Marine biofouling - microbial adhesion to non-solid gel surfaces

by

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A thesis submitted in partial fulfillment of the requirements for the academic title

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SUMMARY

The scope of this work was to develop rapid assays for enumerating microorganisms on gels, and to test whether fouling of gel surfaces is principally different from that of solid substrata. For this purpose, a standard set of different gels were selected, based on biocompatibility, polymer charge and gel strength.

Bacterial adhesion to gels could be conveniently enumerated by first staining with SYBR^R Green I nucleic acid gel stain. Images were then collected using a confocal scanning laser microscope, followed by image analysis to determine the percent coverage of bacteria. Diatom adhesion could be quantified using a fluorescence scanner recording the fluorescent chlorophyll, showing a clear correlation between average fluorescence signals and cell density determined by counting. This method was successfully tested on glass, gels, a painted surface and an antifouling coated surface.

Adhesion of the marine bacterium *Pseudomonas* sp. NCIMB 2021 on gels decreased at higher shear rates. At low shear rates, adhesion varied significantly between different gels in the following descending order: alginate > agarose > chitosan > PVA-SbQ. Lowest cell coverage at all shear rates was recorded on the most hydrophobic gel, PVA-SbQ. Earlier work has shown that this organism adhere better to solid hydrophobic than solid hydrophilic surfaces. Thus, other properties than the surface free energy may be more important for bacterial adhesion to the gels.

The marine diatom *Amphora coffeaeformis* was applied in three different adhesion assays under different shear conditions. At high shear, cells adhered better to highly ionic polymer gels alginate and chitosan than to the low charge polymer gels agarose and PVA-SbQ. At very low shear, *A. coffeaeformis* developed a film even on agarose equivalent to that on the charged polymer gels. Adhesion to PVA-SbQ remained low at all shear rates. As observed for solid substrata, low charge density led to reduced attachment.

Settlement of *Balanus amphitrite* cypris larvae was tested at different polymer concentrations of the hydrogels. All gels inhibited cypris settlement compared to solid polystyrene controls. Gels consisting of 2.5% PVA-SbQ or 0.5% agarose showed the most promising antifouling properties. In all gel experiments, most of the non-settled larvae were able to settle when transferred and

offered a suitable solid substratum. Results indicated that the gel strength was an important factor for cyprid settlement on gels, while the surface wettability seemed to be of minor importance.

A few preliminary field experiments were carried out. These tests suggested that marine bacterial biofilm development is more readily on glass than on a PVA-SbQ gel surface, in accordance with monoculture lab experiments. However, similar amounts of photosynthetic organisms adhered to gels of agarose, alginate, chitosan and PVA-SbQ tested in an outdoor seawater basin during spring bloom. Finally, barnacle settlement was delayed on PVA-SbQ gels exposed in the open sea. After incubation for a full summer season, even those gels became as covered with marine fouling organisms as any other non-toxic surface.

In conclusion, no universal antifouling effects of hydrogels were found. However, this work suggests that both adhesion of a bacterium and settlement of barnacle cypris larvae on gel surfaces may be principally different from solid substrata. Diatom adhesion, on the other hand, was lower on gels with a low charge density, as observed for solid substrata. In general, the most hydrophobic gel, PVA-SbQ, was the least attractive surface for all three organisms.

LIST OF APPENDIX PAPERS

1. Rasmussen K, Vogelsang C, Østgaard K (1997) Biofilm formation on non-solid gel surfaces. In: Verachtert H, Verstraete W (eds) *International Symposium on Environmental Biotechnology, April 1997.* Technological Institute, Oostende, 393-395.

2. Rasmussen K, Østgaard K (2001) Marine biofouling: adhesion of the marine bacterium *Pseudomonas* sp. NCIMB 2021 to hydrogel surfaces. Submitted for publication in *Water Research.*

3. Rasmussen K, Østgaard K (2000) *In situ* autofluorescence detection of a fouling marine diatom on different surfaces. *Biofouling* **15**: 275-286.

4. Rasmussen K, Østgaard K (2001) Adhesion of the marine fouling diatom *Amphora coffeaeformis* to non-solid gel surfaces. *Biofouling* **17**: 103-115.

5. Rasmussen K, Willemsen P R, Østgaard K (2001) Barnacle settlement on gel surfaces. Revised version in prep. for *Biofouling.*

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LIST OF ABBREVIATIONS AND SYMBOLS

a	contact radius
ANOVA	Analysis of variance
ATP	Adenosine-tri-phosphate
BCl	Benzalkonium chloride
DAPI	4',6-diamidino-2-phenylindole
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DDT	1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane
E	Young's modulus
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(BETA-aminoethyl ether)-N,N'-tetraacetic acid
EPS	Extracellular polymeric substances
F_A	Fraction of acetylated units
G	α-L-guluronic acid
G'	Elastic- or storage modulus
G''	Viscous- or loss modulus
G_c	Critical fracture energy
HSL	Homoserine lactone
IMO	International Maritime Organization
KDO	3-deoxy-D-manno-octulosonic acid
LES	Low energy surface
M	β-D-mannuronic acid
MQ	Milli-Q water
n.d.	No data
OD_{660}	Optical density at 660 nm
P_c	Critical pull-off force
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PMMA	Polymethylmethacrylate
PS-A	Polysaccharide-A

PVA-SbQ	Polyvinyl alcohol substituted with stilbazoleum groups
t	thickness
TBT	Tributyltin
θ	Contact angle
ΔG	Gibbs free energy
δ	Phase angle
γ_0	(Shear) stress
γ_{lg}	Surface tension of a liquid in equilibrium with its saturated vapor
γ_{sg}	Surface tension of a solid in equilibrium with the saturated vapor
γ_{sl}	Interfacial tension between a solid and a liquid
γ_c	Critical surface free energy
σ_0	(Shear) strain
ν	Poisson ratio

1 INTRODUCTION

1.1 Marine biofouling

Biofouling is "the undesired deposition of microorganisms and the formation of biofilms" (Flemming *et al.*, 1996). Characklis and Marshall (1990) define a biofilm as "a surface accumulation, which is not necessarily uniform in time or space, consisting of cells immobilized at a substratum and frequently embedded in an organic polymer matrix of microbial origin" (see Figure 1.1). In addition, "a biofilm may be composed of a significant fraction of inorganic or abiotic substances held together by the biotic matrix."

Marine biofilms can for instance occlude fish-cage netting, which may result in oxygen deficiency or destruction of the cage by currents. Microbial induced corrosion of ships' hulls, sensors, oil rigs and other marine installations is another detrimental effect of biofilms. A layer of algal slime only 1 mm thick will increase hull friction by 80% and cause a 15% loss in ship speed (MER, 1996). According to Evans (2000), an unprotected ship which has an underwater surface area of 40 000 m^2 may gather 6000 tonnes of biological matter in less than six months. This may be a high estimate, at least for cold climates such as in Norway where the average seaweed

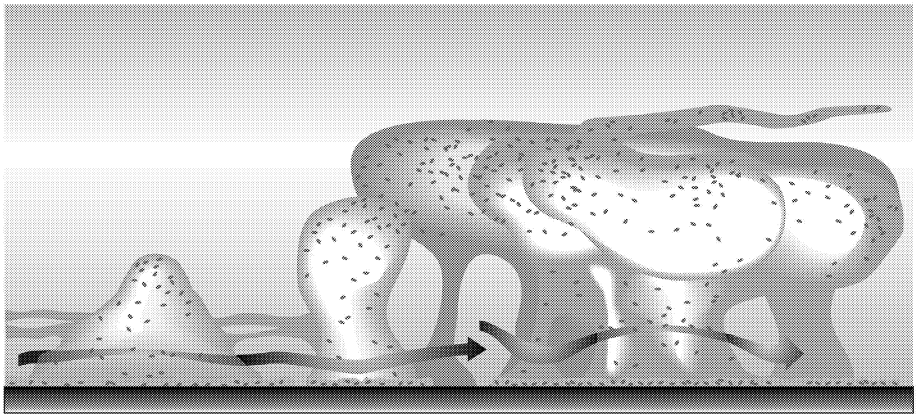

Figure 1.1 Biofilm clusters with cells immobilized in an organic matrix. Water flow through interstitial water channels is indicated by arrows (Center for Biofilm Engineering, Montana State University - Bozeman.).

biomass along the coast is 1kg m^{-2} (Jensen *et al.*, 2000). However, it is obvious that fouled ships may bring invading organisms to other parts of the world where they may outcompete native populations (Eno, 1997).

Prevention of fouling in general and biofouling in particular is called antifouling. It is believed that the ancient Phoenicians and Carthaginians used pitch and possibly copper sheeting on their ships' hulls (Laidlaw, 1952). Lead sheeted timber was found on a wreck of a Phoenician galley from about 700 BC discovered on the seabed off the coast of western Sicily (Lunn, 1974).

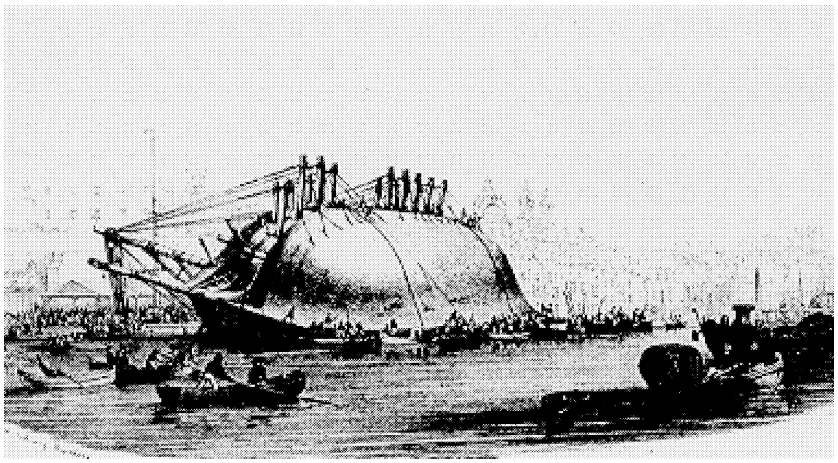

Figure 1.2 HMS *Formidable*, hauled down at Malta for removal of fouling, first half of the nineteenth century (Lunn, 1974).

The Greeks used tar or wax, and applied lead sheeting at least as early as the 3rd century BC (Laidlaw, 1952). In spite of its corrosive action on iron, lead sheeting was perhaps the material most frequently tried for protection of ship bottoms (Laidlaw, 1952). The antifouling properties of lead were poor, and it is therefore believed that it was applied to protect the wood against ship worms (Lunn, 1974). The first field experiment with copper sheeting as an antifoulant was on the H. M. S. Alarm, a 32-gun frigate, in 1758 (Laidlaw, 1952; Lunn, 1974). Copper was found to be an effective antifoulant. However, detrimental effects on iron in contact with copper were revealed. The galvanic oxidation of iron by copper required new antifouling paints when iron and steel boats were introduced in the late 18th century. Slow release paints became available after half a century, containing cuprous oxide, organo-merqury, lead and arsenical compounds, and

DDT (Evans, 2000). Being potentially harmful to both the environment and to human health, these compounds were replaced by the synthetic organotin tributyltin (TBT) in the 1960's (Bennett, 1996). Most major shipping companies applied TBT-based antifoulants and they were also widely used by small boat owners (Evans, 2000). However, the first paint formulations were costly to both the ship owners and the environment in that the release rates were very high. New paint had to be applied every one or two years. The major antifouling breakthrough came with the introduction of self-polishing copolymer paints. These paints provided for microbially clean and smooth surfaces for more than five years before dry-docking was necessary (Bosselman, 1996).

Detrimental effects of TBT-based antifouling paints appeared already in the mid-1970s in the *Crassostrea gigas* oyster farms of the Arcachon Bay, France (Alzieu, 1998). High levels of TBT resulted in failure to reproduce, and anomalies occurring in the shell calcification of adult oysters leading to stunted growth (see Figure 1.3). Serious effects were also observed in England where adult female dog-whelks of *Nucella Lapillus* developed imposex (Bryan *et al.*, 1987). Similar effects were observed all around the world, and it turned out that TBT had become a global contaminant (Ellis & Pattisina, 1990). See also an excellent review by Evans (2000).

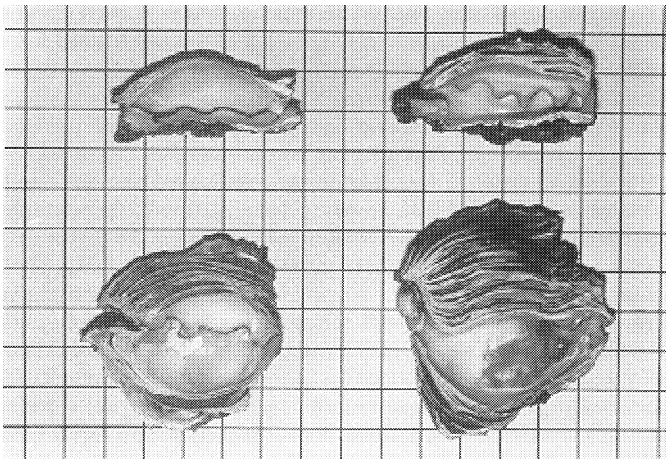

Figure 1.3 Examples of TBT induced shell thickening in Pacific oysters *Crassostrea gigas* from New Zealand (de Mora, 1996).

The International Maritime Organization (IMO) is set to adopt an international convention on the control of harmful anti-fouling systems at a conference to be held in October 2001. The convention comes in response to an IMO resolution of November 1999, which proposes a global prohibition on the application of organotin compounds on ships by 1 January 2003, and a prohibition on the presence of organotin compounds on ships by 1 January 2008.

On this background, a lot of research effort has been put in to finding alternative ways of preventing biofouling of ship hulls. The most promising technology is biocide-free fouling-release coatings based on silicone elastomers (Holm *et al.*, 2000; Brady & Singer, 2000; Ho *et al.*, 1993; Berglin & Gatenholm, 1999). However, these coatings perform best on high-speed vessels. At this time, no biocide-free alternative exists for slow-moving boats, that is with speeds less than 15 knots, including most ocean-going vessels (Berge & Walday, 1999).

1.2 Adhesion

Characklis and Marshall (1990) define adhesion as an interaction between a cell and a substratum which is more or less irreversible, frequently mediated by an adhesin such as an extracellular polymer. Literally no cells are irreversibly adsorbed to a surface, and the authors point out that it is often useful to refer to an assay technique for an operational definition of adhesion. They define attachment, on the other hand, as an interaction between bulk liquid components and biofilm components, implying that adhesion precedes attachment.

Mechanisms of bacterial biofilm development have been reviewed by Busscher and van der Mei (2000) see Figure 1.4. Within seconds after submerging a surface, macromolecules will be adsorbed to it and form a conditional film. Due to their smaller size, diffusional transport of macromolecules to a substratum is much faster than that of microorganisms. According to these authors, initial microbial adhesion is a result of non-specific attractive Lifshitz-van der Waals forces starting to act at several hundred nanometers from the surface. Electrostatic repulsive forces are operative at distances around 10-20 nm. Finally, at distances less than 5 nm, distinct features on the surfaces of the cell and substratum, such as specific adhesion receptors, become effective and facilitate strong adhesion.

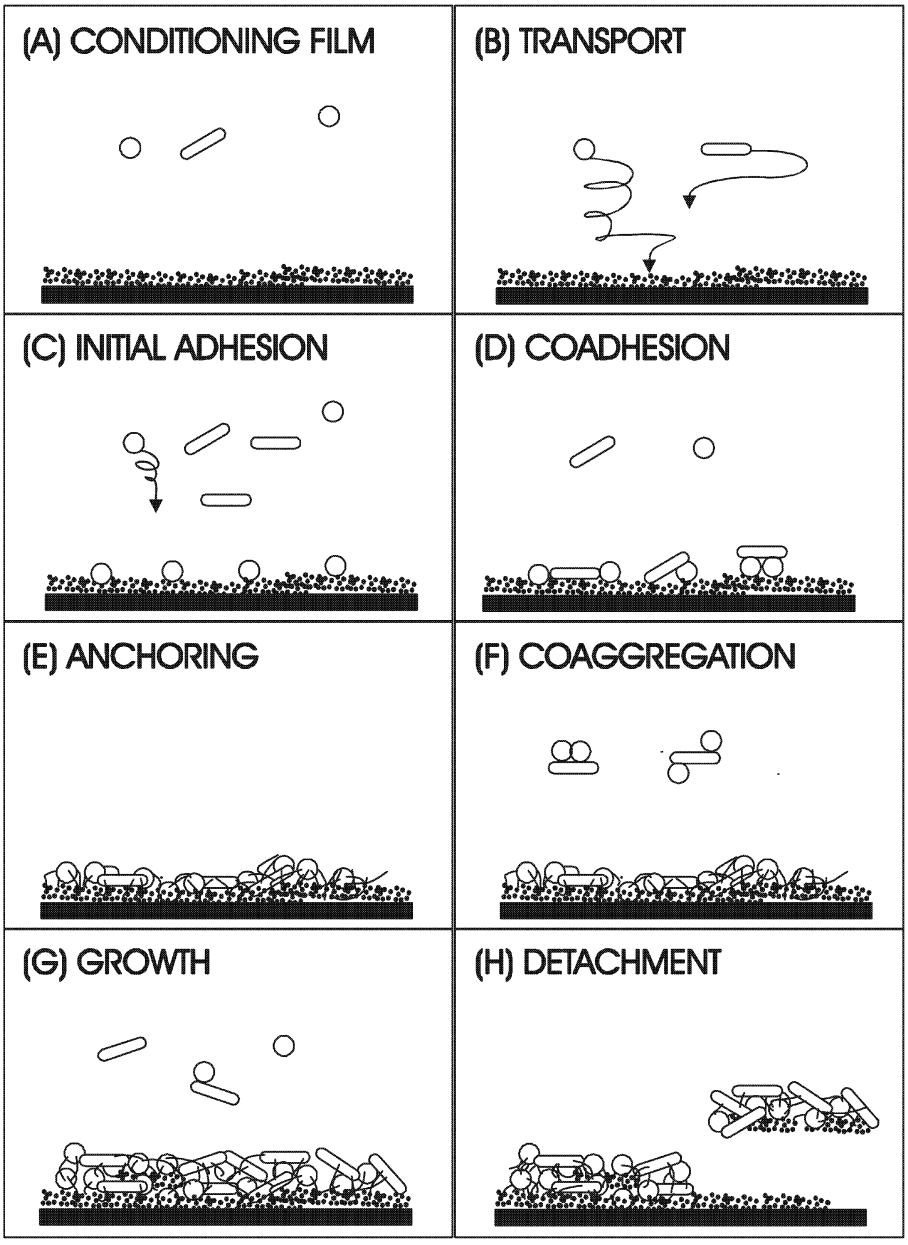

Figure 1.4 Sequential steps in biofilm formation. (A) Conditioning film formation; (B) microbial mass transport; (C) initial adhesion; (D) coadhesion between different strains; (E) anchoring of adhering organisms; (F) coaggregation between organisms in suspension; (G) growth of adhering organisms; (H) detachment of parts of the biofilm (redrawn from Busscher & van der Mei, 2000).

The initial adhesion is generally reversible, but it becomes less reversible within minutes due to the progressive removal of water from in-between the cell and the substratum. Initial adhesion is carried out by primary colonizers, secondary colonizers coadhere with organisms already adhering to the surface. Subsequently, growth is the main source of microbial accumulation in the biofilm. Naturally occurring shear forces control the thickness of the accumulating layer.

1.2.1 Adhesion from a thermodynamical point of view

The thermodynamic approach to describe cell adhesion is to assume that microbial cells behave like colloids. Liftshitz-van der Waals interactions, hydrophobic interactions, and electrostatic interactions are all, to a variable extent, involved when a cell adheres to a surface (Busscher *et al.*, 1990; Van Oss, 1991; Mayer *et al.*, 1999, Nielsen *et al.*, 2001). The condition allowing spontaneous adhesion, taking all of the above interactions into account, is (Van Oss, 1991):

$$\Delta G^{TOT} = \Delta G^{LW} + \Delta G^{AB} + \Delta G^{EL} < 0 \qquad (1.1)$$

where ΔG^{TOT} is the Gibbs free energy of adhesion, ΔG^{LW} is the energy of Liftshitz-van der Waals interactions, ΔG^{AB} is the energy of polar (electron-acceptor-electron-donor) or Lewis acid-base interactions, and ΔG^{EL} is the energy of electrostatic interactions. For a more detailed description see Van Oss (1994).

The Liftshitz-van der Waals interactions comprise three different phenomena (Van Oss, 1994): Keesom interactions which are randomly orienting dipole-dipole interactions; Debye interactions which are randomly orienting dipole-induced dipole interactions; and London interactions which are fluctuating dipole-induced dipole interactions. The resulting force may be attractive, zero or repulsive (Van Oss, 1994).

Polar (electron-acceptor-electron-donor) or Lewis acid-base interactions are based on electron-acceptor–electron-donor interactions between polar moieties in polar media (Van Oss, 1994). In aqueous media, the polar interactions mainly consist of interactions between hydrogen-donors and hydrogen-acceptors forming hydrogen bonds. These interactions include the attractive

hydrophobic interactions and the repulsive counterpart hydration pressure. In aqueous media it is the entropy of water, *i.e.* the reluctance of water molecules to be arranged in an ordered fashion around hydrophobic molecules, that stabilizes the hydrophobic interactions. Van Oss (1994) points out that the word "hydrophobic" is misleading since water is always attracted to a surface, even the most hydrophobic one. Israelachvili (1985) clarified the meaning of the word "hydrophobic" in the following way: "Originally coined to describe the water-hating properties of these substances. It is well to note that their interaction with water is actually attractive, due to the dispersion force, though the interaction of water itself is much more attractive. Water simply loves itself too much to let some substance get in its way."

Finally, electrostatic interactions occur between charged groups such as phosphate groups, carboxyl groups and amino groups (James, 1991).

1.2.2 Adhesion from a physiological point of view

Zobell (1943) was one of the pioneers in biofilm research. He showed that oxygen consumption increase with increased surface area in seawater due to bacterial growth on walls, the phenomenon that was later defined as a biofilm. He also demonstrated that in dilute nutrient solutions organic matter adsorbed on solid surfaces enhances bacterial activity. This is now referred to as a conditioning film. Earlier, Heukelekian and Heller (1940) observed that *Escherichia coli* failed to grow when the concentration of organic nutrients was less than 0.5 - 2.5 mg l^{-1} except in the proximity of solid surfaces. Another substantial finding by Zobell (1943) was the two successive steps in bacterial biofilm development; reversible initial adhesion followed by irreversible attachment.

The extracellular polymeric substances (EPS) are believed to play a vital role in most biofilm matrixes. The amount of EPS in biofilms may range from 50-90% of the total organic carbon (Bakke *et al.*, 1984). In some literature, the abbreviation EPS is also used for exopolysaccharide (Sutherland, 2001a,b; Allison, 1998). However, since other polymeric substances such as proteins, nucleic acids and lipids have been found as major extracellular constituents of biofilms, the former definition has been adopted by several groups (Flemming & Wingender, 2001;

Strathmann, 2001; Kreft & Wimpenny, 2001). In this text I will use "EPS" as an abbreviation for the mixture of polysaccharides, proteins, lipids, nucleic acids and other polymeric substances.

According to the reviews of Sutherland (2001a, b) bacterial polysaccharides may be neutral, polyanionic and less commonly polycationic. Negative charge may originate from uronic acids, inorganic substituents, pyruvate ketals or succinyl half-esters. The polysaccharides can have both hydrophilic and hydrophobic properties, and the phenotype of the polysaccharides can change in response to changes in the surrounding growth environment (Allison, 1998). Bacterial adhesion has been associated with subsequent increase in polysaccharide production for several strains. Sutherland (2001b) suggested that the observed increase in polysaccharide production might be a stress response. Excess carbon and limitations in nutrients such as nitrogen, potassium and phosphate can promote polysaccharide production. It has been shown that the increased polysaccharide production is part of a major change in gene expression. Selected mutants unable to synthesize polysaccharides were unable to form biofilms (Allison & Sutherland, 1987; Watnick & Kolter, 1999). On the other hand, cells unable to produce polysaccharides may still be part of a biofilm through coadhesion with primary colonizers (James *et al.*, 1995). Thus, the proportions of different polysaccharides in mixed biofilms do not necessarily reflect the proportions of the different bacteria present (Skillman *et al.*, 1999). Different polysaccharides may also contribute differently to the structure and properties of the resulting biofilm.

A general structural feature of many biofilm-associated polysaccharides is a high molecular weight, yielding highly viscous solutions. Some may also form weak gels through entanglement. Others may interact with ions to form networks yielding higher viscosity or even gelation. This adds mechanical stability to the film. Substituents may affect the polysaccharide conformation. Acetylated polymers of gellan and alginate form weak gels compared to the non-acetylated polymers (Chandrasekaran & Thailambal, 1990; Skjåk-Bræk *et al.*, 1989).

According to Flemming and Wingender (2001), proteins can constitute more than 30% of the EPS mass of a *Pseudomonas aeruginosa* biofilm. Interactions between polysaccharides and proteins may occur. According to Sutherland (2001a) both polysaccharases and proteases can affect adhesion or initiate detachment of bacteria. However, and fortunately, the presence of other

bacterial species and other macromolecules may block the effect of polymer degrading enzymes (Christensen *et al.*, 2001). As pointed out by Flemming and Wingender (2001): "The generation of a strain capable of degrading any given EPS matrix in a short time would lead to an environmental disaster because it would destroy natural microconsortia which perform a crucial role in the biogeochemical cycles of carbon, oxygen, nitrogen, sulphur and others".

The fact that bacteria may prefer to stick together has been known for a long time. Their ability to "talk" (see Figure 1.5), on the other hand, is a rather new discovery (Rumbaugh *et al.*, 2000). Cell-to-cell communication or quorum sensing is a cell-density-dependent bacterial intercellular signaling mechanism that enables bacteria to coordinate the expression of certain genes. Acylated homoserine lactones (HSL) are used for this purpose in Gram negative bacteria. Some of these molecules are able to diffuse through bacterial membranes, whilst others are actively transported. Inside a neighboring cell the HSL may interact with transcriptional activators and induce transcription of certain sets of genes. Davies *et al.* (1998) showed that cell-to-cell communication is involved in the development of *P. aeruginosa* biofilms. A mutant unable to produce a specific signaling molecule formed a flat and undifferentiated biofilm that was, in contrast to the wild-type, sensitive to sodium dodecyl sulphate. When a synthetic HSL was offered to the mutant the biofilms appeared normal (Davies *et al.*, 1998).

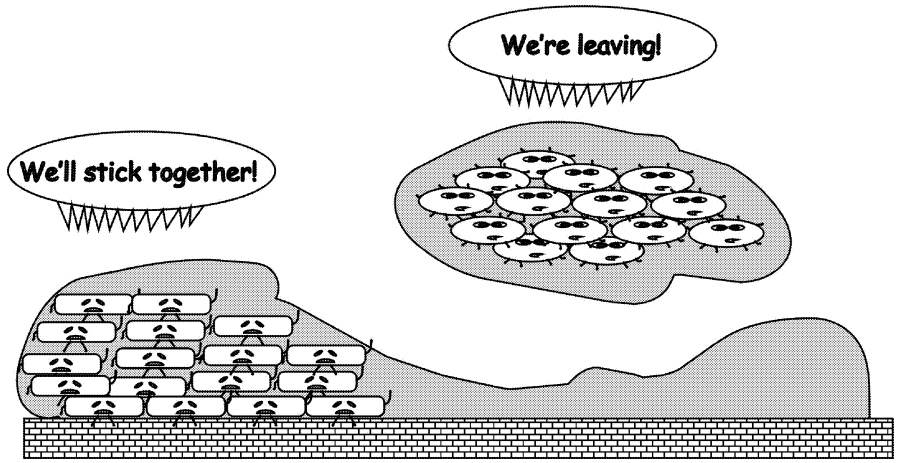

Figure 1.5 Cell to cell communication.

In conclusion, when considering bacteria as colloids (see section 1.2.1) one should always keep in mind that that they are not passive particles. A complete thermodynamic description of adhering microorganisms should include all ATP-driven reactions occurring inside the cells. Moreover, recent developments in the field of microbial behavior (Dusenbery, 1996) show that bacteria should rather be considered as "smart colloids", that is with the ability of choosing a physiological response to a sensed change in the environment.

1.2.3 Biofilm ecology

Natural biofilms consist of multiple species of bacteria. Living close together may be the question of life or death for some populations that supply each other's nutritional needs such as those comprising syntrophic consortia (Atlas & Bartha, 1998).

A large number of other organisms than bacteria are found in marine biofilms. Traditionally, marine fouling has been described as an ecological sequential process (Abarzua & Jakubowski, 1995; Lawrence *et al*, 1995; Little & DePalma, 1988), see Figure 1.6. Within seconds, a sub-

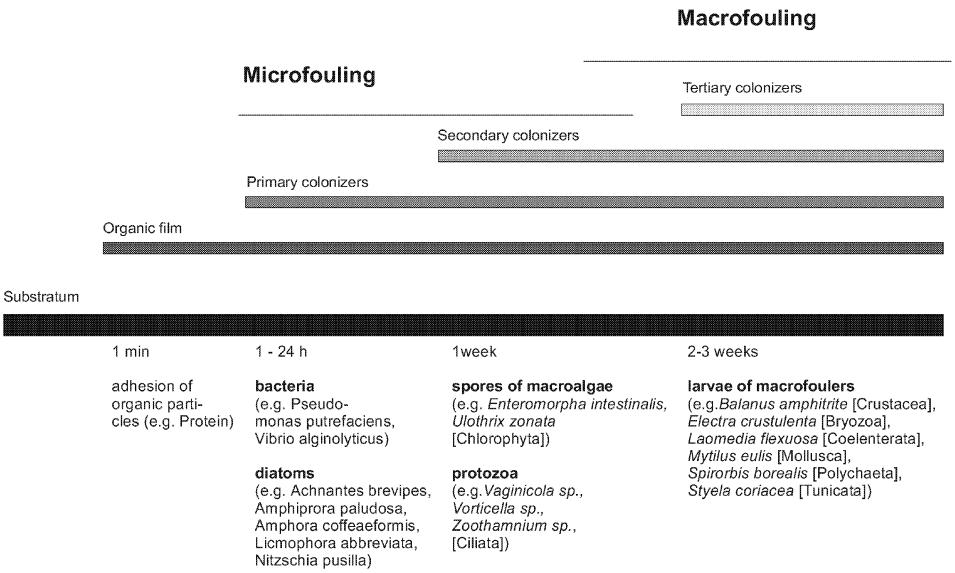

Figure 1.6 Temporal structure of settlement (redrawn from Abarzua & Jakubowski, 1995).

merged surface will be covered by a conditioning film consisting of organic polymers (Busscher & van der Mei, 2000; Loeb & Neihof, 1975; Baier *et al.*, 1968). During the first 24 hours, bacterial colonization will occur. Other microfoulers such as microalgae, fungi and protozoa may join the life in the biofilm the next couple of days (Little & Depalma, 1988). Finally, within a few weeks, barnacles, macroalgae and other macrofoulers will invade the surface (Abarzua & Jakubowski, 1995). It should be noted, as pointed out by Little (1984), that there is no evidence that microfouling is a prerequisite to subsequent settlement although it may in some instances be favorable (Maki *et al.*, 1988; Peterson & Stevenson, 1989; Neal *et al.*, 1996).

1.3 Model organisms

As pointed out in Section 1.2.3, most biofilms in general and marine biofilms in particular, may contain a large number of different organisms. According to Lunn (1974), the Woods Hole Oceanographic Institution has listed more than 600 species from the plant kingdom, and over 1400 of the animal kingdom which are all fouling organisms. Organisms of different kingdoms, divisions, phyla, species and strains may have distinct ways of adhesion, and thus, unequal affinity with respect to adhesion strength to various surfaces. Hence, when selecting model test organisms these should display dissimilar modes of adhesion, and be known to adhere well to submerged marine surfaces. Three different organisms applied in this study, one prokaryote, and a single- and a multiple celled eukaryote, are described below.

1.3.1 Pseudomonas sp. NCIMB 2021

Pseudomonas sp. NCIMB 2021 was isolated by Madilyn Fletcher in the Menai Straits, Anglesey, Wales, and Ruthenium red staining showed that the bacterium was surrounded by a primary acidic polysaccharide (Fletcher & Floodgate, 1973). Immobilized cells were surrounded by a secondary fibrous acidic polysaccharide. The extracellular polymeric substances (EPS) were isolated by Fletcher (1980) using ethanol precipitation. She reported that the EPS consisted of 50-80% protein and a carbohydrate fraction containing mannose, glucose, glucosamine, rhamnose, galactose and ribose, but no uronic acids. However, the presence of acidic groups in the carbohydrate fraction of this marine pseudomonad was confirmed by Christensen *et al.* (1985).

They found a protein fraction consisting of only 26%, and two carbohydrate fractions, PS(polysaccharide)-A and PS-B in the EPS of suspended *P.* sp. NCIMB 2021. PS-A consisted of glucose, galactose, glucuronic acid, and galacturonic acid, and it was mainly produced in the late exponential growth phase. PS-B, on the other hand, was only released in the very late growth phase and in the stationary phase. It consisted of N-acetyl glucosamine, an unidentified 6-deoxyhexose, and 3-deoxy-D-manno-octulosonic acid (KDO). They later extracted a cell-surface polysaccharide (PS-C) which contained PS-B and several other unidentified components (Christensen *et al.*, 1987). PS-C differed physically from PS-B in that it was less water-soluble and gave more viscous solutions. In addition, the ability to adhere to hydrophobic surfaces was correlated to the production of PS-C.

It is generally believed that the EPS play an important role in the adhesion of bacteria to surfaces. Numerous works have been done to explore the adhesion mechanisms of *P.* sp. NCIMB 2021. Fletcher (1977) showed that the adhesion rate to polystyrene petri dishes decreased from the exponential growth phase, through the stationary phase to the death phase. She explained the results by a higher proportion of motile cells in the log phase or possible changes in quality or quantity of cell surface polymers. Fletcher and Loeb (1979) compared the adhesion of the marine pseudomonad to hydrophobic and hydrophilic surfaces. Bacteria were most abundant on hydrophobic surfaces, and the number of attached cells decreased with the wettability of those surfaces. Thus, the surface charge seemed to be a crucial factor for adhesion to hydrophilic surfaces, where moderate numbers of cells attached to metals with a positive or neutral charge (platinum and germanium), and very few bacteria were detected on negatively charged surfaces such as glass, mica, and oxidized plastics.

Fletcher (1980b) concluded that bacteria have both active and passive attachment mechanisms. The conclusion was based on an experiment where inhibitors of energy production and protein synthesis inhibited attachment of *P.* sp. NCIMB 2021 to hydrophobic polystyrene petri dishes, while adhesion to more hydrophilic tissue culture petri dishes was unaffected. Furthermore, differing results were obtained when testing the effects of proteins, bacterial culture supernatant and EPS on the adhesion of the same bacterium to polystyrene petri dishes and tissue culture petri dishes (Fletcher & Marshall, 1982). The authors, therefore, suggested the existence of at least two possible modes of attachment, involving either two separate adhesive polymers or two adsorption

sites on the same polymer. The work of Wiencek and Fletcher (1997), applying self-assembled monolayers constructed from alkanethiols, also showed that *P.* sp. NCIMB 2021 adhered in greater abundance to hydrophobic than to hydrophilic surfaces. In addition, desorption of cells decreased with increasing surface hydrophobicity. The role of EPS in cell adhesion to surfaces was further substantiated by Kalaji and Neal (2000) who showed that capsular EPS from *P.* sp. NCIMB 2021 absorbed in greater amounts to a hydrophobic than to a hydrophilic surface. Gubner and Beech (2000) isolated EPS released into the culture medium as well as capsular- and biofilm associated EPS. All fractions had a positive effect on adhesion of the pseudomonad to one out of two stainless steel types tested.

Several research groups have tried to find ways of preventing *P.* sp. NCIMB 2021 from developing a biofilm. Humphries *et al.* (1986 and 1987a) showed that ethoxylated surfactants had anti-adhesive effects on hydrophobic but not on hydrophilic surfaces. It was suggested that the hydrophobic fraction of the molecule absorbed to the hydrophobic surface, and the hydrophilic chain pointing outwards provided a steric barrier that inhibited the adhesion of bacteria. Later, it was shown that the antiadhesion effect could also be obtained on hydrophilic surfaces by synthesizing surfactants with charged backbones (Humphries *et al.,* 1987b). Christensen *et al.* (1990) showed that biofilm removal could be accomplished by attacking the extracellular polymers with hydrogenperoxide and ferric ions, resulting in oxidative reductive depolymerization.

1.3.2 Amphora coffeaeformis

Diatoms are generally planktonic organisms (Round *et al.*, 1992). However, the diatom *Amphora coffeaeformis* is a common constituent of marine biofilms (Udhayakumar & Chongdar, 1998), and it can tolerate relatively high concentrations of toxins found in self-polishing paints (Robinson *et al.*, 1985). Since it is so widespread and resistant, *A. coffeaeformis* is a common problem as well as a common test organism in antifouling research (Becker, 1996; Beveridge *et al*, 1998; Callow & Finlay, 1995; Clarkson & Evans, 1995; Cooksey & Cooksey, 1986; Finlay & Callow, 1996; Thomas & Robinson, 1986; Wigglesworth-Cooksey & Cooksey, 1996).

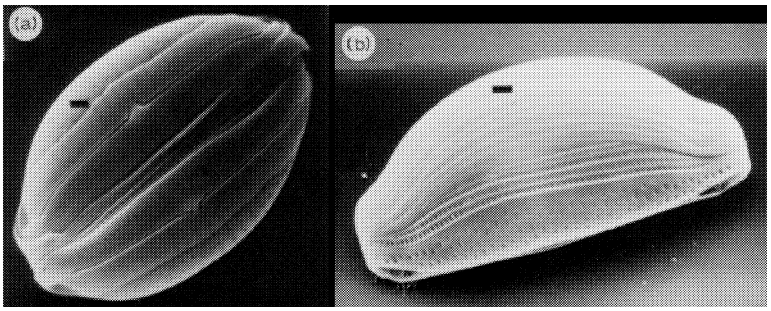

Figure 1.7 (A) SEM photomicrograph of cleaned frustrule of *A. coeffeaeformis.* Ventral view showing raphe openings. Bar = 1 μm. (B) *A. coeffeaeformis* adhered to a glass surface. Bar = 1 μm. (Cooksey & Cooksey, 1986).

Several research groups have studied the mechanisms for adhesion of diatoms to surfaces. Cooksey (1981) found that *A. coffeaeformis* required Ca^{2+} for adhesion and motility on glass. When Sr^{2+} was substituted by Ca^{2+}, the cells would adhere but motility was inhibited. This pointed to an external role of calcium, which was later confirmed by using EGTA (Cooksey & Cooksey, 1986). This Ca^{2+}-chelator resulted in detachment of *A. coffeaeformis*, leaving raphe imprints on the glass. It was suggested that Ca^{2+} could act externally as a bridge between negatively charged cells and substrata or as a stabilizing agent of external acidic polymers. From experiments applying various drugs and metabolic inhibitors, the same authors concluded that Ca^{2+} is needed both inside and outside the cell for adhesion, and that the process requires metabolic energy, protein and glycoprotein synthesis. Lind *et al.* (1997) raised monoclonal antibodies to cell surface proteoglycans of the diatom *Stauroneis decipiens.* One of the antibodies bound to proteoglycans on the cell surface, the raphe (see Figure 1.7), as well as the adhesive trails causing inhibition of adhesion and motility. A different antibody adhered only to proteoglycans on the outer surface of the frustrule and did not inhibit adhesion or motility. It was suggested that there existed two different pools of proteoglycans, one which was only present on the cell surface and a different one present on the raphe and trails as well as the cell surface. Diatom gliding can only occur after the cell has adhered to a surface, and the two processes are closely related. Edgar and Pickett-Heaps (1984) proposed a model for diatom locomotion in which actin is involved in mucilage secretion through the raphe. Webster *et al.* (1985) showed that antiactin drugs inhibited motility and thus, confirmed the presence of actin in *A. coffeaeformis.* Poulsen *et al.* (1999) produced further evidence for the involvement of actin in diatoms by exposing *Craspedostauros australis*

to various anti-actin drugs. In addition, they found that butanedione monoxime, a myosin inhibitor, inhibited gliding. Their hypothesis is that myosins are responsible for creating the gliding force by translocating along actin bundles.

The extracellular polymeric substances (EPS) of diatoms are essential for adhesion, and more information on the EPS composition could lead us closer to a way of preventing fouling. Bhosle *et al.* (1996) characterized the EPS from *A. coffeaeformis* var. *perpusilla* (Grunow) Cleve and found approximately 93% carbohydrate (including pyruvate and uronic acids) and 6% protein. Glucose was the most abundant monosaccharide. In addition, mannose and galactose was detected. Wustman *et al.* (1997) found that the capsule polysaccharides of *A. coffeaeformis* Ag. Kütz. (no. 2080) consisted of 40% glucose and almost equal amounts of glucuronic acid, galactose, mannose, xylose, fucose, and rhamnose.

The hydrodynamic shear can also be a crucial factor in biofouling of some surfaces. Peterson and Stevenson (1989) compared diatom adhesion to ceramic tiles. They used clean tiles and tiles covered by a bacterial biofilm in slow and fast current regime. Diatom adhesion to clean tiles in the fast current was 10 times lower than in slow, whereas this ratio for the conditioned tiles was only 3. Becker (1996) determined the adhesion strength and EPS production of *A. coffeaeformis* on test surfaces with different surface tensions in a radial flow reactor. The attachment strength increased with time for all surfaces except glass, and it was weaker on substrata with surface tensions between 20-25 mN m^{-1}. EPS production was not higher on materials that initially provided weak adhesion. Becker (1998) repeated the detachment experiments with the same test surfaces exposed in the field. Again, diatoms increased their attachment strength to all surfaces with time. After 6 hours and 2 days, the lowest retention rates were observed on substrata with surface tensions between 20-25 mN m^{-1}. After 8 days, there was no significant difference in attachment strength.

1.3.3 Balanus amphitrite

Barnacle cypris larvae, as the one shown in Figure 1.8, are common test organisms in antifouling research. Barnacle cypris settlement is influenced by a number of environmental factors such as

the hexose concentration (Neal & Yule, 1996), cyprid age and storage temperature (Rittschof *et al.*, 1984; Satuito *et al.*, 1996), and microtexture (Berntsson *et al.*, 2000) to name a few. For detailed reviews, see Crisp (1984) and Walker (1995).

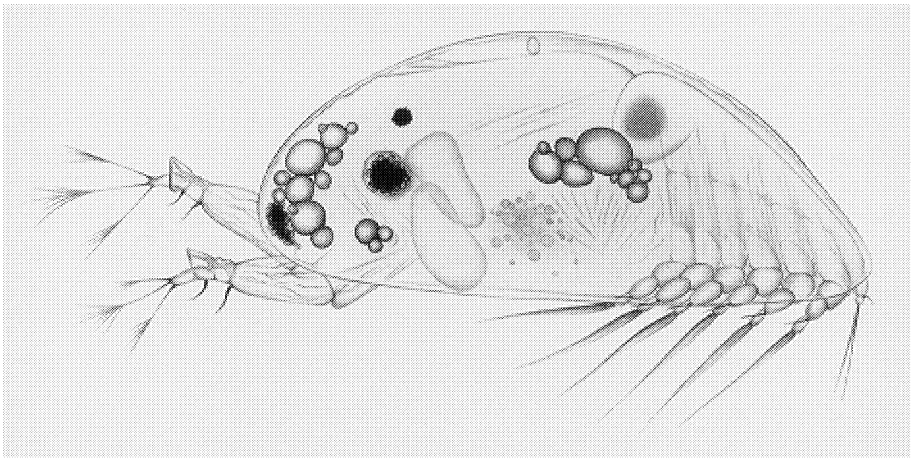

Figure 1.8 A drawing of a cyprid larvae of *Balanus improvisus* (Dahlström *et al.*, 2000).

The surface energy may affect the adhesion strength of barnacles (Swain *et al.*, 1998). Berglin and Gatenholm (1999) compared the antifouling properties of polymethylmethacrylate (PMMA) and polydimethylsiloxane (PDMS) surfaces. A PDMS surface has a low glass transition temperature resulting in flexible polymer chains and a low surface free energy, both contributing to good fouling-release properties. In addition, it was suggested that uncrosslinked PDMS chains on the surface also prevented firm adhesion of *B. improvisus*. Holm *et al.* (2000) compared the performance of three different silicone fouling-release coatings. Rate of accumulation of fouling varied on the three surfaces, as well as removal stress of *B. amphitrite*.

There are always several factors present affecting settlement, and one or several of these may be dominated by others. In addition, different barnacle species may respond in different ways to various cues or external interacting factors such as microbial films (Holmström *et al.*, 1992; Avelin Mary *et al.*, 1993; Keough & Raimondi, 1995; Wieczorek *et al.*, 1995; O'Connor & Richardson, 1998; Lau & Qian, 2000). Maki *et al.* (1992) showed that a bacterial film of *Delaya*

marina increased the surface wettability, but inhibited attachment of *B. amphitrite* cypris larvae. They later showed that there was no correlation between surface wettability and temporary adhesion strength to unfilmed polystyrene, tissue-culture polystyrene or glass (Maki *et al.*, 1994). A hypothesis based on this work was that *D. marina* inhibited fixation of *B. amphitrite* through cyprid chemoreceptors. Bacterial films may stimulate, be neutral or inhibit adhesion of barnacle cypris larvae (Maki *et al.*, 1988; Neal *et al.*, 1996). Other properties than the bacterial species may determine the effect of a biofilm on barnacle settlement. Neal *et al.* (1996) showed that both settlement and settlement behavior of *Eliminus modestus* Darwin cypris larvae were higher on a biofilm developed at high shear compared to a low shear biofilm and a biologically clean glass surface.

Thompson *et al.* (1998) discussed the difficulties of performing lab experiments to solve problems in the field. They report that both traces and presence of adult barnacles of *Semibalanus balanoides* were more important for cyprid settlement than the presence of biofilms on rocks in the field. In lab experiments, the presence of adult barnacles showed no effect at all. However, larvae preferred to settle on filmed surfaces rather than on newly exposed rocks. On the other hand, Qian *et al.* (2000) obtained similar results in the field and laboratory when they tested the effect of flow and surface characteristics on the attachment of barnacle, bryozoan and polychaete larva. The organisms from the three taxa revealed different settlement optima with respect to flow velocity, and also preference to different surface characteristics. Olivier *et al.* (2000) performed field experiments with PMMA panels. *B. amphitrite* cyprid settlement decreased with biofilm age and density. Finally, Raghukumar *et al.* (2000) showed that the fungoid protists, thraustochytrids, appearing on submerged surfaces within 24 hours may have a significant effect on settlement of barnacles. Metamorphosis of *B. amphitrite* was higher in the presence of the thraustochytrids than on both arthropodin-coated surfaces and polystyrene.

1.4 Gel surfaces

Hydrogels are viscoelastic polymer networks dominated by elastic rather than viscous properties, with the ability to bind large amounts of water. On macroscale, these gels may be recognized as surfaces, since particles and molecules larger than the pore size cannot penetrate the gel surface.

However, small molecules may diffuse in and out of the gel matrix. Biofilms are often described as gel-like structures. On microscale, biofilms and hydrogels have obvious differences. Biofilms may contain microcolonies consisting of densely packed bacteria, in this context solid surfaces, with individual diameters in the order of 1 μm (10 000 Å). Hydrogels, on the other hand, are open networks with pore diameters in the range of 50-1500 Å, as observed for Ca-alginate gels (Andresen *et al.,* 1977). In addition, biofilms are both compositionally heterogeneous with respect to macromolecules and structurally heterogeneous in that they may contain voids and water channels (Stoodley *et al.*, 1994).

1.4.1 Gel classification

Smidsrød and Moe (1995) classify polymer networks in the three following groups based on mechanical strength and type of crossbonds; (1) covalent, (2) strong physical, and (3) weak physical. Chromatography matrices are typically formed by chemically crosslinking of the polymer chains resulting in stable gels belonging to the first group (Moe, 1992). Other examples are gels of chitosan crosslinked with glutaraldehyde and PVA-SbQ gels described in Section 1.5. Strong physical networks are formed when alginate is crosslinked with *e.g.* calcium ions (See Section 1.5.3). However, these gels may easily be disintegrated by a sequestrant such as EDTA. Entanglement gels are weak gels typically formed when very long polysaccharide strands are present in high concentrations such as in a bacterial biofilm. These gels may dissolve upon dilution.

1.4.2 Gel properties

Rheological properties of gels include viscoelastic properties and mechanical strength (Mitchell, 1980). A viscoelastic material is a material whose properties are neither purely elastic nor purely viscous, but rather a combination of the two. Mechanical spectroscopy is applied to determine the viscoelastic nature of a sample such as a gel or a viscous solution. The material is subjected to small and reversible oscillatory deformations while the strain response is recorded. Elastic and

viscous contributions can be quantified as the elastic- or storage modulus, G', and the viscous- or loss modulus G" (Ferry, 1980):

$$G' = \frac{\sigma_0}{\gamma_0}\cos\delta \tag{1.2}$$

$$G'' = \frac{\sigma_0}{\gamma_0}\sin\delta \tag{1.3}$$

where γ_0 and σ_0 are the amplitudes of the applied stress and recorded strain, respectively, and δ is the angular delay in response or the phase angle. The phase angle, δ = arctan(G"/G'), gives the ratio between the viscous and the elastic character of the sample. A phase angle of 90°, *i.e.* G"≫G', corresponds to a liquid dominated by viscous properties, whereas a phase angle of 0°, *i.e.* G'≫G", corresponds to an ideal elastic material. The transition from the liquid to the gel state occurs at a phase angle of 45°, *i.e.* when G" = G' (Smidsrød & Moe, 1995).

Mechanical strength of gels can be measured by applying a stress and record the resulting strain:

$$Stress = \frac{force}{area} = \frac{F}{A} \tag{1.4}$$

$$Strain = \frac{change\ in\ length}{original\ length} = \frac{\Delta L}{L_0} \tag{1.5}$$

The ratio between the stress and strain is called Young's modulus, E, and its value depends only on the material (Giancoli, 1989):

$$E = \frac{Stress}{Strain} = \frac{F/A}{\Delta L/L_0} \tag{1.6}$$

Thus, the gel strength can be determined by calculating Young's modulus from the initial slope of the force versus deformation curve (Smidsrød *et al.*, 1972).

The surface free energy has been defined as the potential energy from unsatisfied bonds of molecules present at the surface of a bulk solid (Fletcher, 1980a; Israelachvili, 1992). Several types of forces may contribute to the free energy of a surface, and these include dispersion,

dipole, electrostatic and metallic forces. When a liquid is adsorbed onto a solid, the bonding potential of both phases can only to some extent be satisfied, the remaining unsatisfied bonding potential is known as the interfacial tension or interfacial free energy (Fletcher, 1980a). This interfacial tension can affect adhesion of both dissolved components and microorganisms onto the surface.

The surface free energy can be determined by measuring contact angles (θ) of a series of liquids with a range of surface tensions (Zisman, 1964). When a drop of liquid is placed on a solid surface (see Figure 1.9) and the surface tension of the liquid is larger than the surface tension of the solid, it makes a definite angle, the contact angle (θ), between the liquid and solid phases (Chan, 1994).

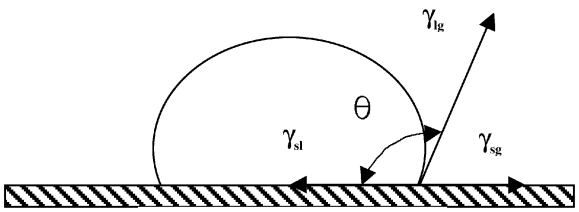

Figure 1.9 A drop of liquid on a surface, illustrating the definition of the contact angle θ.

When the same liquid is placed on surfaces of increasing surface tension, the contact angle decreases until total wetting ($\theta \leq 0$) occurs. Young's equation describes the force balance at the three-phase boundary (Chan, 1994):

$$\gamma_{lg} \cos\theta = \gamma_{sg} - \gamma_{sl} \tag{1.7}$$

where γ_{lg} is the surface tension of the liquid in equilibrium with its saturated vapor, γ_{sg} is the surface tension of the solid in equilibrium with the saturated vapor, γ_{sl} is the interfacial tension between the solid and the liquid (Chan, 1994).

1.5 Model gels

Both surface charge and surface free energy have shown to affect fouling of solid surfaces (Section 1.3). Model gels with different surface charge and hydrophobicity were, therefore, selected. The biocompatibility of the gels was also a crucial factor in the selection process. In order to reveal possible interactions between the fouling organisms and the polymer network, toxic substances could not be present. Gels made of agarose (neutral), modified polyvinyl alcohol PVA-SbQ (very low cationic), alginate (highly anionic) and chitosan (highly cationic) were chosen.

1.5.1 Agarose

Agarose consists of repeating units of 4-linked 3,6-anhydro-α-L-galactose and 3-linked β-D-galactose. Accordingly, this polymer gel should be free of charge. When a hot polymer solution is cooled down, double helixes are formed which will associate to create microcrystalline junction zones as illustrated in Figure 1.10 (te Nijenhuis, 1997). Agarose gels were made by autoclaving a solution of agarose (Sigma Chemicals Co., USA) in distilled water for 20 minutes and subsequent cooling in a water bath at 45°C.

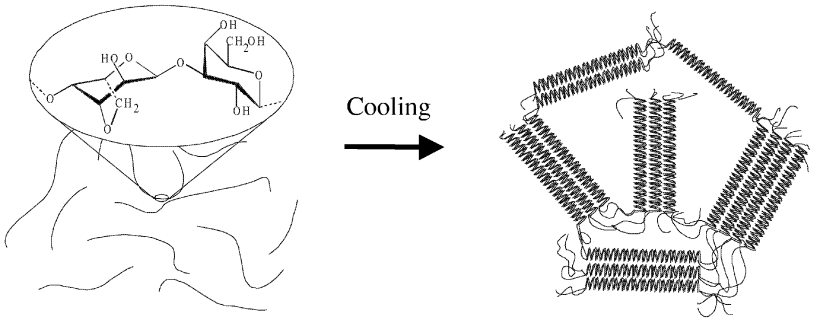

Figure 1.10 Formation of an agarose gel (redrawn from te Nijenhuis, 1997).

1.5.2 PVA-SbQ

The polyvinyl alcohol (PVA-SbQ, Toyo Gosei Kogyo Co., ltd., Japan) used contained PVA with a polymerization degree of 1700, an 88 % degree of saponification and 1.3 mole-% stilbazoleum groups introduced as photosensitive functional sites. The polymer has a low positive charge, which originates from the stilbazoleum groups, see Figure 1.11. A stock solution was diluted using Milli Q (MQ) water to obtain the desired concentration. Gelling of the polymer solutions occurred under bright white light, which initiated formation of a cyclobutane ring between two SbQ units (Ichimura & Watanabe, 1982).

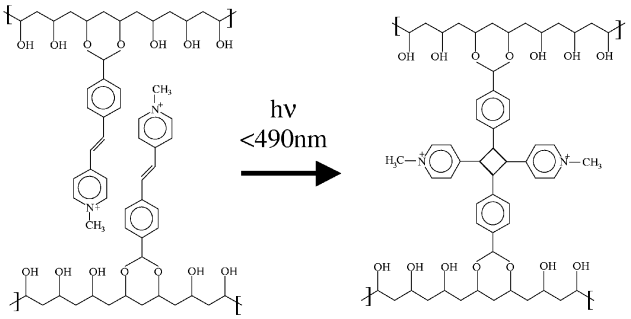

Figure 1.11 Crosslinking of PVA-SbQ chains (redrawn from Vogelsang, 1999).

1.5.3 Ca-alginate

Alginate extracted from brown algae is a binary copolymer of 1-4 linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) arranged in homopolymeric G- and M-blocs of different lengths as well as heteropolymeric MG sequences (Moe *et al.*, 1995). A carboxyl group on each monomer gives a high negative charge of this polymer. Calcium ions enables gel formation by selective and cooperative ionic interaction between different G-blocks along the alginate chains, as illustrated in Figure 1.12. Ca-alginate gels were prepared by controlled dissociation of $CaCO_3$ by glucono-δ-lactone in an alginate (LF10/60, Pronova Biopolymer A/S, Norway) solution (Draget *et al.*, 1990).

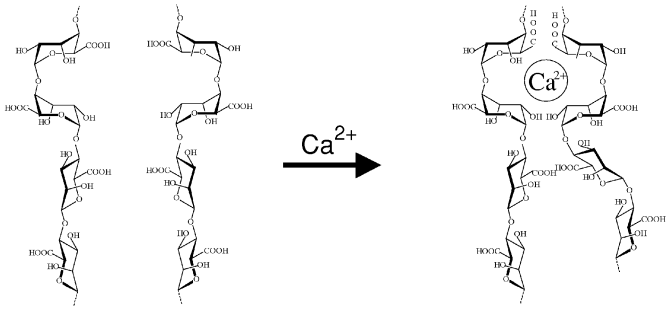

Figure 1.12 Crosslinking of alginate strands with calcium ions (redrawn from Vogelsang, 1999).

1.5.4 Chitosan

Chitosan is completely- or partially deacetylated chitin, and thus consists of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. The aminogroups provide the positive charge of this polymer at acidic to neutral pH. As shown in Figure 1.13, two chitosan chains can be linked through the Schiff's base reaction with glutaraldehyde. Chitosan gels (1%) were prepared by the method described by Draget (1996). Briefly, the gels were prepared by mixing 20 grams of a chitosan solution (F_A 0.17, Pronova Biopolymer A/S, Norway) with 3 ml of a 1 M MES-buffer (pH 6), and then finally 7 ml of a 5 mM glutaraldehyde solution were added.

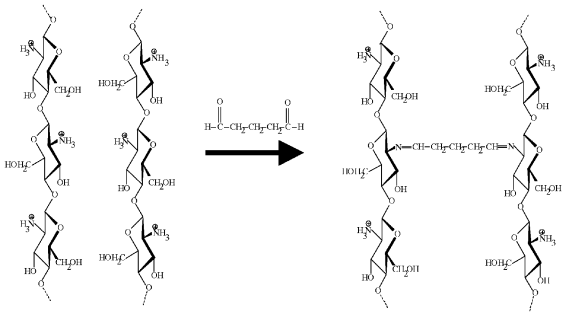

Figure 1.13 Crosslinking of chitosan with glutaraldehyde (redrawn from Smidsrød & Moe, 1995).

1.6 Biofouling of gels

As illustrated in Section 1.3, biofouling of solid surfaces has been studied intensively. Less attention has been given to non-solid surfaces such as hydrogels, consisting of 90-99 % water.

It has already been shown that hydrogels may be suitable for incorporating bioactive materials. Gatenholm *et al.* (1995) immobilized a marine bacterium in a polyacrylamide gel. The bacteria produced a chemical substance that leached out of the gel and was lethal to *B. amphitrite* cypris larvae. Henrikson and Pawlik (1998) incorporated extracts from four different marine organisms into gellan gels. Gels containing extracts from the sponge *Aplysilla longispina* were effective against fouling organisms. However, there was no significant difference in fouling of control gels containing methanol instead of extract compared to that of plexiglass.

Cook *et al.* (1993) found that the amount of *Pseudomonas aeruginosa* adhering to poly-hydroxyethyl methacrylate based hydrogels decreased with increasing water content of the gel. His *et al.* (1996) found no toxic effects on embryonic *Crassostrea gigas* oysters from benzalkonium chloride (BCl) applied in a PHEMA hydrogel. This antifouling system remained visually free of microfouling from 12 weeks up to 5 months in the field (Cowling *et al.*, 2000). Increasing the water content of the basic hydrogel (without BCl) did not improve the performance. It was suggested that the dual action of BCl as an antimicrobial agent and as a surfactant modifying the surface properties of the hydrogel contributed to the antifouling properties of the system.

An apparent distinction between a gel and a solid surface is the softness or mechanical strength. Recently, Brady and Singer (2000) stressed the importance of softness for the performance of fouling-release coatings. These authors used a fracture mechanics approach to describe removal of fouling organisms from such coatings. The mathematical model derived by Kendall (1971) describes the force needed to pull off a rigid body glued to a film of elastic glue on a rigid substrate (Brady & Singer, 2000):

$$P_c = \pi a^2 \sqrt{\frac{2G_c E}{3t(1-2\nu)}} \tag{1.8}$$

where P_c is the critical pull-off force, a is the contact radius, G_c is the critical fracture energy per unit area, E is the Young's modulus of the glue, t is the thickness of the glue, and ν is the Poisson's ratio. G_c is proportional to the critical surface free energy, γ_c. According to this model, the pull off force is a function of the softness (E) of the elastic film as well as the film thickness (t) in addition to the surface energy (γ_c). Brady and Singer (2000) applied Kendall's model on the relative amount of bioadhesion taken as a function of the critical surface free energy of a polymer surface according to Baier and DePalma (1971). They found a good correlation between bioadhesion and the square root of the product between the Young's modulus and critical surface free energy. Minimum adhesion coincided with the lowest elastic modulus, which did not correspond to the lowest surface energy tested (Brady & Singer, 2000).

1.7 Scope

The general scope of this work can be divided in two parts; (1) to develop suitable test assays for detection and quantification of microorganisms on gels (Chapter 2), and (2) to test if the non-solid nature of hydrogels represents a selective factor of relevance for the development of marine antifouling systems (Chapter 3).

Experimental aspects

The aim was to develop rapid test assays for enumerating bacteria and diatoms on gels. The open network of a hydrogel containing up to 99% water pose several constrictions to a biofilm detection assay. Conventional stains such as crystal violet and methylene blue will stain the gel. Gel turbidity may prohibit conventional light microscopy. Fluorescent stains such as DAPI and acridine orange may also diffuse into the gel and thereby enhance background levels. In addition, light scattering and autofluorescence of some gels may also contribute to higher background levels. It was therefore important to map such effects, and then determine how they could be avoided or minimized.

It should be emphasized that testing of the antifouling properties of the gels has received the highest priority. Rapid screening tests require a large number of different test surfaces and short

incubations. Thus, biochemical tests were excluded due to small sample surfaces and low amount of adhered material. In addition, the nature of the gels may impede biochemical assays by *e.g.* absorbing the analyte.

Adhesion studies

The main aim of this work was to test whether fouling of gel surfaces is principally different from that of solid substrata. If so, they could represent an additional selective factor in biofouling ecology. Fouling of three different organisms; one prokaryote, and a single- and a multiple celled eukaryote was studied. In order to reveal possible interactions with the polymeric gel network, cationic, anionic as well as neutral gelling substances were included. Restricting the study to biocompatible gel formation systems, alginate (highly anionic), chitosan (highly cationic), modified polyvinyl alcohol PVA-SbQ (very low cationic) and agarose (neutral) were chosen for the test program.

2 EXPERIMENTAL ASPECTS

In order to reveal the essential features of adhesion to the gel structures it was necessary to characterize some properties of the model gels presented in Section 1.5. Furthermore, as pointed out in the Scope (Section 1.7), gels have properties different from a solid surface that may complicate the detection of attached organisms such as bacteria and diatoms. This chapter will therefore focus on the gel characteristics as well as the establishment of assays for quantifying bacteria and diatoms on gel surfaces. Data discussed in this chapter is collected from several of the Appendix Papers in addition to some previously unpublished results.

2.1 Gel properties

2.1.1 Mechanical strength

The stiffness of gel materials may be measured and expressed by Young's modulus, that is the ratio between applied stress and resulting strain described in Section 1.4.2.

In Appendix Paper 5, Young's moduli for different concentrations of agarose, PVA-SbQ, alginate and chitosan gels have been presented. These data are shown in Figure 2.1. The stiffness of the gels increased with increasing polymer content as expected. Comparing gels with 2% polymer content reveals that Young's modulus of agarose was approximately 1, 2 and 3 orders of magnitude higher than the moduli of alginate, chitosan, and PVA-SbQ, respectively. PVA-SbQ stands out as the softest gel. For comparison, Young's modulus of the common fouling-release material polydimethylsiloxane is $2x10^6$ Nm^{-2} (Brady & Singer, 2000).

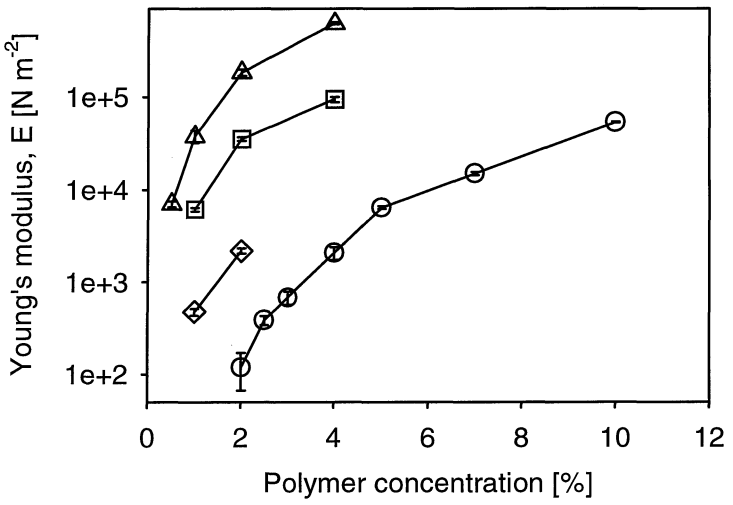

Figure 2.1 Young's moduli [N m^{-2}] calculated from the initial slope of the force-deformation curve for agarose (△), PVA-SbQ (○), alginate (□), and chitosan (◇) gels. Error bars represent the 95% confidence intervals (Appendix Paper 5).

2.1.2 Surface hydrophobicity

Water contact angles may indicate the hydrophobicity of a surface: A hydrophobic surface is recognized by a large contact angle with water and has, therefore, a low surface free energy as described in Section 1.4.2. On high-energy surfaces such as metals, a water droplet will collapse and total wetting occurs.

Water contact angles were measured on gels consisting of different concentrations of agarose, PVA-SbQ, alginate and chitosan (Appendix Paper 5). These data are presented in Figure 2.2. The wettability of PVA-SbQ and chitosan gels decreased with increasing polymer content. The opposite effect was observed for agarose gels. Water contact angles on 1, 2, and 4% alginate gels were approximately 20°, indicating highly wettable gels, opposed to PVA-SbQ gels with contact angles between 80 and 100°, clearly indicating more hydrophobic surfaces.

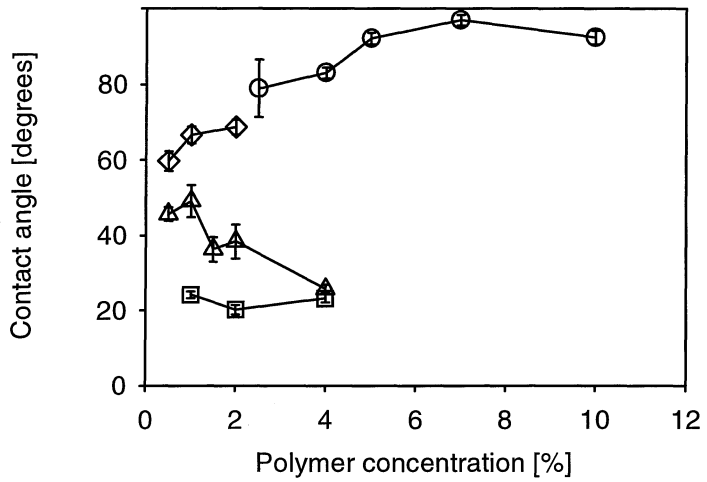

Figure 2.2 Static water contact angles measured on gels of agarose (△), PVA-SbQ (○), alginate (□), and chitosan (◇). Error bars represent the 95% confidence intervals (Appendix Paper 5).

2.1.3 Viscoelasticity

The physical transition between a liquid- and gel state may be defined by the state when the loss- and storage moduli G" and G', are equal implying a phase angle of 45°, as explained in Section 1.4.2. As shown in Figure 2.1, PVA-SbQ formed very soft gels, and at low concentrations it may be difficult to decide whether it is a gel or a viscous liquid. The loss- and storage moduli of different concentrations of PVA-SbQ gels were measured on a StressTech Rheometer from Reologica, Lund, Sweden. Measurements were carried out at 20°C on a 40 mm serrated plate/plate geometry with 1 mm gap. Previously unpublished phase angles for the PVA-SbQ gels recorded at different frequencies are presented in Figure 2.3. Generally the phase angle decreased with increasing polymer content. Polymer solutions of 1.5% or less will apparently not form gels.

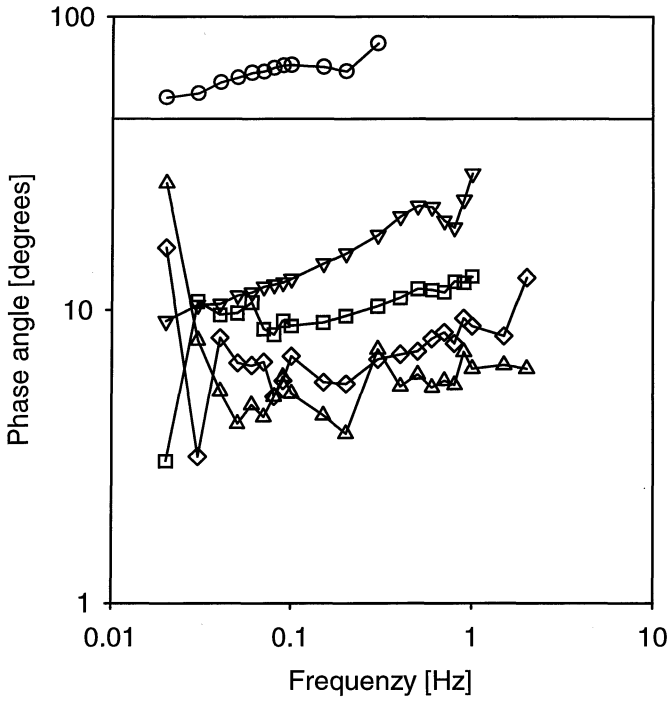

Figure 2.3 Phase angles of 1.5% (O), 1.6% (∇), 1.7% (□), 1.8% (◇) and 1.9% (△) PVA-SbQ. Solid horizontal line indicate a phase angle of 45° (unpublished results).

The 1.6% gel was close to the transition, and those of 1.7% or more were clearly dominated by elastic properties.

2.2 Leaching of soluble compounds

Biocompatible gel systems were pursued in this study to avoid disturbances of adhesion by toxic substances. According to Gåserød (1998), reports on antimicrobial effects of chitosan exist. However, the polymers of agarose, alginate, and chitosan can be considered as non-toxic. The synthetic polymer PVA-SbQ has been applied by others for gel entrapment of different microbes and cells. It has been shown that plant cells, microalgae and bacteria were not inhibited by immobilization inside the PVA-SbQ gel matrices (Nakajima *et al.*, 1986; Hertzberg *et al.*, 1995;

Vogelsang *et al.*, 1999). Glutaraldehyde, on the other hand, the crosslinking agent in chitosan gels, is a well-known toxic compound. Chitosan gels were, therefore, thoroughly washed with PBS before incubation with the test organisms.

Settlement assays with *B. amphitrite* cyprid larvae required a more extensive testing of potential leachate from the gels. It has been reported that excess calcium ions, used for alginate crosslinking, may inhibit *B. amphitrite* cyprid settlement (Clare, 1996; Rittschof *et al.*, 1986). In addition, unlinked low molecular weight polymer chains could leach out of the gels and possibly affect settlement. As described in Appendix Paper 5, a prescreening of potential toxic effects of leaching water from the gels was carried out with both *B. amphitrite* cypris larvae and naupliar larvae of the brine shrimp *Artemia salina.* The results of this test are presented in Table 2.1.

Table 2.1 Prescreening of inhibitory or toxic effects of leaching water from non-treated agarose, PVA-SbQ, alginate and chitosan gels on (A) *B. amphitrite* settlement and (B) *A. salina* mortality. C.I. = 95% confidence interval; P-value = P-value resulting from Dunnett's test (ANOVA) with polystyrene as control (Appendix Paper 5).

A *B. amphitrite*	Gel type	Settlement inhibition [%]	C.I.	ANOVA, *p*-value
	Control	0	15	
	Agarose	39	23	0.0531
	PVA-SbQ	100	0	< 0.0005
	Alginate	2	31	1.00
	Chitosan	59	25	0.00200
B *A. salina*	Gel type	Mortality [%]	95% C.I.	ANOVA, *p*-value
	Control	11	8	
	Agarose	14	6	0.975
	PVA-SbQ	7	6	0.866
	Alginate	10	7	1.00
	Chitosan	11	8	1.00

None of the leaching water samples showed any effect on *A. salina* mortality (Table 2.1B). Analysis of variance revealed significant effect of leaching water from both PVA-SbQ ($p < 0.00050$) and chitosan gels ($p = 0.0020$) on settlement of *B. amphitrite* cypris larvae (Table 2.1A).

No significant effects were detected for agarose or alginate gels. Further testing was carried out to determine if the polymers in solution would inhibit barnacle settlement, and if so, at which concentrations, see Appendix Paper 5. In the case of PVA-SbQ, both a fresh polymer solution and leachate from a PVA-SbQ gel were tested to see if non-reacted light sensitive groups would give a higher degree of inhibition. Settlement inhibition of *B. amphitrite* cypris larvae by diluted solutions of PVA-SbQ, alginate and chitosan are shown in Figure 2.4.

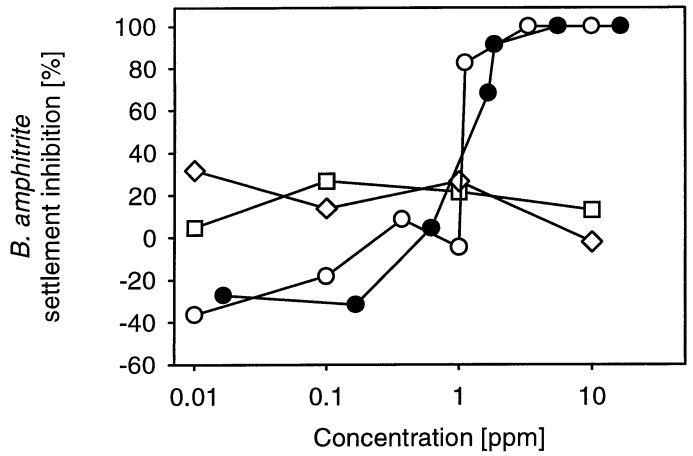

Figure 2.4 Settlement inhibition [%] of *B. amphitrite* cypris larvae in different concentrations of PVA-SbQ polymer solutions (○), leachate from PVA-SbQ polymer gel (●), alginate solutions (□) and chitosan solutions (◇) (Appendix Paper 5).

Based on these data, the 24 hour EC_{50} (± 95% confidence interval) for PVA-SbQ leaching water and diluted PVA-SbQ polymer were determined to be 1.1 ± 0.9 and 0.70 ± 0.50 ppm, respectively (Appendix Paper 5). This shows that the molecules leaching out of the PVA-SbQ gel had the same effect as the diluted polymer with un-reacted stilbazoleum groups. Clearly, the concentration of PVA-SbQ should not exceed 0.4 ppm for *B. amphitrite* settlement tests to avoid inhibitory effects. Analysis of variance indicated no significant effect of the diluted alginate or chitosan solutions. Thus, the effect of leaching water from chitosan gels (Table 2.1A) was most likely due to excess glutaraldehyde. In conclusion, both PVA-SbQ and chitosan gels should be rinsed thoroughly and leaching water should be tested before conducting settlement experiments.

2.3 Gel surface stability

Physico-chemical properties of the media such as pH and ionic strength may affect the gel structures. In addition, when working in open systems bacterial contamination may occur. The influences of conditioning films and bacterial contamination were not directly investigated, but such effects cannot be excluded. Possible interference with the gel surface structures is discussed below.

Chitosan is a positively charged polymer at neutral pH. However, at the pH of seawater the amino groups are discharged and the gel matrix may even collapse. This did not have any dramatic effects on the gels used in the bacterial adhesion experiments where the pH of the artificial medium ranged from 6.9 to 7.2 (Appendix Paper 2). The medium used for diatom adhesion experiments was based on natural seawater with a pH of typically 8.2, and a clear contraction of the gel matrix on microscope slides was observed, see Appendix Paper 4. Natural seawater was also used in the barnacle settlement experiments. Cracks appeared in some of the 1% and 2% gels, and the volumes of all chitosan gels were clearly diminished, see Appendix Paper 5.

No visual changes in gel structure were observed on agarose, PVA-SbQ or Ca-alginate gels used in bacterial and diatom adhesion experiments. There were no visual damages to or changes of the agarose gels used in barnacle adhesion assays (Appendix Paper 5). In the wells with PVA-SbQ, a white precipitate attaching to larvae and barnacles was observed from day 5 on 10% gels and to a less extent on 7% gels. It was absent on 4 and 2.5% gels. On alginate gels, a white precipitate appeared from day 3, also covering the front and back of the cypris larvae, and on the top of the juvenile barnacles. At the end of the experiment, the gels appeared slimy, as if they were approaching a viscous rather than a gel state. The disintegration of alginate gels could possibly affect settlement through immobilization by polymers sticking to the larvae, making settlement more difficult (Appendix Paper 5). It is possible that alginate lyases of *e.g.* bacterial origin caused the degradation of these gels (Sutherland, 1995). Another possibility is that the cyprids themselves produced alginate-degrading enzymes. On chitosan gels, a white precipitate was observed on day 3. The amount was higher on 2% gels than on 0.5% gels (Appendix Paper 5).

As pointed out in Section 1.2.3, adsorption of a conditioning film consisting of natural polymers is regarded as the first step in the colonization of a submerged surface (Busscher & van der Mei, 2000; Loeb & Neihof, 1975; Baier *et al.*, 1968). This may modify the wettability of the surface (Dexter, 1978). Polymers were not present in the media used for bacteria or diatom adhesion (Appendix Papers 2, 3 and 4). However, some excretion products of these organisms may have been present. The natural seawater used in experiments with barnacles may have contained polymers in addition to possible polymers originated from the cypris larvae (Appendix Paper 5). Since only a monomolecular layer is necessary to change the physico-chemical surface properties of a material, the effect of a conditioning film cannot be excluded in any of the experiments.

The adhesion experiments with bacteria and diatoms lasted in general less than 24 hours, and contaminating organisms should be of minor disturbance (Appendix Papers 2, 3 and 4). Maximum incubation time for the barnacle cypris larvae was 8 days, and microbial contamination could not be avoided during incubation of larvae (Appendix Paper 5). Interactions between microbial biofilms and barnacle settlement are complex and not easily predetermined (Maki *et al.*, 1992, 1994; Neal *et al.*, 1996; Olivier *et al.*, 2000; Thompson *et al.*, 1998). This is clear from the work of Maki *et al.* (1988), who tested the effect of 18 bacterial species on *B. amphitrite* settlement. Most of the bacteria either inhibited or were neutral, and only one species stimulated settlement. However, in the work presented in Appendix Paper 5 bacteria would also have been present in the controls where settlement was clearly uninhibited.

2.4 Quantification of attached bacteria

2.4.1 Conventional methods

There are numerous methods and procedures for detecting bacteria attached to a surface (Fletcher, 1990; Gerhardt *et al.*, 1994). Direct counting can be carried out using a light microscope after staining the bacteria with basic dyes such as crystal violet or methylene blue. Crystal violet stained bacteria attached to a transparent glass slide can also be quantified by measuring the absorbance through the colonized surface (Fletcher, 1976). Commonly used fluorescent stains interacting with bacterial RNA or DNA are acridine orange and DAPI (4',6-

diamidino-2-phenylindole). Propidium iodide is another fluorescent stain that will interact with nucleic acids, however, it will only enter dead cells with compromised membranes (Swope *et al.*, 1995). Propidium iodide used in combination with fluorescent stains targeting both viable and dead cells, such as FITC (fluorescein isothiocyanate), or only viable cells, such as CTC (5-cyano-2,3-ditolyl tetrazoleum chloride) (Rodriguez *et al.*, 1992), allows determination of the ratio between living and dead cells.

According to Fletcher (1990), electron microscopy can give information about the mechanism of adhesion, since structures such as fimbriae, flagella or polymeric adhesives may be resolved. Bacterial species- or strain-specific methods include fluorescent labeled antibodies and nucleic acid probes labeled either with radioisotopes or fluorescent dyes (Fletcher, 1990; Amann, 1995). Cells can also be prelabeled with a radiotracer such as ^{14}C-leucine before the adhesion assay (Pringle & Fletcher, 1983). The number of adhered cells can then be determined by scintillation counting.

A number of cell constituents can be extracted from adhered cells and quantified as biochemical markers of the biomass. According to Fletcher (1990), cell constituents such as ATP, DNA, lipopolysaccharide (LPS), lipid phosphate, lipid A, teichoic acids, and muramic acid have been used as a measure of bacterial biomass. Alternatively, adhered cells can simply be scraped off a surface, suspended in a buffer, and counted under a microscope after staining with *e.g.* acridine orange.

Microelectrodes can be used to measure a range of constituents as well as fluid flow within a biofilm (Rasmussen & Lewandowski, 1998). Vrany *et al.* (1997) used attenuated total reflection Fourier transform infrared spectroscopy (ATR/FT-IR) to study the transport of antimicrobial agents into bacterial biofilms.

2.4.2 Selecting an applicable method

The following criteria were the most important when selecting the method for quantification of bacteria on gels: (1) duration - the method should be rapid and allow testing a multitude of

surfaces in the same experiment; (2) sensitivity - bacteria should be detectable in low numbers after short periods of incubation; (3) compatibility - the method should be applicable to the convenient size and shape of test slides.

The number, size and nature of the test surfaces will generally limit the applicable methods for bacterial quantification. Methods applying biochemical markers such as ATP and DNA, or scraping of the biomass, require relatively large test surfaces or thick biofilms (Fletcher, 1990). In the case of hydrogels, biochemical markers may be absorbed in the gel matrix during extraction instead of diffusing into the liquid phase. Hydrogels will be completely stained by *e.g.* crystal violet and methylene blue. Fluorescent stains such as DAPI, acridine orange and FITC may also be absorbed in the gel matrix, creating high background levels that will make fluorescent microscopy or spectrophotometry unsuitable. Tests showed that background levels due to DAPI differed in magnitude between the four model gels (Rasmussen, 1998b). In addition, the stain was more easily removed from some gels than others. Furthermore, autofluorescence of PVA-SbQ gels caused an even higher background level when using stains such as DAPI, with absorption maximum at 360 nm and emission maximum at 460 nm. This is illustrated in Figure 2.5 showing emission scans of a PVA-SbQ gel exposed to en excitation beam of 360 nm (unpublished results).

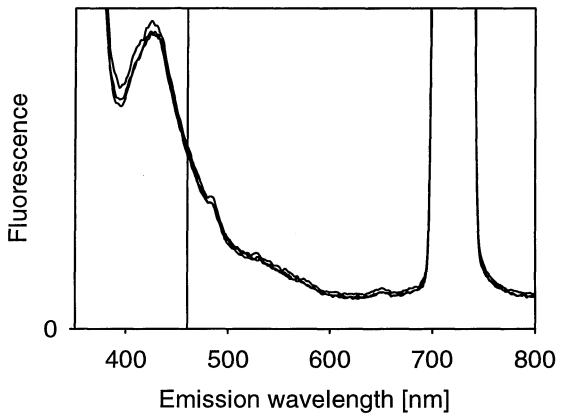

Figure 2.5 Emission scans recorded in three locations on a PVA-SbQ gel. The excitation wavelength was 360 nm. The solid vertical line indicate the emission wavelength for DAPI at 460 nm (unpublished results).

A lot of effort has been put into finding a suitable fluorescent stain (Rasmussen, 1998 a, b). Briefly, the first method tested was carried out by staining the adhered cells with DAPI and measure the fluorescence in a few locations using a fluorescence spectrometer with a front surface accessory, see Appendix Paper 1. The method was later improved by installing a plate reader accessory (see Appendix Paper 3) which allowed collecting data from selected arrays of sampling points, and thus reveal the distribution of cells on the surface. However, due to problems with light scattering, autofluorescence and stain absorption, the method was not found sensitive enough for comparing bacterial adhesion to different hydrogel test surfaces.

The final solution was to apply fluorescence microscopy and image analysis as described in Appendix Paper 2. A number of different stains were tested. The most suitable was SYBR^{R} Green I nucleic acid gel stain, which has a low fluorescence level when it is not associated to nucleic acids. In addition, this fluorophore is excited by light of 490 nm, which is less energetic than that of DAPI causing high background or PVA-SbQ autofluorescence levels. The quantification process was carried out by collecting several images from each test slide, and determining the average coverage using an image analysis software as described in Appendix Paper 2.

2.5 Quantification of attached diatoms

Butterwick *et al.* (1982) compared eight methods for estimating suspended algal biomass. The methods were based on (1) visual and electronic cell counting; (2) optical properties *in vivo* of scattering, attenuance and fluorescence; and (3) chemical estimations of reducing capacity and solvent-extracted chlorophyll *a*.

Methods for quantifying attached diatoms include direct counting through a light microscope (Buzzelli *et al*, 1997; Cooksey, 1981; Cooksey & Cooksey, 1988; Garduno *et al*, 1996). Evans' blue can be used to distinguish between living and dead cells (Clarkson & Evans, 1995). The chlorophyll *a* can be extracted from adhered cells and determined fluorimetrically (Cooksey, 1981). Others have used image analysis for monitoring diatoms (Wigglesworth-Cooksey & Cooksey, 1996; Beveridge *et al.*, 1998; Clarkson *et al.*, 1999).

Butterwick *et al.* (1982) concluded that visual cell counts was the best method for quantifying suspended algae due to low detection limits, small sample volumes, and assessment of cell condition. However, for large experimental series and screening tests, cell counting becomes impractical. For the purpose of studying adhesion of *A. coffeaeformis* to the model gel surfaces, a method based on recording the *in vivo* chlorophyll autofluorescence *in situ* was developed and tested.

This method has been described in detail in Appendix Paper 3. Briefly, a microscope slide based test surface with attached *A. coffeaeformis* was placed in a sample tray. Filtered seawater with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to enhance the fluorescence signal. DCMU blocks the energy flow through the photosynthesis apparatus resulting in an increased amount of energy emitted as fluorescence (Hipkins & Baker, 1986). The fluorescence was then recorded in an array containing 7 x 25 measuring points using a fluorescence spectrometer equipped with a plate reader accessory. Both clean microscope slides and slides covered with PVA-SbQ gels were tested. Background levels caused by light scattering and autofluorescence had to be determined for the test surfaces. A standard average background signal was determined for clean glass surfaces. In the case of glass covered with gels, however, the background intensity varied clearly between different gel samples. Because of this variation, the background had to be determined for each sample. Subsequent to enumeration, diatoms were therefore removed with a spatula and the emission was measured again in each of the 175 points. These intensities were averaged and used as a background value for subtraction. Microscopical investigations verified that no cells could be detected on or inside the gels after this treatment.

Direct cell counts were performed on both glass and gel samples after fluorescence measurements to test the method (Appendix Paper 3). In Figure 2.6, *in situ* data from adhesion and growth experiments on glass and PVA-SbQ gels are used to plot the average fluorescence signal for each biofilm sample versus the areal cell density determined by counting. There is a clear correlation between recorded fluorescence and visual cell counts (Figure 2.6). A plot of the fluorescence of a solution versus concentration of the emitting species should be linear at low concentrations (Skoog *et al.*, 1992). At higher concentrations, linearity is lost due to self-quenching. This phenomenon occurs when molecules in the solution reabsorb fluorescence emitted by other molecules. In addition, when the concentration is high, the excitation light intensity may become

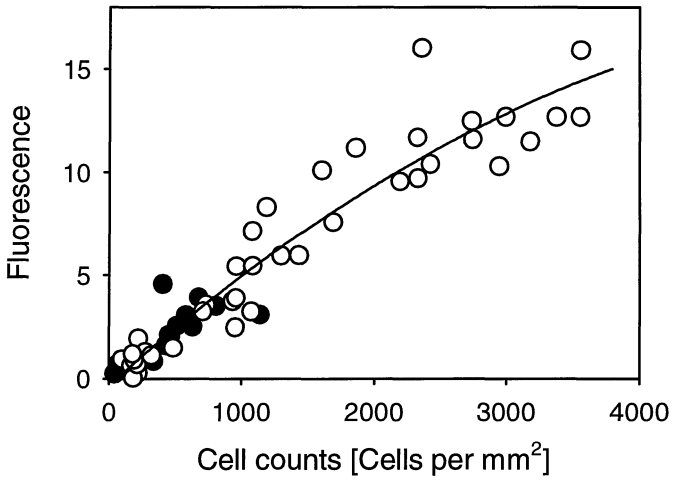

Figure 2.6 Data from growth and adhesion experiments of *A. coffeaeformis* on glass (O) and PVA-SbQ gel (●) where the average fluorescence is plotted versus the concentration of cells [cells per mm^2] determined by visual counts (Appendix Paper 3).

limiting to parts of the sample far away from the light source. It is evident from Figure 2.6 that such quenching occur when fluorescence is recorded from diatoms attached to a surface. When the diatom biofilm is dense, emitted fluorescence may be reabsorbed by other pigments resulting in a lower fluorescence to cell number ratio. A second order polynomial equation was fit to the data (Appendix Paper 3). The second order component of the equation is considered significant when its value exceeds 10% of the total. At this limit, linearity between fluorescence intensity and cell concentration is lost. According to the resulting equation, this happened at a concentration of 1100 cells per mm^2, corresponding to an average fluorescence intensity of 5.4. The detection limit for *A. coffeaeformis* on glass and PVA-SbQ gels was estimated to be approximately 200 cells mm^{-2}, see Appendix Paper 3.

The method can also be applied to other non-transparent surfaces as illustrated in Figure 2.7, where the spatial distribution of *A. coffeaeformis* was recorded on surfaces covered with a black enamel paint and an antifouling paint in addition to a PVA-SbQ gel and a glass slide (Appendix Paper 3). Compared to PVA-SbQ gel (Figure 2.7d) and black paint (Figure 2.7b), the adhesion on glass (Figure 2.7a) was evenly distributed. On PVA-SbQ gel, the majority of the adhered cells were found near the edges. As expected, the antifouling paint leaked toxic material that killed the

inoculum, and hence, no biomass could be detected on these surfaces (Figure 2.7c). Finally, on black enamel painted surfaces, high densities of diatoms were generally observed on the whole surface, with a more scattered distribution than on glass.

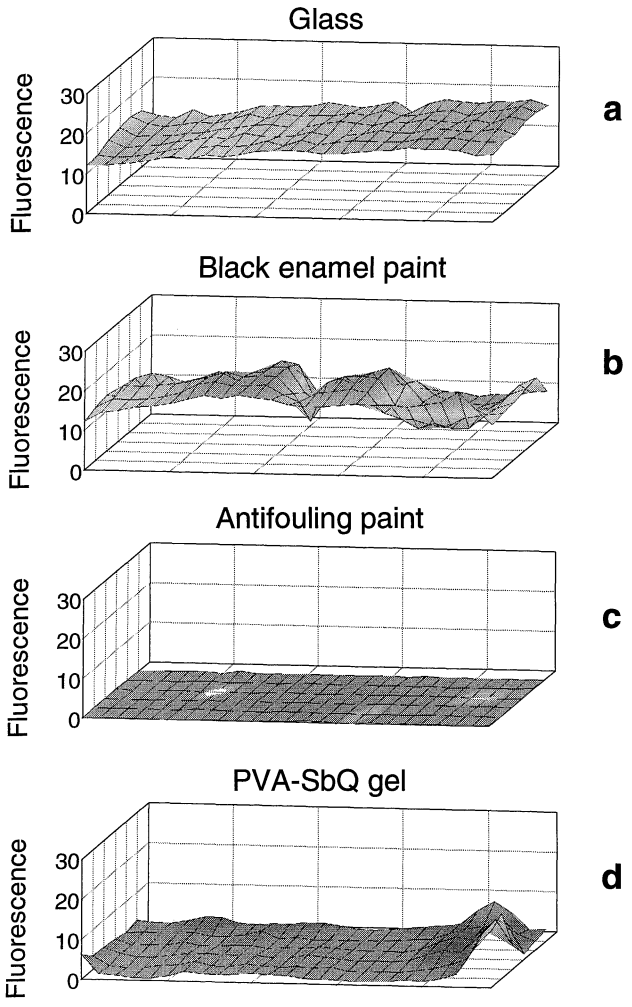

Figure 2.7 Fluorescence signals of *A. coffeaeformis* adhered to clean glass (a), black enamel paint (b), antifouling paint (c) and PVA-SbQ gel (d) after correction for respective background signals (Appendix Paper 3).

3 ADHESION STUDIES

3.1 Bacteria

As discussed in Section 1.2.3, bacteria are typically the first colonizers of a surface in the marine environment. A large number of bacteria show a greater tendency to adhere to hydrophobic surfaces rather than hydrophilic (Fletcher & Loeb, 1979; Otto *et al.*, 1999; Doyle, 2000). Hence, bacterial films are readily developed on hydrophobic fouling-release antifouling coatings (D. Williams, International Coatings, personal communication). As discussed in section 1.6, Brady and Singer (2000) showed that properties such as Young's modulus *i.e.* the material softness may be important for this new generation of antifouling coatings. This section will summarize the results obtained on adhesion of the model organism, *Pseudomonas* sp. NCIMB 2021 to the model gels described in Section 1.5.

3.1.1 Experimental

Preliminary experiments showed that *P.* sp. 2021 adhered in greater numbers to glass than to a PVA-SbQ gel surface (Appendix Paper 1). However, the preliminary tests were performed under uncontrolled shear conditions, and the detection method was not satisfactory as discussed in Section 2.4.2.

The growth medium and the preparation of test surfaces have been described in detail in Appendix Paper 2. Figure 3.1 shows the general experimental setup. A log phase culture of *P.* sp. NCIMB 2021 was transferred to the sterilized rotating annular biofilm reactor described in Appendix Paper 4. Briefly, the reactor consists of two stationary outer cylinders, and a rotating inner cylinder. The inner cylinder had been modified to contain removable casting moulds for gels. Water from a waterbath was recycled in the space between the two outer cylinders to maintain a constant temperature. After 1 hour in batch mode, medium was added continuously. The recirculation rate was approximately 10 times higher than the flow rate to ensure a well-mixed system. Test surfaces were sampled after a total of 14 hours in the reactor. Slides were

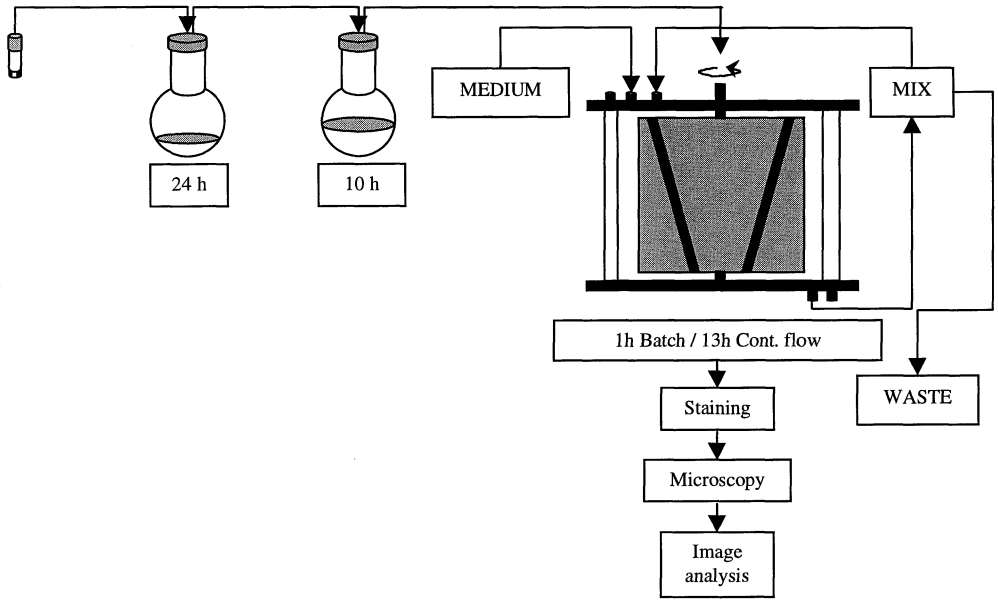

Figure 3.1 Experimental setup (Appendix Paper 2).

then rinsed and stained with a fluorescent nucleic acid stain solution, and images were collected from each slide using a confocal scanning laser microscope. The percent coverage by bacteria of each image was determined using an image analysis software as described in Appendix Paper 2.

3.1.2 Physiological conditions

As mentioned in Section 1.3.1 the composition of the extracellular polysaccharides and motility of *P.* sp. 2021 may vary in different growth phases, and this was shown to affect the degree of adhesion (Christensen *et al.*, 1987; Fletcher, 1980a). Furthermore, Fletcher (1977) showed that adhesion of this pseudomonad depended on culture concentration, temperature, and time allowed for adhesion. Hence, it was essential that the physiological conditions of the bacteria were the same in each experiment. The pH and the optical density were measured 1 and 14 hours after incubation. In addition, the optical density of the inoculum was recorded. The data are presented in Table 3.1. For details, see Appendix Paper 2.

Table 3.1 Shear rate, pH and optical density data for each experiment (Appendix Paper 2).

Shear rate [s^{-1}]	pH 1 hour after incubation	pH 14 hours after incubation	OD_{660} of inoculum	OD_{660} 1 hour after incubation	OD_{660} 14 hours after incubation
5.80	7.03	6.93	0.082	0.016	0.042
			0.081	0.015	0.041
13.7	7.05	6.98	0.076	n.d.	0.044
			0.075	n.d.	0.043
20.6	7.06	7.01	0.082	0.016	0.046
			0.080	0.016	0.046
27.5	7.04	6.99	0.070	0.017	0.042
			0.069	0.016	0.042

The cell densities and growth conditions of the experiments were clearly reproducible (Table 3.1). A separate growth experiment in batch showed that logarithmic growth ceased when the optical density exceeded 0.05 (unpublished results). Hence, cells in the inocula of Table 3.1 were in the transition between exponential growth and the stationary phase. In spite of a dilution rate of 1 h^{-1}, that is higher than the maximal specific growth rate observed in batch (0.5 - 0.7 h^{-1}), the cell density increased in the reactor during the continuous flow mode. However, the optical density did not exceed 0.05, and the cells were thus maintained in the log phase (Appendix Paper 2).

3.1.3 Adhesion of P. sp. NCIMB 2021 to hydrogels

Appendix Paper 2 presents relative adhesion of *P.* sp. 2021 to different gels and glass as a function of controlled shear rate. These data are shown in Figure 3.2.

In all cases, cell coverage decreased at higher shear rates (Figure 3.2). There was a significant difference in adhesion to all gels at a shear rate of 5.8 s^{-1}. Adhesion was lowest on PVA-SbQ, followed in increasing order by chitosan, agarose, and alginate. At the highest shear rate tested, 28 s^{-1}, only adhesion to alginate was significantly higher than to the other three gels. Earlier work has shown that *P.* sp. NCIMB 2021 prefer to adhere to hydrophobic rather than hydrophilic solid surfaces (Fletcher & Loeb, 1979; Wiencek & Fletcher, 1997; Kalaji & Neal, 2000). Figure 3.2, on

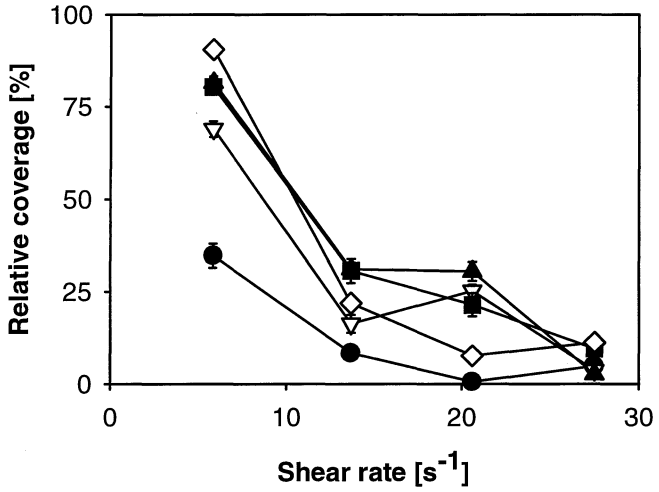

Figure 3.2 Relative adhesion of *P.* sp. 2021 to (■) glass, (▲) 1% agarose, (●) 5% PVA-SbQ, (◊) 1% alginate, and (▽) 1% chitosan as a function of shear rate. Each data point represents the average relative coverage of three gel slides or four glass slides where 12 images were collected on each slide. Error bars represent the standard error of the mean (Appendix Paper 2).

the other hand, shows that other properties than the surface free energy are determining for cell coverage on non-solid surfaces. The most hydrophobic gel, PVA-SbQ (see Section 2.1.2), supported the lowest number of cells at all shear rates tested. The surface charge was crucial for adhesion to hydrophilic surfaces in the work of Fletcher and Loeb (1979), and cells seemed to be electrostatically repelled by negatively charged surfaces. The results presented in Figure 3.2 suggest that the polymer charge of the model gels have minor effect on adhesion. Adhesion to alginate gels was somewhat higher than on chitosan gels at 5.8 s^{-1} and 28 s^{-1}, it was the other way round at 21 s^{-1}, and there was no significant difference at 14 s^{-1}. However, as discussed in Section 3.2, the pK_a for chitosan is approximately 6.6, meaning that amino groups may be discharged at a pH ranging from 6.9 to 7.2.

Both PVA-SbQ and agarose contain only very low amounts of charged groups. In addition, agarose is less hydrophobic than PVA-SbQ gels (Section 2.1.2). However, three experiments show that cell coverage was significantly higher on agarose than on PVA-SbQ (Figure 3.2). This

should be related to Young's moduli of the two gels (Figure 2.1), revealing that 1% agarose gels were more rigid. Thus, the nature of these agarose gels is closer to a solid surface than that of the 5% PVA-SbQ gels.

3.1.4 Concluding remarks

Adhesion of *P.* sp. NCIMB 2021 to glass and all four gels tested was dramatically reduced at increased shear rates in the interval tested (5.80 - 27.5 s^{-1}). At low shear rates, adhesion varied significantly between different gels in the following descending order: alginate > agarose > chitosan > PVA-SbQ. At the highest shear rate tested (27.5 s^{-1}), only adhesion to alginate remained significantly higher than to the other gels. The hydrophobicity of the hydrogel surfaces did not enhance adhesion of *P.* sp. NCIMB 2021 as observed previously for solid surfaces. On the contrary, the most hydrophobic gel was the least attractive for cell adhesion. The 5% PVA-SbQ gel showed the most promising antifouling properties, since adhesion to this gel was generally lower than to all other surfaces tested.

3.2 Diatoms

More than 10 000 diatom species are registered, but only 10 of these are known as major foulers (M. E. Callow, University of Birmingham, personal communication). However, as discussed in Section 1.3.2, fouling diatoms are widespread and they can tolerate relatively high concentrations of toxins found in antifouling paints (Robinson *et al.*, 1985). The resistance to cuprous oxide is for some species so high that they may turn blue and still survive (M. E. Callow, University of Birmingham, personal communication). Biocide free solutions for these target organisms are obviously strongly needed. This section will summarize the results obtained on adhesion of the fouling diatom *Amphora coffeaeformis* presented in Section 1.3.2 to gel surfaces.

3.2.1 Experimental

The experimental systems shown in Figure 3.3 have been described in detail in Appendix Paper 4. Briefly, experimental system 1 consisted of gels cast in a cell culturing well plate for comparing initial adhesion as well as long term biofilm development in the absence of shear. In experimental system 2, microscope slide based test surfaces were tested in aquaria under low shear conditions, see also Appendix Paper 3. A rotating annular biofilm reactor was applied to obtain high and controlled shear rates in system 3. Clean polystyrene wells were used as reference surfaces in experimental system 1. In experimental systems 2 and 3, both glass and a low energy surface paint (LES) were used for comparison to the gels.

Culture preparation and experimental procedures are explained in detail in Appendix Paper 4. Briefly, aliquot portions of a logarithmic phase culture of *A. coffeaeformis* were transferred to the test wells of experimental system 1. The diatoms were allowed to settle for 10 minutes before suspended cells were removed and replaced with sterile medium. Daily removal of suspended diatoms and recording of those attached were carried out for 6 days. Diatoms applied in experimental system 2 were allowed to settle for 1 hour before the test surfaces were transferred to an aquarium with sterile medium placed on a rocking platform shaker. Cells attached to the microscope slide based test surfaces were recorded after 24 hours. Each experiment applying the rotating annular biofilm reactor of experimental system 3 was also run for 24 hours before recording of attached cells. The quantification of attached diatoms by measuring the *in situ* autofluorescence is described in Section 2.5, see also Appendix Paper 3.

3.2.2 Adhesion of A. coffeaeformis to hydrogels

Appendix Paper 4 presents the fluorescent intensities of attached *A. coffeaeformis* in the wells of experimental system 1. These data are shown in Figure 3.4. Initial adhesion was highest on Ca-alginate and polystyrene. On PVA-SbQ, the amount of diatoms retained on the gels was below the detection limit at days 1, 2 and 5. High standard deviations occur when relatively low fluorescence signals are close to the background values.

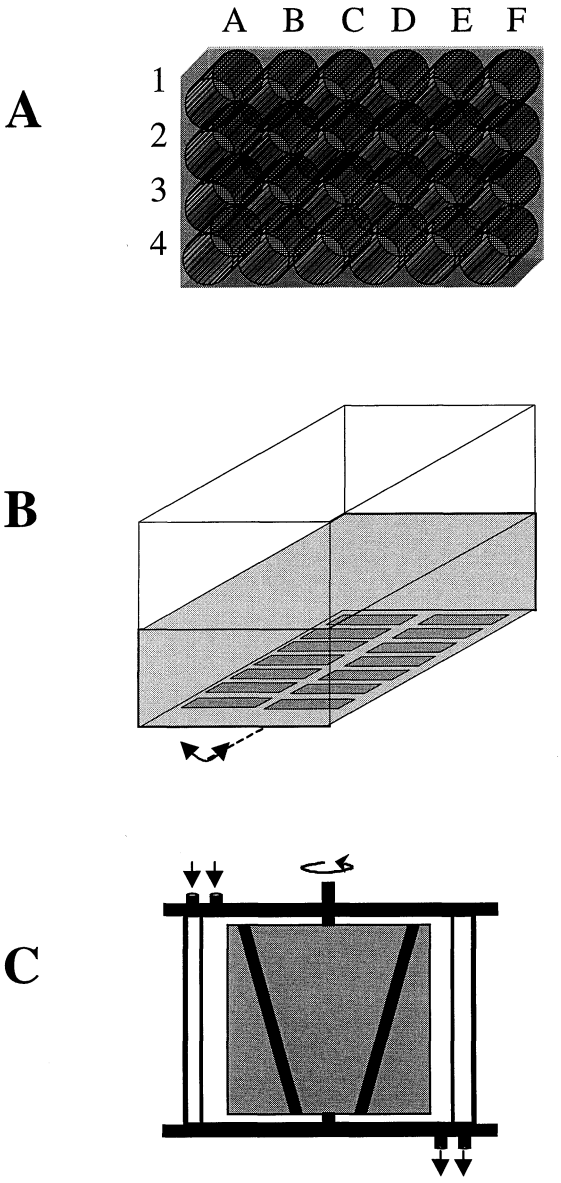

Figure 3.3 The experimental systems are (A) a 24 well cell culturing plate, (B) a glass aquarium with 14 microscope slide based test surfaces, and (C) a rotating annular biofilm reactor (Appendix Paper 4).

In experimental system 2, some shear was exerted on the test surfaces. The results presented in Appendix Paper 4 are also shown in Figure 3.5. Highest diatom cell adhesion was observed on Ca-alginate and chitosan. In contrast to system 1 (Figure 3.4), adhesion to agarose was significantly lower than to Ca-alginate and chitosan (Figure 3.5). Again, lowest adhesion occurred on the PVA-SbQ gels.

Adhesion of *A. coffeaeformis* to different gels as a function of shear rate is shown in Figure 3.6, see also Appendix Paper 4. In general, the amount of diatoms on the different surfaces decreased with increasing rotational speed. As observed in experimental system 2 (Figure 3.5), most diatoms were recorded on Ca-alginate and chitosan. At a shear rate of 10 s^{-1} and above, adhesion to PVA-SbQ and agarose was very low, while adhesion to LES became low at and above 17 s^{-1}. A similar shear effect could be detected on chitosan and alginate above 17 s^{-1}. Adhesion to glass was low in all experiments (Appendix Paper 4).

The well plates used in experimental system 1 were designed for cell cultivation, and adhesion to these surfaces was high as expected. Microscope slides used in system 2 were made hydrophilic by rinsing in methanol, see Appendix Paper 4. In spite of the charged nature of the glass, *A. coffeaeformis* adhered relatively weakly to these surfaces (Figure 3.5), leading to occasional losses when samples were removed from the reactor. These cases were excluded from the dataset. Adhesion to LES was clearly higher than to glass in system 2 and in system 3 at shear rates lower than 17 s^{-1}, corresponding to a relative surface speed of 1.5 knots. Low energy surface paints are applied on high speed vessels, and firm attachment of fouling organisms is prevented in partial by the hydrophobic properties (Berglin & Gatenholm, 1999; Brady & Singer, 2000). The low adhesion that was observed on glass in system 3 could be due to some adsorption of organic contaminants creating a hydrophobic film (Appendix Paper 4).

The gels applied in this work contained from 90 to 99% water. Molecules can enter the gels and become physically entrapped in the gel matrix. However, the chances for *A. coffeaeformis* entering the gels are rather low. Andresen *et al.* (1977) observed pore diameters of Ca-alginate gels in the range 5-150 nm whereas the size of *A. coffeaeformis* is in the range 5 - 10 μm. No diatoms could be detected inside the gels by microscopical investigations (Appendix Paper 4).

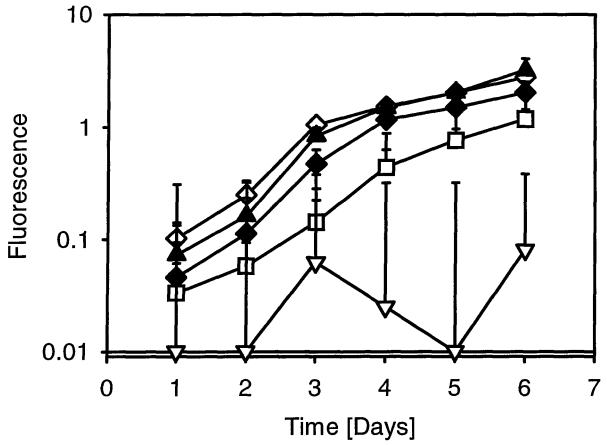

Figure 3.4 Adhesion and growth of *A. coffeaeformis* on gels of 1.5% agarose (◆), 10% PVA-SbQ (▽), 1% alginate (▲), 1% chitosan (□) and polystyrene (◇) as reference in a well plate, see Figure 3.3A. Cells were inoculated at day 1. Error bars indicate the standard deviation. The solid horizontal line indicates the fluorescence detection limit (Appendix Paper 4).

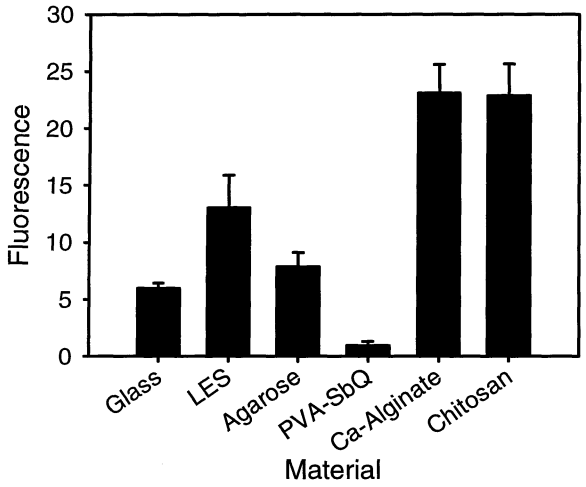

Figure 3.5 Average fluorescence intensity from *A. coffeaeformis* on glass, LES, 1.5% agarose, 10% PVA-SbQ, 1% Ca-alginate, and 1% chitosan sampled from a glass aquarium, see Figure 3.3B. Error bars indicate the standard error of the mean (Appendix Paper 4).

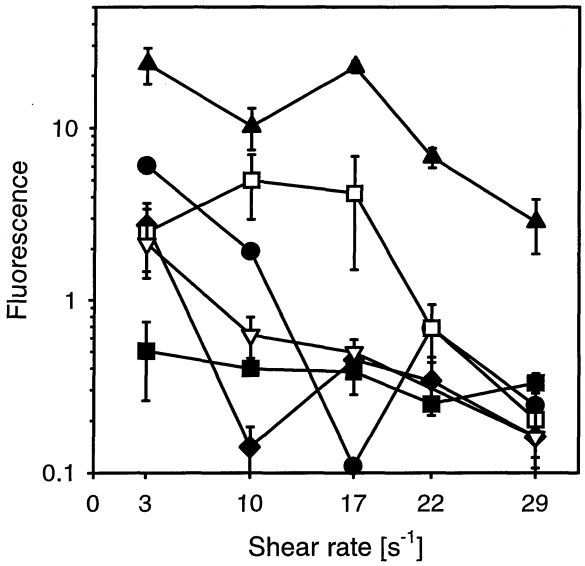

Figure 3.6 Adhesion of *A. coffeaeformis* to glass (■), LES (●), 1.5% agarose (◆), 10% PVA-SbQ (▽), 1% alginate (▲), and 1% chitosan (□) at different shear rates in the rotating annular biofilm reactor, see Figure 3.3A. Error bars indicate the standard deviation (Appendix Paper 4).

Changing the polymer concentration affects several parameters such as the gel strength (rigidity and fracture point), pore size, surface energy or wettability, and the concentration of functional groups at the surface. One or several of these variables may affect diatom adhesion. However, no significant concentration dependent difference in adhesion to Ca-alginate gels was observed, see Appendix Paper 4. A significantly higher adhesion was only obtained for PVA-SbQ gels with polymer concentrations as low as 2.5% and 2% (Appendix Paper 4). Rheological studies showed that the critical transition in viscoelastic properties occurred around 1.7% (Figure 2.3). Proper gelling will not occur at this concentration, and the PVA-SbQ polymer solution may behave rather like glue. This may explain why *A. coffeaeformis* showed a higher affinity to PVA-SbQ when approaching the critical gelling concentration.

Wigglesworth-Cooksey *et al.* (1999) observed that *A. coffeaeformis* Ag. Kutz behaved differently on hydrophobic surfaces compared to hydrophilic. The diatoms moved backwards and forwards

with no net movement, and detachment and reattachment occurred frequently. Edgar and Pickett-Heaps (1984) report that diatom adhesion to teflon was low compared to other surfaces, but their gliding speed was higher. This may explain the difference in adhesion observed on agarose and PVA-SbQ. Agarose and PVA-SbQ both represent polymer networks with very low charge density, and adhesion to both gels was low at shear rates above 3 s^{-1} (Figure 3.6). However, the PVA-SbQ gel is significantly more hydrophobic than the agarose gel according to the water contact angles (Figure 2.2), and a significant difference in adhesion was observed in the absence of shear (Figure 3.4) as well as at low shear rates (Figure 3.5). It should be emphasized that the surface free energy may have different effects on different strains of *A. coffeaeformis*. Callow *et al.* (1993) showed that adhesion of *A. coffeaeformis* var. *perpusilla* was stronger on a silane-coupled hydrophobic coating than to acid-washed glass.

A. coffeaeformis adhered easily to alginate and chitosan at low shear rates (Figures 3.4, 3.5 and 3.6). Only at shear rates above 17 s^{-1}, diatom adhesion was reduced on these gels. As discussed in Section 2.3, chitosan is a positively charged polymer at neutral pH, whilst at the pH of seawater amino groups are discharged and the gel matrix may even eventually collapse. Ca-alginate is negatively charged and obviously attractive to the diatoms. The chemistry of certain functional groups on the polymers could also affect adhesion, notably since the alginates of the gel matrix are crossbound by calcium. Cooksey (1981) found that Ca^{2+} is necessary for adhesion and motility of *A. coffeaeformis*, and a proposed mechanism for calcium was to stabilize external acidic polysaccharides. On alginate gels, Ca^{2+} could possibly act as a link between the gel and the EPS of the diatoms. Strong adhesion to both positively and negatively charged gels could reflect some cell surface patchiness or presence of more than one adhesive polymer, as found for *Stauroneis decipiens* by Lind *et al.* (1997).

3.2.3 Concluding remarks

A. coffeaeformis adhered better to the charged polymer gels of alginate and chitosan at high shear rates than to the low charge polymer gels, agarose and PVA-SbQ. In the system where shear was absent, *A. coffeaeformis* developed a biofilm on agarose equivalent to the charged polymer gels. Adhesion to PVA-SbQ was low at all shear rates. It is concluded that non-solid surfaces did not

represent an obstacle to settling and growth of this particular organism. As observed for solid surfaces, low charge density led to reduced attachment, particularly at high shear.

3.3 Barnacles

Crisp (1974) divided the exploration period of barnacles prior to metamorphosis in three phases; "broad exploration", "close examination" and "inspection". In the inspection phase, barnacle cyprids use their six hind legs for swimming or walking and the two antennules in front for surface inspection, see Figure 1.8. The antennules are equipped with an adhesive disk responsible for adhering the "foot" to the surface. The adhesive is produced in unicellular antennulary glands (Nott & Foster, 1969), secreted through the disks, and left behind as proteinacious foot prints (Walker & Yule, 1984; Clare *et al.*, 1994). According to Naldrett (1993), the hydrophobic adhesive is crosslinked through cysteine linkages. Crisp and Meadows (1962) proposed that cyprids respond to arthropodins as a positive settlement factor and, moreover, that the larvae can chemically sense the suitability for settlement. Clare and Nott (1994) believe that the fourth antennular segment sense the surface through flicking during the exploration period.

Barnacle cyprid larvae are much larger than both bacteria and diatoms, and thus, easy to monitor. However, preventing the cyprids from settlement without using biocides seems to be a much greater task. This section will summarize the results obtained on settlement of *B. amphitrite* cypris larvae to the hydrogels described in Section 1.5.

3.3.1 Experimental

An experimental layout is presented in Figure 3.7, for details see Appendix Paper 5. As described in Section 2.2, preliminary experiments were performed to test whether any substances leaching out of the gels could inhibit cyprid settlement. It was concluded that thorough washout was necessary for PVA-SbQ and chitosan gels. In addition, both cyprid settlement and naupliar mortality should be tested in leaching water from gels as a quality control before the settlement assays on gels were carried out, see Appendix Paper 5.

Different concentrations of the model gels were prepared in 24-well plates as described in Appendix Paper 5. After thorough leaching, *B. amphitrite* cyprid settlement and *B. amphitrite* naupliar mortality were tested in the leaching water. The results presented in Table III of Appendix Paper 5 confirmed that leaching was sufficient and no substance in the liquid phase should affect settlement. A detailed description of the settlement assays is given in Appendix Paper 5. Briefly, 15 cyprids was added to 8 replicates of each gel concentration as well as clean polystyrene control wells. The number of metamorphosed larvae and attached cyprids was monitored for 8 days. To assess the quality of viable but non-settled larvae, a sub-sample was transferred on day 6 to clean polystyrene wells containing seawater.

3.3.2 Settlement of B. amphitrite cypris larvae on hydrogels

Settlement of *B. amphitrite* cyprids on gels of agarose, PVA-SbQ, Ca-alginate and chitosan was studied as a function of time in Appendix Paper 5. These data are also presented in Figure 3.8.

All agarose gels inhibited settlement compared to polystyrene (Figure 3.8 A). Settlement was delayed on 4% and 0.5% gels compared to 1% and 2% gels. The number of settled larvae had stabilized within 8 days, and the level was significantly lower on the gels than on controls. Lowest settlement was achieved on 0.5% gels.

PVA-SbQ gels also inhibited settlement compared to polystyrene (Figure 3.8 B). There was no significant difference in relative settlement between the gels on day 1. The number of settled larvae increased significantly on 10 and 7% gels between days 1 and 2. The 2.5% gels were the least attractive for settlement.

Alginate gels were also clearly less attractive as substrata for *B. amphitrite* cypris settlement than polystyrene (Figure 3.8 C). The number of settled larvae leveled out after day 1 on all gels. A maximum of 20% and 40% of the larvae settled on 1% and 2% gels, respectively, whilst settlement on 4% was somewhere in between. Statistical analyses revealed no significant difference in settlement on alginate gels on days 1 or 6.

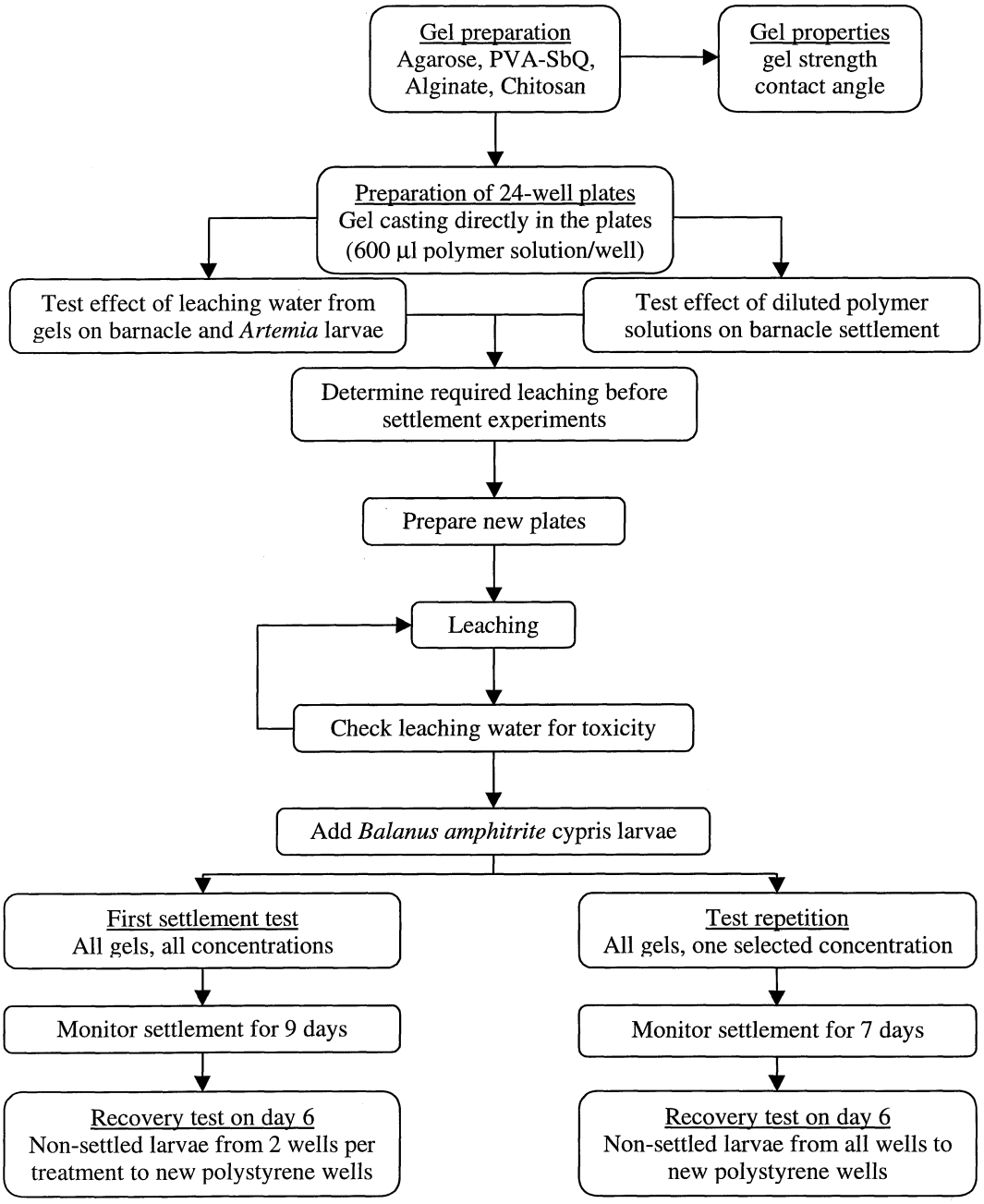

Figure 3.7 Experimental layout (redrawn from Appendix Paper 5).

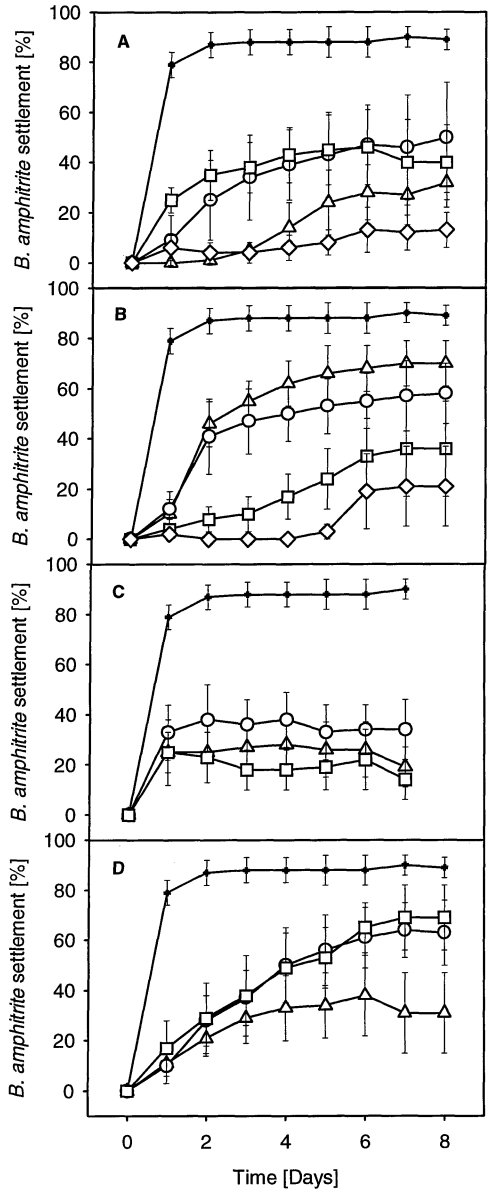

Figure 3.8 Settlement of *B. amphitrite* cypris larvae on polystyrene (✚) and (A) 0.5 (◇), 1(□), 2(○), and 4%(△) agarose gels; (B) 2.5 (◇), 4(□), 7(○), and 10% (△) PVA-SbQ gels; (C) 1(□), 2(○), and 4%(△) alginate gels; (D) 0.5 (□), 1(○), and 2% (△) chitosan gels. Each data point from day 1 through day 6 represents the mean of 8 replicates. Two replicates were withdrawn from the quality test on day 6, hence, data points on days 7 and 8 represent means of 6 replicates. Error bars indicate 95% confidence intervals (Appendix Paper 5).

Finally, chitosan gels also inhibited settlement compared to polystyrene (Figure 3.8 D). Settlement on 0.5% and 1% gels increased linearly for 6 days and leveled out at approximately 65-70%. On 2% gels, only minor increase in settled larvae occurred from day 3, culminating at approximately 35%.

The results presented in Figure 3.8 show that *B. amphitrite* cypris larvae settled more easily to polystyrene that to gels of agarose, PVA-SbQ, alginate, and chitosan. In addition, settlement was different on different concentrations of the gels. Possible factors contributing to these results are discussed below.

Settlement increased with increasing polymer concentration, and thus with increasing stiffness of the PVA-SbQ gels in accordance with the findings of Brady and Singer (2000) discussed in Section 1.6. Non-settled larvae adhered to the 2.5% gels although the antennules were not connected to the surface. In addition, metamorphosis was inhibited on these gels (Appendix Paper 5). This could be due to PVA-SbQ being sticky at low concentrations as observed for *Amphora coffeaeformis*, see Appendix Paper 4.

Settlement on agarose gels was not correlated to the Young's modulus (Appendix Paper 5). Agarose gels were generally rigid compared to the gels of PVA-SbQ (Figure 2.1), and cues other than the stiffness have obviously influenced settlement on these gels. Neither on alginate nor on chitosan gels could settlement be correlated to gel strength. However, the Young's moduli of chitosan gels shown in Figure 2.1 are not representative for the gels during the experiments due to contraction of the gel network in seawater as discussed in Section 2.3.

As discussed in Section 3.2.2, the pore diameters of the model gels are small compared to the size of a cyprid larva. However, this does not imply that the cyprids recognize the gels as solid surfaces. Settlement on agarose, PVA-SbQ, and alginate was lowest on those gels with the lowest polymer content, and hence, the largest polymer interdistances. It may be possible that the cypris larvae can sense the polymer density and thus reject surfaces based on polymer interdistances.

Water contact angles measured on PVA-SbQ gels shown in Figure 2.1 indicate a low surface free energy. The surface free energy decreased with increased polymer concentration. Compared with

the settlement results shown in Figure 3.8 B, settlement seemed to increase with decreased surface free energy on PVA-SbQ gels. It is, therefore likely that other factors, such as gel strength discussed above, are more important for *B. amphitrite* settlement than the surface free energy. The surface free energy of agarose increased with increased polymer concentration. Thus, the 0.5% gels had both the lowest surface free energy and gel strength, which resulted in low settlement (Figure 3.8 A). However, this does not explain why 4% gels gave lower settlement than both 1 and 2% gels.

The percentage of settled larvae on gels never reached the same level as on polystyrene. Non-settled larvae were transferred to new polystyrene wells containing seawater to test if they had lost their ability to settle or if it was the gels that prevented settlement (Appendix Paper 5). Larvae from all gels settled to polystyrene. Hence, the cyprids had not lost their settling ability, moreover, the gels were just not suitable for settlement. Although asepsis was pursued, the influence of bacterial or fungal contamination cannot be completely excluded in the long-term experiments, particularly if the polymer gels may have acted as a carbon source for such infections. However, there are several indications that such effects were small, if present. First of all, non-settled larvae were still able to settle on polystyrene surfaces even after long term incubations with gels. Secondly, the precipitate appearing in the wells of PVA-SbQ and chitosan gels was clearly time and concentration dependent. Finally, there are no irregular changes in the settlement data presented in Figure 3.8, as would have been expected if growing infections became significant.

3.3.3 Concluding remarks

In conclusion, it has been shown that all the gels tested inhibited settlement of *B. amphitrite* cypris larvae (Appendix Paper 5). The polymer network influenced the degree of inhibition with respect to both polymer density and type. None of the polymers were directly toxic to *B. amphitrite* cypris larvae or *A. salina* nauplius larvae. The majority of non-settled larvae in all gel experiments was able to settle when offered a suitable solid substratum, see Appendix Paper 5.

3.4 Field testing

Field experiments are invaluable when assessing the actual antifouling properties of a new coating (Thompson *et al.*, 1998). As pointed out by Brady and Singer (2000), nature does not rely on one mechanism for adhesion, but uses many, and all of them must be defeated at the same time. A typical example is the work of Kavanagh *et al.* (2001), showing that oil incorporation in silicone coatings decreased the adhesion strength of barnacles whilst the adhesion of tubeworms and oysters to the same surfaces became even more firm than without the oil. This section will summarize data obtained from some very preliminary pilot studies. In these studies, the model gels described in Section 1.5 were exposed to natural seawater in different test systems simulating different aspects of natural conditions.

3.4.1 Experimental

Three different experimental systems were applied:

Experimental system 1 was an indoor flow-through system without illumination. Appendix Paper 1 describes an experiment where glass and 10% PVA-SbQ gel surfaces were tested. Seawater was continuously pumped from a depth of 90 meters in the Trondheimsfjord, and through an open 100 L tank containing microscope slide based test surfaces. The absence of illumination favoured chemotrophic bacterial growth opposed to photosynthetic growth. Samples were stained with DAPI, and attached bacteria were quantified by measuring the resulting fluorescence, see Appendix Paper 1.

Experimental system 2 was an outdoor flow-through system with natural illumination. In this case, gels of 1.5% agarose, 10% PVA-SbQ, 1% alginate and 1% chitosan were prepared on microscope slides as described in Appendix Paper 4. Clean microscope slides were used as reference surfaces. 12 replicates of each gel and clean slides were mounted on a custom-made test panel as shown in Figure 3.7. Two panels were prepared and vertically submerged in an outdoor test basin so that the upper slides were located 1 meter below the surface. Seawater was

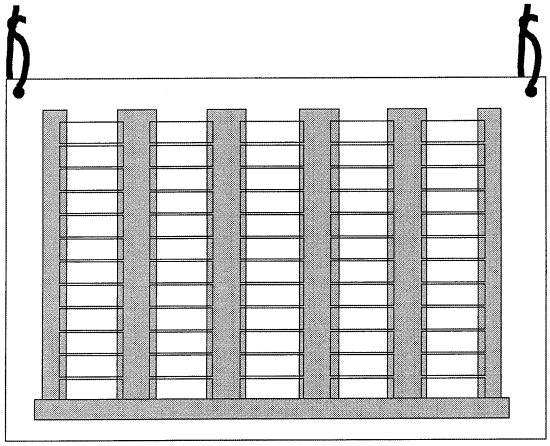

Figure 3.7 Panel for 5 x 12 microscope slide based test surfaces.

continuously pumped from a depth of 90 meters of the Trondheimsfjord, and run through the basin. Two replicates, one from each panel, were sampled once a day for 12 days. The experiment was repeated with sampling intervals of one or two days. Fouling was quantified by measuring the *in situ* chlorophyll *a* autofluorescence as described in Section 2.5, see also Appendix Paper 3.

Experimental system 3 was an *in situ* system where gels were exposed to natural conditions in open sea on a test raft outside Sandefjord (Appendix Paper 1). Only 5% PVA-SbQ gels were tested in July 1995. Those were examined visually after 2-3 weeks. A second series was performed from May until August 2001. Gels of 1% agarose, 1% alginate, 1% chitosan and 4% PVA-SbQ were tested.

3.4.2 Fouling of gel surfaces

Appendix Paper 1 presents the DAPI-fluorescence recorded on glass and PVA-SbQ gels applied in the indoor pilot study. These data are shown in Figure 3.8. The results suggest that bacterial films developed more readily on glass than on a PVA-SbQ gel surface in this case. This is in

accordance with the lab experiments applying the marine bacterium *P.* sp. NCIMB 2021 presented in Section 3.1 (see also Appendix Paper 2).

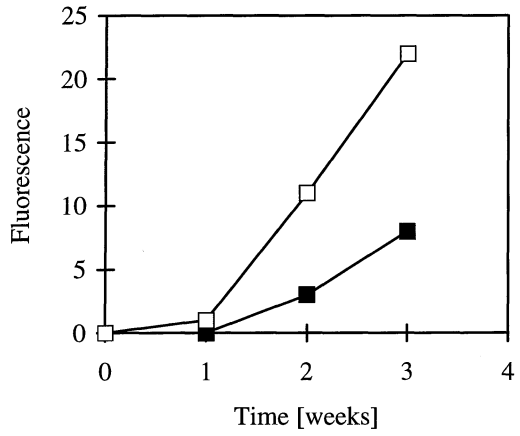

Figure 3.8 Fluorescence from DAPI stained bacteria on glass (□) and PVA-SbQ gels (■) after incubation in an indoor tank with natural seawater (Appendix Paper 1).

Two successive experiments where gels were tested in the outdoor basin were carried out between March 23rd and April 25th 1999. This is generally the season of the first plankton bloom in the Trondheimsfjord (Sakshaug, 1978). No barnacles were observed by visual or microscopical inspection on any of the surfaces in this period, as expected. The average fluorescence of two replicates for each test material is shown in Figure 3.9. Fouling by photosynthetic organisms was generally higher in the first period (Figure 3.9 A) than in the second period (Figure 3.9 B). The results suggest that chitosan gels were the most attractive surfaces, however these gels collapsed after 5-6 days in seawater, see also Section 2.3. Both glass and alginate seemed to be in the lowest range of algal fouling. However in general, there were only minor differences in fluorescence recorded on different gels. In the lab tests using the diatom *A. coffeaeformis* the most attractive and unattractive surfaces were alginate and PVA-SbQ gels, respectively, see Section 3.2. In the field, numerous different strains and species are present and a natural selection of periphyton that adhere easily to gels may be expected to occur. Such phenomena can only be documented by detailed taxonomic analyses of the community structures (Gustavson &

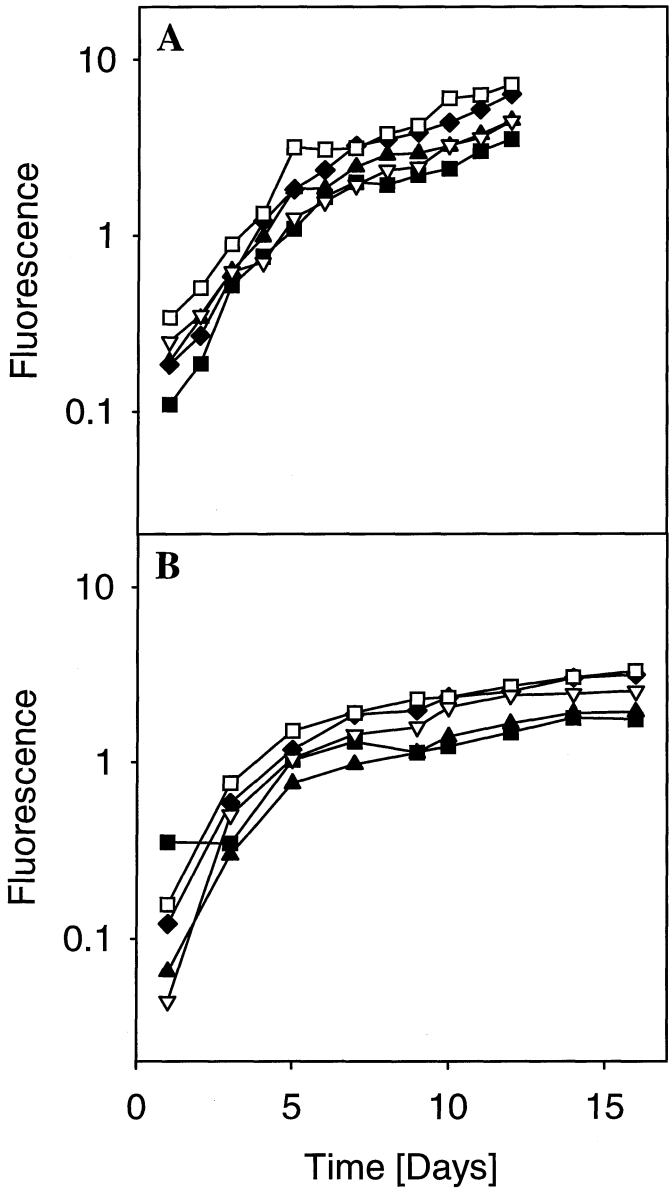

Figure 3.9 Average fluorescence from two replicates of glass (■) and gels consisting of 1.5% agarose (◆), 10% PVA-SbQ (▽), 1% alginate (▲) and 1% chitosan (□). The two experiments lasted from (A) March 27th until April 8th, and (B) from April 9th until April 25th (previously unpublished results).

Wängberg, 1995; Dahl & Blanck, 1996). Dahl and Blanck (1996) tested the effect of long term exposure of different concentrations of the antifouling algicide Irgarol 1051 on the periphyton community structure. They found that at 1nM a number of taxa showed decreased abundance while others were present in greater numbers than on control surfaces. Thus, there was a shift towards more tolerant species. In a similar experiment three series with TBT, Irgarol and Seanine, respectively, and one series with all three together were tested. On control surfaces 60 species were detected and additional 69 "new" species appeared on surfaces exposed to the four different treatments (H. Blanck, Göteborg University, Personal communication).

In the open sea raft experiments, no barnacles were observed on PVA-SbQ gels after 2-3 weeks in 1995 (Appendix Paper 1). However, numerous barnacles had settled on the panels supporting the test slides. In the lab assays applying *B. amphitrite* cypris larvae, they did settle on PVA-SbQ gels, see Section 3.3 and Appendix Paper 5. In the field, however, the cypris larvae had the option to select between different surfaces and to choose the most favourable. Another possible explanation is that cyprids settled on the gels in the field as well, but due to weak adhesion natural shear forces caused detachment. Microfouling and algal attachment seemed to be unaffected on the PVA-SbQ gels, see also Appendix Paper 1. As expected, the gels of agarose, alginate and chitosan were destroyed within 3 months in the open sea in 2001, while PVA-SbQ gels remained stable. In this test series fouling was somewhat delayed on PVA-SbQ gels compared to control surfaces after 3 months, as illustrated in Figure 3.10. However, after 4 months, all surfaces were completely covered by organisms including algae, barnacles and large populations of the blue mussel *Mytilus edulis* (for further details, see Willemsen, 2001).

3.4.3 Concluding remarks

Representativity and reproducibility are always a problem in field studies, and particularly in this case, were only data from a few preliminary pilot studies are available. Without attempting to generalize these findings, the field data available so far may be summarized as follows: Marine bacterial biofilm development was more readily on glass than on a PVA-SbQ gel surface, in accordance with the mono-culture lab experiments. Similar amounts of photosynthetic organisms adhered to gels of agarose, alginate, chitosan and PVA-SbQ tested in an outdoor seawater basin

during the spring season of algal blooming. Mussel and barnacle settlement was delayed on PVA-SbQ gels exposed in the open sea, while microfouling seemed relatively unaffected.

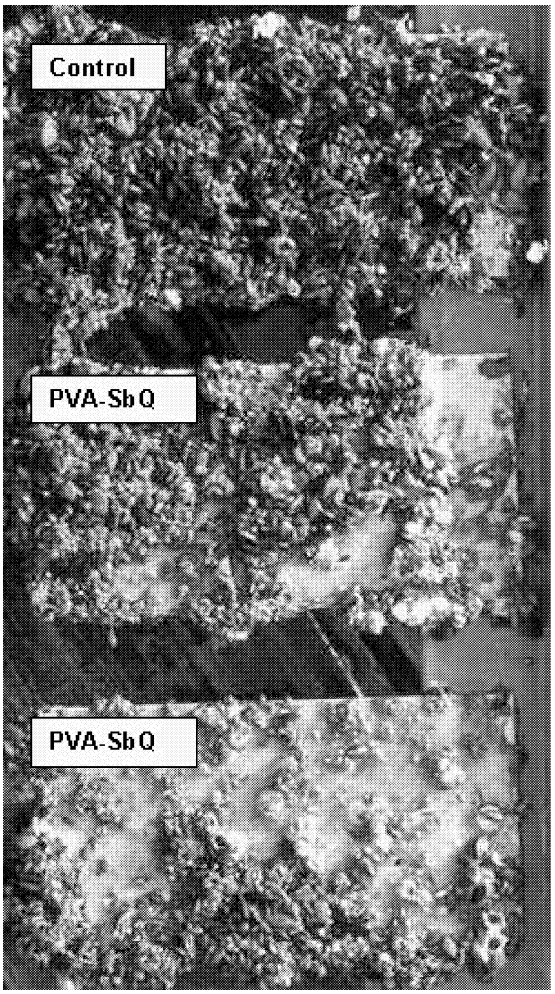

Figure 3.10 Fouling of a control surface and two 4% PVA-SbQ gel surfaces after a 3-month exposure in open sea. Fouling consists of mussels, barnacles and some algae.

4 GENERAL DISCUSSION AND CONCLUSIONS

4.1 Main findings

Method development (Chapter 2) showed that bacterial adhesion to gels could be conveniently enumerated by first staining with SYBR^R Green I nucleic acid gel stain. Images were then collected using a confocal scanning laser microscope, followed by image analysis to determine the percent coverage of bacteria (Section 2.4; Appendix Paper 2). Diatom adhesion to gels could be quantified using a fluorescence scanner recording the chlorophyll *a* autofluorescence of adhered cells, showing a clear correlation between average fluorescence signals and cell density determined by counting (Section 2.5; Appendix Paper 3). This method was successfully tested on glass, gels, a painted surface and an antifouling coated surface.

Bacterial adhesion on gels decreased at higher shear rates (Section 3.1; Appendix Paper 2). At low shear rates, adhesion varied significantly between different gels in the following descending order: alginate > agarose > chitosan > PVA-SbQ. Lowest cell coverage at all shear rates was recorded on the most hydrophobic gel, PVA-SbQ. Accordingly, properties other than the surface free energy are more important for bacterial adhesion to the gels.

The marine diatom *Amphora coffeaeformis* was applied in three different adhesion assays under different shear conditions (Section 3.2; Appendix Paper 4). At high shear, cells adhered better to the ionic polymer gels alginate and chitosan than to the low charge polymer gels agarose and PVA-SbQ. At very low shear, *A. coffeaeformis* even developed a film on agarose equivalent to that on the charged polymer gels. Adhesion to PVA-SbQ remained low at all shear rates. As observed for solid substrata, low charge density led to reduced attachment.

All gels inhibited settlement of *B. amphitrite* cyprid larvae compared to solid polystyrene controls (Section 3.3; Appendix Paper 5). Gels consisting of 2.5% PVA-SbQ or 0.5% agarose showed the most promising antifouling properties. Most of the non-settled larvae in all gel experiments were able to settle when transferred and offered a suitable solid substratum. Results indicated that the

gel strength was an important factor for cyprid settlement on gels, while the surface wettability seemed to be of minor importance.

Preliminary field tests suggested that marine bacterial biofilm development was more readily on glass than on a PVA-SbQ gel surface, in accordance with monoculture lab experiments (Section 3.4). However, similar amounts of photosynthetic organisms adhered to gels of agarose, alginate, chitosan and PVA-SbQ tested in an outdoor seawater basin during spring bloom. Finally, when exposed in the open sea, PVA-SbQ gels showed a delayed fouling particularly with respect to barnacles. However, after a full summer season of 4 months, even those gels became as covered as any other non-toxic surface.

The main aim of this work was to test whether fouling of gel surfaces is principally different from that of solid substrata (Section 1.7). No universal antifouling effects of hydrogels were found. However, in this work both adhesion of a bacterium and settlement of barnacle cypris larvae on gel surfaces seemed different from solid substrata. Diatom adhesion, on the other hand, was lower on gels with a low charge density, as observed for solid substrata. In general, the most hydrophobic gel, PVA-SbQ, was the least attractive surface for all three organisms.

4.2 General evaluation

According to Murphy's law on technology §21, "The only exact sciences are mathematics and belated wisdom". Thanks to hindsight, I can now say that quite a few things could have been done differently, and a lot of work may then have been avoided. Some of the early decisions that may seem questionable in the light of Murphy's law are discussed in this section.

4.2.1 Selection of test gels

Some of the gels selected for testing in this work are obviously unsuitable as antifoulants. Full-scale mechanical strength requirements would probably not be fulfilled by any of them. Furthermore, alginate gels are vulnerable for biodegradation (Vogelsang, 1999), as experienced by Gatenholm *et al.* (1995) who immobilized a bacterium producing a settlement-inhibitor in

alginate gels. After a one-day exposure in the sea, the gels had disappeared. According to the authors, this was either a result of enzymatic degradation or feeding by marine organisms. The biopolymer chitosan would also be a short-lived success in the marine environment, loosing its cationic charge and collapsing at such high pH. However, the model gels were selected based on their biocompatibility. This decision was made of two reasons; (1) in order to reveal properties of the hydrogel network that impair adhesion, the polymers should not have interfering toxic effects towards the test organisms and (2) at some later stage, gel entrapment of organisms producing fouling inhibitors would only be possible in biocompatible systems.

Gels may also be unsuitable in long-term lab experiments, as discussed above for the barnacle experiments (Section 3.3; Appendix Paper 5). A general qualitative description of gel stability as a function of polymer concentration, duration, and external conditions may be illustrated as shown in Figure 4.1. For a practical range of polymer concentrations, moderate external con-

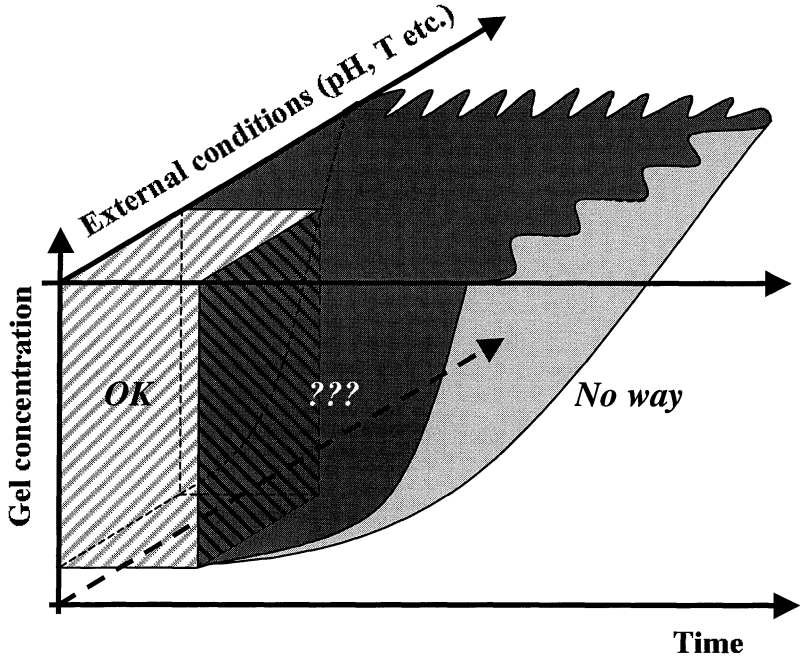

Figure 4.1 Qualitative illustration of gel stability as a function of gel concentration, duration and external conditions.

ditions and a limited test time, the gels may be assumed stable. This combination of conditions is expected to be fulfilled in the cube of Figure 4.1. After long-term incubations and at more extreme environmental conditions, there is an outer zone where the gels have lost their original properties. A collapsed chitosan gel and a dissolved alginate gel belong to this zone. However, between the "definitely OK" zone and the "absolutely no way" zone, there is a vague gray area where some sort of modification of the original gel surface may have occurred, but the effect on the experimental results are still negligible. Such alterations could be a conditioning film, bacterial contamination or some sort of precipitation. A white precipitate was indeed observed on some gels applied in barnacle cypris larvae experiments (Section 3.3; Appendix Paper 5). The problem is then to decide when to stop collecting data, and moreover, which data to include and which to exclude in a presentation. This dilemma can be illustrated by the curves representing cyprid settlement on gels of agarose, PVA-SbQ and chitosan. As shown in Figure 3.8, they did not reveal any irregular changes. Hence, possible interferences such as precipitates observed on some of these gels did not have any obvious effect on the results. Contraction of chitosan gels, on the other hand, may have enhanced settlement on these gels (Figure 3.8D). Settlement on alginate gels leveled out after only 1 day (Figure 3.8C). Larvae were clearly immobilized by dissolved gel material, and a prolonged time series was not necessary.

In conclusion, it is always useful to bring an experimental system outside its limits of validity as part of a test program. However, the data should then be interpreted with extreme caution if the actual location of these limits cannot be precisely defined.

4.2.2 Selection of test organisms

Modeling and simulation are two contrasting research approaches, but both equally important in a product development process. In modeling, the system is simplified to a controllable level, and preferably down to a level where the effects of single parameters can be tested separately. In this work, simple bioassays were applied to test hydrogel surfaces. To be of some general relevance, three ecologically and phylogenetically different test organisms were selected. Simulation, on the other hand, means that the experimental conditions should be as close to natural conditions as practically possible. Then, the model organisms should be representative for those of a fouling

community in the marine environment. Both *Amphora coffeaeformis* and *Balanus amphitrite* are well known foulers. The marine prokaryote *Pseudomonas* sp. was selected since it has already been widely used in other adhesion studies. Thorough and extensive simulations can only be performed in the field where ecology plays a significant role. Some fouling organisms may promote adhesion of others. In addition, preventing a model organism from adhering to a surface in the lab may at best mean that the same organism will fail to adhere to the surface when exposed in the field. However, a natural selection towards other fouling organisms may occur. As pointed out in Section 3.4, upon long term exposure to common antifouling biocides, similar amounts of "new" phytoplankton species appeared compared to the numbers recorded on control surfaces (H. Blanck, Göteborg University, personal communication; see also Dahl & Blanck, 1996). Finally, obtaining reduced adhesion strength of one organism to a surface does not imply that all other foulers will form weaker bonds. This was illustrated by Kavanagh *et al.* (2001). As already mentioned in Section 3.4, they showed that a specific oil incorporated in a fouling-release coating decreased the adhesion strength of a barnacle whilst a tubeworm and an oyster became more firmly attached.

In conclusion, a multitude of experimental systems will be necessary to establish a reliable connection between the lab and field situation, ranging from modeling to simulation and from pure cultures to ecosystems.

4.2.3 Selection of experimental systems

Experimental results may be affected by the incubation system itself, and obviously by experimental conditions such as limiting nutrients, temperature and pH. Three different incubation systems were applied for testing adhesion of the diatom *A. coffeaeformis* (Section 3.2; Appendix Paper 4). This was done mainly to obtain different shear conditions, but also to see if the actual incubation system was a critical factor for the results. In this case, the results were generally consistent. Pros and cons for each incubation system have been discussed in detail in Appendix Paper 4. The rotating annular reactor was applied in the bacterial adhesion studies where controlled shear was critical (Appendix Paper 2). For cyprid settlement the well plate

system was more appropriate, since the cyprids should not be allowed to choose between different surfaces (Appendix Paper 5).

For practical reasons, the temperature applied in bacterial- and diatom adhesion experiments were carried out at 20°C. Barnacle cyprid larvae were incubated at 28°C. Obviously, these temperatures are not representative for Norwegian coastal waters at higher latitudes.

Natural seawater and a seawater based medium were applied for cyprid settlement and diatom adhesion experiments, respectively. However, bacterial adhesion tests were carried out with an artificial seawater medium where pH ranged from 6.9 to 7.1. The pH of natural seawater is typically around 8.2. An advantage of the low pH of the bacterial growth medium was lower contraction of the chitosan gel network, see Section 2.3. However, the results may not be directly representative for what would occur under natural conditions.

The degree of rinsing will affect the amount of biomass left on a test surface. Pitt *et al.* (1993) found that bacteria remained in their original positions when surfaces were rinsed with saline for 3 minutes followed by 95% ethanol for 3 minutes before exposure to air. When surfaces were rinsed with saline only, the air -liquid interface disrupted the spatial distribution of the bacteria. In the bacterial adhesion assay described in Section 3.1 (see also Appendix Paper 2), slides were dipped in PBS and 96% ethanol for 2 minutes before staining. According to Pitt *et al.* (1993), the rinsing with saline and ethanol should occur in the reactor itself to avoid disruption by an air-liquid interface, but this was impractical due to the large volume of the rotating annular reactor employed in my work. However, test surfaces in this work were incubated for 14 hours resulting in a stronger adhesion than after 1-hour incubations applied by Pitt *et al.* (1993). Gómez-Suárez *et al.* (2001) presented a detailed theoretical analysis of the detachment of bacteria adhering to surfaces upon passage of air-liquid interfaces. They concluded that *in situ* enumeration should be performed to avoid the problem. Alternatively, dipping should be rapid since detachment by a passing air-liquid interface is smallest when the interface moves at higher velocity. At high velocities, no time is allowed for a three-phase contact between air-liquid-bacterium to form (Gómez-Suárez *et al.*, 2001).

Long-term steady-state biofilms were generally not obtained with diatoms or bacteria. However, as already pointed out, the aim of this work was not to test biofouling effects of the organisms, but rather to compare the properties of different gel surfaces. Rapid bioassays for initial adhesion were therefore pursued. In addition, gel stability had to be considered (Figure 4.1).

4.3 Future studies

Future work can go in two directions. The first is to pursue a more fundamental understanding of interactions between fouling organisms and gel networks, and the other is to move on to more applied aspects of gels as antifoulants.

One approach in such basic research would be to focus on a single gel system. This network should be as stable as possible and of course non-toxic. The results presented in this thesis suggest that fouling organisms should be tested separately since there seem to be no universal antifouling effect of hydrogels. *In situ* observations of adhering bacteria and diatoms in systems such as a flat plate reactor where the bottom is covered with a gel would be interesting. Wave development or vibrations of the gel network should also be investigated. The gel thickness and configuration would affect such phenomena. Simultaneous measurements of the gel strength would reveal the mechanical stability of the gel system. Effects of shear on- or adhesion strength of barnacles settled on gels should be tested.

Field testing of gels should only include stable polymer networks such as PVA-SbQ (Vogelsang, 1999) as well as more economic alternatives such as polyacrylamide. At present, it is not likely that anyone will try to cover an oil tanker with a gel, but it may have a future potential for other devices such as marine sensors.

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Appendix Paper I

BIOFILM FORMATION ON NON-SOLID GEL SURFACES

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ABSTRACT

An experimental gel surface model system has been developed, based on photo-induced gelation of the synthetic polymer PVA-SbQ, to make gel slabs that will stick directly to rubbed glass slides. In pilot studies, bacterial attachment, growth and respiration were quantified by direct epi-fluorescence spectrometry after staining with DAPI and CTC. The turbidity of multiple biofilms was also recorded in a custom made continuous lab reactor system. The adhesive marine bacteria *Pseudomonas* sp. NCMB 2021 showed reduced adhesion and growth on PVA-SbQ gels compared to glass slides. A similar difference was observed for natural bacterial biofilms formed in darkness in a continuous flow of natural seawater from 90 m depth. When directly exposed in the open sea, the gel surfaces showed complete absence of barnacles, reduced bacterial growth, and apparently unaffected algal attachment compared to glass slides.

INTRODUCTION

Biofilm formation on solid surfaces has been studied intensively. Less attention has been given to non-solid surfaces such as hydrogels, consisting of 90 - 99 % water. Here we present an experimental model system based on photo-induced gelation of the synthetic polymer PVA-SbQ, which is polyvinyl alcohol substituted with light-sensitive styrylpyridinium groups [1-3]:

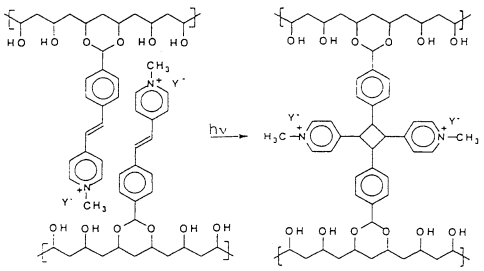

Fig. 1 The photo-cross linking of the synthetic PVA-SbQ

The advantages of this experimental model system are: Low charge density of the polymer network, high resistance against biodegradation in open environments, suitability for testing of gel entrapped non-leaching biocides, suitability for testing slow release of low-solubility biocides, and biocompatibility during gelation - so that gel entrapment of any microorganism to form artificial biofilms with known structure is possible.

The preliminary pilot studies of the model system presented here include controlled laboratory studies with the adhesive bacterium *Pseudomonas sp.* NCMB 2021 [4] as well as testing of biofilm formation directly in the open sea.

MATERIALS AND METHODS

Gel formation

The PVA-SbQ used in this work (13.19 % W/W solution, pH 6.4 purchased from Toyo Gosei Kogyo Co., ltd., Toshihanyaesudori bld., 1-9-6, Hacchoubori, Chuo-Ku, Tokyo 104 Japan) contained PVA with a polymerization degree of 1700, an 88 % degree of saponification and 1.3 mol% styrylpyridinium groups introduced as photo-sensitive functional sites [3].

Rubbed glass slides were placed on a slide tray (KEBO Lab, Oslo, Norway) with the rubbed side facing upwards. A 10 % aqueous solution of the polymer was poured onto the slides creating a thin liquid layer (1 mm). The coated slides were then exposed to bright white light over night in a water saturated atmosphere to avoid dehydration.

Fluorescent staining and recording

Bacterial attachment was quantified by direct epi-fluorescence spectrometry after staining exposed slides with DAPI (4'.6-diamidino-2-phenylindole) [5]. Respiration activity was estimated by staining with CTC (5-cyano-2,3-ditolyl tetrazolium chloride) [6]. A Perkin Elmer model 3000 fluorescence spectrometer with a fixed-angle adapter was used to measure the surface epifluorescence obtained in both cases, at excitation and emission wavelength combinations of 330 nm / 440 nm and 420 nm / 620 nm,

respectively. Additional optical filters were applied to reduce the severe interference of light scattering.

Reactor system

The biofilm growth of the adhesive marine bacteria *Pseudomonas* sp. NCMB 2021 was studied in a continuous flow of synthetic seawater medium, see Fig. 2. The reactor was a 130 ml stirred glass vessel with a fixed rack for a maximum of 12 microslides. The turbidity (absorbance units at 670 nm) of the total reactor system with slides was monitored by a custom made diode system [7].

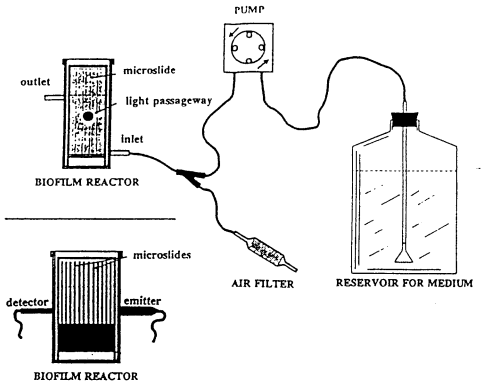

Fig. 2 Reactor system

The reactor was first run as a batch (7-14 h), followed by complete washout for biofilm development studies. The absorbance data are corrected for sampling of slides for staining.

Testing in open sea

Gel slides were also directly exposed in the sea outside Sandefjord (Southern Norway) in July 1995, a period with warm and sunny weather. Slides were mounted facing the sun or downwards. After 2-3 weeks the slides were examined visually.

In a different experiment, slides were exposed indoor to seawater continuously pumped up from 90 meters depth with a constant temperature of 5-6 °C. The biofilm development was studied by taking out samples for DAPI and CTC staining once a week.

RESULTS

Lab

As shown in Fig. 3, *Pseudomonas* sp. NCMB 2021 showed a delayed biofilm formation and a reduced growth on PVA-SbQ gels compared to glass slides exposed to seawater in the lab system:

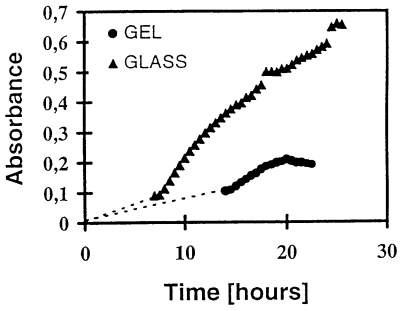

Fig. 3 Biofilm formation on glass and gel slides measured as turbidity.

A similar time-dependent development was observed by quantifying DAPI- and CTC staining (results not included). Fig. 4 shows the results after 27 hours of cultivation, glass and gel surfaces being simultaneously exposed in the same reactor.

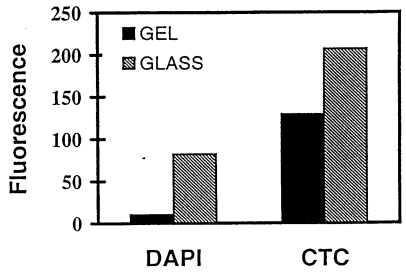

Fig. 4 Fluorescence measurements on gel and glass following DAPI- and CTC staining. The slides were exposed in the lab reactor for 27 hours before the measurements were performed.

Open sea

Indoor exposure to seawater indicated reduced bacterial growth on gels compared to glass slides, confirming the results from the reactor experiment. See Fig. 5.

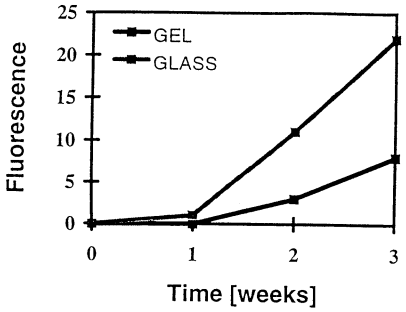

Fig. 5 Fluorescence from DAPI stained bacteria the first three weeks of exposure to seawater.

When exposed in the open sea, the gel surfaces showed complete absence of barnacles, but the algal attachment was apparently unaffected when compared to glass slides. Slides facing light had a high algal growth (results not included).

DISCUSSION

Biofouling or biofilm formation on solid surfaces is the result of ecological factors favoring adhesive growth. Improved nutrient supply due to surface adsorption as well as water flow may be important factors. A non-solid aqueous gel surface may therefore represent a selective factor that will slow down biofilm formation in general, and most important, reduce the diversity of microorganisms able to participate in the process.

This system may be combined with eco-compatible biocides to obtain an effective antifouling-system, that is biocides that do not leach (NLBs), or controlled release of biocides that are easily degraded in the open environment.

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Appendix Paper II

Marine biofouling: adhesion of the marine bacterium *Pseudomonas sp.* NCIMB 2021 to hydrogel surfaces

Short title: Marine bacterial adhesion to hydrogels

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Abstract

Adhesion of *Pseudomonas sp.* NCIMB 2021 was tested on different non-solid hydrogel surfaces under different shear conditions. Gels consisting of alginate (highly anionic), chitosan (highly cationic), modified polyvinyl alcohol PVA-SbQ (very low cationic) and agarose (neutral) were casted in moulds custom-made for a rotating annular biofilm reactor. Cells were stained with SYBR^R Green I nucleic acid gel stain, and images were collected using a confocal laser scanning microscope. Relative adhesion was quantified by determining percent cell coverage using image analysis. Bacterial adhesion on gels decreased at higher shear rates. At low shear rates, adhesion varied significantly between different gels, in the following descending order: alginate > agarose > chitosan > PVA-SbQ. Only adhesion to alginate remained significantly higher than to the others at high shear rates. Lowest cell coverage at all shear rates was recorded on PVA-SbQ gels. Clearly, the hydrophobicity of the hydrogel surfaces did not enhance adhesion as observed for solid surfaces. A 5% PVA-SbQ gel showed the most promising antifouling properties.

Keywords: Biofouling; non-solid surfaces; biofilm reactor; SYBR^R Green I; image analysis

Introduction

Submerged surfaces, including ships' hulls, are quickly covered with microfoulers such as bacteria, diatoms and protozoa, as well as macrofoulers such as barnacles and mussels, causing increased frictional resistance and biodeterioration (Little & DePalma, 1988). The importance of bacteria in marine colonization of surfaces has been known for a long time (Zobell & Allen, 1935). A marine bacterium that has been studied intensively for the purpose of solving problems related to biofouling is *Pseudomonas sp.* NCIMB 2021. This strain was isolated by Madilyn Fletcher in the Menai Straits, Anglesey, Wales (Fletcher & Floodgate, 1973).

It is generally believed that the extracellular polymeric substances (EPS) play an important role in the adhesion of bacteria to surfaces. Numerous works have been done to explore the adhesion mechanisms of *P. sp.* NCIMB 2021 and how to prevent it from developing a biofilm (Fletcher & Floodgate, 1973; Fletcher, 1977 and 1980; Fletcher & Loeb, 1979; Christensen *et al.*, 1987 and 1990; Humphries *et al.*, 1987; Wiencek & Fletcher, 1997; Kalaji & Neal, 1999; Gubner & Beech, 2000). Fletcher (1977) showed that the adhesion rate to polystyrene petri dishes (PD) decreased in cultures passing from the exponential growth phase, through the stationary phase to the death phase. One explanation to this phenomenon was given by Christensen *et al.* (1987) who showed that this organism produce one polysaccharide in the exponential growth phase and a different polysaccharide in the stationary phase. Fletcher and Loeb (1979) compared directly the adhesion of this marine pseudomonad to hydrophobic and hydrophilic surfaces. Bacteria were most abundant on hydrophobic surfaces, and the number of attached cells increased inversely with the wettability of those surfaces. The surface charge seemed to be a crucial factor for adhesion to hydrophilic surfaces, where moderate numbers of cells attached to metals with a positive or

neutral charge (platinum and germanium), and very few bacteria were detected on negatively charged surfaces (glass, mica, and oxidized plastics). Fletcher (1980) concluded that bacteria have both active and passive attachment mechanisms.

As illustrated above, bacterial adhesion to solid surfaces has been studied intensively. Less attention has been given to non-solid surfaces such as hydrogels, consisting of 90-99 % water. Hydrogels are polymer networks with the ability to bind large amounts of water. On macroscale, these gels may be recognized as surfaces since particles and molecules larger than the pore size cannot penetrate the gel surface. However, small molecules may diffuse freely in and out of the gel matrix. It has already been shown that hydrogels may be suitable for incorporating bioactive materials (Gatenholm *et al.,* 1995; Henrikson & Pawlik, 1998; His *et al.*, 1996; Cowling *et al.*, 2000). Cook *et al.* (1993) found that the amount of *Pseudomonas aeruginosa* adhering to poly-hydroxyethyl methacrylate (PHEMA) based hydrogels decreased with increasing water content of the gel. Preliminary experiments showed that PVA-SbQ gels revealed antiadhesive effects towards *P. sp.* NCIMB 2021 compared to glass slides (Rasmussen *et al.*, 1997). Recently, we have shown that non-solid surfaces did not represent an absolute obstacle to settling and growth of the diatom *Amphora coffeaeformis* (Rasmussen & Østgaard, 2001). However, clearly reduced attachment was observed on gels with low charge density, particularly at high shear. The same gels also inhibited settlement of *B. amphitrite* cypris larvae compared to polystyrene controls (Rasmussen *et al.*, 2001). Also in the case of barnacle settlement, gels with low charge density revealed the most promising antifouling properties.

The aim of this work was to test bacterial adhesion to non-solid surfaces in relation to the physical and chemical properties of the gels. In order to reveal possible interactions between the

bacteria and the polymeric gel network, cationic, anionic as well as neutral gelling substances were included. Restricting the study to biocompatible gel formation systems, alginate (highly anionic), chitosan (highly cationic), modified polyvinyl alcohol PVA-SbQ (very low cationic) and agarose (neutral) were therefore chosen for the test program. Glass served as a solid surface reference.

Materials and methods

Culture

A pure culture of *Pseudomonas sp.* NCIMB 2021 (National Collections of Industrial Marine, Food and Industrial Bacteria, Aberdeen, Scotland) was grown at 20°C in the medium applied by Christensen *et al.* (1990) with the following modifications: KH_2PO_4 and K_2HPO_4 were exchanged with 10 mM BIS-TRIS (Sigma Chemicals Co., USA) and 6.2 µM NaH_2PO_4 x H_2O. In addition, the yeast extract applied in our work was not dialyzed. The pH was adjusted to 7.2. Cell densities were monitored by measuring the optical density at 660 nm using a UV/visible spectrophotometer (Ultraspec. 2000, Pharmacia Biotech).

Test surfaces

As summarized in Table 1, the following gels were tested for bacterial adhesion: agarose (Sigma Chemicals Co., USA), polyvinyl alcohol substituted with light-sensitive stilbazolium groups (PVA-SbQ, Toyo Gosei Kogyo Co., ltd., Japan), alginate (LF10/60, Pronova Biopolymer A/S, Norway), and chitosan (F_A 0.17, Pronova Biopolymer A/S, Norway). Glass was used as a solid

surface reference material. The choice of polymer test concentration and the preparation of the gels has been treated in detail elsewhere (Rasmussen & Østgaard, 2001). Gelation occurred directly in moulds custom-made for the reactor. PVA-SbQ and chitosan gels were leached in PBS for 48 hours, whilst agarose and alginate gels were leached for 24 hours to remove soluble components (Rasmussen *et al.*, 2001). Glass slides were washed in a 10% HCl solution and then in 70% ethanol. They were rinsed and stored in distilled water prior to use.

Gel strength was determined by calculating the Young's modulus (E) from the initial slope of the force versus deformation curve (Smidsrød *et al.*, 1972) measured by a Stable Micro Systems TA-XT2 Texture analyzer (Stable Micro Systems, England). A Dynamic Contact Angle and Absorption Tester (DAT 1121, Fibro system AB, Sweden) was used to determine static water contact angles on non-treated gels. The angle was recorded 10 seconds after the water drop had been applied on the surface.

Experimental setup

The experimental setup is pictured in Figure 1. A culture flask containing 100 ml of sterile medium was inoculated with 0.5 ml of a *P. sp.* NCIMB 2021 stored at -80°C. After 24 hours, 1 ml of the new culture was transferred to another culture flask containing 200 ml of medium. This culture was allowed to grow for 10 hours before the optical density (OD) was measured, and the whole culture was transferred to the sterilized rotation annular biofilm reactor (Biosurface Technologies Corporation, MT, USA). The reactor has been described elsewhere (Rasmussen & Østgaard, 2001). Briefly, the reactor consisted of two stationary outer cylinders, and a rotating inner cylinder. The inner cylinder had been modified to contain 16 removable casting moulds for

gels. Water from a waterbath was recycled in the space between the two outer cylinders to maintain a constant temperature of 20°C. Three slides of each gel type and four glass slides were used in each experiment. Medium was recycled through a recycle loop containing a mixing chamber. A liquid sample was collected for pH and OD measurements after the reactor had been running for 1 hour in batch mode. Afterwards, medium was continuously added at a dilution rate of 1 h^{-1}. The recirculation rate was approximately 10 times higher than the flow rate to ensure a well-mixed system. Test surfaces were sampled after a total of 14 hours in the reactor. The pH and OD were also measured at this time.

Slides were then submerged 3 times in PBS, 2 minutes in 96% ethanol (Pitt *et al.*, 1993), and finally stained for 15 minutes in a SYBR^{R} Green I nucleic acid gel stain solution (Molecular Probes, Leiden, The Netherlands) prepared by diluting a 10 000X concentrate 400 times (Noble and Fuhrman, 1998) with PBS. 12 images were collected from each slide using a confocal laser scanning microscope (MRC-600, BIO-RAD Microscience Division, England), see also Figure 2. The percent coverage (Verran *et al.*, 1980) by bacteria on a 64 μm x 64 μm square of each image was determined using Matrox^{R} Inspector (version 3.0, Matrox Electronic Systems Ltd., USA). Experiments were repeated 3 times at different shear rates, calculated from the angular velocity of the rotating inner cylinder.

Statistical methods

Analysis of variance (ANOVA) was performed on adhesion data using MINITAB^{TM} (version 13.1, Minitab Inc.). A multiple comparison of different gels for each shear rate was done using Tukey's test (Walpole *et al.*, 1998).

Results and discussion

In order to get an indication of the hydrophobicity of the gels, water contact angles were measured. Results in Table 1 show that the PVA-SBQ was more hydrophobic than the chitosan gel. The alginate gel was the most hydrophilic gel, followed by agarose.

Results from pH and OD measurements for each experiment are presented in Table 2, showing that the cell densities and growth conditions of the experiments were reproducible. A separate growth experiment in batch showed that logarithmic growth phase ceased when the OD exceeded 0.05 (results not included). Hence, cells in the inoculum were in the transition phase between exponential growth and stationary phase. In spite of a dilution rate of 1 h^{-1}, that is higher than the maximal specific growth rate observed in batch (0.5 - 0.7 h^{-1}), the cell density increased in the reactor during the continuous flow mode. However, the OD did not exceed 0.05, and the cells were thus maintained in the log phase.

Figure 2 shows a typical example of growth patterns recorded on glass and a PVA-SbQ gel tested at the lowest shear rate (5.8 s^{-1}). Cell coverage on glass was clearly higher than on the PVA-SbQ gel. Another difference between the two surfaces was the presence of cell clusters on PVA-SbQ as visualized. However, cell clustering was also observed on the other gels, and even on glass at higher shear rates.

Relative adhesion of *P. sp.* 2021 to different gels and glass as a function of shear rate is summarized in Figure 3. Cell coverage decreased at higher shear rates. Analysis of variance

indicated significant difference in adhesion to all gels at a shear rate of 5.8 s^{-1}. Adhesion was lowest on PVA-SbQ followed in increasing order by chitosan, agarose, and alginate. At the highest shear rate tested, 28 s^{-1}, only adhesion to alginate was significantly higher than to the other three gels. There was no significant difference in adhesion to agarose, PVA-SbQ and chitosan gels at these conditions.

Earlier work has shown that *P. sp.* NCIMB 2021 prefer to adhere to hydrophobic rather than hydrophilic solid surfaces (Fletcher & Loeb, 1979; Wiencek & Fletcher, 1997; Kalaji & Neal, 1999). Our work, on the other hand, shows that other forces are determining for cell coverage on non-solid surfaces. The most hydrophobic gel, PVA-SbQ, supported the lowest number of cells at all shear rates tested. The surface charge was crucial for adhesion to hydrophilic surfaces in the work of Fletcher and Loeb (1979). Cells seemed to be electrostatically repelled by negatively charged surfaces. The polymer charge of the gels in our work seemed to have minor effect on adhesion. Adhesion to alginate gels was higher than on chitosan gels at 5.8 s^{-1} and 28 s^{-1}, it was the other way round at 21 s^{-1}, and there was no significant difference at 14 s^{-1}. However, it should be emphasized that the pK_a for chitosan is approximately 6.6, meaning that amino groups may be discharged at a pH ranging from 6.9 to 7.2.

Both PVA-SbQ and agarose contain low amounts of charged groups. In addition, agarose is less hydrophobic than PVA-SbQ gels. However, three of our experiments show that cell coverage was significantly higher on agarose than on PVA-SbQ (Figure 3). This should be related to the Young's moduli of the two gels (Table 1), revealing that 1% agarose gels were more rigid. Thus, the nature of these gels is closer to a solid surface than the 5% PVA-SbQ gels.

Conclusions

1) Adhesion of *P. sp.* NCIMB 2021 to glass and all four gels tested was reduced at increased shear rates in the interval tested (5.80 - 27.5 s^{-1}).

2) At low shear rates adhesion varied significantly between different gels in the following descending order: alginate > agarose > chitosan > PVA-SbQ.

3) At the highest shear rate tested (27.5 s^{-1}), only adhesion to alginate remained significantly higher than to the other gels.

4) The hydrophobicity of the hydrogel surfaces did not enhance adhesion of *P. sp.* NCIMB 2021 as observed previously for solid surfaces. On the contrary, the most hydrophobic gel was the least attractive for cell adhesion.

5) A 5% PVA-SbQ gel showed the most promising antifouling properties, since adhesion to this gel was generally lower than all other surfaces tested.

Acknowledgements

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TABLE 1 Description of the test surfaces applied.

Surface type	Surface material	Type	Standard concentration [%]	Gel strength, E [N/m^2]	Contact angle [degrees] (s.d.)	Gelling principle
Solid reference	Glass	-	-	-	n.d.	-
Test gel	Agarose	Neutral	1.0	38 000	49 (7)	Thermic; cooling
Test gel	PVA-SbQ	Low cationic	5.0	6 400	92 (3)	Photoinduced crosslinking
Test gel	Alginate	Cationic	1.0	6 800	24 (2)	Ca^{2+} crosslinking
Test gel	Chitosan	Anionic	1.0	1 600	67 (5)	Glutardialdehyde crosslinking

TABLE 2 Shear rate, pH- and optical density data for each experiment.

Shear rate [s^{-1}]	pH 1 hour after incubation	pH 14 hours after incubation	OD_{660} of inoculum	OD_{660} 1 hour after incubation	OD_{660} 14 hours after incubation	Dilution rate [h^{-1}]	Resirc-ulation rate	Temp-erature [°C]
5.80	7.03	6.93	0.082	0.016	0.042	1	10	20
			0.081	0.015	0.041			
13.7	7.05	6.98	0.076	n.d.	0.044	1	10	20
			0.075	n.d.	0.043			
20.6	7.06	7.01	0.082	0.016	0.046	1	10	20
			0.080	0.016	0.046			
27.5	7.04	6.99	0.070	0.017	0.042	1	10	20
			0.069	0.016	0.042			

Figure legends:

Figure 1 Experimental setup.

Figure 2 Growth patterns recorded on (A) a PVA-SbQ gel and (B) glass tested at a shear rate of 5.80 s^{-1}.

Figure 3 Relative adhesion of *P. sp.* 2021 to (■) glass, (▲) agarose, (●) PVA-SbQ, (◊) alginate, and (▽) chitosan as a function of shear rate. Each data point represents the average relative coverage of three gel slides or four glass slides where 12 images were collected on each slide. Error bars represent the standard error of the mean.

Figure 1

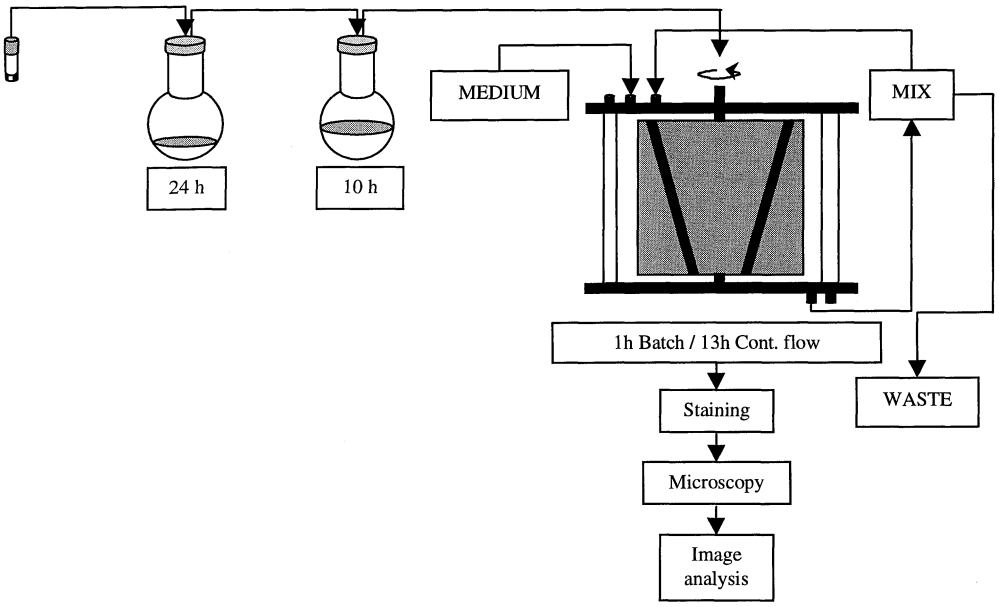
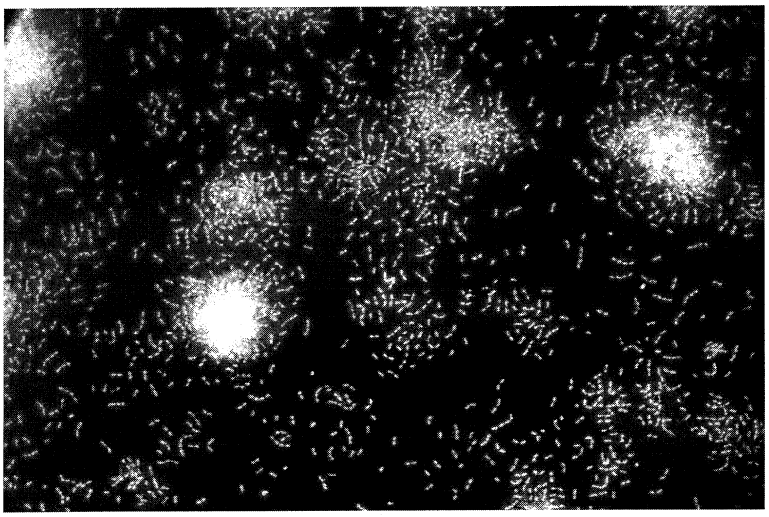

24 h
10 h
MEDIUM
MIX
1h Batch / 13h Cont. flow
WASTE
Staining
Microscopy
Image analysis

Figure 2

A



B

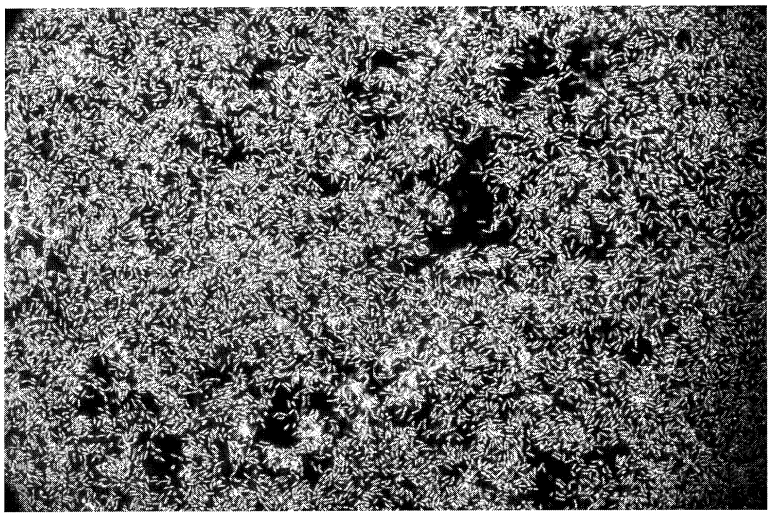
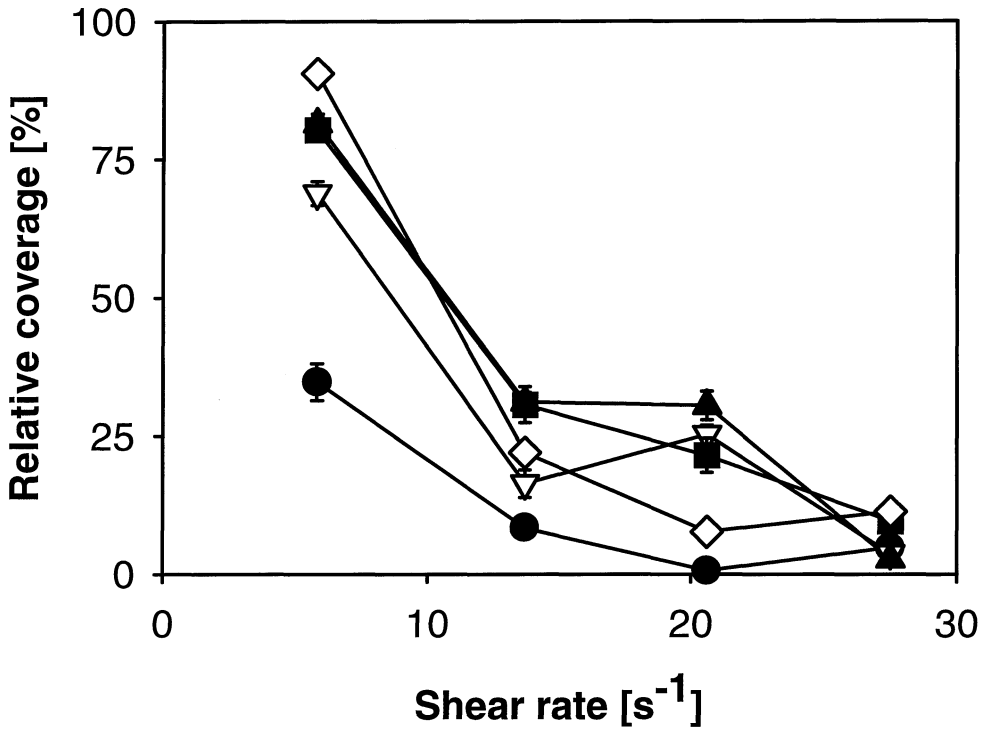


Figure 3



Appendix Paper III

Paper III is not included due to copyright.

Appendix Paper IV

Paper IV is not included due to copyright.

Appendix Paper V

Barnacle settlement on hydrogels

Short version: Barnacle settlement on hydrogels

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Abstract

Settlement of cultured *Balanus amphitrite* cypris larvae was tested on different non-solid hydrogel surfaces. Gels consisting of alginate (highly anionic), chitosan (highly cationic), polyvinyl alcohol substituted with light-sensitive stilbazolium groups (PVA-SbQ; very low cationic) and agarose (neutral) were applied in cell culture multi-well plates. Polystyrene served as a solid surface reference. Preliminary experiments were performed to test if any substances leaching out of the gels could inhibit barnacle settlement. Leachate from the gels revealed no toxicity towards *Artemia salina* nauplius larvae, whilst PVA-SbQ in solution at and above a concentration of 0.4 ppm inhibited *B. amphitrite* cypris settlement. Gels were, therefore, washed to avoid such effects during further testing, applying toxicity and settlement tests with *B. amphitrite* nauplii and cyprids, respectively, to verify that washing was effective. Settlement was tested directly on the different test materials, followed by a quality test of non-settled larvae. All gels inhibited barnacle settlement compared to polystyrene controls. Gels consisting of 2.5% PVA-SbQ or 0.5% agarose showed promising antifouling properties. Although some settlement occurred on 2.5% PVA-SbQ gels, metamorphosis was clearly inhibited. Only 10% of the larvae had settled on 0.5% agarose gels after 8 days. Less than 40% settlement occurred on alginate gels as well as 2% chitosan gels. Quality testing showed that the majority of remaining non-settled larvae in all gel experiments were able to settle when they were offered a suitable solid substratum.

Introduction

Submerged surfaces, including ships' hulls, are quickly covered with microfoulers such as bacteria, diatoms and protozoa, as well as macrofoulers such as barnacles and mussels, causing increased frictional resistance and biodeterioration (Little & DePalma, 1988). The development of self-polishing antifouling paints containing organotin derivatives was a major breakthrough in the search for new ways of protecting ships' hulls from marine colonizers. However, it has been shown that organotin derivatives may be harmful to the marine environment and its biodiversity, as reviewed by Evans (2000). The International Maritime Organization (IMO) has, therefore, agreed that tributyltin (TBT) will be phased out between 2003 and 2008. Hence, alternative ways of preventing biofouling are needed.

Barnacles are common test organisms in antifouling research. Barnacle cypris settlement is influenced by a number of environmental factors such as the hexose concentration (Neal & Yule, 1996), cyprid age and storage temperature (Rittschof *et al.*, 1984; Satuito *et al.,* 1996), and microtexture (Berntsson *et al.*, 2000) to name a few. For detailed reviews, see Crisp (1984) and Walker (1995).

The surface energy may affect the adhesion strength of barnacles (Swain *et al.*, 1998). Berglin and Gatenholm (1999) compared the antifouling properties of PMMA and polydimethylsiloxane (PDMS) surfaces. A PDMS surface has a low glass transition temperature resulting in flexible polymer chains and a low surface free energy, both contributing to good fouling release properties. In addition, it was suggested that uncrosslinked PDMS chains on the surface also prevented firm adhesion of *B. improvisus*. Holm *et al.* (2000) compared the performance of three

different silicone foul-release coatings. Rate of accumulation of fouling varied on the three surfaces, as well as removal stress of *B. amphitrite*. There are always several factors present affecting settlement, and one or several of these may be dominated by others. In addition, different barnacle species may respond in different ways to various cues such as microbial films (Avelin Mary *et al.*, 1993; Holmström *et al.*, 1992; Keough & Raimondi, 1995; O'Connor & Richardson, 1998; Wieczorek *et al.*, 1995; Lau & Qian, 2000). Maki *et al.* (1992) showed that a bacterial film of *Delaya marina* increased the surface wettability, but inhibited attachment of *B. amphitrite* cypris larvae. They later showed that there was no correlation between surface wettability and temporary adhesion strength to unfilmed polystyrene, tissue-culture polystyrene or glass (Maki *et al.*, 1994). A hypothesis based on this work was that *D. marina* inhibited fixation of *B. amphitrite* through cyprid chemoreceptors. Bacterial films may stimulate, be neutral or inhibit adhesion of barnacle cypris larvae (Maki *et al.*, 1988; Neal *et al.*, 1996). Other properties than the bacterial species may determine the effect of a biofilm on barnacle settlement. Neal *et al.* (1996) showed that both settlement and settlement behavior of *Eliminus modestus* Darwin cypris larvae were higher on a biofilm developed at high shear compared to a low shear biofilm and a biologically clean glass surface.

Thompson *et al.* (1998) discussed the difficulties of performing lab experiments to solve problems in the field. They report that both traces and presence of adult barnacles of *Semibalanus balanoides* over-ruled cues from biofilms on rocks in the field, whilst the presence of conspecifics showed no effect in the lab experiments. However, larvae preferred to settle on filmed surfaces rather than on newly exposed rocks. On the other hand, Qian *et al.* (2000) obtained similar results in the field and laboratory when they tested the effect of flow and surface characteristics on the attachment of barnacle, bryozoan and polychaete larva. The organisms from

the three taxa revealed different settlement optima with respect to flow velocity, and also preference to different surface characteristics. Olivier *et al.* (2000) performed field experiments with PMMA panels. *B. amphitrite* cyprid settlement decreased with biofilm age and density. Finally, Raghukumar *et al.* (2000) showed that the fungoid protists, thraustochytrids, appearing on submerged surfaces within 24 hours may have a significant effect on settlement of barnacles. Metamorphosis of *B. amphitrite* was higher in the presence of thraustochytrids than on both arthropodin coated surfaces and polystyrene.

As illustrated above, barnacle settlement on solid surfaces has been studied intensively. Less attention has been given to non-solid surfaces such as hydrogels, consisting of 90-99 % water. Hydrogels are viscoelastic polymer networks dominated by elastic rather than viscous properties, with the ability to bind large amounts of water. On macroscale, these gels may be recognized as surfaces, since particles and molecules larger than the pore size cannot penetrate the gel surface. However, small molecules may diffuse in and out of the gel matrix. Biofilms are often described as gel-like structures. However, on microscale biofilms and hydrogels have obvious differences. Biofilms may contain microcolonies consisting of densely packed bacteria, in this context solid surfaces with individual diameters in the order of 1 μm = 10 000 Å. Hydrogels, on the other hand, are open networks with pore diameters in the range of 50-1500 Å as observed for Ca-alginate gels (Andresen *et al.,* 1977). In addition, biofilms are both compositionally heterogeneous with respect to macromolecules and structurally heterogeneous in that they may contain voids and water channels (Stoodley *et al.*, 1994).

It has already been shown that hydrogels may be suitable for incorporating bioactive materials. Gatenholm *et al.* (1995) immobilized a marine bacterium in a polyacrylamide gel. The bacteria

produced a chemical substance that leached out of the gel and was lethal to *B. amphitrite* cypris larvae. Henrikson and Pawlik (1998) incorporated extracts from four different marine organisms into gellan gels. Gels containing extracts from the sponge *Aplysilla longispina* were effective against fouling organisms. However, there was no significant difference in fouling of control gels containing methanol instead of extract compared to that of plexiglass. Cook *et al.* (1993) found that the amount of *Pseudomonas aeruginosa* adhering to poly-hydroxyethyl methacrylate (PHEMA) based hydrogels decreased with increasing water content of the gel. His *et al.* (1996) found no toxic effects on embryonic *Crassostrea gigas* oysters from benzalkonium chloride (BCl) applied in a PHEMA hydrogel. This antifouling system remained visually free of microfouling from 12 weeks up to 5 months in the field (Cowling *et al.*, 2000). Increasing the water content of the basic hydrogel (without BCl) did not improve the performance. It was suggested that the dual action of BCl as an antimicrobial agent and as a surfactant modifying the surface properties of the hydrogel contributed to the antifouling properties of the system. Recently, Rasmussen and Østgaard (2001) showed that non-solid surfaces did not represent an absolute obstacle to settling and growth of the diatom *Amphora coffeaeformis*. However, clearly reduced attachment was observed on gels with low charge density, particularly at high shear.

The aim of this work was to test barnacle settlement on non-solid surfaces in relation to the physical and chemical properties of the gels. Preliminary tests included toxicity testing of leached material from gels. Effects of leaching water and diluted polymer solutions on barnacle settlement were also assessed. Such effects had to be eliminated before settlement on gels could be tested. After sufficient washout, settlement was tested directly on the different test gels, followed by a quality test for non-settled larvae. In order to reveal possible interactions with the polymeric gel network, cationic, anionic as well as neutral gelling substances were included.

Restricting the study to biocompatible gel formation systems, alginate (highly anionic), chitosan (highly cationic), modified polyvinyl alcohol PVA-SbQ (very low cationic) and agarose (neutral) were therefore chosen for the test program. Polystyrene served as a solid surface reference. These test gels were casted under sterile conditions, and should therefore be considered as empty and open porous polymer networks. Initially, they were completely devoid of solid structures at the size of a bacterium or larger. However, during long-term incubation at non-axenic conditions, purely chemical processes as well as microbial contamination may affect gel surface structure stability. This has to be taken into account, both by observations during incubation as well as in the final evaluation of the results.

Materials and methods

The complete experimental layout is summarized in Figure 1.

Gel preparation and application

As summarized in Table I, gels made of the following materials were tested for barnacle settlement: agarose (Sigma Chemicals Co., USA), polyvinyl alcohol substituted with light-sensitive stilbazolium groups (PVA-SbQ, Toyo Gosei Kogyo Co., ltd., Japan), alginate (LF10/60, Pronova Biopolymer A/S, Norway), and chitosan (F/A 0.17, Pronova Biopolymer A/S, Norway). Polystyrene was used as reference solid surface material.

Gelling mechanisms are pictured in Figure 2 and summarised in Table 1. Agarose consists of repeating units of 4-linked 3,6-anhydro-α-L-galactose and 3-linked β-D-galactose. When a hot

polymer solution is cooled down, double helixes are formed which will associate to create microcrystalline junction zones (te Nijenhuis, 1997). Agarose gels were made by autoclaving a solution of agarose in distilled water for 20 minutes and subsequent cooling in a water bath at 45°C. The PVA-SbQ used contained PVA with a polymerization degree of 1700, an 88 % degree of saponification and 1.3 mol% stilbazolium groups introduced as photo-sensitive functional sites. This solution was diluted using Milli Q (MQ) water to obtain the desired concentration. Gelling of the polymer solutions occurred under bright white light which initiated formation of a cyclobutane ring between two SbQ units (Ichimura & Watanabe, 1982) as shown in Figure 2. Alginate extracted from brown algae is a binary copolymer of 1-4 linked α-L-guluronic acid (G) and β-D-mannuronic acid (M), arranged in homopolymeric G- and M-blocs of different lengths as well as heteropolymeric MG sequences (Moe *et al.*, 1995). Calcium ions enables gel formation by selective and cooperative ionic interaction between different G-blocks along the alginate chains, as illustrated in Figure 2. 1% Ca-alginate gels were prepared by controlled dissociation of 15 mM $CaCO_3$ by 30 mM glucono-δ-lactone (Draget *et al.*, 1990). The concentrations of $CaCO_3$ and glucono-δ-lactone were increased 2 and 4 times in 2% and 4% alginate gels, respectively. Chitosan consist of completely or partially deacetylated chitin, which is a polymer of β(1→4) linked N-acetylglucosamine. As shown in Figure 2 two, chitosan chains can be linked through the Schiff's base reaction with glutardialdehyde. Chitosan gels (1%) were prepared by the method described by Draget (1996). Briefly, the gels were prepared by mixing 20 grams of a polymer solution with 3 ml of a 1 M MES-buffer (pH 6), and then finally 7 ml of a 5 mM glutaraldehyde solution were added.

Gel strength was determined by calculating the Young's modulus (E) from the initial slope of the force versus deformation curve (Smidsrød *et al.*, 1972) measured by a Stable Micro Systems TA-

XT2 Texture analyzer (Stable Micro Systems, England). A Dynamic Contact Angle and Absorption Tester (DAT 1121, Fibro system AB, Sweden) was used to determine static water contact angles on non-treated gels. The angle was recorded 10 seconds after the water drop had been applied on the surface.

Barnacle settlement tests were carried out in polystyrene 24-well plates (Nunclon Delta Multidish, Nalge Nunc International). For this purpose gels were casted directly in the plates, applying approximately 600 µl polymer solution in each well, see Fig. 1.

Larval culture

The *B. amphitrite* cyprids were cultured according to standard procedures (Willemsen, 1994 adapted from Rittschof *et al.*, 1992). Briefly, adult *B. amphitrite* brood stocks, maintained in plastic 10-liter carboys, with aeration and controlled temperature at 28°C on a 15h:9h light:dark cycle, were fed on a daily diet of *Skeletonema costatum* and naupliar larvae of the brine shrimp *Artemia salina*. Adults maintained under such conditions spontaneously release nauplii within 30 minutes to a few hours upon changing the water, removing the aeration and providing a concentrated light source. The photopositive larvae, collected by pipette and transferred to 8-liter carboys at a density of 1-2 larvae ml^{-1}, were fed daily with 500 ml *S. costatum* at 1-3 mill. cells ml^{-1}. The carboys were kept at a temperature of 28°C and a 15h:9h light:dark photo period. To prevent bacterial growth, a mixture of the antibiotics streptomycin and penicillin was added to the vessels at the start of the culture. When more than 75% of the larvae had reached the cyprid stage, usually after 4-5 days, the culture was filtered through plankton sieves in order to separate the cyprids from the nauplii. Cyprids were kept at 2-5°C in the dark in filtered seawater before

settlement assays were carried out. Both metamorphosed larvae and cypris larva attached to a surface by their anntennules were counted as settled. Filtered (0.2 μm) seawater was used in all experiments.

Prescreening of toxic effects of leaching water

Replicate gels of agarose (1.5%), PVA-SbQ (10%), chitosan (1%) and alginate (1%) were prepared in 24-well plates. Phosphate buffer (PBS) was added to the wells when gellation was completed. The buffer was removed after five days. Seawater was then added and changed after 24 hours. Samples were collected after 48 hours to determine the effect of leachate on barnacle settlement and *A. salina* mortality. 100 μl leachate was transferred from 8 replicates of each gel to separate wells in a 96-well plate (Nunclon Delta MicroWell Plates, Nalge Nunc International). Additional 95 μl seawater was added before precisely 10 *B. amphitrite* cypris larvae, with approximately 5 μl seawater, were transferred to each well using a pasteur pipette. Control settlement was tested in 8 wells containing 200 μl seawater. An identical test plate was prepared for testing of *A. salina* mortality. Approximately 10 *A. salina* nauplius larvae were transferred to each well. The number of permanently settled *B. amphitrite* and dead *Artemia* larvae was determined after 24 and 48 hours, respectively.

Effect of polymers in solutions

B. amphitrite settlement was tested in both leaching water and diluted polymer solutions of PVA-SbQ. Leaching water was prepared by adding seawater to a 500 ml glass bottle containing 40 ml of a 10% PVA-SbQ gel. The concentration of PVA-SbQ in solution was determined after five

days by measuring the absorbance at 340 nm using a UV/visible spectrophotometer (Ultraspec 2000, Pharmacia Biotech). A 13.17% polymer solution was diluted to 0.5% using MQ water to avoid precipitation. Both solutions were diluted to 10, 1.0, 0.10, and 0.010 ppm with seawater. Four replicates of 200 µl were prepared for each concentration in a 96-well plate. *B. amphitrite* cypris settlement was tested in the diluted leaching water, polymer solutions and in 4 control wells containing 200 µl seawater. The commercial antifouling biocide SeaNine-211 [4,5-dichloro-2-octyl-3(2H)isothiazolone] was included as a reference. The experiment was repeated with a narrower concentration range; 10, 3.3, 1.1, and 0.37 ppm.

Alginate and chitosan solutions (1.5%) were diluted to 10, 1.0, 0.10, and 0.010 ppm with seawater. Four replicates of 200 µl were prepared for each concentration in a 96-well plate. *B. amphitrite* cypris settlement was tested in the diluted polymer solutions and in 4 control wells containing 200 µl seawater. Agarose was not tested in solution due to low solubility in seawater at 28°C.

Settlement on gels

See Fig. 1. Gels were prepared in 24-well plates at the concentrations indicated in Table I. Since polystyrene is an attractive target for cyprid settlement, the wellplates were rotated by hand to obtain a gel coating of the walls as well as the bottom of the wells. However, if settled cyprids or barnacles were still observed on exposed polystyrene, those individual organisms were excluded from the data set.

PVA-SbQ gels were leached for 20 days in 70% seawater (filtered and autoclaved) changed once or twice a day, to reach an acceptable leachate level below 0.4 ppm. Since the effect of leaching water from agarose and alginate gels was insignificant (see Table II and the results section), these gels were only leached for 5 and 3 days, respectively. Chitosan gels were leached for 12 days with PBS instead of seawater to avoid collapse of the gel, which may occur at high pH.

After this pre-treatment, tests were performed to verify that the leaching was sufficient. Two replicates of 200 µl from each gel concentration and each well plate were prepared in a 96-well plate. Precisely 10 *B. amphitrite* cypris larvae were transferred to the first replicates, and approximately 15 *B. amphitrite* nauplii were transferred to the others. Four controls with seawater were prepared for both cyprids and nauplii.

Then, 1 ml seawater was added to 8 replicates of each gel concentration. In addition, 8 control wells located in the same well plate as the alginate gels and 8 control wells in a new well plate were used. Precisely 15 *B. amphitrite* larvae from a single batch stored for 8 days in the refrigerator were added to each well. Seawater was changed for all treatment after 2, 4, and 7 days by carefully removing and adding 800 µl 3 times. Non-settled larvae were removed from two of the eight replicates on day 6 for quality testing as described below. The other wells were monitored for a total of 9 days.

The experiment was repeated with one concentration of each polymer in 6 replicates: 4% PVA-SbQ and 1% agarose, chitosan, and alginate gels. A younger *B. amphitrite* batch, stored for only one day in the refrigerator, was applied in this experiment. Seawater was changed each day as described above. After 7 days non-settled larvae were removed for quality testing (see below).

Quality of non-settled larvae

In the first experiment, where different gel concentrations were tested, all non-settled larvae from 2 replicates were transferred on day 6 to a single well in a 24-well reference plate containing 1 ml seawater. Immediately after the transfer, seawater was changed 3 times as described above. Settled and non-settled *B. amphitrite* larvae were counted after 1, 2, and 3 days. Non-settled larvae from the six replicates in the repeated experiment were transferred to separate wells in a 24-well reference plate on day 7. Settlement percentage was assessed after 1, 3, and 5 days.

Statistical methods

Analysis of variance (ANOVA) was performed on settlement data using MINITAB™ (version 13.1, Minitab Inc.). Dunnett's test applies to experiments where different treatments are compared to a control (Walpole *et al.*, 1998). This test was, therefore, applied on experimental data where effects of leaching water on *B. amphitrite* settlement and *A. salina* mortality were assessed as well as results from testing *B. amphitrite* settlement in diluted polymer solutions of alginate and chitosan. Significant differences in settlement of *B. amphitrite* on different gels were also assessed (Walpole *et al.*, 1998). The analysis was performed on two separate days, in addition, the different gels were treated separately. Percent settlement in 8 replicates for each treatment i.e. gel concentration were put into the "General Linear Model" of Minitab™, and a pairwise comparison was run applying Tukey's test (Walpole *et al.*, 1998).

Results

Gel characteristics

The gel strength was determined by estimating the Young's modulus for each gel concentration. Figure 3 A shows that there was a significant increase in stiffness with increasing polymer content for all gels. Comparing gels with 2% polymer content reveals that the Young's modulus of agarose was approximately 1, 2 and 3 orders of magnitude higher than the moduli of alginate, chitosan, and PVA-SbQ, respectively.

In order to estimate the surface free energy, wettabilities of the different gels were determined by contact angle measurements. Figure 3 B shows that the wettability of PVA-SbQ and chitosan gels decreased with increasing polymer content. The opposite effect was observed for agarose gels. Water contact angles on 1, 2, and 4% alginate gels were approximately 20°, indicating highly wettable gels, opposed to PVA-SbQ gels with contact angles between 80 and 100°, indicating a more hydrophobic surface.

Prescreening of toxic effects of leaching water

Glutaraldehyde, the crosslinking agent in chitosan gels, is a well-known toxic compound, and unreacted molecules could inhibit or kill the cyprid larvae. It has also been reported that excess calcium ions, used for alginate crosslinking, may inhibit *B. amphitrite* cyprid settlement (Clare, 1996; Rittschof *et al.*, 1986). In addition, unlinked low molecular weight polymer chains could

leach out of the gels and potentially affect settlement. For the above reasons, a prescreening of potential toxic effects was necessary.

Table II shows the results of testing *B. amphitrite* settlement inhibition and *A. salina* mortality in leaching water from agarose, PVA-SbQ, alginate and chitosan gels. Analysis of variance revealed significant effect of leaching water from PVA-SbQ ($p < 0.00050$) and chitosan ($p = 0.0020$) on settlement of *B. amphitrite* cypris larvae. No significant effects were detected for agarose ($p = 0.053$) or alginate gels ($p = 0.99$). The same leaching water showed no effect on *A. salina* mortality (see Table II).

Effects of polymers in solution

Barnacle settlement was tested in polymer solutions of PVA-SbQ, chitosan and alginate at known concentrations. In addition, leaching water from a PVA-SbQ gel was prepared to see if material leaching out of the gel affected the larvae in a different manner than the non-gelled polymer. The larval activity in both PVA-SbQ leaching water and the non-gelled polymer solutions was remarkably high compared to controls. Figure 4 A shows the inhibition of *B. amphitrite* cypris larvae at different concentrations of leaching water from a PVA-SbQ gel as well as diluted polymer solutions and SeaNine-211 as reference. The 24 hour $EC_{50} \pm 95\%$ confidense interval for leaching water and diluted polymer, determined according to the trimmed Spearman-Karber method (Hamilton *et al.*, 1977), were 1.1±0.9 and 0.70±0.50 ppm, respectively. This shows that the molecules leaching out of the gel have the same effect as the diluted polymer. Hence, a diluted polymer solution can be used as standard for determining the content of PVA-SbQ in leaching water for the purpose of these experiments. Moreover, the concentration of PVA-SbQ should not exceed 0.4 ppm for *B. amphitrite* settlement tests to avoid inhibitory effects.

Figure 4 B shows the relative inhibition of *B. amphitrite* cypris larvae at different concentrations of diluted polymer solutions of chitosan and alginate compared to control wells. Analysis of variance indicated no significant effect of the two polymers. The effect of leaching water from chitosan gels (see Table II) is, therefore, most likely due to excess glutaraldehyde. In conclusion, both PVA-SbQ and chitosan gels should be rinsed thoroughly and leaching water should be tested before conducting settlement experiments.

Quality testing of leaching water before settlement testing on gels

Since it has been shown that *B. amphitrite* nauplii are more sensitive than *Artemia sp.* to several compounds (Saskikumar *et al.*, 1995), we tested both *B. amphitrite* cypris settlement and *B. amphitrite* nauplii mortality in the leaching water from gels applied in the settlement experiments, see below. These results are presented in Table III. Highest mortality was observed in leaching water from 0.5% chitosan gels, where the average survival was 96%. Cyprid settlement was normal in all wells. It was, therefore, concluded that leaching was sufficient to run the settlement tests on gels without toxic interference.

Gel surface stability

Although gels were casted under sterile conditions, microbial contamination could not be avoided during incubation of larvae. Long-term incubation at non-axenic conditions necessitated visual inspection of the gel surfaces. No visual damages to or changes of the agarose gels were observed. In the wells with PVA-SbQ a white precipitate attaching to larvae and barnacles was

observed from day 5 on 10% gels and to a less extent on 7% gels. It was absent on 4 and 2.5 % gels. On alginate gels, a white precipitate appeared from day 3, also covering the front and back of the larvae, and on the top of the juvenile barnacles. At the end of the experiment, the gels appeared slimy, and it seemed as if they were approaching a viscous rather than a gel state. Alginate gels tended to dissolve in the repeated experiment as well. On chitosan gels, a white precipitate was observed on day 3. The amount was higher on 2% gels than on 0.5% gels. Cracks appeared in some of the 1 and 2% gels, and the volumes of all chitosan gels were clearly diminished.

Settlement on gels

Agarose

Settlement of *B. amphitrite* cypris larvae on different concentrations of agarose gels and polystyrene controls is shown in Figure 5 A. All agarose gels inhibited settlement compared to polystyrene. Settlement was delayed on 4% and on 0.5% gels by approximately 3 and 5 days, respectively, compared to 1% and 2% gels. The number of settled larvae had stabilized within 8 days, and the level was significantly lower on the gels than on controls. Lowest settlement was achieved on 0.5% gels. Analysis of variance showed that settlement on 1% gels was significantly higher than on any of the other gels on day 1. On day 6, the number of settled larvae on 0.5% gels was significantly lower than on the other gels. Both non-settled cypris larvae and the barnacles remained active in the agarose wells throughout the experiment.

PVA-SbQ

Settlement of *B. amphitrite* cypris larvae on the four PVA-SbQ gels is shown in Figure 5 B. PVA-SbQ gels also inhibited settlement compared to polystyrene. Analysis of variance indicated no significant difference in percent settlement between the gels on day 1. The number of settled larvae increased significantly on 10 and 7% gels between days 1 and 2. On 4% gels, larvae settled gradually until day 7. Settlement on 2.5% gels was delayed by approximately 4 days. On day 6, settlement on 10% gels was significantly higher than on both 2.5 and 4% gels. The number of settled larvae was significantly higher on 7% gels than on 2.5%. All *B. amphitrite* cypris larvae were active during the first three days. Activity declined, particularly in wells with 10 and 7% gels. Metamorphosis was very low on 2.5% gels.

Alginate

Settlement on the three alginate gels is shown in Figure 5 C. Alginate gels were clearly less attractive as a substratum for *B. amphitrite* cypris settlement than polystyrene. The number of settled larvae leveled out after day 1 on all gels. Maximum 20% and 40 % of the larvae settled on 1% and 2% gels, respectively, whilst settlement on 4% was somewhere in between. Analysis of variance revealed no significant difference in settlement on alginate gels on days 1 or 6.

Chitosan

B. amphitrite cypris settlement on chitosan gels is shown in Figure 5 D. Chitosan gels inhibited settlement compared to polystyrene. Settlement on 0.5% and 1% gels increased linearly for 6 days and leveled out at approximately 65-70%. On 2% gels, only minor increase in settled larvae occurred from day 3, culminating at approximately 35%. Analysis of variance revealed no significant difference in relative settlement on chitosan gels on day 1. On day 6, settlement on

0.5% gels was significantly higher than on 2% gels. Two replicates were harvested for the quality test on day 6. Due to a higher settlement in these wells, an apparent decline in settlement is shown in the recordings between days 6 and 7 on the remaining 2% gels (Fig. 5 D). Larval activity was generally lower in the presence of chitosan gels than agarose and PVA-SbQ. Larvae on 0.5% and 1% gels were more active than those on 2% gels.

Selected concentrations

Settlement of *B. amphitrite* cypris larvae on polystyrene and selected concentrations of agarose, PVA-SbQ, alginate and chitosan gels is shown in Figure 6. All gels inhibited settlement. Settlement was very low on all gels after 1 day incubation, while control settlement was 70%. On chitosan and agarose gels, settlement was delayed with 1 and 2 days, respectively. Analysis of variance indicated no significant difference in settlement on gels on day 1. The number of settled larvae after 7 days on chitosan and agarose was 85% and 50%, respectively. There was no significant difference in the number of settled larvae on PVA-SbQ and alginate on day 7, with a settlement of less than 10%. Larvae were generally active on all gels.

Quality of non-settled larvae

To assess the quality of viable but non-settled larvae, a sub-sample was transferred on day 6 to pure polystyrene wells. In the case of alginate gels; the larvae were pumped in and out of a pasteur pipette several times to break any precipitate coating and remove adhered polymers before transfer to new wells for quality testing. Data for larvae originating from incubations with gels of agarose, PVA-SbQ, alginate and chitosan are presented in Table IV A. Settlement after 3 days is indicated as well as total number of larvae tested. Even if larvae are offered an attractive

surface such as polystyrene, not all of them will be expected to settle as shown in Figures 5 and 6. For this reason, samples with less than 8 larvae were discarded from the dataset. Larvae from all gels settled on polystyrene.

Table IV B presents the results from the quality test of non-settled larvae from the repeated experiment. Relative settlement after 3 days is indicated as well as the sample size. Settlement on chitosan gels was high (see Figure 6), and due to a low number of non-settled larvae, chitosan was excluded from the quality test. Non-settled larvae from the other three gels settled well on polystyrene as indicated.

Discussion

Crisp (1974) divided the exploration period of barnacles prior to metamorphosis in three phases; "broad exploration", "close examination" and "inspection". In the inspection phase, barnacle cypris larvae walk on the surface using the antennules. These are equipped with an adhesive disk responsible for adhering the "foot" to the surface. The adhesive is produced in unicellular antennulary glands (Nott & Foster, 1969), secreted through the disks, and left behind as proteinacious foot prints (Walker & Yule, 1984; Clare *et al.*, 1994). According to Naldrett (1993), the hydrophobic adhesive is crosslinked through cysteine linkages. Crisp and Meadows (1962) proposed that cyprids respond to arthropodins as a positive settlement factor and, moreover, that the larvae can chemically sense the suitability for settlement. Clare and Nott (1994) believe that the fourth antennular segment sense the surface through flicking during the exploration period. In our work *B. amphitrite* cypris larvae settled more easily to polystyrene than to gels of agarose, PVA-SbQ, alginate, and chitosan, suggesting a much stronger interaction

between the adhesive and polystyrene than to the hydrogels. Possible factors contributing to these results are discussed below.

Effect of polymers in solution

Others have applied PVA-SbQ gels for gel entrapment of different microbes and cells. It has been shown that plant cells, microalgae and bacteria were not inhibited by immobilization inside the PVA-SbQ gel matrix (Nakajima *et al.*, 1986; Hertzberg *et al.*, 1995; Vogelsang *et al.*, 1999). Thus the reactivity of the light sensitive substituents does not seem to interfere with cell activity.

In our experiments PVA-SbQ in solution inhibited settlement of *B. amphitrite* cypris larvae at concentrations above 0.4 ppm. The swimming activity was higher than in controls when exposed to PVA-SbQ, indicating a stimulatory action. This polymer has a low net positive charge, and it may interact with the adhesive of the cyprids as was suggested for different hexoses and a pentose by Neal and Yule (1996). Although alginate consists of the polar hexoses α-L-guluronic acid and β-D-mannuroic acid units, no inhibitory interaction was detected. Chitosan is a positively charged polymer at neutral pH. At the pH of seawater, the amino groups are discharged and the polymer has no net charge. This may result in aggregation or adsorption to surfaces, and thus will not necessarily interact with swimming cyprids.

Gel surface stability

Adsorption of a conditioning film consisting of natural polymers is generally regarded as the first step in the colonization of a submerged surface (Little & DePalma, 1988; Loeb & Neihof, 1975).

This will increase the wettability of the surface (Dexter, 1978). In our work, polymers originating from the natural seawater applied prior to and during the experiments, may have adsorbed to the gels, if present. In addition, excretion products from the larvae and settled barnacles may have been present, and since only a monomolecular layer is necessary to change the physico-chemical surface properties of the material, some effects of a conditioning film cannot be completely excluded. Interactions between such a bacterial contamination and barnacle settlement are complex and not easily predetermined (Maki *et al.*, 1992, 1994; Neal *et al.*, 1996; Olivier *et al.*, 2000; Thompson *et al.*, 1998). This is clear from the work of Maki *et al.* (1988), who tested the effect of 18 bacterial species on B. amphitrite settlement. Most of the bacteria either inhibited or were neutral, and only one species stimulated settlement. However, in our work conditioning films and bacteria would also have been present in the controls where settlement was high. Since settlement varied strongly between different surfaces, the effects of such films should be negligible.

The disintegration of alginate gels affected settlement possibly through immobilization by polymers sticking to the larvae. In addition, it seemed as if the gels were approaching a viscous rather than a gel state at the end of experiment, making settlement more difficult. It is possible that alginate lyase of e.g. bacterial origin caused the degradation of these gels (Sutherland, 1995).

Chitosan is a positively charged polymer at lower pH. At the pH of seawater, the amino groups are discharged and the gel matrix may eventually collapse. Gels will therefore, upon exposure to seawater, slowly become more and more rigid until they collapse and end up as a solid film, in accordance with our observations. The transition from non-solid to more rigid structures may have increased the settlement on these surfaces.

Gel stiffness

Settlement increased with increasing polymer concentration, and thus with increasing stiffness of the PVA-SbQ gels, in accordance with the model applied for elastomeric coatings by Brady and Singer (2000). An opposite effect was actually observed for adhesion of the diatom *Amphora coffeaeformis* to these gels (Rasmussen & Østgaard, 2001). In this recent study, it was found that the critical transition in viscoelastic properties occurred around 1.7% (results not included). Below this concentration, the polymers will not form a gel, and the polymer solution may behave rather like a glue. Gels consisting of 5-10% PVA-SbQ are highly elastic. When the concentration approaches the critical transition, the viscous properties become more prevailing. This may explain why non-settled larvae adhered to the soft 2.5% gels although the antennules were not connected to the surface, and why metamorphosis was inhibited among settled cypris larvae.

Settlement on agarose gels was not correlated to the Young's modulus. Gels consisting of 1 and 2% agarose attained a higher settlement than 0.5 and 4% gels. Agarose gels were generally rigid compared to the gels of PVA-SbQ, and cues other than the stiffness have obviously influenced settlement on these gels. Neither on alginate nor on chitosan gels could settlement be correlated to gel strength. However, the Young's moduli of chitosan gels shown in Figure 3 A are not representative for the gels applied in the experiments, see gel surface stability above.

Pore size

The gels applied in this work contained from 90 to 99.5% water. Øyaas *et al.* (1995) reported effective diffusion coefficients for mono- and disaccharides in 2% Ca-alginate gel beads to be

85% of their respective diffusion coefficients in water. Moreover, on a microscale, the gel can be regarded as a compartment of stagnant water where mass transport occurs by diffusion only. Pore diameters of Ca-alginate gels in the range 5-150 nm were observed by Andresen *et al.* (1977). It is obvious that a barnacle cypris larva approximately 500 μm in length will not be able to enter such a gel matrix. However, this does not imply that the cyprids recognize the gels as solid surfaces. Settlement on agarose, PVA-SbQ, and alginate was lowest on those gels with the lowest polymer content, and hence, the largest polymer interdistances. It may be possible that the cypris larvae can sense the pore size of the gels, e.g. by flicking of the fourth segment of the antennules during the exploration period, and thus reject surfaces based on polymer interdistances.

Surface free energy

Swain *et al.* (1998) showed that the surface energy affects the adhesion strength of barnacles. The positive antifouling properties of polydimethylsiloxane based coatings was attributed to their low surface free energy and low glass transition temperature by Berglin and Gatenholm (1999). On the other hand, Maki *et al.* (1994) found no correlation between surface wettability and temporary adhesion strength of *B. amphitrite* to unfilmed polystyrene, tissue-culture polystyrene and glass. Water contact angles measured on PVA-SBQ gels shown in Figure 3 B indicate a low surface free energy. In addition, the surface free energy decreased with increased polymer concentration. Compared with the settlement results shown in Figure 5 B, settlement seemed to increase with decreased surface free energy on PVA-SbQ gels. It is, therefore, likely that for these gels other factors, such as gel strength, are more important for *B. amphitrite* settlement than the surface free energy. The surface free energy of agarose increased with increased polymer concentration. Thus, the 0.5% gels had both the lowest surface free energy and gel strength,

which resulted in low settlement. However, this does not explain why 4% gels gave lower settlement than both 1 and 2% gels.

Quality of non-settled larvae

The percentage of settled larvae on gels never reached the same level as on polystyrene. Non-settled larvae were transferred to new polystyrene wells containing seawater to test if they had lost their ability to settle or if it was the gels that prevented settlement. Larvae from all gels settled to polystyrene (Fig. 7). Hence, the cyprids had not lost their settling ability, moreover, the gels were just not suitable for settlement. Although asepsis was pursued, the influence of bacterial or fungal contamination cannot be completely excluded in the long-term experiments, particularily if the polymer gels may have acted as a carbon source for such infections. However, there are several indications that such effects were small, if present. First of all, non-settled larvae were still able to settle on polystyrene surfaces even after long term incubations with gels. Secondly, the precipitate appearing in the wells of PVA-SbQ and chitosan gels was clearly time and concentration dependent. Finally, there are no irregular changes in the settlement data presented in Figures 5 and 6, as would have been expected if growing infections became significant.

Concluding remarks

In conclusion, it has been shown that all the gels tested inhibited settlement of *B. amphitrite* cypris larvae. The polymer network influenced the degree of inhibition with respect to both polymer density and type. None of the polymers were toxic to *B. amphitrite* cypris larvae or *A.*

salina nauplius larvae. The majority of non-settled larvae in all gel experiments was able to settle when offered a suitable solid substratum. As pointed out by Thompson *et al.* (1998), field experiments are invaluable when assessing the antifouling properties of new coatings. All four gels are, therefore, currently being tested for *in vivo* antifouling performance on a test raft in Sandefjord, Norway.

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Table I Gel characteristics: Polymer charge, gelling principle and polymer concentrations of gels applied in this work.

Polymer	Charge	Gelling principle	Polymer concentrations tested [%, wt/wt]
Agarose	Neutral	Thermic; cooling	0.50; 1.0; 2.0; 4.0
PVA-SbQ	Low cationic	Photoinduced crosslinking	2.5; 4.0; 7.0; 10
Alginate	Anionic	Ca^{2+} crosslinking	1.0; 2.0; 4.0
Chitosan	Cationic	Glutardialdehyde crosslinking	0.50; 1.0; 2.0

Table II Prescreening of inhibitory or toxic effects of leaching water from non-treated agarose, PVA-SbQ, alginate and chitosan gels on (A) *B. amphitrite* settlement and (B) *A. salina* mortality. C.I. = 95% confidence interval; P-value = P-value resulting from Dunnett's test (ANOVA) with polystyrene as control.

A. *B. amphitrite*	Gel type	Settlement inhibition [%]	C.I.	ANOVA, *p*-value
	Control	0	15	
	Agarose	39	23	0.0531
	PVA-SbQ	100	0	< 0.0005
	Alginate	2	31	1.00
	Chitosan	59	25	0.00200
B. *A. salina*	Gel type	Mortality [%]	95% C.I.	ANOVA, *p*-value
	Control	11	8	
	Agarose	14	6	0.975
	PVA-SbQ	7	6	0.866
	Alginate	10	7	1.00
	Chitosan	11	8	1.00

Table III Quality testing of leaching water. Effect of leaching water from thoroughly washed gels of agarose, PVA-SbQ, alginate and chitosan on *B. amphitrite* settlement and *B. amphitrite* nauplii mortality.

Gel type	Polymer concentration	*B. amphitrite* cyprid settlement [%]	*B. amphitrite* nauplii mortality [%]
Control		73	0
Agarose	4	65	0
Agarose	2	60	0
Agarose	1	77	0
Agarose	0.5	60	0
PVA-SbQ	10	60	0
PVA-SbQ	7	65	0
PVA-SbQ	4	63	0
PVA-SbQ	2.5	75	0
Alginate	4	70	0
Alginate	2	60	0
Alginate	1	80	1.5
Chitosan	2	70	0
Chitosan	1	73	2.8
Chitosan	0.5	60	4

Table IV Quality of non-settled *B. amphitrite* larvae from (A) settlement experiment with different gel concentrations and (B) repeated experiment with one concentration of each gel.

	Gel type	Polymer concentration [%]	Sample size	Settlement after 3 days [%]
A.	Agarose	4	11	27
	Agarose	2	9	44
	Agarose	1	5	n.d.[1]
	Agarose	0.5	9	11
	PVA-SbQ	10	1	n.d.
	PVA-SbQ	7	7	n.d.
	PVA-SbQ	4	7	n.d.
	PVA-SbQ	2.5	14	21
	Alginate	4	13	54
	Alginate	2	16	38
	Alginate	1	17	35
	Chitosan	2	11	18
	Chitosan	1	7	n.d.
	Chitosan	0.5	10	40
B.	Agarose	1	32	47
	PVA-SbQ	4	65	41
	Alginate	1	84	88
	Chitosan	1	<8	n.d.

[1] n.d. = no data; samples with less than 8 larvae were discarded from the dataset, see text.

Figure legends

Figure 1 Experimental layout.

Figure 2 Gelling mechanisms of agarose, PVA-SbQ, alginate, and chitosan. See text for details.

Figure 3 Gel characteristics. (A) Youngs moduli [N m^{-2}] calculated from the initial slope of the force-deformation curve for agarose (△), PVA-SbQ (○), alginate (□), and chitosan (◇) gels. Error bars represent the 95% confidence intervals. (B) Static water contact angles measured on gels of agarose (△), PVA-SbQ (○), alginate (□), and chitosan (◇). Error bars represent the 95% confidence intervals.

Figure 4 Settlement inhibition [%] of *B. amphitrite* cypris larvae in (A) different concentrations of PVA-SbQ polymer solutions (○), leachate from PVA-SbQ polymer gel (●), SeaNine-211 (✚), and (B) different concentrations of alginate- (□) and chitosan (◇) polymer solutions.

Figure 5 Settlement of *B. amphitrite* cypris larvae on polystyrene (✚) and (A) 0.5 (◇), 1(□), 2(○), and 4%(△) agarose gels; (B) 2.5 (◇), 4(□), 7(○), and 10% (△) PVA-SbQ gels; (C) 1(□), 2(○), and 4%(△) alginate gels; (D) 0.5 (□), 1(○), and 2%

(△) chitosan gels. Each data point from day 1 through day 6 represents the mean of 8 replicates. Two replicates were withdrawn from the quality test on day 6, hence, data points on days 7 and 8 represent means of 6 replicates. Error bars indicate 95% confidence intervals.

Figure 6 Settlement of *B. amphitrite* cypris larvae on gels consisting of 1% agarose (△), 4% PVA-SbQ (○), 1% alginate (□), 1% chitosan (◇) and polystyrene (✚) as reference. Each data point represents the mean of 6 replicates. Error bars indicate 95% confidence intervals.

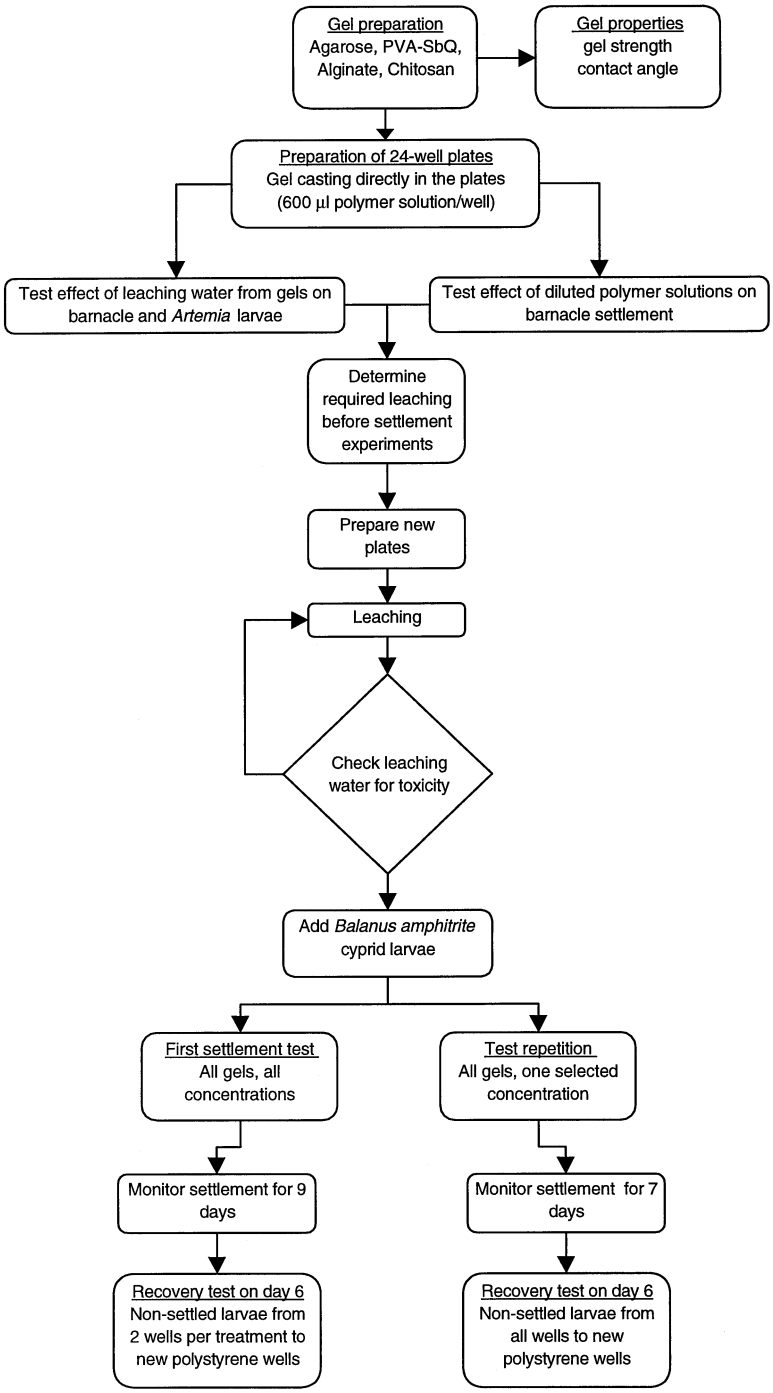

Gel preparation
Agarose, PVA-SbQ, Alginate, Chitosan
Gel properties
gel strength
contact angle
Preparation of 24-well plates
Gel casting directly in the plates
(600 µl polymer solution/well)
Test effect of leaching water from gels on barnacle and Artemia larvae
Test effect of diluted polymer solutions on barnacle settlement
Determine required leaching before settlement experiments
Prepare new plates
Leaching
Check leaching water for toxicity
Add Balanus amphitrite cyprid larvae
First settlement test
All gels, all concentrations
Test repetition
All gels, one selected concentration
Monitor settlement for 9 days
Monitor settlement for 7 days
Recovery test on day 6
Non-settled larvae from 2 wells per treatment to new polystyrene wells
Recovery test on day 6
Non-settled larvae from all wells to new polystyrene wells

Figure 1

Figure 2

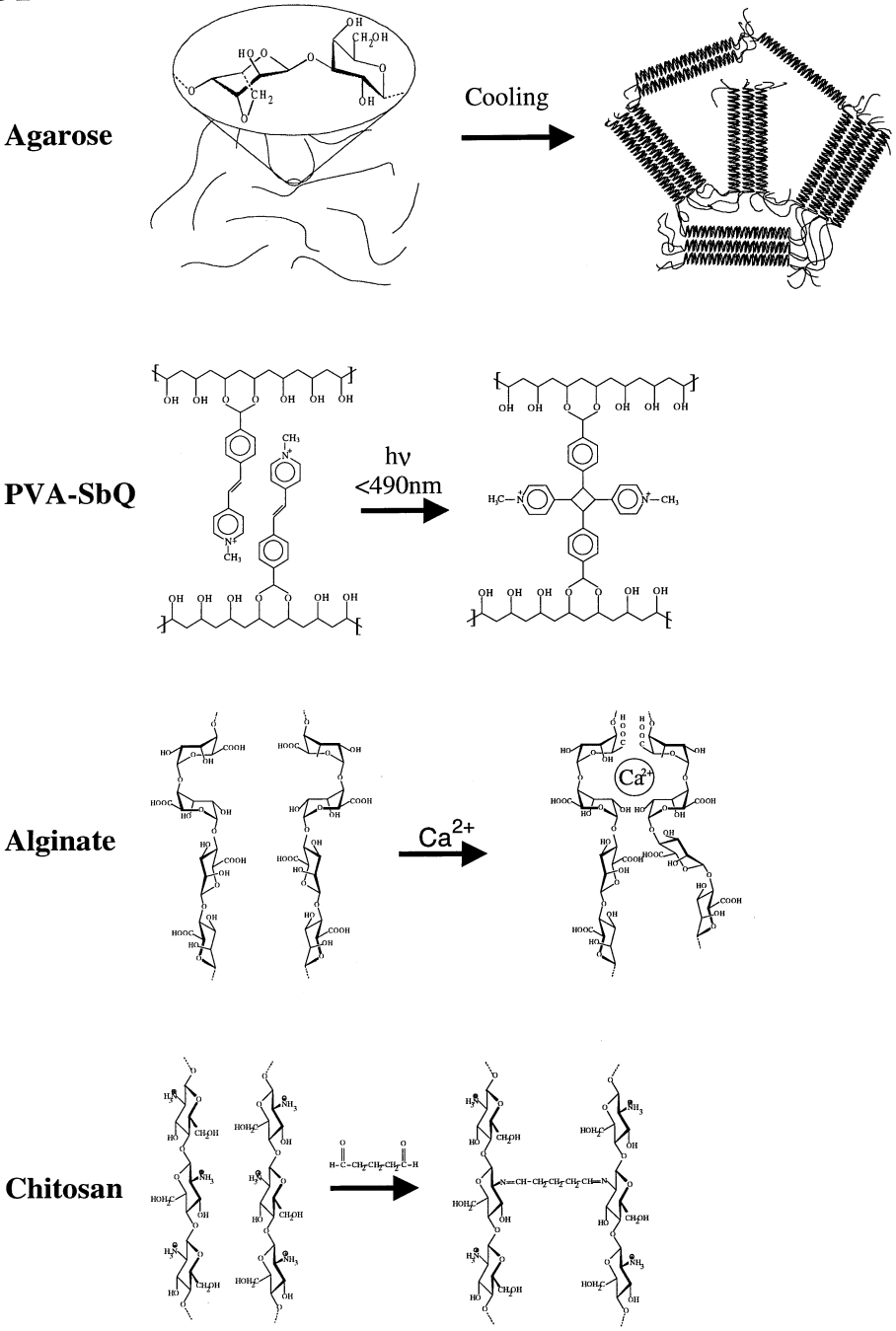


Figure 3

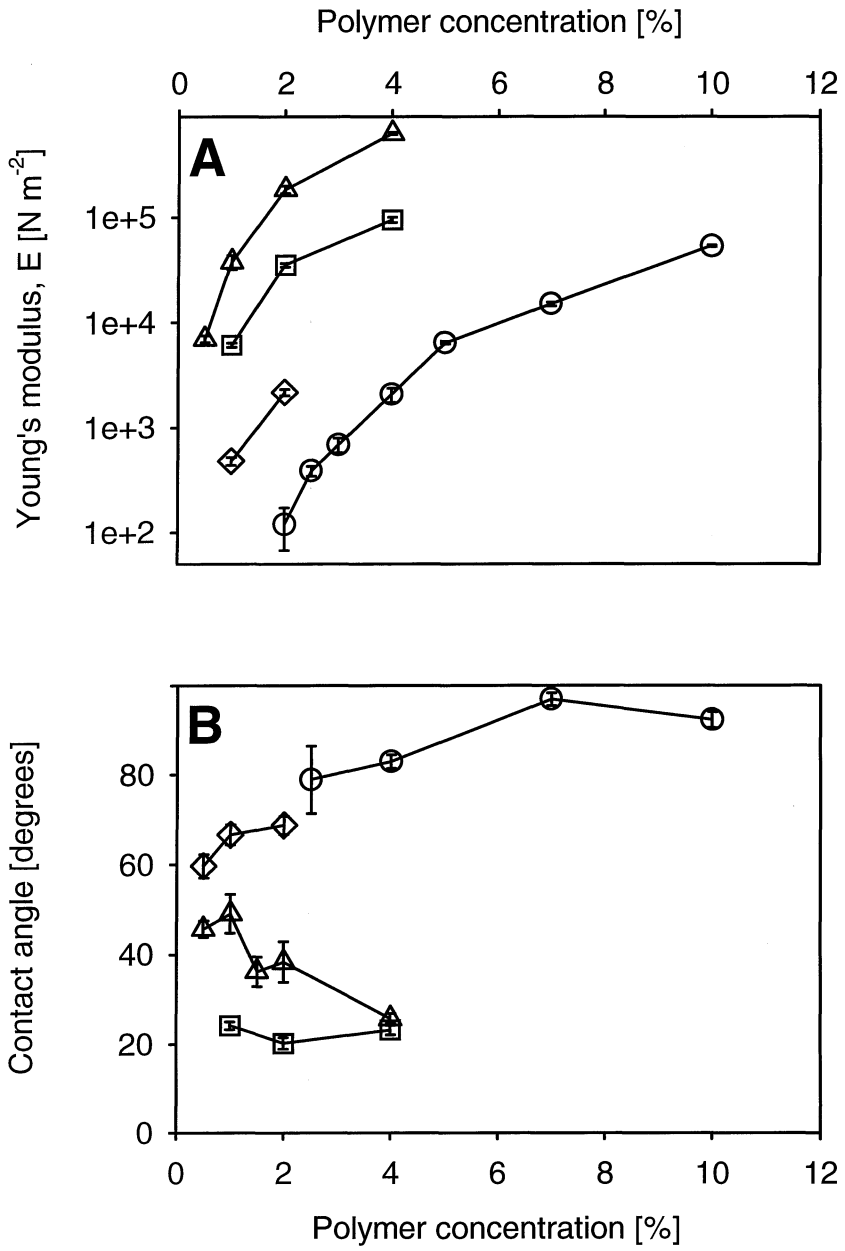
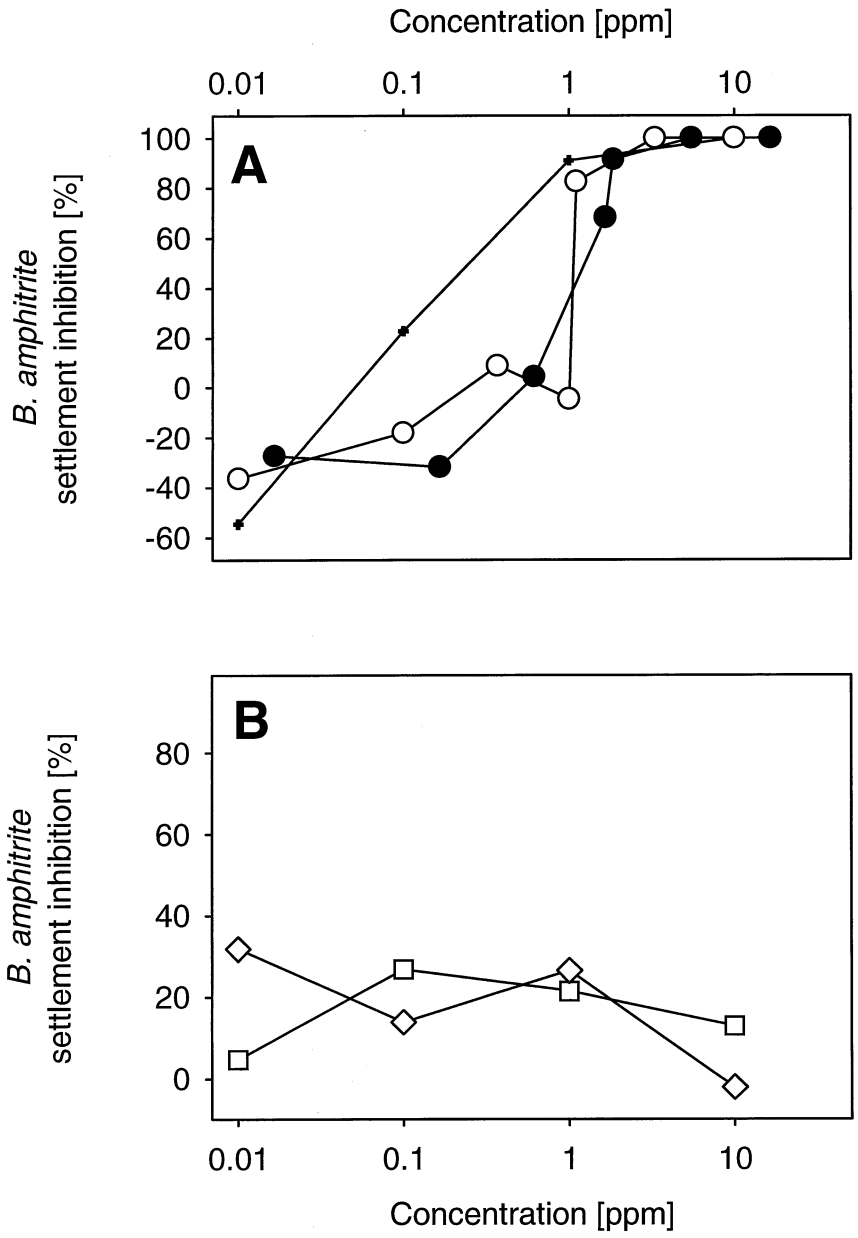

Polymer concentration [%]
0
2
4
6
8
10
12
A
Young's modulus, E [N m-2]
1e+5
1e+4
1e+3
1e+2
B
Contact angle [degrees]
80
60
40
20
0
Polymer concentration [%]

Figure 4


Concentration [ppm]
0.01
0.1
1
10
A
B. amphitrite
settlement inhibition [%]
100
80
60
40
20
0
-20
-40
-60
B
80
60
40
20
0
0.01
0.1
1
10
Concentration [ppm]

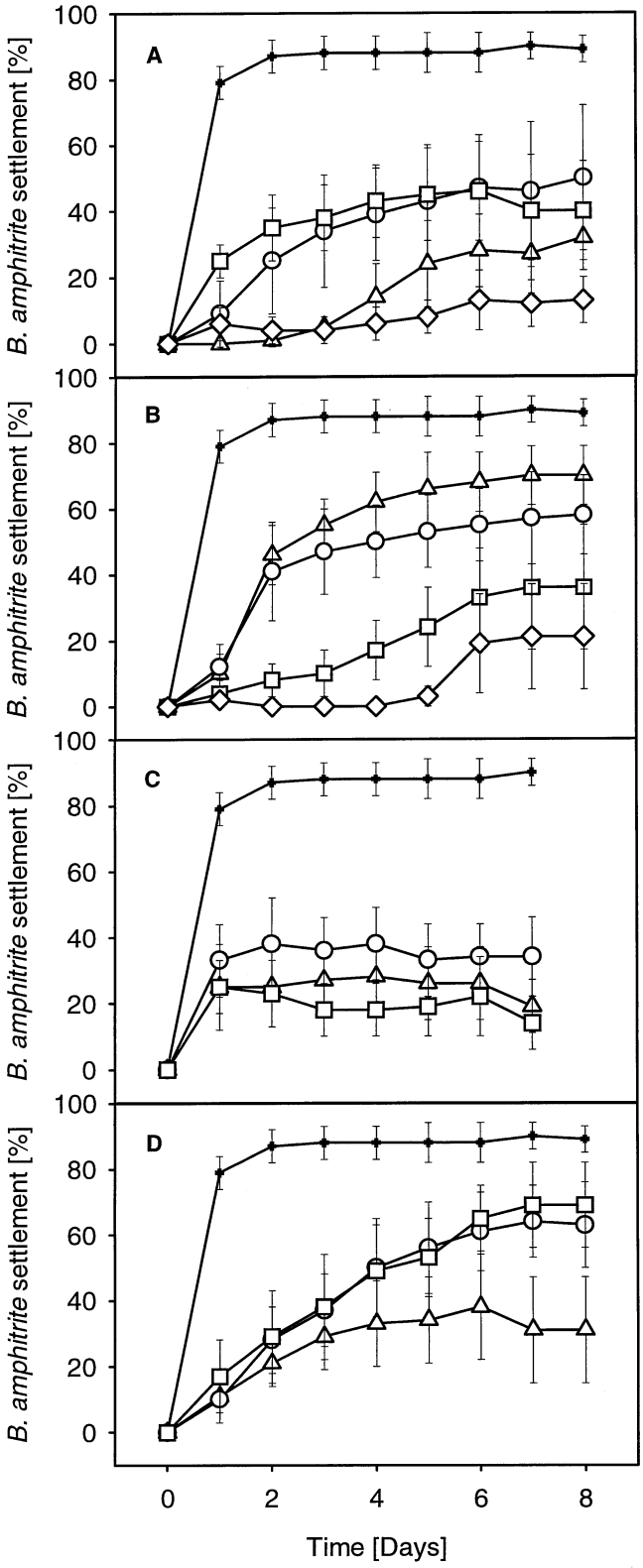

A
B
C
D
B. amphitrite settlement [%]
0
20
40
60
80
100
0
2
4
6
8
Time [Days]

Figure 5

Figure 6