7.1 Preparation of Samples

The quaternary ammonium compound benzalkonium chloride was obtained from various suppliers and in total six types were tested.

The materials and suppliers are listed below :

Trade Name	Supplier / Manufacturer	Supplied Form / Purity
Benzalkonium chloride	Aldrich	white/cream hygroscopic powder (100%)
Benzalkonium chloride	Sigma	cream semi-solid (100%)
Quadrilan BC	Akcros	straw coloured liquid (50%)
Arquad B-50	Akzo Nobel	straw coloured liquid (50%)
Arquad DMMCB-50	Akzo Nobel	straw coloured liquid (50%)
Arquad DMHTB-75	Akzo Nobel	white hygroscopic powder (75%)

Table 2.7.1 Benzalkonium chlorides

(Note, the purity values are not guaranteed by the suppliers and are only approximate. Chapter 3 reports the results of HPLC analysis of these samples from which more accurate purities are calculated.)

The homologue distributions were obtained, if possible from the suppliers, and are listed for the six benzalkonium chlorides :

1. Aldrich Chemicals Ltd

Benzalkonium chloride containing the alkyl chain from C₈-C₁₈.

No purity value available from supplier, assumed to be approximately 100%.

Supplied as a creamy white hygroscopic powder.

2. Sigma Chemical company Ltd

Benzalkonium chloride.

Alkyldimethylbenzylammonium chloride containing mainly C₁₂ and some C₁₄ and C₁₆.

No purity value available from supplier, assumed to be approximately 100%.

Supplied as a semi-solid.

3. Akcros Chemicals Ltd

Benzalkonium chloride.

Trade name 'Quadrilan BC'.

Contains approximately 90% C₁₂ and C₁₄ according to supplier.

Purity 50%. Supplied as a liquid

4. Akzo Nobel Chemicals Ltd supplied the following materials

I. Cocobenzyltrimethylammonium chloride (fractionated coco-alkyl)

Trade name DMMCB-50.

Purity 50%. Supplied as a liquid.

Homologue values

$$C_{12} = 68\%$$

$$C_{14} = 29\%$$

$$C_{16} = 3\%$$

II. Cocobenzyltrimethylammonium chloride

Trade name Arquad B-50

Purity 50%. supplied as a liquid.

Homologue values

$$C_8 = 5\%$$

$$C_{10} = 6\%$$

$$C_{12} = 50\%$$

$$C_{14} = 19\%$$

$$C_{16} = 10\%$$

$$C_{18} = 10\%$$

III. Tallowbenzyltrimethylammonium chloride (hydrogenated tallow-alkyl)

Trade name Arquad DMHTB-75

Purity 75%. Supplied as a creamy/white solid.

Homologue values

$$C_{12} = 1\%$$

$$C_{14} = 4\%$$

$$C_{16} = 31\%$$

$$C_{18} = 64\%$$

Solutions of each of the benzalkonium chlorides were prepared using distilled water. The solutions prepared were 5%w/v based on quantitative analysis (see Chapter 3). All produced clear solutions which did not crystallise out on standing except for the DMHTB-75 material which, due to its high concentrations of the C_{16} and C_{18} homologues is fairly insoluble.

The hydrogels were loaded with benzalkonium chloride by soaking them in the 5%w/v solutions. The purities of each of the benzalkonium chlorides was determined by using a standard obtained from the USP [4].

In order to determine a suitable solvent in which the DMHTB-75 could be dissolved, the following solvents were used to prepare 5%w/v solutions in which small pieces of hydrogel were soaked and their appearance noted after 24 hours :

Solvent	Material solubility	Hydrogel appearance
Distilled water	partially	normal
Acetone	partially	gel becomes sticky
Methanol	partially	gel becomes swollen
Chloroform	soluble	not miscible with water
Iso-propyl alcohol	soluble	gel becomes swollen

Table

As expected, DMHTB-75 is more soluble in non-polar solvents but as these have an adverse effect on the hydrogel they are unsuitable for this application.

Due to the difficulties experienced in preparing a useful solution of DMHTB-75, a method for incorporating this material at the hydrogel preparation stage was

developed. However, a sample was also prepared by soaking the hydrogel in a 5% w/v solution of DMHTB-75. Details of the experimental procedure are given below.

Preparation of Loaded Hydrogel - Soaking Method

It was possible to prepare a 5%w/v solution by warming the mixture and thus a piece of hydrogel was added to this solution. The solution remained clear for 24 hours after which time it began to crystallise out.

The hydrogel was left soaking for 8 weeks after which time a slightly opaque sample was then exposed at the UMBSM.

The remaining benzalkonium chloride solutions were transparent and pale straw in colour. Hydrogel material was soaked in them for 8 weeks and the resultant samples were completely transparent. These were then exposed at UMBSM.

Preparation of Loaded Hydrogel - Incorporated at Hydrogel Preparation

It was only necessary to use this method for the DMHTB-75 material due to its insolubility.

The method used for the preparation of hydrogel material involves a clean-up process in which the hydrogel is heated in distilled water until all the ethylene glycol is released and the material becomes transparent. As the DMHTB-75 becomes more soluble when heated, heat would not be applied when cleaning up hydrogel which had DMHTB-75 incorporated at the production stage.

Method of Preparation [41]

The following materials were added, by pipette, to a beaker and mixed well.

- 7.5 ml distilled water
- 15 ml ethylene glycol
- 15 ml 2-hydroxyethylmethacrylate
- 0.15 ml tetraethylene glycol dimethacrylate
- 1.5 ml 6% aqueous ammonium sulphate
- 1.5 ml 12% aqueous sodium metabisulphate

The mixture was stirred for a few minutes and the pH recorded

$$\text{pH} = 4.00$$

To this mixture 2.00 g of DMHTB-75 was added. This was stirred until it dissolved and the pH of this mixture was again recorded.

$$\text{pH} = 3.80$$

The mixture was now a white, cloudy viscous liquid.

It was then poured into a 150 mm² Perspex mould and covered with a glass lid. The hydrogel was cured in a 60°C oven overnight.

The hydrogel was released from the mould and soaked in distilled water at room-temperature. The distilled water was changed 4 times within the following 5 days by which time the hydrogel had been cleaned up.

To determine the quantitative amount of DMHTB-75 in the hydrogel, HPLC analysis was carried out. Analysis was done on duplicate samples. Injections were done in duplicate with $\geq 98\%$ agreement accepted.

2.7.2 HPLC Analysis

Two 25 mm diameter discs were cut from the same piece of hydrogel that was used in the marine exposure trial and these discs were analysed in order to obtain the zero time content.

Results (Chapter 3 gives full details of all HPLC analysis)

From the quantitative analysis the quantities of DMHTB-75 found to be present in the hydrogel were

$$\text{Disc 1} = 2.70\%w/w$$

$$\text{Disc 2} = 2.51\%w/w$$

From this result it appears that a considerable amount of DMHTB-75 has remained in the hydrogel after the clean-up process. This result is what would have been expected due to the hydrophobic nature of the C₁₈ homologue of the DMHTB-75 material.

Analysis of Homologue Distribution

Using the same samples that were prepared for the above quantitative analysis, RP-HPLC analysis was carried out in order to determine the percentage of each homologue present in the hydrogel (Method in given in Chapter 3). Samples and injections were carried out in duplicate with $\geq 98\%$ agreement accepted. (This was the case for all the results reported in this section).

The homologue distribution was as follows :

C ₁₂	C ₁₄	C ₁₆	C ₁₈
1.49%	3.94%	30.43%	63.71%

Which is the same homologue distribution as the original material and therefore the hydrogel material containing the DMHTB-75 is representative of the material.

DMHTB-75 Wash Residue

A sample of the last portion of the distilled water (200 ml) in which the loaded hydrogel had been stored prior to marine exposure was analysed for DMHTB-75 content. This result would indicate how much active substance was being lost before the test had actually begun.

Result

All the alkyl chains were present on the chromatogram. The actual amount was calculated. The mean result is given below.

$$\text{Value} = 0.0036\%w/v$$

The result shows that a small proportion of the active material is lost in the clean-up stage. However, this amount is very little in comparison to the amount left in the hydrogel.

It should be noted that the chromatogram showed that the amount of C_{18} released compared to the overall amount present is very small. This would be as expected due to the hydrophobic nature of C_{18} .

Marine Results From DMHTB-75 Samples

Both samples were exposed at UMBSM on the 15th March 1995 i.e. the soaked sample and the sample prepared with DMHTB-75 included at production.

Soaked Sample : Algal fouling appeared at 12 weeks

Preparation Sample : Algal fouling appeared at 16 weeks

The rack containing these samples was left out at UMBSM for 27 weeks. The sample which contained the DMHTB-75 (preparation) was analysed. (Mean result of duplicate samples is given below).

$$\text{Content} = 2.08\%w/w$$

This was 79.69% of the original content measured at the beginning of the experiment.

From this result it is possible to say that the release rate for this active material is slow compared to the benzalkonium chloride with higher percentages of C_{12} and C_{14} which was used in the MAST II Project [41]. Fouling occurs even although the active substance is still in the hydrogel. Detailed release studies for all the marine trials can be found in Chapter 3.

Further Marine Samples

From these results DMHTB-75 appeared to be an effective antifouling material and the following samples were prepared for future marine trials.

DMHTB-75 Incorporated at Production

Two samples were prepared for marine trials.

DMHTB-75 Content : 0 Time

1) 2.59%w/w

2) 2.81%w/w

After 18 weeks marine exposure

1) 1.98%w/w

2) Not monitored at this time point.

The amount left in the sample is in the same order which was retained by the first trial sample.

DMHTB-75 Sample which was Soaked

Two samples were prepared for marine trials.

DMHTB-75 Content : 0 Time

1) 3.15%w/w

2) 3.67%w/w

After 18 weeks marine exposure

1) 1.93%w/w

2) Not monitored at this time point

Although a high proportion of the DMHTB-75 remained in the sample it began to foul at 10 weeks. (All analysis was done in duplicate and the mean value quoted. Details of experimental procedure are given in Chapter 3).

2.7.3 Purification of DMHTB-75

The marine studies carried out used the DMHTB-75 as supplied.

From the analytical work carried out the content of benzalkonium chloride has been found to be 63.32%w/v based on the USP Standard. based on duplicate injections with $\geq 98\%$ agreement of results.

In order to improve the purity of the material supplied some was dried at room-temperature in a vacuum oven. After drying the material was weighed and 84.25% of the original weight was present therefore about 17% had been evaporated off.

The supplier stated in the Certificate of Analysis that 17% of the material was propan-2-ol and 8% was water.

The sample was then analysed using HPLC and found to have a benzalkonium chloride of 74.88%w/v.

Effect on Homologue Distribution of Vacuum Drying

After vacuum drying the homologue distribution was as follows :

C_{12}	C_{14}	C_{16}	C_{18}
2.80%	4.01%	33.91%	59.19%

The homologue distribution in the original material was as follows :

C_{12}	C_{14}	C_{16}	C_{18}
1.86%	5.05%	32.29%	60.87%

From this result it can be seen that vacuum drying has practically no effect on the homologue distribution. Due to the extent of time which had to be spent vacuum drying it was decided at this point that marine exposure samples would be prepared using the material obtained directly from the supplier.

2.7.4 pH Measurements of Hydrogel Samples containing Benzalkonium Chloride

Surface pH measurements were made on hydrogel samples containing both the benzalkonium chloride supplied by Aldrich and the DMHTB-75 supplied by Akzo Nobel.

BAC Hydrogel pH = 3.7

DMHTB-75 Hydrogel pH = 3.9

After 5 weeks in the marine environment surface pH measurements were again taken.

BAC Hydrogel pH = 7.5

DMHTB-75 Hydrogel pH = 7.5

All measurements were done in triplicate and the mean value has been quoted.

It appears that when hydrogel is immersed in seawater the pH increases to that of nearly the seawater itself. It should be noted that the surface pH measurements are difficult to take due to the nature of the hydrogel's surface and therefore the pH's quoted are based on values ranging from 7-8 with regard to the seawater samples. From this result it appeared that the seawater has changed the surface pH to that similar to its own i.e. the water content of the hydrogel now has seawater in it.

2.8 Cetrimide

2.8.1 Preparation of Materials

The quaternary ammonium compound cetrimide (alkyltrimethylammonium bromide) was obtained from various suppliers. A total of four materials were obtained. Table 2.8.1 shows the details of each, given by the suppliers.

Name	Supplier	Supplied Form	Homologues Present
Cetyltrimethylammonium bromide (CTAB)	Aldrich	Creamy/white powder	Mainly C ₁₆ , with some C ₁₂ and C ₁₄
Mixed alkyltrimethylammonium bromide (ATAB)	Sigma	Creamy/white powder	Mainly C ₁₄ , with some C ₁₂ and C ₁₆
Hexadecyltrimethylammonium bromide (HTAB)	Sigma	Creamy/white powder	Mainly C ₁₆ , with some C ₁₂ and C ₁₄
Hexadecyltrimethylammonium bromide (HTAB)	Fluka	Creamy/white powder	Mainly C ₁₆ , with some C ₁₂ and C ₁₄

Table 2.8.1 Cetrimide materials

2.8.2 Preparation of Cetrimide for the Loading of Hydrogel

Aqueous solutions of cetrimide require to be prepared in order that the hydrogels can be soaked in these solutions and the uptake of the active substance can take place.

The following 1%w/v aqueous solutions were prepared by simultaneously heating and stirring the mixture.

Material	4 hours	24 hours	4 days
Aldrich CTAB	clear	crystallisation	crystallisation
Fluka HTAB	clear	crystallisation	crystallisation
Sigma HTAB	clear	crystallisation	crystallisation
Sigma ATAB	clear	clear	clear

Table 2.8.2 Cetrimide solubility

From the above results it can be seen that cetyltrimethylammonium / hexadecyltrimethylammonium bromide (Cetrimide USP) which is predominantly C_{16} does not stay in solution. This would be expected due to the predominance of the C_{16} homologue which exhibits hydrophobicity.

Sigma Chemical Company Ltd state in their catalogue that the product CTAB, which they supply, is 10% soluble in water. From the results table it can be seen that this is not the case and although the material will initially go into solution at 1%w/v, with heating, it crystallises out on standing after a few days. Figure 2.8.1 shows the amount of material which came out of solution in 24 hours at room-temperature.



Figure 2.8.1 Crystallisation in the HTAB sample (Sigma) after 24 hours.

The material which was used as cetrimide in this research was ATAB. A 5% w/v solution could be prepared which would stay in solution and therefore could be used to soak a hydrogel thus loading it with active material. This result could be expected as the three other cetrimide samples were mainly composed of the C_{16} homologue which is only slightly water soluble. The hydrogel material was soaked for 8 weeks in a 5% w/v solution. It remained transparent during this time with no notable change in its character i.e. it did not swell, which would have made it mechanically weak.

In the initial marine trial a sample of cetrimide was immersed on Rack 1, 1995. When the loaded hydrogel was immersed in seawater it remained transparent. This material did not show particularly good anti-fouling action and more details of its performance are given in section 4.5. Further marine trials in 1996 reinforced this result.

2.9 Chlorhexidine

2.9.1 Experimental Work

The research into the anti-fouling properties of chlorhexidine concentrated on the material in the gluconate form and some work was done on the less soluble chlorhexidine acetate. Chlorhexidine gluconate 20%w/v was supplied by Zeneca Pharmaceuticals (trade name for this material is "Hibitane") and chlorhexidine acetate was supplied by Sigma Chemical.

Chlorhexidine Gluconate - Solution Preparation

Chlorhexidine gluconate (CG) was supplied as a 20%w/v solution which was straw-coloured and sticky to the touch. It was used "as supplied" and also as a 5%w/v solution, which was prepared by dilution of the 20%w/v solution using distilled water.

Hydrogels in Solutions

When the hydrogel material was soaked in 20%w/v and 5%w/v solutions of CG it became swollen and mechanically weak after 24 hours and was therefore not suitable for use in marine trials. If the hydrogel was left in these solutions in excess of 6 days it was so greatly swollen that it tore easily on being handled. It was found that by pretreating the swollen hydrogel in seawater in the laboratory before marine trials, the hydrogel shrunk to nearly its original size. An experiment was carried out in order to quantify the swelling and subsequent shrinkage.

2.9.2 Swelling/Shrinking of Hydrogel

Two discs were cut from a previously prepared sheet of hydrogel material and weighed. These discs were then soaked in 150 ml of 20%w/v CG. The weights were

recorded for 17 days and are shown in table 2.9.1. Figure 2.9.1 shows weight increase in the hydrogel material against time.

Time	Weight Hydrogel Disc 1 (g)	Weight Hydrogel Disc 2 (g)
0 time	0.5059	0.5500
24 hours	0.8327	0.8628
4 days	1.7584	1.7997
5 days	1.8760	1.9816
6 days	1.9789	2.1031
17 days	1.9895	2.1629

Table 2.9.1 Weight of two hydrogel discs soaked in chlorhexidine gluconate

After 17 days no further weight increase was observed.

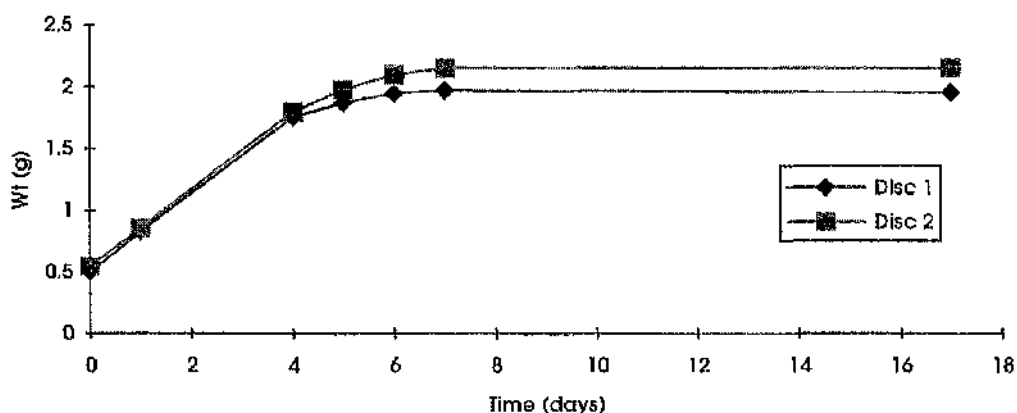


Fig. 2.9.1 Hydrogel weight gain due to swelling in chlorhexidine gluconate

The two discs were then transferred into 200 ml of filtered seawater and their weight changes were recorded. The results are shown in table 2.9.2.

Time	Weight Hydrogel Disc 1 (g)	Weight Hydrogel Disc 2 (g)
0 time	1.9895	2.1629
24 hours	1.0734	1.3430
3 days	0.7887	0.9160
7 days	0.7550	0.8357

Table 2.9.2 Shrinkage of material in seawater.

From the results it can be seen that after 24 hours the hydrogel samples halve in weight and after 3 days they appear to reach a weight after which there is little change. In practice the samples were exposed at sea after 24 hours shrinkage and did not subsequently tear in their support frame.

2.9.3 Preparation of Hydrogel Samples for Marine Exposure Trials

Hydrogel pieces were soaked in both 5%w/v and 20%w/v solutions of chlorhexidine gluconate. They were left for 6 days in these solutions after which time they had become swollen and thus mechanically weak. They were then transferred to seawater for 24 hours before they were put on marine trials. In seawater they became opaque and the surface had a white precipitate on it. Figure 2.9.1 shows such discs which were soaked in seawater.

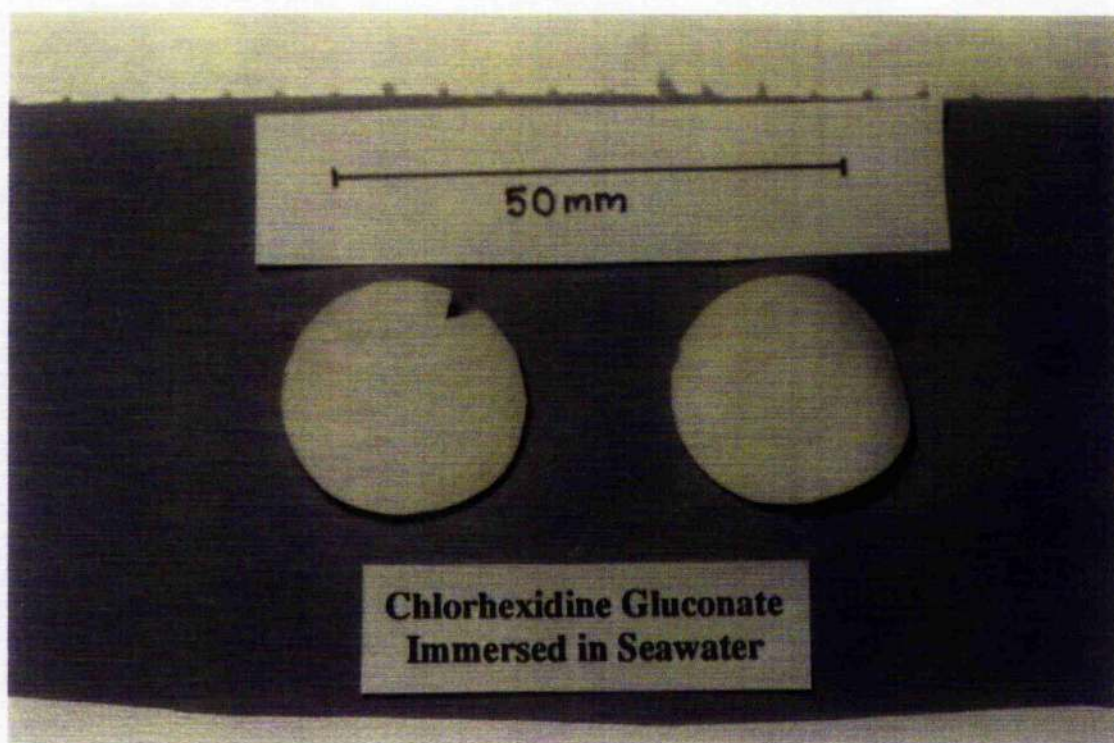


Fig. 2.9.1 Chlorhexidine gluconate soaked hydrogel after immersion in seawater.

These reactions occurred each time samples containing chlorhexidine gluconate were immersed in seawater. In order to determine what the white salt-like material which was precipitated onto the hydrogel surface was, the following experiment was set-up.

2.9.4 Experimental - Method

Into 30 ml bottles, 10 ml of 20%w/v chlorhexidine gluconate was added and to each solution the following quantities of seawater was added :

1. 0.1 ml
2. 0.2 ml
3. 0.5 ml
4. 0.7 ml

The bottles were sealed and left at room-temperature for 24 hours. The experiment was carried out in triplicate

Results

After 24 hours a white precipitate had formed in the solutions containing 0.5 ml and 0.7 ml of seawater.

In order to identify this white precipitate another experiment was set-up where 10 ml of chlorhexidine gluconate was added to 10 ml of filtered seawater. The volume of seawater was increased in order that sufficient precipitate would be formed to analyse. The white precipitate formed was filtered through Whatman No. 1 paper and dried in the oven overnight at 60°C.

Weight of the precipitate = 1.3464g

The precipitate was analysed using Fourier Transforms infra-red spectroscopy (FT-IR). When this scan was compared to that of chlorhexidine salt infra-red scans in the British Pharmacopoeia it was found to match that of chlorhexidine dihydrochloride. Chlorhexidine dihydrochloride (Sigma) was then scanned and compared with the unknown and the scans were found to be identical. The white precipitate can therefore be identified as chlorhexidine dihydrochloride which is extremely insoluble in water.

Figures 2.9.2 and 2.9.3 show the FT-IR scan of the white precipitate and chlorhexidine dihydrochloride (Sigma) respectively.

Discussion

Various facts relating to chlorhexidine must be considered in order to explain the results obtained when chlorhexidine gluconate was immersed in seawater. The first being that the antimicrobial activity of chlorhexidine is pH-dependent. The optimum range is between pH 5.5 to 7.0. However, in solution it is active within the pH range from 5.0 to 8.0 [21]. At pH 8.0 chlorhexidine begins to precipitate out and, in addition, if the water soluble gluconate form has been used and is then exposed to inorganic anions such as carbonates, chlorides, etc. then precipitation occurs. Therefore, two effects occur in the marine environment which prevent chlorhexidine producing its optimum antimicrobial action.

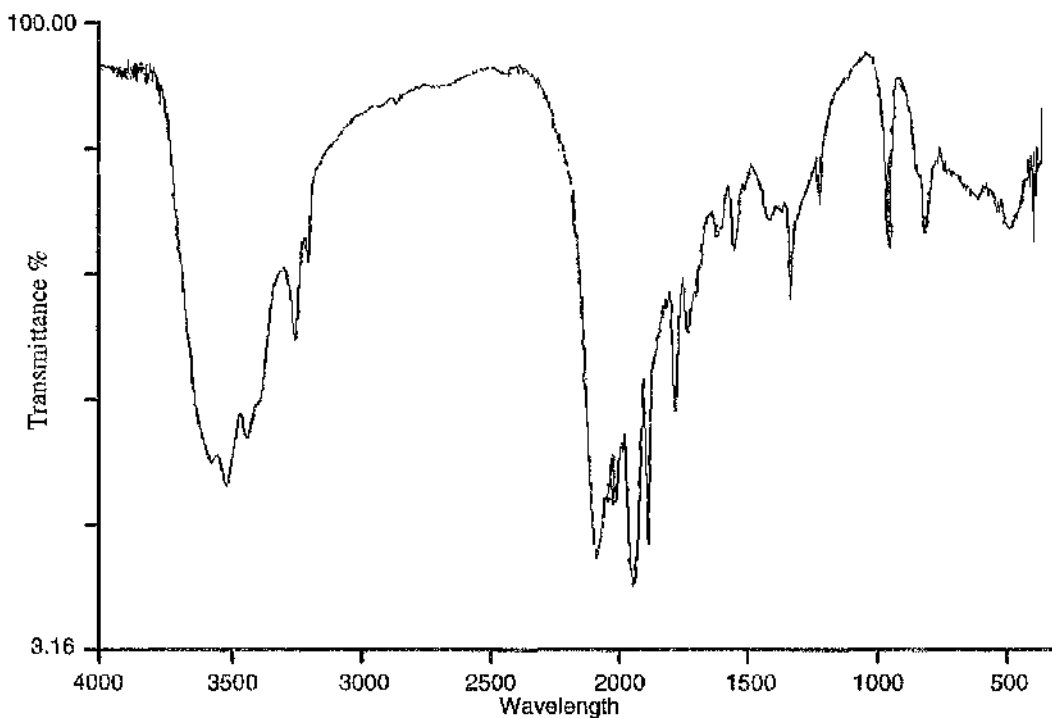


Figure 2.9.2 Chlorhexidine Dihydrochloride
(result from adding chlorhexidine gluconate to seawater)

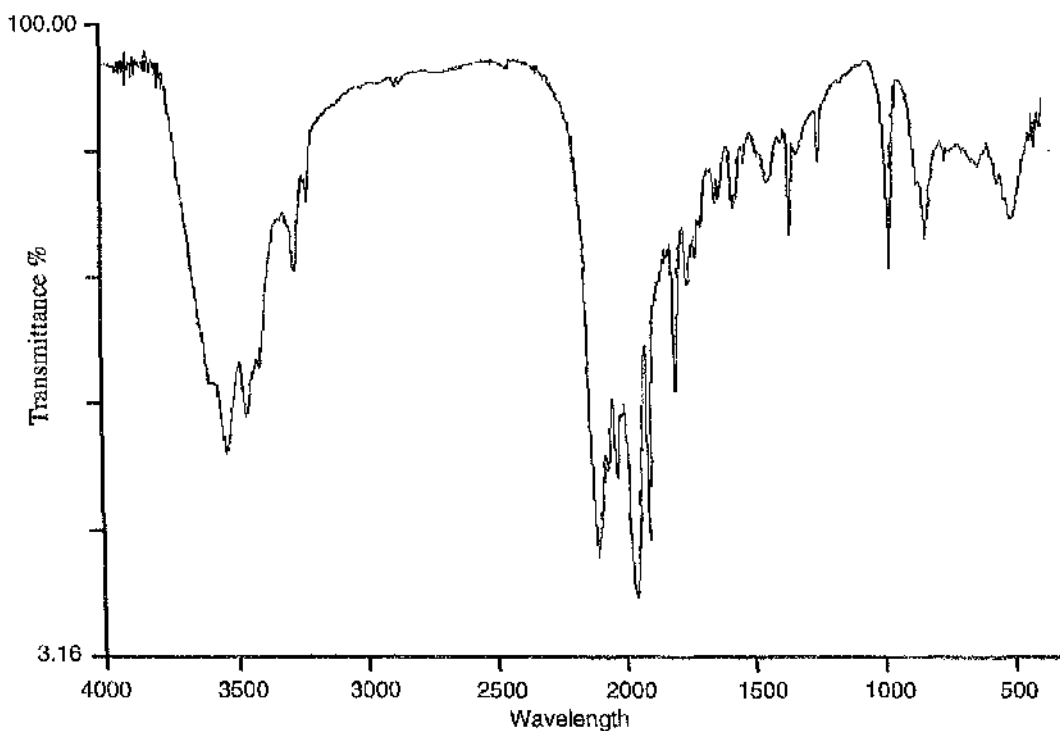


Figure 2.9.3 Chlorhexidine Dihydrochloride (Sigma)

2.9.5 Chlorhexidine Acetate

Marine trials using chlorhexidine acetate are detailed in Chapter 4 and analysis of the samples using HPLC is detailed in Chapter 3.

Preparation of Samples

1.5 g of chlorhexidine acetate was dissolved in 100 ml of distilled water. A piece of hydrogel was soaked in this solution and any changes to its character noted. The hydrogel did not swell and the chlorhexidine acetate remained in solution at room-temperature. Three pieces of hydrogel were prepared for marine trials using this soaking method.

2.10 Benzalkonium Chloride and Ethylenediaminetetraacetic Acid - Experimental Work

2.10.1 Initial Work

A 200 ml solution containing 5%w/v benzalkonium chloride (BAC) and 2.5%w/v tri-sodium ethylenediaminetetraacetic acid (tri-EDTA) was prepared and stored at room-temperature. This solution was monitored and it remained clear for 7 days. At this point a piece of hydrogel material, 120 mm², was soaked in the solution for 10 weeks. After this period the hydrogel appeared to have remained the same shape and size as it was originally. The sample was very clear and shiny and was observed to be crystal clear. When this sample was exposed to the marine environment it became opaque (details of its marine behaviour can be found in Chapter 4).

As this initial sample had been shown to be useful as an anti-fouling material further work on the BAC/EDTA combination was begun and solutions prepared as described below.

Preparation of Solutions

EDTA itself is practically insoluble in water, however, it forms a series of mono-, di-, tri-, and tetrasodium salts which increase in water solubility as well as in pH.

200ml of the following solutions were prepared and the pH of each recorded :

Solution	pH
5% BAC	5.75
5% BAC / 2.5% di-EDTA	4.40
5% BAC / 2.5% tri-EDTA	7.88
5% BAC / 2.5% tetra-EDTA	10.50

Hydrogel pieces were soaked in these solutions for 3 weeks. After this time a marine trial was begun with 2 samples from each of the above solutions. When the rack was examined after 2 weeks the 6 hydrogels containing the sodium salts of EDTA were all opaque. In Chapter 4 details of the behaviour of each of these hydrogels in the marine environment is given. From this trial the addition of tri-sodium EDTA gave the superior anti-fouling performance and further trials using the benzalkonium chloride/ tri-EDTA combination were carried out.

2.10.2 Complex Formation

The opaque nature of samples containing the mixture of benzalkonium chloride and tri-sodium EDTA indicated that a practically insoluble complex was forming in the system when it was introduced to seawater. Hydrogel samples which had been soaked in tri-sodium EDTA alone remained transparent in seawater and so the presence of benzalkonium chloride was essential to the complex formation. An experiment was set-up in the laboratory where each of the three sodium salts of EDTA were added to seawater both with and without benzalkonium chloride. The details of the experiment are shown.

Experiment - Complex Formation

100ml solutions of the following were prepared using filtered seawater and stored at a temperature of 5°C for two weeks.

1. 1g di-sodium EDTA
2. 1g tri-sodium EDTA
3. 1g tetra-sodium EDTA
4. 1g di-sodium EDTA and 1g BAC
5. 1g tri-sodium EDTA and 1g BAC
6. 1g tetra-sodium EDTA and 1g BAC

Results

After 2 weeks the samples were filtered through Whatman No1 filter paper and the white residue was dried in the oven for 48 hours at 40°C. The dried material was then weighed.

Material	Weight (g) of white residue
1g di-sodium EDTA	1.0567g
1g tri-sodium EDTA	0.4408g
1g tetra-sodium EDTA	0g
1g di-sodium EDTA and 1g BAC	1.0412g
1g tri-sodium EDTA and 1g BAC	0.3790g
1g tetra-sodium EDTA and 1g BAC	0g

Table 2.10.1 Weights of white residues.

Discussion

From the pKa values of EDTA this result would be expected as the pH of the seawater was 7.93. In this experiment the presence of the benzalkonium chloride made no difference to the amount of complex formed (the experiment was repeated and the results were found to be the same). However, in the hydrogel system the

benzalkonium chloride did affect the complex formation and so this laboratory experiment did not fully reflect what was happening in the marine trials.

2.10.3 Preparation of Marine Samples (Winter 1995)

Hydrogel samples containing DMHTB-75 were soaked in 2.5% (aq) tri-sodium EDTA for 1 week. After this time the hydrogel had a patchy appearance i.e. areas of white, cloudy and clear. The pH of the surface of this material was 8.19 which is much more alkaline than when DMHTB-75 alone is incorporated into hydrogel. Table 2.10.2 below shows the pH measurements and hydrogel appearance of the four variations of the samples which were put on marine trials on the 19th November 1995. The hydrogels containing the benzalkonium chloride and the benzalkonium chloride/tri-EDTA were prepared by soaking in the appropriate solutions. Three samples of each were prepared.

Material	pH	Comment
DMHTB-75	4.40	material opaque/matt white
DMHTB-75/EDTA	8.19	material patchy i.e.opaque/patchy/clear
BAC	3.69	clear
BAC/tri-EDTA	7.14	clear

Table 2.10.2 Surface pH measurements of hydrogels containing different types of benzalkonium chlorides.

After marine immersion it was found that the pH of each sample (three of each) was now approximately 7.5, near to that of seawater.

2.10.4 Preparation of Samples (Spring 1996)

The experiment described in section 2.10.3 was repeated and this time hydrogel containing tri-sodium EDTA was also included in the marine trials in order to

establish if it had any effect on anti-fouling on its own. The results of the anti-fouling behaviour of these trials is detailed in Chapter 4.

The pH measurements of each sample was recorded before sea immersion and a mean value for each sample is shown below :

Material	pH
DMHTB-75/tri-EDTA	8.02
DMHTB-75	4.00
BAC	4.80
BAC/tri-EDTA	7.54
tri-EDTA	7.12

The pH measurement of each sample was taken after 1 week in seawater and samples now had a pH of about 7.50. From the work done on monitoring the surface pH of hydrogels it appears that the pH of seawater dominates any pH effects that the active substances might have.

2.11 Experimental Work - Mixed Surfactant Systems

The materials used in this work were supplied by :

Benzalkonium chloride	-	Aldrich
Cetrimide	-	Sigma
Chlorhexidine gluconate	-	Zeneca

2.11.1 Benzalkonium Chloride and Chlorhexidine Gluconate

Two 100 ml solutions containing 5%w/v benzalkonium chloride and 0.5%w/v chlorhexidine gluconate were prepared. Pieces of hydrogel were soaked in these solutions for 2 weeks. There was no visible change in the hydrogel appearance and

thus samples were used directly in marine trials without pretreatment in seawater in the laboratory.

The experiment was repeated for solutions containing 5%w/v benzalkonium chloride and 2%w/v chlorhexidine gluconate.

The hydrogels had a slight translucent appearance after 2 weeks in the marine environment.

2.11.2 Benzalkonium Chloride and Cetrimide

A 100 ml solution containing 5%w/v benzalkonium chloride and 5%w/v cetrimide was prepared. It remained clear and a piece of hydrogel was soaked in this solution for 8 weeks and then put on marine trials. The hydrogel remained clear after immersion at UMBSM.

From the marine trial results reported in Chapter 4 it can be seen that combinations of surfactants did not improve the anti-fouling ability beyond that of benzalkonium chloride on its own and therefore further work was not carried out on this branch of the research.

2.12 Conclusions

From the details of the experimental work it can be seen that some technical difficulties were experienced in preparing loaded hydrogel material for the anti-fouling trials. However, most of these were overcome and satisfactory alternatives were found.

The surface pH measurements proved that the active substances, in conjunction with the hydrogel were not able to maintain their own pH as the pH of the seawater was the dominant effect.

At the end of each of the sections comments have been made and so there is no need for further explanations at this stage. In Chapter 5 there is a drawing together of all the findings and areas for possible future research are discussed.

2.13 Chapter 2 - References

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Chapter 3

The quantitative analysis of potential anti-fouling materials

- 3.1 History and Theory of High Performance Liquid Chromatography
- 3.2 Method for Homologue Separation of Benzalkonium Chloride
- 3.3 Quantitative Analysis of Benzalkonium Chloride
- 3.4 Quantitative Analysis of Cctrimide
- 3.5 Quantitative Analysis of Chlorhexidine
- 3.6 Conclusions
- 3.7 References

3.1 High Performance Liquid Chromatography

Chapter three is divided into six sections. The first deals with the historical development of high performance liquid chromatography (HPLC), which was the analytical technique chosen for analysis of the successful anti-fouling materials. Details of the development and practicalities of HPLC operation are dealt with in some depth, as it is useful to have a clear picture of the mechanisms involved when considering the method development. Section 3.2 deals with the method chosen for the analysis of the homologue distribution of the benzalkonium chlorides, the successful anti-fouling materials. The theory of the method is described as well as why it was preferred to other analytical techniques and other published HPLC methods. Sections 3.3 to 3.5 deal with the quantitative analysis of the prospective anti-fouling materials. The last section summarises the results obtained and their relevance with regard to the anti-fouling studies.

3.1.1 History and Origins

Liquid chromatography in its original form, now known as Classical Liquid Chromatography, was first reported in 1903 by Tswett, a Russian botanist who utilised the technique to separate plant pigments. In 1906 he published work in which he stated that "Chromatography is a method in which the components of a mixture are separated on an absorbent column in a flowing system" [1,2]. (Tswett used the term "chromatography" to describe his work, the word being derived from the Greek for colour, chroma and from write, graphein). Although his doctoral thesis covered this work he continued his career as a botanist, not as a chromatographer, and nothing more was heard of Tswett in chromatography. After Tswett's work was documented, little more on this technique was recorded until the 1930's, after which time many researchers became interested in various forms of chromatography.

In 1941 Martin and Synge [3] carried out partition chromatography of amino acids. However, it was not until 1952, when the same two researchers applied a mathematical theory (theoretical plate count) to their work, that the understanding of the mechanisms of chromatography became clearer. Great strides were made during the 1940-50's and in the early 1950's Martin and James achieved a major milestone in their development of gas-liquid partition chromatography when they applied a gaseous mobile phase to an involatile liquid-coated solid support which led to widespread use of gas chromatography [4]. This method brought with it automation,

making it much more acceptable, and thus gas chromatography became routine in the analytical laboratory.

It was during 1960-70's, however, when high performance liquid chromatography (HPLC) became a useful analytical tool, as it was found to be applicable to materials for which gas chromatography could not be used e.g. non-volatiles or thermally unstable materials. Various researchers produced papers and books on the subject during this period, included among these are Horvath, Kirkland and Knox who made major contributions to the understanding of HPLC [5-8].

3.1.2 Operation of Methods

In this section a brief description of the operational aspects of the predecessor of HPLC is given. This is followed by the operation and theoretical background of HPLC.

Classical Column Chromatography

In classical column chromatography, open glass tubes of lengths 15-20cm and diameters ranging from 1-4cm were filled with packing materials such as alumina or silica. The sample was added to the top of the column as was a suitable solvent, to "carry" the sample, which would now be termed a mobile phase. As the sample moved down the column its separation could be detected as a series of coloured bands which appeared as each component in the sample is retained by the column packing. These coloured bands became known as "chromatograms", a term which is still used today to describe the printout from the integrator or PC in modern HPLC systems.

In order to extract each of the components the packing material was removed and the component extracted from it. This was a difficult and time consuming business for which a high degree of analytical skill was required. By 1930, however, the extracts were collected as liquid fractions as they emerged from the column. Although this was an improvement it was still difficult to achieve useful results unless the procedure was performed by an experienced chromatographer. Despite these drawbacks, considerable work was done in the separation of natural products and the building bricks of Modern HPLC were laid. Figure 3.1.1 is a diagrammatic representation of classical column chromatography.

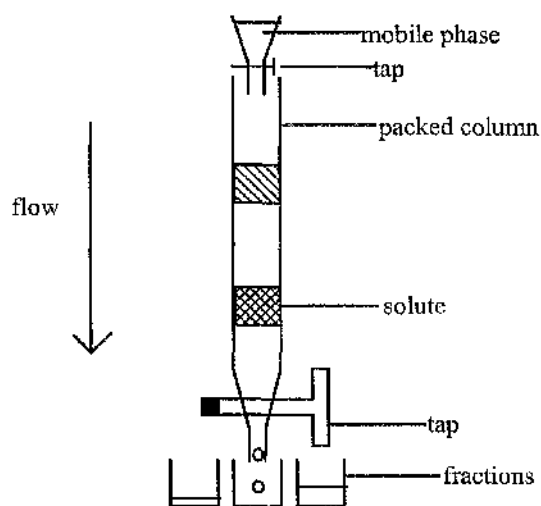


Fig. 3.1.1 Diagrammatic representation of classical column chromatography

Modern High Performance Liquid Chromatography (HPLC)

Prior to the 1960's much work had been done on gas chromatography which had by then become a major analytical tool, however, as greater understanding of the mechanisms involved in liquid chromatography were found, researchers turned their attention back to this type of work. Various reasons were given for the improvements in liquid chromatography resulting in the emergence of HPLC. The main problem which researchers had faced was that of poor quality packing materials resulting in the column bleeding after a few injections. Therefore, the introduction of small porous silica particles reduced this problem as it led to less "dead volume" of the mobile phase within the column. On the technical side the use of high pressure via a pump greatly speeded up the technique. In short, as technology moved forward the system became more automated and thus useful for routine laboratory analysis on a large scale. However, it was not until the 1970's that HPLC became fully accepted as an analytical technique.

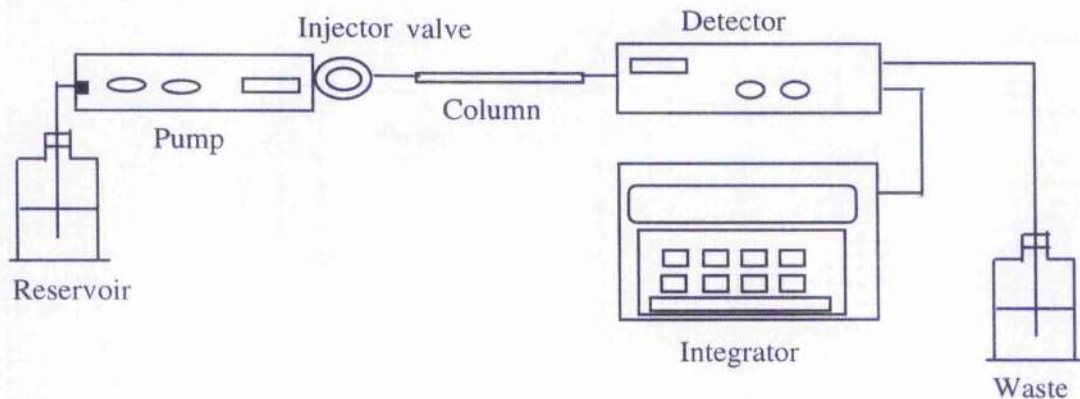


Fig. 3.1 2 Diagrammatic representation of an Isocratic HPLC System

Figure 3.1.2 shows the basic components of HPLC. Detection is mostly by ultra-violet (UV) although fluorescence, mass spectrometry and refractive-index are also used if the analyte of interest does not absorb in the UV range. Figure 3.1.3 shows one of the two systems on which this research was carried out.

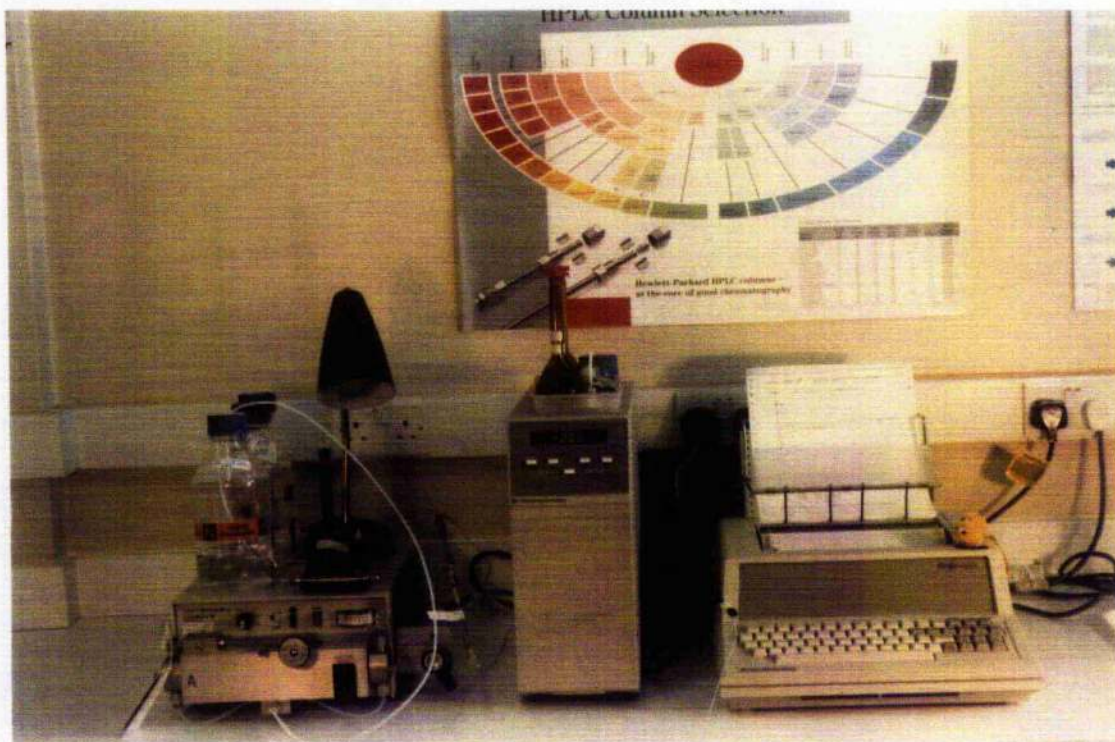


Fig. 3.1.3 Photograph of the instrumentation of HPLC.

Mechanisms of HPLC

The separation process is dependent on the distribution between two phases, the mobile phase (solvent) and the stationary phase (column). The mobile phase and stationary phase are chosen depending on the analysis required. Initially, unmodified silica-based polar columns with a non-polar mobile phase were used, this was known as Normal Phase HPLC. However, when chemical supports were bound to this silica its character was changed significantly i.e. the column packing could be made to be non-polar or slightly polar. This became known as Reverse Phase HPLC. (The word "polar" in chromatography is used as an index of the ability of compounds to interact with one another [9]). The description polar is applied to solutes, stationary phase and mobile phase and the more polar a molecule, the more strongly it can interact with other molecules through various mechanisms which are discussed in the next section. The most commonly used solvents for mobile phase are acetonitrile and methanol which are used in Reverse-Phase-HPLC (RP-HPLC) which accounts for about 70% of all HPLC analysis, due to fast re-equilibration times during solvent change over. In a typical HPLC system the mobile phase is pumped through the system at high pressures, typically 2000psi (SI units 13MNm^{-2}). The flow-rates through the system vary but are usually in the order of 1-2ml/min. It is into this liquid flow that the analyte is injected via a syringe through the "Rheodyne Valve" which has a fixed volume loop which issues an accurate volume. When the analyte reaches the silica packing of the column it interacts and is held there by a mixture of mechanisms. These mechanisms are :

- Adsorption
- Partition
- Ion-exchange

These mechanisms are complex and much work is still being done in order to understand the exact nature of reaction between analyte, column and mobile-phase [9,10].

As the analyte is eluted from the column it is then qualitatively and/or quantitatively detected by one of the following detectors :

- UV/VIS
- Fluorescence
- Mass Spectroscopy
- Refractive Index

The most commonly used detector is ultra-violet (UV) due to its quantitative accuracy, its ease of use and fairly reasonable price.

The output from the detector is then translated to a meaningful result by an integrator, PC package or digital recorder. In figure 3.1.4 a typical chromatogram from a UV detector is shown.

The description given is only a general mechanism, details of the more complex variations in HPLC have not been explained, e.g. gel permeation-exclusion or affinity, which use chemistries not required for the analysis of the compounds of interest in this work.

3.1.3 Considerations for Method Development

When developing a method its usefulness is frequently determined by chemical and physical factors i.e. the results obtained from HPLC must adhere to certain criteria in order for these results to be analytically acceptable. The two chemical factors which are used to determine if a method is useful are capacity and selectivity.

Capacity Factor, k' (Retention)

The capacity factor, k' , which is also known as the retention factor, is a measure of how well the sample molecule is retained by the column during an isocratic separation. The capacity factor is affected by a change in mobile phase strength and column packing. It is calculated from the chromatogram by equation 3.1.1.

$$k' = \frac{V_R - V_o}{V_o} = \frac{t_R - t_o}{t_o}$$

Equation 3.1.1 Capacity Factor k' .

Where V_0 is the void volume of the system and V_R is the volume of the retained analyte of interest. The time (t) taken for the peaks to be eluted can also be used to calculate the capacity factor. The capacity factor should be between 2 and 10. If the value is lower than 2 then analyte is not being adequately retained and if the value is greater than 10 the analysis is taking too long. k' in the range between 2 and 5 represents a good balance between analysis time and resolution. The chromatogram shown below is one from the separation of the homologues of USP Standard benzalkonium chloride. The capacity factors for this particular example are $k'_1 = 2.61$ and $k'_2 = 3.17$ thus this method is satisfactory in that it meets the criteria for the capacity factor.

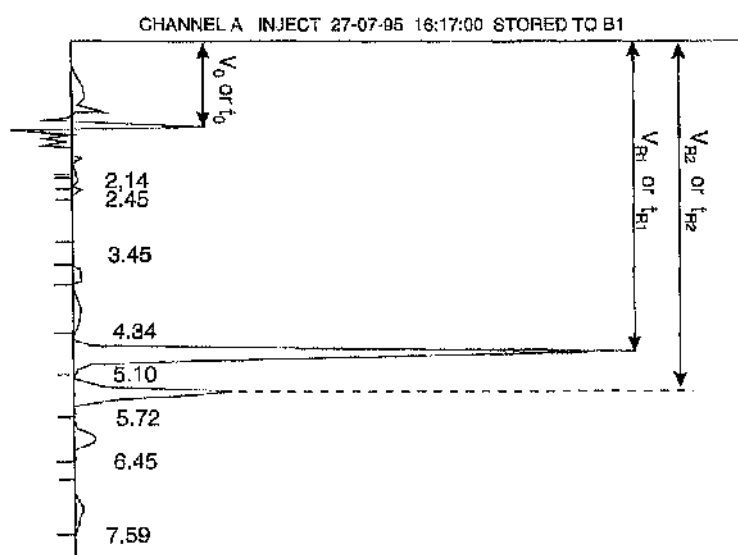


Fig. 3.1.4 A Chromatogram

Selectivity Factor, α

Selectivity is a measure of the relative retention of two components in a mixture. It can be affected by changes in the following :

- Mobile Phase
- Stationary Phase
- pH
- Temperature

In order to calculate the selectivity factor the following equation is used.

$$\alpha = \frac{k'_2}{k'_1} = \frac{V_2 - V_0}{V_1 - V_0}$$

Equation 3.1.2 Selectivity Factor, α

The selectivity factor (α) must be greater than or equal to 1 for the method to be acceptable, as less than 1 would mean that the peaks were not being properly resolved. For the USP Standard benzalkonium chloride example, shown in figure 3.1.4, $\alpha = 1.21$.

The physical factor which is measured is the Efficiency. Efficiency varies with the linear velocity which is proportional to flow rate. A measure of column efficiency is calculated from the theoretical plate count. If the method developed meets these chemical and physical criteria then it is suitable for use. However, there are practical considerations which must be looked at :

column cost

mobile phase cost and ease of preparation

method robustness

In Section 3.2 the method developed for the separation of the homologues of benzalkonium chloride will give details of these characteristics.

3.2 HPLC Method for the Separation of the Homologues of Benzalkonium Chloride

The importance of the carbon chain lengths to the properties of benzalkonium chloride has been detailed previously (Chapter 2), thus the development of a robust method was required in order that the percentage of each chain length could be detected.

Various gas chromatography methods have been employed in the past but degradation of the column makes these methods unfavourable [11-14]. Many papers have been published on HPLC methods for the analysis of benzalkonium chloride.

The method is required to be :

- ◇ Reproducible
- ◇ Fast, i.e. not more than approximately 12 minutes, as longer would make routine analysis very time consuming.
- ◇ Cost effective, therefore an inexpensive column is required due to large sample batches.
- ◇ Able to be used with a mobile phase that is easily prepared and stable at room temperature.

The methods considered were reproducible and had statistical data to back them up. However, many of them had analysis times of greater than 12 minutes [15-19].

Benzalkonium chloride exhibits three wavelength (λ) maximums at 257, 263 and 269nm and these are useful down to levels of about 0.004%. A shoulder appears at about 220nm and much greater absorbance occurs at lower wavelengths after this point. Many methods utilise 214nm as the absorbing wavelength as this can be used for trace analysis (wavelengths lower than this suffer from interference from mobile phase absorbance, i.e. UV cut-off limit).

Two methods were assessed, one developed by Meyer [20] and the other by Gomez-Gomar [21].

The chromatographic conditions for both of these methods are shown :

Method 1, Meyer

HPLC Conditions

Column	Techopak 10CN (HPLC Technology)
Column Temp	ambient
Flow Rate	2.00ml/min
Mobile Phase	acetonitrile / 0.1M sodium acetate 60 / 40 pH = 5.03 adjusted using glacial acetic acid
Wavelength	254nm
Detection	0.5AUFS (attenuated units full scale)

The Techopak 10CN column is 30cm long. The silica used in the production of this column is 10 μ m irregular silica. Separation efficiency of this column is 25,000plates/m (manufacturer's value).

Method 2, Gomez-Gomar

HPLC Conditions

Column	Techsphere 5CN (HPLC Technology)
Column Temp	ambient
Flow Rate	2.00 ml/min
Mobile Phase	acetonitrile / 0.1% triethylamine (TEA) 40 / 60 pH = 2.50 adjusted using orthophosphoric acid
Wavelength	214nm
Detection	0.5AUF5

The Techsphere 5CN column is 25cm long. The silica used in the production of this column is 5 μ m regular silica. Separation efficiency of this column is 60,000plates/m (manufacturers value). The conditions above are slightly changed from the original method [21] as the column used was one that was not available in the U.K. The one used was an equivalent. The temperature was held at 40°C in the original method, (probably to keep it constant rather than to improve the analysis), and at ambient temperature in this work since a column oven was not available for use.

Both methods were tried. However, it was decided that the second method was generally more useful for the current application as it was sensitive to lower detection limits. This was due to the absorbing wavelength i.e. there is more absorbance at 214nm than at 254nm.

3.2.1 Chemistry of Method

The chemistries involved in the method chosen for the separation of the alkyl chains of benzalkonium chloride are described in some detail as it was noted that, although the literature covers this area in different works, it was not possible to find a comprehensive study in one single paper.

Column and Mobile Phase Chemistry

The column used is prepared using 5 μ m diameter silica which has cyano propyl bound to the silanol groups.

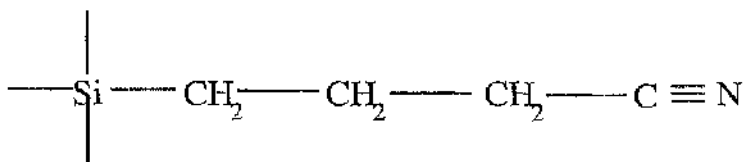


Fig. 3.2.1 Diagram of cyano propyl bonded to silica support.

From the manufacturer's information the column has a minimum efficiency of 60,000 plates/metre, when new. The value was calculated in the laboratory when the column was purchased and found to be 64,000 plates/metre using equation 3.2.1

$$N = 5.54 \left(\frac{t_r}{W_{0.5}} \right)^2$$

Equation 3.2.1 Theoretical number of plates, i.e. efficiency.

Where t_r is the retention time and $w_{0.5}$ is half the column width.

Although the silanols have the cyano propyl groups attached to them some remain unbound as it is extremely difficult to completely eradicate all of the charge. The negative charge which remains unbound is free to bind to the analyte (solute), which results in unfavourable peak tailing.

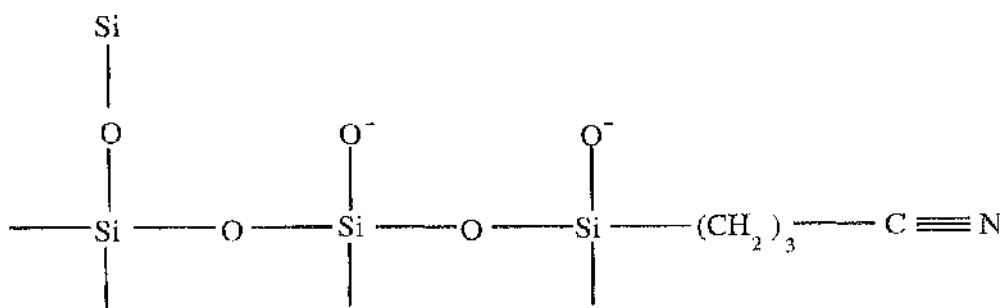


Fig. 3.2.2 Diagram showing negative charge remaining on column support surface.

To eliminate the effects of the residual negative charges the modifier triethylamine (TEA) is used.

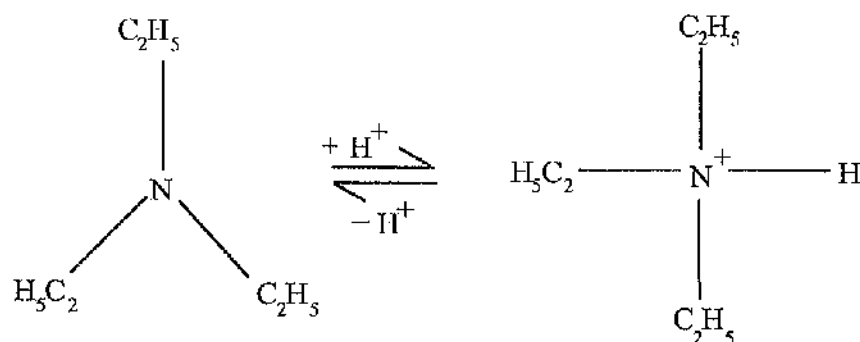
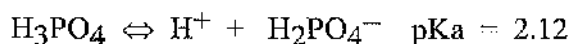


Figure 3.2.3 Diagram of triethylamine.

Orthophosphoric acid is used to adjust the pH to 2.5.

Orthophosphoric acid is a tribasic acid which is ionised over the entire pH range.



The above equations show the successive dissociation equilibria of orthophosphoric acid. The ion which would predominate here would be H_2PO_4^- .

At pH 2.5 the TEA will be fully protonated and available to neutralise the negative sites on the silica column thus virtually eliminating the peak tailing.

Action of the Amine Modifier

The amine modifier triethylamine (TEA) used in this homologue separation was examined in great detail by Kiel et al [22]. The action of the amine appears to be three-fold when used in the mobile phase for the analysis of basic analytes.

The stationary phase used in Kiel's work was a C_{18} silica column, whereas in the work done here, a CN column was used.

The three actions taking place are reported to be :

- Hydrophobic interaction
- Ion exchange
- Hydrogen bonding

The character of the column is altered by the amine modifier resulting in the analysis of the quaternary ion being improved for the following reasons :

The hydrophobic interaction will result in localised positive charge on the surface of the stationary phase which will repel the positively charged solute, thus decreasing analysis time.

This action will depend on the length of the alkyl chain bound to the silica and thus this effect will be less pronounced on the cyano propyl column than in a C_{18} column.

Ion exchange between the amine modifier and the stationary phase will depend on the balance between hydrophobic and silanophilic effects.

The method used in this homologue separation of benzalkonium chloride is more affected by silanophilic effects as the cyano propyl column has a short chain compared with that of the C_{18} bounded phase investigated by Kiel.

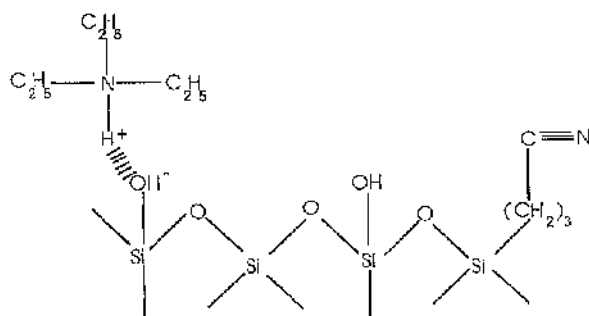


Fig. 3.2.4 Diagram showing hydrogen bonding of the amine modifier TMA.

Hydrogen bonding was the main cause of peak tailing when analysis of amines were being investigated and it can be assumed that hydrogen bonding will have a major effect on the analysis of benzalkonium chloride. The TMA acts by binding to the sites where hydrogen bonding may occur and rendering them neutral. Recent work by various HPLC column manufacturers has led to the development of double

endcapping which results in a sterically protected silica surface, this work is directed at preventing peak tailing when acidic and basic analytes are being analysed and should theoretically prevent the need for column modifiers such as TEA. However, until more work is done in this area the use of modifiers still seems to be the most useful alternative.

3.2.2 Application of Method in the Analysis of the Benzalkonium Chlorides

The benzalkonium chlorides being examined in this work have, as previously detailed (Chapter 2, Section 2.2), various alkyl chain lengths. As the chain length increases the material becomes more hydrophobic.

e.g. Akzo-Nobel product Arquad B50 is mainly C₁₂ and C₁₄

Akzo-Nobel product Arquad DMHTB-75 is mainly C₁₆ and C₁₈

The method operates such that the most polar homologue will come off the column first.

Benzalkonium chloride, which contains 60% of the C₁₈ homologue, is practically insoluble in water and it evokes a more hydrophobic effect on the column than the polar C₁₂ homologue would. This is demonstrated in the chromatograms of the homologue separations as the more polar alkyl chains have sharper peaks. Figures 3.2.5 and 3.2.6 show the homologue distribution of Arquad B-50 and Arquad DMHTB-75. This analysis was carried out using Method 2 described in section 3.2.

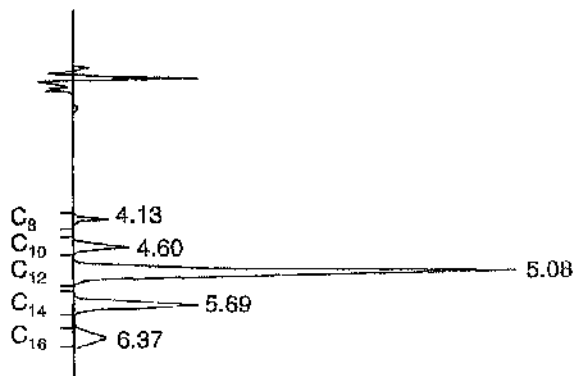


Fig. 3.2.5 Arquad B-50, homologue separation

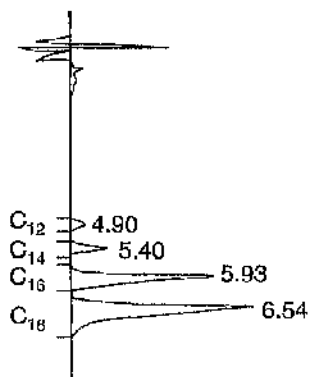


Fig. 3.2.6 Arquad DMHTB-75, homologue separation

3.3 Quantitative Analysis of Benzalkonium Chlorides

Introduction

The quantitative analysis of benzalkonium chloride is divided into sections. The first deals with the benzalkonium chlorides in their "as supplied" state. The purities of these materials are calculated as well as the percentages of the alkyl chain lengths, i.e. the homologue distribution. The subsequent section deals with the quantitative analysis of the benzalkonium chlorides and also the homologue distribution in the hydrogel material for the marine trials. Only the benzalkonium chlorides which showed initial promise as fouling free surfaces were quantitatively analysed. The analysis was done rack by rack from the marine exposure experiments. Hydrogels

were exposed in marine trials in either duplicate or triplicate. From these samples one hydrogel was used for quantitative analysis while the other or others were used for the monitoring of fouling. Quantitative analysis was done by taking two cork bores as described (Section 3.2.2). All the quantitative analysis was done using duplicate injections with $\geq 98\%$ agreement i.e. $\pm 2\%$. Results were shown as a mean of the two results as in table 3.3.4. As there was limited hydrogel material available due to the variety of samples which had to be compared on each rack, this method was adopted for all the marine analysis throughout this chapter. From the results obtained it seemed reasonable to use this method as the variation seen was not significant i.e. the trends remained the same for release. (See figures 3.3.1-3.3.4).

3.3.1 Quantitative Analysis of Benzalkonium Chlorides from Suppliers - Purities

The benzalkonium chlorides supplied are given a purity value from their manufacturer/supplier. However, this is only a nominal value which is not guaranteed by a Certificate of Analysis. In order to obtain an accurate value each sample was analysed using HPLC. The benzalkonium chloride standard (Lot J, Cat.No.05100) used to quantify these active substances was one obtained from the United States Pharmacopoeia (USP). It was supplied as a 10% aqueous solution.

The HPLC method was that used in the Mast II Project [23]. However, to improve the analysis i.e. peak shape, the composition of the mobile phase was changed (organic content was increased from 60 to 80). The details of the chromatographic conditions are shown below :

HPLC Conditions

Instruments	Datajet Integrator, Thermo Separating Products (TSP) Spectra Series UV100, TSP Constametric III metering pump, LDC / Milton Roy
Column	Techsphere 5CN (25cm), HPLC Technology
Column Temp	ambient
Flow Rate	2.00 ml / min
Mobile Phase	acetonitrile / 0.06M NaH_2PO_4 80 / 20 pH = 7.00
Wavelength	214nm
Detection	0.5AUFS

The method produces a single peak which includes all the homologues. The percentage purity is calculated using the external standard method.

Purity Results

Name	Manufacturer/Supplier	Purity Value
Benzalkonium Chloride	Aldrich	91.72%w/v
Benzalkonium Chloride	Sigma	90.51%w/v
Quadrilan BC	Akcros	47.23%w/v
Arquad B-50	Akzo Nobel	43.23%w/v
Arquad DMMCB-50	Akzo Nobel	48.13%w/v
Arquad DMHTB-75	Akzo Nobel	63.32%w/v

Table 3.3.1 Manufacturer/Suppliers' Purity Values.

From these purity results 5%w/v solutions were prepared in which hydrogel material was soaked prior to marine trials. This was with the exception of DMHTB-75, which was added at the production stage as it had proved difficult to produce a 5%w/v solution of this material. A full description of the preparation of loaded hydrogels is given in Chapter 2, Section 2.7.

Homologue Separations of Benzalkonium Chlorides

This method is able to separate the alkyl chains of benzalkonium chloride and thus give information about the composition as well as quantifying it.

The six benzalkonium chlorides were analysed using the following HPLC conditions.

HPLC Conditions

Instruments	Datajet Integrator, Thermo Separating Products (TSP) Spectra Series UV100, TSP Constametric III metering pump, LDC / Milton Roy
Column	Techsphere 5CN (25cm), HPLC Technology
Column Temp.	ambient
Flow Rate	2.00 ml / min
Mobile Phase	acetonitrile / 0.1% triethylamine (TEA) 40 / 60 pH was adjusted to 2.3 using orthophosphoric acid
Wavelength	214nm
Detection	0.5 AUFS

Results

BAC	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈
USP Std.	-	-	69.32%	25.83%	5.03%	-
Quad. BC	-	-	73.52	27.97	-	-
B - 50	3.14%	5.48%	65.39%	20.36%	5.68%	-
DMMCB-50	-	0.73%	73.09%	23.59%	2.63%	-
Sigma BAC	-	-	63.88%	36.08%	-	-
Aldrich BAC	-	-	70.27%	29.63%	-	-
DMHTB-75	-	-	1.86%	5.05%	32.29%	60.87%

Table 3.3.2 Homologue distributions from HPLC analysis. (- not detected)

The suppliers values are shown in Table 3.3.3.

BAC	C ₈	C ₁₀	C ₁₂	C ₁₄	C ₁₆	C ₁₈
USP Std	-	0.1%	67%	26%	7%	-
B - 50	-	3%	54%	22%	10%	10%
DMMCB-50	-	-	68%	29%	3%	-
DMHTB-75	-	-	1%	4%	16%	64%

Table 3.3.3 Homologue distribution - Suppliers Values (- not detected)

The agreement between the values obtained from the analysis and the suppliers' values is good. The suppliers do not give any guarantee with their values and therefore the homologue values calculated by the HPLC method will be used for comparison with future work. (Detailed analysis of benzalkonium chloride (Aldrich) found differences of up to 10% in the homologue distribution [24]. All the work here was carried out using a batch in which the C₁₂ / C₁₄ distribution was 70% / 30%).

3.3.2 Analysis of Hydrogel Samples

The work in this thesis was done as part of a research project, which looked at the anti-fouling action of hydrogels containing quaternary ammonium compounds. Within the research project it was found, due to the heterogeneous nature of the hydrogel material, that the zero time content of the quaternary ammonium compound

experience already gained within the research project this was considered to be acceptable, as it did not reflect major differences in the fouling ability of the material.

The results are presented from each rack in chronological order.

Rack 1 1995

No analysis was carried out on the benzalkonium chloride samples on this rack as this was an initial trial of potential anti-fouling agents. Samples which proved to be successful anti-fouling agents were used in subsequent trials and quantitative analysis of these was performed.

Rack 2 1995

This rack contained chlorhexidine gluconate samples and the results of quantitative results are given in Section 3.5.

Rack 3 1995

The samples containing the DMHTB-75 on Rack 1 1995 proved to be successful in deterring fouling and therefore four samples were prepared containing this form of benzalkonium chloride and were immersed on this rack. Two of the samples were prepared by adding the DMHTB-75 to the hydrogel at the production stage (prep.). This was done in the proportions of 1 g DMHTB-75 to 10 ml of hydrogel. The other two samples were prepared by soaking (soak) the previously prepared hydrogel in a 5%w/v solution of DMHTB-75 and keeping this solution warm to stop crystallising-out of the DMHTB-75 (about 25-30°C). Zero time analysis of all four samples was done by taking two discs of hydrogel cut from the sheet using a cork bore with a diameter of 20 mm. These were weighed and then soaked in methanol (about 50 ml) for 24 hours to swell the hydrogel and thus encourage the active substance to flow into the methanol. After 24 hours the discs were cut into small pieces and crushed using a mortar and pestle and returned to the methanol. The mixture was then warmed and stirred on a magnetic hot-plate for 15 minutes after which it was put in the ultrasonic bath for 15 minutes. The mixture was then filtered through Whatman No. 1 filter paper into a 100 ml volumetric flask which was made up to the mark using methanol [23]. When the discs being analysed were for the zero time-point a dilution

of this was made as the initial preparation was too concentrated for the method. The injections were done in duplicate and agreement between area under the peaks of $\geq 98\%$ was accepted. The results for the zero time analysis are shown in table 3.3.4.

Sample Position on Rack	Concentration of DMHTB-75 (%w/w)
Position 1 (soak)	3.14
Position 4 (prep)	2.58
Position 5 (prep)	2.81
Position 7 (soak)	3.67

Table 3.3.4 Zero time analysis results.

The samples soaked in the solution have absorbed greater quantities of DMHTB-75. However this value may have been elevated as some liquid may have been present on the hydrogel surface since the discs were not washed before analysis.

After 18 weeks the samples were analysed quantitatively. The results are shown in table 3.3.5.

Sample Position on Rack	Concentration of DMHTB-75 (%w/w)
Position 1 (soak)	2.13
Position 4 (prep)	2.18

Table 3.3.5 DMHTB-75 content after 18 weeks.

From the results it can be seen that the rate of release of the DMHTB-75 is very slow. Samples were not taken from positions 5 and 7 as these samples were being used to monitor fouling.

As well as the quantitative analysis the samples (positions 1 and 4) were analysed for homologue composition. This was done in order to determine the difference in retention of the various alkyl chain lengths in the hydrogel material.

Results of Homologue Retention

Sample	Time	C ₁₂ (%)	C ₁₄ (%)	C ₁₆ (%)	C ₁₈ (%)
Position 1	0	Not done	Not done	Not done	Not done
Position 1	18 weeks	0.739	2.21	36.43	60.97
Position 4	0	1.49	3.94	30.43	63.71
Position 4	18 weeks	0.305	0.947	29.67	69.20

Table 3.3.6 Variation in homologue distribution at zero time and at 18 weeks.

Discussion

From the results in Table 3.3.6 it can be seen that the more hydrophobic alkyl chains remain in the hydrogel while the more hydrophilic alkyl chains release at higher rates. This result could indicate that the material is actually binding to the hydrophobic areas of the hydrogel. It should be noted that the percentages given for C₁₈ homologue do not mean that the value has increased rather that the distribution of the values has changed, i.e. the lower homologues have been released.

Rack 4 1995

This rack was immersed at UMBSM on the 14th December 1995 when the sea temperature was 10°C (the rack layout is shown in Chapter 4 Section 4.2). This rack contained the most successful anti-fouling materials from the previous marine trials. The purpose of this rack was to monitor the release of the different benzalkonium chlorides i.e. Benzalkonium chloride (Aldrich) and DMHTB-75 (Akzo). In addition to this tri-sodium ethylenediaminetetraacetic acid (tri-EDTA) was added to both benzalkonium chlorides and its effect on release was also monitored. Fouling was not monitored closely as it was the winter season and little algal fouling flourishes during this time of the year at the chosen location. Samples for HPLC analysis were taken from the same four hydrogel samples throughout the trial.

Release of Benzalkonium Chlorides

Position/Material	0 Time (%w/w)	5 weeks (%w/w)	12 weeks (%w/w)
6-BAC (Aldrich)	6.93	3.60	2.52
11-BAC (Aldrich) + tri-EDTA	9.78	8.85	3.69
10-DMHTB-75	4.95	4.93	5.29
2-DMHTB-75 + tri-EDTA	5.67	5.26	5.49

Table 3.3.7 Release of benzalkonium chlorides from December 1995 to March 1996 (Rack 4 1995)

Discussion

BAC is approximately half its original value after 5 weeks however, the rate of release slows down between 5 and 12 weeks.

BAC and tri-EDTA releases slowly from 0 to 5 weeks but the release rate increases from 5 to 12 weeks. The presence of tri-EDTA appears to slow down the rate at which the BAC is released initially but this action reduces with time. From the fouling results (Chapter 4 Section 2.10) the samples containing BAC and tri-EDTA are initially opaque. This changes with time leaving the sample clear, at which point algal fouling occurs. It could be that a fairly insoluble complex is being formed due to the tri-EDTA, which prevents the release of the benzalkonium chloride. As this complex slowly dissolves the benzalkonium chloride is able to be released. (Further experimental work would be required for complete understanding of this system.) The presence of the tri-EDTA in the original benzalkonium chloride soaking solution increases the quantity absorbed initially. This suggests that the structure of the hydrogel is slightly altered by the addition of the tri-EDTA allowing it to absorb more of the BAC.

DMHTB-75 appears to stay constant in this trial and the value actually rises. This increased value can probably be explained by the fact that the hydrogel shrinks slightly in seawater [25] and therefore the concentration of the active substance increases in a given area.

DMHTB-75 and tri-EDTA appears to stay constant throughout the 12 weeks.

Results of Homologue Retention

The samples were analysed for their homologue content at the same time points as the quantitative analysis. (As in table 3.3.6, the C_{18} does not increase, but the homologue distribution changes due to release of the lower homologues).

Position/ Material	Time	C_{12} (%)	C_{14} (%)	C_{16} (%)	C_{18} (%)
2 - DMHTB-75 + tri-EDTA	0	0.862	4.10	31.83	63.19
	5 weeks	0.400	2.56	29.76	67.33
	12 weeks	0.477	1.57	28.46	69.41
6 - BAC (Aldrich)	0	72.00	27.50	-	-
	5 weeks	72.94	26.86	-	-
	12 weeks	66.44	33.61	-	-
10 - DMHTB-75	0	0.633	4.10	31.61	63.72
	5 weeks	0.360	2.10	29.74	68.07
	12 weeks	0.389	1.71	29.02	68.68
11 - BAC + tri-EDTA	0	70.04	30.01	-	-
	5 weeks	62.97	37.06	-	-
	12 weeks	62.02	37.71	-	-

Table 3.3.8 Homologue distribution changes from zero time to 12 weeks.

Rack 1 1996

No quantitative analysis was carried out as this rack was used purely to compare anti-fouling abilities of the active substances and to show duplication of the results of the 1995 season.

Rack 2 1996

This rack was immersed at UMBSM on the 20th March 1996 when the sea-temperature was 6°C. The layout of the rack is shown in Chapter 4, Section 4.2. Quantitative analysis was carried out on the four active materials at zero time, 5 weeks and 12 weeks (as in Rack 4 1995).

Release of Benzalkonium Chlorides

Position/Material	0 Time (% w/w)	5 weeks (% w/w)	12 weeks (% w/w)
1-BAC (Aldrich)	6.18	3.59	2.04
4-BAC (Aldrich) + tri-EDTA	13.81	11.52	5.52
3-DMHTB-75	3.72	5.51	4.54
15-DMHTB-75 + tri-EDTA	5.65	5.93	5.28

Table 3.3.9 Release of benzalkonium chlorides from March 1996 to June 1996 (Rack 2 1996)

Discussion

The pattern for release is the same as that of Rack 4 1995. The amount of BAC initially absorbed in the sample containing tri-EDTA is higher than that which the sample on Rack 4 1995 contained, however the absorption into hydrogels is known to vary [25]. The release rate was very similar to that in Rack 4 1995.

Comparison of Release of Benzalkonium Chlorides in Different Seasons

The following graphs show the similarities in release over two different seasons i.e. Winter 1995-1996 and Spring 1996.

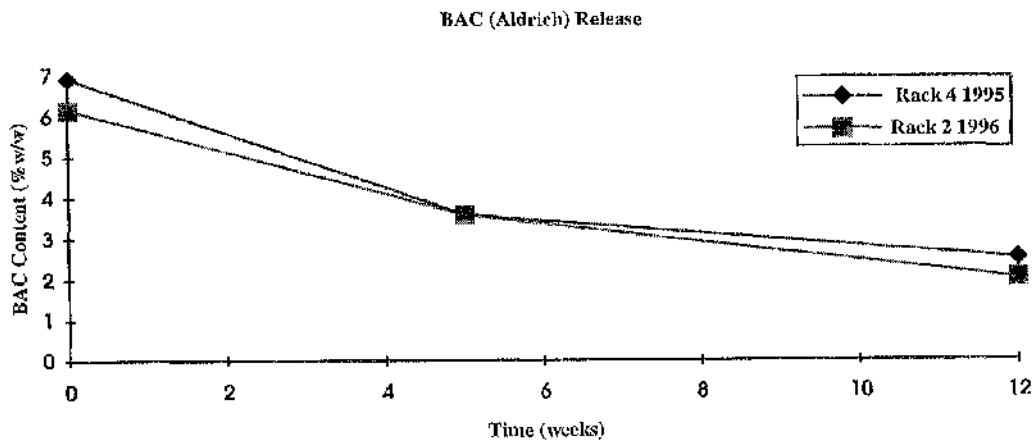


Fig. 3.3.1 BAC release over 12 weeks.

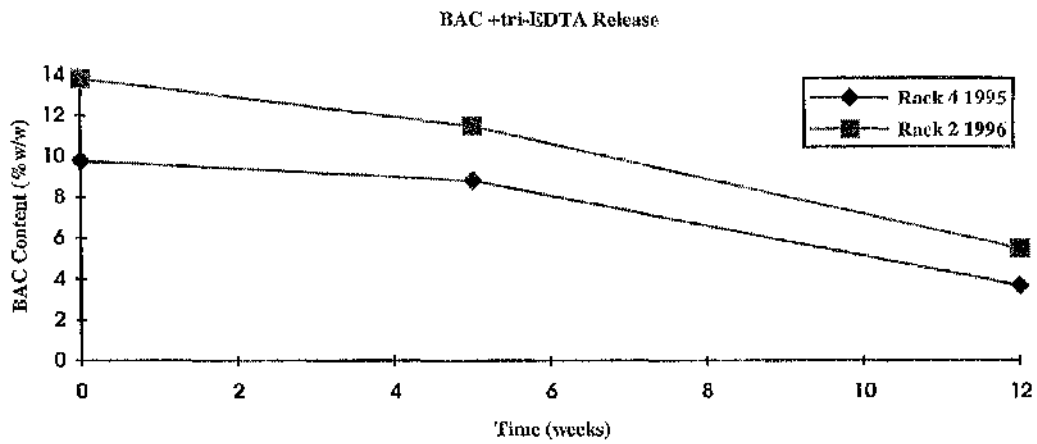


Fig. 3.3.2 BAC + tri-EDTA over 12 weeks.

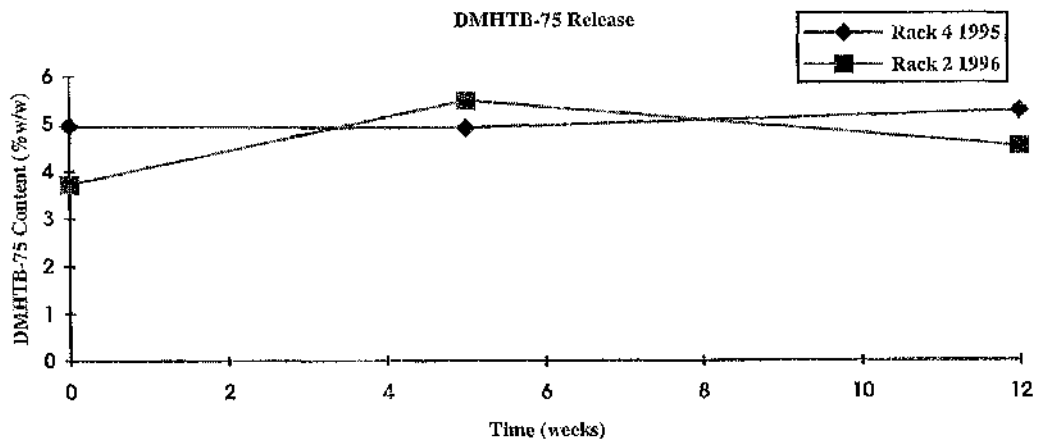


Fig. 3.3.3 DMHTB-75 over 12 weeks.

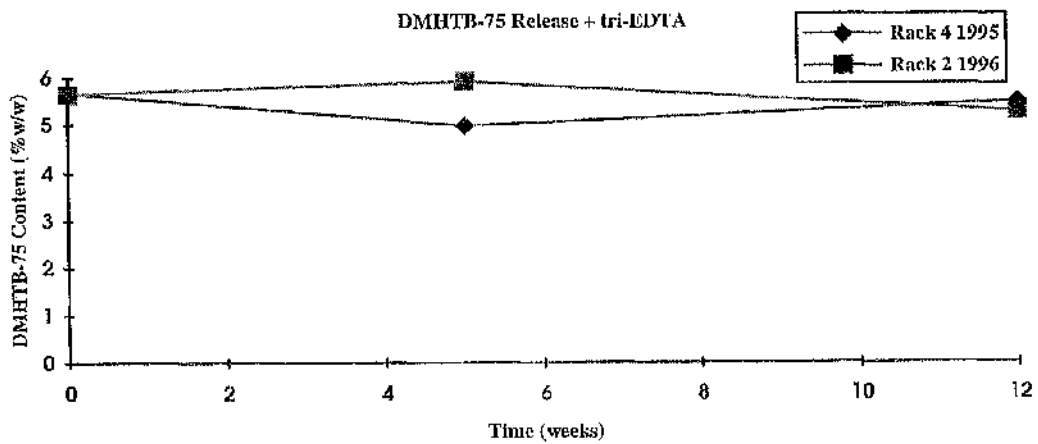


Fig. 3.3.4 DMHTB-75 + tri-EDTA over 12 weeks.

From the graphs it is possible to conclude that for the two different seasons examined, little effect on the release of the benzalkonium chlorides was noted.

Discussion

BAC

This material has a useful anti-fouling lifetime when incorporated into the hydrogel, as it can be visually clear of fouling for up to 10-12 weeks (fouling results Chapter 4). The BAC releases to less than half its original content by 12 weeks but although some BAC remains at this time the surface begins to foul.

BAC + tri-sodium EDTA

The inclusion of the chelating agent tri-EDTA increases the uptake of the BAC into the hydrogel. The release is slowed down over the first 5 weeks compared with BAC on its own. However, this rate increases and by 12 weeks the amount left is less than half the original value.

DMHTB-75

The DMHTB-75 does not appear to decrease in value over the 12 weeks in fact the opposite is true. Although this increase may be attributed to the slight shrinkage which the hydrogel undergoes in the marine environment.

DMHTB-75 + tri-sodium EDTA

The inclusion of tri-EDTA into the samples containing DMHTB-75 made little difference to the release. It did, however, cause the hydrogel to swell and bulge slightly thus distorting the flat even surface and for this reason it would not be very useful as an anti-fouling material.

3.4 Analysis of Cetrимide

The method development for the analysis of cetrимide was restricted to a literature review as cetrимide was not successful as an anti-fouling material.

Methods in the Literature

The British Pharmacopoeia (BP) [26] and International Pharmacopoeia (IP) [27] analyse cetrime by titrating with potassium iodide, a non-specific method. The method is time consuming and can only be used at relatively high concentrations of cetrime and would not be a suitable method for trace analysis. Methods have been developed using gas chromatography [28,29]. However, in practice these methods proved difficult to use for routine analysis as the cetrime degrades the column after a few injections, resulting in it requiring to be repacked during analysis. The quality of the peaks is also poor making quantitative results unreliable. The ideal method of analysis for cetrime would be HPLC. However, cetrime does not have a chromophore i.e. it does not absorb in the ultra-violet (UV) range and so HPLC/UV methods for the analysis of cetrime involve the use of ion-pair reagents such as p-toluene sulphonic acid which result in a negative UV peak. This is recorded for quantitative analysis [30]. Initial experimental work was done using the Indirect Photometric Chromatography (IPC) method but it was not found to be reproducible and slight variations in the mobile phase composition resulted in huge variations in results. Therefore, the method was not suitable for large batch analysis as it was not sufficiently robust. HPLC with Refractive Index (RI) methods have also been published [31,32]. The main drawback of such methods is the inaccuracies in RI detection as changes in temperature, pressure, minor pump pulsations and mobile phase greatly affect the readings [33] i.e. a very stable environment is required. This is difficult to achieve in a normal laboratory and therefore RI methods were not considered as useful for this work.

3.5 Analysis of Chlorhexidine

3.5.1 Available Methods

The British Pharmacopoeia (BP) [26] analysis of chlorhexidine is done by titration (non-aqueous) for samples of greater than 1g. The titration involved lends itself to auto-titration as manual titrations are laborious and frequently error-laden. However, the concentrations which require to be analysed in this work will be less than 1 g and therefore other methods of analysis have to be employed. The BP analyse samples of low concentrations using a spectrophotometric technique involving the reaction with

brown colour. This method is useful, although when high volumes of samples have to be analysed it is extremely time consuming. Therefore, it would be desirable to find a more straightforward method. Chlorhexidine can be analysed using HPLC [33,34]. These methods use ion-pairing techniques and require extensive preparation of samples and mobile phase. From the initial marine trials (see Chapter 4 Section 4.6), chlorhexidine did not appear to be a very useful anti-fouling material and therefore only initial method development was done.

3.5.2 Method Development

UV Spectroscopy

The following standards were prepared from a 0.1%w/v stock solution (Ex Zencca).

1. 0.004%w/v
2. 0.002%w/v
3. 0.001%w/v
4. 0.0005%w/v

An absorbance wavelength of 254nm was set as chlorhexidine gluconate absorbs highly at this wavelength. (This was determined by scanning a chlorhexidine gluconate sample. The scan is shown in figure 3.5.1).

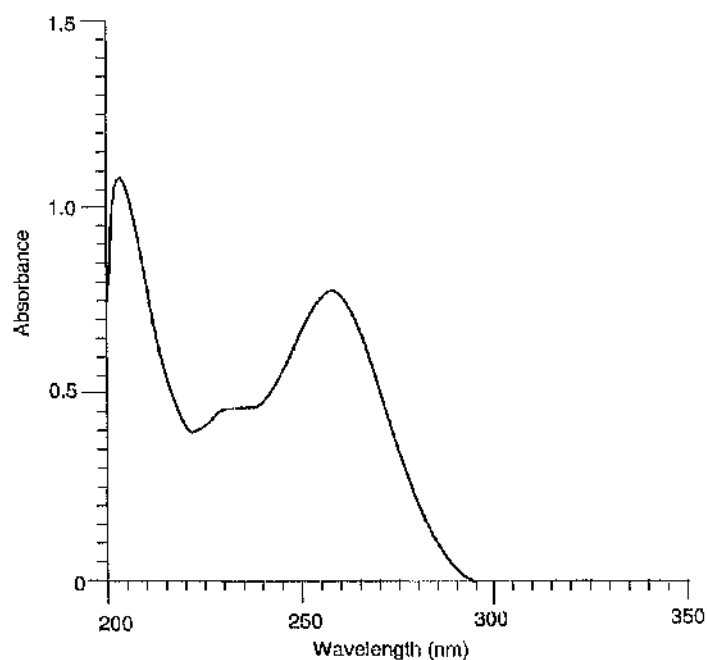


Fig. 3.5.1 UV scan of Chlorhexidine Gluconate

Results

Std conc (%w/v)	Absorbance
0.004	1.315
0.002	0.678
0.001	0.322
0.0005	0.18

Fig 3.5.2 shows a diagrammatic representation of the graph plotting the absorbance values against the concentrations of the standards.

UV Absorbance of Chlorhexidine Gluconate

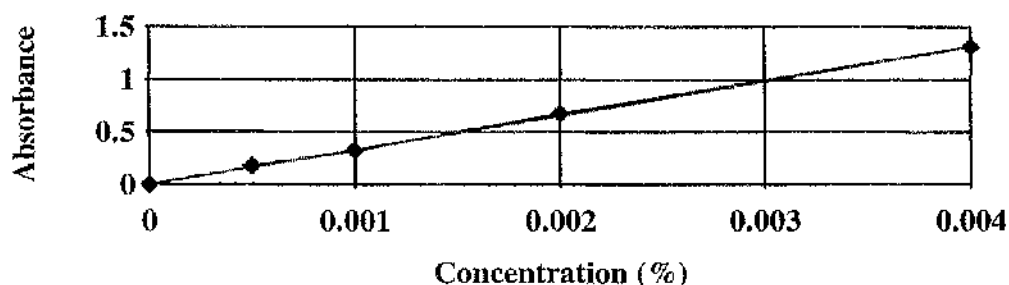


Fig. 3.5.2 UV Absorbance of Chlorhexidine Gluconate

This graph was linear in the working range. The correlation coefficient was 0.995.

HPLC

A method for the quantitative analysis of chlorhexidine was developed in the laboratory. It was based on the cationic nature of chlorhexidine at pH 7. The details of the conditions are shown below.

Column	:	Hypersil BDS C18
Column Temperature	:	ambient
Wavelength	:	254nm
Flow Rate	:	2.00ml/min
AUFS	:	0.08
Mobile Phase	:	80 acetonitrile : 20 0.06M NaH ₂ PO ₄ pH=7.00

The capacity factor (k') of chlorhexidine using this method was 1.2 which is lower than what is usually considered acceptable. However, the method produced sharp, reproducible peaks and the method was robust. A calibration graph was plotted of chlorhexidine concentration against area under the peak

The following standards were prepared from a 20%w/v solution of chlorhexidine gluconate and made up in methanol.

1. 0.0430%w/v
2. 0.0215%w/v
3. 0.0107%w/v
4. 0.0054%w/v

Results

As the chlorhexidine solution was only 20%w/v concentration the actual concentration is 1/5 of the values of the standards.

Std conc.(%w/v)	Peak Area
8.60×10^{-3}	4620864
4.30×10^{-3}	2273845
2.14×10^{-3}	1076637
1.08×10^{-3}	510532

Fig 3.5.3 shows a diagrammatic representation of the graph plotting the peak area against the chlorhexidine concentrations of the standards.

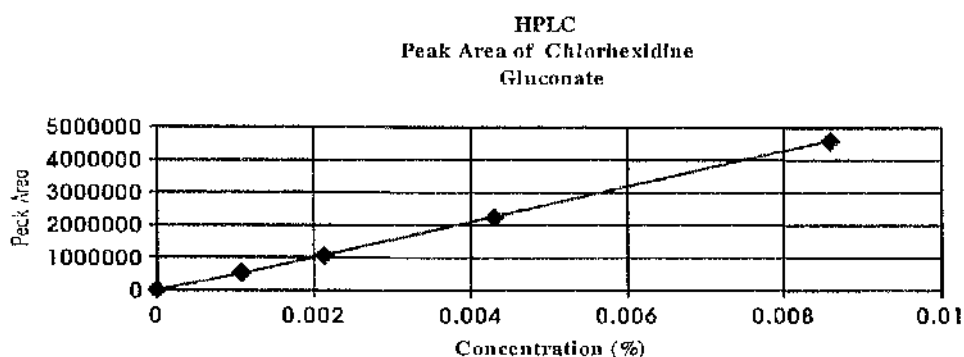


Fig. 3.5.3 HPLC Peak Area of Chlorhexidine Gluconate

This graph was linear in the working range. The correlation coefficient was 0.999.

Limited method development of chlorhexidine was done as it was unsuccessful as an anti-fouling material.

3.5.3 Quantitative Analysis of Chlorhexidine

Chlorhexidine gluconate and chlorhexidine acetate were both used in marine trials as prospective anti-fouling materials. The chlorhexidine gluconate was supplied in a 20%w/v solution by Zeneca Pharmaceuticals and the chlorhexidine acetate was supplied in powder form (98%) by Aldrich. Further preparation of soaking solutions used purity values when calculating solution concentrations.

Marine Samples - Chlorhexidine Content

Preparation of Samples for Analysis

Discs of hydrogel were cut from the sheet using a cork borer with a diameter of 20 mm. These were weighed and then soaked in methanol (about 50 ml) for 24 hours to swell the hydrogel and thus encourage the active substance to flow into the methanol. After 24 hours the discs were cut into small pieces and crushed using a mortar and pestle and returned to the methanol. The mixture was then warmed and stirred on a magnetic hot-plate for 15 minutes after which time it was put in an ultra-sonic bath for 15 minutes. The mixture was then filtered through Whatman No. 1 filter paper

into a 100 ml volumetric flask which was made up to the mark using methanol [24]. When the zero time discs were analysed a dilution of this was made as the initial preparation was too concentrated for the method.

Rack 1 1995

Samples which had been soaked in 5%w/v and 20%w/v chlorhexidine gluconate were then soaked in seawater for 24 hours prior to immersion at UMBSM (see Chapter 2, Section 2.9 for details). Discs were taken (2 from each sample) from these samples and prepared for HPLC analysis as described.

Chlorhexidine Gluconate (CG) Zero Time Content :

The standard used for this analysis was the same material as was used in the samples. In the calculations the purity used is 20%w/v as stated by the supplier.

Soaked for 6 days in 5%w/v CG 9.65%w/w

Soaked for 4 days in 20%w/v CG 12.55%w/w

This was merely an initial analysis but it indicated that a reasonable quantity of chlorhexidine gluconate had been absorbed by the sample and was present when it was immersed at sea.

Rack 2A and 2B 1995

Chlorhexidine Gluconate (CG) zero time analysis :

Soaked for 7 days in 20%w/v CG 18.64%w/w

The longer soak dramatically increased the amount of chlorhexidine gluconate absorbed by the hydrogel. (Chapter 2 Section 2.9 gives details of the hydrogel's reaction to chlorhexidine gluconate).

Rack 3 1996 Samples Containing Chlorhexidine Acetate

A sheet of hydrogel was soaked for 7 days in 1.5%w/v chlorhexidine acetate solution (100 ml). After this time four discs were cut and analysed using HPLC as per the method described for the chlorhexidine gluconate. The standard used for this analysis was the same material used for the samples (99%purity).

1. 2.59%w/w
2. 2.84%w/w
3. 2.78%w/w
4. 2.81%w/w

Samples from this hydrogel were immersed at UMBSM during April 1996.

No further analysis of chlorhexidine was carried out as it was not a useful anti-fouling material due to the formation of the insoluble hydrochloride salt which resulted in the surface of the hydrogel becoming rough and thus prone to fouling.

3.6 Overall Conclusions

3.6.1 Methods

The method developed for the separation of the alkyl chain lengths of benzalkonium chloride has shown itself to be robust and therefore useful for the analysis of this material.

The methods reviewed for the analysis of cetrimide show that although there is a wide choice of analytical techniques none of these are ideal as the sample and/or reagent preparation is prone to error and the chromatographic methods are such that they are not sufficiently robust.

Chlorhexidine can be analysed by various techniques and therefore when choosing which to use it is important to pick one, which is easily used and does not involve complicated preparation. From the initial work done it appears that ultra-violet spectroscopy was a useful method for quantitative analysis as it was simple to operate and requires an instrument which is available in most analytical laboratories.

3.6.2 Results

Conclusions were drawn at the end of each piece of work. The main point that was evident through the marine release trials was that, although substantial amounts of benzalkonium chloride remained in the hydrogel material, fouling still did eventually occur. The balance has therefore altered and there is no longer sufficient deterrent to prevent fouling. This problem is prevalent in all areas where the prevention of bacterial growth has to be controlled [36]. Details of the fouling results can be found in Chapter 4.

The benzalkonium chloride content of the hydrogel can be retained longer by including the chelating agent tri-sodium ethylenediaminetetraacetic acid and also by changing the alkyl chain length to the more hydrophobic C₁₆ and C₁₈ homologues.

A full discussion of the relationship between release and fouling is given in Chapter 5.

3.7 Chapter 3 - References

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Chapter 4

The results of marine anti-fouling trials carried out on the surface-active agents.

- 4.1 Introduction
- 4.2 Technical Details
- 4.3 Anti-fouling performance of Benzalkonium Chloride
- 4.4 Anti-fouling performance of Cetrimide
- 4.5 Anti-fouling performance of Benzalkonium Chloride with the addition of EDTA
- 4.6 Anti-fouling performance of Chlorhexidine.
- 4.7 Anti-fouling performance of mixed surfactant systems.
- 4.8 Conclusions
- 4.9 References

Marine Studies

This chapter is divided into nine sections of which the first two are the introduction and experimental details while the other six deal with the marine studies carried out on each of the potential anti-fouling active substances which are described in detail in Chapter 2. The last section lists the references.

4.1 Introduction

The results from the MAST II Project [1] indicated that the quaternary ammonium compound benzalkonium chloride was able to resist marine fouling for up to 12 weeks making it a potentially useful anti-fouling material and thus further study was required. In Chapter 2 details are given of the different properties of the various homologues, which make up the compound benzalkonium chloride and thus it is obvious that the "umbrella" name benzalkonium chloride can encompass materials with very different properties. These different forms of benzalkonium chlorides were put on marine trials and their anti-fouling abilities studied and compared. A further quaternary compound known as cetrimide was also investigated for its anti-fouling ability. The characteristics of cetrimide are detailed in Chapter 2.

Section 4.5 deals with the anti-fouling effect of adding the chelating agent ethylenediaminetetraacetic acid (EDTA) to benzalkonium chloride as it has been suggested that a synergistic effect may occur from the combination [2,3]. Full details of this effect are included in Chapter 2.

Section 4.6 deals with the fourth active substance to be investigated as a possible anti-fouling agent the biguanide, chlorhexidine. This was examined in the acetate and gluconate forms.

Section 4.7 considers the effect of mixing surfactants. A mixture of benzalkonium chloride and cetrimide, as well as the benzalkonium chloride and chlorhexidine gluconate mixtures were investigated. The fundamental idea behind these mixtures was that the separate anti-fouling actions may be additive i.e. synergistic. [4,5].

Finally, in Section 4.8 the anti-fouling results for all the surfactants are summarised and conclusions reached.

4.2 Marine Trials - Technical Details

4.2.1 Construction of Test Racks

Racks were constructed using Perspex (acrylic sheet) with a thickness of 6mm. The supporting back was solid Perspex to which rectangular frames were secured at the four corners using screws. These frames allowed the hydrogel sample to be held in place. The area of sample under test measures 60 x 80mm. This size was chosen in order to eliminate edge effects (fouling) which occur when the sample size is too small. Rubber frames were placed between the Perspex frames and the backing support to prevent the edges of the frame cutting through the hydrogel material. The racks used varied in size; the smallest had 4 samples and the largest had 16 samples

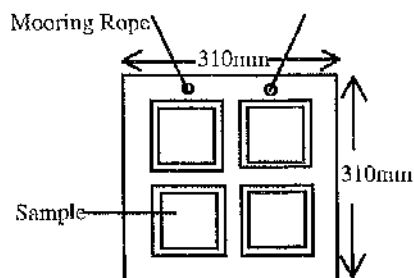


Fig 4.2.1 Diagram of rack containing 4 samples.

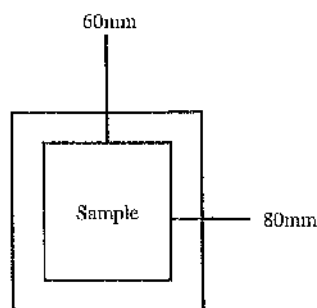


Fig 4.2.2 Perspex frame which holds sample in place.

Rack sizes are shown below :

4 sample rack 310mm²

12 sample rack 440mm x 570mm

16 sample rack 570mm²

Figure 4.2.3 shows a rack containing 16 samples just before it was immersed in the sea.



Fig. 4.2.3 Rack containing 16 samples

The racks were suspended from the pier using nylon rope and were weighed down using lead weights. Approximately 7 metres of rope was used and the racks were suspended to a depth of 3 metres below the sea surface depending on the tides. At no time were they exposed above the waterline.

Keppel pier is situated on the Firth of Clyde directly in front of University Marine Biological Station Millport (UMBSM) and the racks were situated 30-40 metres from

the shoreline. The minimum sea temperature recorded was 5°C during January 1995 with the maximum recorded was 16°C during September 1995. The pH of the seawater in this area is about 8. Although this site shows variations in fouling pressure, even in adjacent racks, it is still indicative of how successful an antifouling coating is behaving in that the acrylic rack itself acts as a control and will foul and thus be a guide to the fouling pressure exerted on each rack. Due to the pier supports, hydrodynamic effects also influence the fouling. However, even considering these variations the site is still extremely useful for test racks. The marine trials were carried out from March 1995 until November 1996. Figure 4.2.4 shows Keppel Pier. The racks were suspended over the near (southerly) side between the 9th and 11th vertical poles.



Fig. 4.2.4 Keppel Pier, January 1996.

Racks

This section describes the sample arrangement of each of the seven racks which were used in the marine trials.

1	5	9	13
2	6	10	14
3	7	11	15
4	8	12	16

Fig. 4.2.5 Rack 1 1995

1. 20% Chlorhexidine gluconate (24 hrs soak in IO before immersion)
2. 5% Cetrimide
3. 5% Cocotrimethylammonium chloride (Akzo)
4. 5% Benzalkonium chloride (Aldrich)
5. 5% DMMCB-50 (Akzo)
6. 5% DMHTB-75, soak (Akzo)
7. 5% B-50 (Akzo)
8. 5% Benzalkonium chloride (Sigma)
9. 5% Benzalkonium chloride (Aldrich) + 5% Cetrimide
10. 5% Chlorhexidine gluconate (24 hrs soak in IO before immersion)
11. 5% Benzalkonium chloride (Aldrich) + 2.5% di-EDTA
12. 5% Benzalkonium chloride (Aldrich) + 0.5% Chlorhexidine gluconate
13. 5% Benzalkonium chloride (Aldrich)
14. 5% Quadrilan BC (Akcros)
15. 5% DMHTB-75, incorporated at production (Akzo)
16. 5% DMMCB-50 (Akzo)

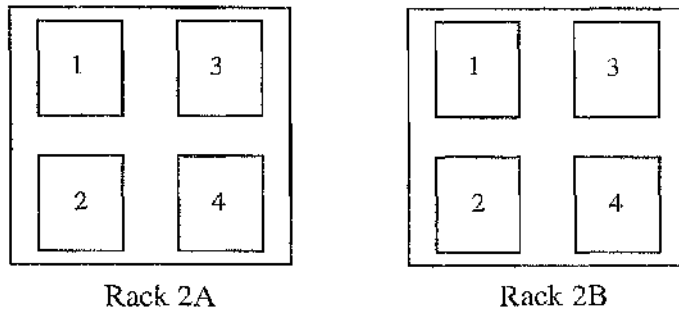


Fig. 4.2.6 Rack 2A and 2B 1995

1. 20% Chlorhexidine gluconate
2. 5% Benzalkonium chloride (Aldrich) + 0.5% Chlorhexidine gluconate
3. 5% Benzalkonium chloride (Aldrich) + 2% Chlorhexidine gluconate
4. 20% Chlorhexidine gluconate

Rack 2B is as Rack 2A except positions 2 and 3 are reversed.

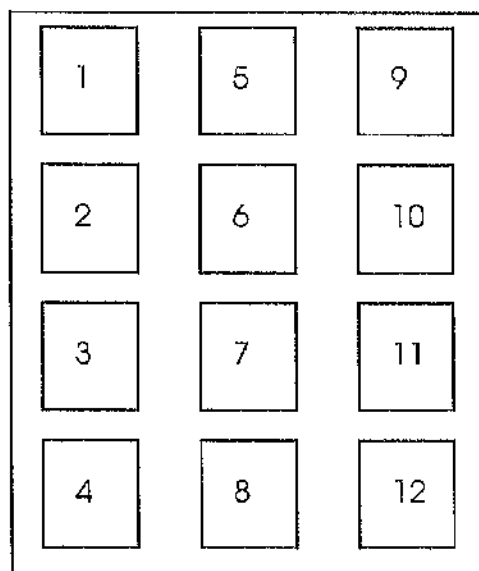


Fig. 4.2.7 Rack 3 1995

1. 5% DMHTB-75 (Akzo) sample soaked
2. 5% Benzalkonium chloride (Aldrich) + 2.5% di-EDTA
3. 5% Benzalkonium chloride (Aldrich)
4. DMHTB-75 (Akzo) sample prepared at production stage.
5. DMHTB-75 (Akzo) sample prepared at production stage.
6. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
7. 5% DMHTB-75 (Akzo) sample soaked
8. 5% Benzalkonium chloride (Aldrich) + 2.5% tetra-EDTA
9. 5% Benzalkonium chloride (Aldrich)
10. 5% Benzalkonium chloride (Aldrich) + 2.5% tetra-EDTA
11. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
12. 5% Benzalkonium chloride (Aldrich) + 2.5% di-EDTA

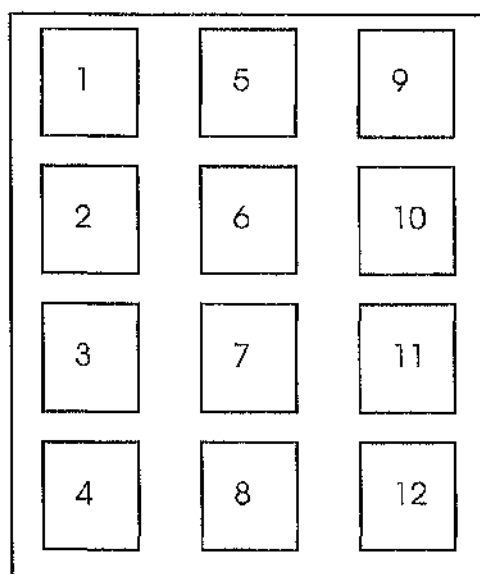


Fig. 4.2.8 Rack 4 1995

1. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
2. 5% DMHTB-75 (Akzo) + 2.5% tri-EDTA
3. 5% DMHTB-75 (Akzo)
4. 5% Benzalkonium chloride (Aldrich)
5. 5% DMHTB-75
6. 5% Benzalkonium chloride (Aldrich)
7. 5% DMHTB-75 (Akzo) + 2.5% tri-EDTA
8. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
9. 5% Benzalkonium chloride (Aldrich)
10. 5% DMHTB-75 (Akzo)
11. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
12. 5% DMHTB-75 (Akzo) + 2.5% tri-EDTA

This rack was not used to monitor fouling as it was immersed in the winter months. It was used to measure release of the active substances.

1	5	9	13
2	6	10	14
3	7	11	15
4	8	12	16

Fig 4.2.9 Rack 1 1996

1. 5% Benzalkonium chloride (Aldrich) + 5% cetrimide
2. 5% Benzalkonium chloride (Sigma)
3. 5% B-50 (Akzo)
4. 5% Quadrilan BC (Akcros)
5. 5% Cetrimide
6. 5% Benzalkonium chloride (Aldrich) + 5% Cetrimide
7. 5% Benzalkonium chloride (Sigma)
8. 5% B-50 (Akzo)
9. 5% Quadrilan BC (Akcros)
10. 5% Cetrimide
11. 5% Benzalkonium chloride (Aldrich) + 5% Cetrimide
12. 5% Benzalkonium chloride (Sigma)
13. 5% B-50 (Akzo)
14. 5% Quadrilan BC (Akcros)
15. 5% Cetrimide
16. Blank, Acrylic

1	5	9	13
2	6	10	14
3	7	11	15
4	8	12	16

Fig. 4.2.10 Rack 2 1996

1. 5% Benzalkonium chloride (Aldrich)
2. 2.5% tri-EDTA
3. 5% DMHTB-75 (Akzo) + 2.5% tri-EDTA
4. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
5. 5% DMHTB-75 (Akzo)
6. 5% Benzalkonium chloride (Aldrich)
7. 2.5% tri-EDTA
8. 5% DMHTB-75 (Akzo) + 2.5% tri-EDTA
9. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
10. 5% DMHTB-75 (Akzo)
11. 5% Benzalkonium chloride (Aldrich)
12. 2.5% tri-EDTA
13. 5% DMHTB-75 (Akzo) + 2.5% tri-EDTA
14. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
15. 5% DMHTB-75 (Akzo)
16. Blank, Acrylic

1	5	9	13
2	6	10	14
3	7	11	15
4	8	12	16

Fig. 4.2.11 Rack 3 1996

1. 20% Chlorhexidine gluconate
2. 1.5% Chlorhexine acetate
3. 0.5% Chlorhexidine gluconate
4. 2% Chlorhexidine gluconate
5. 5% Chlorhexidine gluconate
6. 20% Chlorhexidine gluconate
7. 1.5% Chlorhexidine acetate
8. 0.5% Chlorhexidine gluconate
9. 2% Chlorhexidine gluconate
10. 5% Chlorhexidine gluconate
11. 20% Chlorhexidine gluconate
12. 1.5% Chlorhexidine acetate
13. 0.5% Chlorhexidine gluconate
14. 2% Chlorhexidine gluconate
15. 5% Chlorhexidine gluconate
16. Blank, Acrylic

4.3 Marine Trials - Benzalkonium Chloride

The hydrogels were prepared either by soaking them in 5%w/v benzalkonium chloride solutions or in the case of the DMHTB-75 material by introducing the active substance at the hydrogel production stage. (The prefix %w/v indicates the concentration of the soaking solutions and not the actual amount absorbed by the hydrogel).

4.3.1 Initial Study - Rack 1

This rack was immersed from Keppel Pier at University Marine Biological Station Millport (UMBSM) on the 15th March 1995. The total number of samples in the rack was 16, since active substances other than benzalkonium chloride were being tested at this stage.

The mean weekly sea temperature at UMBSM for that particular week was 6°C.

Samples Prepared by Soaking in 5% w/v Solutions.

The samples below were soaked for 8 weeks, however subsequent samples were soaked for only 2-3 weeks as results from the MAST II Project [1] indicated that a longer soaking time was not necessary.

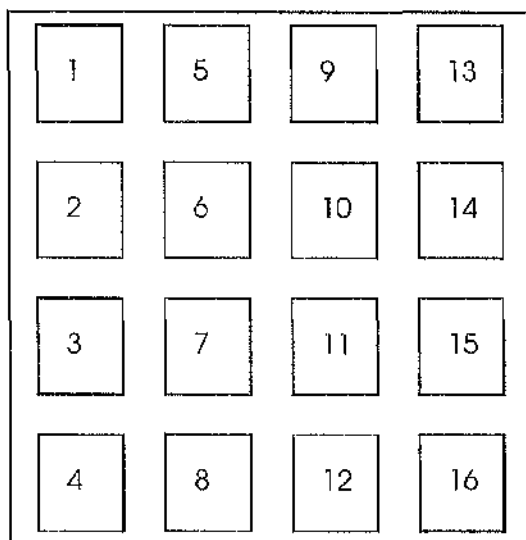
Number of Samples	Benzalkonium Chloride Type i.e. suppliers name for material	Supplier of Benzalkonium Chloride
2	Benzalkonium chloride	Aldrich
2	Arquad DMMCB-50	Akzo Nobel
1	Arquad B -50	Akzo Nobel
1	Benzalkonium chloride	Sigma
1	Quadrilan BC	Akcros
1	Arquad DMHTB-75	Akzo Nobel

Table 4.3.1 Soaking solutions

Sample Prepared at Hydrogel Production Stage

In this sample 2g of DMHTB-75 was added at the hydrogel production stage.

The layout of samples on the first rack is shown in Fig 4.3.1. In this section the suppliers name is only given in order to indicate which benzalkonium chloride was used and is not used if the type of benzalkonium chloride has a specific trade name.



- Positions 4 and 13 - 5%w/v Benzalkonium chloride (Aldrich)
- Positions 5 and 16 - 5%w/v DMCCB-50
- Position 6 - 5%w/v DMHTB-75, soaked sample
- Position 7 - 5%w/v B-50
- Position 8 - 5%w/v Benzalkonium chloride (Sigma)
- Position 14 - 5%w/v Quadrilan BC (Akcros)
- Position 15 - 5%w/v DMHTB-75, prepared at hydrogel production

Fig 4.3.2 Layout of Rack 1 1995

Figure 4.3.2 shows a photograph of the Rack GMTC 1 1995 taken before initial immersion. It can be seen that some samples are opaque. In this rack these are the benzalkonium chloride samples which contain predominantly the C₁₆ and C₁₈ alkyl chains.

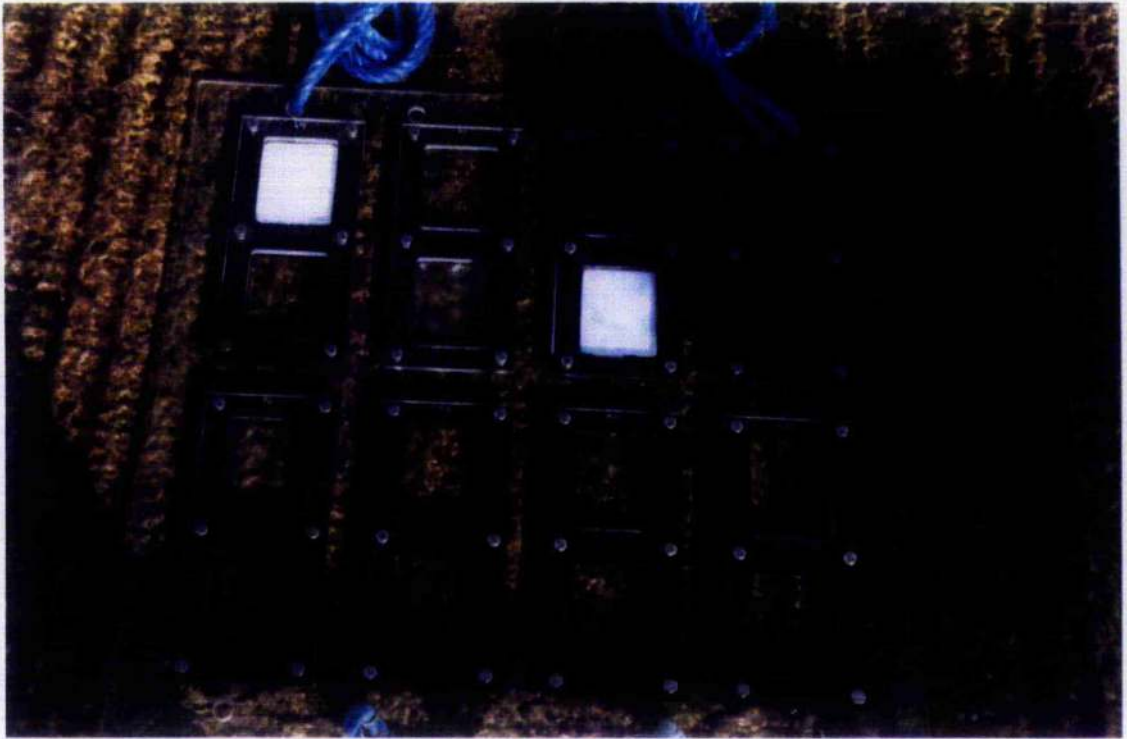


Fig. 4.3.2 Rack 1 1995 prior to initial immersion.

Fouling Results

Table 4.3.2 shows the fouling recorded for up to a 12 week period. The DMHTB-75 sample (position 15) remained clean until week 17, making it the most effective fouling resistant coating tested on this rack. The rack itself was very heavily fouled by week 8. Figure 4.3.3 shows Rack 1 1995 at the 8 week time-point.

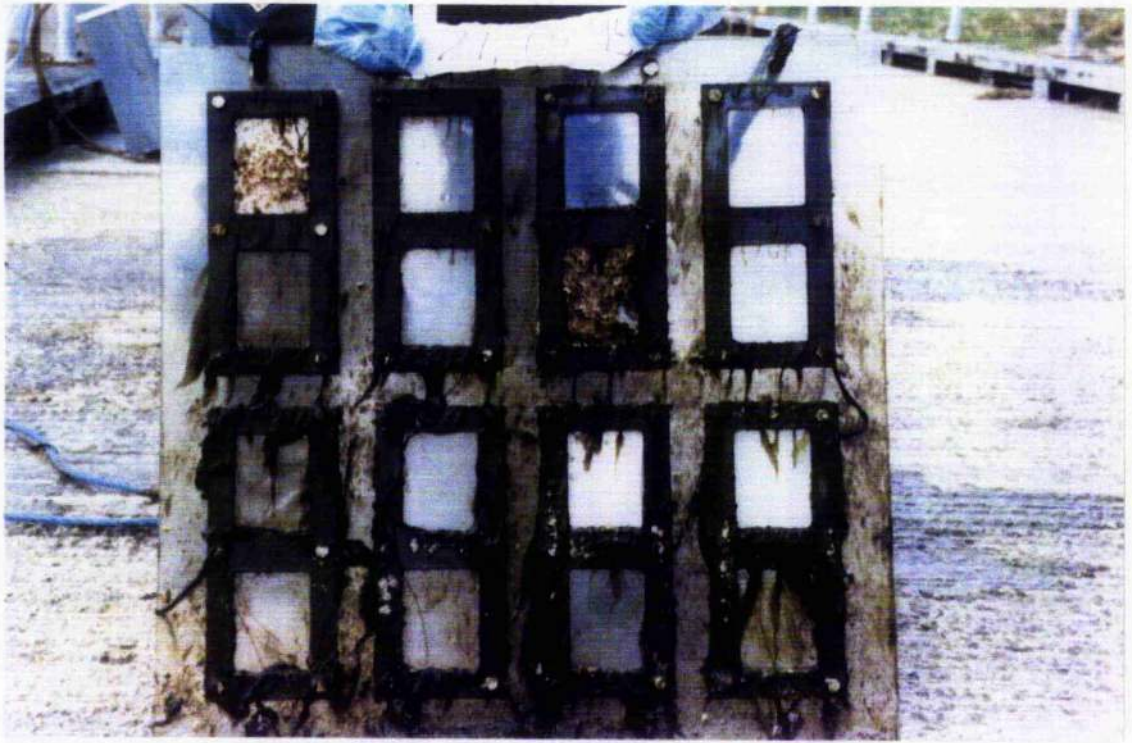


Fig. 4.3.3 Rack 1 1995 after 8 weeks marine exposure.

Rack Position	Benzalkonium Chloride Sample - Soaking Solution	Fouling Result
4	5%w/v Benzalkonium chloride (Aldrich)	Light algal fouling at 10 weeks
5	5%w/v DMMCB-50	Algal fouling at 12 weeks
6	5%w/v DMITB-75	Clean
7	5%w/v B-50 Akzo	White film over surface, by 10 weeks, identified as biofilm
8	5%w/v Benzalkonium chloride (Sigma)	Algal fouling at 10 weeks
13	5%w/v Benzalkonium chloride (Aldrich)	Slight algal fouling at 12 weeks
14	5%w/v Quadrikan BC	Slight algal fouling at 12 weeks
15	5%w/v DMHTB-75 Active substance included at production.	Very clean at 12 weeks
16	5%w/v DMMCB-50	Light algal fouling at 8 weeks

Table 4.3.2 Fouling results

Conclusions

From the initial results it was possible to pick the most successful samples and do further marine trials with these.

4.3.2 Marine Trials - Rack 3 1995

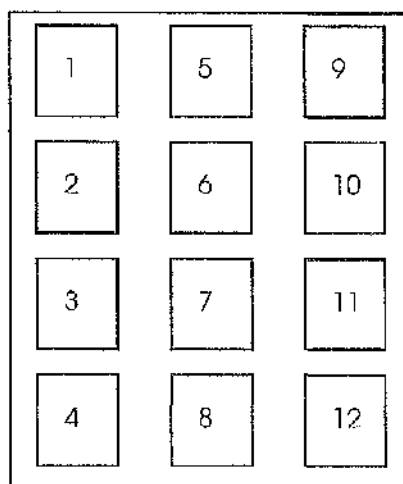
This rack was immersed at UMBSM on the 18th December 1995. This rack contained 12 samples, 6 of which were benzalkonium chlorides. The mean weekly sea temperature at UMBSM for that particular week was 8°C.

Samples prepared by soaking - The following samples were prepared by soaking them for 3 weeks in 5%w/v solutions.

Number of samples	Benzalkonium chloride Type i.e. suppliers name for product	Supplier of material
2	Benzalkonium chloride	Aldric
2	DMHTB-75	Akzo Nobel

Table 4.3.3 Samples prepared by soaking

Samples prepared at production - 2g of DMHTB-75 was included in each of the two hydrogels produced.



Positions 1 and 7 - 5%w/v DMHTB-75 soaked sample

Positions 4 and 5 - DMHTB-75 samples prepared at production stage.

Positions 3 and 9 - 5%w/v Benzalkonium chloride (Aldrich).

Fig. 4.3.4 Rack 3 1995

Summary of Fouling Results - Rack 3 1995

Rack Position	Benzalkonium chloride type	Fouling Result
1	5%w/v DMHTB-75 (soak)	Light algal fouling at 12 weeks
3	5%w/v Benzalkonium chloride	White surface film at 4 weeks, biofilm
4	5%w/v DMHTB-75 (prep)	Small pieces of brown filamentous algae noted at 6 weeks
5	5%w/v DMHTB-75 (prep)	Light algal fouling at 10 weeks, top 25% of sample
7	5%w/v DMHTB-75 (soak)	Algal fouling noted on 50% of at 10 weeks
9	5%w/v Benzalkonium chloride	White surface film at 4 weeks, biofilm

Table 4.3.4 Summary of Fouling Results for Rack 3 1995

Conclusions

The hydrogel material containing DMHTB-75 gave the best fouling resistance in this trial.

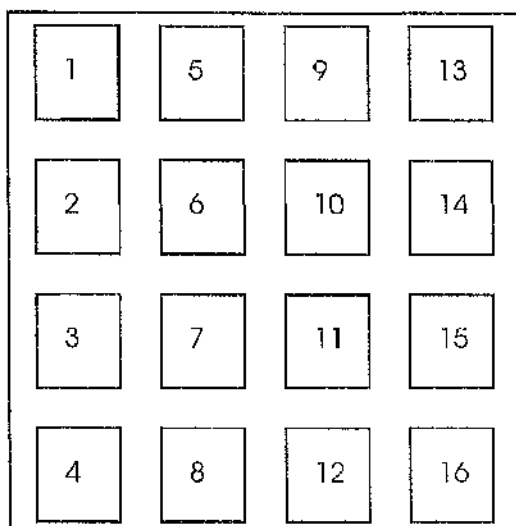
4.3.3 Marine Trials 1996

In order to confirm the results obtained during the Spring and Summer of 1995 further racks were prepared which contained the most successful anti-fouling samples from that period. Two new racks were immersed at Keppel Pier at UMBSM on the 20th March 1996. The temperature that day was 6°C.

Rack 1 1996

This rack contained benzalkonium chlorides of slightly different homologous composition, as well as cetrimide and samples combining benzalkonium chloride and

cetrimide. All samples were prepared by soaking them in 5%w/v solutions for 2-3 weeks.



1. 5% Benzalkonium chloride (Aldrich) + 5% Cetrimide
2. 5% Benzalkonium chloride (Sigma)
3. 5% B-50 (Akzo)
4. 5% Quadrilan BC (Akcros)
5. 5% Cetrimide
6. 5% Benzalkonium chloride (Aldrich) + 5% Cetrimide
7. 5% Benzalkonium chloride (Sigma)
8. 5% B-50 (Akzo)
9. 5% Quadrilan BC (Akcros)
10. 5% Cetrimide
11. 5% Benzalkonium chloride (Aldrich) + 5% Cetrimide
12. 5% Benzalkonium chloride (Sigma)
13. 5% B-50 (Akzo)
14. 5% Quadrilan BC (Akcros)
15. 5% Cetrimide
16. Acrylic panel

Fig. 4.3.5 Layout of Rack 1 1996

Summary of Fouling Results - Rack 1 1996

The results of the benzalkonium chlorides tested on this rack indicate that small differences in chain length give no obvious improvement to the anti-fouling ability of benzalkonium chloride. Table 4.3.5 shows the fouling results from March -June 1996.

Sample Position	Benzalkonium Chloride Type Name and Supplier	Fouling Result
2	Benzalkonium Chloride, Sigma	Light algal fouling at 8 weeks
7	Benzalkonium Chloride, Sigma	As 2 above
12	Benzalkonium Chloride, Sigma	As 2 above
3	B-50, Akzo	Algal fouling at 8 weeks
8	B-50, Akzo	Light algal fouling at 8 weeks
1	B-50, Akzo	Light algal fouling at 8 weeks
4	Quadrilan BC, Akcros	Light algal fouling at 8 weeks
9	Quadrilan BC, Akcros	Very light algal fouling at 8 weeks
14	Quadrilan BC, Akcros	Light algal fouling at 8 weeks

Table 4.3 5 Summary of Fouling Results Rack 1 1996

It can be seen from the photograph shown in figure 4.3.6 that the rack itself is extremely heavily fouled at 8 weeks and although the samples containing benzalkonium chloride showed light algal fouling they have in fact resisted fouling well at this test site compared with the acrylic of the rack. Position number 16 was an acrylic test panel and is also extremely heavily fouled with dense filamentous algae. The results for samples containing a mixture of materials are reported in section 4.7.

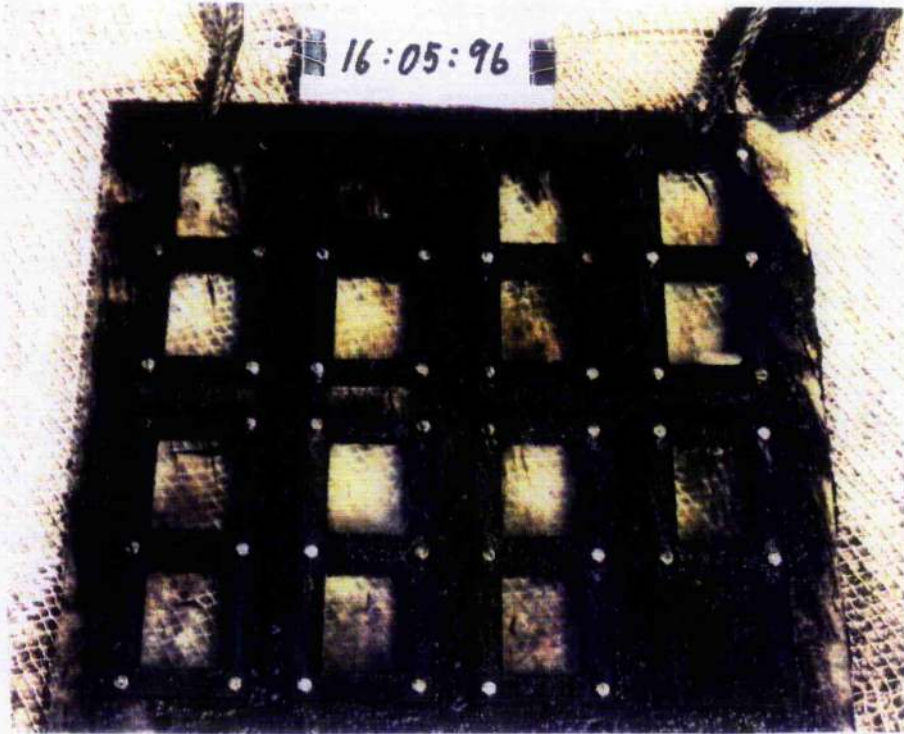


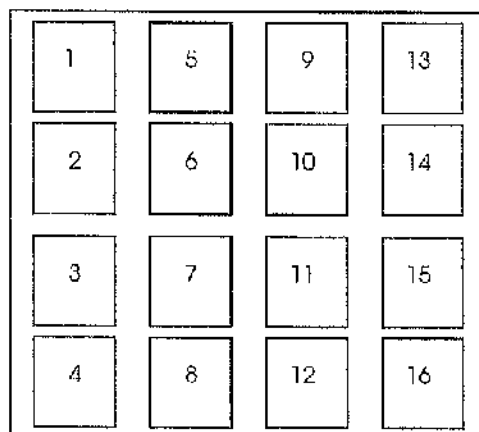
Fig 4.3.6 Rack 1 1996 after 8 weeks.

Rack 2 1996

This rack contained samples of benzalkonium chloride with chain lengths C_{12} and C_{14} and benzalkonium chloride (Trade name Arquad DMHTB-75) with chain lengths C_{16} and C_{18} in order to make a direct comparison of these two different forms of benzalkonium chloride. The further 9 samples on the rack are discussed in section 4.4

Marine Trial - Rack 2 1996

The marine conditions for this rack were as Rack 1 1996.



1. 5% Benzalkonium chloride (Aldrich)
5. 5% DMHTB-75
6. 5% Benzalkonium chloride (Aldrich)
10. 5% DMHTB-75
11. 5% Benzalkonium chloride (Aldrich)
15. 5% DMHTB-75
16. Acrylic panel

Fig 4.3.7 Layout of Rack 2 1996

Summary of Fouling Results - Rack 2 1996

The table below summarises the results obtained from March - June 1996.

Sample Position	Benzalkonium Chloride Type Name and Supplier	Fouling Result
1	Benzalkonium Chloride (Aldrich)	100% light algal fouling at 8 weeks
6	Benzalkonium Chloride (Aldrich)	100% light algal fouling at 8 weeks
11	Benzalkonium Chloride (Aldrich)	50% light algal fouling at 8 weeks
5	Arquad DMHTB-75 (Akzo Nobel)	light, patchy fouling, 20% cover at 8 weeks
10	Arquad DMHTB-75 (Akzo Nobel)	light, patchy fouling, 10% cover at 8 weeks
15	Arquad DMHTB-75 (Akzo Nobel)	sample clean at 8 weeks

Table 4.3.6 Summary of fouling results for Rack 2 1996.

Here the samples containing the longer chains C_{16} and C_{18} resisted overall fouling for the longest time. Figure 4.3.7 below shows the rack at the 8 week time-point.

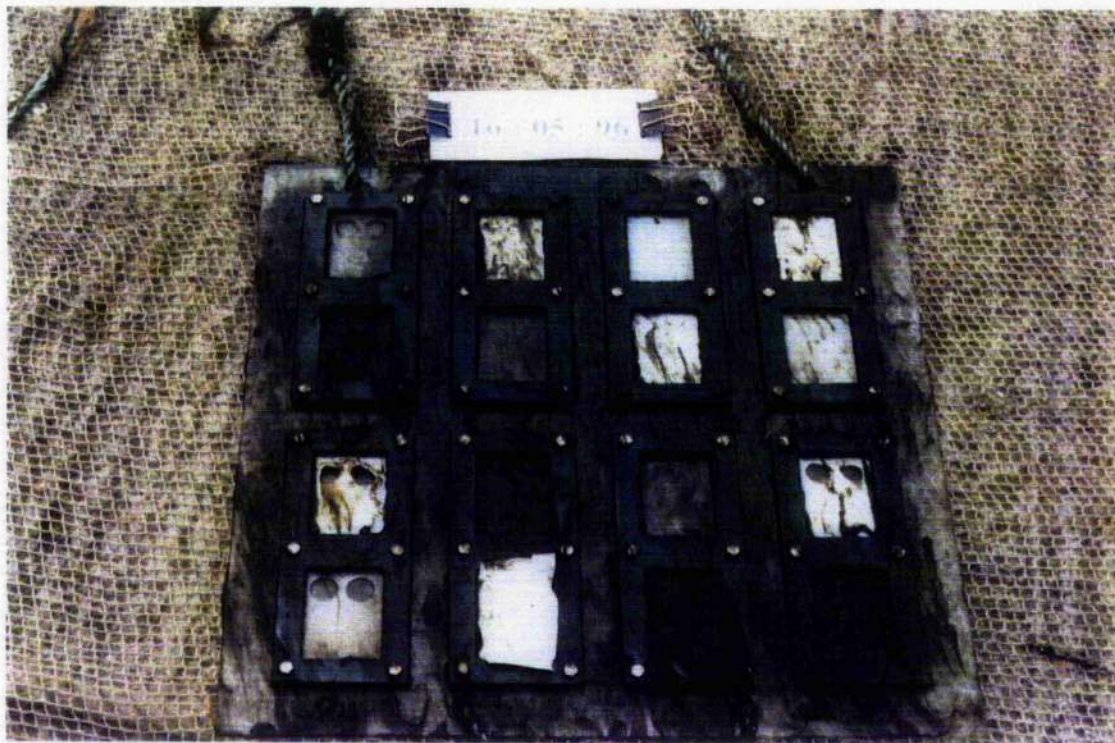


Fig. 4.3.7 Rack 2 1996 after 8 weeks.

The results from both 1995 and 1996 have shown the benzalkonium chloride containing the C_{16} and C_{18} chains to be the most successful type of benzalkonium chloride for anti-fouling action.

4.4 Marine Trials - Cetrimide

Hydrogel material was loaded with cetrimide by soaking it in 5%w/v solutions. Initially this was done for 8 weeks. However, in subsequent trials this time was reduced to 2-3 weeks as this was sufficient time for loading the sample. The sample produced was transparent and remained so in seawater. In Chapter 2 details are given concerning the preparation of the cetrimide solutions and the reasons for the choice of cetrimide being used. The cetrimide used in the marine trials was supplied by Sigma and is described as "mixed alkyltrimethylammonium bromide, which is

predominantly C_{14} but also contains C_{12} and C_{16} homologues". This material complies with the British Pharmacopoeia description of cetrimide.

4.4.1 Initial Study - Rack 1 1995

The sample prepared was immersed at UMBSM on the 15th March 1995, all details of the trial site conditions are given in section 4.3.1 The cetrimide sample was in position 2 on Rack 1 1995.

Fouling Results

At the 8 week time point the cetrimide sample was fouled by brown algae.



Fig. 4.4.1 Rack 1 1995 after 8 weeks of marine conditions.

Conclusion

In comparison to benzalkonium chloride, cetrimide behaved less efficiently as an anti-fouling material in this trial.

4.4.2 Second Study - Rack 1 1996

In this study three samples containing cetrinide were prepared by soaking them in 5%w/v solution. Each sample was soaked in 100 ml of solution for 2-3 weeks. All the samples produced were transparent and they remained transparent when they were immersed in seawater.

Rack 1 1996

The layout of this rack is shown in section 4.2 with the details of the exposure conditions given in section 4.3.3. The cetrinide samples were in positions 5, 10 and 15.

Fouling Results

The fouling results for the cetrinide samples are summarised in Table 4.4.1. Figure 4.4.2 shows the extent of fouling on the cetrinide samples at the 8 week time-point.

Sample Position	Active Substance Supplier	Fouling Result
5	Cetrinide - Sigma	Dense, brown algal fouling at 8 weeks
10	Cetrinide - Sigma	Dense, brown algal fouling at 8 weeks
15	Cetrinide - Sigma	Brown algal fouling at 8 weeks

Table 4.4.1 Fouling results for cetrinide samples on Rack 1 1996

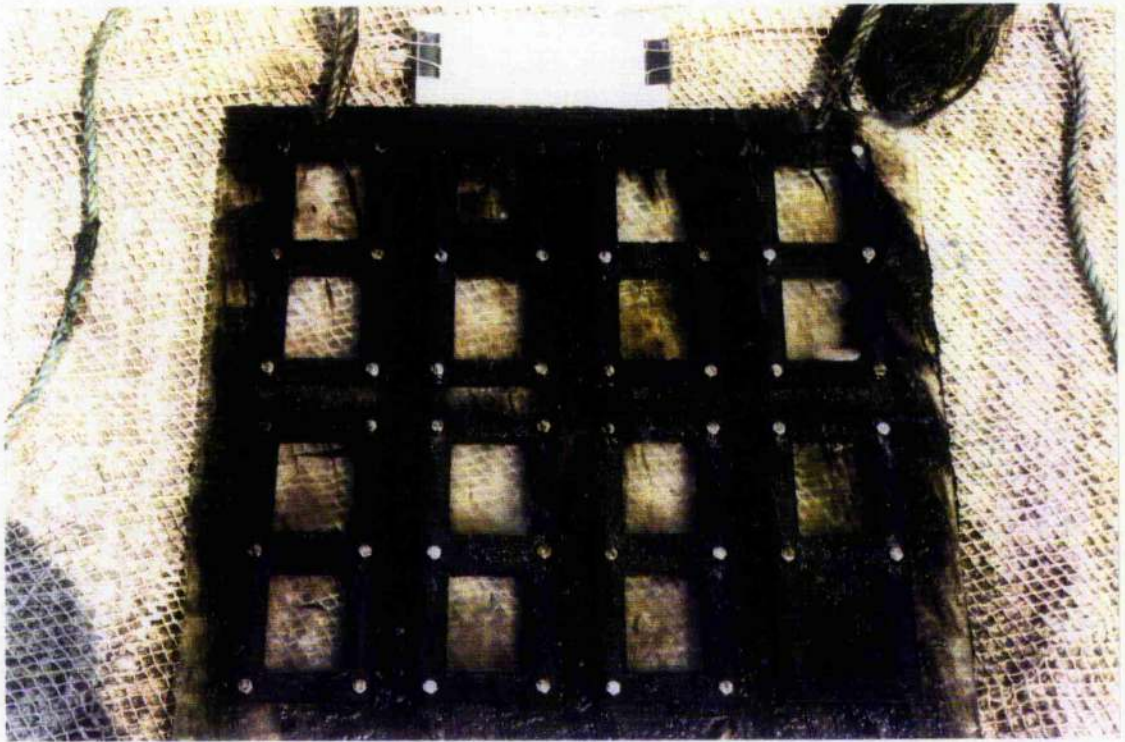


Fig. 4.4.2 Rack 1 1996 at the 8 week time-point

Conclusions

The results obtained from this rack confirm the previous results from the 1995 trial. Cetrimide is less able to deter fouling, when incorporated into the hydrogel material, than benzalkonium chloride.

4.5 Benzalkonium Chlorides and Ethylenediaminetetraacetic Acid

4.5.1 Initial Marine Trial - Method for Preparation

A 100 ml solution of 5% benzalkonium chloride and 2.5% ethylenediaminetetraacetic acid disodium salt dihydrate (di-EDTA) was prepared. This solution was clear. A piece of hydrogel 120 mm² was soaked in this solution for 10 weeks. After this period the hydrogel appeared to have remained the same shape and size as it was originally. The sample was very clear and shiny and was observed to be "crystal clear".

The sample was immersed at UMBSM on the 16th March 1995 when the sea temperature was 6°C.

Marine Results

The sample was photographed and examined visually at 2 week intervals.

Time (weeks)	Comments
0	Sample transparent
2	Sample opaque (white). No fouling
4	Sample opaque. No fouling
6	The sample still opaque except for 3 bubbles which were transparent and had diameters of 3-4mm. No fouling.
10	The transparent air bubbles are now larger with diameters of 10-12mm. The remainder of the sample is still opaque. No fouling.
12	The lower 1/3 of the sample is now transparent. No fouling.
14	The sample is now transparent. Algal fouling has developed on the lower 1/3 of the sample.
17	Sample transparent and fouled.

From the results obtained from this initial marine trial the addition of di-sodium ethylenediaminetetraacetic acid (di-EDTA) appeared to have an effect on the anti-fouling properties of the hydrogel benzalkonium chloride system, since a control sample containing only BAC on the same rack without the di- EDTA fouled at 10 weeks.

4.5.2 Second Marine Trial

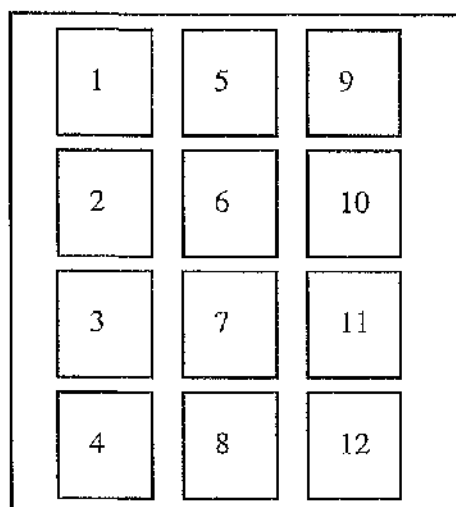
From the initial result it could be seen that the presence of disodium ethylenediaminetetraacetic acid (di-EDTA) improved the anti-fouling ability of benzalkonium chloride. In the second trial di-, tri- and tetra- sodium ethylenediaminetetraacetic acid (EDTA) were all tested since each has a different dissociation constant and thus this may affect its action in seawater. Samples soaked in the di-, tri- and tetra- sodium salts of EDTA were all immersed at UMBSM. Samples soaked in 5% benzalkonium chloride were also immersed as control samples.

The pH of each of these soaking solutions was recorded and these values were found to range from 4.40 to 10.50, i.e. from a fairly acid solution to an alkali solution.

Solution (200ml)	pH
5% Benzalkonium chloride	5.75
5% Benzalkonium chloride + 2.5% di-EDTA	4.40
5% Benzalkonium chloride + 2.5% tri-EDTA	7.88
5% Benzalkonium chloride + 2.5% tetra-EDTA	10.50

Two pieces of 120 mm² hydrogel material were soaked in each of the 200ml solutions for 2 weeks. (At this stage it was decided that 2 weeks would be the soaking time for hydrogel in benzalkonium chloride, as per the MAST II Protocol [1]).

The samples were immersed at UMBSM on the 28th June 1995 when the sea temperature was 14°C. The rack was designated Rack 3 1995.



1. 5% DMHTB-75, soak (Akzo)
2. 5% Benzalkonium chloride (Aldrich) + 2.5% di-EDTA
3. 5% Benzalkonium chloride (Aldrich)
4. 5% DMHTB-75, 2g incorporated at production (Akzo)
5. 5% DMHTB-75, 2g incorporated at production (Akzo)
6. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
7. 5% DMHTB-75, soak (Akzo)
8. 5% Benzalkonium chloride (Aldrich) + 2.5% tetra-EDTA
9. 5% Benzalkonium chloride (Aldrich)
10. 5% Benzalkonium chloride (Aldrich) + 2.5% tetra-EDTA
11. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
12. 5% Benzalkonium chloride (Aldrich) + 2.5% di-EDTA

Fig. 4.5.1 Diagram of Rack 3 1995

Summary of Marine Results (Rack 3 1995)

Table 4.5 1 gives details of the change in appearance of the hydrogel from opaque grey/white to transparent. This occurred with time in the marine environment. Initially, clear bubbles appeared on the hydrogel at about 6 weeks and these had a diameter of 2-3mm. This size increases to 10mm by 10 to 12 weeks. By 12 weeks the di- and tetra-EDTA samples became nearly clear, however the tri-EDTA remained opaque apart from at the location of the bubbles.

Time	di-EDTA	tri-EDTA	tetra-EDTA
0 Time	(2) crystal clear (3) (12) crystal clear	(6) crystal clear (11) crystal clear	(8) crystal clear (10) crystal clear
2 weeks	(2) opaque (12) opaque	(6) opaque (11) opaque	(8) opaque (10) opaque
4 weeks	(2) opaque (12) opaque	(6) opaque (11) opaque	(8) opaque (10) opaque
6 weeks	(2) opaque (12) opaque, 2 clear bubbles	(6) opaque, 5 clear bubbles (11) opaque, 10 clear bubbles	(8) opaque, 5 clear bubbles (10) opaque, 10 clear, bubbles
10 weeks	(2) clear, light fouling (12) clear, light fouling	(6) opaque, 10 clear bubbles (11) opaque, 8 clear bubbles	(8) top 33% clearing (10) opaque, 10 large bubbles
12 weeks	(2) clear, light fouling (12) clear, brown algal fouling	(6) opaque, bubbles (11) opaque, bubbles	(8) clear, film on surface (10) sample clearing

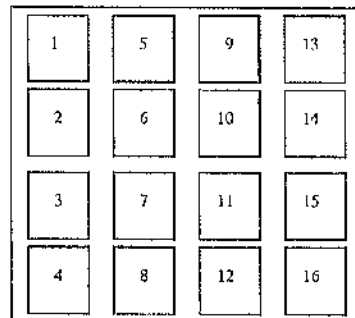
Table 4.5.1 Fouling Results of Rack 3 1995 (The sample positions on the rack are shown in brackets)

Conclusions

From these results the addition of tri-EDTA to benzalkonium chloride deters the fouling of the hydrogel surface for the longest period. The marine trials incorporating tri-EDTA into benzalkonium chloride all indicated that an improvement in anti-fouling performance resulted. Thus further trials to confirm this finding were carried out during 1996.

4.5.2 Marine Trials 1996 - Rack 2 1996

This rack contained a total of 16 samples. The rack was immersed at Keppel Pier at UMBSM on the 20th March 1996. The sea temperature on that day was 6°C.



1. 5% Benzalkonium chloride (Aldrich)
2. 2.5% tri-EDTA
3. 5% DMHTB-75 + 2.5% tri-EDTA
4. 5% Benzalkonium chloride (Aldrich) + 2.5% tri-EDTA
5. 5% DMHTB-75
6. 5% Benzalkonium chloride (Aldrich)
7. 2.5% tri-EDTA
8. 5% DMHTB-75 + 2.5% tri-EDTA
9. 5% Benzalkonium chloride + 2.5% tri-EDTA
10. 5% DMHTB-75
11. 5% Benzalkonium chloride (Aldrich)
12. 2.5% tri-EDTA
13. 5% DMHTB-75 + 2.5% tri-EDTA
14. 5% Benzalkonium chloride + 2.5% tri-EDTA
15. 5% DMHTB-75
16. Acrylic panel

Fig. 4.5.2 Diagram of Rack 2 1996

Fouling Results

From the diagram of the rack it can be seen that 3 samples of each material were exposed.

The results are shown below:

The frame of the rack was fouled by brown filamentous algae within 5 weeks.

5% w/v benzalkonium chloride (Aldrich) - the 3 samples remained clean until 8 weeks when an all-over light brown layer of algal fouling was noted on each of them.

5% w/w DMHTB-75 (Akzo) - the 3 samples remained clean until 8 weeks when light, brown patchy algal growths were noted on all 3 samples, however, large clean areas remained on these samples.

5% w/v benzalkonium chloride (Aldrich) + 2.5% tri-EDTA - the 3 samples remained visually clean at the 12 week time-point and only began to show light patchy fouling at 17 weeks.

5% w/w DMHTB-75 (Akzo) + 2.5% tri-EDTA - the 3 samples produced were not completely smooth as the previous 9 above and the irregular surface is more attractive to fouling. At 8 weeks the samples were free of algal fouling but one sample had 2 barnacles attached.

2.5% tri-EDTA - the 3 samples were fouled by brown algae at the 3 week time-point.

Conclusions

This trial confirmed the results obtained during 1995. Figure 4.5.3 overleaf shows the rack at the 10 week time-point. Samples in positions 4, 9 and 14 are those containing 5% benzalkonium chloride with 2.5% tri-EDTA and positions 1, 6 and 11 are those containing benzalkonium chloride alone.

The order of the anti-fouling ability from the results obtained is :

BAC + tri-EDTA > DMHTB-75 > DMHTB-75 + tri-EDTA > BAC

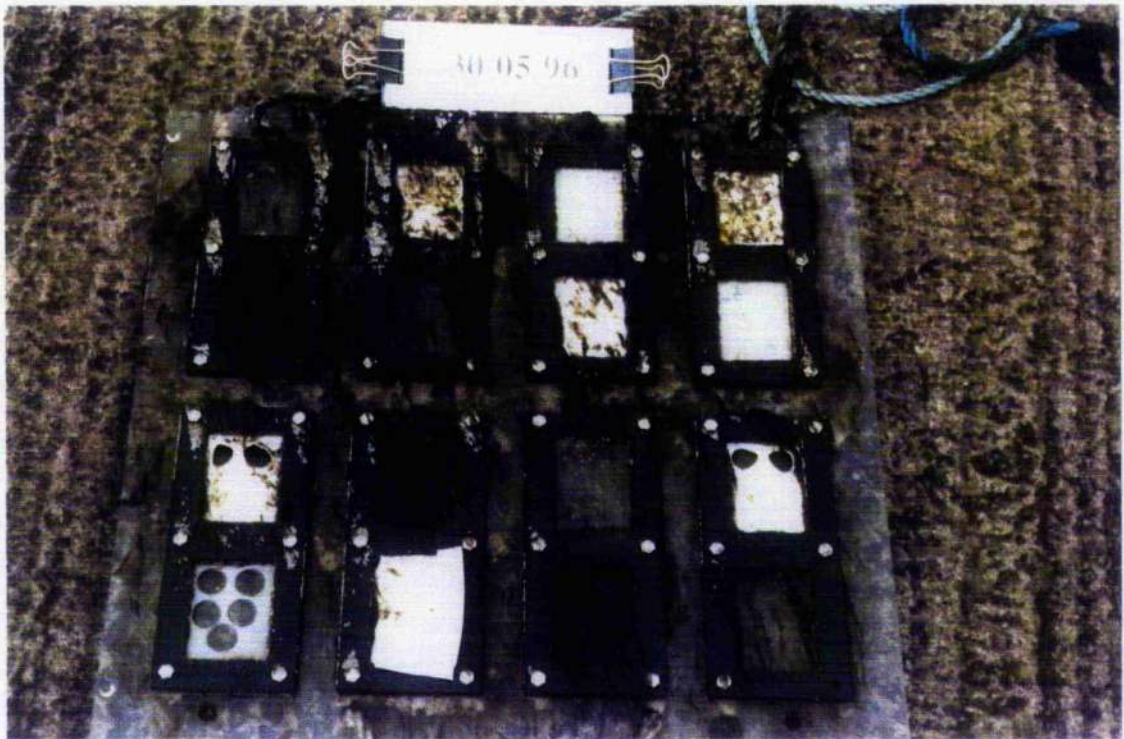


Fig. 4.5.3 Rack 2 1996 at the 10 week time-point.

4.6 Marine Trials - Chlorhexidine Gluconate

The hydrogel samples were prepared by soaking them in solutions of 5%w/v and 20%w/v solutions of chlorhexidine gluconate (CG). They were then pre-treated in artificial seawater ("Instant Ocean", IO) for 24 hours before being put on marine trials.

4.6.1 Initial Study

This rack was immersed on the 15th March 1995. The weekly mean sea temperature was 6.°C.

Rack 1 1995

This rack, as described in section 4.2, contained various antimicrobial agents as it was the initial study in order to determine the most useful agents. There were 16 samples in all. Details of the rack can be found in section 4.2 and marine conditions at immersion can be found in section 4.3.1.

This rack contained 2 samples of chlorhexidine gluconate.

1. Position 1 - sample soaked in 20%w/v Chlorhexidine gluconate.
2. Position 10 - sample soaked in 5%w/v Chlorhexidine gluconate.

Fouling Results

At the 4 week time-point both samples had tiny barnacles attached to their surface.

Second Trial Racks 2A and 2B 1995

These racks were immersed at UMBSM on the 24th May 1995. The mean sea temperature for that week was 10°C.

Both racks contained 4 samples in total. Out of these 4, 2 were samples which had been soaked for 7 days in 20%w/v chlorhexidine gluconate and pre-treated in seawater for 24 hours prior to marine immersion.

Again tiny barnacles were noted on all chlorhexidine gluconate samples within 4 weeks of immersion.

Conclusions - Racks 1, 2A and 2B

From the above trials it seems that barnacles are able to colonise and grow very efficiently on samples of this nature. No algal growth was noted on any of the samples up to and beyond a 12 week period. This is probably due to the fact that algae do not flourish in areas adjacent to barnacles.

4.6.2 Marine Trials - Rack 3 1996

This rack contained chlorhexidine samples which were all prepared by soaking them in the following solutions :

20%w/v	Chlorhexidine gluconate
5%w/v	Chlorhexidine gluconate
2%w/v	Chlorhexidine gluconate
0.5%w/v	Chlorhexidine gluconate
1.5 %w/v	Chlorhexidine acetate

Samples were soaked in the chlorhexidine gluconate for 1 week. Those that had been in 5% and 20% solutions were pre-treated in seawater prior to immersion at sea due to the swelling which occurred in the chlorhexidine gluconate solutions, (Chapter 2 details this). The other samples showed little or no swelling and were immersed straight from their soaking solutions. The rack was immersed on the 24th April 1996 when the sea temperature was 8°C.

Below in figure 4.6.1 the layout of Rack 3 1996 is shown.

1	5	9	13
2	6	10	14
3	7	11	15
4	8	12	16

1. 20% Chlorhexidine gluconate
2. 1.5% Chlorhexidine acetate
3. 0.5% Chlorhexidine gluconate
4. 2% Chlorhexidine gluconate
5. 5% Chlorhexidine gluconate
6. 20% Chlorhexidine gluconate
7. 1.5% Chlorhexidine acetate
8. 0.5% Chlorhexidine gluconate
9. 2% Chlorhexidine gluconate
10. 5% Chlorhexidine gluconate
11. 20% Chlorhexidine gluconate
12. 1.5% Chlorhexidine acetate
13. 0.5% Chlorhexidine gluconate
14. 2% Chlorhexidine gluconate
15. 5% Chlorhexidine gluconate
16. Acrylic panel

Fig. 4.6.1 Layout of Rack 3 1996

Fouling Results

At the 3 week time-point all the samples, with the exception of positions 4, 8 and 13, were fouled by barnacles. The positions which were clean and clear were the samples prepared by soaking in 0.5% chlorhexidine gluconate. At the 7 week time-point these samples were still clean despite the fact that the rack itself was heavily fouled with filamentous brown algae. Figure 4.6.2 shows this.

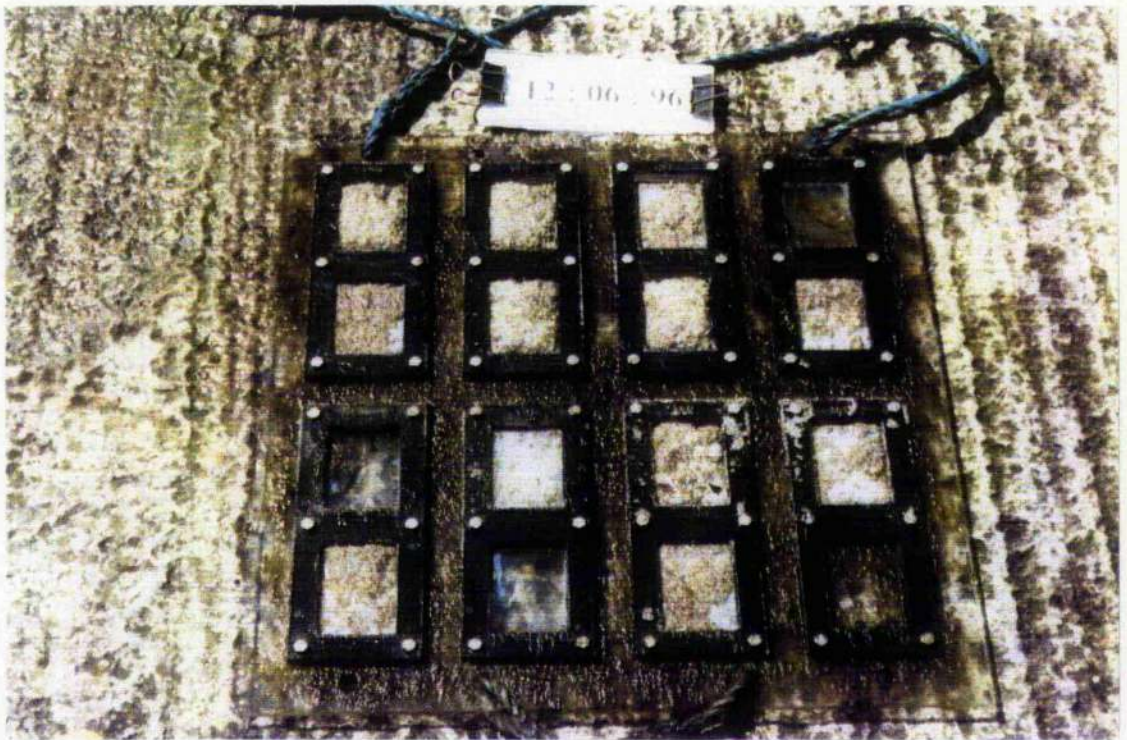


Fig. 4.6.2 Rack 3 1996 at the 7 week time-point.

Conclusions

When samples which have been soaked in concentrations of chlorhexidine gluconate greater than 1%w/v are added to seawater immediately they go white and produce a grainy surface. The slightly roughened surface is attractive to barnacles and thus they settle there [6]. This is discussed in greater detail in Chapter 2.

4.7 Marine Trials - Mixed Surfactant Systems

4.7.1 Initial Study - Rack 1 1995

Initially two mixed solutions were prepared. These were

1. 5%w/v benzalkonium chloride (Aldrich) + 5%w/v alkyltrimethylammonium bromide, which will be called cetrimide (Sigma)

2. 5%w/v benzalkonium chloride (Aldrich) + 0.5%w/v chlorhexidine gluconate (Zeneca)

Hydrogel samples were prepared by soaking them in the above solutions for 8 weeks, however, subsequent samples were soaked for only 2-3 weeks as quantitative analysis (MAST II Project) indicated that as long a period as 8 weeks for soaking was not necessary. The samples prepared were transparent and remained so after immersion in seawater.

Number of Samples	Material Type	Supplier of Material
1	5%w/v Benzalkonium chloride + 5%w/v Cetrimide	Aldrich Sigma
1	5%w/v Benzalkonium chloride + 0.5%w/v Chlorhexidine gluconate	Aldrich Zeneca

Table 4.7.1 Preparation of samples.

The rack was immersed at UMBSM on the 15th March 1995. The total number of samples on the rack was 16 as various potential anti-fouling materials were being tested in this initial trial. Full details of the conditions are given section 4.2. The mean weekly sea temperature for that particular week was 6°C.

Fouling Results

Rack Position	Active Substance	Fouling result
9	5%w/v Benzalkonium Chloride + 5%w/v Cetrimide	Slight algal fouling was noted at 12 weeks
12	5%w/v Benzalkonium Chloride + 0.5%w/v Chlorhexidine Gluconate	White film over sample at 12 weeks

Table 4.7.2 Fouling results.

Conclusions

The two samples appear to be able to resist fouling to the same extent as benzalkonium chloride on its own.

Second Marine Trials Racks 2A and 2B

A further two racks, each with 4 sample positions was prepared and immersed at UMBSM on the 24th May 1996. The weekly mean sea-temperature for that week was 12°C. On each rack the following samples were exposed

Rack 2A 1996

Position 2 - 5%w/v Benzalkonium chloride + 0.5%w/v Chlorhexidine gluconate

Position 3 - 5%w/v Benzalkonium chloride + 2%w/v Chlorhexidine gluconate

Rack 2B 1996

Position 2 - 5%w/v Benzalkonium chloride + 2%w/v Chlorhexidine gluconate

Position 3 - 5%w/v Benzalkonium chloride + 0.5%w/v Chlorhexidine gluconate

The other four samples were all prepared by soaking them in 20%w/v chlorhexidine gluconate. These samples have been discussed in Section 4.6. Racks 2A and 2B were attached back-to-back. Figure 4.7.3 shows a photograph of the arrangement.

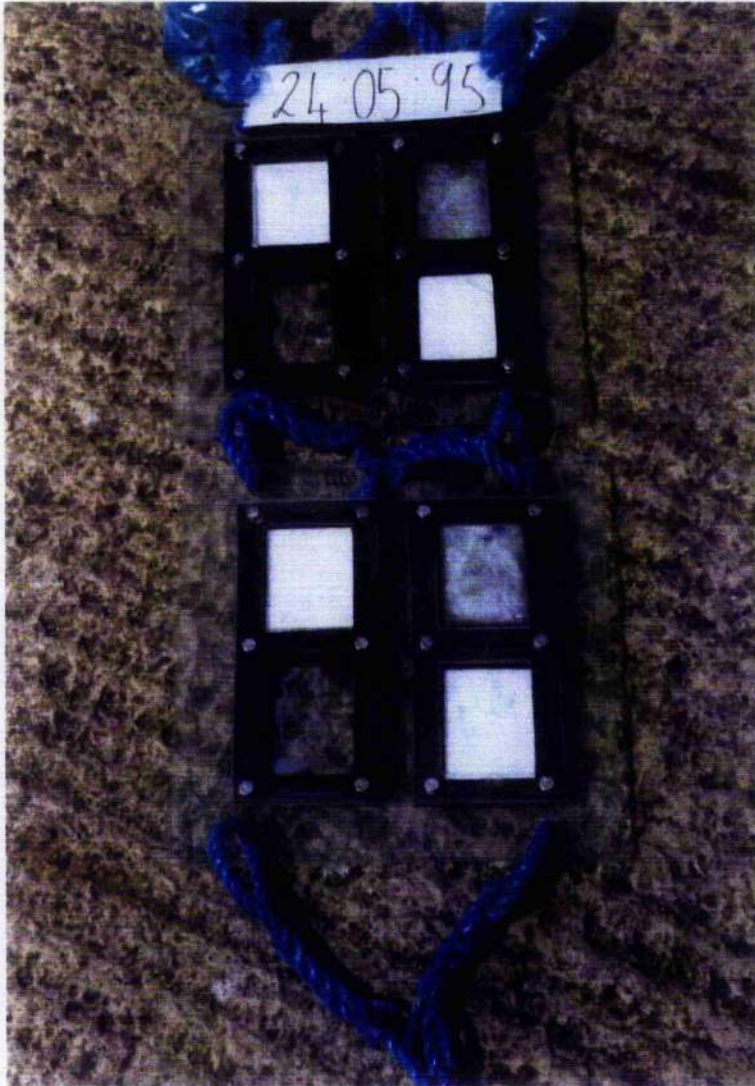


Fig. 4.7.2 Arrangement of racks 2A and 2B.

Fouling Results

Table 4.6.3 shows a summary of the fouling on the four samples.

Sample Position	Active Substance	Fouling
Rack2A	(2) 5%w/v Benzalkonium chloride + 0.5%w/v Chlorhexidine gluconate.	Brown algal fouling at 11 weeks
Rack 2A	(3) 5%w/v Benzalkonium chloride + 2%w/v chlorhexidine gluconate	Brown algal fouling at 11 weeks
Rack 2B	(2) 5%w/v Benzalkonium chloride + 2%w/v Chlorhexidine gluconate.	Brown algal fouling on bottom 20% at 11 weeks
Rack 2B	(3) 5%w/v Benzalkonium chloride + 0.5%w/v Chlorhexidine gluconate	Brown algal fouling at 9 weeks

Table 4.7.3 Summary of fouling on Racks 2A and 2B. (The sample positions on the rack are shown in brackets)

Conclusions

Samples containing the above mixtures only increased the anti-fouling lifetime of benzalkonium chloride very slightly.

4.7.2 Marine Trials - Rack 1 1996

Further samples were prepared and immersed at UMBSM on the 20th March 1996. Full details of the trial conditions are given in section 4.3.3.

Three samples were prepared by soaking each in 100 ml of 5%w/v benzalkonium chloride and 5%w/v cetrimide for 2-3 weeks. The samples produced were transparent

and remained that way when immersed in seawater. The samples were in positions 1, 6 and 11 of Rack 1 1996.

Fouling Results

Sample Position	Active Substances	Fouling Result
1	5%w/v Benzalkonium chloride + 5%w/vCetrimide	Light algal fouling at 8 weeks
6	5%w/v Benzalkonium chloride + 5%w/v Cetrimide	Light algal fouling at 8 weeks
11	5%w/v Benzalkonium chloride + 5%w/v Cetrimide	Light algal fouling at 8 weeks

Table 4.7.4 Summary of results from Rack 1 1996.

The mixture of quaternary ammonium compounds does not appear to improve the anti-fouling performance as the three samples above. It was not any more effective than the benzalkonium chloride alone.

4.8 Conclusions

Each section includes a summary of marine results and a brief conclusion. The fouling recorded was all visual and no microscopy was done in these trials. Notes were taken on the fouling at each time-point, as were photographs. In this section the conclusions are given as well as a brief discussion about the usefulness of each potential anti-fouling material.

The results of the trials carried out during 1995 and 1996 show that increasing the chain length of the benzalkonium chloride from being predominantly C_{12} and C_{14} to material containing C_{16} and C_{18} (DMHTB-75), increased the anti-fouling lifetime of the hydrogel. The manner in which the hydrogel fouled when it contained these longer chains was patchy as opposed to the all-over fouling which the hydrogel containing the shorter chains exhibited. The release results in Chapter 3 indicate that practically all of the longer chains remain in the hydrogel throughout the trial.

However, the material does eventually foul despite this. As the C₁₈ chain is hydrophobic in nature it may be repelling fouling by static charge as opposed to antimicrobial action shown by the shorter chains.

Cetrimide did not deter fouling as well as benzalkonium chloride in the hydrogel system. As this result was observed in the initial trial no release studies were carried out.

The addition of the chelating agent, tri-sodium ethylenediaminetetraacetic acid (tri-EDTA), to the hydrogel containing benzalkonium chloride (Aldrich C₁₂ and C₁₄) has the most noted improvement on the anti-fouling behaviour. From the release results in Chapter 3 it can be seen that the presence of tri-EDTA retards the release of benzalkonium chloride. It is possible that the tri-EDTA forms a stable complex in the hydrogel material thus reducing the ease of escape of the benzalkonium chloride. Further work is necessary to establish the exact nature of the mechanism.

Chlorhexidine was not successful as an anti-fouling agent as it formed insoluble chlorhexidine dihydrochloride, a white grainy substance, on the hydrogel material which made the surface rough and attractive to barnacles [6]. The samples which were soaked in 0.5%w/v chlorhexidine gluconate did not go completely opaque and the hydrogel surface remained smooth. These samples did not have barnacles on their surface and remained free of algal fouling until 13-14 weeks. This was not a significant improvement on benzalkonium chloride which the MAST II Project had used and therefore at this stage this line of research was not pursued.

The mixed surfactant systems were able to deter fouling to the same extent as benzalkonium chloride on its own and were therefore not worth following up as a more attractive alternative.

4.9 Chapter 4 - References

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Chapter 5

Discussion, Conclusions and Future Work

- 5.1 Introduction
- 5.2 Suitability of Cationic Surfactants as Anti-fouling Agents
- 5.3 Why Antimicrobials Fail
- 5.4 Antimicrobials and Surfactants
- 5.5 Micelles
- 5.6 Methods of Analysis
- 5.7 Surface Energy
- 5.8 Final Conclusions
- 5.9 References

5.1 Introduction

When the research began the aim was to develop a non-toxic anti-fouling coating which would protect sensors from marine fouling when they were deployed from remote buoys in the ocean. Within the research it was also hoped that a better understanding of quaternary ammonium compounds, the potential anti-foulers, would be achieved, both in their action in the marine environment and also in their analysis. In this chapter the progress towards these aims is discussed. The results obtained show that fouling in the marine environment, was as expected, highly complex. Many additional problems occur in seawater such as anion and cation interference, pH and solubility which make the task of any anti-fouling agent more challenging.

5.2 Suitability of Cationic Surfactants as Anti-fouling Agents

5.2.1 Benzalkonium Chloride

The hydrogel loaded with benzalkonium chlorides, both the material which was predominantly C_{12} and C_{14} alkyl chains (benzalkonium chloride, Aldrich) and DMHTB-75 which was predominantly C_{16} and C_{18} alkyl chains, were suitable as fouling protection in the marine environment as no obvious degradation of the hydrogel was noted during the trials.

The use of the benzalkonium chlorides showed that the various alkyl chain lengths had different effects when they were incorporated into the hydrogel and also when the loaded hydrogel was immersed in the sea. The results showed that by using benzalkonium chloride which contained mainly C_{16} and C_{18} alkyl chains the long term anti-fouling ability was greater than for that of the benzalkonium chloride which was mainly C_{12} and C_{14} alkyl chains.

From the release graph shown in chapter 3, figure 3.3.1, the benzalkonium chloride, C_{12} and C_{14} alkyl chains, had released from the hydrogel to less than half the original amount over the twelve week test. At about ten weeks the samples had begun to foul and thus although there was active compound present, it was not in great enough quantities to prevent the onset of fouling. The release rate of this type of benzalkonium chloride was faster than the more

hydrophobic DMHTB-75, which is practically zero, but it indicates that not all the benzalkonium chloride was present in the water portion of the hydrogel as the amount does not fall to zero. As the hydrogel is a methacrylate, its structure is such that there are lone pairs available in the non-water portion to which some irreversible bonding or extremely slow release of the benzalkonium chloride is possible. It has been suggested by Walters et al [1] that ion-ion interactions between the ionised carbonyl groups of the hydrogel and the benzalkonium chloride take place and therefore this would explain the release results obtained. Previous work has been published on the diffusion of quaternary ammonium compounds in gels using NMR Pulsed Field Gradients [2]. Studies of this type may help explain the behaviour seen in the release results. However, such studies were not within the remit of this particular research.

The small to zero release of the DMHTB-75 from the hydrogel throughout all the marine trials can be attributed to the more hydrophobic nature of the C₁₆ and C₁₈ alkyl chains. It can be assumed that these chains have a preference to remain in the hydrophobic portions of the hydrogel as opposed to the polar water portion of the hydrogel.

5.2.2 Benzalkonium Chloride with Ethylenediaminetetraacetic Acid, Sodium Salts

The inclusion of the chelating agent ethylenediamine tetraacetic acid, tri-sodium salt (tri-EDTA) extended the anti-fouling lifetime of the benzalkonium chloride, C₁₂ and C₁₄ alkyl chain length, by four to five weeks. By including the tri-EDTA in the benzalkonium chloride soaking solution the actual amount of benzalkonium chloride absorbed by the hydrogel increased. The release of benzalkonium chloride was also slower from hydrogel containing this combination than from that which contained benzalkonium chloride alone. This slow release can be attributed to the probable complex formation between the calcium ions (413ppm present in seawater) and the tri-EDTA in the water portion of the hydrogel. The calcium tri-EDTA complex is fairly stable and it is likely to be able to reduce the flow of the benzalkonium chloride due to its presence at the hydrogel surface (when cut in cross section a white layer is noted at the surface, below which the section is transparent). It was noted

(chapter 4, section 4.5) that as the white complex began to clear the hydrogel began to foul and thus this theory is likely. An additional factor in this reduced release rate is the fact that the previously mentioned lone pairs present on the methacrylate portion of the hydrogel will bind with the benzalkonium chloride as the calcium ions would not compete for these sites due to the presence of the tri-EDTA.

5.2.3 Chlorhexidine

In chapter 2, the experimental section, it was reported that chlorhexidine gluconate was not useful in the marine environment, despite its antimicrobial action, as it formed the insoluble salt chlorhexidine dihydrochloride at pH 8. The presence of this salt made the surface rough and thus prone to fouling.

5.3 Why Antimicrobials Fail

In this section a few common reasons are given as to why antimicrobials fail. It is important to accept that no one mechanism is likely to prevent marine fouling indefinitely and it is control and an extended lifetime that is the anticipated outcome of research such as this.

There are many reasons why antimicrobials fail and when consideration is given to the challenging environment in to which these materials are introduced it is not surprising that eventually they are unable to stop the proliferation of bacteria, etc. However, control for extended periods of time can be achieved if the correct antimicrobial agent is chosen.

Problems occur for a variety of reasons. The most common causes of antimicrobial failure are due to :

pH, which may prevent the active species from being useful e.g. prevent ionisation.

The concentration of the antimicrobial being too weak to interact with the outer layers of the cell.

The formation of biofilms contribute greatly to the resistance of populations.

Organisms eventually adapt to resist attack i.e. build an immunity.

5.4 Antimicrobial and Surfactant Actions

In chapter 2 details of the properties of the antimicrobial agents tested are given but it is probably worth mentioning again the two properties which make these particular materials unique in their actions as it has been suggested that no one mechanism will prevent biofouling [3]. These properties are antimicrobial and surface activity. From the marine fouling results it can be seen that the ability to be surface-active enables quaternary ammonium compounds to be fairly successful in preventing fouling as the long alkyl chains of the DMHTB-75 demonstrated. In order for this mechanism to be understood, further studies of the material would have to be undertaken where the microbial build-up was monitored microscopically. The literature already provides a lot of information on antimicrobial action but very little of this information looks at the marine application and so comparative studies for the shorter chains i.e. C₁₂ and C₁₄ benzalkonium chloride would give a clearer picture as to why these materials are successful as marine anti-foulers.

The surface active properties have been given little attention in the past, however, a recent publication by Landa et al [4] has suggested that the surfactant action of mouth washes plays a more important role in plaque removal than the antimicrobial action.

The use of benzalkonium chloride for cleaning soft contact lenses has been thoroughly investigated. However, again little attention has been paid to its surface-active properties as most of the work has been in the antimicrobial area. Studies have, however, looked at the irreversible binding of quaternary ammonium compounds such as benzalkonium chloride to contact lenses when used as cleaning agents. Walters et al [1] suggested that surface active ability induced the adherence of benzalkonium chloride to hydrogel like material. Obviously a clearer understanding of the benzalkonium chloride hydrogel

combination would be required in order to state that the surfactant role is more crucial than the antimicrobial role in an anti-fouling role, although the marine trials do suggest that the surface activity of the materials have been successful.

5.5 Micelle Formation

The formation of micelles has been dealt with in chapter 2 section 2.6. This physical ability associated with quaternary ammonium compounds will most certainly have an effect on their release rate. As a micelle is much larger than an ion it can be assumed that it will have greater difficulty releasing from hydrogel than a single ion would. The formation of such clusters of ions therefore make the release of quaternary ammonium compounds slower than for their ionised counterparts such as hypochlorites.

5.6 Methods of Analysis

The HPLC method developed for the analysis of the homologues of benzalkonium chloride has been shown to be robust, fast and cost effective (Chapter 3, section 3.2) and therefore useful in this application.

5.7 Surface Energy

The effects of surface energy on attachment have long been investigated. One of the most widely accepted uses of such a phenomenon is the "non-stick" frying pan, which operates by coating the pan with a material that has a very low surface free energy. The application of low surface energy coatings to anti-fouling has been extensively investigated [5-7]. Research has found that these coatings are unable to maintain their surface characteristics due to the formation of a "conditioning film" [8]. Such a film is the fore-runner to biofilm formation (details of biofilm formation are given in chapter 1). This type of anti-fouling protection is therefore unable to prevent the small algal foulers which are pigmented and thus have a derogatory effect on light transmission. Its application for the protection of light sensors is therefore not practical. It should be noted that despite these problems non-stick type surfaces do not allow larger fouling to attach well and are easy to clean making them useful for the protection of larger underwater structures.

Initial investigative work into the surface energy of hydrogels has been carried out. Due to their hydrophilic nature these materials do not come into the category of low-energy surfaces. However, use of the "Cahn Balance" to determine surface energies of hydrogels containing benzalkonium chloride (Aldrich) and DMHTB-75 showed that although the total surface energies are similar, 55.20dynes/cm and 50.33dynes/cm respectively, the polar component of the benzalkonium chloride is greater than that for the DMHTB-75. Further work on surface energy measurements may help to explain the anti-fouling actions associated with these materials.

5.8 Final Conclusions

The outcome of all the aims within this research have been encouraging and so various strands will be furthered within GMTC. The relative ease of hydrogel production coupled with the ready availability of quaternary ammonium compounds such as benzalkonium chloride make this anti-fouling coating a favourable choice. The environmental restrictions also make this coating a good choice as legislation is reluctant to license new products and benzalkonium chloride is already an accepted antimicrobial.

The research has been run as part of other anti-fouling projects within GMTC. The application of loaded hydrogel to sensors is now being carried out as part of one of these projects and results so far have shown that such protection is suitable and is able to extend the lifetime of these sensors [9].

5.9 Chapter 5 - References

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