BEHAVIOURAL BIOASSAYS FOR NON-BIOCIDAL COATINGS

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ABSTRACT

Within hours, any undefended structure immersed in the marine environment will become fouled: a term known as biofouling. This phenomenon causes substantial economic losses and affects not only shipping vessels but also static structures. Although metal biocides added to coatings have proved very effective against biofouling, there were increasing concerns about the detrimental effects these were having on non-target species. The problem facing manufacturers of these coatings is the lack of available testing methods for non-biocidal antifouling coatings. This thesis aims to develop a framework for a suite of behavioural bioassays to investigate the efficiency of non-biocidal coatings.

The research represents methodological investigations coupled to pilot studies. Three fouling species were investigated, *Spirorbis borealis*, *Balanus amphitrite*, and *Balanus improvisus*. All three species showed significant differences in behaviour on non-biocidal coatings supplied by Akzo Nobel and these behaviours could be used to discriminate between coatings.

Immersion trials were carried out in Sweden, Singapore and the UK, in order to ascertain whether behavioural parameters of the larvae in the laboratory could be used to predict fouling observed in the field. All three species demonstrated that aspects of their behaviour could be used to predict fouling at least at one location, with both *Spirorbis borealis* and *Balanus amphitrite* displaying behaviour that could be used to predict fouling in all three sites.

The research showed that the behavioural bioassays have the potential to be developed into an acceptable commercial screening test. From the conclusions a final protocol for filming, digitising and analysing larval behaviour, in order to predict field fouling is presented. Development of this protocol could lead to a rapid commercial screening test for non-biocidal antifouling coatings.

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CHAPTER 1

INTRODUCTION

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Biological fouling, or biofouling, is a term used to describe the accumulation of aquatic organisms including micro-organisms, plants, and animals, on artificial surfaces (Wahl 1989). As chemical fouling and fouling by non-biological material start the colonisation process (Baier 1972), these processes are usually encompassed when using the term biofouling. Within hours of immersion any un-defended surface will become fouled (Abarzua and Jakubowski 1995). This universal phenomenon affects every structure that one places in the marine environment and causes substantial economic losses (Clare 1995). It affects not only ships' hulls, causing increase drag and hence increases fuel consumption (Champ 1999), but also static objects such as underwater pipes and heat exchangers (Thompson *et al.* 2000). Biofouling is commonly divided into microfouling and macrofouling. Microfoulers such as bacteria, protists and diatoms initiate the fouling process allowing the macrofoulers (e.g. barnacles, limpets and seaweeds) to gain a foothold (Davis and Williamson 1995). Nearly 4,000 species have been listed as part of fouling communities (Anderson 2000).

In an attempt to minimise biofouling various antifouling strategies have been tried. Metallic sheeting was initially used (Callow 1990) and this led the way to coatings containing metal or organometalic substances such as copper and tin. These coatings proved very successful against invertebrate macrofoulers, especially those surfaces containing tributyltin (TBT). However they were found to not only have toxic effects on fouling organisms but also on a wide range of non-target species (Thain and Waldock 1986, Alzieu 1991, Dyrynda 1992, Gianguzza *et al.* 1996, Nicholson and

Evans 1997). Therefore the use of TBT has been restricted to boats over 25m long in many European countries (Waldock *et al.* 1988, Matthiessen *et al.* 1995). The use of copper in coatings is now increasing (Christoffersen 2000) with added herbicides such as triazine in Irgarol 1051 (Hall *et al.* 1999), but again there is growing concern about the detrimental effects of such herbicides on non-target species (Davis and Williamson 1995, Hall *et al.* 1999, Evans *et al.* 2000, Thomas *et al.* 2000, Okamura *et al.* 2000, Connelly *et al.* 2001, Martinez and Barcelo 2001, Sakkas *et al.* 2002).

As many marine organisms have evolved antifouling mechanisms, these natural alternatives are now being investigated (for review see Clare 1996a). Antifouling manufacturers are also designing non-toxic coatings based on biological findings; low surface energy coatings, containing primarily silicones, limit the adhesion of macrofoulers and consequently these can be easily removed (Swain and Schultz 1996). Although such silicones hold great potential, considerable improvements are still required in order for these nontoxic alternatives to be as efficient as the previous biocide based coatings. Such improvements are restricted by the testing periods required, as most testing is dependent on immersion trials that take between at least 1 and 2 months to perform.

This chapter aims to review research past and present, that has been carried out in relevant areas. It discusses the monetary value of the biofouling problem, together with certain antifouling strategies and their effectiveness, and explains the need for non-biocidal alternatives. Fouling succession with an emphasis on macrofouling is then presented together with a discussion on known settlement cues of macrofoulers. Finally the overall aims of the research are stated.

Economic Importance of Biofouling

As early as the 4th century Aristole noted that "small fish" slowed down the speed of ships (Fischer *et al.* 1984). The increased drag caused by fouling organisms not only decreases the speed of boats but also increases fuel consumption, and the degree of vibration and propeller noise (Callow 1990). Slight to severe barnacle fouling alone, could increase fuel costs of the world commercial fleet by $0.03 and $0.61 billion respectively (Thomason *et al.* 1998) and an increase in roughness of just 10μm has been estimated to increase fuel consumption by 1% (Champ 1999). Extensive research has gone into designing hulls of naval ships that reduce the running noise and evade sonar systems, however, as soon as macrofoulers have established themselves the noise increases and tends to degrade the ships sensor performance for sonar systems (Haderlie 1984). Ships consequently have to spend more time out of water for cleaning purposes. The lack of dry-docking facilities and this increasing demand makes this a major economic problem for ship owners. Extended dry-docking fees for world-wide commercial fleets have been estimated at $800 million (Milne and Abel 1991). Likewise fouling of navigational buoys and their attachment chains, primarily by hydroids, barnacles and algae (Thompson 1977), causes them to dip further into the water due to excess weight. This makes them difficult to see and therefore they need continuous cleaning. Fouling also interferes with the working of oceanographic equipment such as cameras, current meters, hydrophones and sensors (Haderlie 1984). Biofouling of offshore platforms is inevitable; antifouling coatings are not usually used on such structures, as reapplication at regular intervals is not possible. Therefore foulers continue to accumulate during their lifetime (Cox 1980) and this increases the loading forces on the supports, thus endangering them during storms and high waves. Fouling, mainly by mussels, of underwater pipes and heat exchangers also occurs (Thompson *et*

al. 2000, Matsui *et al.* 2002). Fouling also accelerates corrosion of metallic and other surfaces; an estimated cost of $200 million has been incurred by the US Navy from damage to wharf structures along the US coastline (Fischer *et al.* 1984). Due to the widespread problem of marine biofouling considerable effort and money has been put in to antifouling strategies, world-wide annual costs of antifouling substances are estimated to be at least $1.4 billion (Clare 1995).

Antifouling strategies

Techniques and coatings

There has been a constant battle against fouling organisms ever since we invaded the seas. Almost 2000 years ago effective antifouling strategies were sought after, many of which keep re-appearing through time. Early preventative methods included a layer of animal hair mixed with tar or nailing copper or iron nails with large heads to the wooden sheathing to produce a metallic cladding. Mixtures such as arsenic or sulphur with oil and tar, and brimstone and grease were also in use (Evans 1988). Lead sheathing was first introduced by the Romans and in the 1500s its use spread to Spain and later France and England. This became the most frequently used antifouling method before the 1700s. Copper sheathing was first used by the ancient Phoenicians and Carthaginians, which they attached to the wooden hulls of their ships to prevent shipworm and fouling. Due to the corrosive effect of lead on iron hulls the use of copper soon became re-established as a preventative method and by 1780 it was in general use by the British Navy (Callow 1990). Between 1835 and 1865 it has been estimated that around 300 patents for antifouling measures were taken out in the UK (Evans 1988) the most notable being a metallic soap "McInnes" patented in 1860. It contained the toxin copper sulphate which was applied hot over an iron oxide and resin varnish primer

(Major 1970 *in* Evans 1988*)*. As chemical technology improved so did the antifouling *compositions being manufactured, coatings containing oxides of copper and mercury* and solvent based resins were soon developed and formed the bases of the antifouling coatings which are in common practice today.

Current biocidal antifouling coatings can be separated into three major groups, soluble matrix (conventional coatings), contact leaching or diffusion types, and self-polishing copolymers or ablative coatings (Evans 1988). All three types incorporate metallic or organametallic biocide, and/or herbicide which leach out into the surrounding waters forming a concentrated toxic film within the boundary layer preventing settlement of foulers (Fischer *et al.* 1984, Evans 1988).

Conventional coatings contain cuprous oxides or sometimes other biocides such as arsenic, mercury and lead, mixed into a rosin binder (rubber matrix). Rosin is a substance obtained from trees it has been used for well over a century in antifouling coatings as it is cheap, easily obtainable and slightly soluble in seawater (Anderson 2000). On contact with seawater, the acidic rosin dissolves to release the biocide. Due to surface erosion the coatings have a limited life span and after about a year re-coating is required (Bowmer and Ferrari 1989, Callow 1990). Diffusion type coatings have a slightly longer life span as the biocide dissolves through the insoluble matrix of the coating. Release of the toxin is extremely rapid at first but as the concentration of the biocide within the matrix decreases so does its release. The active life of this type of antifouling agent is around 15 months (Callow 1990) after which the coating has to completely removed and replaced (Bowmer and Ferrari 1989).

To overcome the problems of variable biocide release and the limited life span of conventional and contact leaching antifouling agents, a self-polishing copolymer was developed. In this system biocide release is restricted to a thin layer of about 5 μm on the surface of the coating. Biocides are added to a copolymer formed between tributylin methacrylate and methyl methacrylate. When in contact with seawater the copolymer hydrolyses (polishes) releasing tributylin tin and a water-soluble sodium copolymer. The initial rough surface of the coating becomes smoother over time, which increases the effectiveness of the coating, as the smoother surface reduces macrofoulers. The ability of this type of agent to respond to the water flow in such a way, also means that, regardless of the age of the coating, the surface chemistry and efficient release of the biocide is not altered. The active life is thus related to the thickness in which it is applied and can be up to five years (Brady *et al.* 1987, Callow 1990). Although hydrolysis is dependent on temperature, pH and speed of the vessel through the water, alterations in the basic formulation can alter the polishing characteristic to suit a variety of service conditions (Callow 1990). Any defect however on the surface will foul as readily as an unpainted surface; maintenance is therefore needed to ensure that the coverage of the coating is not impaired in any way.

Many antifouling methods other than coatings have been under investigation, some of which are in widespread use, details of these can be seen in Table 1.1. Despite the numerous methods employed none have been as effective as the use of biocides. However in the last 20 years there has been growing concern regarding the detrimental effects these toxins have on non-target organisms, especially tributyltin (TBT) (*see below*), thus many countries have banned the use of such toxins. France was the first to regulate the use of TBT; as small coastal vessels were thought to be the main source of contamination, due to the high concentration found in coastal waters and around

harbours and marinas, the use of TBT on boats less than 25 m was banned in 1982. The UK followed suit in 1987 (Waldock *et al.* 1988, Matthiessen *et al.* 1995) and now most Western European countries, Japan, New Zealand, Australia, US and Canada operate similar regulations. Japan has banned entirely the use of TBT on all vessels and the application of all TBT antifouling coatings is to be banned worldwide by 2003. By 2008 a complete ban on the presence of TBT antifoulants on ships hulls is proposed (Champ 1999, Evans 1999, Evans *et al.* 2000), although it is unclear whether this will entail complete removal of the coating or merely sealing it with an alternative coating (Anon 2001).

Method	Mechanism	Effectiveness	Reference
Ultrasonics	Repulsion	Promising for specialised areas requires more investigation.	Branscomb and Rittschof 1984
Scrubbing	Mechanical removal	Can be 100% efficient, but limited longevity and some applications can be expensive, e.g. need for dry docking	Cologer *et al.* 1977
Chlorine	Oxidation	Can prevent all fouling organisms, but has limited application and can be hazardous, causing pollution.	Thompson *et al.* 2000
Electrical Fields	Inhibits biological growth	Short term effectiveness against bacteria.	Abou-Ghazala and Schoenbach 2000
Magnetic Fields	Changes membrane properties	Short term effectiveness against bacteria; requires more research.	Genenscer *et al.* 1962
Ultra violet	Disrupts cellular components	Effective against macro and microfoulers. Expensive and requires large power source.	Benson *et al.* 1973
Colour	Inhibits settlement	Short lived and selectively effective.	Hodson *et al.* 2000
Thermal-control	Coagulates cellular proteins	Application limited, and has negative impact from effluents.	Characklis 1980, Rajagopal *et al.* 1999
Air bubbles	Prevents Settlement	Ineffective around area of indention, impractical for some structures.	Houghton 1970
Osmotic control (fresh water)	Osmotic disruption	Effective against macrofoulers but leaves dead organisms attached. Economically unfeasible for some ship to re-route, reoccurs in marine environment.	Benson *et al.* 1973
Flocking	Inhibits settlement by adding electrostatically charged fibers to an adhesive coated surface	Effective against brown and green algae and encrusting animals, no effect on stoloniferous animals and red algae. Increased recruitment of tube-building polychaetes and solitary ascidians. More research needed.	Phillippi *et al.* 2001

Table 1.1 Antifouling strategies, other than the use of coatings, with their reported effectiveness.

Detrimental effects of biocides

Tributyltin (TBT) has been described as the most toxic substance ever to be deliberately introduced into the marine environment (Goldberg 1986). It can be highly toxic at even low concentrations (Evans and Leksono 1995) and as it does not degrade quickly (Evans and Clarkson 1993) it therefore persists in the marine environment long after contamination has occurred. The detrimental effects this toxin was having on non-target species has escalated since the mid-1970s. Malformation of oyster shells on the west coast of France, making them unmarketable, was linked to the contamination from the high use of pleasure boats used in the area (Alzieu *et al.* 1986). Similar findings are evident in the UK (Dyrynda 1992) and now there is increasing evidence of impacts on a range of other aquatic biota including fish (Seligman *et al.* 1990, Wester *et al.* 1990, Tiano *et al.* 2001), squid (Yamada *et al.* 1997), and crustaceans (Langston *et al.* 1990), but the most noticeable effect has been on a number of gastropod species, especially *Nucella lapillus* (Goldberg 1986, Douglas *et al.* 1993, Evans *et al.* 1996, Santos *et al.* 2000).

A condition known as imposex has been linked with TBT poisoning in *N. lapillus* and other gastropods species, such as *Littorina littorea* (Matthiessen *et al.* 1995), *Buccinum undatum* (Nicholson and Evans 1997) and *Nassarius reticulates* (Barroso *et al.* 2002). The female grows male reproductive characters such as a penis and vas deferens. The latter ultimately grows over the female reproductive organs and can cause sterility by occluding the opening of the oviduct and preventing egg release. The severity of this phenomenon is colossal, and imposex has been implicated as the causal factor behind the decline of *N. lapillus* in south-west England (Bryan *et al.* 1986). It is however promising to note that since the ban of TBT based antifouling coatings on small vessels

the concentration of this toxin has decreased in the water column and sediment (Evans *et al.* 1995, Matthiessen *et al.* 1995), high concentrations are now restricted to areas used by larger boats, such as dry docks and ports (Waldock *et al.* 1988). Incidents of oyster shell malformation (Alzieu 1991) and imposex in dogwhelks (Evans *et al.* 1991) have declined, and although recovery is not universal (Evans *et al.* 1995), restrictions on the use of antifouling agents containing TBT have evidently had a positive effect on the marine environment.

Nontoxic foul-release coatings

With the apparent success of the ban of biocides in antifouling coatings, there is increasing pressure on government bodies to further tighten regulations on their use. Consequently, there is a need for non-toxic alternatives. Significant research has gone into "non-stick" or foul release coatings with low surface energies. The theory being, that the ultra smooth, hydrophobic surface of these coatings, prevents macrofoulers from attaching or allowing them only to weakly attach (Candries *et al.* 2000). Consequently, they are dislodged at high speeds or can easily be removed by mechanical cleaning processes. The main types of non-stick coatings that have been under development are fluoropolyurethanes and silicones (Callow 1990, Brady 1997). Fluoropolyurethanes filled with polytetrafluoroethylene (PTFE) have been extensively studied as nontoxic alternatives and form a basis of the $4 billion antifouling research programme of the US Naval research office (Brady *et al.* 1987). Silicone coatings based on polydimethylsiloxane (PDMS) also hold promise. Although fouling does occur, adhesion strength is low (Swain and Schultz 1996) and this in turn enhances cleaning by biotic disturbances such as grazing and predation (Swain *et al.* 1998). It can be self

cleaning on fast vessels (Schultz *et al.* 1999), but care has to be taken during mechanical removal of macrofoulers, as the coating is relatively soft (Brady *et al.* 1987).

Natural alternatives

The search for an alternative to the toxic self-polishing coatings has also led to the close examination of a range of naturally occurring products. Many organisms have evolved their own strategies to prevent fouling and it is these organisms that have been under close scrutiny. Among the methods employed by nature is an array of chemicals that repel the settlement of fouling organisms to otherwise potential hosts. Many of these have been isolated, identified and tested for potential antifouling agents (Table 1.2): for a more detailed list see Clare 1996a. However, before any of these natural occurring alternatives can replace the synthetic biocides used today, mass screening processes have to be carried out (Kjaer 1992). The toxins have to be shown to work against a variety of species whilst ensuring that it will also not be detrimental to the environment (Willingham and Jacobson 1996). Such processes can take up to 3 years and cost well over $1 million dollars (Clare 1995). Furthermore, after this screening process it will require manufacturing in commercial quantities. It maybe for these reasons some commercial manufactures are reluctant to pursue such potential products. However others believe this approach may hold good hope in finding the answer to the fouling problem and prospective investors are increasing (Clare 1995). Whether or not such products will provide a commercially competitive solution still remains to be seen and a considerable amount of work is still required before any assessments can be made.

Source	Active Substance	Organisms used for Bioassay	Reference
Crella incrstans	Lyso-platelet activating factor	Ascidians, bryozoans, barnacles and Algae	Butler *et al.* 1996
Phyllospongia papyracea	Furospongolide	*Balanus amphitrite*	Goto *et al.* 1993
Acanthella cavernosa	Terpenoids	*Balanus amphitrite*	Hirota *et al.* 1996
Echinogorgia complexa & Dendronephthya (Morchellana) sp	Crude extracts	Diatoms	Wilsanand *et al.* 2001
Caribbean Sponges	Crude extracts	*Balanus amphitrite*	Willemsen 1994
Renilla reniforms	Renillafoulins	Barnacles Bryozoans	Standing *et al.* 1984, Rittschof *et al.* 1986, Rittschof *et al.* 1988
Leptogorgia virgulata	Diterpene	Barnacles Bryozoans	Standing *et al.* 1984, Rittschof *et al.* 1986, Rittschof *et al.* 1988
Ulva reticulata	Polysachcharide, protein or glycoconjugate	*Hydroides elegans*	Harder and Qian 2000
Ircinia oros, I. variabilis and *I. spinosula*	Organic extracts	Algae, mussels	Tsoukatou *et al.* 2002
Zoobotryon pellucidum	Tribromogramire	*Balanus amphitrite*	Kon-Ya *et al.* 1994a
Eudistoma olivaceam	Eudistomin	Bryozoan	Davis and Wright 1990
Delisea pulchra	Halogenated furanomes	*Balanus amphitrite* and *Ulva lactuca*	Steinberg and De Nys 1994
Bifurcaria bifurcata	Diterpenes	Bacteria, fungi, diatoms, spores and zygotes of macroalgae and *Mytilus edulis*	Hellio *et al.* 2001

Table 1.2 Examples of natural substances being screened for possible use as commercial antifouling agents

Succession of fouling communities

Fouling communities consist of almost all major groups of marine invertebrates. Thirteen of the 17 familiar invertebrate phyla have been associated with such assemblages (Benson *et al.* 1973). Although the most troublesome are sessile invertebrates, resisting water flow and removal by firmly cementing their secreted calcareous exoskeletons to the substratum, their attachment forms only a small part of the sequence which occurs during fouling colonisation. The fouling sequence has been divided into four main stages (Wahl 1989, Davis and Williamson 1995); firstly biological conditioning, then two stages of microfouling, by bacteria and unicellular organisms, which all make up what is known as the biofilm, and then finally the macrofouling stage by multicellular eukaryonts.

Biological conditioning occurs within seconds of a solid making contact with a water body (Loeb and Neihof 1975, Baier 1984). Dissolved organic matter such as polysaccharides and protein molecules accumulate on the surface of the immersed solid and chemically attract motile bacteria from within the water body (Fletcher and Loeb 1979). The electrostatic forces by which the bacteria cling to the surface are dependent on its composition and some bacteria have evolved physical or chemical mechanisms by which they attach (Wahl 1989). The extracellular polymer substances produced by the bacteria results in a number of consequences. It acts as a strong adhesive and can trap swimming larvae on the surface. The barrier that it creates may protect other fouling organisms from the effects of any antifouling coating present on the surface. Also the properties of the wetted surface can be altered such as colour, pH and electrical potential (Di Salvo and Daniels 1975). The bacterial film becomes encapsulated within the exudations forming a "zoogleal" slime (Zobell 1939) which resists most mechanical

cleaning processes (Marshall *et al.* 1971). Colonisation of bacteria continues until the whole surface area is saturated, which may take days or weeks. The composition and dominance of the bacterial species involved in forming this initial fouling layer is dependent on microtopography of the substratum and geographical location.

The next phase during the biofouling process is colonisation by unicellular eukaryonts and may occur within a few days of immersion. This layer is made up predominantly of diatoms (Marshall *et al.* 1971, Caron and Sieburth 1981), but also includes other protists. It is unclear whether the bacterial slime is a prerequisite for diatom colonisation. Some studies have shown that this is the case (Little 1984). However, some strains of bacteria have been shown to inhibit diatom settlement (Maki *et al.* 1988) and colonisation has also been observed in the absence of a bacterial film (Sieburth and Tootle 1981). Regardless of the order of succession, the secreted mucus of the benthic diatoms aids their attachment (Cooksey *et al.* 1984). They grow intermixed with the bacterial film and consequently the thickness of the slime layer increases; a diatom layer of up to 500μm thick has been observed growing on supertankers (Characklis and Cooksey 1983).

The fourth stage in this succession is the attachment of multicellular eukaryonts. This final stage in the formation of fouling communities occurs from days to weeks after the preceding events of the biochemical conditioning. It is the longest phase and begins with the recruitment of algal spores and meroplankton (Wahl 1989). Again there has been dispute as to whether organisms in this phase actually require the slime film before adhering to the surface. Many organisms such as *Spirorbis borealis* (Knight-Jones 1951, Crisp and Ryland 1960, De Silva 1962, Meadows and Williams 1963), *Ciona intestinalis* (Szewzyk *et al.* 1991, Wieczorek and Todd 1997), numerous species of

barnacles (Meenakumari and Nair 1994, O'Connor and Richardson 1996) and bryozoans (Miller *et al.* 1948, Wisely 1958, Ryland 1974, Mihm *et al.* 1981, Brancato and Woollacott 1982) have been shown to have a preference for slimed surfaces, yet some such preferences vary with bacterial composition (Neal and Yule 1994), origin (Raimondi 1988, Keough and Raimondi 1995) and age (Maki *et al.* 1988, Maki *et al.* 1989, Maki *et al.* 1992, Keough and Raimondi 1995, Wieczorek *et al.* 1995) of the biofilm layer. Although the slime layer ultimately mediates the stimulatory characteristics of the immersed solid (Fischer *et al.* 1984), most research has primarily focused on the physico-chemical properties of the biofilm layer that affects larval settlement, in particular surface energy. This has been shown to be one of the key factors affecting settlement (Rittschof and Costlow 1989a, Roberts *et al.* 1991, Holm *et al.* 1997) however, other aspects of the bacterial film may also cause either a facilitative or inhibitory response. It has been suggested that the adhesive of bryozoan larvae used for temporary attachment may bind differently to extracellular material of different bacteria, thus this exudate may also play a role in determining larval settlement (Maki *et al.* 1989). Other suggestions of properties of the bacterial film encouraging settlement include, trapping larvae, increasing the food source for the settlers and favouring the deposit of adhesive substances by the increased alkalinity (Zobell 1939). Although due to the buffering capacity of seawater the latter seems unlikely. There are however numerous chemical and physical factors (see below) that have been shown to affect the settlement of sedentary marine invertebrates (for reviews see Crisp 1974, Pawlik 1992, Rodriguez *et al.* 1993). So although a bacterial slime layer does affect settlement of some species, these other factors may act in synergy with the biofilm to facilitate or inhibit settlement or alternatively override it completely, lessening the importance of the preconditioned layer.

Factors affecting settlement and settlement cues

Light and gravity

Gravity and light are hard to differentiate between, as a negative geotrophic response is similar to a positive phototactic response when the polarised light is from above, as in a water column. Most of the literature is therefore concerned with the latter. Evidence of photaxis is numerous in the literature (Lynch 1947, Ryland 1960, Gee 1963, Millar 1971, Crisp 1974, Svane and Dolmer 1995, Maldonado and Young 1996). Responses to light are more apparent in swimming larvae than those about to settle, and the generalisation is that there is a photopositive behaviour that reverses just before settlement. This behaviour will affect the chance of meeting a suitable substratum on which to explore, thus ultimately affecting settlement. However, the response during exploratory behaviour is not so well documented and may well not be as influential during settlement as other cues. Laboratory observations also do not always correspond to the settlement behaviour seen in the field; *Bugula. neritina* was found to remain photopositive in the laboratory but in the field this species mostly settles in parts where there is little incident light (McDougall 1943). Species of differerent phototactic behaviour also settle on similar substrata like the epiphytics on *Fucus serratus* (Crisp 1974) thus implying that phototactic behaviour is of small importance when choosing a suitable settlement site. On smooth surfaces cyprids tend to settle more strongly on illuminated side but this decreases with rugosity (Crisp and Barnes 1954) again suggesting the phototaxis has little influence over other settlement cues when active behaviour is involved. However most larvae do in fact show preference for dark shaded places (Crisp 1974) and when given the choice of a light or dark substratum they preferentially settle on the dark (black) surfaces (Dahlem *et al.* 1984). Hence, when considering light independently for other settlement cues it can be seen that light does influence settlement of marine larvae.

Hydrodynamics

On a large scale the oceanic currents and local tides play the most important role in determining the locality of surfaces on which the larvae settle. Larvae have a limited degree of choice on a large scale due to their relatively poor swimming ability and the settlement time window (Anderson and Underwood 1994) in relation to these hydrodynamic processes. It is therefore the passive component of hydrodynamics, i.e the disposition of the larvae caught in flow that mostly affects settlement patterns (Wethey *et al.* 1988). Many authors agree that on a smaller scale hydrodynamic consequences become less important (Walters 1992a, Gregoire *et al.* 1996, Hills and Thomason 1996, Bourget and Harvey 1998). It is unclear at exactly at what scale this passive process becomes an active behavioural response whereby the larvae exercises substratum choice; active behaviour determining the spatial variation for cyprids along the shore has been estimated at scales less than 100m (Pineda 1994), however it has been found that passive settlement process are sufficient to explain recruitment patterns at scales greater than 3cm (Bourget and Harvey 1998). At whatever scale this active process becomes important, the speed of water past an object is only important in so far as it affects the velocity gradient in the boundary layer (Crisp 1955). At high velocities the larvae cannot maintain a position along a substratum for long enough to temporally attach and start to explore, however, more initial contacts by chance are made in faster flows (Mullineaux and Butman 1991). It has been found that cyprids reject surfaces more frequently in a fast flow compared to a slower flow, but rejection does occur in shear stress well below one that would result in dislodgement (Mullineaux and Butman 1991). This rejection of the substratum in fast flow is probably due to the mass stream flow velocity and not related to the fine scale near surface hydrodynamics. Therefore passive transport activates initial contact but then the larvae display a directional active behavioural response in which hydrodynamics has only a small influence.

Gregarious and territorial settlement

Gregariousness is the tendency to settle within a group, whereas territorial behaviour is shown when individuals space out slightly from their neighbours during settlement. Both laboratory and field experiments have shown that many marine larvae of sessile invertebrates tend to settle around adult conspecifics (Knight-Jones 1951, 1953b, Knight-Jones and Crisp 1953, Crisp 1974). There are many advantages to this kind of settlement, a habitat inhabited by conspecifics is more likely to support postlarval growth compared to an alternative site with no other settlers (Jensen 1989). The sessile nature of these invertebrates limits the proximity in which sexual reproduction can take place, thus gregarious settlement will increase the chance of successful fertilisation in both internally fertilising and free spawning species (Crisp 1979). Aggregated individuals can also benefit from a higher overall fecundity as the life span of gregarious adults is also believed to be longer than that of individuals settling alone (Wilson 1974). On a small scale (< 1.5cm) this preference of cyprids to settle near conspecifics is postulated to be more important than heterogeneity of the substratum (Chabot and Bourget 1988). However, gregarious settlement will result in more competition for food and this will increase as the distance from their neighbour decreases. Thus some species, e.g. *S. borealis* (Wisely 1960) and barnacle cyprids (Knight-Jones and Moyse 1961) display territorial behaviour and settle at a distance far enough away from conspecifics. This behaviour allows space for growth and minimises intraspecific competition at least when juvenile. Aggregation of different species also occurs during settlement. This maybe due to restricted dispersal (Keough 1989), hydrodynamics (Havenhand and Svane 1991) or a preference for a certain living substrata, e.g. *Fucus* sp., on which there is limited space (Pawlik 1992).

Surface characteristics

SURFACE ENERGY

It is well documented that a biofilm can influence the settlement of marine larvae, as already discussed. However, as this layer influences the surface chemistry of the substratum such as surface energy it is difficult to differentiate between the two factors, i.e. are the larvae responding to characteristics of the biofilm itself or is the larvae responding to the altered surface chemistry. More recent studies use silanisation (addition of saline reagents to a surface) to alter the surface energy of a substratum while maintaining other properties such as rugosity, colour and more importantly presence or absence of biofilm. These studies have shown that surface energy does indeed influence the settlement behaviour of some marine larvae (Rittschof and Costlow 1989a, Roberts *et al.* 1991). In general barnacles have a preference for high surface energies, bryozoans have a preference for lower one (Holm *et al.* 1997, Rittschof and Costlow 1989a) and hydroids appear to hold no preference (Rittschof *et al.* 1998). Surface energy influences the effectiveness of adhesives used for attachment (Baier 1984). The larvae themselves have surface properties which may influence settlement; these depend on larval type and stage (Rittschof and Holm 1997). Generally larvae have high surface energies but settling cyprids can have a low surface energy (Rittschof *et al.* 1998) and thus will be trapped in a surface film more easily (Rittschof and Holm 1997), and forced to settle on un-preferred surfaces.

RUGOSITY

The rugosity or roughness of a surface can be divided into surface texture (heterogeneity of the substratum operating on a scale below that of the larvae) and surface contour (heterogeneity of the substratum operating on a scale above that of the

larvae) (Le Tourneux and Bourget 1988). Both of these can influence the settlement of larvae. It is well documented that *Semibalanus balanoides* display a clear preference for pits and cracks (Crisp and Barnes 1954, Crisp 1961, Wethey 1984, Le Tourneux and Bourget 1988, Hills *et al.* 1999a) as well as some *Balanus* species (Walters and Wethey 1996). *S. borealis* also display a preference for crevices created by the midrib of *F. serratus* (Wisely 1960) and some bryozoan species have also displayed a preference for small crevices (Ryland 1959). This preference is possibly because these create a suitable refuge site from predators and high shear, and consequently enhances survival (Walters and Wethey 1996) and it may afford stronger antennular adhesion (Le Tourneux and Bourget 1988) or may prevent desiccation (Raimondi 1990). In contrast, some negative settlement responses have been documented for some barnacle species; Crisp and Barnes (1954) found panels with grooves and ridges recruited low numbers of *S. balanoides* cyprids. *B. improvisus* has also been shown to be negatively affected by surface texture in particular on the micron scale (Andersson *et al.* 1999, Berntsson *et al.* 2000a, Berntsson *et al.* 2000b, Dahlström *et al.* 2000, Petronis *et al.* 2000) profile heights of 30-45μm reduced settlement in the field by 92% (Berntsson *et al.* 2000b).

Cyprids seem to respond to a certain scales of surface texture (Le Tourneux and Bourget 1988, Walters and Wethey 1996, Hills and Thomason 1998a and b). *S. balanoides* can discriminate between textured surfaces with a step size of 35μm, and showed a preference to settle on a rugosity between 35μm–1mm (Le Tourneux and Bourget 1988). Hills and Thomason (1998a), similarly found *S. balanoides* prefer a scale of <0.5mm roughness compared to either smoother or rougher substrata; this scale corresponds to their body size and showed that the density of settlement on a range of manufactured surfaces was related to the number of pits and crevices at this scale. In general cyprids prefer small pits to large pits and large pits to grooves (Wethey 1986).

Wethey (1984) concluded that contour was the dominant factor influencing *S. balanoides* settlement densities. Thus both surface texture and contour play important roles influencing settlement of marine larvae.

Chemicals

Chemicals influence the settlement of marine larvae and these are believed to be of greater importance than other influential factors (Pawlik 1992). Chemicals have been shown to be important in gregariousness settlement (see above), exploring barnacle larvae leave behind a trail of adhesive substances from their antennules known as 'footprints' (Walker and Yule 1984, Clare *et al.* 1994), which contains a pheromone termed settlement-inducing protein complex (SIPC) by Matsumura *et al.* (1998b). This high molecular mass glycoprotein complex (Matsumura *et al.* (1998b), influences settlement of later arriving cyprids (Clare *et al.* 1994, Matsumura *et al.* 1998a); it is believed to make the surface more attractive resulting in gregarious settlement even in the absence of conspecifics (Matsumura *et al.* 1998a). Exactly how they work remains to be established (Clare 1995) however research has indicated that such chemical responses are mediated by chemoreception (Rittschof *et al.* 1998).

Chemicals from the substratum may also affect settlement, many species settle on living biota, including biofilms, and these give out chemicals to which the larvae respond. *S. borealis* has shown to hold a great preference for settling on *F. serratus* and it is hypothesised that an interaction between its own adhesive mucus and the algal extracts induces settlement (Crisp and Williams 1960, Crisp 1984). Many other species have also been shown to respond to chemical signatures, and this chemical response is postulated to be the most specific and influential factor affecting settlement (Crisp

1974). Other chemicals are used in natural antifouling strategies (Table 1.2) therefore chemicals not only induce settlement but also inhibit as well.

Larval age

The 'desperate larvae' hypothesis is a term used to describe the phenomenon that older larvae show less discrimination over settlement cues, in particular presence of conspecifics (Knight-Jones 1953a, Branscomb and Rittschof 1984, Jarrett 1997, Satuito *et al.* 1996). It is believe that these larvae have not found a suitable substratum on which to settle and cannot prolong planktonic life any further due to lack of resources, or cells becoming incompetent to allow metamorphosis (Jarrett and Pechenik 1997). A more recent hypothesis that explains the lack of discrimination is given by Toonen and Pawlik (1994) which suggests that there are certain larvae that act as founders and therefore do not settle in response to conspecifics and act to disperse the population. However, as less discrimination in older larvae has been shown to be associated with factors other that the presence of conspecifics, e.g. low frequency sound (Branscomb and Rittschof 1984), it seems evident that age does influence settlement behaviour, regardless of whether founders exists in the larval population.

Pre-settlement movement behaviour of larvae

Settlement behaviour between apparently diverse taxa of sessile marine invertebrates is remarkably similar (Crisp 1974). On contact with the substratum temporary attachment is followed by an exploration stage, and if certain requirements are met by the surface, fixation and metamorphosis occur, if not the larvae will break contact with the substratum and swim off in search for a more suitable attachment site. Some researchers have reported settlement of cyprids can occur without any exploratory behaviour (Hills

et al. 1998, Hills *et al.* 2000), however, other work has identified three phases of this exploration stage: broad exploration, close exploration and inspection (Crisp 1974). During broad exploration the larvae travels along the surface in a relative straight line with infrequent turns, which may result in the larvae breaking contact and swimming off or entering the next phase. During this close exploration phase behaviour changes to short steps which frequently change direction. Just before metamorphosis occurs the larva enters the inspection phase in which it rotates and moves to and fro within its own body length (Crisp 1961, Knight-Jones and Crisp 1953). Between species there is a slight variation in the behaviour within these three stages, these will be discussed for the species included in this research in each relevant chapter.

Methods available for testing efficiency of non-biocidal coatings.

In light of the changing attitudes regarding biocide based antifouling coatings, non-toxic alternatives must be found, tested and manufactured for commercial purposes. The largest problem facing industries is the lack of suitable rapid testing methods. Traditional methods of assessing toxic compounds were based on lethal dose or concentration 50 tests (LD_{50} and LC_{50}). However, such testing methods, based on mortality, are inapplicable to the testing of non-biocidal antifouling coatings. Methods available for non-biocidal testing include immersion trails and adhesion measurements. Immersion trails which involve the examination of the extent of fouling after a suitable submersion period are widely used. These however are lengthy, may be restricted by deployment time (Nandakumar 1996, Thomason *et al.* 2000), in addition percent fouling data can not always be used to differentiate performance of foul-release formulations (Swain *et al.* 2000). Measurements of adhesion of macro fouling

organisms (Callow *et al.* 1988, Swain *et al.* 1992, Swain *et al.* 2000, Darkangelo Wood *et al.* 2000) are also available. This method involves measuring the force required to remove isolated, live organisms with a hand held force gauge (Swain *et al.* 2000). However, although this method has proved useful for a comparison of foul-release coatings, it does not reveal information on how organisms are responding to certain aspects of the surface.

Analysis of larval behaviour seen in the laboratory has revealed that behavioural studies can be extremely useful and provide an insight into how non-biocidal coatings work. (Berntsson *et al.* 2000a) Exploratory behaviour in the field (Matsumura *et al.* 2000, Thomason *et al.* 2002) has been used to interpret recruitment patterns (Hills *et al.* 2000) and discriminate between different antifouling coatings (Thomason *et al.* 2002). Therefore, it is postulated that an understanding of the behavioural responses to certain surface cues would allow subtle changes to be made to coatings in order to improve their efficiency. Furthermore, the behaviour changes could be analysed in order to allow a quick, reliable method for assessing the performance of such coatings.

Bioassays

A bioassay is 'a quantitative determination of a substance by measuring its biological effect on e.g. growth that is the use of an organism to test the environment' (Walker 1998). Bioassays have been used in many fields of research for the testing of a broad range of different substances; some focus on mortality levels of the organism tested, others are based on the sublethal effects of toxicants, while other bioassays focus on changes in behaviour for interpretation.

Bioassays have been used in a broad range of research; from medical research (Oku *et al.* 2002, Takahashi 2002) and water assessment (Dos Santos *et al.* 2002, Ulitzur *et al.* 2002, Geffard *et al.* 2001, Johnson 1988) to pesticide research (Edelson *et al.* 2002, Scott *et al.* 2002, Stevens *et al* 2002, Isman 1993), and other general chemical effects (Sherwood *et al.*1991, Knols *et al.* 1997, Lokkeborg *et al.* 1995). Antifouling research has also been reliant on a range of bioassay techniques, including toxicology of biocide coatings (e.g. Persoone and Castritsicatharios 1989, Meador *et al.* 1984) and testing non-toxic natural alternatives (e.g. Sera *et al.* 2000, Willemsen 1994, Takasawa *et al.* 1990).

When looking at behaviour it should be realised that it is influenced by both external (stimulus) and internal (physiological) factors (Baker and Cardé 1998) and steps must be taken when designing a behavioural bioassay to minimise all other factors not under investigation. Stebbings *et al.* (1980) set out criteria for a viable bioassay; the test organism should be available for as much of the year as possible, it should be ecologically significant and economically important and the bioassay itself should be easy to learn, cost effective and short term. Variation in organisms used for experimentation should be minimised as possible to maximise sensitivity, and in addition satisfy the usual statistical criteria.

Aims of research

The aim of this research was to study and analyse the exploratory behaviour of a selection of marine invertebrate fouling species, and to use these behaviours to develop a laboratory based screening bioassay which could be used to predict the performance of novel non-biocidal coatings prior to field trials. Much of the work presented represents methodological advances coupled to pilot results and conclusions; various

methodologies were tried and tested to attempt to produce a robust behavioural bioassay for non-biocidal coatings. A collection of fouling organisms were selected and tested for suitability, availability, and reliability. The developed bioassays were used experimentally to determine the effect of a diversity of unknown non-biocidal surfaces on the variation in a range of behavioural indices. To determine the validity of the bioassay for prediction of antifouling efficacy of the tested non-biocidal surfaces, behavioural bioassay results were compared to data supplied from standard field immersion trials. The final section of the work assesses the value of the behavioural bioassay technique developed for the rapid screening of potential non-biocidal formulations.

CHAPTER 2

SPECIES AND COATINGS USED

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SPECIES AND COATINGS USED

Introduction

This chapter describes the species and antifouling coatings used through out the research. Firstly details are given of all the species initially considered and reasons for use or dismissal and a summary of this can be seen in Table 2.1. The following section outlines the coatings used and includes how the test panels were prepared. Two physical properties were investigated for all of the coatings; rugosity and surface energy. To evaluate the bioassays, immersion trials on some of the coatings were also carried out, this is described together with the results. The final section outlines the proceeding experimental chapters.

Species selection.

Initially a considerable time was spent in search of suitable species to be included in the bioassays. Factors considered included ease of use, reliability of source, cost and availability. A number of fouling organisms were considered:

- Urochordata, Ascidiacea: *Ciona intestinalis* (Linnaeus) and *Ascidiella aspersa* (Müller).
- Bryozoa, Gymnolaemata, Cheilostomatida: *Bugula neritina* (Linnaeus) and *Bugula fulva* (Rylan).
- Annelida, Polycheata, Serpulidea: *Spirorbis borealis* (Daudin).

- Crustacea, Cirripedia, Thoracia : *Balanus amphitrite* (Darwin) and *Balanus improvisus* (Darwin).
- Mollusca Pelecypoda Mytiloida: *Mytilus edulis* (Linnaeus).

Species considered but rejected

CIONA INTESTINALIS AND ASCIDIELLA ASPERSA

The ascidians *Ciona intestinalis* and *Ascidiella aspersa* were considered as a test organism as, although ascidians generally are not considered of great economic importance as a fouling taxa, they are a major part of fouling communities world-wide (Millar 1971). Artificial fertilisation of them is also readily accomplished almost all year round (Berrill 1947).

A cheap and reliable source of *C .intestinalis* could not be located, however a reliable source was found for sexually mature *A. aspersa.* These were purchased from Loch Fyne Sea Farms, Loch Fyne, Scotland, UK and kept at the Dove Marine Laboratory, Cullercoats, UK, in large sinks with a constant flow through of unfiltered natural seawater. Artificial fertilization was carried out using method described by Dr A. McDougall, Newcastle University (pers. comm.): The eggs and sperm of sexual mature adult (recognisable by white sperm ducts and reddish oviducts) were removed after careful removal of the test; by puncturing the sperm duct with a fine tipped syringe through the body wall and gently squeezing the specimen, sperm was then syringed out of the individual. The oviduct lies just to one side of the sperm duct and another puncture allowed the release of the eggs. These were then mixed in a 5cm petri dish containing 4ml of 0.4μm filtered natural seawater and 5mmol solution of TAPs (N-

ris[Hydroxymethyl]methyl-3-amino-propanesulfonic acid) and left to develop into tadpole larvae for 1-2 days in a temperature controlled room at 10±2°C.

Artificial fertilisation was carried several times during February and September, using a variety of different solutions:

- Filtered natural seawater with and without TAPS solution
- Autoclaved filtered seawater with and with out TAPS
- Both of the above with the addition of Streptomycin sulphate (50mg l^{-1}) and penicillin G (60mg l^{-1}) as per Wieczorek and Todd (1997).

A ~1% success rate of producing larvae was achieved for the first solution without TAPs. No other solution yielded any tadpoles, although it was evident that a most of eggs had been fertilised but not developed further. It is possible that the purchased adult stock was too mature and their eggs were no longer viable (McDougall pers. comm.). The complete transparent nature of the tadpoles also caused concern due to visibility problems during the bioassay. As a reliable source of larvae could not be obtained ascidians were dismissed as a suitable test organism.

BUGULA NERITINA

The bryozoan *Bugula neritina* was considered as a test organism. *B. neritina* is most commonly found in harbours and ports, fouling piers, buoys and ship's hulls and is common in fouling communities. It has been described as "one of the most serious fouling organisms" (Ryland and Haywood 1977), as it freely grows within the intake pipes of ships and condenser chambers. Due to this, settlement and metamorphosis has been extensively studied (Lynch 1947, Woollacott and Zimmer 1971).

Another reason for considering *B. neritina* is the fact that this species broods its embryos within an ovicell and therefore a culturing method is not required. If the colonies are kept in running sea water in total darkness, as described by Ryland (1960), illumination either by artificial light or natural daylight will stimulate the liberation of the larvae after about 30-40 minutes (Lynch 1947). This method has also been employed by other researchers (Maki *et al.* 1989, Rittschof and Costlow 1989). Adults can be also be maintained in the lab on a diet of diatoms (Maki *et al.* 1989).

Unfortunately a suitable source of live brooding specimens could not be found. An alternative *Bugula* sp. was also considered (*B. fulva*) but again a suitable reliable source could not be found. These bryozoans were therefore dismissed as viable test organisms.

Species used

BALANUS AMPHITRITE

Balanus amphitrite (Figure 2.1) was chosen for the behavioural bioassay due to its abundance and importance within biofouling communities. It has featured in many bioassays concerned with inhibition and facilitation of larvae settlement thus much information has been documented regarding its settlement cues (Holmström *et al.* 1992, Kon-Ya and Miki 1994, Maki *et al.* 1988, Mullineaux and Butman 1991, O'Connor and Richardson 1998, Rittschof *et al.* 1984, Rittschof and Costlow 1989, Wieczorek *et al.* 1995). Culturing techniques are also well documented and rearing larvae to the cyprid stage can be performed with relative ease. A regular stock of laboratory reared cyprids was kindly provided by Dr Anthony Clare, Newcastle University.

BALANUS IMPROVISUS

Balanus improvisus is the main fouling barnacle in Swedish waters. It is cultured successfully at Tjärnö Marine Biological Laboratory, Sweden, and an invitation was gained to work at this laboratory using their stock. Although *B. amphitrite* was also being used, the preference of *B. improvisus* for smooth surfaces on the microscale (μm) (Berntsson *et al.* 2000a and b) as opposed to the pits and grooves shown by *B. amphitrite* (Walters 1992) was thought to be an interesting comparison.

SPIRORBIS BOREALIS

This species can be found all over Britain in large numbers in association with the brown algae *Fucus serratus* (Hayward *et al.* 1996), therefore the relative ease of collection was the predominant factor behind the choice of *S. borealis*. Adults were collected from Cullercoats bay, Northumberland. Larvae (Figure 2.2) are also easily attainable, as *S. borealis* will liberate them under illuminated conditions (Knight-Jones *et al.* 1971, Nott 1973, Williams 1964) especially during peak periods (De Silva 1962). If the adults are kept overnight in air, larvae should be liberated the following morning, after a period of illumination and immersion (Nott 1973). It has also been demonstrated that even without the immersion period, i.e. just with a suitable period of darkness, larvae can be obtained (Williams 1964). Due to the strong phototactic behaviour of the larvae they can be collected relatively easily as they accumulate around the light source. Usually twenty to thirty larvae can be produced by a single adult (Knight-Jones 1951) and the pelagic stage can be between 15 minutes and 2 hours, although it differs with different broods (Knight-Jones 1951, 1953a). Therefore *S. borealis* was an ideal specimen to be included in the behavioural bioassays.

MYTILUS EDULIS

Mytilus edulis, the common or blue mussel is abundant and widely distributed throughout Europe (Hayward *et al.* 1996). It is one of the major fouling species causing serious economic problems (Richmond and Seed 1991). Juvenile *M. edulis* have featured in many antifouling bioassays (Takasawa *et al.* 1990, Satuito *et al.* 1993, Kitajima *et al.* 1995, Etoh *et al.* 1997, Sera *et al.* 2000) as they are capable of assessing the suitability of a substratum using chemoreceptors (Morse 1990, Woodin 1991) and actively crawl over the substratum, testing it with the foot. (Crisp *et al.* 1985, Satuito *et al.* 1993). They were chosen as the test organism, due to their abundance and availability through both winter and summer and juveniles were collected from Black Middens, North Sheilds, Tyne and Wear, UK. Work using *M. edulis* was being carried out as part of an honours project and due to the potential of this work, further work was undertaken as part of this thesis. This part of the thesis (Chapter 3) represents a standalone entity and describes a choice test bioassay that can be used for testing the efficiency of non-biocidal coatings.

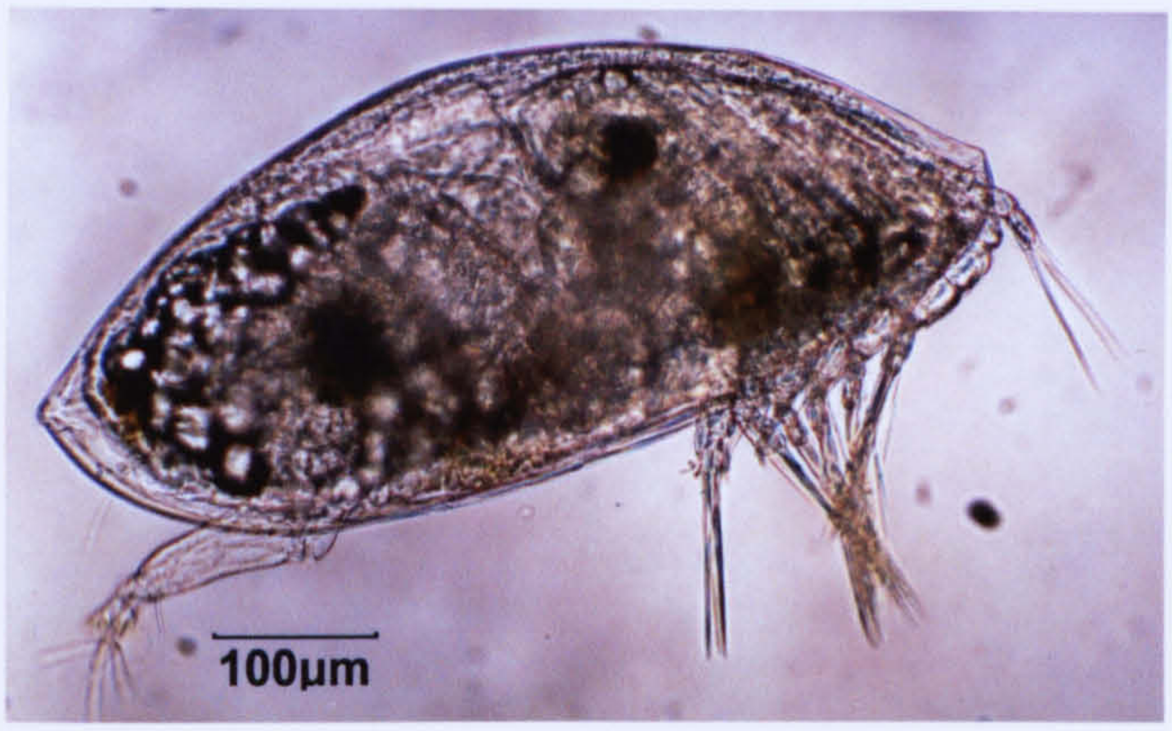


Figure 2.1 *Balanus amphitrite* non-feeding cypris stage.

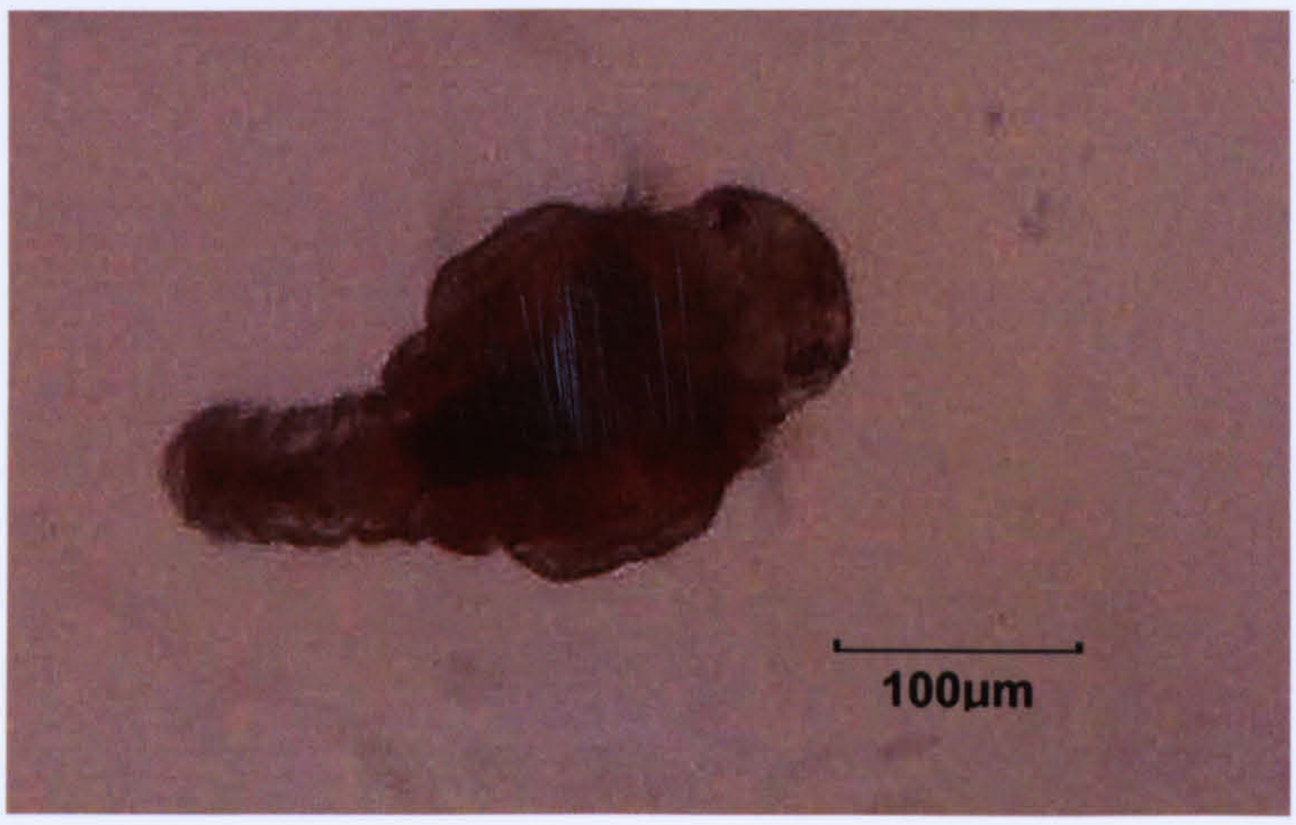


Figure 2.2 *Spirorbis borealis* larva.

Species	Main Reasons for dismissal or approval	Dismissed or approved
Ciona intestinalis & *Ascidiella aspersa*	Artificial fertilization unsuccessful.	Dismissed
Bugula neritina & *Bugula fulva*	Suitable source could not be located.	Dismissed
Spirorbis borealis	Ease of collection, larvae liberated readily, short pelagic life.	Used
Balanus amphitrite	Serious fouling species, cultured in University.	Used
Balanus improvisus	Invitation gained to work with cultured stock in Sweden.	Used
Mytilus edulis	Ease of collection, availability.	Used (for choice bioassay)

Table 2.1 Summary of the main reasons for either dismissing species or including them in the bioassay.

Coatings

All coating used for this research were provided by Akzo Nobel. Some were already available commercial antifouling coatings and others were experimental coatings. For reasons of confidentiality no chemical compositions can be given for each coating and therefore codes are used, details are given in Table 2.2.

Preparation of test panels

Panels were made from either aluminium, glass or manufactured watch glasses, although all were prepared in the same manner. The panel media used with each method is stated in the relevant sections. All panels coated were primed with a tie coat VAL, using a 110mm Harris mini roller, and left overnight to air dry before the coating was

applied. In order to obtain the best contrast between the larvae and coated panel while using Ethovision® (*for details see* Method 2 below) all type two arenas were primed with white VAL before the clear coating was applied. Panels used for type 1 arenas and the mussel arenas (Chapter 4) used the standard grey formulation of VAL. The coatings were applied using a 25mm pure bristle Harris paint brush apart from coating 615 which was rolled using a 110mm Harris mini roller (Figure 2.3). The panels were left to air dry at room temperature for at least two weeks prior to use for any residual solvent to leave the coating.

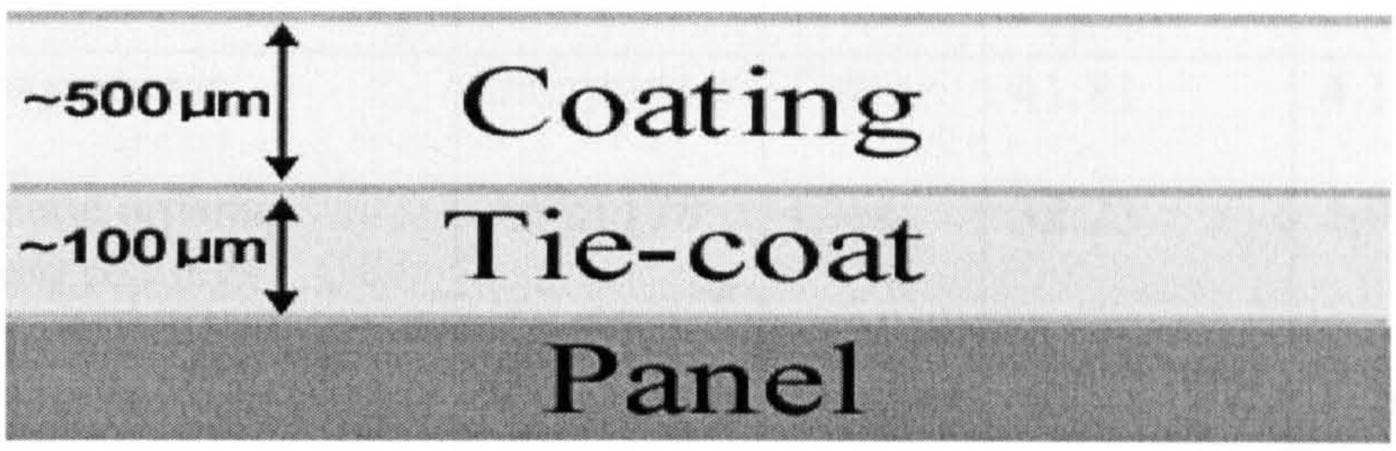


Figure 2.3 Schematic diagram of the arrangement and approximate thickness of the different layers of coatings used to prepare a test panel. Panels were of variable thickness depending of the media used.

Coating code	Coating description	Name or formulation code	Colour	Total Surface energy dy/cm	Roughness (R_a) μm	Relevant chapters
615	Epoxy	N/A	White	53.47	4.483	4,5,6,7,8
616	Silicone/organic hybrid coating	FR355	Milky white	83.47	1.027	3,4,6,7, 8
617	Experimental silicone	2807 -12A	Clear	51.63	0.880	3,4,6,7,8
618	Experimental fluoropolymer	2789-75C	Clear	40.97	7.363	3,4,6,7, 8
619	Experimental silicone	2807-75	Clear	32.82	1.270	4,6,7,8
ITS	Commercially available silicone.	Intersleek	Clear	39.77	1.203	3,4,6,7, 8
ITP	Epoxy primer	Interprotect	Grey	41.81	4.183	3
AF2	Silicone/organic hybrid coatings	AF21178	Clear	32.23	1.950	3
VAL	Acrylic polymer based coating	Valkyrie	White or grey	39.84	1.707	As a primer in 4,6,7,8
VRD	Commercially available silicone.	Veridian	Clear	38.71	0.747	5,3
19A	Experimental Fluoropolymer	2789-19A	Clear	33.68	12.540	5
19C	Experimental Fluoropolymer	2789-19C	Clear	26.31	10.593	5
PXC	Experimental hydrophobic textured surface, based on a hydrophobically modified coating	N/A	White	Not available	Not available	3

Table 2.2 - A description of coatings used through out this research. Physical properties measured are given as well as the chapters the coating feature in. Coatings in **bold** represent the main coatings used throughout.

Physical properties

As physical properties of the substratum have been shown to effect settlement of larvae (see Chapter 1) two such properties of the coatings used were established.

RUGOSITY

The coatings were painted on a standard glass microscope slide. Rugosity of each was measured by a laser profilometer (UMB UBC12), which has a z resolution of <0.1μm. Three areas, 20mm x 10mm, were scanned for each coating. One hundred datum points/mm were measured on the x axis and 1 point/mm measured on the y axis (Figure 2.4). The arithmetic mean of all points measured within the overall sample (R_a) is given for each coating in Table 2.2.

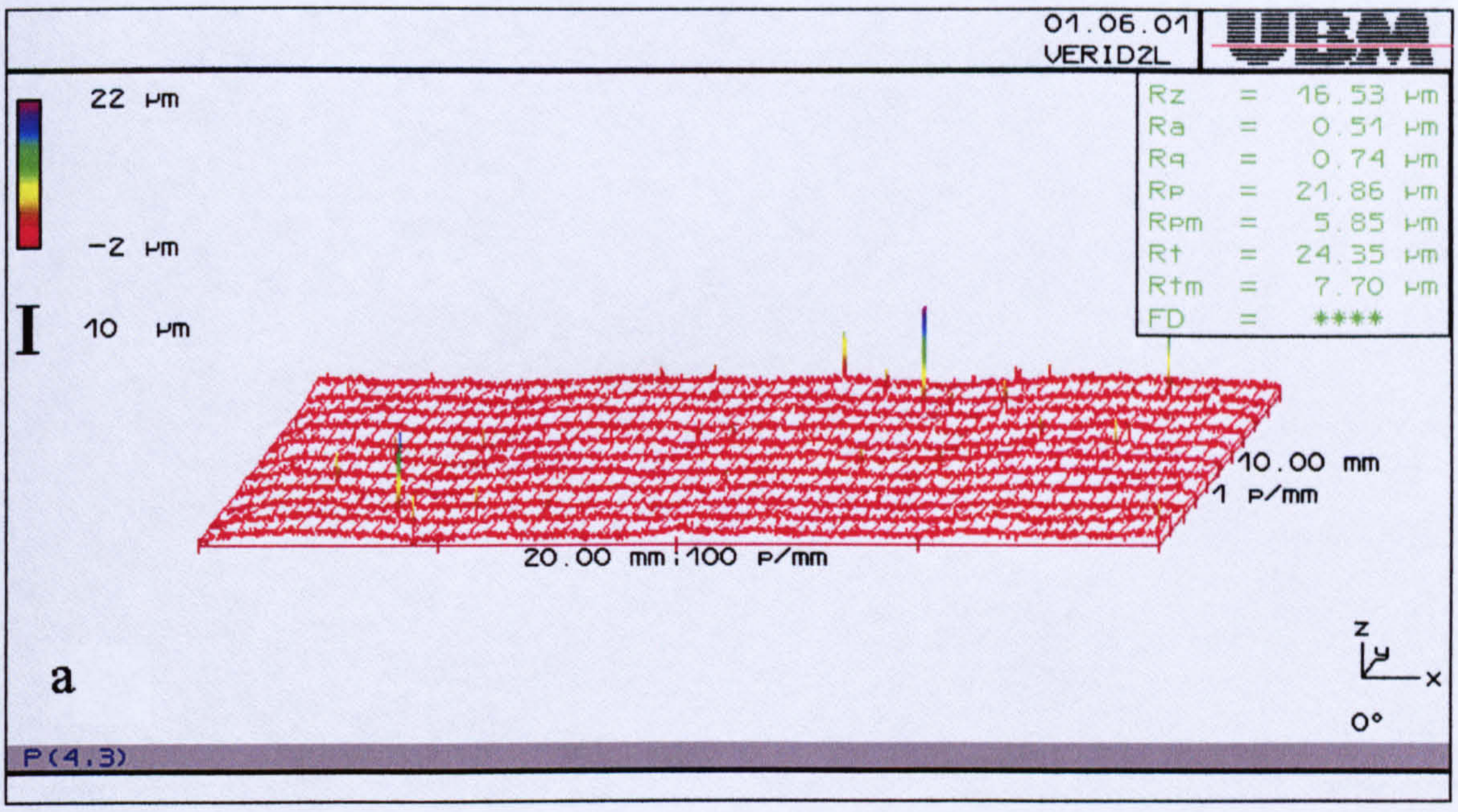


Figure 2.4 Examples of the surface profiles given by the profilometer of two different coatings. A) A coating with a low R_a value (0.51µm) - relatively smooth, b) A coating with a higher R_a value (4.5µm) - shown to have a rougher surface. *Note different scales.*

SURFACE ENERGY

The contact angle (Figure 2.5) of each coating was measure in air using the sessile drop method (Spelt *et al.*1987, Rittschof and Costlow 1989, Noda 1992, Fernández Estarlich *et al.* 2000) with both water and methylene iodide. Coatings were painted on standard glass microscope slides pre-cleaned using ethanol. A drop of approximately 2mm was dropped onto the coating through a fine tipped syringe positioned approximately 1 cm above the slide. The drop was left for 2 minutes at room temperature to equilibrate. Although the contact angle of surfaces including silicones and fluorosilicones (Fernández Estarlich *et al.* 2000, Petronis *et al.* 2000) are known to be highly dynamic and will alter over time and at different humidities and temperatures, time did not allow for a full investigation. The results therefore are only for comparative purposes and each coating may have giving a different value if left for longer or performed at a different temperature. The image was filmed using a Pulnix video camera (PE2015) fitted with a Computar Macro10x lens. The video camera connected to a Compaq Pentium 1 PC. The contact angle was determined using an in house computer program (Contact angle v3), based on LabView 6 and Ni-imaq, provided by Akzo Nobel. Six replicates for each coating and each liquid were performed. The average angles produced for both liquids were then used to calculate the surface energy according to Owens-Wendt geometric mean (Park and Jin 2001, Pisanova *et al.* 1998, Scheikl and Dunky 1998, Correia *et al.*1997, Owens and Kobayashi 1994) using DemoFTA200 (in house surface energy program). Values for each coating are given in Table 2.2.

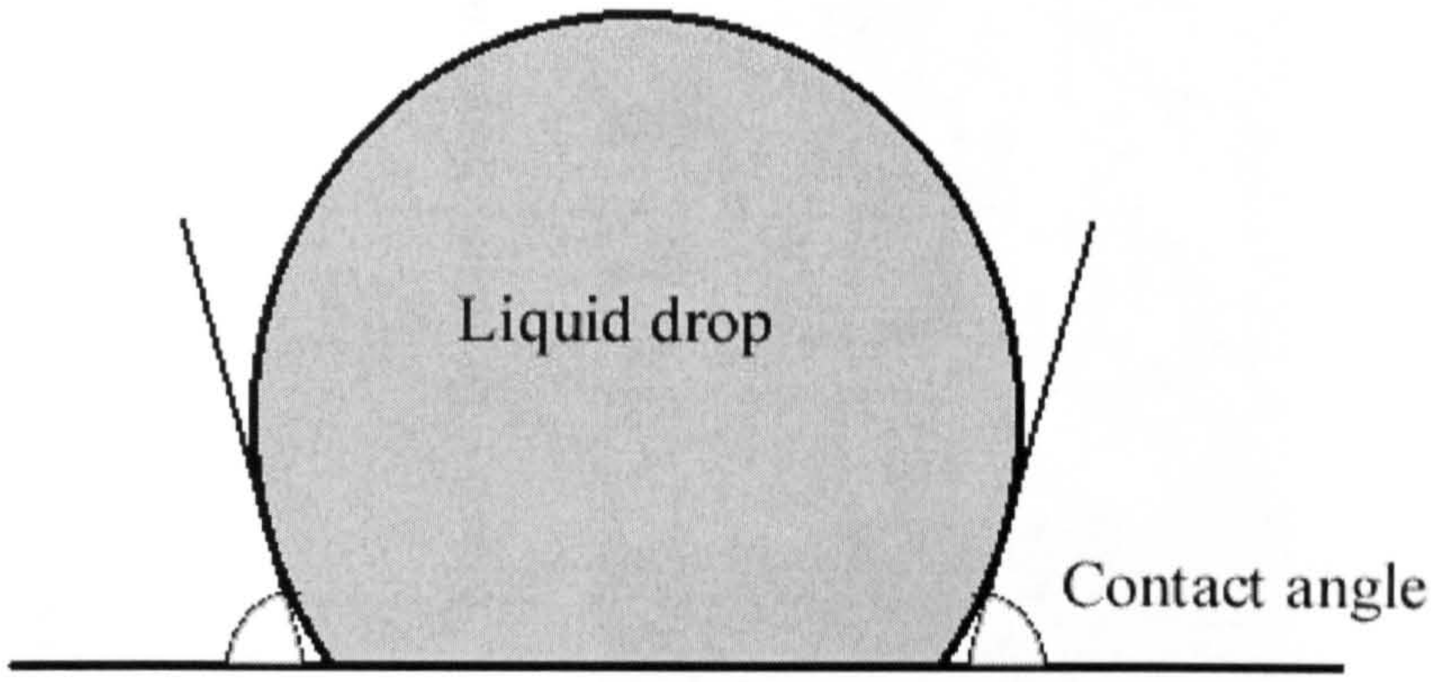


Figure 2.5 Schematic diagram showing the measured contact angle made by a liquid on a surface.

Immersion Trials

METHOD

In order to obtain data from the field, that could be used to relate to the laboratory behavioural data, immersion trails were performed on six of the main coatings: ITS, 615, 616, 617, 618 and 619. A marine plywood board (610mm x 610mm x 7mm) was primed with white VAL and then divided into thirty-six 9cm x 9cm parts. Six replicates of each coating were then painted on the board into these parts. The arrangement of the coatings used was a variant on a latin square design so that replications were placed on each row, i.e. at different depths (Figure 2.7). The boards were sent to 3 different raft locations (Figure 2.6) and immersed in May 2001 so that the top of the panel was ~18in below the waterline:+

Site 1 – Newton Ferrers, Devon UK

Site 2 – Changi, East Singapore

Site 3 – Bratton, West Coast of Sweden

Figure 2.6 World map showing the geographical location of all three sites. More detailed maps for each site are also shown.

After approximately 5 months the boards were assessed (Figure 2.8); the percentage fouling was visually estimated (Meese and Tomich 1992), by trained evaluators (Akzo Nobel employees). Four categories of fouling, microfouling, weed, soft-bodied animals, and hard bodied animals were quantified in terms of percent cover and recorded. All data were arcsine transformed before analyses. In order to establish an overall community fouling value, principle component analysis (PCA) using a covariate matrix was carried out using the four fouling categories, firstly, for all sites and then for individual sites. To compare fouling burden between sites MANOVA was used with a nested model, the hierarchical arrangement being sites then coatings. All statistical analyses were carried out using Minitab v12. To summarise the total percent fouling and community fouling burden of all three sites, the mean PCA values for axis 1 of the coatings, were standardised using a scale 0-25 (0-high fouling, low performance 25-low fouling, high performance), depending on the size of the neighbouring value, so that values of similar size were positioned closely together. To establish if the bioassay could be used as a predictor of antifouling performance the PCA axis 1 scores and total percent fouling values were then used in multiple regression with the behavioural data gained from the bioassays (for details see bioassay analysis below). Results for this are given in each of the relevant chapters.

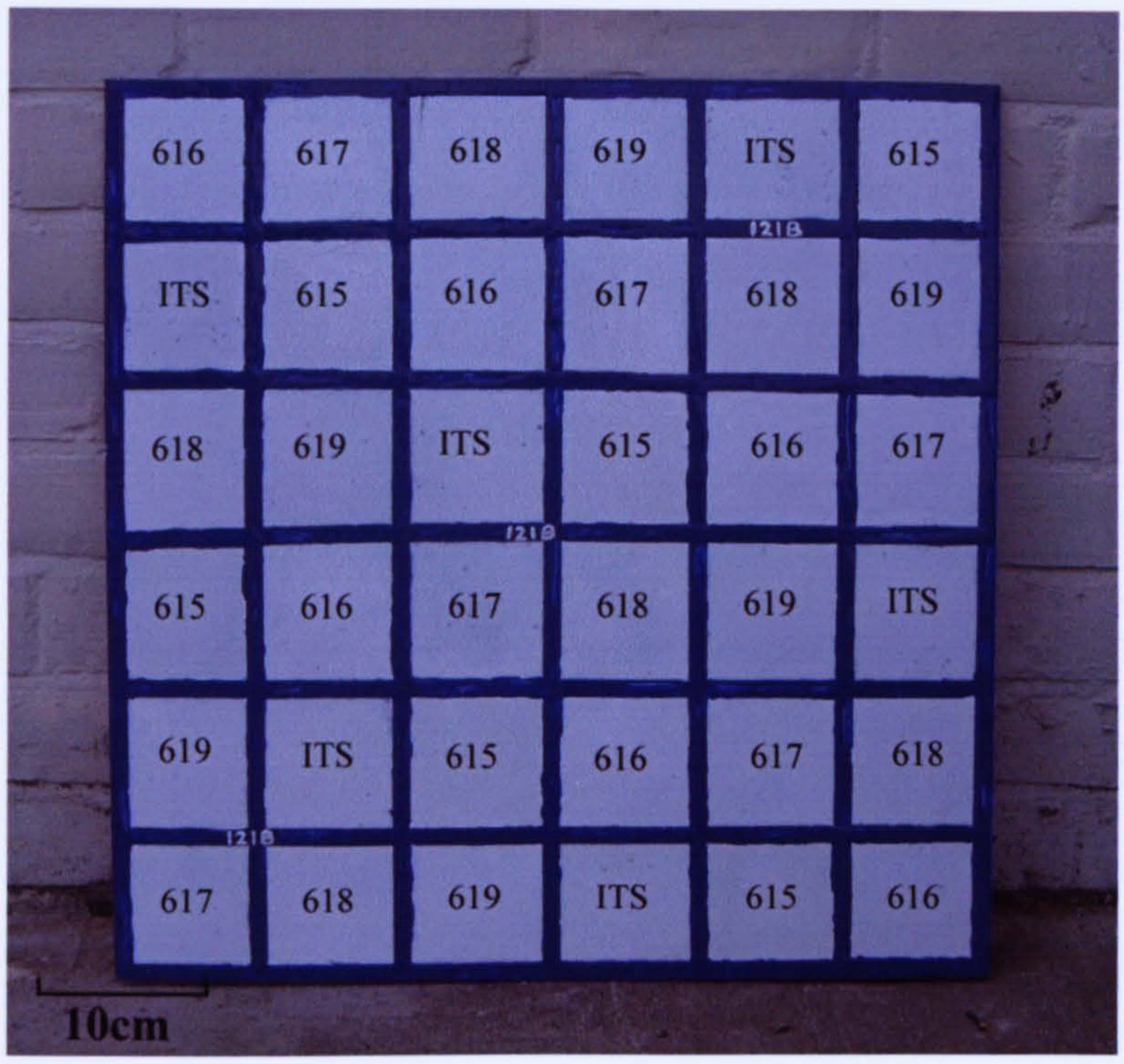


Figure 2.7 Painted board used in immersion trails. The arrangement of coatings is shown, this arrangement is such that a coating type is never adjacent to another coating of the same type and replications of coatings fall on different rows.

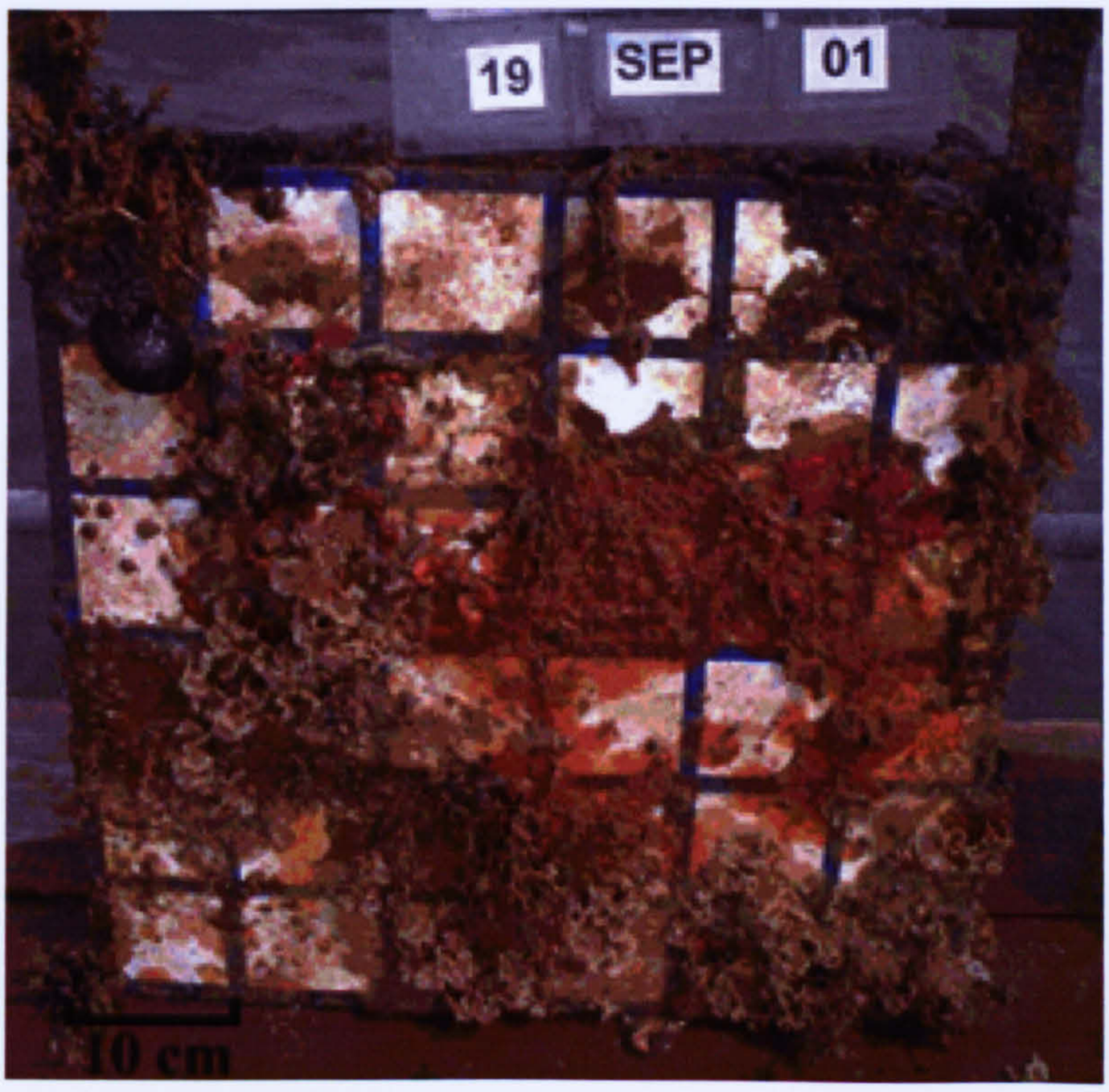


Figure 2.8 Example of board after immersion period, used to assess antifouling performances of each coating.

RESULTS OF IMMERSION TRIALS

The fouling data collected at the sites in UK, Singapore and Sweden, broken down by fouling category and coating types are shown in Figure 2.9. The data shows that weed fouling was minimal at all the sites, whereas most sites had a degree of hard bodied fouling. Sweden was dominated by hard fouling and very little microfouling was found on any of the coatings, Coatings found at the UK site however were covered to a large degree by microfoulers with hard bodied fouler becoming less important with in the community coverage. These data suggest that at different sites the fouling community is very different. These data were analysed (see below) in order to establish how the data were to be managed and used in the multiple regressions carried out with the behavioural data from the bioassays. The data were combined for all sites first, however this showed that fouling at each site was significantly different, therefore data sets from sites were separated.

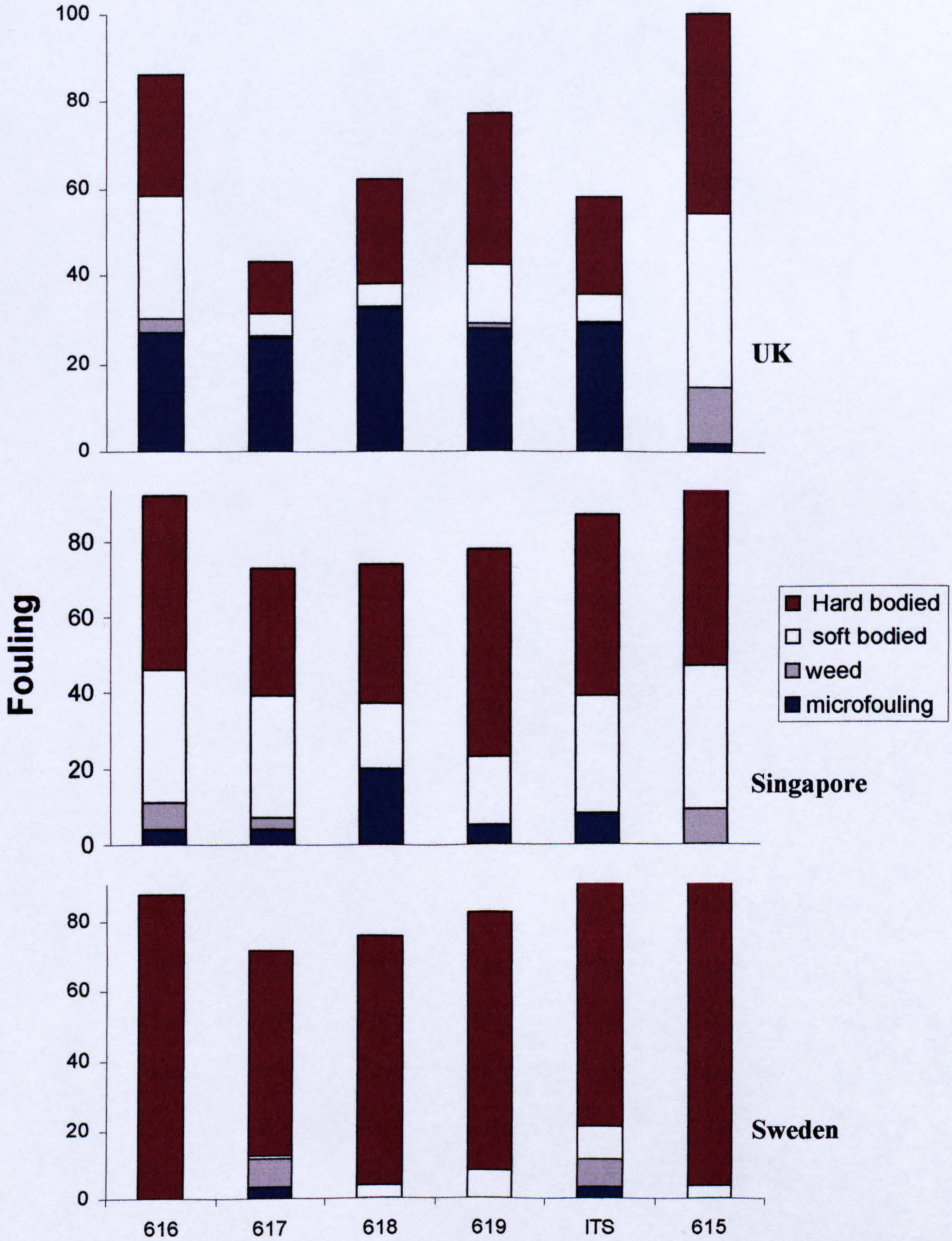


Figure 2.9 Percentage fouling on each coating used in the immersion trials for each site.

All sites combined

The PCA analysis was carried out for the data at all three sites combined, to get a global community fouling score for each coating. The first axis of this analysis explained 65% of the variation in the field fouling data. The scores for axis 1 and 2 shows that both UK and Sweden form two distinct groups (Figure 2.10) suggesting that the fouling at these two sites is different. The data from Singapore tended to be scattered between the Swedish and UK data. To determine if there was a significant relationship between coatings within sites a MANOVA was carried out by sites and by coating. Both sites (MANOVA Wilk's $\gamma = 0.31$ $p < 0.001$) and coatings within sites (MANOVA Wilk's $\gamma = 0.25$ $p < 0.001$) showed significant differences for these data. The percentage of hard fouling on different coatings within each site and the percentage of weed fouling overall at each site were the only factors showing no significant differences (Table 2.3). This analysis suggests that there were consistent differences between coatings between sites for soft bodied fouling, weed fouling and microfouling. Examples of such differences in fouling burden between such geographically different sites can be found in the literature (Wood *et al.* 2000, Swain *et al.* 2000). However, the identified significant relationship between coating type between significantly different sites, suggests a generic pattern to fouling burden by coating between geographically distinct sites. This pattern of consistency of fouling burden between coatings was therefore analysed using separate PCA analysis for each site.

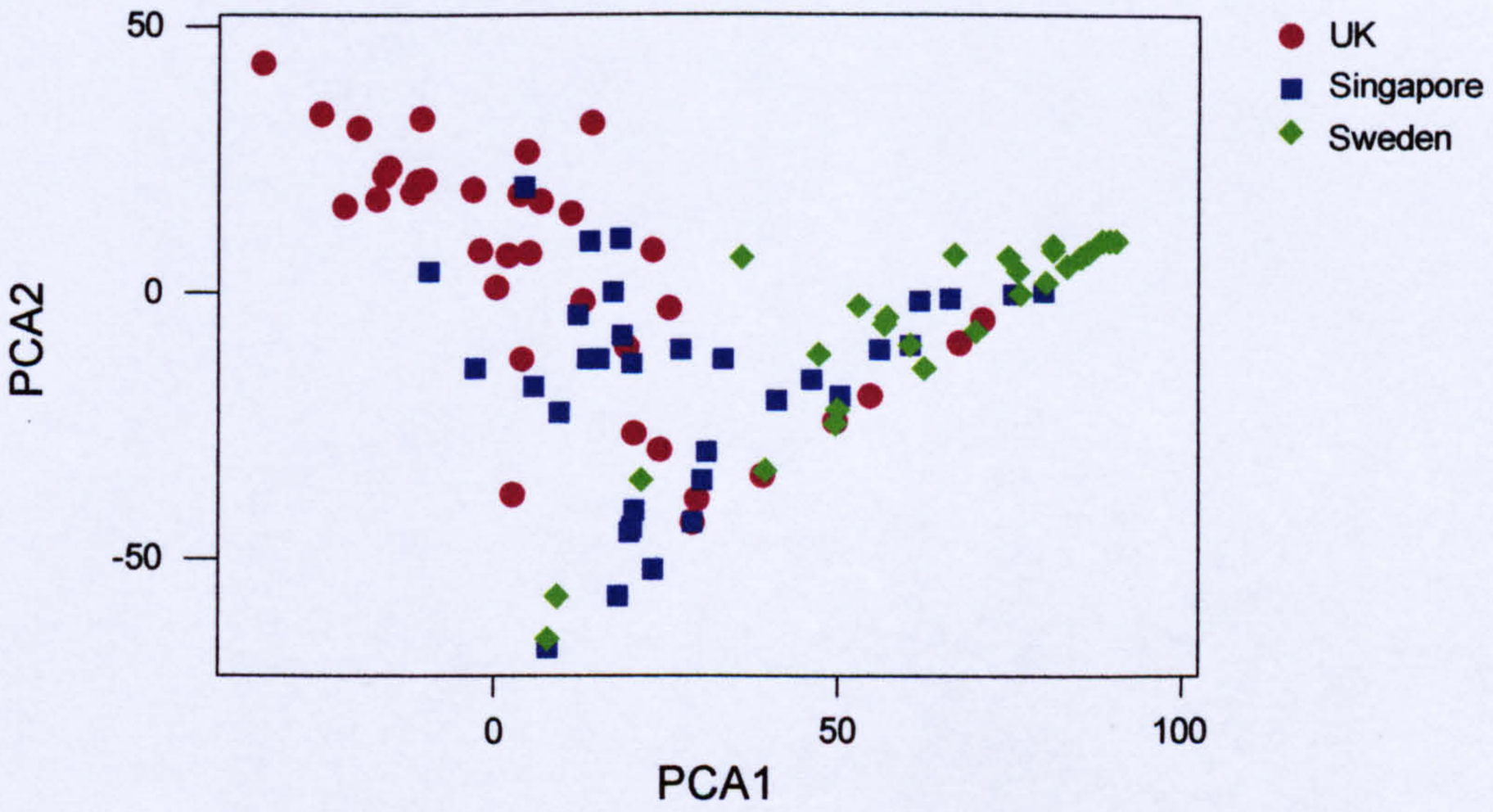


Figure 2.10 Principle component analysis score plot, showing the overlapping relationship between the sites.

	df	F	p
Microfouling			
Site	2	39.88	<0.001
Coating within site	15	2.09	0.017
Weed fouling			
Site	2	1.94	0.150
Coating within site	15	4.24	<0.001
Soft fouling			
Site	2	14.62	<0.001
Coating within site	15	2.84	0.001
Hard fouling			
Site	2	55.50	<0.001
Coating within site	15	1.70	0.066

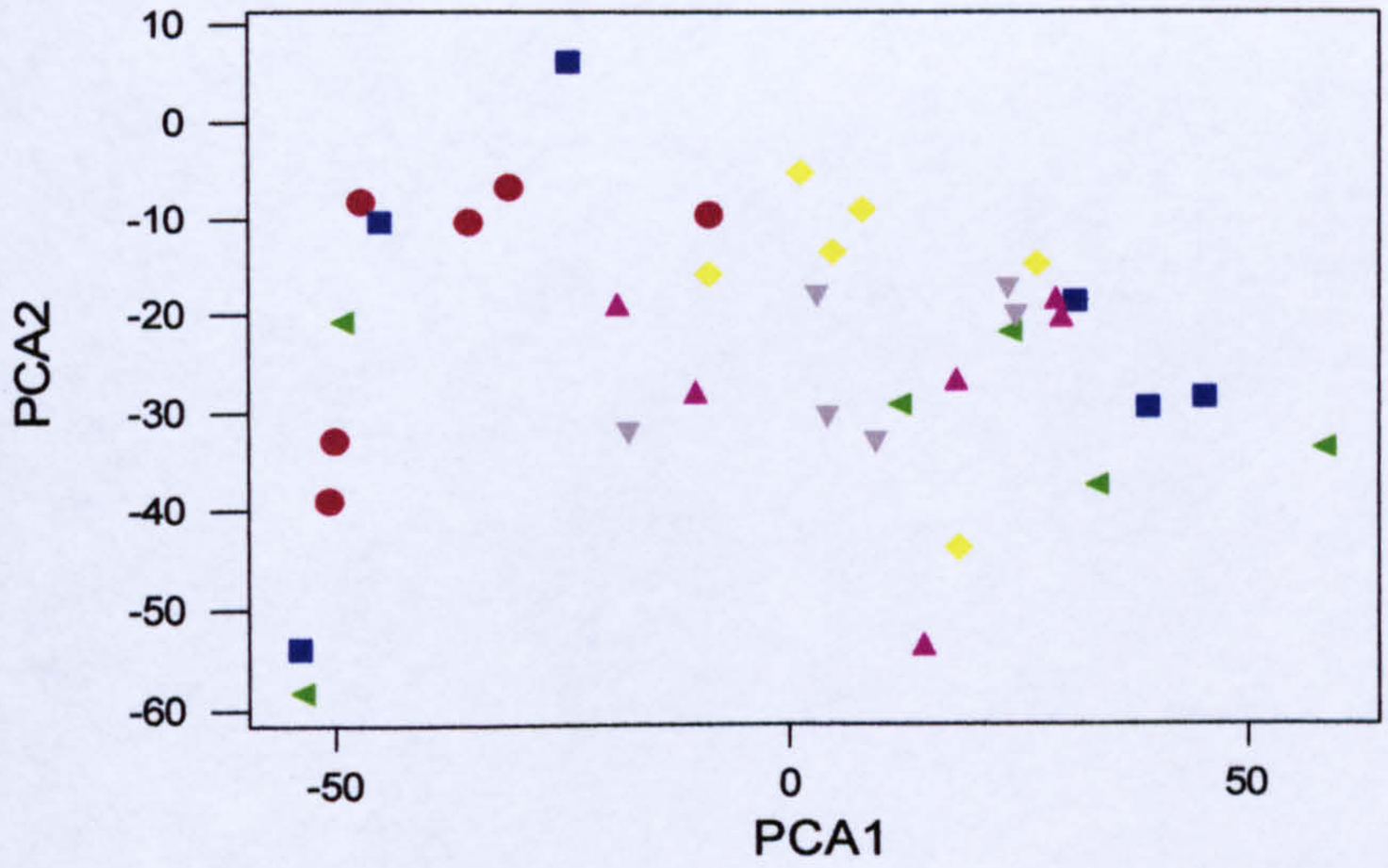
Table 2.3 Univariate ANOVA table from MANOVA analysis for each fouling category. Degrees of freedom (df), F ratio (F) and significance (p) are presented.

Sites separated

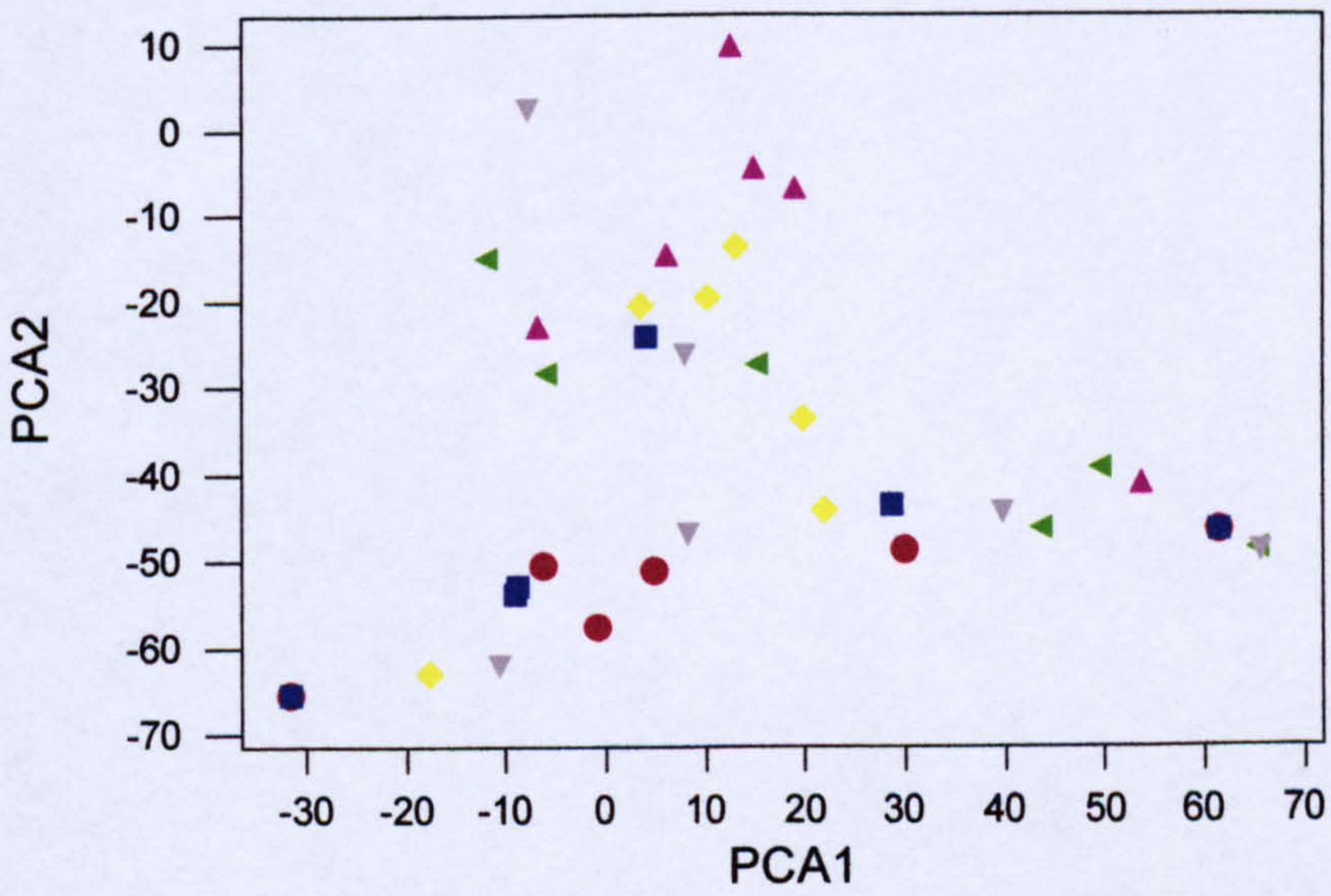
To investigate the relationship between coatings at different sites, PCA analysis was carried out for each of the three sites separately. Seventy five percent of the variation in the data gained for the UK site can be explained by the PCA score for axis 1 (Table 2.4). The scores for axis 1 and 2 are shown in Figure 2.11. There is a tendency for some grouping for coatings 615, ITS and 617 (Figure 2.11). MANOVA was used to investigate the significance of these groupings. UK shows an overall significant difference in fouling burden of coatings (MANOVA Wilk's $\gamma = 0.171$ $p < 0.001$). Weed and soft bodied fouling showed significant differences (Table 2.5) suggesting that these categories of fouling were significantly influenced by coating type. However microfouling and weed fouling were not significantly affected by coating type (Table 2.5).

For the Singapore data the PCA scores for axis 1 and 2 can be seen in Figure 2.11. The first axis of this analysis, for the Singapore data, explains 61% of the variation (Table 2.4). There is a tendency for some groups to be apparent for some coatings (Figure 2.11) also at this site, mainly coatings 618 and 615. MANOVA was used to investigate the significance of this grouping shown in the PCA analysis. Overall, coatings at the Singapore site show a significant difference (MANOVA Wilk's $\gamma = 0.30$ $p = 0.016$). There were significant differences between coatings for micro and weed fouling at this site (Table 2.5), suggesting that coating type is significantly influencing the micro and weed fouling burden at this site. However both soft and hard bodied fouling showed no significant differences (Table 2.5) suggesting that coating type was not significantly influencing these categories of fouling burden at this site.

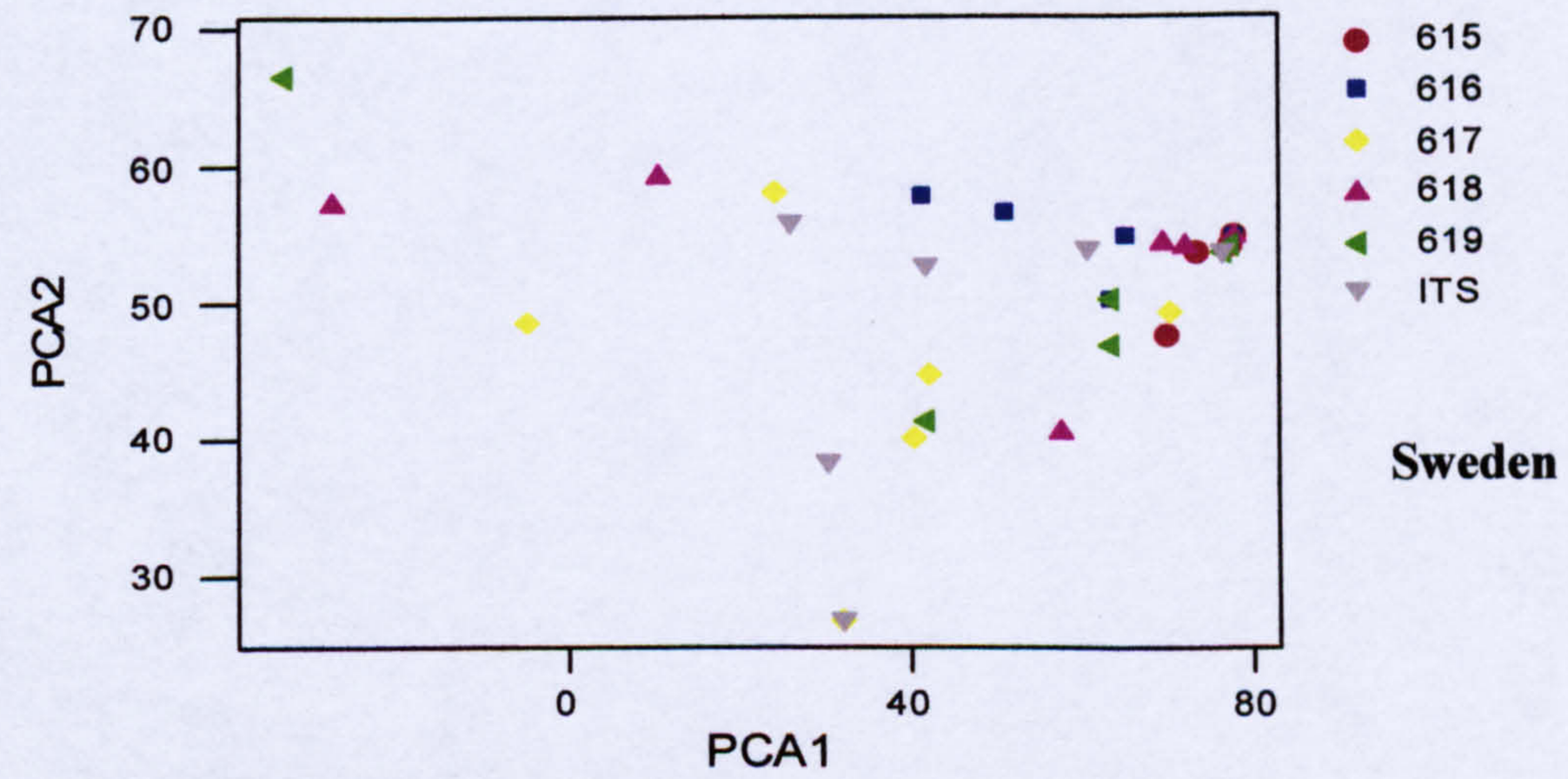
The PCA scores for axis 1 and 2 for the Swedish data can be seen in Figure 2.11, axis 1 explains 89% of the variation (Table 2.4). Some degree of grouping between coatings 615, 616, 619 is apparent at this site (Figure 2.11). However the MANOVA carried out to investigate the significance of this grouping showed that overall coatings there was no significant difference ($p > 0.05$) between coating type. Univariate analysis however did show significant differences among coating type for hard bodied fouling, but not for the other categories of fouling (Table 2.5). This suggests that only hard bodied fouling burden is influenced by the coating type at this site.



UK



Singapore



Sweden

Figure 2.11 Principle component analysis score plots comparing all three sites, and showing the scattered relationship between the different coatings.

UK	**PCA 1**	**PCA2**
Eigenvalue	1023.8	208.4
proportion	0.728	0.148
cumulative	0.728	0.876
variable		
Microfouling	0.690	-0.396
Weed fouling	-0.068	0.153
Soft fouling	-0.430	0.418
Hard fouling	-0.578	-0.803
Singapore		
Eigenvalue	710.33	388.40
proportion	0.616	0.337
cumulative	0.616	0.953
variable		
Microfouling	-0.096	0.534
Weed fouling	-0.018	-0.074
Soft fouling	-0.595	-0.704
Hard fouling	0.798	-0.462
Sweden		
Eigenvalue	875.14	70.10
proportion	0.890	0.712
cumulative	0.890	0.961
variable		
Microfouling	-0.025	-0.395
Weed fouling	-0.044	-0.188
Soft fouling	-0.625	0.712
Hard fouling	0.779	0.549

Table 2.4 Eigenanalyis of the covariance matrix of PCA for all three sites showing the Eigenvalue and influences of all categories of fouling given.

UK	**df**	**F**	**p**
Microfouling	5	1.67	0.173
Weed fouling	5	12.03	<0.001
Soft fouling	5	6.16	<0.001
Hard fouling	5	1.39	0.256
Singapore			
Microfouling	5	3.51	0.013
Weed fouling	5	4.36	0.004
Soft fouling	5	2.46	0.056
Hard fouling	5	0.39	0.849
Sweden			
Microfouling	5	0.80	0.558
Weed fouling	5	0.64	0.673
Soft fouling	5	1.36	0.268
Hard fouling	5	3.12	0.022

Table 2.5 Univariate analysis of variance table showing the degrees of freedom (df), F ratio (F) and significance (p) calculated for each coating and each fouling category at the three different sites.

SUMMARY OF RESULTS FROM IMMERSION TRIALS

To summarize the community fouling data, as described by the PCA analysis, and to determine the trends between coatings by site, the PCA axis 1 scores for each site was standardised between 0 and 25 (0- high fouling, low performance 25- low fouling, high performance). The rank order along the standardised axis is similar between the UK and Sweden (Figure 2.12). In Singapore coating 615 and 616 are highly fouled as in the other two sites. However the variation in the standardised fouling burden for the other coatings is generally dissimilar to the other 2 sites.

The total percent fouling as seen in Figure 2.9 was also summarized using a standardized scale between 0 and 25 (0- high fouling, low performance 25- low fouling, high performance) to determine trends between coatings and to establish any differences

in antifouling performance as compared to PCA analysis. When using these data, coatings from Sweden and the UK site show approximately the same trends in antifouling efficiency as using the PCA scores (Figure 2.13). Coating 617 and 618 at the Singapore site however perform a lot better if just the total percent fouling is considered. Coatings at the Singapore site show relatively the same trend pattern as the other two sites using these data.

Both total percentage and PCA scores for each site separately were used to investigate any link between the field data and laboratory behavioural data.

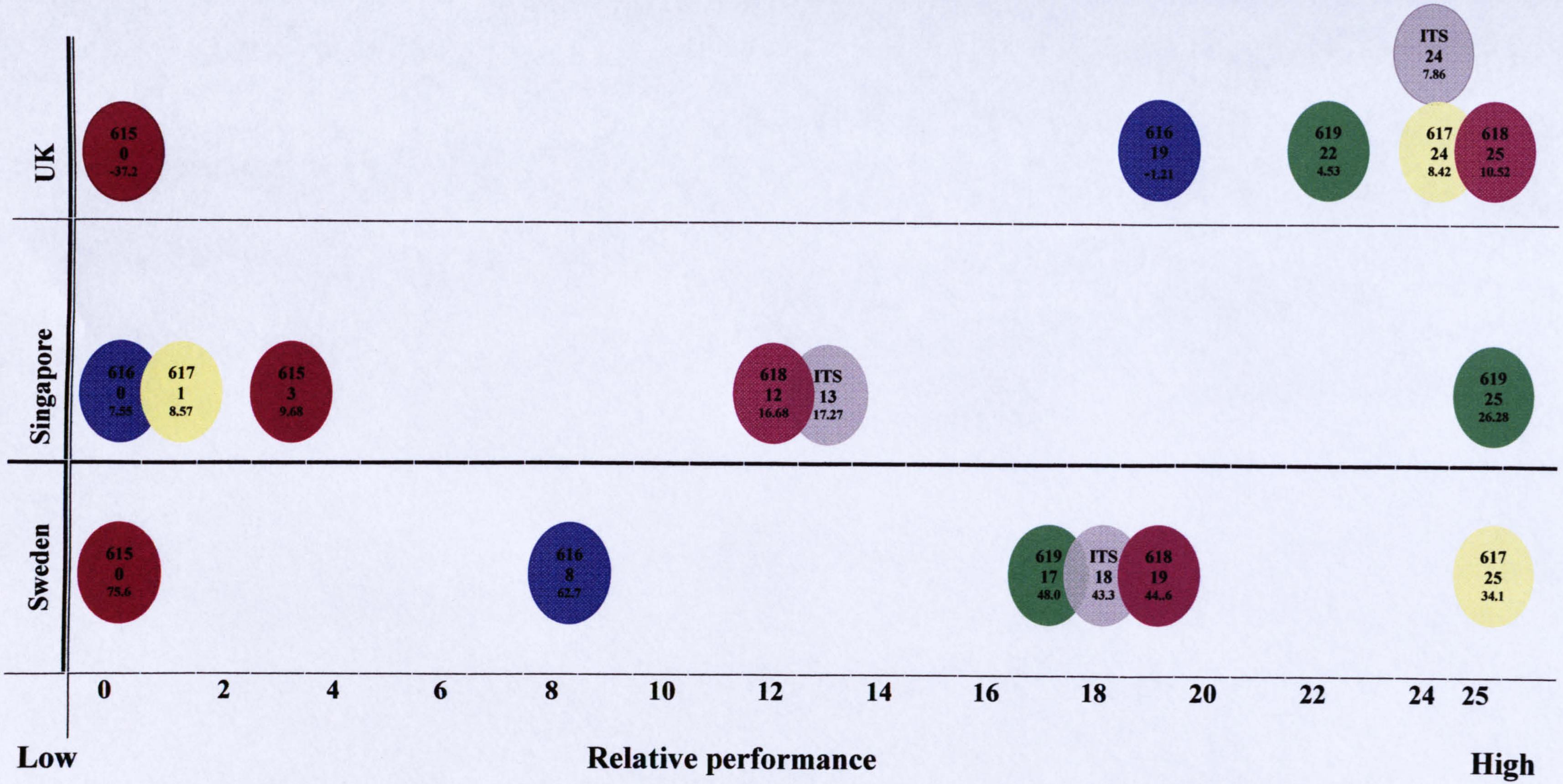


Figure 2.12 Antifouling performance of the coatings for each site on a standardised axis. PCA analysis of community fouling was used to position the coatings along the axis. A score is given for each which shows the relative distance between each coating. (0- High fouling, low performance 25- low fouling, high performance).

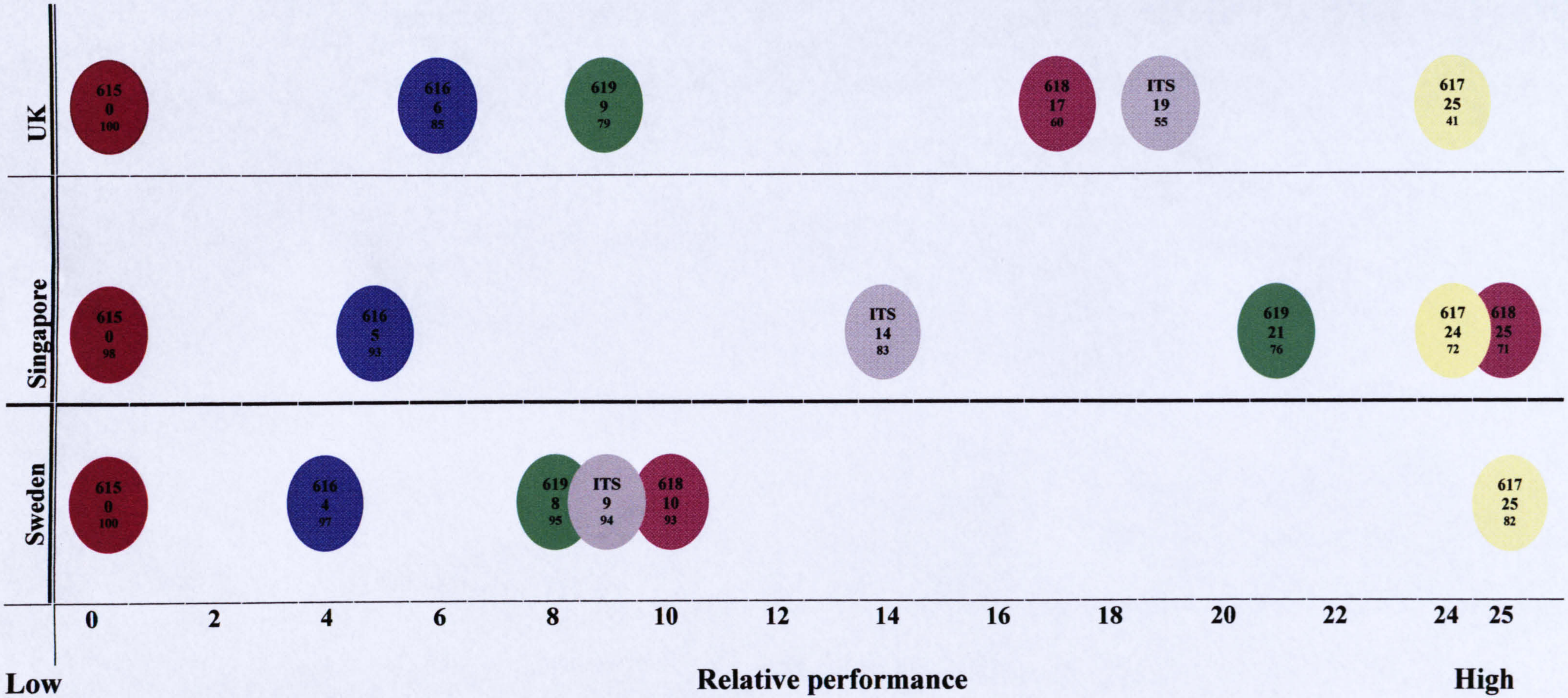


Figure 2.13 Antifouling performance of the coatings for each site on a standardised axis. Mean total percentage fouling was used to position the coatings along the axis. A score is given for each which shows the relative distance between each coating. (0- High fouling, low performance 25- low fouling, high performance).

Outline of experimental chapters

The following chapter (Chapter 3) describes a bioassay using the choice test method using *Mytilus edulis*. The initial part of the research outlined in Chapter 3 was carried out in collaboration with a student carrying out a BSc honours project, and due to the potential of this work further work research was undertaken as part of this thesis. Chapter 4 outlines work using a standard settlement technique, carried out in order to establish a rank order of efficiency of the coatings seen in the laboratory. The bioassay procedure, the main part of this thesis, using the species specified above, is described in Chapters 6-8. Methodology of this procedure is given in Chapter 5.

CHAPTER 3

CHOICE TEST

CHAPTER 3

A BEHAVIOURAL BIOASSAY USING *MYTILUS EDULIS* – THE CHOICE TEST METHOD

Introduction

Mytilus edulis (Linnaeus), the common or blue mussel, is widely distributed and abundant throughout Europe (Hayward *et al.* 1996). It is currently widely used as a species for detecting and monitoring anthropogenic pollution and contamination (e.g. Goldberg *et al.* 1978, Johnson, 1988), as literature on physiology and ecology is extensive (Chipperfield 1953, Bayne 1964, Board 1983, Johnson 1988, King *et al.* 1990, Petraitis 1991, McGrath *et al.* 1994, Reimer *et al.* 1995, Dobretsov and Railkin 1996, Hunt and Scheibling 1998, Cote and Jelnikar 1999). *M. edulis* is also one of the major fouling species causing serious economic problems (Richmond and Seed 1991) and as such, extensive work has been carried out on physical and biological influences on mussel settlement (e.g. Board 1983, Igić 1988, King *et al.* 1990, Hunt and Scheibling 1996, Pulfrich 1996).

Laboratory based bioassays have also been developed using *M. edulis* in order to screen novel antifouling substances such as the 'blue mussel' assay (Harada *et al.*1984). This assay used the production of byssus threads as an indicator of repellent activity of the active antifouling substance under investigation. Other authors also agree that *M. edulis* juveniles are ideal subjects in screening bioassays (Takasawa *et al.* 1990, Satuito *et al.* 1993, Kitajima *et al.* 1995, Etoh *et al.* 1997, Sera *et al.* 2000). *Mytilus edulis* juveniles were chosen as the test organism for this bioassay, due to their abundance and availability through both winter and summer. They are capable of assessing the

suitability of a substratum using chemoreceptors (Morse 1990, Woodin 1991) and juveniles have also been shown to actively crawl over the substratum, testing its suitability with the foot. (Crisp *et al.* 1985, Satuito *et al.* 1993).

In choice, or preference testing, the test organism is given a choice of two or more conditions and allowed to move freely between them. This allows an insight into what the test organism can discriminate between and can be used to assess the relative value of the particular environment or condition selected. A complex environment can be broken down into individual parts and the attractiveness of each component evaluated (Dawkins 1978). Each component can be ranked in order of preference (Heizmann *et al.* 1998). Such choice testing techniques have been used in many different fields of research: Animal behaviour (Jones *et al.* 1996, Jensen 1999, Kopp *et al.* 1999) including environment improvement or enriching (Blom *et al.* 1992, Heizmann *et al.* 1998), pharmacology (Wilson *et al.* 1997), physiology (Czech 1999) and parsitology (Adams and Fell 1997). Choice testing has also been used in larval settlement experiments, looking at gregariousness (Knight-Jones 1951), species discrimination (Wilson 1970) and in particular substratum choice (Ryland 1959, Crisp and Williams 1960, Williams 1964, Knight-Jones *et al.* 1971). Laboratory choice testing is relatively simplistic, after the choice chamber has been set up, and are easy to monitor and evaluate. However they cannot distinguish between a preference for a condition or environment, or an avoidance for it. Also as with all laboratory testing there is always concern that the results may not be applicable to the real environment as animals often behave unnaturally under artificial conditions (Crowe and Underwood 1998). Therefore relating the findings to the field often gives more convincing results.

The aim of this work was to design a simple, yet accurate and precise, bioassay, which could be carried out on manufactured coatings by any persons with minimum training and in non-specialist laboratories. This bioassay is not designed to replace submersion trials, but be used as a screening test, to permit an initial indication as to how effective the antifouling properties of the coatings are. Effective coatings subsequently being selected for expensive and time consuming field trials

Materials and method

Test organism

Juvenile *Mytilus edulis* of shell length 5 ± 2mm were collected from the Black Middens, North Shields, Tyne and Wear, UK [55° 00'N, 1°25'W] from December 1998 through to May 2000. Clumps of adults were carefully teased apart, as to not tear the byssal threads and cause damage to the mussels. The juveniles were collected from among the adults. A clump of about 20 adults on average yielded around five juveniles. They were gently rinsed in seawater and placed in a small plastic container with seawater collected from the site. The container was placed in a controlled temperature room at 10 ± 1°C prior to experimentation. The mussels were kept in these conditions for between three to five days during which time no aeration or food was given. Only mussels showing crawling behaviour or extension of the muscular foot were chosen for the bioassay.

Test choice chambers

Two types of the test chamber were used. Type I was initially used but proved expensive due to increased replication, thus Type II was developed as an alternative.

Although constructed differently, altering only in size and materials used, both made the same basic chamber. Each chamber unit constructed made two independent choice chambers.

TYPE I

Two aluminium panels rollered with different coatings were placed at the bottom of glass staining troughs (10.2cm x 10.2cm x 7.3cm). A Perspex partition was used to divide the troughs into two equal halves (Figure 3.1). Prior to experimentation the test chambers (excluding test panels) were dipped in Sigmacote® (Sigma Chemical Co. ltd.) which coats the glass with a silicone layer and prevents the mussels attaching to the sides of the troughs with out any detrimental effects (Kitajima *et al.* 1995). The chamber was left to air dry for 24hrs before use. After use the chambers were machine washed using a commercial acid rinse, Deconmatic, rinsed in clean water and re-used.

TYPE II

Five pieces of glass (two, 14.6cm x 5cm and three, 9cm x 5cm) were put together using aquarium grade silicone to make the outer arena. Test coatings were rollered on to a glass panel (10cm x 15cm) so that the coating on one half of the panel differed from the other half. Finally the glass arena was placed on top of the panel as to complete the test chamber (Figure 3.2).

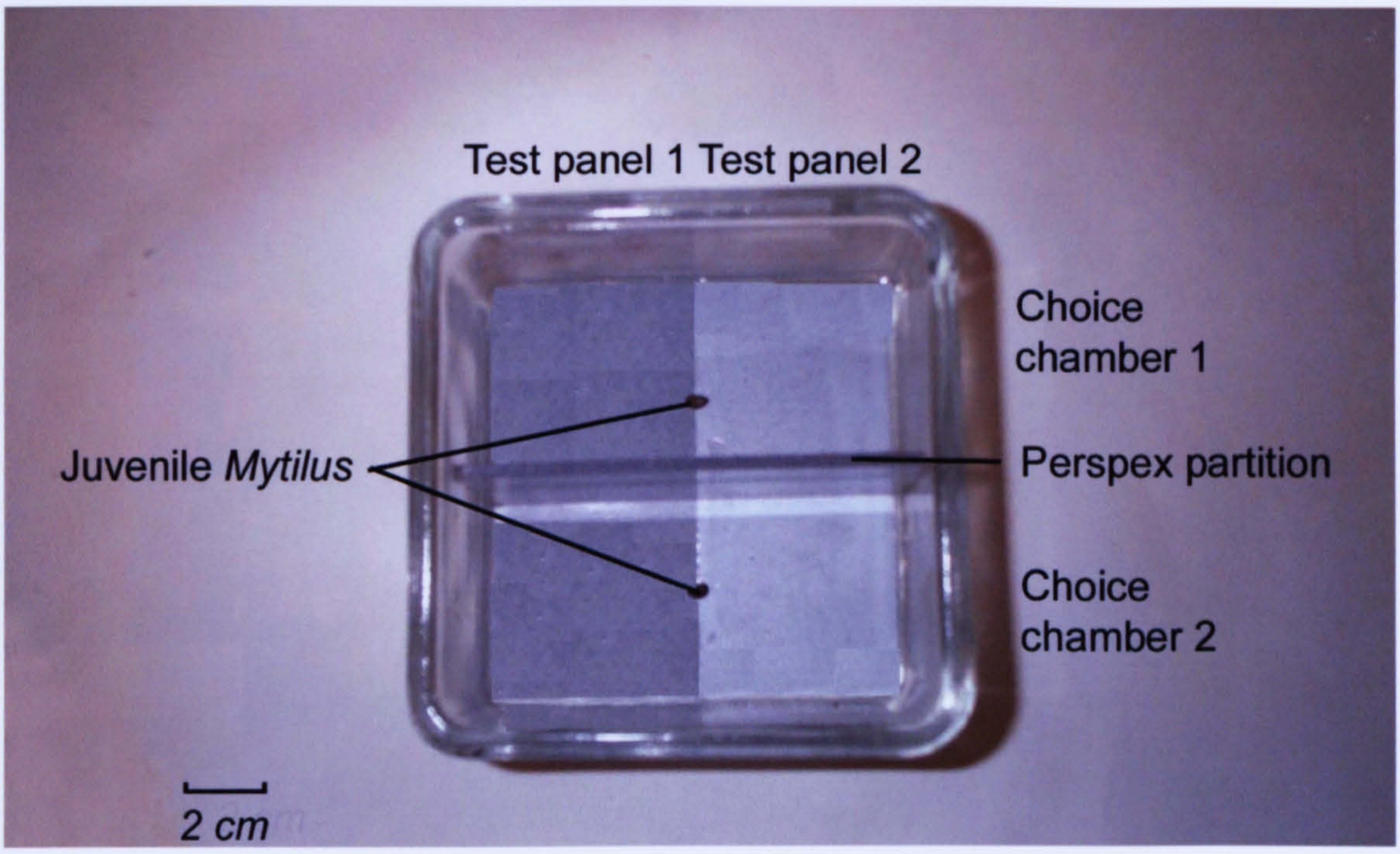


Figure 3.1 Type I choice chamber, showing test panels and starting position of juvenile mussels.

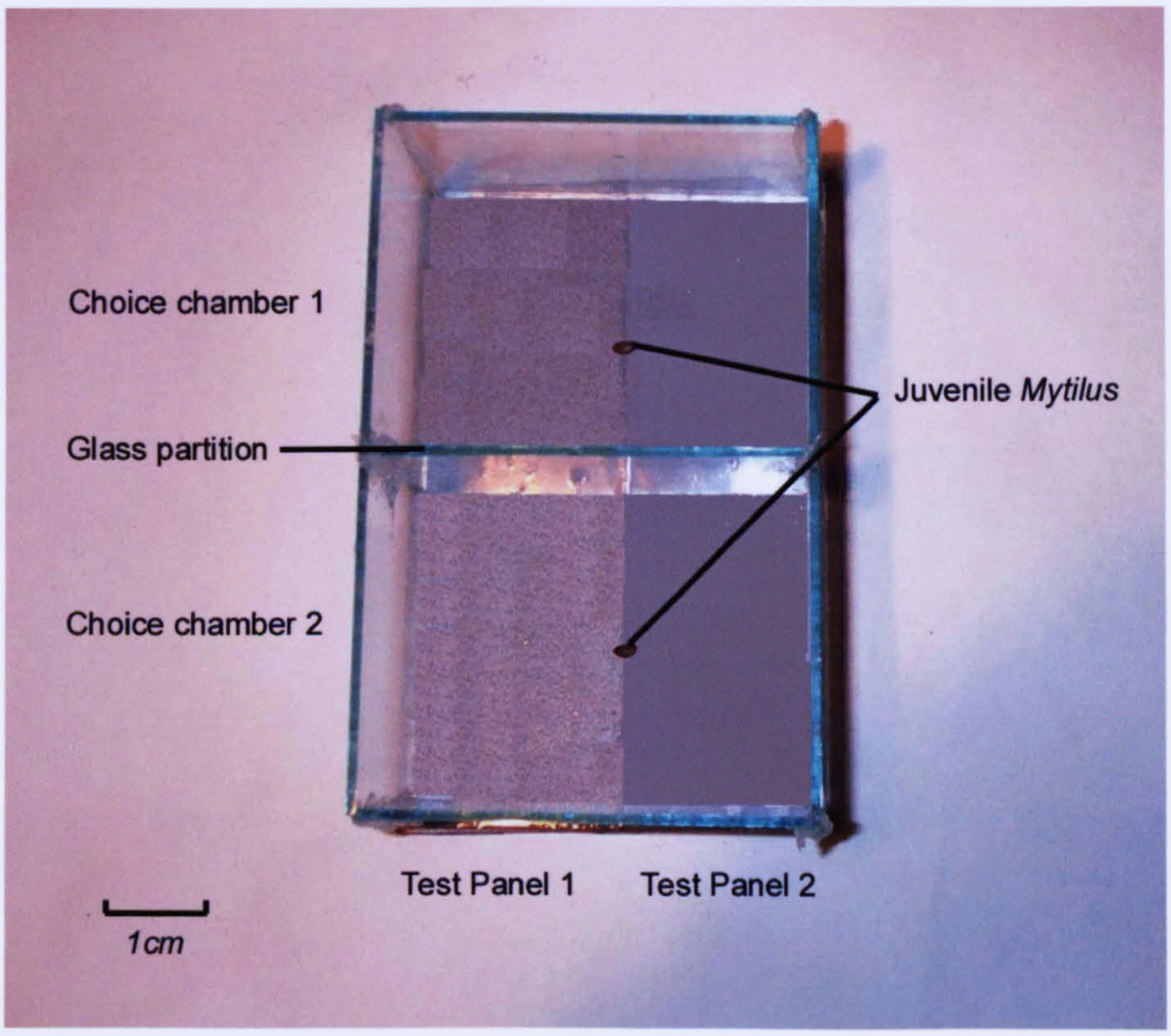


Figure 3.2 Type II choice chamber, showing test panels and starting position of juvenile mussels

Akzo Nobel performed chemical analysis on Sigmacote® in order to find a cheaper alternative. 10% Glassclad® 6C in heptane was used instead of Sigmacote®. The glass arenas (excluding test panel) were dipped and left to dry for 24hrs as for Type I. After use the chambers were machine washed with Deconmatic, rinsed thoroughly and re-used.

It was found when comparing coatings 617 and 618 against ITS, that a significant number of mussels crawled up the sides ($\chi^2 = 9.102$ $p < 0.001$). As the antifouling efficiency rating for these coatings is high (Akzo Nobel pers. comm.), it was believed that the Glassclad® was not sufficient to deter the mussels from the sides. To overcome this problem the sides of the chambers used for the comparison of these coatings, were painted with the coatings under investigation. For example the sides of choice chamber comparing ITS against 617 would have been coated half with ITS and half with 617 respective to the coating on the bottom test panel.

Bioassay Method

One juvenile mussel was placed in the middle of the choice chamber where the two coatings met. Seawater from the collection site was filtered through a 0.2µm Millipore® filter and placed in the choice chamber to give a depth of ≈1.5cm. In order to seal the water within the chamber Type II chambers were placed in a rectangular container (60cm x 30cm x 20cm). This was then filled with the filtered water to give a depth in the chambers of ≈1.5cm. To prevent any phototactic behaviour the chambers were left in the dark, at 10 ± 1°C for 16 hours. After this period the coating on which the mussel had settled on was recorded. Replication varied from 8 using Type I chambers

to 20 using Type II. Data was analysed using χ^2 tests with degrees of freedom for all tests as 1. Yates' continuity correction was used according to Caswell (1982).

COMPARISON OF COATINGS

A range of coatings were investigated and the results compared with known antifouling efficiency ratings.

Trial 1 – Using Type 1 arenas, ITP (high fouling, low performance), ITS (low fouling, high performance) and two intermediate coatings PXC and VRD were compared.

Trial 2 - Using Type II arenas 616, AF2, 617 and 618 (see Table 2.2) were compared. ITP and ITS were compared again along with these coatings to test for any differences due to arena type.

During both trials only two different coatings were compared at any one time (Table 3.1) as the mussels leave a mucus trail as they move over the surface by their foot (Bayne 1976). Such chemical trails have been shown to affect settlement choice in other species, in particular barnacles (Clare *et al.* 1994, Clare *et al.* 1998, Matsumura *et al.* 1998a), therefore test panels were only used once. Although the test chambers were reused after removing any protein deposits by using an acid rinse, this method was not used for the test panels, due to the concern of either altering the chemical nature of the coating or leaving a deposit on the surface of the coating.

	PXC	**616**	**AF2**	**VRD**	**ITS**	**617**	***618***
ITP	8	20	20	8	8	-	-
PXC		-	-	8	8	-	-
616			20	-	20, 40*	-	-
AF2				-	20	-	-
VRD					8	-	-
ITS						20	20,60*
617							20

Table 3.1 showing the pair wise comparisons of coatings investigated in both trials, as indicated by the number of replication made for each: 8 replications-trial 1, 20 or >20-trial 2. A re-run, increasing sample size, of the investigation is indicated by * .

Results

Trial 1

This trial was a pairwise comparison of ITS and ITP and two intermediate coatings VRD and PCX. The results can be seen in Figure 3.3.

These results show that ITP was preferred to ITS ($\chi^2 = 8.125$, $p = 0.004$) and VRD ($\chi^2 = 8.125$, $p = 0.004$) VRD was chosen in preference to ITS ($\chi^2 = 4.625$ $p = 0.039$). No significant differences were found for PXC against any other coating. From these results the rank order of the coatings is as follows: ITS>VER> ITP from the highest antifouling performance to the lowest. This corresponds to findings in the field (Akzo Nobel pers. comm.)

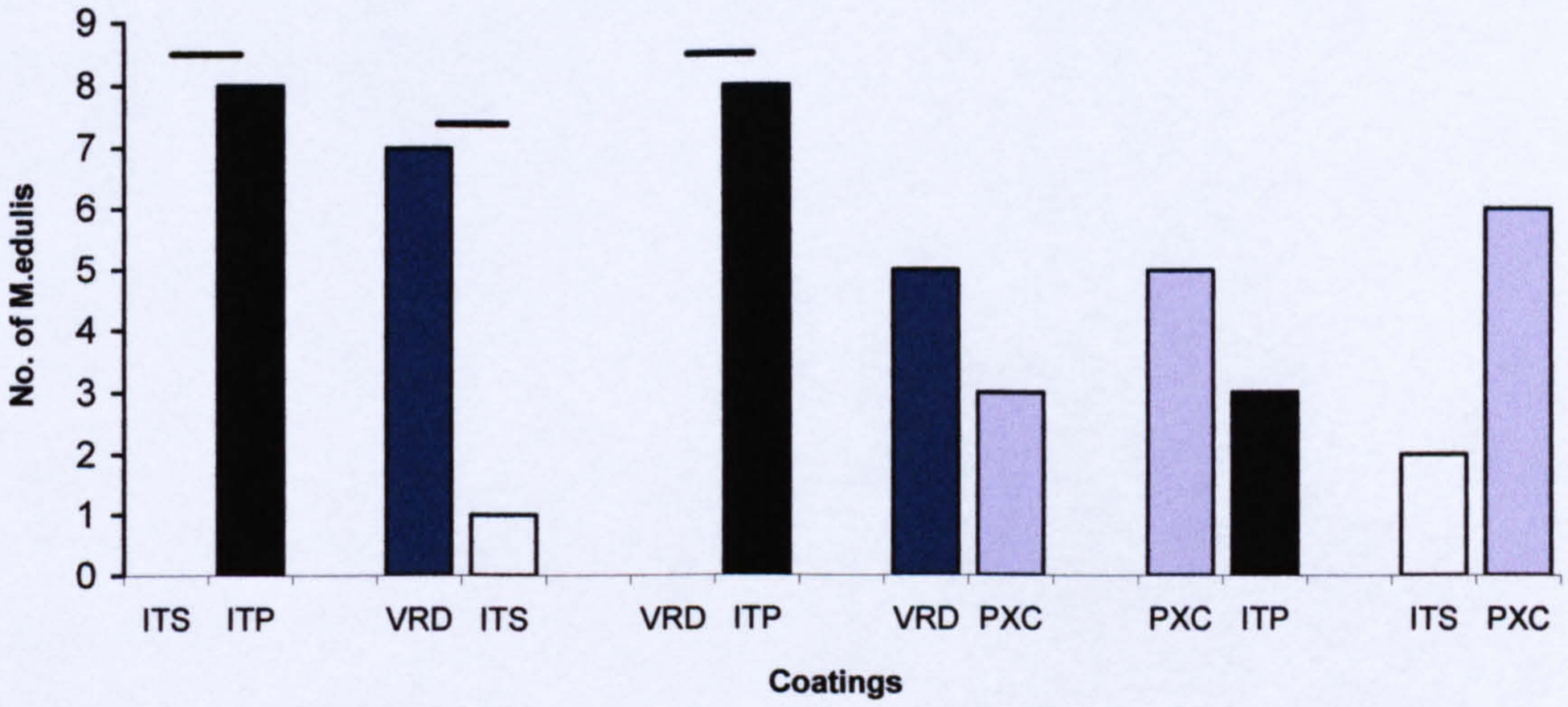


Figure 3.3 Pairwise comparisons of different test coatings used in trial 1, showing number of *M. edulis* settled on each coating after 16 hrs. Bars indicate significant differences at $p<0.05$.

Trial 2

ITS and ITP were compared again for the next set of coatings (Figure 3.4), and this enabled Type I and Type II arenas to be compared. ITP was preferred to ITS (χ^2 = 3.912, p = 0.051) as before. This suggests that chamber type was not having a significant effect on larval choice. AF2 was preferred to ITS (χ^2 = 5.466, p = 0.023) but no other significant preferences for a coating type were found. 616 and ITS choice were re-run with a sample size of 40 to establish if a higher sample size would show a significant difference. In this comparison ITS was chosen in preference to 616 (χ^2 = 9.355, p = 0.003).

When comparing coatings with high efficiency ratings (Figure 3.5), it was only when a sample size of 60 was used, that the mussels showed a significance preference for ITS over coating 618 (χ^2 = 11.811, p < 0.001). With a sample size of 20 no significant differences were found (p > 0.05), therefore at this sample size, no statistically significant preference for the three coatings could be detected.

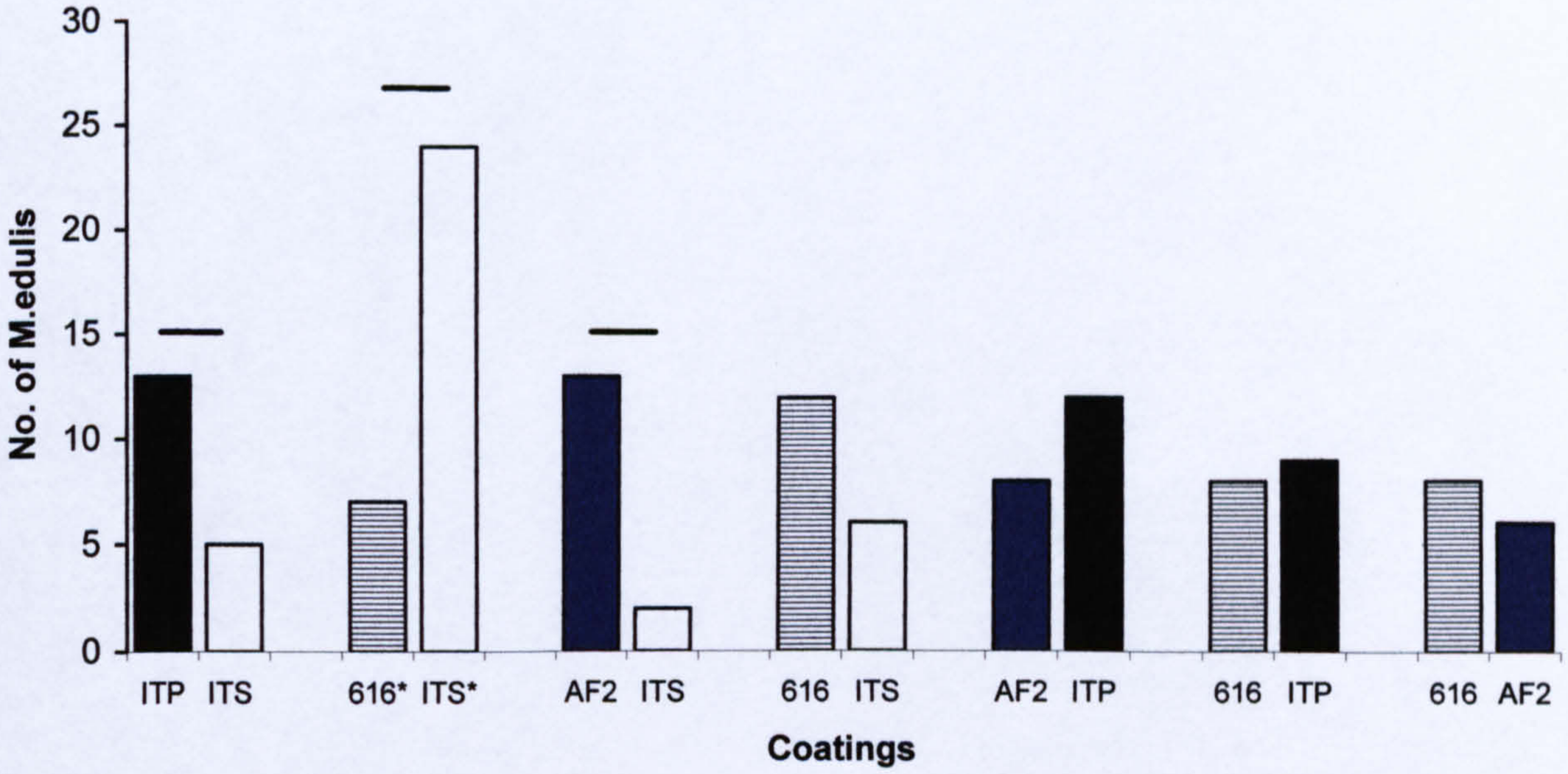


Figure 3.4 A pair wise comparison of the coatings included in trial 2. ITP, ITS, 616, and AF2, used Sigmacote on the sides of the arenas. *Re-run increasing sample size from n = 20 to n = 40. Bars indicate significant differences at $p < 0.05$.

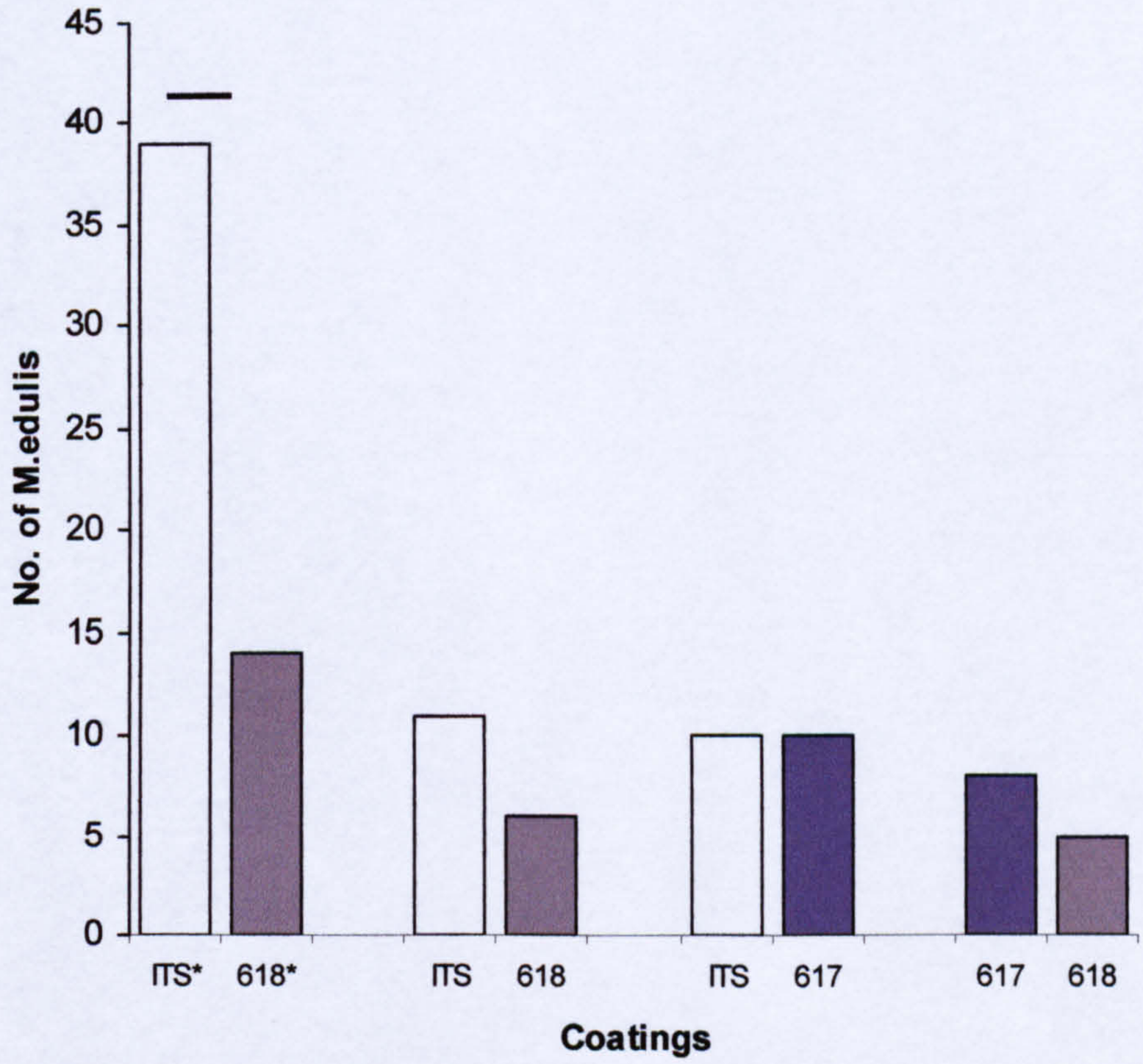


Figure 3.5 Pairwise comparison of coatings included in trial 2, using arenas coated with the appropriate coating, ITS, 618, and 617, *Re-run increasing sample size from n = 20 to n = 60. Bars indicate significant differences at $p < 0.05$.

Discussion

The developed choice test using *M. edulis* can provide an indication of antifouling efficiency of manufactured coatings. ITP is used as a tiecoat and has no antifouling properties, thus when compared with ITS, the leading non-toxic antifouling coating, *M. edulis* consistently showed a preference for ITP. The method allowed a rank order to be established for the coatings used in trial one, apart from coating PXC, which are consistent with previous field data (Akzo Nobel pers. comm.). There is always a concern that laboratory based experiments do not fully represent the patterns and processes found in nature (Crowe and Underwood 1998), however, within this trial the results have corresponded to findings within the field. On the other hand the results for trial two were too inconsistent to establish a ranking order. It is believed that the intermediate coatings used in this trial are very closely related in respects to antifouling performance and that the sensitivity of this method was not sufficient enough to allow for this.

Byssal thread production

Byssal threads are secreted by the byssal gland located in the muscular foot of *M. edulis,* and they are the means by which the mussels attaches itself to the substratum. The byssus consist of three parts; the stiff stem which remains attached to the distal part of the foot, the thread which extends into the pliable elastic pad which adheres to the substratum. Extensive work has been carried out regarding the molecular structure of the thread itself and the adhesive protein which binds the pad to the substrate (Mascolo and Waite, 1986, Rzepecki *et al.* 1992, Qin and Waite 1995, Qin *et al.* 1997, Coyne *et al.* 1997, Waite *et al.* 1998). Despite the production of the threads *M. edulis* is by no

means immobile and can easily break them and reattach new ones (Bayne 1967, 1976, Crisp *et al.* 1985,) thus choice may not be as critical as true sessile species such as a barnacle and consequently subtle differences in coating type, discussed above, may not be detected. On the other hand, it was noted during the bioassay very few byssal threads were laid down on some coatings compared to others, it is therefore proposed that the inclusion of a byssal thread count (Harada *et al.* 1984, Crisp *et al.* 1985, Etho *et al.* 1997, Sera *et al.* 2000) may indeed increase the sensitivity of the test.

Sample size

Another proposed way of increasing the sensitively of the bioassay method is increasing the sample size. When the comparisons were re-run with a larger sample size, both gave significant results. As with all experiments by increasing sample size at some point any differences found will become significant (Figure 3.5), however experimental constraints are always of concern and the key to a good study is to find optimal sample size. It is therefore important to establish what differences shown represent a difference in coating performance, and thus adapt the sample size accordingly, i.e. a three to one difference needs a sample size of 24 to give a significant result however for a five to one difference to be significant a sample size of 10 is needed (Figure 3.6).

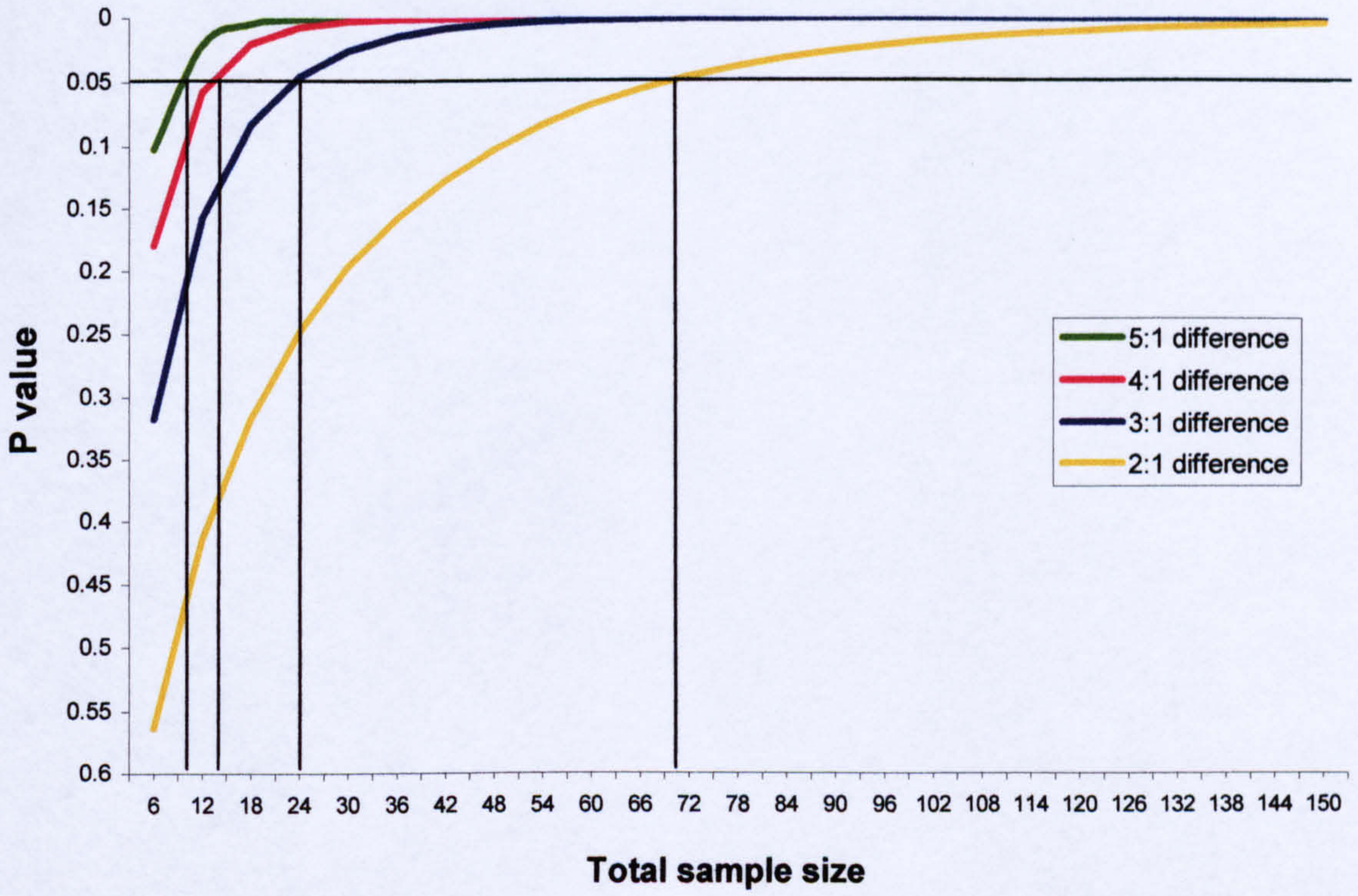


Figure 3.6 Graph to show theoretical varying differences and how the p value, of these differences, changes as sample size increases. P = 0.05 is indicated as well as the sample number needed for significance.

The recommended protocol is to carry out a first trial with a sample size of twenty and if required, to carry out a second trial with a sample size of 70, in order to establish differences of coatings with similar antifouling performances, as any subtle differences will be detected, with this sample size. Although this number may seem relatively high the simplistic method of the bioassay proposed here allows an increase of sample size with minimal effort after the arenas have been constructed.

Coatings with high efficiency

When using coatings with predicted high performance, as in trial 3, Glassclad® is not sufficient to deter the mussels from settling on the vertical sides of the chambers, thus the coating had to be painted on these sides. This process was quite laborious and increased the time of setting up the bioassay. Due to the chemical trail that mussels leave behind while exploring the surface with their foot (Bayne 1976, Crisp *et al.* 1995) the chambers could not be used more than once, thus new ones had to be constructed and painted before each bioassay. The aim of this work was to produce a simple technique that was relatively easy to set up and administrate and it is believed that an alternate method needs to be found to prevent the mussels from crawling up the sides when dealing with predicted high performance coating, in order to meet this criteria.

Evaluation and comparisons of other techniques

The increasing demand for environmentally safe alternative antifouling coatings has led to the corresponding need for effective means of appraisal of these alternatives. Conventional methods (*see* Swain and Schulz 1996) are not, at present, sufficient to

meet the required speed and accuracy needed for the rapid development of such coatings. Laboratory based bioassays using *M. edulis* have been developed to screen novel antifouling substances; the blue mussel assay first developed by Harada *et al.* (1984) used adult mussels which were fixed on the edge of a sample zone which was soaked with the substance under investigation. The quantity and location of byssus thread formation after 24 hours was used as an indicator of repellent activity of the substance. This method was improved by increasing number of mussels used and shortening observation time (Ina *et al.* 1989) and has obtained satisfactory results for screening active agents of possible antifouling coatings (Takasawa *et al.* 1990), However this method still has its drawbacks, it requires a running seawater system and complex preparation requiring large amounts of sample material. Moreover the conditions are highly artificial, as they do not utilise the avoidance/attraction behaviour of the mussel. The method can also not be used during the winter as insufficient byssal threads were produced. These drawbacks were recognised by Kitajima *et al.* (1995) who devised a toxicity and repellent assay using 4 juvenile mussels, which were free to explore the test plate enriched with the test sample. This method does have the advantage of not requiring a running seawater but preparation of test plates is complex and the use of more than one mussel is considered as a major draw back due to the gregarious nature of *M. edulis* (Bayne 1964, Satuito *et al.* 1993, *personal observation*) which may be influencing the findings.

Other bioassays using *M. edulis* have also been published: Etoh *et al.* (1997) used 15-25mm mussels placed in a PVC assay pipe with the test substance absorbed on paper lining the inner edges of the pipe. This method again requires running seawater, complex preparation and takes two days to carry out. Hayashi and Miki (1996), Sera *et al.* (2000) use the retraction of the foot of fixed mussels as an indicator of the repellent

activity of the test sample. Hellio *et al.* (2000) measured the phenoloxidase activity in the byssal gland of *M. edulis* in the presences of different antifouling substances, this method can only be carried out by trained evaluators and uses very specialised equipment. A brief comparison of all these methods and the proposed method in this paper can be seen in Table 3.2. It is noted however that all these methods above were designed to test active components of possible antifouling substances and concentrations needed for repellent activity, and not already manufactured antifouling coatings, like the method proposed here.

Method	Complexity of preparation	Use of specialist equipment	Running sea water requirement	Duration of bioassay	Over all complexity of method
Kitajima *et al.* 1995	2	1	NO	16-24 hrs	2
Etoh *et al.* 1997	2	1	YES	2 days	3
Ina *et al.* 1989	2	1	YES	3hrs	3
Hellio *et al.* 2000	4	4	NO	<1hr	4+
Harada *et al.* 1984	2	1	YES	Overnight	3
Sera *et al.* 2000	2	2	YES	3hrs	3
Hayashi and Miki 1996	2	2	YES	<1hr	3
Choice Test Method	**1**	**1**	**NO**	**16hrs**	**1**

Table 3.2 Comparison of key attributes of bioassays developed using *M. edulis*. 1-5 ratings of some attributes are given. 1 very low – 4 high

Summary

The choice test method proposed in this paper fulfils the criteria set out for a viable bioassay as stated by Stebbings *et al.* (1980); the test organism should be available for much of the year as possible, be ecologically significant and economically important and the bioassay itself should be easy to learn, cost effective and short term. Although the results show that refinements of the method are needed to increase sensitivity of the bioassay, with the improvements suggested, it is believed the authors are well on their way to producing a simple yet effective bioassay. This bioassay will determine the efficiency of non-toxic antifouling coatings, giving an accurate comparison of such coatings and allow an order of efficiency to be established, which will relate to field findings.

CHAPTER 4

SETTLEMENT ASSAYS

CHAPTER 4

SETTLEMENT ASSAYS FOR COATING EVALUATION USING *BALANUS AMPHITRITE*.

Introduction

Balanus amphitrite (Darwin) has been consistently studied for many years. This is possibly due to its wide geographical distribution and also the ability to culture large numbers of competent *B. amphitrite* larvae in the laboratory (Rittschof *et al.* 1992, Dr Antony Clare pers. comm.). Many settlement aspects of this species have been studied, for example: settlement in flow (Kelly and Wethey 1986, Wethey *et al.* 1988, Mullineaux and Butman 1991, Walters *et al.* 1999), settlement pheromones (Clare *et al.* 1994, Matsumura *et al.* 1998a, Clare and Matsumura 2000) and the effects of biofilms (Maki *et al.* 1988, Holmstrom *et al.* 1992, Wieczorek *et al.* 1995, Lau and Qian 2000), wettability (Schmidt *et al.* 1987, Rittschof & Costlow 1989a), and age (Crisp 1988, O'Connor and Richardson 1994, Satuito *et al.* 1996) on settlement. Such is the degree of work on this *Balanus* barnacle species that it has taken on the role of a model fouling organism and as such, is used for screening potential antifouling extracts (Standing *et al.* 1984, Sarma *et al.* 1991, Goto *et al.* 1993, Kon-Ya *et al.* 1994b, Willemsen 1994, Hirota *et al.* 1996). The similarity in life-cycle, physiology and some settlement behaviours to many other barnacle species, and to marine invertebrates in general, coupled to the ongoing culturing of this species in a number of laboratories worldwide (e.g. University of Newcastle and TNO, Netherlands) maintains its importance as a model organism for fouling research.

The competence of fouling organisms to settle and metamorphose has long been used to investigate settlement cues and antifouling potential of substances. Simple laboratory settlement assays have been developed, such as the standard dish settlement assay (Rittschof *et al.* 1984, 1992). Such assays involve known amounts of larvae, to minimise larval-larval interactions (Clare *et al* 1994, Wieczorek *et al* 1995) and take place in a static petridish with a fixed time period and light regime. These assays determine the attractiveness/inhibitory character of the substance under investigation by assessing the settlement percentage of the larvae after the experimental period. The larvae are either given a single treatment, a choice of two, or multi- treatments on which to settle. Other more elaborate assays have also been described (e.g. Crisp & Meadow 1962, 1963), in which the larvae are given a choice of differently treated slate substrata in a rotating dish in which a jet of air provides a counter current. Such assays, or variations on the assays, have been used to investigate effects of larval age (Rittschof *et al.* 1984, Dineen and Hines 1992, O'Connor & Richardson 1994), biofilms (Maki *et al* 1990, Keough and Raimondi 1995, Wieczorek *et al* 1995), larval interactions (Clare *et al* 1994, Dineen and Hines 1994, Clare 1996b, Matsumura *et al.* 2000), and antifouling potential of substances (e.g. Dahlström *et al.* 2000)

The value of such techniques has also been commercially recognised and some companies are looking into (e.g. TNO, Dr Ritchie Head pers comm.), or have used (e.g. Hempels, Dr Antony Clare pers comm.) the generic approach to evaluate the antifouling potential of new non-toxic antifouling coatings.

A modified method of the standard dish settlement assays (Rittschof *et al.* 1992) using *B. amphitrite* was set up in order to obtain an *in vitro* performance rating of the

coatings used throughout the bioassays (see Table 2.2), to see how these performances relate to field findings, and to enable a comparison of settlement versus behaviour.

Materials and Methods

Thirty glass panels (6.5cm x 6.5cm) were primed with white VAL and left to air dry overnight. The panels were then half coated with ITS and again left to dry overnight. The other half was then coated with each of the six coatings (coatings marked in bold Table 2.2), five replications of each pair wise comparison against ITS (including ITS against ITS) were made up. These were then left on the bench for two weeks for any residual solvent to evaporate.

A circular hole (0.5cm) was drilled into the bottom of thirty sterile polystyrene petridishes (Falcon no. 1006, 5cm diameter). These were inverted and placed on the coated glass panel; aquarium grade silicone was used to secure them. The coated panel with petridish was then placed in a 7.3cm x 7.3cm food grade polythene square dish (manufactured by A.W. Gregory & Co Ltd no. 6021). 10ml of FSW was placed within the inverted circular petridish through the drilled hole and 20ml of FSW was placed within the surrounding square petridish in order to maintain the water level in case of any leakage through the silicone layer. *Balanus amphitrite* cyprids were cultured at Newcastle University according to Clare (1996). To minimize larval-larval interactions 20 cyprids were placed within each of the inverted dishes (Holm *et al.* 2000). These were covered and placed in a controlled temperature room at 25°C for 5 days. Number of cyprids settled, not settled and mortality was recorded as a percentage of cyprids found at the end of this period.

No statistical analysis was carried out as it became apparent that there was a problem with the procedure.

Results for assay 1

All cyprids found after five days on coating 617 were dead and for all other coatings mean percentage of dead cyprids was above 70%, apart from coating 618 where mortality was 41% (Figure 4.1). This apparent high mortality level found after five days indicated that something was having detrimental effects on the larvae in all treatments. In order to ascertain whether this was due to the experimental set up or the coatings themselves, another settlement assay was set up using the above method but with the following treatments:

- Five uncoated glass panels with an inverted petridish secured by silicone.
- Five panels coated with half ITS and half 616 constructed as stated above.
- Five petridishes.

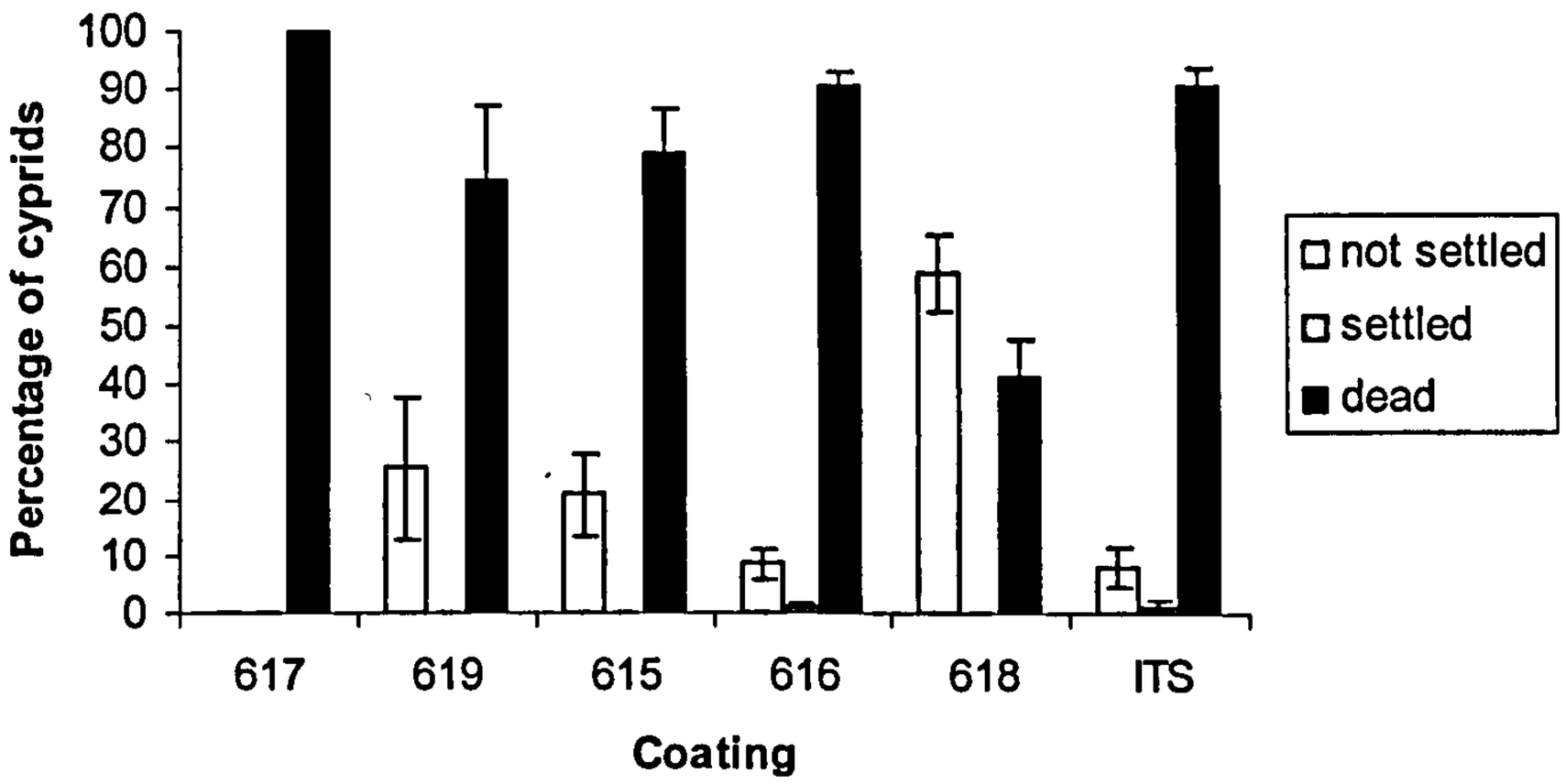


Figure 4.1 The mean percentage *B. amphitrite* cyprids settled, not settled and dead after a five day settlement assay (n=5). Error bars are shown indicating the standard error. *All treatments included one half of the treatment plate coated with ITS as a control.*

Results for assay 2

Figure 4.2 shows that the treatment with the coated glass (616/ITS) was the cause of the detrimental effect. However, as this was coated with a combination of coatings (ITS (control) and 616), so that the larvae had a choice of coatings on which to settle, it was still not clear which of the coating were causing the high levels of mortality (Figure 4.2), 616 or ITS. In order to ascertain the precise cause of the high levels of mortality, another settlement assay was run with the only one coating per treatment.

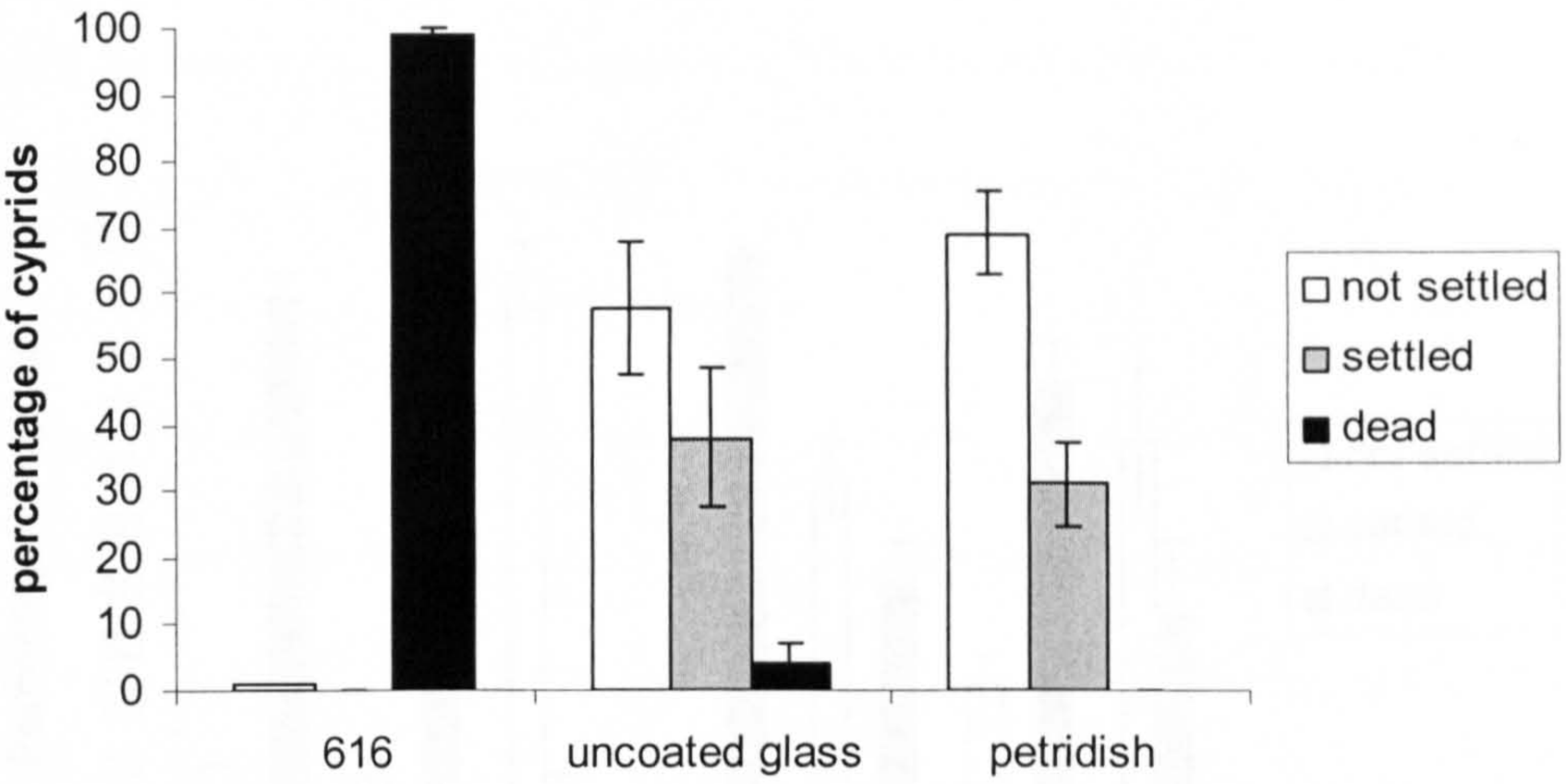


Figure 4.2 The mean percentage of *B. amphitrite* cyprids settled, not settled and dead for different experimental setups after a five day settlement assay (n=5). Error bars are shown indicating the standard error. 616 treatments included one half coated with ITS as a control.

Results for assay 3

The results from assay 3 (Figure 4.3), showed all coatings appeared to be leaching something into the surrounding water which was having a detrimental effect on the larvae, apart from 615. Although time was allowed for any solvents to evaporate prior to use of the coatings, it was thought that residual solvents may have been responsible.

In order to determine the exact nature of the chemical residue causing the larval mortality, Gas Chromatography-Mass Spectrometry (GC-MS) of the water from the petridishes was carried out by Akzo Nobel. Although the sensitivity of this technique is dependent upon what compounds are to be detected, for aromatic organic solvents such as are used in coating formulation the threshold would be approximately 50ppm. Akzo Nobel concluded from this investigation of the water samples taken after the end of the settlement trial, that there was no detectable organic solvent, at this sensitivity level, present in 0.5ml of the sample water.

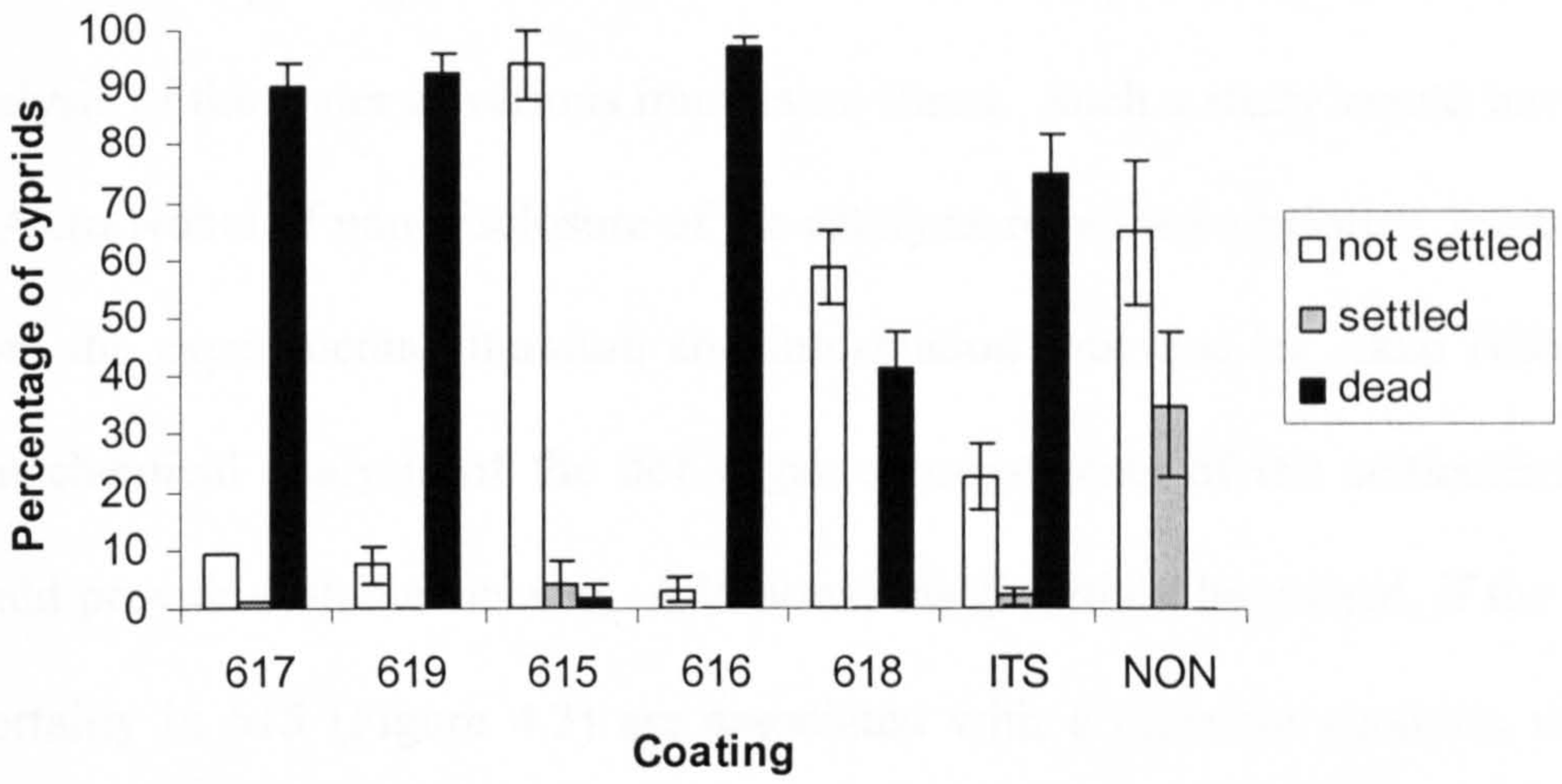


Figure 4.3 Mean percentage of *B. amphitrite* cyprids settled not settled and dead on separate coatings after a five-day settlement assay (n=5). Error bars are shown indicating standard error. (NON – uncoated glass panel).

Discussion

It is apparent from these results that the source of mortality in the larvae was not determined from the mass spectrometry carried out by Akzo Nobel. Mortality of this kind on silicone foul release coatings has been observed before (Dr Antony Clare pers. comm.) although this was believed to be caused by traces of Di-butyltin di actetate.

Metal catalysts are needed for curing the tested coatings, and they may well have been present in the small amounts in the coatings tested, although no chemical compositions were disclosed by Akzo Nobel. It was stated that not all coatings contained the same catalyst (Akzo Nobel pers. comm.) However, the degree to which the catalysts differed was not disclosed. Consequently, it is possible that, although catalytic variants were used in the coatings, some generic chemical effect from the catalytic variants in the coatings caused the observed mortality.

At this stage there remains the apparent paradox of high levels of larval mortality caused by non-biocidal coatings during the settlement assays. Clearly, a further in-depth chemical study is required in order to overcome this problem such as further analytical analysis of the water at various immersion times. Such a study would have to be based at Akzo Nobel, if non-disclosure of the catalysts remained a priority. From the evidence from the experiments, literature and information provided by Akzo Nobel, it appears that chemical analysis of the non-organic components of the settlement assay water could provide suitable answers. Additional insight might be gained, if the low levels of mortality in 615 (Figure 4.3) are associated with a different catalytic variants to the other tested coatings, which is quite likely as this is an epoxy primer rather than a silicone antifouling coating.

The resolution of this paradox is an important issue if such settlement assays are to be commercially developed for non-biocidal coatings. However, the apparent toxic nature of the non-biocidal coatings could largely be an artefact of the long time exposures to the surfaces (5 days) and the low volume of stagnant water in the experimental chambers. Consequently, these are not issues that impact upon the behaviour of the coating in the field, but they do at present prevent standard style bioassay techniques on

these types of coatings and therefore at present the only option of a suitable screening test lies within the following proposed research.

CHAPTER 5

BIOASSAY METHOD

CHAPTER 5

BEHAVIOURAL BIOASSAY EQUIPMENT AND METHODOLOGY

Many considerations were taken into account when planning the detail of the basic experimental procedure: Handling of organisms, size and therefore visibility of larvae, temperature and lighting and chemical cues or other factors which may affect settlement behaviour: Consequently prior to laboratory work a literature survey was carried out to determine possible errors and sources of variation. This survey provided information which was taken into account when designing the bioassay technique outlined later in the chapter.

Practicalities of the bioassay design.

It has been suggested that pipetting can influence behaviour of larvae (Ryland 1960) thus care was taken when transferring the larvae into the test arena (petridish) and the handling was kept to a minimum. The size of the larvae creates a need for high magnification camera; initially a square arena was chosen in order to obtain the largest field of view on the computer monitor. Temperature changes (Kon-Ya and Miki 1994) and variations in water volume (Wieczorek and Todd 1998), may also alter larval behaviour; a standard temperature was used throughout the bioassays for each species. *S. borealis* and *M. edulis* was run at 10°C ± 2°C which was maintained using cooler blocks stored in the freezer. To overcome the strong phototactic behaviour shown by the larvae (see Chapter 1) a circular lighting tube was designed to fit around the test arena,

thus by using this lighting arrangement the larvae should not be drawn to any particular side of the dish as the light intensity will be equal on all sides of the arena.

Spontaneous metamorphosis on the water surface has been observed for *S. borealis* (Knight-Jones 1951, Wieczorek and Todd 1998). Although the exact reason for this is not known, it has been suggested that this seems to be due to overdue liberation of larvae (Knight-Jones 1951). Hence to minimise this, adult spirorbids were collected on a regular basis and not kept in the laboratory for long periods without stimulation of larval release. If this occurred during the bioassays, trials were disregarded and run again. Variation of behaviour in *S. borealis* has been observed depending on the time of day the bioassays were run (Knight-Jones 1953a), observations of *B. amphitrite* also show less settlement during hours of darkness (Walton-Smith 1948) whether this is due to a phototactic response or part of a circadian rhythm remains unclear, however these variations should be eliminated by sufficient replication and were not considered a major problem.

Some larvae such as *S. borealis* (Knight-Jones 1953a) *S. balanoides* (Jarrett and Pechenik 1997) and *B. amphitrite* (Rittschof *et al.* 1984) show decreased substratum discrimination as they age. Therefore the same number of different age groups was run on each coating in order to minimise age effects.

Chemical trails from exploring larvae have been shown to affect settlement choice in some species, in particular barnacles (Yule and Walker 1983, 1985, Clare *et al.* 1994, Clare *et al.* 1998, Matsumura *et al.* 1998a and b). The arenas were washed in commercial acid rinse (Decomatic) to remove any traces of protein. Due to concern of

altering the surface of the test coating or leaving an acid residue, the test panels were only used once.

It is postulated that larva-larva interactions can be of great influence when running the bioassays. The gregariousness nature of barnacles (Knight-Jones 1955, Clare *et al.* 1994) and *S. borealis* (Knight-Jones 1951) may well override any influence of the substratum under investigation (Gotelli 1990). Also the problem of pseudo-replication would have occurred with numerous larvae in one test arena. Although low larval numbers results in low statistical power (Wieczorek and Todd 1998), these effects were thought to be important enough to only allow one larvae per bioassay. High numbers of replicates were therefore needed to increase the statistical power, but this replication eliminated any variation created by the founder effect (Toonen and Pawlik 1994).

Materials and Method 1

Test chambers used in Method 1

Four glass slides (4cm x 2cm) were place together using aquarium grade silicone to make a bottomless square box. This was placed on top of a glass panel (6.5cm x 6.5cm), on which the test coating had been painted. The glass panel was then placed in a square food grade polythene petridish (A.W. Gregory & Company Ltd), (Figure 5.1). Forty millilitres of 0.2μm filtered seawater (FSW) was placed in the petridish and one larva was placed within the glass arena prior to filming. The glass panel with the test coating was discarded after use but the glass arena and petridish were cleaned using a commercial acid rinse (Deconmatic), rinsed thoroughly and reused.

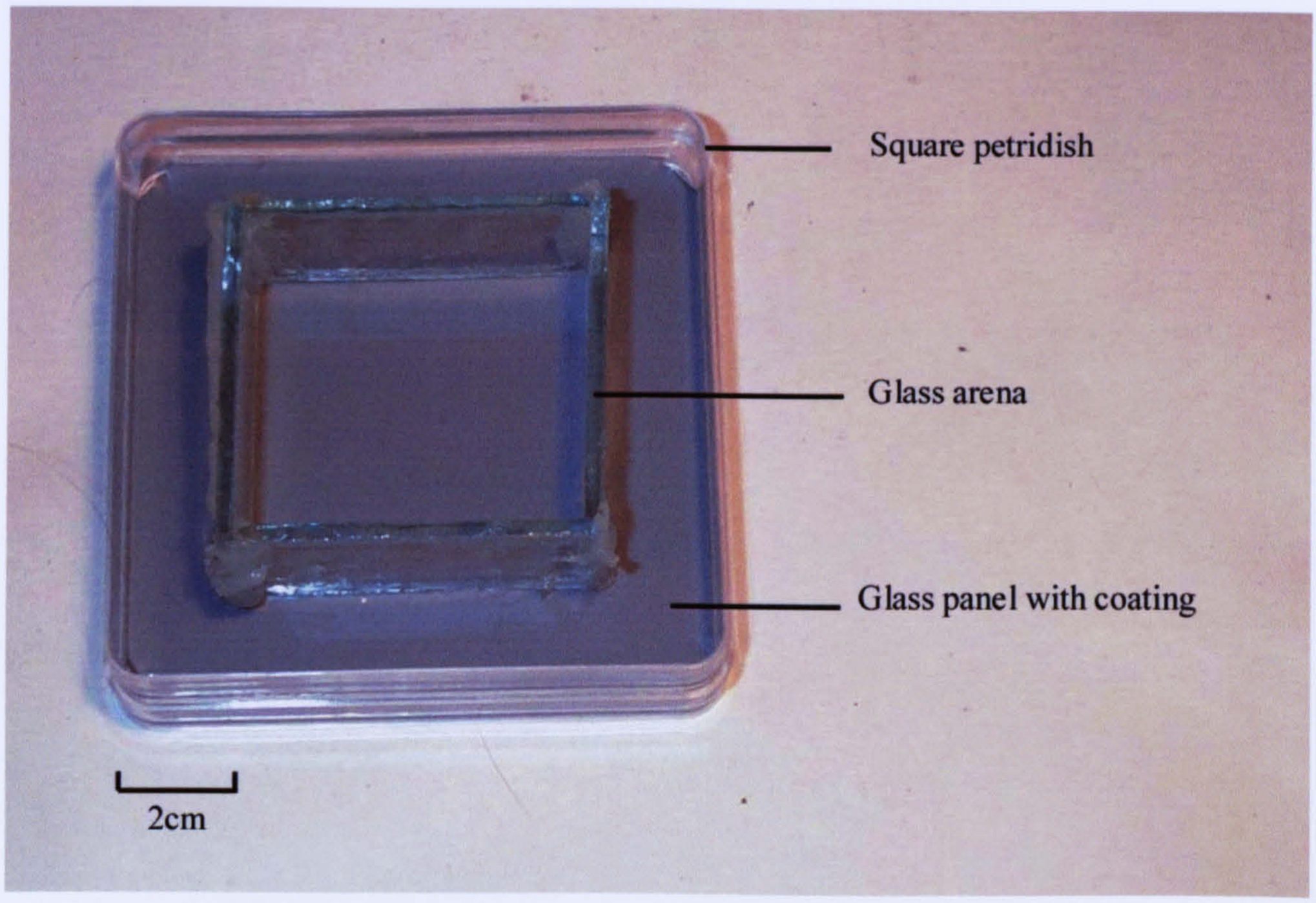


Figure 5.1 Type 1 test chamber (arena) showing different components.

Bioassay technique for Method 1

Each larva to be filmed was placed within the filming unit (Figure 5.2). The filming unit comprised of a plywood box (110cm x 64cm x 67cm) to excluded any incidental light. The box contained the Kaiser RS1 vertical camera stand with attached black and white camera (JVC, model TK S3503E). The camera was connected via a Movie star® frame grabber to a Pentium II 350mhz PC, on to which the moving image was recorded. The moving image was run through LookC™ software (v2, distributed by ATM) that stored the AVI files on the hard disks. At the end of the trial these were then transferred to compact disk, which were used for analysis. An in house built variable intensity circular light (Philips TLE 32W, 12in) was used supported by a stack of square dishes. This arrangement enabled the height and illumination to be altered with relative ease depending on the image produced. Domestic cooler blocks frozen at –12°C were placed within the filming unit and under the test arenas for *S. borealis* (Chapter 6) in order to maintain a temperature of 10°C ± 2°C. Trials using either *Balanus* species were performed at room temperature (22°C ± 2°C) and therefore cooler blocks were not used. Once the larva was placed under the camera it was given 2 minutes to acclimatise then movement was recorded at 1Hz for 30 minutes.

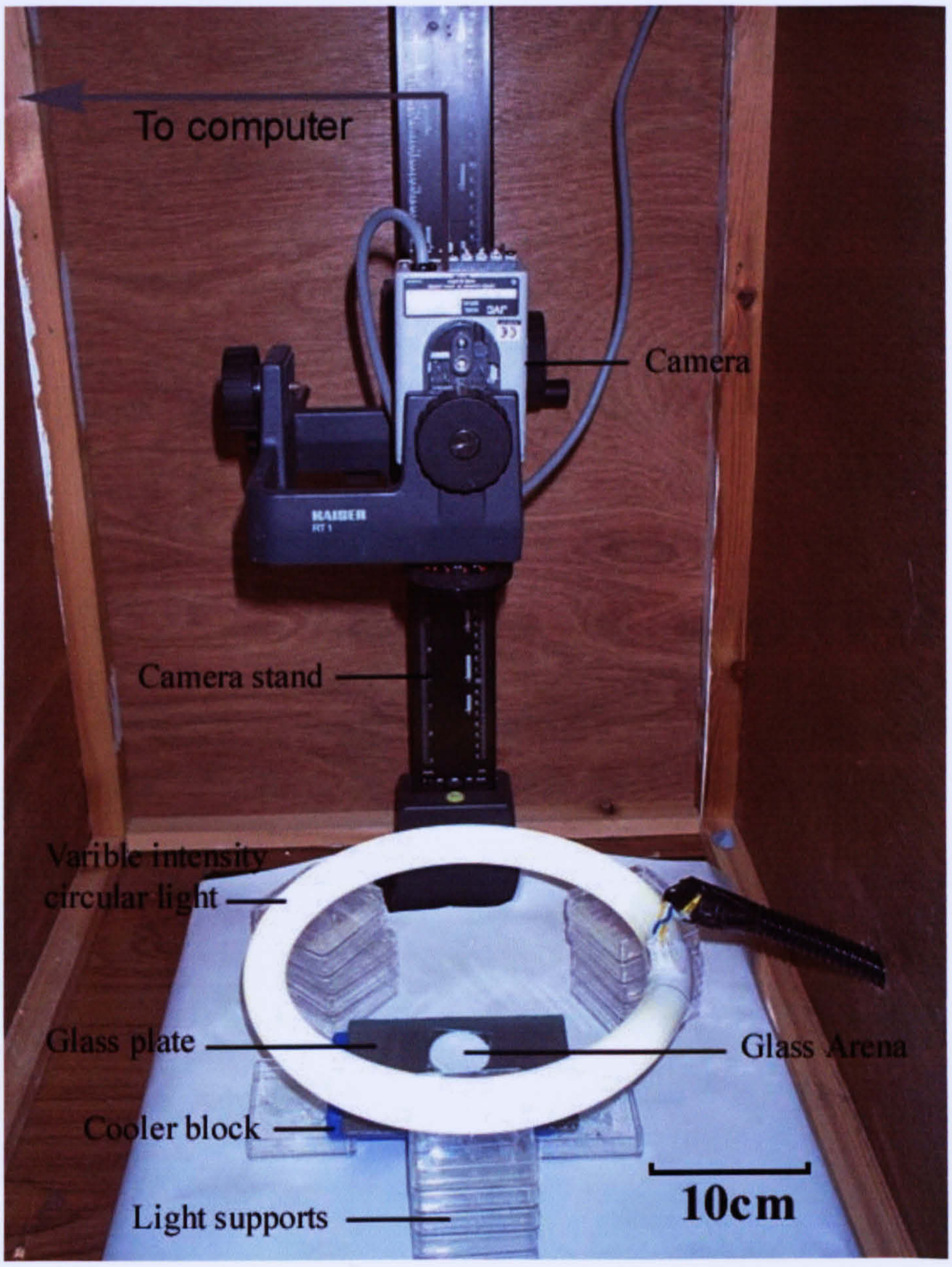


Figure 5.2 The filming unit showing the arrangement of equipment used for the bioassay.

Analysis for Method 1

The video record of each bioassay was stored on compact discs. These were played back frame by frame through computer software, Video point v2.1 (distributed by Pasco Scientific). This program translates the movement of the mouse into measurements of the x y coordinates relative to the track. The coordinates were then used to calculate certain behavioural parameters (Table 5.1) using an in house template written in Excel 2000. The behavioural parameters were then analysed using Minitab v12. ANOVA GLM was used for each parameter after suitable Box-Cox transformations had been carried out.

Parameter	Units	Description
Total distance travelled	cm	*Distance over entire track*
Mean velocity	cm/sec	*Average rate of movement*
Stationary time	sec	*Periods of inactivity*
Moving time	Sec	*Periods of activity*
Crow flies distance (beeline)	cm	*Distance between first and last point on the track*
Mean turn rate	degree/sec	*Track average of the absolute turn values/time between sample points (x and y co-ordinate)*
Mean meander	degree/cm	*Track average of turn/distance at each sampling point (x and y co-ordinate)*
Index of straightness	N/A	*The linearity of travel or coefficient of a straight line (calculated by dividing the crow flies distance and actual length of track)*

Table 5.1 Description of parameters used in method one, that were calculated from the x and y co-ordinates of the larva track (adapted from Varley *et al.* 1994).

Pilot study for Method 1

Balanus improvisus was used as the test organism and work was carried out in Tjärnö Marine Biological Laboratory, Sweden. The method was carried out as stated above. Coatings VRD, 19A and 19C (for details see Table 2.2) were investigated and compared to a control surface, Plexiglass. This smooth surface is known to foul readily with *B. improvisus* when immersed (Berntsson *et al.* 2000a and b) and therefore was thought to be a suitable control. Seven replications were performed for each coating including the control.

Results for Method 1

When comparing parameters for coatings 19A, 19C and Plexiglass no significant differences were found. However all parameters showed a significance difference between Plexiglass and VRD; total distance (ANOVA, $F = 18.42$, $p < 0.001$), velocity (ANOVA, $F = 20.41$, $p < 0.001$), crowflies distance (ANOVA, $F = 38.37$, $p < 0.001$), mean rate of turn (ANOVA, $F = 24.78$, $p < 0.001$), mean meander (ANOVA, $F = 24.59$, $p < 0.001$) and activity (ANOVA, $F = 24.54$, $p < 0.001$).

Evaluation of Method 1

Significant differences were found when comparing Plexiglass and VRD, these coatings were used as two extremes, Plexiglass (high fouling, low performance) VRD (low fouling, high performance) and therefore should have shown differences. However the other experimental coatings 19C and 19A showed no differences and therefore the bioassay was believed not to be rigorous enough and alterations to the method had to be

made. The frame rate and duration of the bioassay was determined by the maximum capacity of the storage media and did not take into consideration larval speed and variation in larval behaviour. Observation of an exploring cyprid indicated that it circles at a much faster rate that can be detected by 1Hz, thus an investigation of optimal frame rate for each species taking into consideration constraints of storage and analysis time were proposed. During the pilot study only 7 replications for each coating was used, it became evident that individuals varied enormously within treatments and a much larger sample size was needed. Optimal sample size experiments were also proposed for each species in order to overcome this variation.

The purchase of an automated tracking device in October 2000, Ethovision® Pro, version 1.90 (manufactured by Nodulus Information Technology) greatly reduced analysis time. Ethovision is an integrated system containing both hardware and software, which can be used for video tracking, motion analysis and behaviour recognition. This greatly diminished analysis time and enabled a frame rate of 25Hz to be used with no extra work. It meant that frame rate was no longer an issue and sample size could be dramatically increased.

Materials and Method 2

The arena type in method 1 was unsuitable for the automated tracking devise as some of the larvae were obstructed from view when exploring the corners of the arena and some larvae also escaped from the arena and had to be re-run. In order for the movement to be tracked by Ethovsion®, a different arena was deviced.

Arena for Method 2

Test coatings were applied to 4cm diameter watch glasses (*see* preparation of panels) then secured to a glass platform using bluetack (Figure 5.3). The watch glass was filled with FSW and a square glass lid (4cm x 4cm) was placed on the top after the test organism had been placed into the dish. This eliminated the meniscus line of the seawater allowing the larvae to be seen at all times. One larva was placed within the glass per trial. The coated watch glass was only used once. The glass lid was washed in Deconmatic, rinsed thoroughly and reused.

Bioassay technique for Method 2

The bioassay was the same as stated in method 1. The live moving image from the camera was directly linked to the Ethovision® software program via the integrated frame-grabber. It was recorded at 25Hz, the raw data was stored as x y coordinates in Ethovision® file format. The tracks were visually assessed after for any errors during the automated tracking. These were edited using the edit mode of Ethovision® if necessary.

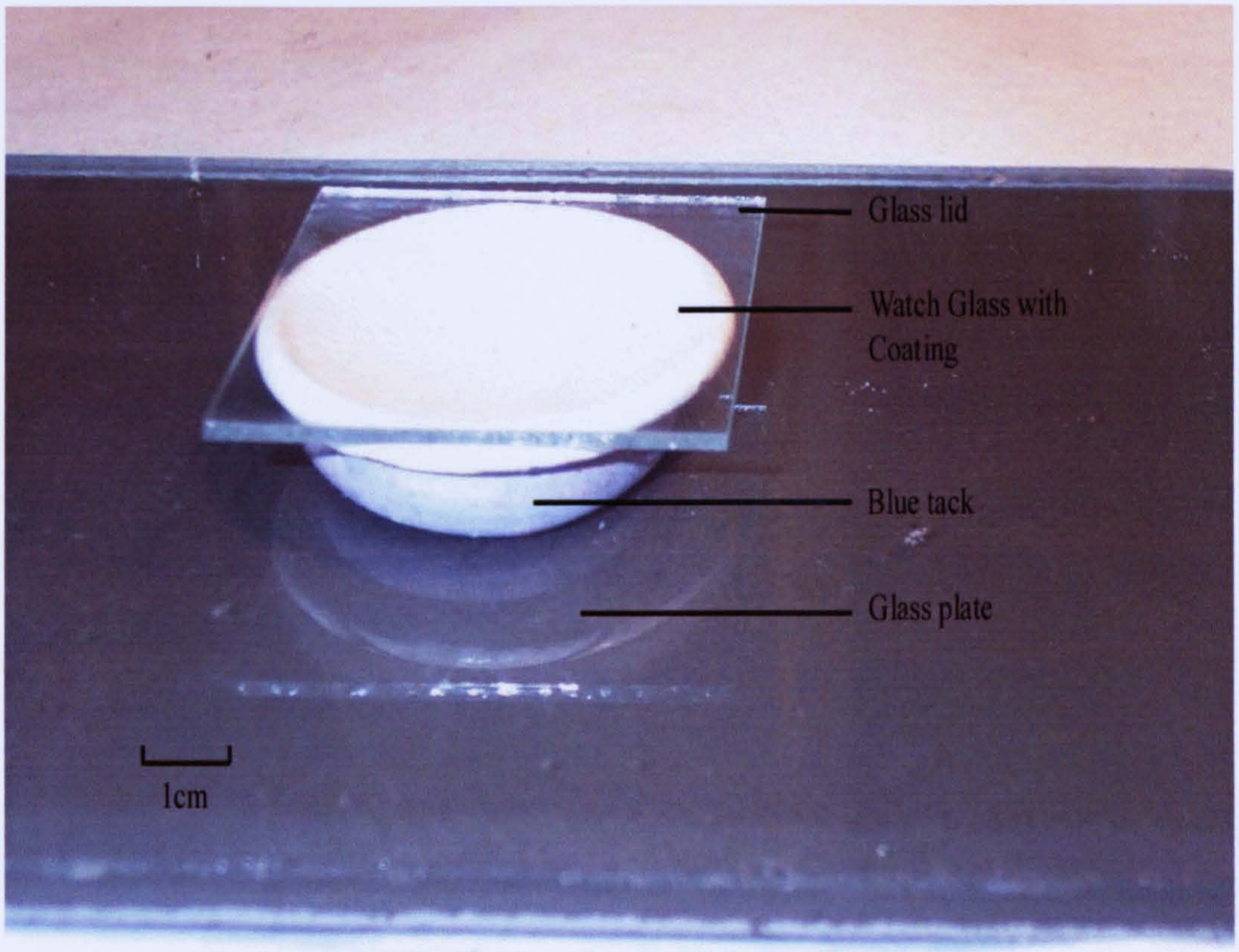


Figure 5.3 Type 2 arena showing the arrangement of components.

Analysis for Method 2

The x y coordinates were used to calculate the parameters within Ethovision® software and then transported into Minitab v12 for further analysis, unless otherwise stated. Parameters used are listed in Table 5.2

Parameter	Units	Description
Total distance travelled	cm	*Distance over entire track*
Mean Distance	cm	*Track average of distance between sample points*
Mean velocity	cm/sec	*Average rate of movement*
Moving time	sec	*Periods of activity*
Mean turn angle	degrees	*Average change in direction of movement between sample points*
Mean turn rate	degree/sec	*Track average of the absolute turn values/time between sample points (x and y co-ordinate)*
Mean meander	degree/cm	*Track average of turn/distance at each sampling point (x and y co-ordinate)*
Heading Angle*	degrees	*The direction of movement in relation to a reference point.*

Table 5.2. Description of parameters used in method two, that were calculated from the x and y co-ordinates of the larval track (adapted from Varley *et al.* 1994) **this was not used in the statistical analysis of differences between coatings, but as an indicator of any phototactic behaviour, results are shown in each relevant chapter*)

The data were transformed using suitable Box-Cox transformations for all parameters showing non-normal distributions by the Anderson-Darling normality test prior to carrying out parametric statistical tests.

The data were analysed to investigate the following: Firstly to determine any significant differences shown in exploratory behaviour on the different coatings; then to

see if the behaviour could be used to discriminate between the coatings, and finally, to see of the laboratory behaviour could be used to predict the data produced in the field for the different sites.

To establish if there were significant differences between coatings, a multivariate balanced MANOVA using a general linear model (GLM) was carried out. All parameters listed in Table 2.7 were used as dependent variables with coating type as a fixed factor. Tukey's pairwise simultaneous test (95% confidence interval) was then used to detect any differences found between coatings for each parameter.

Canonical discriminant analysis (CDA) was carried out to see if the coatings could be distinguished, using SPSS v11. All the behavioural parameters were used as predictors and the coatings were distinct groups. To classify the groups, the prior probabilities of group membership were assumed to be equal and a within group covariance matrix was used. A combined group scatter plot was produced for canonical discriminate functions 1 and 2, together with a summary table of predicted group membership for all coatings.

To see if the behavioural data from the laboratory bioassay, could be used to predict the field data gained by the immersion trails, multiple linear regression was carried out. A stepwise method was used to see which behavioural parameters were the best predictors. Both the PCA axis 1 scores obtained for each site and the total fouling for each site (see immersion trial section above) were used as the dependent values. The behavioural parameter data were used as independent variables and the probability of F was specified to be ≤ 0.05.

CHAPTER 6

SPIRORBIS BOREALIS

CHAPTER 6

BEHAVIOURAL BIOASSAY USING *SPIRORBIS BOREALIS*

Introduction

Spirorbis borealis (Daudin) (=*Spirorbis spirorbis* (Linnaeus)) is the most widespread and abundant species of spirorbid in Northwest Europe and Britain (Hayward *et al.* 1996). This tube dwelling polycheate is hermaphroditic. Fertilisation takes place within the tube either by self-fertilisation, or more commonly, cross-fertilisation whereby the worm takes up sperm previously released into the water body by conspecifics (Daly 1978). Although there is variation in seasonality among differing localities, breeding occurs predominantly during the summer (Hayward *et al.* 1996).

Larvae are released into the water column and are positively phototactic for approximately the first hour, changing to a negative response to light as they search for potential settling sites (Gee 1963). They have a pelagic life of about two hours in favourable conditions (Knight-Jones 1951), although in less favourable conditions they have been seen to continue settling for up to 12 hours (Williams 1964). They are both gregarious and territorial (Knight-Jones 1951), settling preferentially in the linear grooves formed by the midrib of *Fucus serratus* (Wisely 1960) where adults are already present.

Pre-settlement behaviour of *S. borealis* (Figure 6.1) has been well documented; most larvae have been observed to collide head on with the substratum then re-orientate themselves within seconds (Nott 1973). The larvae explore the surface with apical cilia

and move rapidly over the substratum often crawling over obstructions rather than moving round them. The tail like abdomen remains projecting longitudinally occasionally twitching from side to side (Knight-Jones 1951). During exploration they secrete a temporary attachment thread, the strength of which has been suggested to be correlated with the extent of exploration (Nott 1973), as this prevents detachment by water currents (Knight-Jones 1951). When nearing settlement the abdomen flexes turning the trunk of the body sideways and causing a change of direction. After travelling for a short period this movement is repeated and the direction is changed again (Knight-Jones 1951). This may continue for minutes until the behaviour changes to a backward and forward motion covering a distance of about 2 mm, crawling slows and turning increases. A violent change of direction by flexion of the lateral part of the body marks the latter end of their exploration and when a suitable settlement site has been selected, it remains stationary for a short period with only slight wriggling with contractions and relaxation of the body muscle (Knight-Jones 1951, Nott 1973). The larvae finally releases its adhesive secretion from its attachment gland. At this stage metamorphosis is now imminent.

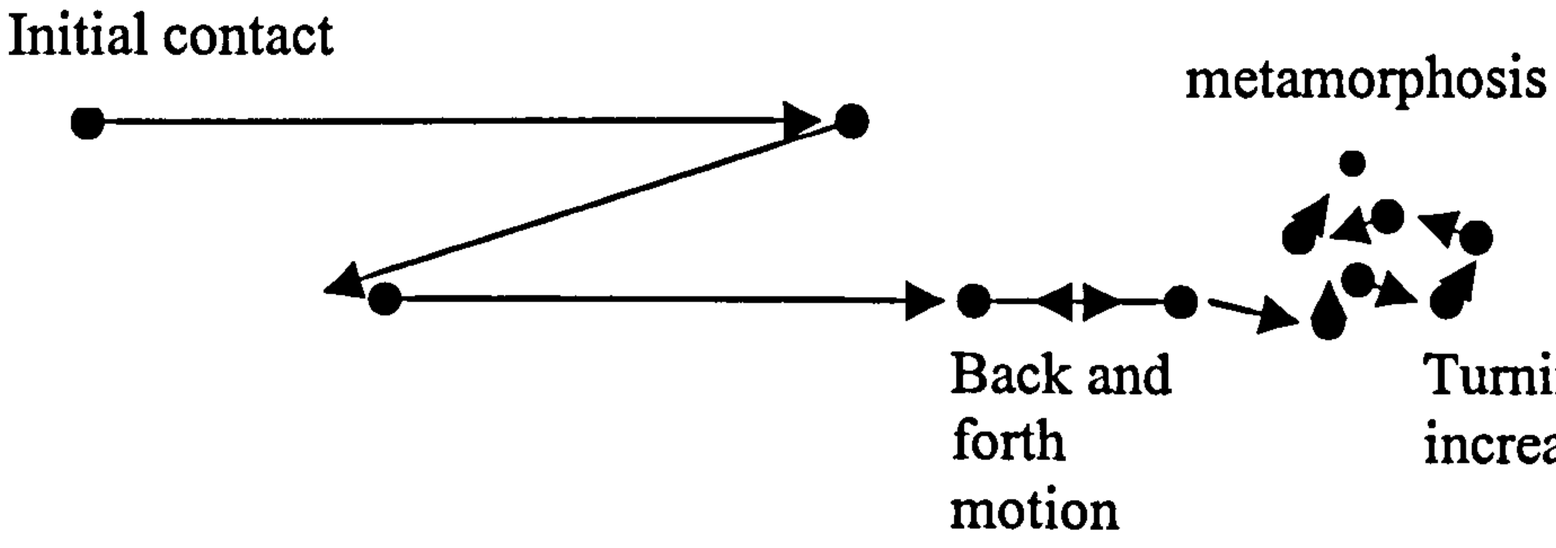


Figure 6.1 Schematic diagram showing pre-settlement behaviour of *S. borealis*

The aim of this investigation was firstly, to determine the suitability of *S. borealis* as a test organism. Secondly, to asses if the behaviour of *S. borealis* was different on different coatings and if this behaviour could be used to distinguish between the coatings used in the bioassay, and finally to determine whether the exploratory behaviour of *S. borealis* could be used to predict the fouling of these coatings, as seen in the field immersion trials.

Details of why *S. borealis* was chosen for the bioassay are given in Chapter 2. The short pelagic life of the larvae, although beneficial for the bioassay, caused concern regarding the length of time the larvae could be used for, due to the desperate larvae hypothesis (Chapter 1). A preliminary experiment was therefore run in order to ascertain the time it took for 50% of the population to settle. After this time more larvae were liberated, if needed, to complete the daily work. Optimal frame rate and sample size for this species were also investigated before the bioassay could be run. The findings of these investigations are discussed within the preliminary section below, before presenting the main body of the work.

Preliminary work

50% Settlement time

Materials and Method

Six 5.5cm pieces long of *Fucus serratus* were cut. Pieces were selected with 5 adult *S. borealis* already settled on each piece. If any piece had more adults settled on them, these were removed using a scalpel blade. The pieces of *F. serratus* were then placed each in a 2l plastic container with 50ml of 0.2μm filtered seawater at 10°C. Ten *S. borealis* larvae (obtained by the method described below) were placed in each container

placed in a refrigerator at 10°C. The number of larvae settled on the *F. serratus* was counted every 15mins using a binocular microscope until five larvae had settled in each container.

The mean time for 50% settlement for all containers was used as the 'shelf' life of the larvae.

RESULTS

The mean time for 50% settlement for all 6 containers was 4hrs ± 34mins. Therefore the larvae were kept for a maximum of 4hrs after which time the collection procedure was performed again to obtain newly liberated larvae for any continuing work.

Optimal Frame Rate

MATERIALS AND METHOD

Method 1 (Chapter 5) was used to determine the optimal frame rate and coating 615 was used on the glass panel. To promote exploration behaviour, extract from adult *S. borealis* and *F. serratus* was used. The extract was made according to Williams (1964) with the addition of 2g wet weight of macerated (using a pestle and mortar) adult *S. borealis*. Four drops, from a glass pipette, were painted over the surface of the coating 615 and oven dried for 20mins at 60°C prior to use. The maximum frame rate that could be established by the frame grabber used in Method 1 (Chapter 5) was 6Hz. At this frame rate only four minutes of moving video could be recorded and stored on to one CD (650mb). Therefore filming was done for four minutes. Parameters described in Table 2.6 were calculated for 6Hz, 3Hz, 1Hz and 0.5H and 7 replicates were carried out for each. To determine the optimal frame rate for Method 1 (Chapter 5) using *S.*

borealis, the point at which a 10% difference in the behavioural parameters from 6Hz was used.

RESULTS

The results are shown in Figure 6.2. At 3Hz four of the six parameters lie within the 10% limits. Although both values for turn rate and moving time are greatly reduced at this frame rate, due to time constraint on analysis it was agreed that a frame rate of 3Hz would be satisfactory as this would cut analysis time by half. It was understood that the results shown are completely dependent on the maximum frame rate at which the bioassay was recorded and if a higher frame rate was used the results would show a different result, however this was the maximum obtainable frame rate achievable by the frame grabber used.

The purchase of the automated tracking device, Ethovision, meant that the frame rate could be increased to 25Hz. The results shown here therefore were not needed for the preceding bioassay, although they do show the importance of such a study.

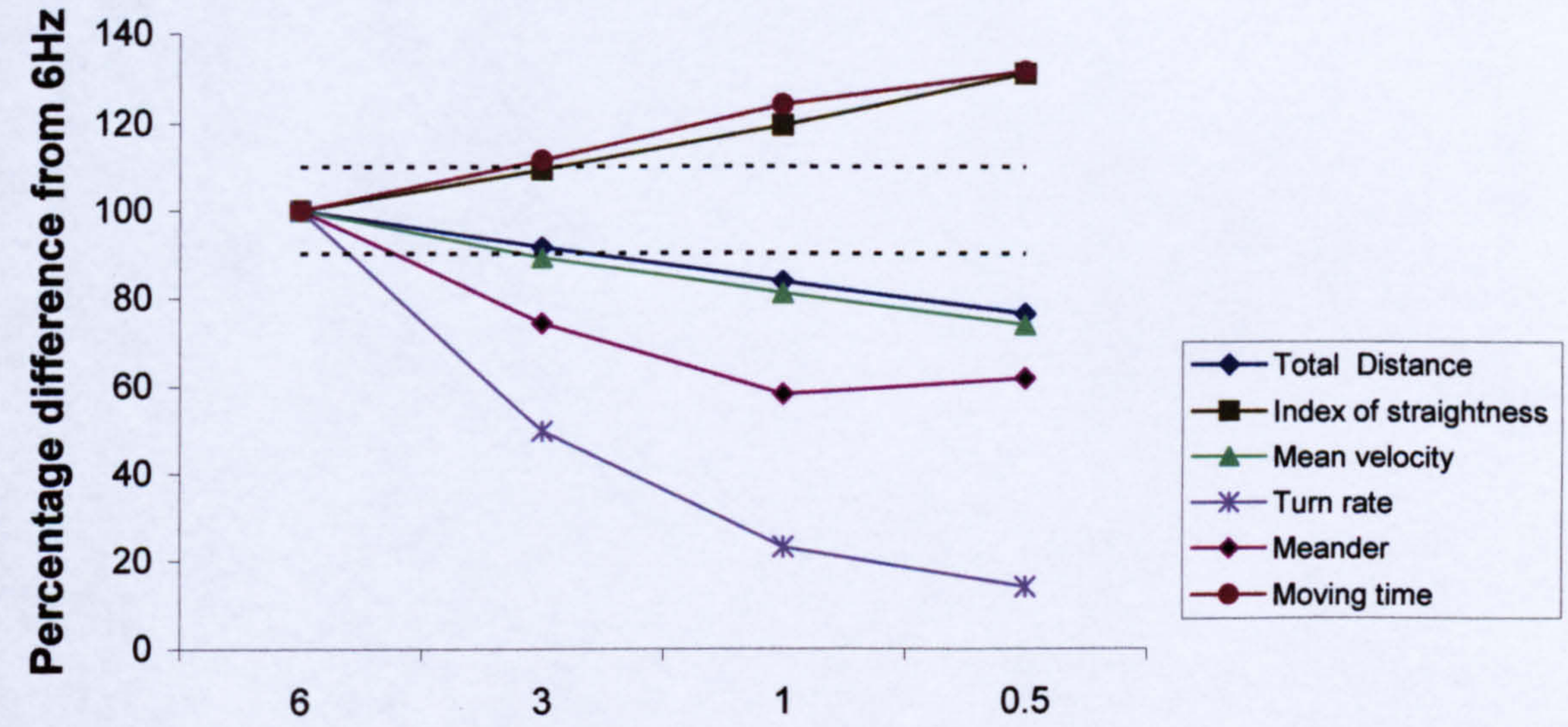


Figure 6.2 The percentage difference in values of the larval behavioural parameters calculated at different frame rates for *S. borealis*. The dotted lines indicate a 10% deviation from the value at 6Hz.

Optimal Sample Size

MATERIALS AND METHOD

Method 2 (Chapter 5) was used to determine optimal sample size. To promote exploration behaviour extract from adult *S. borealis* and *F. serratus* was used (see above). Four drops of extract from a glass pipette were painted onto the watch glasses (arenas) no coating was applied. These were oven dried for 20mins at 60°C prior to use. Eighty replicates were carried out. The running means for meander, turn angle, total distance and turn rate were used to visually assess the optimal sample size.

RESULTS

Figure 6.3 shows the running means of four parameters calculated from the x y co-ordinates of the larval tracks. It can be seen that the minimum sample size which lies within the 95% confidence interval of the sample size of 80 was 40 for most of the parameters. It was therefore agreed that a sample of 40 would be used for this species during the bioassays.

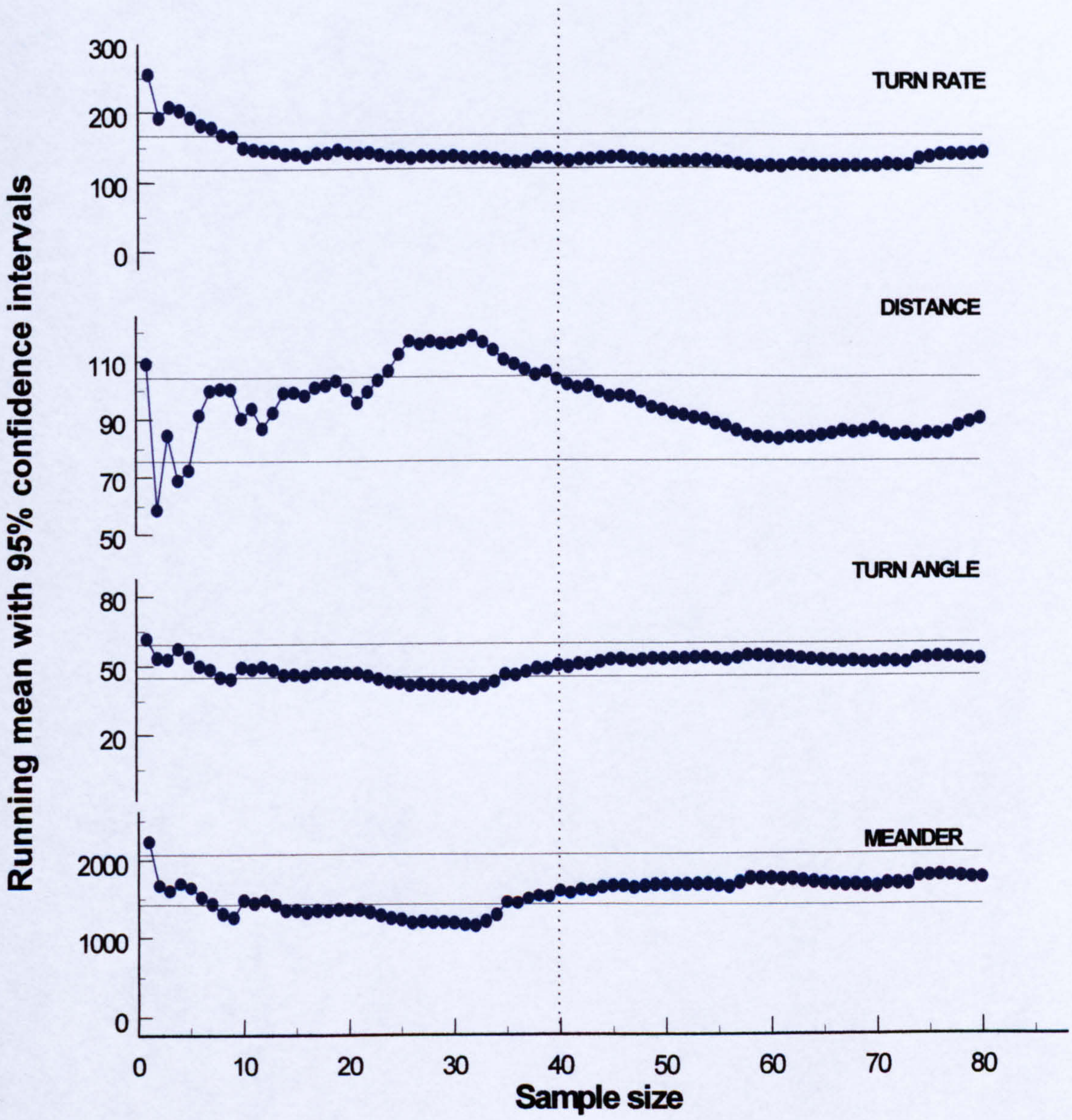


Figure 6.3 Running means of meander, turn angle, distance and turn rate with the 95% confidence interval for the sample size of 80.

The Bioassay

Materials and Methods

COLLECTION AND STORAGE OF LARVAE

Strands of *F. serratus* with adult *S. borealis* settled on them were collected from Cullercoats bay, Northumberland (N55° 02' 05" W 1° 25' 39") during May to September of 1999-2001. They were placed in tanks of a recirculating seawater system at 10°C and fed on Kent Micro•Vert invertebrate food every two to three days. Approximately 500g of *F. serratus* was taken out of the tanks and placed in a black plastic bag, and left under the bench in the laboratory the night before use. The next morning the algae were placed in a container (60cm x 40cm x 30cm) and enough unfiltered seawater from the collection site was added to cover the algae. This was left for 20 minutes. The algae were then removed and the seawater was filtered through a plankton net (150μm) which collected any liberated larvae. The larvae were stored in 0.5μm FSW in a refrigerator at 10°C for a maximum of 4hrs, prior to use.

INVESTIGATION OF THE BEHAVIOUR OF *S. BOREALIS* ON DIFFERENT COATINGS

Method 2 (Chapter 5) was used to investigate the coatings. The coatings used are shown in bold in Table 2.2, 40 replications were carried out for each coating and analysis was carried out as stated in Analysis 2, Chapter 5.

Results

EXPLORATORY BEHAVIOUR OF *S. BOREALIS* ON SIX DIFFERENT COATINGS

The mean values for the behavioural parameters of *S. borealis* on all six coatings are given in Figures 6.4 and 6.5. The total distance of *S. borealis* on coatings 616 and 617 were less than on other coatings, compared to coating 615; *S. borealis* nearly travelled twice as far on this coating compared to 617 (Figure 6.4). *S. borealis* also spent less time moving on 617 and 616 compared to the other coatings and travelled fastest on coating ITS (Figure 6.4). A smaller step size (mean distance) was observed for the larvae on coating 619 compared to the other coatings especially ITS on which ~50% increase in mean distance was observed (Figure 6.4). Furthermore both turn rate and meander of *S. borealis* larvae was greatly increased on coating 619. Compared to the other coatings both turn rate and meander of the larvae on coating 619 were approximately twice as large (Figure 6.5).

Representative tracks made by *S. borealis* on all six coatings are presented in Figure 6.6. It can be seen from the tracks that on coatings 619 and ITS *S. borealis* travelled in a relatively linear fashion compared to the other coatings. The tracks made on coatings 615 and 617 are highly convoluted showing that the larvae explored in a circular manner across the coatings. These tracks of behaviour therefore suggest that coatings 615 and 617 were in some way attractive to the larvae.

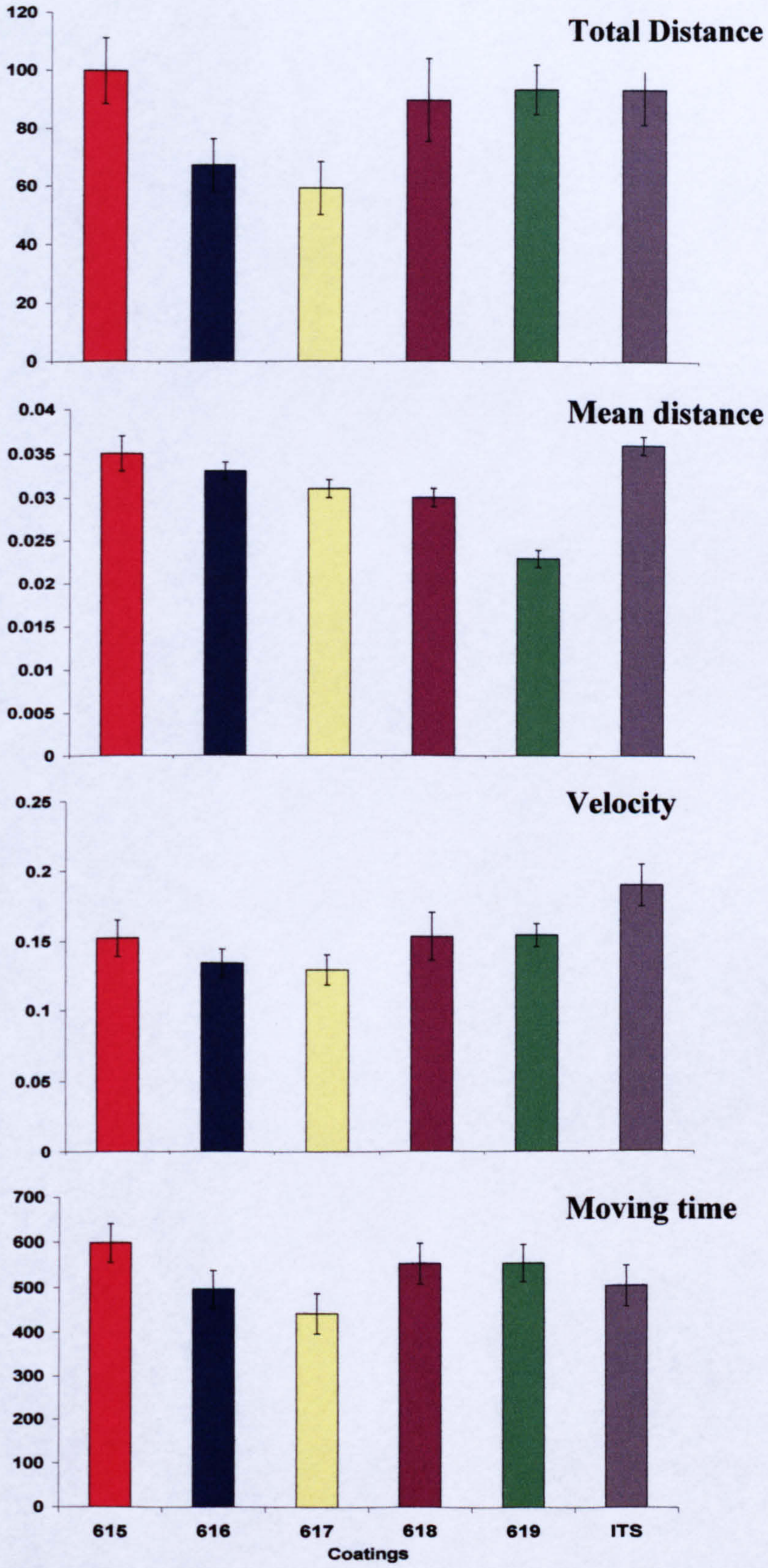


Figure 6.4 A comparison of the mean values (± s.e) of moving time (sec), velocity (sec/mm), mean distance (cm) and total distance (cm) for *S. borealis*, on each coating tested (n = 40).

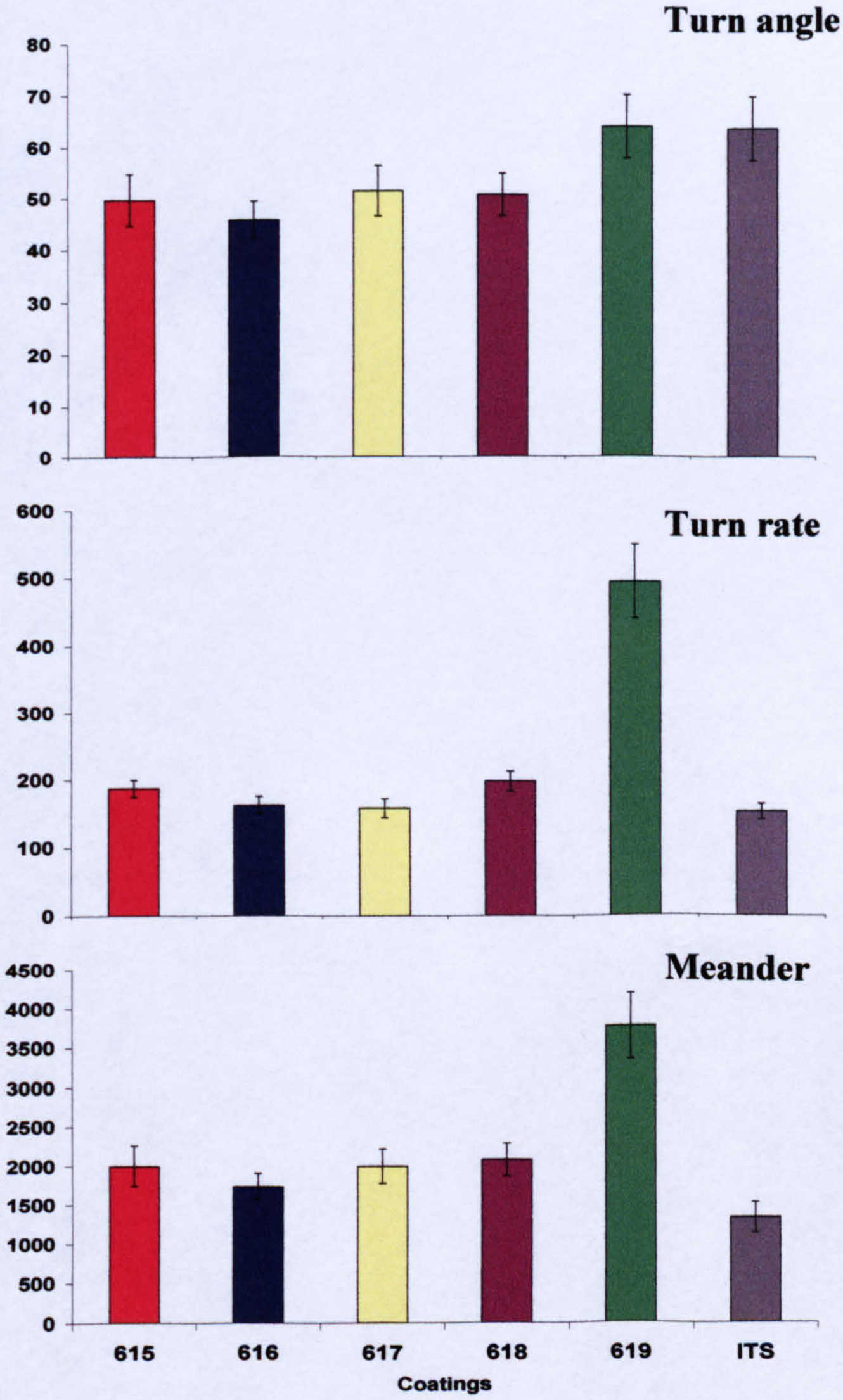


Figure 6.5 A comparison of the mean values (±s.e) of turn angle (degrees), turn rate (degrees/sec) and meander degrees/cm) for *S. borealis*, on each coating tested (n = 40).

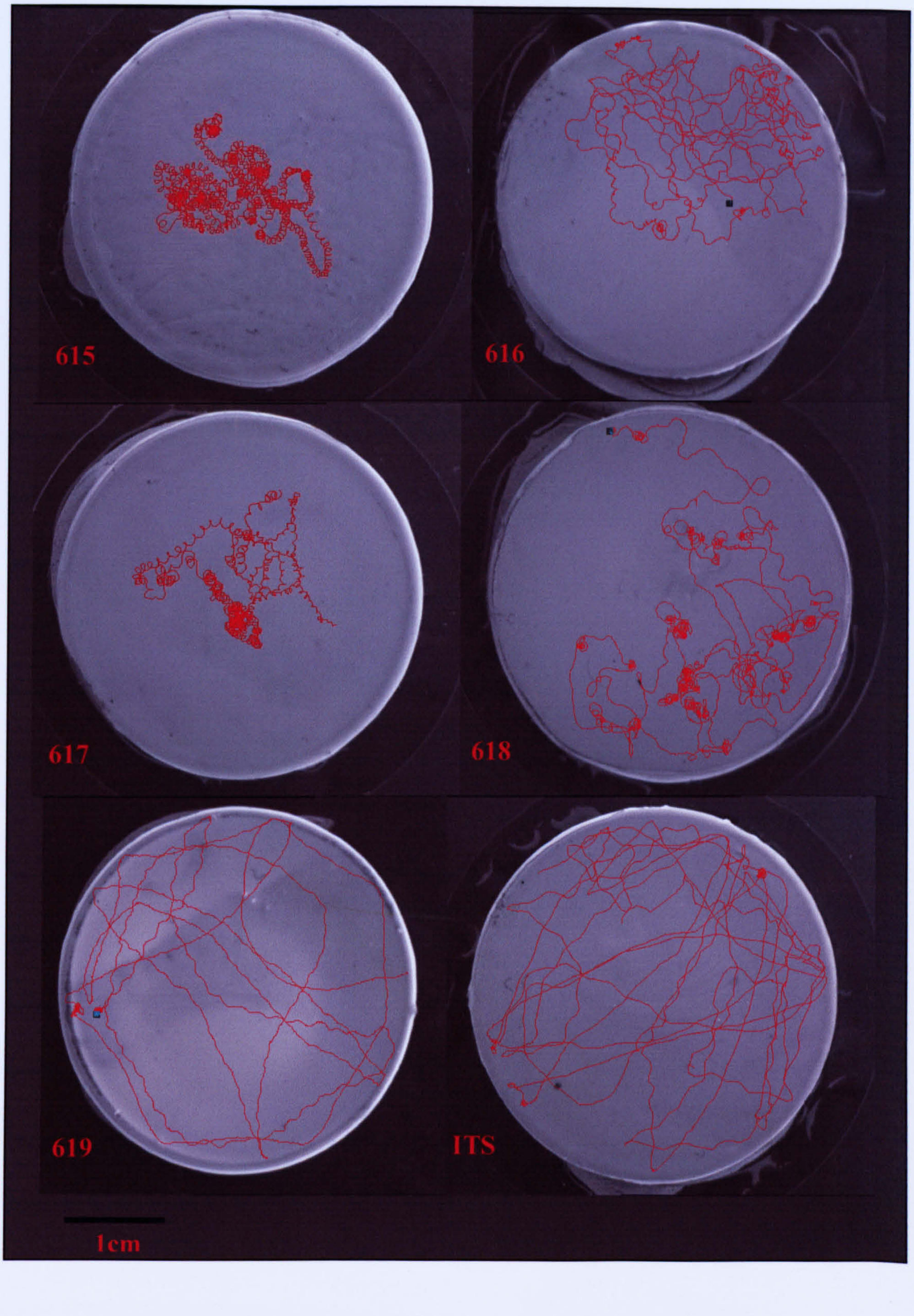


Figure 6.6 Representative tracks made by *S. borealis* on all six coatings, showing the arena used in the trials as the background image. The blue square represents the starting position of the larvae.

DO COATINGS SHOW SIGNIFICANTLY DIFFERENT BEHAVIOUR OF *S. BOREALIS*?

To establish if there were differences between the behavioural parameters due to coating type a GLM MANOVA was carried out (Chapter 5). Overall, there was a significant difference between coatings (MANOVA Wilk's $\lambda = 0.244$, $p < 0.001$). Univariate analysis, showed significant differences for total distance travelled (ANOVA $F = 2.37$, $p = 0.04$), mean distance travelled (ANOVA $F = 9.88$, $p = 0.016$), velocity (ANOVA $F = 2.87$, $p < 0.001$), turn rate (ANOVA $F = 22.28$, $p < 0.001$) and meander (ANOVA $F = 9.16$, $p < 0.001$). No significant differences were found between coatings for turn angle ($p > 0.05$) and moving time ($p > 0.05$) suggesting that these behavioural parameters are not influenced by coating type.

Tukey 95.0% Simultaneous Confidence Intervals were carried out on the parameters that showed a significant difference using ANOVA. Coating 619 was significantly different to all other coatings for all behavioural parameters apart from velocity and total distance (Table 6.1). This suggests that the behaviour of *S. borealis* on coating 619 is much different than on all other coatings. No other coatings showed significant differences for any of the behavioural parameters apart from velocity; *S. borealis* is significantly faster on coating ITS than on coatings 616 and 617. Total distance does not show any differences using this method of analysis suggesting that either the ANOVA produced a type 1 error or a type 2 error was produced by the Tukey analysis.

Total distance	616	617	618	619	ITS
615	0.424	0.105	0.906	1.000	0.998
616		0.979	0.961	0.331	0.700
617			0.631	0.071	0.260
618				0.841	0.990
619					0.992
Mean distance					
615	0.969	0.631	0.300	<0.001	1.000
616		0.974	0.791	<0.001	0.910
617			0.995	0.001	0.479
618				0.003	0.193
619					<0.001
Velocity					
615	0.921	0.781	1.000	1.000	0.285
616		1.000	0.905	0.877	0.026
617			0.755	0.711	0.010
618				1.000	0.310
619					0.350
Turn Rate					
615	0.850	0.671	1.000	<0.001	0.580
616		1.000	0.823	<0.001	1.000
617			0.635	<0.001	1.000
618				<0.001	0.544
619					<0.001
Meander					
615	0.993	1.000	1.000	0.001	0.125
616		0.978	0.973	0.001	0.382
617			1.000	0.002	0.085
618				0.002	0.080
619					<0.001

Table 6.1 The results of Tukey 95.0% Simultaneous Confidence Intervals comparing all coatings. Exact p values are given if >0.001, shaded boxes represent significance.

HEADING ANGLE ANALYSIS

The heading angle was investigated using Oriana v1.06 to determine the preferred direction of movement of *S. borealis* (Figure 6.7). This direction is evenly distributed (Rayleigh uniformity test $p > 0.05$). This indicates that there were no external directional factors, such as light, influencing the movement of *S. borealis* during the bioassays.

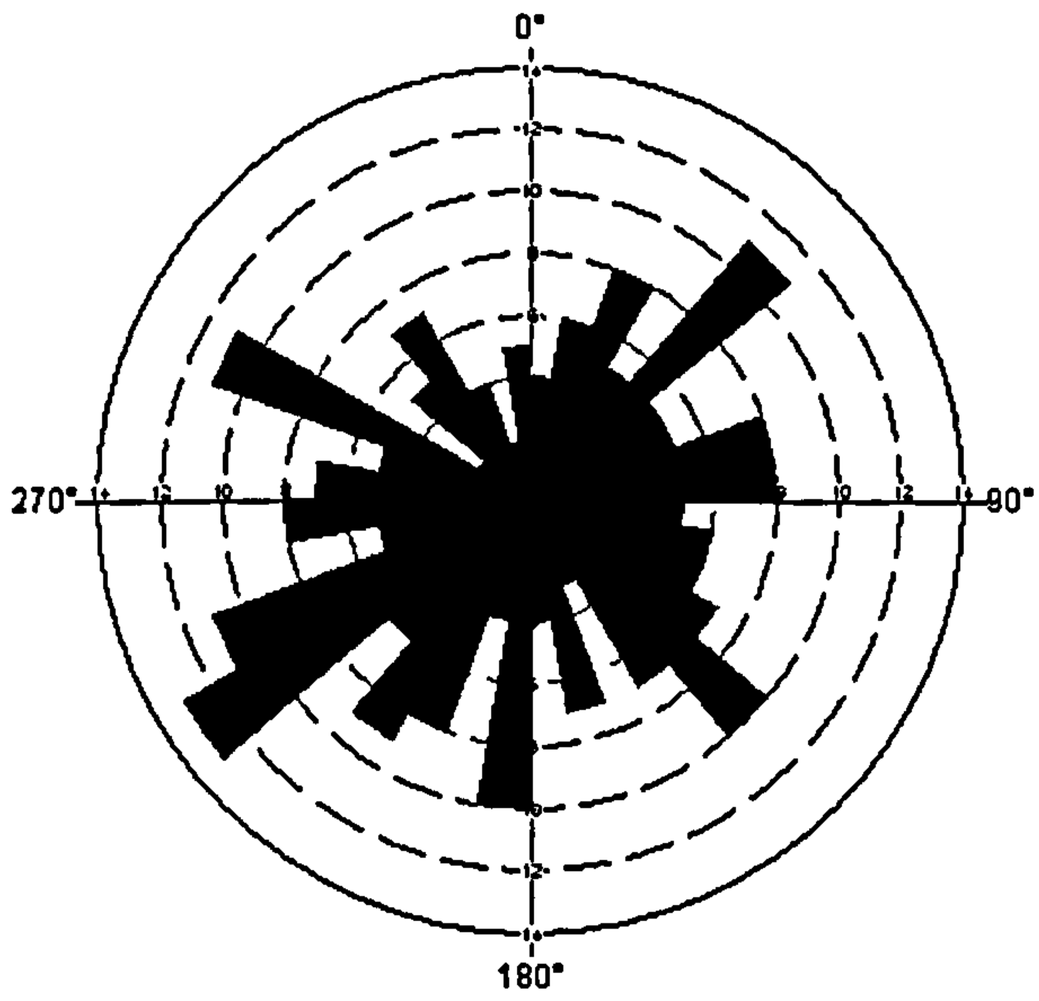


Figure 6.7 Circular histogram of the heading angles of *S. borealis*. Data for all bioassays on all coatings are combined. (n = 240).

CAN THE BEHAVIOUR OF *S. BOREALIS* BE USED TO DISTINGUISH BETWEEN COATINGS?

To determine if the behavioural parameters could be used to distinguish between the coatings, canonical discriminant analysis (CDA) was carried out. Ninety six percent of the variation was described by functions 1 and 2. The structure matrix shows that function 1 is dominated by turn rate (r = -0.515) followed by meander (r = -0.343) and

then mean distance (r = 0.342) all other variables had a lesser influence (absolute value of r < 0.06). Within function 2 mean velocity (r = 0.333), turn angle (r = 2.91) and turn rate (r = 0.29) were the dominant variables with all other parameters having less of an influence (absolute value of r < 0.2). Plotting the functions produced by this analysis (Figure 6.8) only coatings 619 and ITS could be identified as distinct groups. The predicted group membership of the coatings (Table 6.2) shows that coatings could be predicted correctly, on average, 50% of the time. Coating 619 could be predicted correctly 87.5% of the time, suggesting that the behaviour on this coating was very different compared to the other coatings. Coatings ITS and 615 however could also be predicted ≥50% of the time, suggesting that these coatings also had some differences which were affecting the behaviour of *S. borealis*. Coatings 616 and 617 were highly misclassified indicating that the behaviour of *S. borealis* was very similar on these coatings.

		Predicted group membership					
		615	616	617	618	619	ITS
Original grouping	615	**50.0**	10.0	15.0	25.0	.0	.0
	616	30.0	**27.5**	20.0	20.0	.0	2.5
	617	22.5	20.0	**32.5**	25.0	.0	.0
	618	17.5	15.0	20.0	**45.0**	.0	2.5
	619	5.0	.0	2.5	5.0	**87.5**	.0
	ITS	17.5	2.5	10.0	12.5	.0	**57.5**
50.0% of grouped cases correctly classified.							

Table 6.2 The percentage predicted group membership of each replication as given by CDA of all six coatings. Figures shown in **bold** indicate percentage correct for each coating.

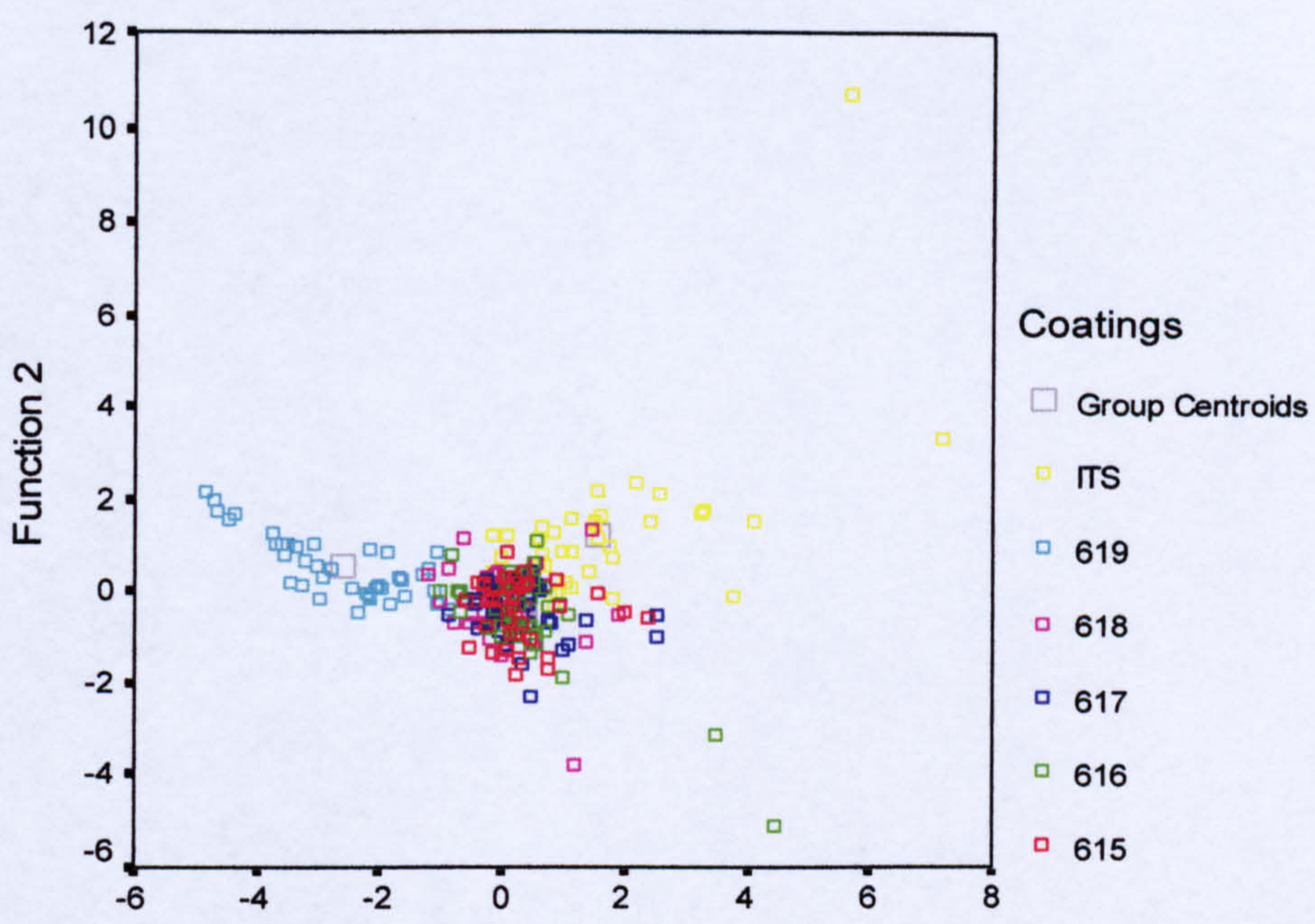


Figure 6.8 Canonical discriminant function scatter plot for all coatings. Group centroids can only be seen for ITS and 619, other centroids are obscured.

CAN THE EXPLORATORY BEHAVIOUR OF *S. BOREALIS* BE USED TO PREDICT FOULING IN THE FIELD?

PCA axis 1 scores generated by the field data and the total fouling data were used in stepwise multiple regression (Chapter 5) to determine which parameters were significantly related to the field data for each site, thus could be used as predictors of fouling burden.

UK

Multiple regression with total percent fouling values.

The behavioral parameters could be used to predict the percent fouling found at Newton Ferrers, UK (R^2_{adj} = 0.081, F = 6.295, p < 0.001). The best predictors found by the stepwise method were turn rate, turn angle, mean velocity and total distance (Table 6.3). This shows that both turn rate and total distance of exploring larvae decreased on coatings with higher antifouling performance. Turn angle and velocity however increase with antifouling performance at this site.

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	70.657		8.870	<0.000
Turn rate	2.440	.306	3.650	<0.000
Turn angle	-3.695	-.217	-2.404	0.017
Mean Velocity	-83.144	-.341	-3.374	0.001
Total distance	1.851	.218	1.996	0.047

Table 6.3 The coefficients calculated for all the parameters used in the regression model of % fouling found at the UK site. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown.

Multiple regression with PCA scores

When using the community fouling score generated by PCA in the regression the only significant relationship (R^2_{adj} = 0.017, F = 5.19, p = 0.024) found was for the behavioural parameter, mean distance:

$$PCA = 9.62 - 112.77 \text{mean distance}.$$

The PCA value increased with antifouling performance at this site (Figure 2.12). Therefore these results suggested that the mean distance of the larvae decreases on coatings of higher antifouling performance.

Singapore

Multiple regression with total percent fouling values.

Mean distance could be used to predict total fouling burden in Singapore. Total percent fouling at this site was related to mean distance (R^2_{adj} = 0.045, F = 12.34, p = 0.001) using linear regression:

$$\text{Total fouling} = 71.953 + 106.68 \text{mean distance}.$$

These results suggested that the mean distance of the larvae increased on coatings of lower antifouling performance (Figure 2.13).

Multiple regression with PCA scores

The behavioural parameters could be related to the community fouling data at the Singapore site by linear regression (R^2_{adj} = 0.389, F = 39.01, p < 0.001). Turn rate, mean distance, moving time, and total distances were found to be the best predictors of the community fouling for the Singapore site (Table 6.4). The PCA value increased with antifouling performance at this site (Figure 2.12). Therefore these results suggested that

both turn rate and total distance of exploring larvae increased on coatings with higher antifouling performance (Figure 2.12). Mean distance and total moving time however decreased with antifouling performance.

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	21.417		8.761	<0.001
Turn rate	.817	.310	5.553	<0.001
Mean distance	-163.543	-.534	-7.903	<0.001
Moving time	-.174	-.711	-6.521	<0.001
Total distance	2.538	.905	7.335	<0.001

Table 6.4 The coefficients calculated for all the parameters used in the regression model of PCA scores for the Singapore site. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown.

Sweden

Multiple regression with total percent fouling values.

Total percent fouling data from the Swedish site could be predicted using moving time (R^2_{adj}= 0.02, F = 5.94, p = 0.015). Total fouling at this site was significantly related to moving time using linear regression:

$$\text{Total fouling} = 91.27 + 0.0328\text{moving time.}$$

These results suggest that the time spent moving by the larvae increased on coatings of lower antifouling performance.

Multiple regression with PCA scores

When using the community fouling score generated by PCA in the regression a significant relationship found was for mean distance ($R^2_{adj} = 0.013$, F = 4.17, p = 0.042):

$$\text{PCA} = 43.31 + 8.4.35\text{mean distance.}$$

The PCA value decreased with antifouling performance at this site (Figure 2.12). Therefore these results suggest that the mean distance of the larvae decreased on coatings of higher antifouling performance.

Discussion

Can the behaviour of S. borealis be used to distinguish between the coatings?

Overall the behaviour of *S. borealis* is shown to differ on the different coatings investigated, however coating 619 consistently shows different behaviours compared to the other coatings. On this coating *S. borealis* travels for the same distance at the same speed than on the other coatings, but turns quicker and more frequently. This twisting and turning behaviour suggests the larvae are exploring the surface in such a way that may ultimately lead to settlement; on exploration of a surface, *S. borealis* moves in a relatively uniform direction at first but as settlement and metamorphosis is nearing this movement changes to shorter steps with frequent changes in direction (Nott 1973, Knight-Jones 1951). Therefore coating 619 may be considered as the most attractive surface for *S. borealis.*

Coating 619 is an experimental silicone with a relatively smooth surface with a lower surface energy than the other coatings (Table 2.2). Rugosity and surface energy are known to affect settlement of marine larvae (for review see Crisp 1974), though the specific effects of these two surface parameters on *S. borealis* have not been well documented. There is little difference in rugosity between 619, 616, 617 and ITS (Table 2.2) suggesting that if *S. borealis* is responding to this surface characteristic any behaviour changes would be consistent on all 4 coatings. *S. borealis* also has been

shown to prefer grooves made by the midribs of *F. serratus* (Wisley 1960) suggesting that the relatively smooth surface of coating 619 should not cause the pre-settlement behaviour shown by *S. borealis* on this coating. However, the rugosity of the coatings (Table 2.2) were measured on a much smaller scale (μm) than the apparent measurement scale of a groove made by the midrib of *F. serratus* (mm). Other marine larvae have been shown to respond significantly to topographic cues at one particular scale (Le Tourneux and Bourget 1988, Hills *et al.* 1998a). Therefore as little is known regarding the micronscale of the groove, it is unclear if the coatings provide a texture that *S. borealis* respond to. Furthermore surface texture may not be an important settlement cue under the bioassay conditions as the preference shown for grooves or crevices has been linked to hydrodynamic cues (Butman 1988, Wethey *et al.* 1988), the still water of the bioassay therefore may eliminate any preferences shown.

The surface energy is lower on 619 compared to the other coatings (Table 2.2). No literature has been found on the preference of *S. borealis* for surface energy, but the results from these bioassays are concomitant with a preference for low surface energy. Behaviour is however extremely complex and as very little is known regarding the composition of the coatings consequently it is impossible to tell what and how the larvae is responding to.

The canonical discriminant analysis also shows that that behaviour on coating 619 is very different allowing nearly an 88% correct prediction of this coating when compared to the others. Coatings ITS and 615 can be predicted with ≥50% success. This suggests that behaviour on these coatings is also quite different. ITS is a commercial non-biocidal foul release coating specified for fouling control on high activity and fast vessels, whereas coating 615 is an epoxy primer used as a positive control and has no

documented antifouling properties (Table 2.2). It is therefore not surprising that *S. borealis* should exhibit different behaviours on these coating if it is responding to any of the antifouling properties present on the coatings. The other coatings are frequently misclassified by the CDA, these coatings maybe too similar in chemical composition for any differences to be detected by *S. borealis'* behaviour. No information from Akzo Nobel was available as to whether these different coatings represent an array of minor chemical variants, or whether they are from a diverse range of base formulations. Consequently, it is difficult to ascertain if either, larval behaviour is not being affected by surface characteristics, or if the surfaces are too similar for behavioural bioassays to permit discrimination. The results do show that when taking all the behavioural parameters into account the coatings can be discriminated between, with a ≥50% success rate.

Can the exploratory behaviour of S. borealis be used to predict field data?

The behavioural data could be used to explain some of the variation seen in the field data, using both PCA community fouling and overall percentage fouling burden although the variation explained (R^2_{adj}) is low at many of the sites. This however, is a function of sample size and variance and such relationships are unlikely to happen by chance alone ($p < 0.05$), therefore a significant amount of the fouling at such sites can be explained by the behavioural parameters. An increase in the amount variables or testing different behavioural parameters may explain more of the variation.

Fouling burden is different at each of the sites (Chapter 2) therefore it is not surprising that different behavioural parameters are important at different sites and with the two ways of interpreting fouling burden. At the Singapore site, the 4 behavioural parameters

that can be used to explain the variation in the community fouling data are turn rate, mean distance moving time and total distance whereas the total percentage fouling burden at the UK site can be explained by turn rate, turn angle, velocity and total distance. Despite these differences mean distance however seems to be an important behavioural parameter as it was consistently identified as having a significant relationship with the field data.

Clearly, if mean distance is short this suggests that the larvae experienced a small part of the surface and then left the vicinity, whereas a long mean distance suggests a more considerable exploration of the coating surface. Surface exploration appears to be a generic feature of marine larval pre-settlement behaviour (Knight-Jones 1951, Crisp 1961, Crisp 1974, Berntsson 2000b), possibly due to the long term consequences of the non-reversible settlement decision (Wethey 1984). Thus, exploration distance could be expected be an important precursor to subsequent pre-settlement behaviour, and consequently commonly linked to longer term community settlement in the field.

Suitability of using S. borealis as a test species.

Despite *S. borealis'* viviparity and short pelagic larval life which lends well to making it a desirable species for the bioassay, its settlement preferences maybe of a disadvantage to this behavioural bioassay; *S. borealis* preferentially settles on *F. serratus* and will only readily settle on inert objects such as stone and glass after a bacterial film has been established (Knight-Jones 1951, De Silva 1962, Meadows and Williams 1963). The preference for settlement on *F. serratus* may even override any influences of the physical characteristics of the surface such as texture and contour (Williams 1964).

S. borealis was seen by the computer software Ethovision as a dot of approximately 15 pixels. This was dependent on the required setup, as the whole arena needed to be included in the field of view. Any slight movement however of the larvae would therefore not have been detected at this magnification. During exploratory behaviour *S. borealis* is known to wave its abdomen from side to side contracting and relaxing its body while otherwise remaining stationary (Nott 1973). This type of behaviour would not be detected at this magnification and the larvae would appear stationary as if not exploring at all. Some subtle and small-scale differences in behaviours therefore may have been missed due to this.

Summary

The ease of collection, viviparous nature and short pelagic life of *S. borealis*, makes it an ideal species to be included in the bioassay. All of the coatings showed significantly different behaviours and these behaviours could be used to distinguish between most of the coatings. Some of the behavioural parameters of *S. borealis* could also be used to predict the fouling burden seen in the field. Although different parameters were important at different sites, mean distance was a key variable in these predictions.

CHAPTER 7

BALANUS AMPHITRITE

CHAPTER 7

A BEHAVIOURAL BIOASSAY USING *BALANUS AMPHITRITE*

Introduction

Balanus amphitrite (Darwin) is one of the most prominent barnacles in fouling communities in warm temperate and tropical waters where it occurs at low water and in the shallow sublittoral zone. In Britain it is restricted to estuaries, ports, such as Swansea docks, and Shoreham harbour where the water is warmed by heated effluents from power stations (Southward 1976).

Settlement of cyprids occurs between May and August in the UK (Crisp and Molesworth 1951 In Rainbow 1984) the behaviour of which is well documented (*for reviews see* Barnes 1971, Crisp 1974) and both chemical and physical settlement cues have been identified which actively influence a cyprids choice of settlement site (see Chapter 1 for details). *B. amphitrite* is both gregarious and territorial (Crisp 1961) showing preferences for high initial wettability (Schmidt *et al.* 1987), darker surfaces (Walton-Smith 1948), grooves and crevices (Walters 1992a) and surfaces with an established biofilm (Wieczorek *et al.* 1995).

On contact with the substratum, temporary attachment is followed by an exploration stage, and if certain requirements are met by the surface, fixation and metamorphosis occurs, if it does not the larvae will then break contact with the substratum and swim off in search of a more suitable attachment site (Rainbow 1984). Three phases within this exploration stage have been described: broad exploration, close exploration and

inspection (Crisp 1974), although more recent work has shown that settlement can occur with very little exploration (Hills *et al.* 1998, Hills *et al.* 2000). *B. amphitrite* is believed to follow the same settlement behaviour generalised for cyprids; during broad exploration the larvae literally "walk" over the substratum using their adhesive antennules (Walker and Yule 1984). These antennules contain many sensory organs (Nott and Foster 1969) and together with sensory receptors on the caudal appendages and carapace (Walker and Lee 1976) are use to explore the surface. They travel along the surface in a relative straight line with infrequent turns, which may result in the larvae breaking contact and swimming off or entering the next phase. During the following close exploration phase behaviour changes to short steps which frequently change direction. Just before metamorphosis occurs the larva enters the inspection phase in which it rotates and moves to and fro within its own body length (Knight-Jones and Crisp 1953). The mode of locomotion does not change throughout the exploration of the substratum, only the degree of twisting and turning increases (Knight-Jones and Crisp 1953, Crisp 1961).

Few attempts have been made to determine the spatial scale of this behaviour and it seems probable that different settlement cues operate at different scales (Le Tourneux and Bourget 1988). It has been estimated that the net distance travelled by *B. amphitrite* during exploration is < 3 cm (Crisp 1961, Mullineaux and Butman 1991). Other work using the cyprid *Semibalanus balanoides* estimated spatial scale for the three phases, broad exploration, close exploration and inspection to be 1 m, 1 mm and <300 μm respectively (Le Tourneux and Bourget 1988). Although contact can be broken at any time during exploration, field experiments have show that exploratory behaviour of cyprids is stereotyped by different factors (Hills *et al.* 2000). It is therefore postulated

that this behaviour, if related to settlement density, could be used to detect antifouling properties of experimental non-toxic antifouling coatings.

Details of why *B. amphitrite* was chosen for the behaviour bioassay are given in Chapter 2. Prior to the investigation of coatings a sample size experiment was run to establish an appropriate sample size that minimised the effect of individual variability. The bioassay at present is restricted to white coatings, which allows maximum contrast of the larvae for automated detection. A staining experiment was run with this species to establish whether it was possible to stain the cyprids for a better contrast. This would allow coatings of different colours to be investigated and/or more replication to be run at once.

The aim of this investigation was firstly, to determine the suitability of *B. amphitrite* as a test organism. Secondly, to assess whether the behaviour of *B. amphitrite* was different on different coatings and if this behaviour could be used to distinguish between the coatings used in the bioassay, and finally to determine whether the exploratory behaviour of *B. amphitrite* could be used to predict the fouling of these coatings, as seen in the field immersion trials.

Preliminary work - Sample Size Experiment.

Materials and Method

Method 2 (Chapter 5) was used to determine optimal sample size. In order to promote exploration a settlement extract was used. No adult *B. amphitrite* were available for this, therefore as different species have shown to promote exploratory behaviour and settlement (Knight-Jones 1955, Larman and Gabbott 1975), *Semibalanus balanoides*

adults were used to make the crude extract; 0.1g of wet weight of adult extracted from the shell, was macerated using a pestle and mortar with 1ml of distilled water. This was stored in a domestic freezer (-10 °C) prior to use. Two drops of this extract from a glass pipette were painted onto the watch glasses (arenas) with no coating applied. These were oven dried for 20mins at 60°C prior to use. Eighty replications were carried out. The running means for meander, turn angle, distance and turn rate were used to assess the optimal sample size.

Results

Figure 7.1 shows the running mean of the four parameters calculated from the x y co-ordinates of the *B. amphitrite* tracks. A sample size of 30 lies with in the 95% confidence intervals for each of the parameters, therefore 30 replicates were used for this species during the bioassay.

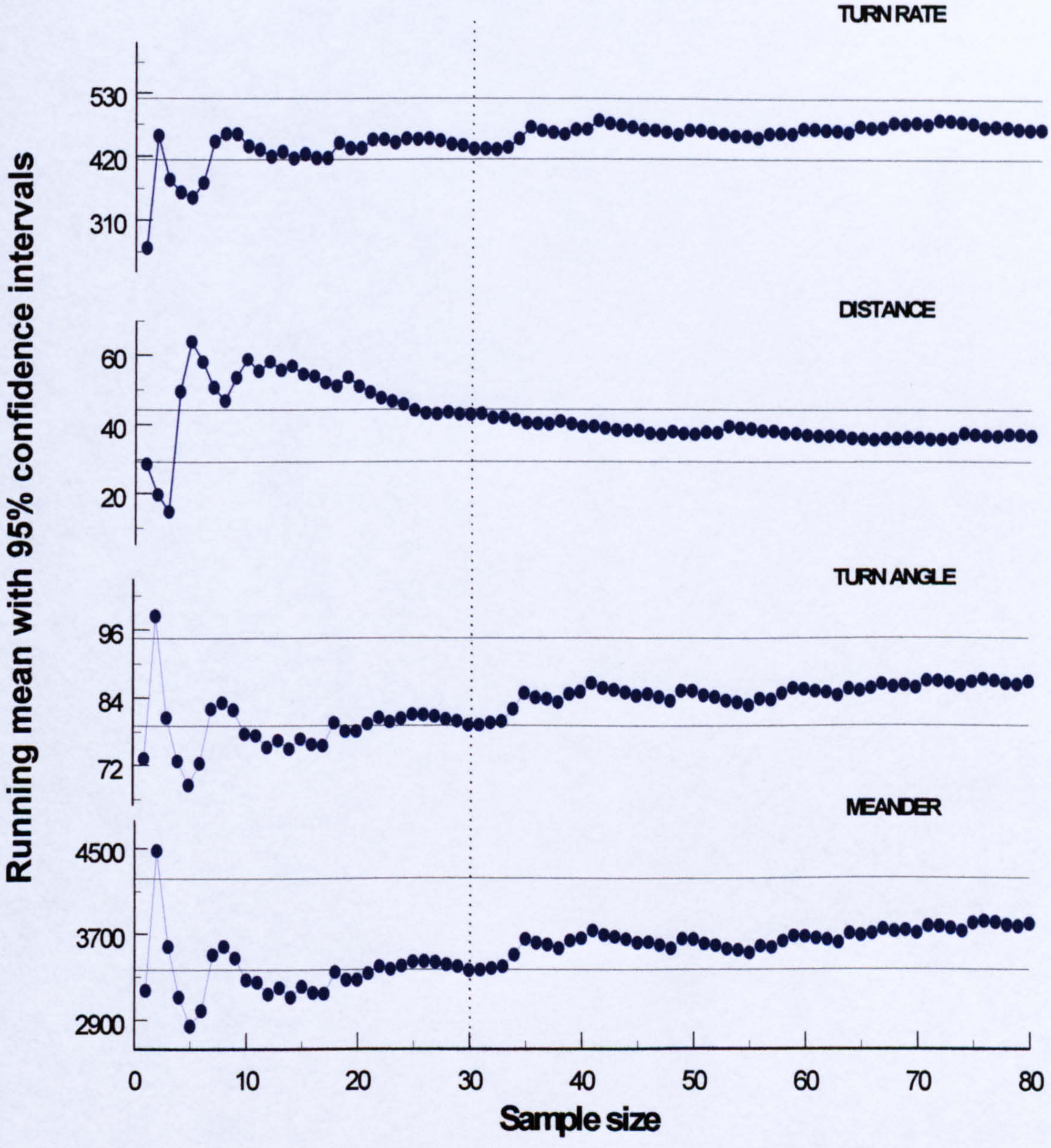


Figure 7.1 Running means of meander, turn angle, distance and turn rate with the 95% confidence interval for a sample size of 80.

Materials and Method

Collection and storage of larvae

B. amphitrite cyprids were cultured from adult broodstock according to Clare (1996b). They were kept in domestic refrigerator at 5 ± 1°C prior to use. Day 1-5 cyprids (incl.) were used for experimentation.

Staining investigation

The stains neutral red (Sigma-Aldrich) and nile blue (Sigma-Aldrich) were chosen to stain the cyprids as both were readily available, and are non toxic vital stains which were readily absorbed by the cyprids (Figure 7.2). Nile red has widely been used, staining starfish (Feder 1955), pelecypods (Loosanoff and Davis 1947), copepods (Anstensrud 1989, Kelly *et al.* 1998) and entire epifaunal collections (Howard 1985) as well as cyprids (Dr A Clare, unpublished data.). Nile blue has been used for also staining starfish (Feder 1955, Loosanoff 1937) as well as sponges (Loosanoff and Davis 1947) and prawns (Dawson 1957); for a review of immersion stains see Smith and Present (1983).

The cyprids were stained on the morning of experimentation. They were removed from the seawater in which they were stored , by filtering it through a small piece of plankton netting (150μm) and placed in a watch glass (4cm) with 2ml of the appropriate stain at room temperature. Cyprids that were stained with neutral red were left for 1 hr in a 0.01mg ml^{-1} solution (Anstensrud 1989, Kelly *et al.* 1998) with filtered seawater (0.2μm). Various concentrations and immersion times of nile blue were tried prior to experimentation in order to use the minimum concentration that gave the best visual

absorption. A 10mg ml^{-1} of nile blue in seawater for 3-4mins (Recek 1956) was not enough time for the stain to be absorbed, so this was increased to 20mins (Dawson 1957). It was then found that 5mg ml^{-1} of nile blue was sufficient to stain the cyprids when immersed for 20mins therefore this concentration and immersion time was used for cyprids that were to be stained with nile blue. Unstained cyprids were treated in the same manner as the ones being stained, but only filtered seawater was used in the watch glass. They were immersed for 30mins. After immersion the cyprids were again filtered through the netting and placed in filtered seawater. They were left at room temperature prior to filming.

The filming method was as per Method 2, Chapter 5. Two cyprids were filmed at once in separate arena. Stained and non stained cyprids were filmed alternatively. Thirty replications for each staining regime were carried out. A comparison of stained and not stained cyprids was carried out using GLM MANOVA after a suitable Box-Cox transformation had been carried out. Behavioural parameters were used as dependent variables and the staining method as a fixed factor. Both nile blue and neutral red were compared against the non stained control cyprids. All analysis was carried out using Minitab v12.

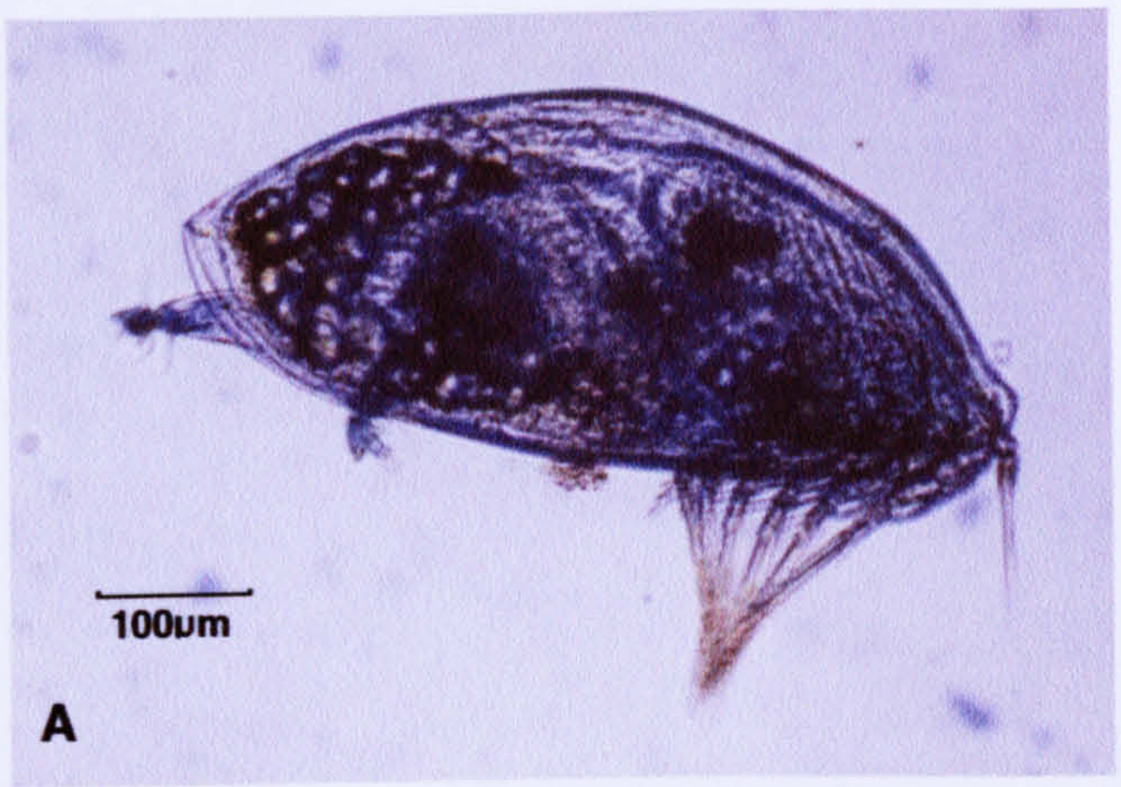


Figure 7.2 Comparison of stained and unstained *B. amphitrite* cyprids; A) Nile blue stain B) Unstained C) Neutral red stain.

Investigation of cyprid behaviour on different coatings

Collection and storage of larvae was as above. The method was carried out as per Method 2 (Chapter 5). The coatings shown in bold in Table 2.2 were tested. The bioassay was carried out 30 times for each coating. Analysis was carried out as stated in Method 2, Chapter 5.

Results

Staining investigation

The mean values for the parameters calculated for the stained and unstained cyprids are given in Table 7.1. It can be seen that the overall distance travelled by the unstained cyprids was far greater compared to the stained ones. The unstained cyprids also travelled faster and for longer compared to the ones stained either by nile blue or neutral red. Also the mean distance and velocity of cyprids stained with neutral red varied greatly in comparison with non stained cyprids (Table 7.1).

The results showed that there were no overall significance effects on behaviour of *B. amphitrite* by neutral red staining ($p > 0.05$). When the behavioural parameters were investigated individually no differences were found ($p > 0.05$ for all) apart from moving time (ANOVA $F = 6.30$, $p = 0.015$). The results therefore show that cyprids stained with neutral red spent less time moving on the surface than cyprids with no stain.

Parameter	Nile blue	No Stain	Neutral red
Total distance travelled (cm)	36.82±5.03	63.56±5.71	55.23±4.79
Mean Distance (cm)	0.022±0.00117	0.024±0.00077	0.069±0.044
Mean velocity (cm/sec)	0.177±0.0103	0.474±0.0034	0.188±0.282
Moving time (sec)	273.2±49.4	474±59.0	315.0±32.4
Mean turn angle (degrees)	129.7±10.4	138.01±5.13	137.47±5.14
Mean turn rate (degrees/sec)	1156.3±66.5	1128.7±45.7	1098.3±52.4
Mean meander (degrees/cm)	6571±401	6306±261	6021±336

Table 7.1 Mean values ± standard error of the behavioural parameters for *B. amphitrite* cyprids, stained with nile blue and neutral red, and unstained.

When investigating the effects of nile blue staining, the stained and unstained cyprids did overall show significant differences in behaviour (MANOVA Wilks $\gamma = 0.63$, $p = 0.001$, $n = 5$). Both moving time (ANOVA $F = 11.64$, $p = 0.001$) and total distance (ANOVA $F = 12.34$, $p = 0.001$) travelled showed significant differences when considering the individual components of the behaviour. The results therefore suggest that cyprids stained with nile blue travelled less distance over the surface and spent less time moving compared to unstained cyprids.

Investigation of coatings

EXPLORATORY BEHAVIOUR OF *B. AMPHITRITE* ON SIX DIFFERENT COATINGS

The mean values for the behavioural parameters of *B. amphitrite* for all six coatings are given in Figures 7.3 and 7.4. The cyprids on coating ITS spent less time moving and travelled less distance compared to other coatings especially coatings 617, 618 and 619 (Figure 7.3). However angular movement of the cyprids on coatings 617 and 619 was less compared to the other coatings especially ITS and 616 (Figure 7.4).

Representative tracks made by *B. amphitrite* on each of the six coatings can be seen in Figure 7.5. It can be seen that very little exploratory behaviour was exhibited by *B. amphitrite* on coating ITS as compared to the other coatings. The tracks made on the other coatings were also highly convoluted suggesting that the larvae explored in a circular manner across them.

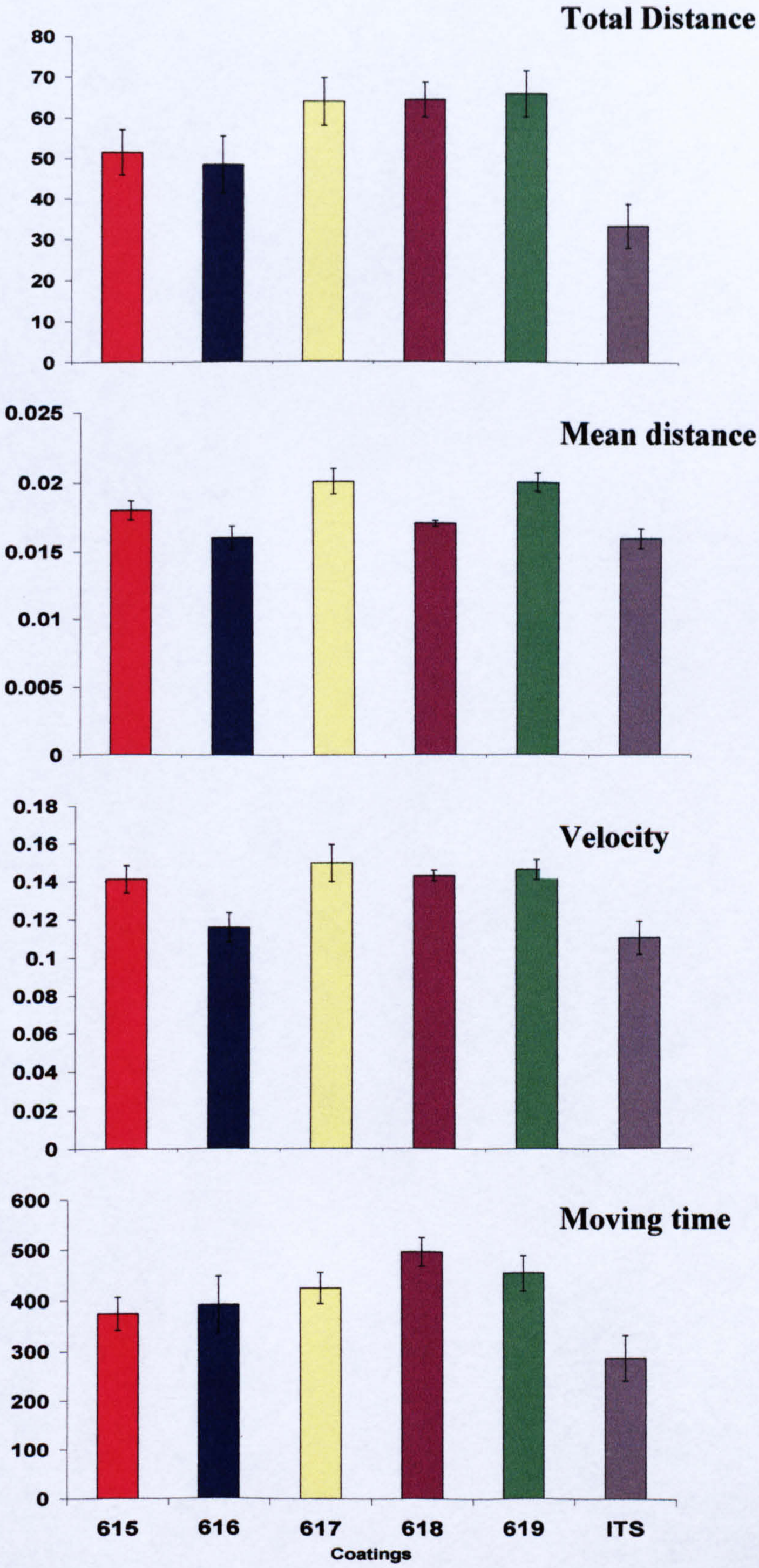


Figure 7.3 Mean values (± s.e) of moving time (sec), velocity (sec/mm), mean distance (cm) and total distance (cm) for *B. amphitrite* cyprids, on each coating tested (n = 30).

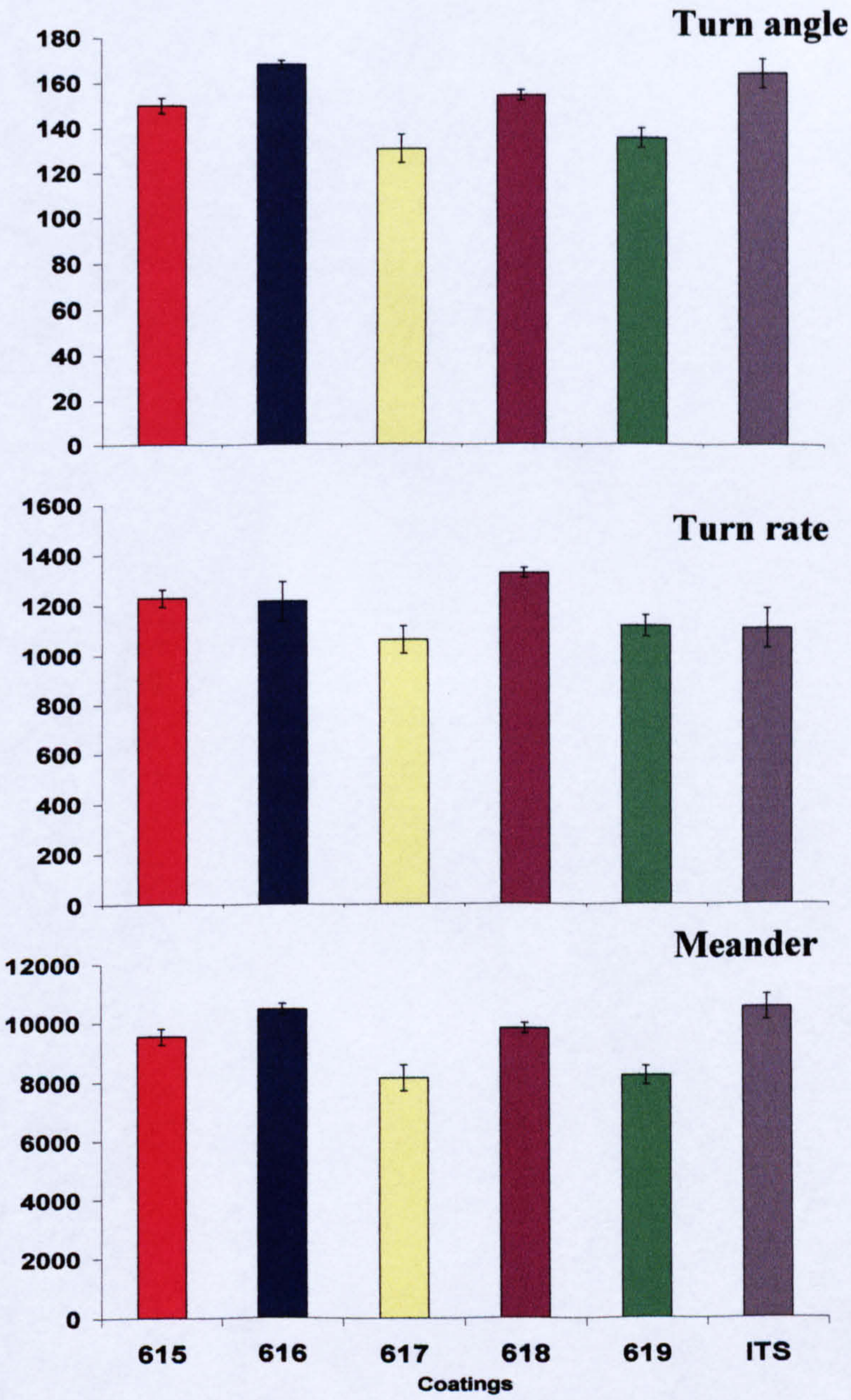


Figure 7.4 Mean values (± s.e) of turn angle (degrees), turn rate (degrees/sec) and meander (degrees/cm) for *B. amphitrite* cyprids, on each coating tested (n = 30).

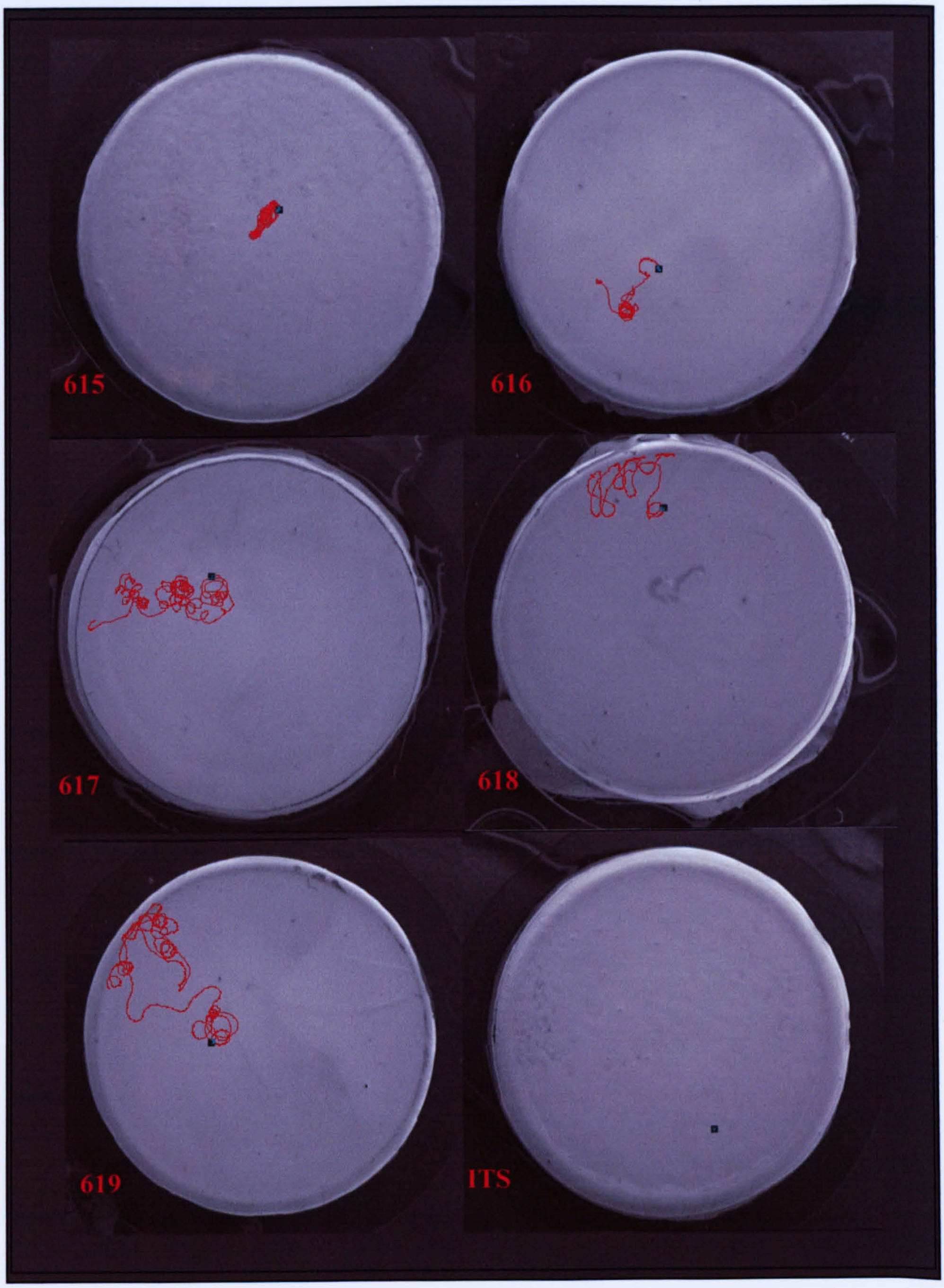


Figure 7.5 Representative tracks made by *B. amphitrite* on all six coatings, showing the arena used in the trials, as the background image. The black square represent the initial position of the cyprid. Note no movement for coating ITS.

DO COATINGS SHOW SIGNIFICANTLY DIFFERENT BEHAVIOUR OF *B. AMPHITRITE?*

A GLM MANOVA was carried out in order to establish if there were differences between the behavioural parameters due to the coating type. There was a significant difference overall between coatings (MANOVA Wilk's $\lambda = 0.396$, $p < 0.001$). Univariate analysis showed significant differences for all parameters; total distance travelled (ANOVA $F = 5.98$, $p < 0.001$), mean distance travelled (ANOVA $F = 7.75$, $p < 0.001$), mean velocity (ANOVA $F = 5.94$, $p < 0.001$), turn angle (ANOVA $F = 16.83$, $p < 0.001$), turn rate (ANOVA $F = 4.13$, $p = 0.001$), meander (ANOVA $F = 13.30$, $p < 0.001$) and moving time (ANOVA $F = 3.97$, $p = 0.002$).

Tukey 95.0% Simultaneous Confidence Intervals were carried out to make pairwise comparisons of the coatings for all parameters (Table 7.2). For all of the behavioural parameters, apart from turn rate, ITS significantly differs from coating 619. Coating 617 also shows significant differences in many of the behavioural parameters compared to ITS. Both 618 and 616 show significant differences for 4 out of the 7 parameters with coating 619 and likewise coating 617 shows significant differences between both 616 and 618. These differences show that the behaviour of *B. amphitrite* is altered in some way depending on coating type.

Total distance	616	617	618	619	ITS
615	0.971	0.685	0.516	0.533	0.105
616		0.223	0.128	0.136	0.461
617			1.000	1.000	0.001
618				1.000	0.001
619					<0.001
Mean distance					
615	0.340	0.453	0.715	0.486	0.057
616		0.002	0.992	0.003	0.963
617			0.018	1.000	<0.001
618				0.021	0.719
619					<0.001
Velocity					
615	0.169	0.958	1.000	0.995	0.014
616		0.018	0.109	0.048	0.936
617			0.987	0.999	0.001
618				1.000	0.007
619					0.002
Turn Angle					
615	0.004	0.030	0.965	0.084	0.009
616		<0.001	0.048	<0.001	1.000
617			0.002	0.999	<0.001
618				0.008	0.093
619					<0.001
Turn rate					
615	0.999	0.184	0.689	0.523	0.805
616		0.073	0.896	0.283	0.558
617			0.003	0.989	0.890
618				0.021	0.076
619					0.998
Meander					
615	0.175	0.027	0.987	0.023	0.066
616		<0.001	0.523	<0.001	0.998
617			0.003	1.000	<0.001
618				0.003	0.275
619					<0.001
Moving time					
615	1.000	0.946	0.271	0.751	0.404
616		0.912	0.218	0.681	0.476
617			0.817	0.997	0.061
618				0.970	0.001
619					0.017

Table 7.2 The results of Tukey 95.0% Simultaneous Confidence Intervals showing a pairwise comparison for all parameters on all coatings . Exact p values are given. Significant values are indicated by the shaded box.

HEADING ANGLE ANALYSIS

The heading angle was used to determine the preferred direction of movement of *B. amphitrite* cyprids during all the bioassays, using Oriana v1.06 (Figure 7.6) This direction is evenly distributed (Rayleighs uniformity test, $p > 0.05$). This indicates that there were no external directional factors, such as light, influencing the movement of *B. amphitrite* cyprids during the bioassays.

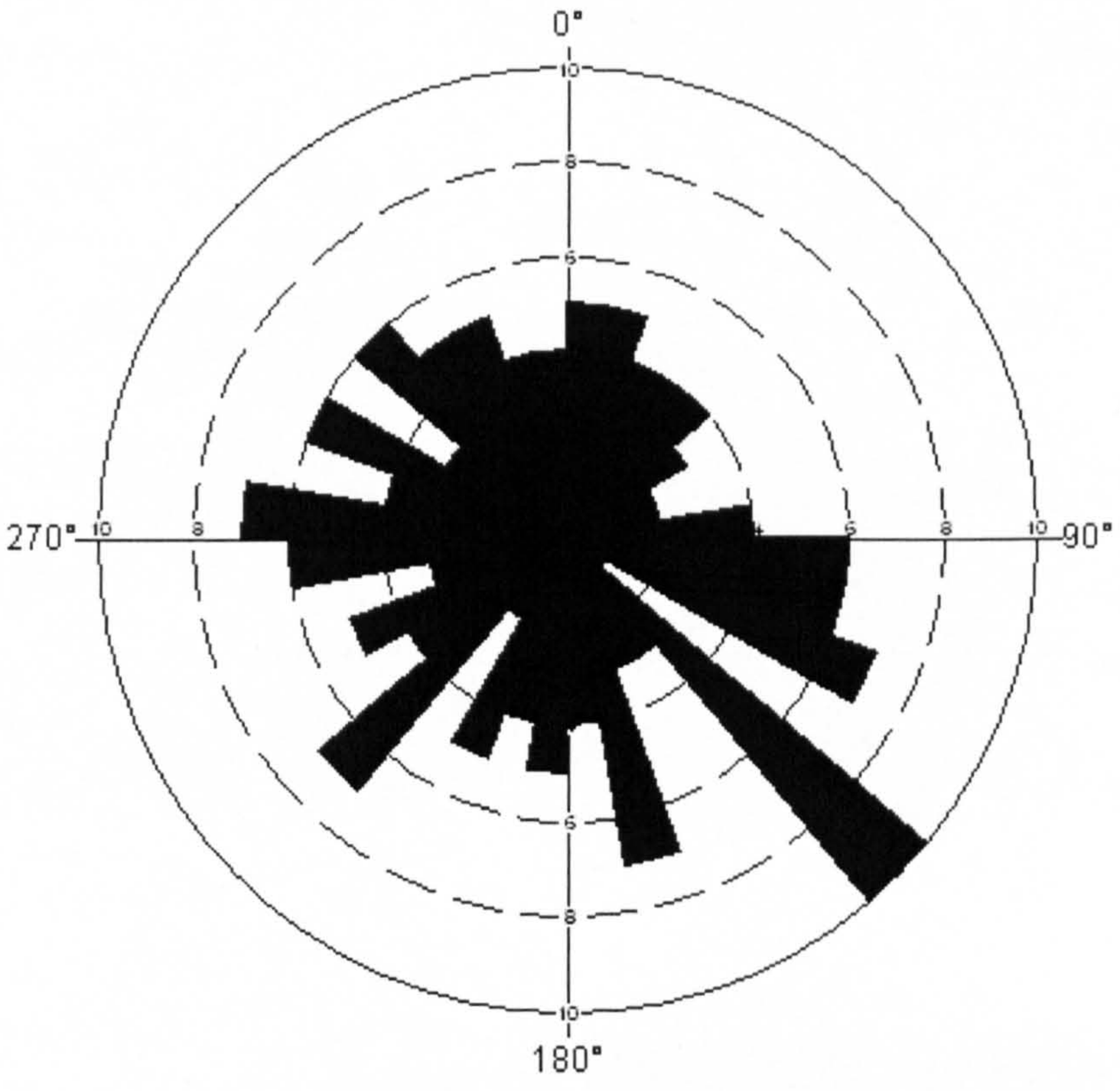


Figure 7.6 Circular histogram of the heading angle of *B. amphitrite* cyprids. All data were combined for all bioassays on all coatings (n = 180).

CAN THE BEHAVIOUR OF *B. AMPHITRITE* BE USED TO DISTINGUISH BETWEEN COATINGS?

In order to see if the behavioural parameters could be used to distinguish between the coatings, canonical discriminant analysis (CDA) was carried out. Eighty seven percent

of the variation was described by functions 1 and 2. The structure matrix of the CDA showed that turn angle (r = -0.722), meander (r = 0.615), mean distance (r = -0.464) and velocity (r = 0.432) dominate function 1 but all other parameters were of a lesser influence (absolute value of r < 0.370). Function 2 was dominated by meander (r = 0.450) followed by mean distance (r = 0.360) and then turn angle (r = -0.277), all other parameter has a lesser influence on function 2 (absolute value of r < 0.21). On plotting the functions produced by this analysis, no distinct groups could be seen (Figure 7.4). This suggests that the behaviour of *B. amphitrite* on the coatings was very similar. The predicted group membership of the coatings (Table 7.3) shows that coatings could be predicted correctly 45 % of the time. Coatings 616 and 618 could be predicted correctly >50% of the time, suggesting that the behaviour on these coating were different compared to the other coatings. However, all other coatings were frequently misclassified; behaviour on 615 is similar to the behaviour of cyprids on 618 (Table 7.3). Similarly 617 was misclassified with coatings 618 and 619and ITS was misclassified with both 616 and 618 (Table 7.3) suggesting that behaviour of *B. amphitrite* was similar on these misclassified coatings to the original group coating.

		Predicted group membership					
		615	**616**	**617**	**618**	**619**	**ITS**
Original grouping	**615**	**40.0**	3.3	16.7	30.0	6.7	3.3
	616	3.3	**60.0**	0.0	13.3	0	23.3
	617	6.7	0.0	**40.0**	26.7	13.3	13.3
	618	23.3	10.0	10.0	**53.3**	0	3.3
	619	10.0	10.0	20.0	23.3	**33.3**	3.3
	ITS	10.0	13.3	6.7	23.3	3.3	**43.3**
45.0 % of grouped cases correctly classified.							

Table 7.3 The percentage predicted group membership of each replication as given by CDA of all six coatings. Figures shown in **bold** indicate percentage correct for each coating.

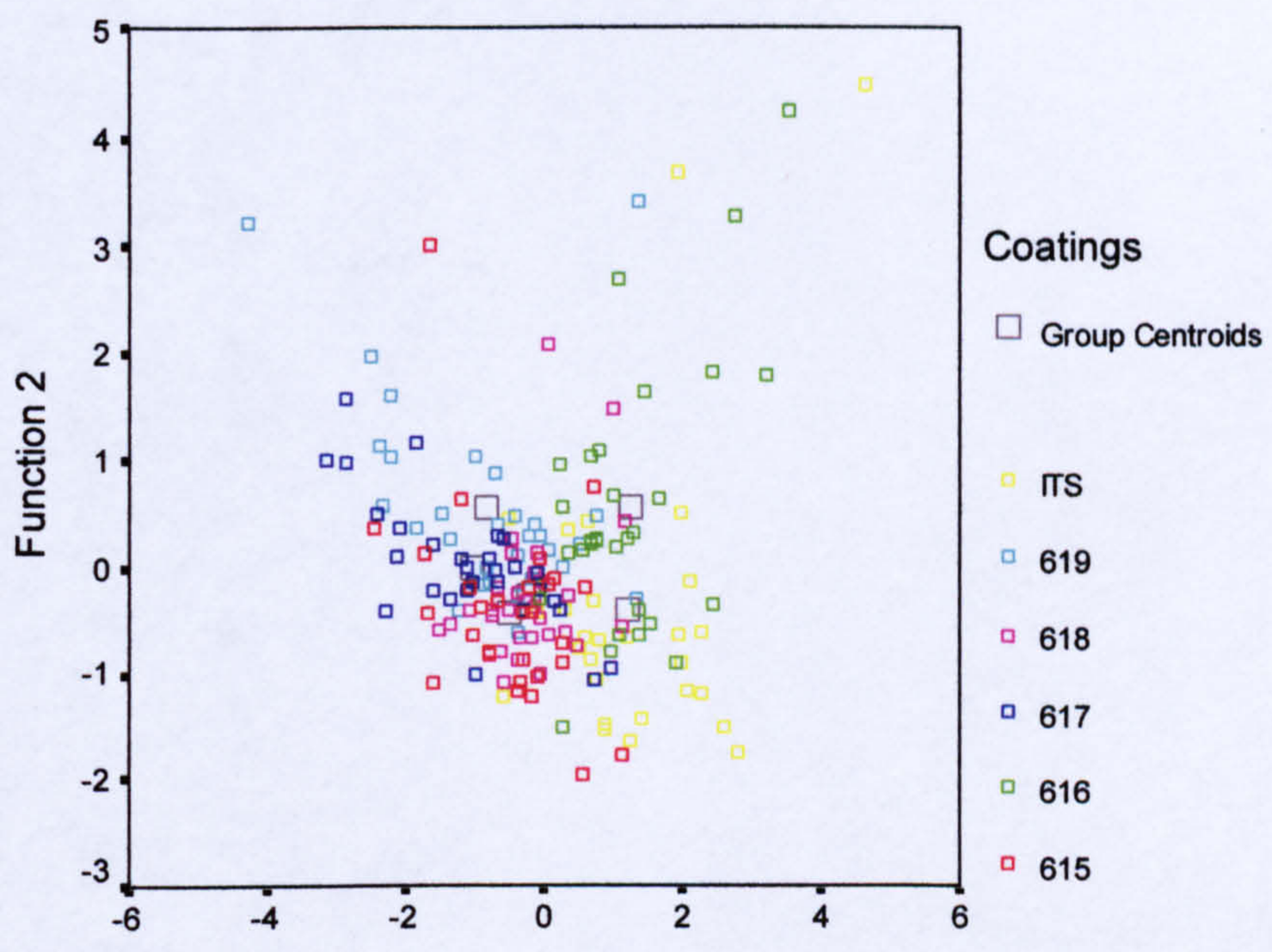


Figure 7.7 Canonical discriminant function scatter plot for all coatings.

CAN THE EXPLORATORY BEHAVIOUR OF *B. AMPHITRITE* BE USED TO PREDICT FOULING IN THE FIELD?

PCA axis 1 scores generated by the field data and the total fouling data were used in stepwise multiple regression (Chapter 5) to see which parameters were significantly related to the field data for each site, and thus could be used as predictors of fouling burden.

UK

Multiple regression with total percent fouling values.

No behavioural parameters could be used to predict percent fouling found at the UK site.

Multiple regression with PCA scores

Turn angle could also be used to predict community fouling as described by the PCA found at the UK site. ($R^2_{adj} = 0.017$, $F = 4.161$, $p = 0.043$) Community fouling was related to turn angle using linear regression:

$$PCA = 17.95 - (3.45 \times 10^{-4})\text{turn angle.}$$

The PCA scores increases with antifouling performance at this site (Figure 2.12). These results therefore suggest that the turn angle of the larvae decreases on coatings with higher antifouling performances.

Singapore

Multiple regression with total percent fouling values.

Turn angle and moving time could both be used to predict total fouling burden found at the Singapore site. Total percent fouling at this site was related to these parameters ($R^2_{adj} = 0.094$, $F = 10.34$, $p < 0.001$) using linear regression:

$$\text{Total fouling} = 75.42 + (9.7 \times 10^{-4})\text{turn angle} - (4.08 \times 10^{-4})\text{moving time}$$

These results suggest that the turn angle of the larvae increases and movement decreases on coatings of lower antifouling performance.

Multiple regression with PCA scores

No behavioural parameters of *B. amphitrite* could be used to predict community fouling at the Singapore site.

Sweden

Multiple regression with total percent fouling values.

Turn angle could be used to predict total fouling burden found at the Swedish site. Total percent fouling at this site was related to turn angle ($R^2_{adj} = 0.07$, $F = 14.43$, $p < 0.001$) using linear regression:

$$\text{Total fouling} = 87.89 + (5.36 \times 10^{-4})\text{turn angle.}$$

These results suggest that the turn angle of the larvae increases on coatings of lower antifouling performance.

Multiple regression with PCA scores

Turn angle could also be used to predict community fouling as described by the PCA found at the Swedish site. ($R^2_{adj} = 0.037$, $F = 6.882$, $p = 0.009$) Community fouling was related to turn angle using linear regression:

$$PCA = 41.72 + (9.25 \times 10^{-4})\text{turn angle.}$$

The PCA scores decrease with antifouling performance at this site (Figure 2.12). These results therefore suggest that the turn angle of the larvae decrease on coatings with higher antifouling performances.

Discussion

Staining investigation

Staining the cyprids increased the contrast with the background and allowed the filming of two replications at once. However, the results of the investigation showed that staining with neutral red significantly affected moving time but the differences were not enough to detect an overall difference in behaviour when analysed using MANOVA. Nile blue staining, on the other hand, had an effect on overall behaviour. Total distance travelled and moving time were the key component of the changes in behaviour due to nile blue. Staining techniques have been used in such fields as population biology (Loosanoff and Davies 1947, Howard 1985, Marini and Ferrari 1998) and behavioural studies (Kelly *et al.* 1998, Dr A Clare, pers. comm.); it is therefore surprising that documentation has not been found on investigations like the one carried out in this Chapter.

Studies of populations using mark and recapture techniques, would be mostly affected by the moving time and total distance travelled, indicating that such studies could be

severely impaired by staining with nile blue at the concentration used. Overall neutral red did not significantly affect the overall behaviour of *B. amphitrite,* and may have a potential as a staining technique to allow investigations of different colour coatings and/or increase number of cyprids filmed at one time in further studies. However, less time was spent moving by the stained individuals, it is therefore recommended that this stain also be used with care. This study here has shown that the staining techniques may have a significant impact on the behaviour of the target species.

Can behaviour of B. amphitrite be used to distinguish between coatings?

B. amphitrite behaves differently on the tested array of coating types, for all the measured behavioural parameters. These behaviours could be used to distinguish between the coatings with an overall 45% success rate.

The pairwise comparisons showed that coating ITS was consistently different from coatings 619, 618 and 617. On ITS *B. amphitrite* spends less time moving, but when moving, takes shorter steps, moves slower, but with a higher degree of turning. This behaviour follows the behaviour described for close exploration and inspection phases of an exploring cyprid (Knight-Jones and Crisp 1953, Crisp 1961, 1974). The frequent turns and short steps of close exploration is followed by the inspection phase, prior to metamorphosis, where the cyprid rotates and moves to and fro it own body length. The results therefore suggest that ITS was preferred over coatings 619, 618 and 617. These results are surprising as coating ITS is a commercially available antifouling coating and *B. amphitrite* is one of the most economically important fouling organisms. However, ITS is a foul release coating, on which antifouling properties are in part linked to the

ease of 'releasing' or removal of fouling organisms from the surface. Consequently, this coating may not be explicitly formulated to deter settlement to begin with, as these coatings have shown to foul in the field (Swain and Schultz 1996, Swain *et al.* 2000). Surface energy is also known to affect barnacle settlement (Rittschof and Costlow 1989a, Roberts *et al.* 1991, Gerhart *et al.* 1992, Becker 1993, Holm *et al.* 1997) and *B. amphitrite* has been shown to have a preference for high surface energy (Schmidt *et al.* 1987). Coating ITS, as with most foul release coatings, rely partly on their low surface energies for their antifouling properties (Candries *et al.* 2000). The results shown here therefore suggest that this preference is being overridden by other influences, or not displayed, on coatings ITS.

Surface rugosity also affects settlement of barnacles (see Chapter 1), and *B. amphitrite* has shown a preference for grooves and crevices (Walters 1992). The coatings studied were investigated for rougosity and differences (largest difference 6.48μm) between coatings could be seen (Table 2.2). However, this surface rugosity was measured at a micron scale (μm) and although cyprids have shown to be able to discriminate between textured surfaces with a step size of 35μm (Le Tourneux and Bourget 1988) *Semibalanus balanoides*, has been shown to preferentially settle on surfaces with a scale of roughness of less than 0.5mm (Hills *et al.* 1999). Any difference of surface rugosity seen in Table 2.2 may not therefore influence settlement choice for the target species.

These results show that the cyprids showed less time exploring coating ITS. The cyprids tended to spend a lot of time lying on their lateral side and moving only their appendages, just as if they were 'stuck' to the surface. This phenomenon, although not documented, has been observed by other researchers on other silicone coatings (Dr A

Clare, Dr K Berntsson, pers. comm.). As little is known regarding the surface chemistry of coating ITS in comparison with the other coatings little explanation of this can be offered. Electrostatic forces may be a cause, as settling cyprids can have low surface energies (Rittschof *et al.* 1998) the low surface energy of coating ITS (This study, Candries *et al.* 2000), may cause the cyprid to be easily trapped in the surface film (Rittschof and Holm 1997). This phenomenon, whatever the cause, may have largely influenced the results and resulted in misinterpretations, as the cyprids could not behave in a natural manner if moving time is restricted by some sort of surface attractant.

The results may also have been misinterpreted due to the magnification of the tracking device, especially on Coatings ITS. The cyprid is seen as a collection of approximately 25 pixels and its xy coordinate was registered as the centre point. During the inspection phase of exploration the cyprid moves to and fro within its own body length (Crisp 1961, Knight-Jones and Crisp 1953b), a behaviour which may have been missed at the magnification used.

Can the exploratory behaviour of B. amphitrite be used to prediction field data?

The behaviour of *B. amphitrite* seen in the laboratory on the six different coatings could be used to predict some of the fouling burdens seen in the field, however variation of the fouling explained by the behavioural parameters (R^2_{adj}) was quite low for all sites, this is discussed in the previous chapter (Chapter 6).

Turn angle was shown to be the key behavioural parameter as it was consistently identified as having a significant relationship with the field data. The relationship

between the behavioural data and fouling suggested that turn angle of the moving cyprids increased on coatings with lower antifouling performance. Turning has been shown to increase during exploration of a potential settlement site by cyprids (Knight-Jones and Crisp 1953, Crisp 1961) consequently this relationship is therefore logical.

The community fouling burden as described by the PCA, at the Singapore site and percent fouling seen in the UK, however, showed no relationship with the behavioural data. It is well documented that fouling assemblages are dynamic undergoing a succession of changing species during development (Allen 1950, Allen and Wood 1950, Turner 1993, Minchinton and Scheibling 1993, Underwood and Anderson 1994, Nandakumar 1996). The fouling communities seen on the immersion panels after approximately 5 months, are a reflection not just of initial settlement, but post-settlement mortality (Gaines and Roughgarden 1985, Walters 1992b), disturbances (Ayling 1981, Paine 1981, Connell and Keough 1985) and inter and intraspecific competition (Russ 1982, Nandakumar and Kikuchi 1993, Underwood and Anderson 1994). The behavioural data gained by the bioassay reflects only initial settlement so if there is further settlement or if the post settlement processes are "strong" any initial settlement patterns reflected in this behaviour will be masked.

Suitability of using B. amphitrite as a test species.

B. amphitrite seemed an ideal specimen due to is economic importance as a fouling species. It has been extensively studied and therefore a lot is documented regarding settlement cues (Maki *et al.* 1988, Rittschof *et al.* 1984, Rittschof and Costlow 1989a, 1989b, Mullineaux and Butman 1991, Holmström *et al.* 1992, Kon-Ya and Miki 1994, Wieczorek *et al.* 1995, O'Connor and Richardson 1998). However, it could only be

included in this work as a supply of cyprids was provided by Dr A. Clare, Newcastle University. The laborious culturing technique requires the work of a full time technician. By culturing stock, cyprids can be made available all year round, however the cultures can be very sensitive and the algae cultures, require for feeding, are prone to crashes (Mrs Sheelagh Henry, pers. comm.). The decreased discrimination of cyprids with age (Chapter 1) does not allow for field collection as it is very hard to age cyprids. If a suitable source could be located or funding available to set up culturing procedures of this species, the importance of it as a fouling organism does warrant the use of this species.

Summary

Staining with nile blue was found to significantly affect behaviour, neutral red overall did not affect behaviour, and may have a potential as a staining technique to allow investigations of different colour coatings and/or increase number of cyprids filmed at one time during further work with this species. The cyprids showed significantly different behaviours on all the coatings tested, and these behaviours allowed a degree of successful prediction of the coatings. At all sites studied, the behavioural parameters could be used to predict some of the fouling seen in the field, with turn angle as the key variable.

CHAPTER 8

BALANUS IMPROVISUS

CHAPTER 8

A BEHAVIOURAL BIOASSAY USING *BALANUS IMPROVISUS*

Introduction

Balanus improvisus (Darwin) is a balanomorph barnacle which can tolerate a wide range of salinities. It is widespread, found throughout the Atlantic coasts and extending to the Indo Pacific and Australasia, where it was believed to be introduced by shipping (Rainbow 1984). It is the only barnacle species found in the Baltic (Rainbow 1984, Hayward *et al.* 1996), and is considered a serious fouling macroorganism in Scandinavian waters (Dahlström *et al.* 2000, Berntsson *et al.* 2000a). It has recently attracted attention from antifouling perspective (Berntsson *et al.* 2000a). In Britain settlement of *B. improvisus* cyprids occurs from May to late September (Rainbow 1984).

As with most other barnacles species both physical and chemical settlement cues (Chapter 1) are known to affect settlement. *B. improvisus* is gregarious (Dineen and Hines 1992) responding to previously settled adults. It has an inhibitory or facilitative response to an established biofilm depending on the substratum (O'Connor & Richardson 1996). In contrast to *B. amphitrite,* a preference for hydrophobic polystyrene over glass has also been reported (O'Connor & Richardson 1994). Although it has been shown that some cyprid species, have a preference for settling in cracks and pits (Crisp 1975, Crisp 1981, Wethey 1986, Le Tourneux and Bourget 1988, Hills and Thomason 1996), *B. improvisus* has been shown to be negatively affected by surface texture (Andersson *et al.* 1999, Berntsson *et al.* 2000a and b, Dahlström *et al.*

2000, Petronis *et al.* 2000); profile heights of 30-45μm reduced settlement in the field by 92% (Berntsson *et al.* 2000b). This finding however has not been observed in the field by all other authors; Köhler *et al.* (1999), report increase abundance of *B. improvisus* in the field on rugosities of 0.5mm and 1mm compared to smooth, 0.1mm and 5mm on artificial substratum and Hills *et al.* (1999b) also report reduced settlement densities in the field on smooth artificially manufactured surfaces as opposed to the fine (<0.5mm) medium (0.5-2mm) and coarse (2-4mm) surfaces. These contradictory findings maybe related to the scale of rugosity at which the studies were based on, as different settlement cues are important at different scales (Le Tourneux and Bourget 1988, Hills and Thomason 1996). It has been suggested that on the micronscale (μm) the microtexture of the surface may interfere sterically with the cyprids behaviour while crawling along on antennules (Andersson *et al.* 1999) as cyprids have been observed spending longer exploring profiled surfaces than the smooth ones, and such profiles seemed to trigger the cyprids to swim off (Berntsson *et al.* 2000b).

Exploratory behaviour of *B. improvisus* has been assumed to follow the same three phases (Berntsson *et al.* 2000b), broad exploration, close exploration and inspection (Crisp 1974) as described, for other barnacles details of which are given in Chapter 7, Introduction.

The aim of this investigation was firstly, to determine the suitability of *B. improvisus* as a test organism. Secondly, to assess whether the behaviour of *B. improvisus* was different on different coatings and if this behaviour could be used to distinguish between the coatings used in the bioassay, and finally to determine whether the exploratory behaviour of *B. improvisus* could be used to predict the fouling of these coatings, as seen in the field immersion trials.

Details of the reasons for choosing *B. improvisus* are given in Chapter 2. Prior to the investigation of coatings, a sample size experiment was run to establish an appropriate sample size that minimised individual variability. This work was carried out during a visit to Tjärnö Marine Biological Laboratory, West coast of Sweden.

Preliminary work - Sample Size Experiment.

Materials and Methods

COLLECTION AND STORAGE OF LARVAE

The cyprids were reared from an adult brood stock as per Dahlström *et al.* (2000); In brief, nauplii released from the brood stock were collected by sieves, fed on *Thaassiosira pseudonana* and *Isochrysis galbana* and kept for 6-7 days at 27±1°C to develop into cyprids. Moulted cyprids were then collected, sieved, and washed to remove algae and detritus before use. Day 1 to 5 cyprids (inclusive) were used for the experiment, these were stored at room temperature prior to use.

SAMPLE SIZE EXPERIMENT

Method 2 (Chapter 2) was used to determine optimal sample size. In order to promote exploration a settlement extract was used; two drops of extract made from adult *B. improvisus* as per Dineen and Hines (1992). The extract was stored at -86°C prior to use, and was painted, using a craft hobby paint brush, onto watch glasses (arenas) with no coatings. The watch glasses were left to dry at room temperature overnight prior to use. Eighty replications were carried out. The running means ± 95% confidence intervals, for meander, turn angle, distance and turn rate were used to visually assess the optimal sample size.

Results

Figure 8.1 shows the running mean of the four parameters calculated from the xy co-ordinates of the *B. improvisus* tracks. A sample size of 50 would lie with in the 95% confidence intervals for each of the parameters. Unfortunately, as this work was carried out during the visit to Tjärnö, and time did not allow for such a replication of coatings, therefore only 40 replicates were used for this species during the bioassay. It can be seen however, that a sample size 40 is within the 95% confidence interval of all the parameters apart from meander, where it lies only slightly outside.

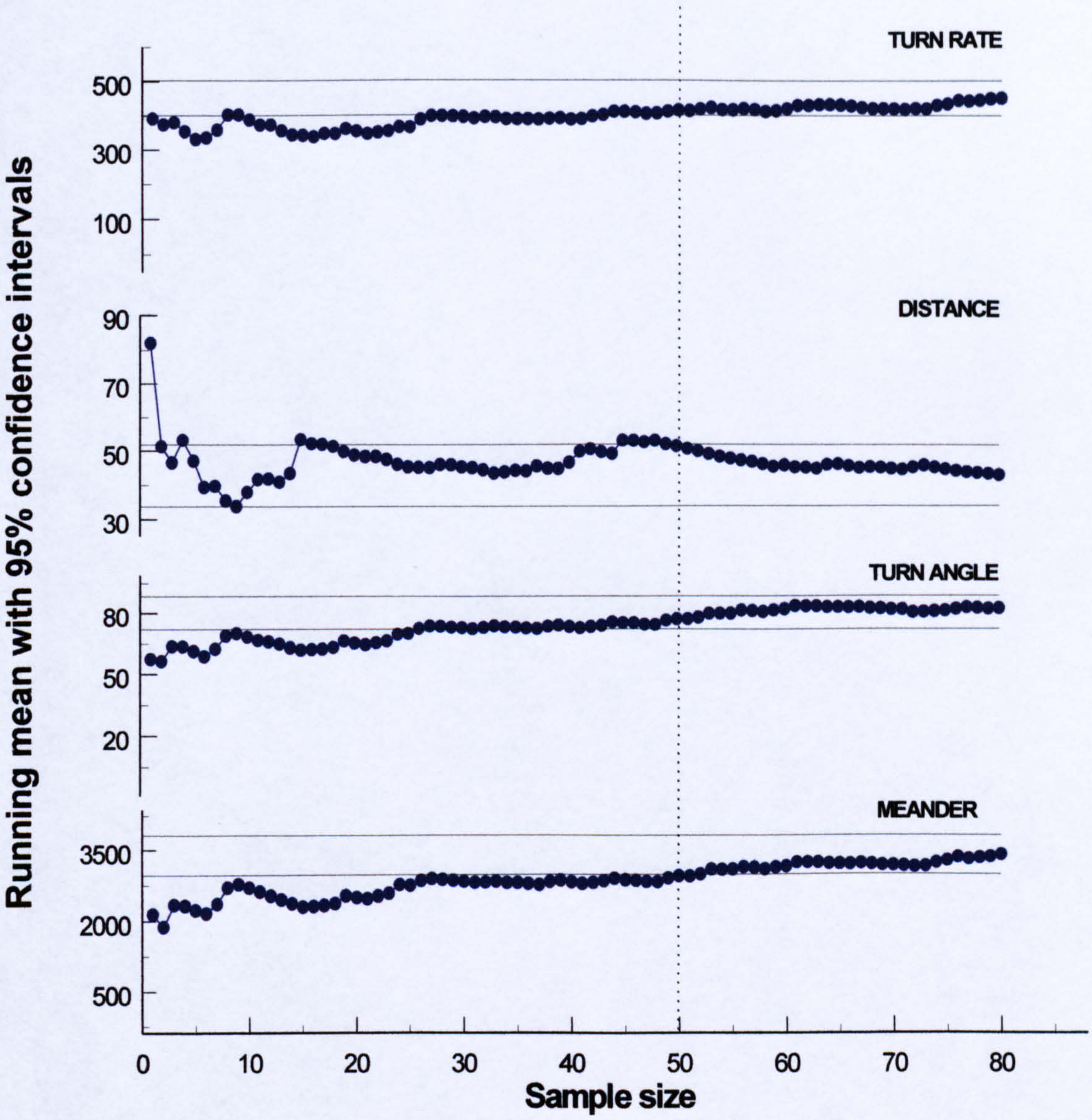


Figure 8.1 Running means of meander, turn angle, distance and turn rate with the 95% confidence interval for a sample size of 80.

Materials and Methods

Collection and storage of larvae

Cypids were obtained from Tjärnö Marine Biological Laboratory, Sweden and cultured as stated above. Day 1-5 cyprids (inclusive) were used for the bioassay and stored at room temperature prior to use.

Investigation of the behaviour of B. improvisus *on different coatings*

The method was carried out as per Method 2 (Chapter 2). The coatings shown in bold in Table 2.2 were tested, apart from coating 615 as this coating was not available prior to the visit. The bioassay was carried out 40 times for each coating. Analysis was carried out as stated in Method 2, Chapter 2.

Results

Investigation of coatings

EXPLORATORY BEHAVIOUR OF *B. IMPROVISUS* ON FIVE DIFFERENT COATINGS

The mean values for the parameters for all five coatings are presented in Figures 8.2 and 8.3. *B. improvisus* travelled further on 617 and 619 than on all other coatings (Figure 8.2). However, similar values are shown for mean distance, velocity and moving time of the cyprids on all coatings (Figure 8.2). The degree of turning of *B. improvisus* on coating 618 is shown to be much larger in comparison to other coatings (Figure 8.3).

Representative tracks made by *B. improvisus* on each of the five coatings can be seen in Figure 8.4. The tracks suggest that less distance was explored on coating 618 and the

most on coatings ITS. All coatings, except 618, show that the cyprids spent some of the time moving in a tight circular motion; this behaviour suggests that the coatings are in some way attractive to the cyprids.

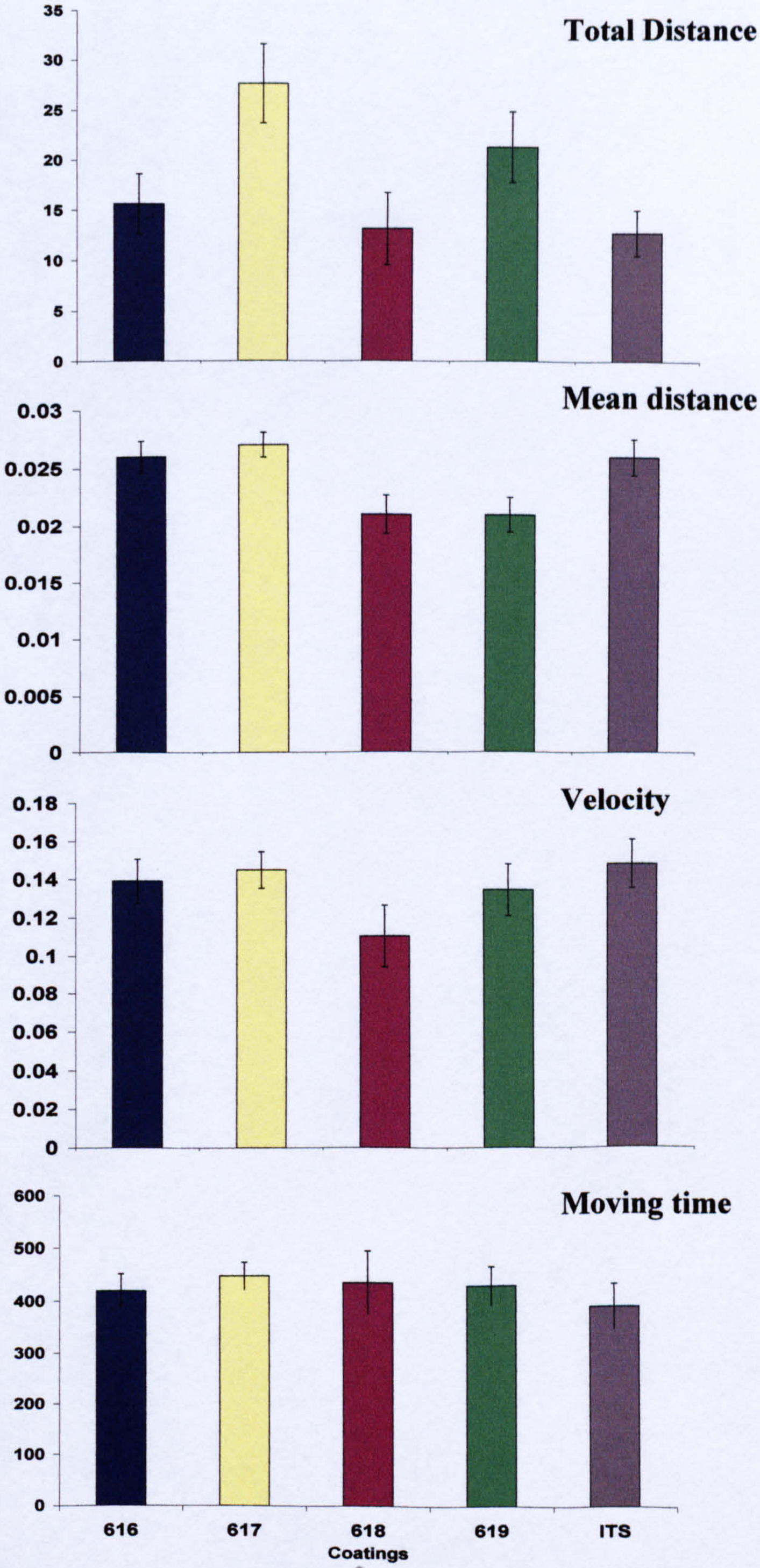


Figure 8.2 Mean values (± s.e) of moving time (sec), velocity (sec/mm), mean distance (cm) and total distance (cm) for *B. improvisus*, on each coating tested (n = 40).

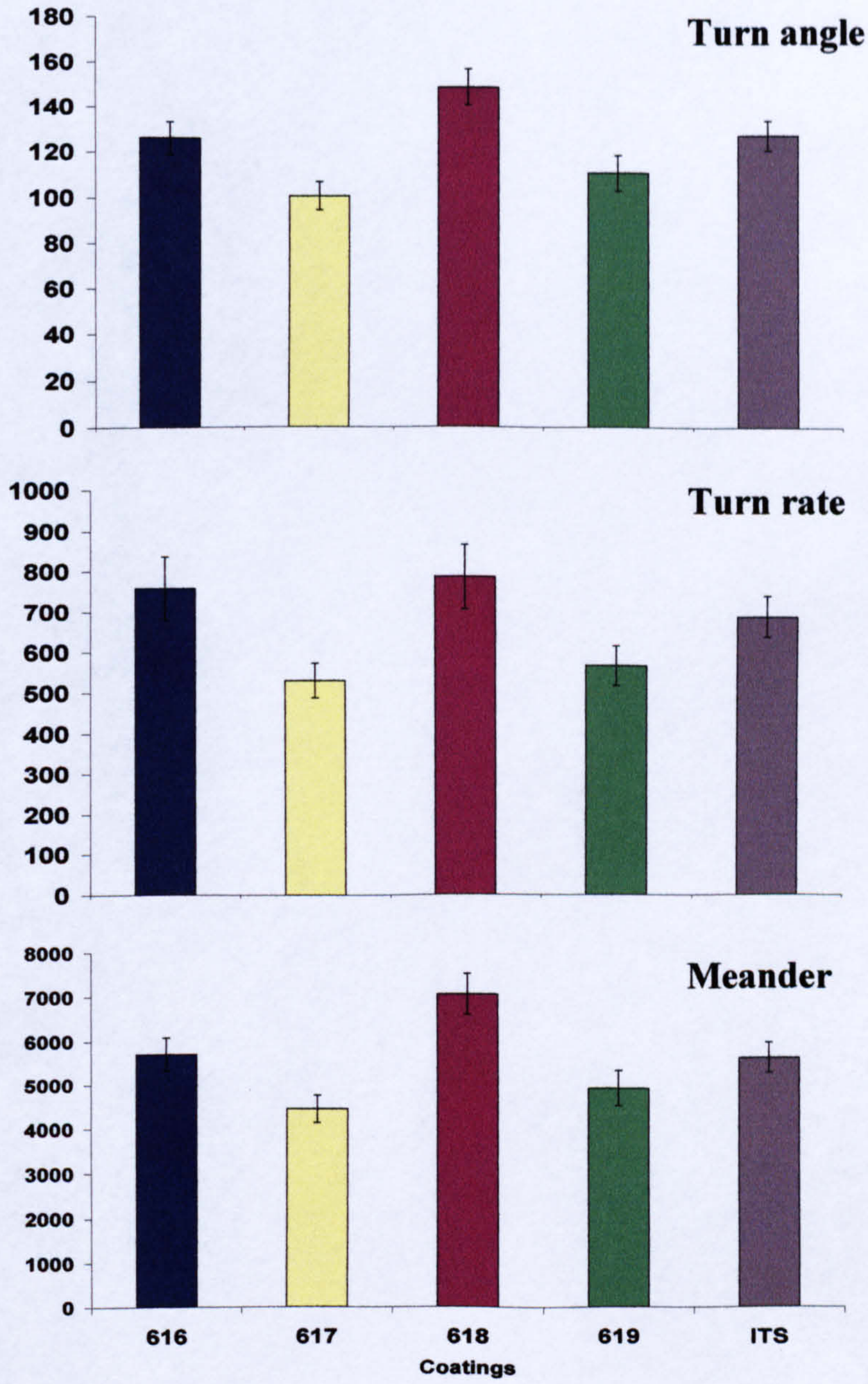


Figure 8.3 Mean values (± s.e) of turn angle (degrees), turn rate (degrees/sec) and meander (degrees/cm) for *B. improvisus*, on each coating tested (n = 40).

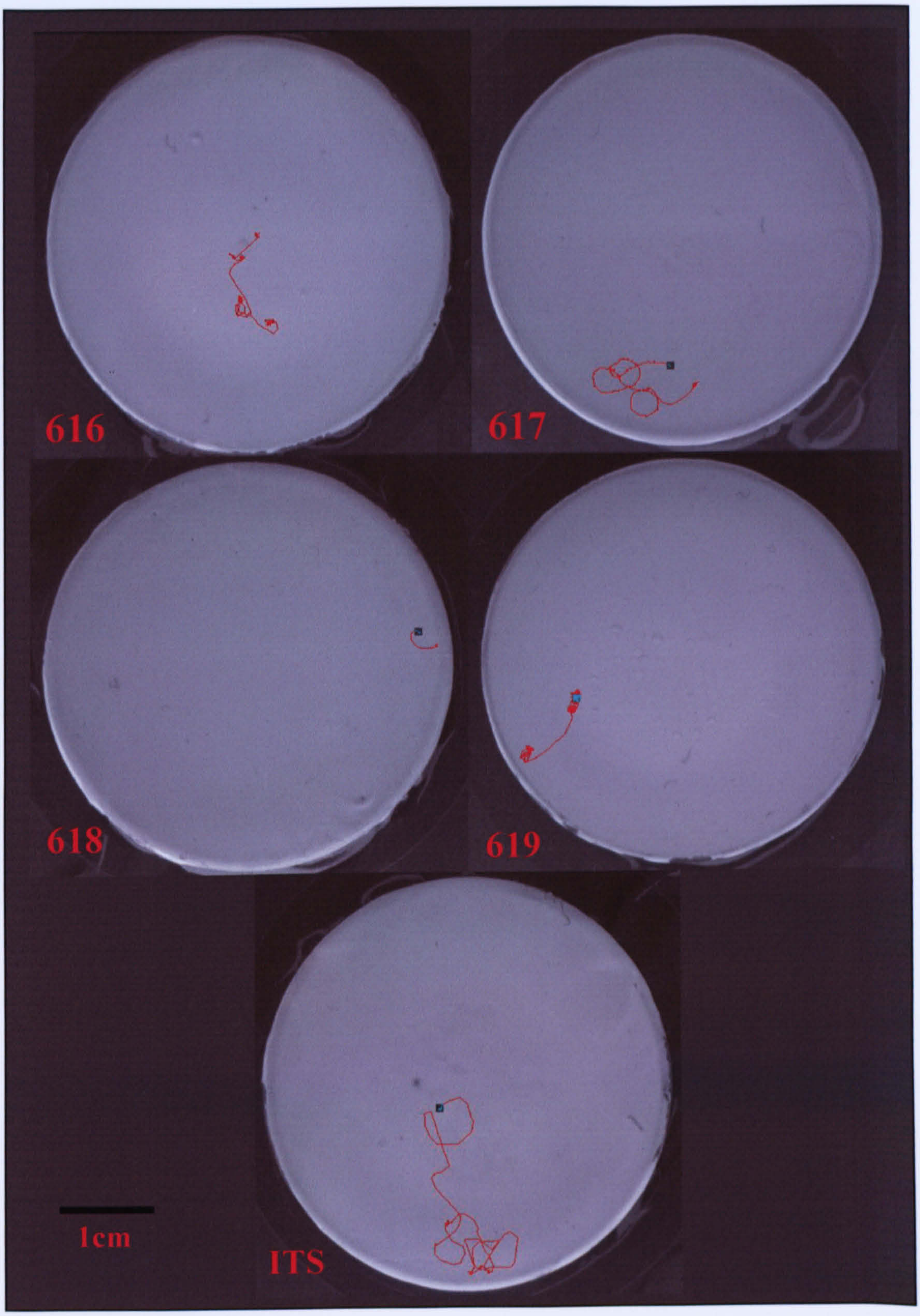


Figure 8.4 Representative tracks made by *B. improvisus* on all five coatings, showing the arena used in the trials, as the background image. The blue square represents starting position of the cyprid.

DO COATINGS SHOW SIGNIFICANTLY DIFFERENT BEHAVIOUR OF *B. IMPROVISUS?*

A GLM MANOVA was carried out to establish if there were differences between the behavioural parameters due to the coating type (Chapter 2). The behaviour of *B. improvisus* overall showed significant differences on all the coatings (MANOVA Wilk's $\lambda = 0.682$, $p < 0.001$). When investigating the behavioural parameters using univariate analysis, significant differences were found for total distance (ANOVA $F = 6.26$, $p<0.001$), turn angle (ANOVA $F = 6.29$, $p < 0.001$), turn rate (ANOVA $F = 2.72$, $p = 0.031$) and meander (ANOVA $F = 5.87$, $p < 0.001$).

Tukey 95.0% Simultaneous Confidence Intervals were carried out to make pairwise comparisons of the coatings for all parameters that were significantly different (Table 8.1). All coatings showed a difference from another coating for total distance travelled suggesting that exploration of the surfaces was not the same on all coatings. Coating 618 showed significant differences from coating 617 and 619, indicating that behaviour of *B. improvisus* was different on 618 than the other 2 coatings Turn rate did not show any significant differences using this method of analysis, which maybe a result of a type 1 error produced by ANOVA or a type 2 error from the Tukey test.

Total distance	617	618	619	ITS
616	0.017	0.621	0.387	0.998
617		0.0001	0.688	0.012
618			0.016	0.724
619				0.310
Turn Angle				
616	0.090	0.198	0.528	0.998
617		0.0001	0.881	0.068
618			0.003	0.261
619				0.450
Turn rate				
616	0.146	0.999	0.315	0.999
617		0.092	0.996	0.266
618			0.214	0.984
619				0.488
Meander				
616	0.141	0.181	0.552	1.000
617		0.0001	0.936	0.177
618			0.003	0.155
619				0.616

Table 8.1 The results of Tukey 95.0% Simultaneous Confidence Intervals showing a pairwise comparison for all parameters on all coatings. Exact p values are given. Significant values are indicated by the shaded box.

HEADING ANGLE ANALYSIS

The heading angle was investigated using Oriana v1.06, to determine the preferred direction of movement of *B. improvisus* during all the bioassays (Figure 8.5). These data were evenly distributed (Rayleigh uniformity test, $p > 0.05$), indicating that there were no external directional factors, such as light, influencing the movement of *B. improvisus* during the bioassays.

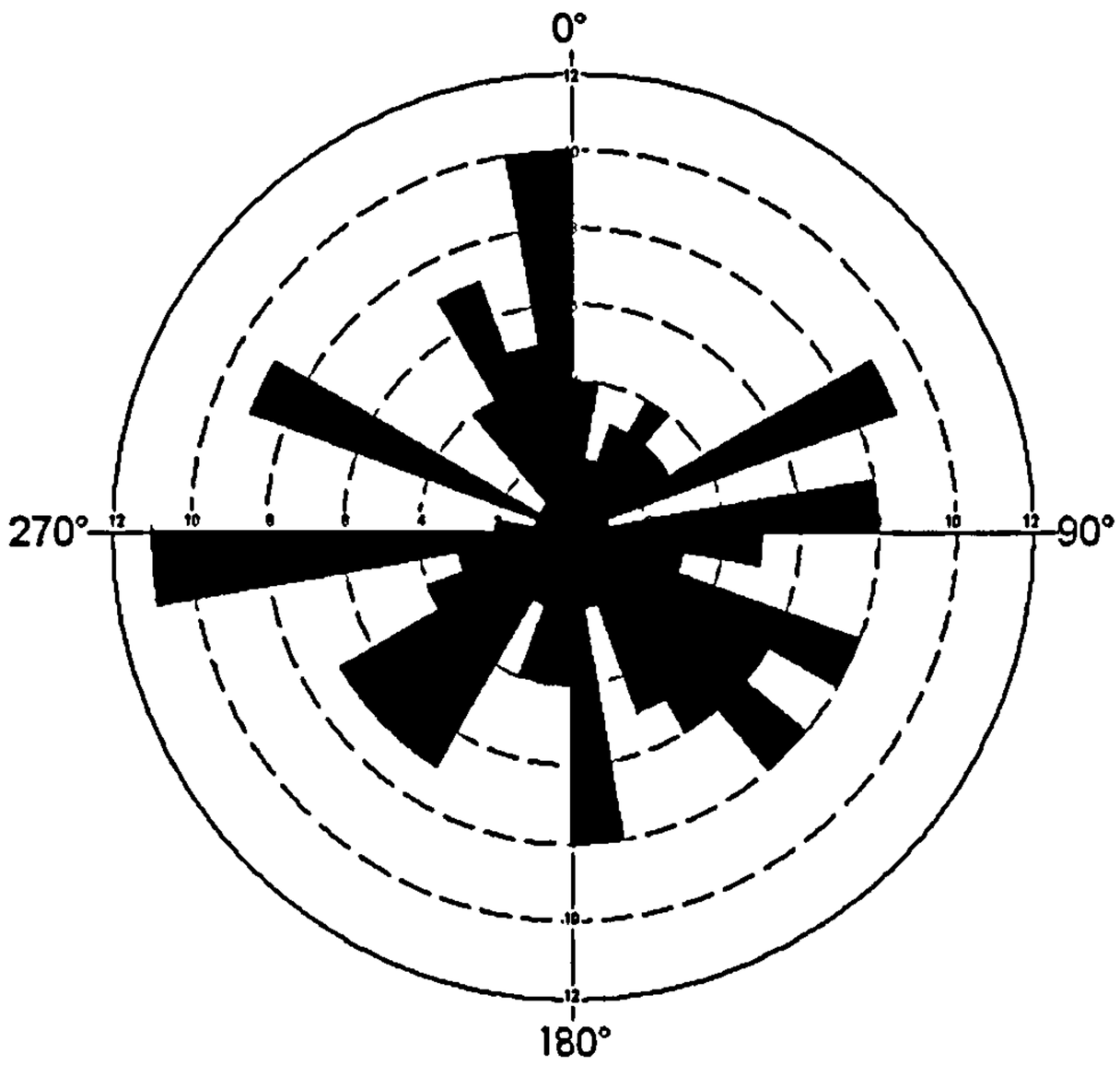


Figure 8.5 Circular histogram of the heading angle of *B. improvisus*. Data are combined for all bioassays on all coatings (n = 200).

CAN THE BEHAVIOUR OF *B. IMPROVISUS* BE USED TO DISTINGUISH BETWEEN COATINGS?

To determine whether behavioural parameters could be used to distinguish between the coatings, canonical discriminant analysis (CDA) was carried out. CDA showed that 87.3% of the variation was described by functions 1 and 2. The structural matrix of the CDA showed that total distance (r = -0.549), turn angle (r = 0.542) and meander (r = 0.513) dominate function 1 with other parameters having a lesser influence (absolute value of r <0.338). Function 2 was dominated by mean distance (r = 0.484) and velocity (r = 0.576) all other behavioural parameters had a smaller influence (absolute value of r < 0.271). When plotting the functions produced by this analysis (Figure 8.6), no distinct grouping can be identified; suggesting behaviour is similar on each coating. The predicted group membership of the coatings (Table 8.2) shows that coatings can be correctly predicted ~41% of the time. Coating 616 was highly misclassified being

predicted correctly only 5.3% of the time, in this group all other coatings were predicted as coating 616 more times than 616. This suggests that some behaviour parameters on this coating were very similar to all other coatings. Coatings 617 and 618 can be predicted correctly over 60% of the time, suggesting that the behaviour of *B. improvisus* on these coatings is different than on the other coatings. Behaviour on coating 619 is shown to be similar to coatings 618 and 617 as these are misclassified in this group as frequently as 619 is predicted correctly, thus behaviour of cyprids on these particular coatings was also similar.

		Predicted group membership				
		616	**617**	**618**	**619**	**ITS**
Original grouping	**616**	**5.3**	23.7	31.6	15.8	23.7
	617	2.6	**66.7**	7.7	20.5	2.6
	618	5.7	5.7	**60.0**	2.9	25.7
	619	5.4	32.4	21.6	**27.0**	13.5
	ITS	10.8	8.1	24.3	10.8	**45.9**
40.9% of original grouped cases correctly classified.						

Table 8.2 The percentage predicted group membership of each replication as given by CDA of all six coatings. Figures shown in **bold** indicate percentage correct for each coating.

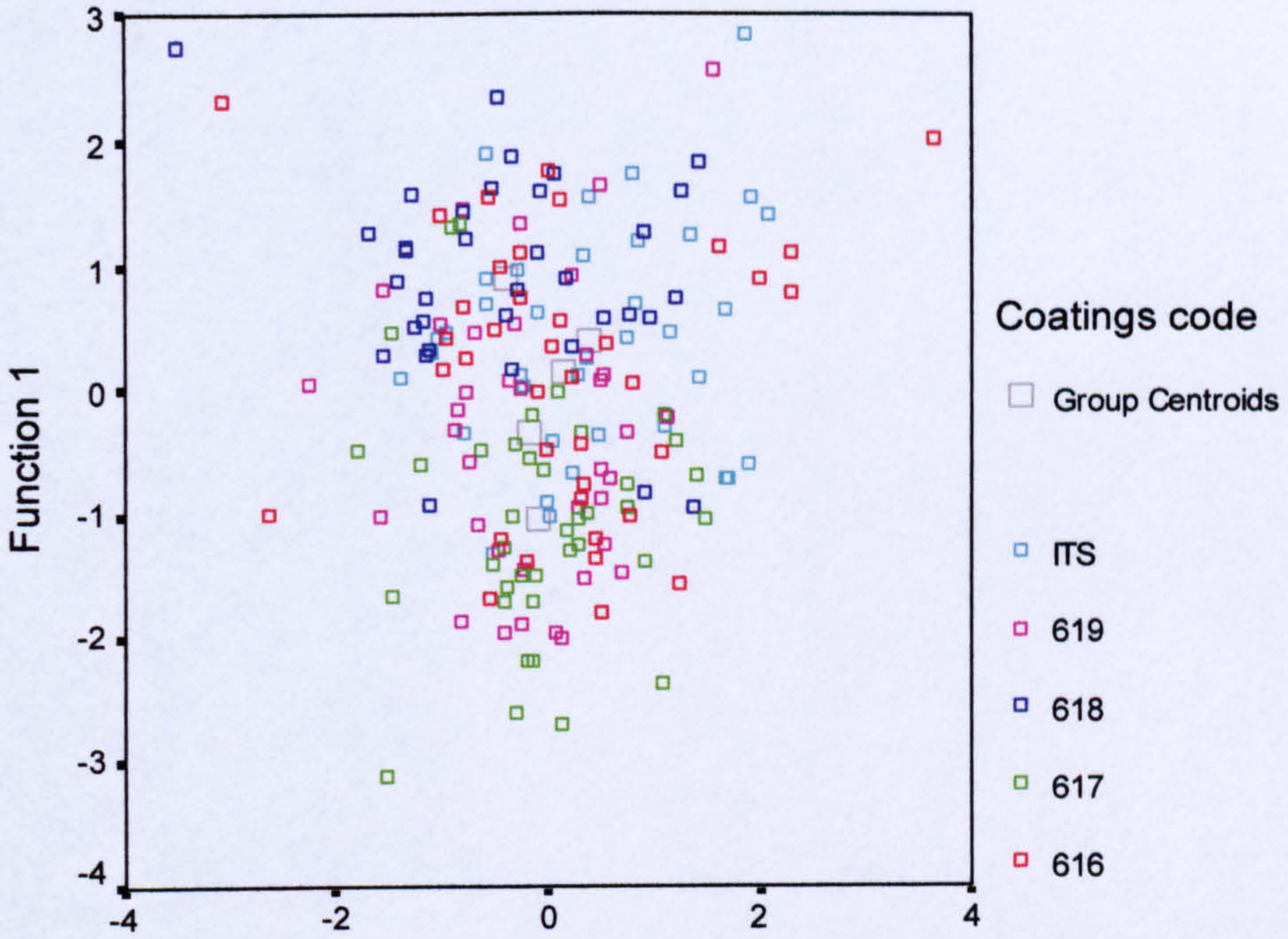


Figure 8.6 Canonical discriminant function scatter plot for all coatings.

CAN THE EXPLORATORY BEHAVIOUR OF *B. IMPROVISUS* BE USED TO PREDICT FOULING IN THE FIELD?

PCA axis 1 scores generated by the field data and the total fouling data were used in stepwise multiple regression (Chapter 2), to see which parameters were significantly related to the field data for each site, and thus could be used as predictors of fouling burden.

No behavioural parameters were able to predict the total fouling found at the UK site ($p > 0.05$ for all parameters) and the Singapore site ($p>0.05$ for all parameters) using linear regression. Community fouling as described by the PCA scores at both these sites ($p > 0.05$ for all parameters for both sites) also showed no relationship to the behavioural parameters. Community fouling at the Swedish site also could not be related to the behavioural parameters ($p > 0.05$ for all parameters). However both total distance and turn angle could be used as predictors of the total fouling burden in Sweden. These parameters were related to fouling using linear regression ($R^2_{adj} = 0.079$, $F = 9.562$, $p < 0.0001$):

$$\text{Total fouling} = 93.434 - 2.035\text{total distance} + 0.031\text{turn angle}.$$

The results therefore showed that total distance of the larvae decreases and turn angle increases on coatings with lower antifouling performance of the coatings, i.e. increased fouling at this site.

Discussion

Can behaviour of B. improvisus be used to distinguish between coatings.

Overall *B. improvisus* behaved differently on different coatings. Total distance travelled was the key parameter displaying the most differences between coatings. Cyprids on coating 618 displayed more twisting and changing of direction than on coatings 617 and 619 however less distance was covered. This behaviour follows the behaviour described for close exploration and inspection phases of an exploring cyprid (Crisp 1974). The frequent turns and short steps of close exploration is followed by the inspection phase, prior to metamorphosis, where the cyprid rotates and moves to and fro in its own body length (Knight-Jones and Crisp 1953, Crisp 1961). Therefore these findings suggest that coating 618 is more attractive to the cyprids than coatings 617 and 619.

As surface rugosity affects settlement of barnacles (Chapter 1), the coatings investigated were investigated for this (Table 2.2). *B. improvisus* is shown to have a preference for smooth surfaces (Andersson *et al.* 1999, Berntsson *et al.* 2000a and b, Petronis *et al.* 2000) it is therefore surprising that coatings 618 was shown to have the roughest surface (Table 2.2). Although the differences in roughness shown in Table 2.2 are on a micron scale (μm) and are slight (largest difference 6.48 μm), an average roughness (R_a) of only 5-10 µm has shown to significantly reduced recruitment and reduce exploratory behaviour of *B. improvisus* (Berntsson *et al.* 2000b). These results have therefore shown that any behavioural response of *B. improvisus* to surface texture was possibly being overridden or integrated with other factors.

Can the exploratory behaviour of B. improvisus be used to predict field data?

The *in vitro* behaviour of *B. improvisus* could be used to predict the total fouling burden recorded in Sweden. At this site it was shown that on coatings with lower antifouling performance the degree of turning increases, this follows as it suggests that cyprids are exploring the surfaces in such as way that indicates a preference to settle (Knight-Jones and Crisp 1953b, Crisp 1961, Crisp 1974). Total distance also increases as performance increases indicating small-scale investigations are lessened on such coatings.

B. improvisus is a common fouling organism in Swedish waters (Berntsson *et al.* 2000a), and consequently is a major component of the field-based settlement panels from Sweden. If, for *B. improvisus,* there is a relationship between settlement behaviour and subsequent fouling then this would show up only in the Swedish data, as the other field locations did not have this species present, in such numbers, in their fouling assemblages.

Furthermore the settlement season in high latitude water tends to be short and intense. Early-season heavy settlement by species such as *B. improvisus* on available space leads to exclusion of other conspecifics and other-species settlement (Minchinton & Scheibling 1993). Consequently, the subsequent community represents mainly a function of the characteristics of an early and intense settlement season (Connell 1985, Menge 2000). Although, other factors can modify the community, such as post settlement mortality (Gaines and Roughgarden 1985, Walters 1992b), there is evidence for an association between settlement characteristics and early season community, though this can be masked later in the season (Connell 1985). Consequently it is possible that, in the Swedish data set, the differences in heavy early settlement of *B.*

improvisus reflected in the behavioural indices were subsequently maintained as differences in recruit densities between panels. Thus, because of space-exclusion, differences in initial settlement densities remained largely unaltered by subsequent settlement over the length of the Swedish field trial data.

Other sites however did not show any relationship between the laboratory behaviour and fouling burden, suggested reasons for this are given in Chapter 7.

Suitability of using B. improvisus as a test species

The pairwise comparisons between the coatings did not indicate that the behaviour of cyprids on coating ITS was significantly different to other coatings. Therefore the phenomenon of cyprids sticking to the surface of some silicones as seen for *B. amphitrite,* (Chapter 7), was not evident for the results reported here, although it has been observed for *B. improvisus* on other silicone surfaces (Berntsson pers. comm.).

The sample size investigation revealed a sample of 50 is needed for a 95% confidence of the mean values. This is a lot more that is needed for *B. amphitrite* (Chapter 7). This maybe because *B. improvisus* does not store as well as *B. amphitrite* (Rittschof *et al.* 1984, Dahlström *et al.* 2000) and the individuals may be affected in different ways during the five day storage. This increased sample size however does mean that more work is needed for this species compared to the other species investigate in this research.

Misinterpretation of results due to magnification as discussed in Chapter 7 also may have influenced the results. Also the logistics of culturing (Chapter 7) also are factors to be considered before the use of this species.

Summary

The behaviour of *B. improvisus* was significantly different on coatings overall and could be used to distinguish between some of them. Only total fouling in Sweden could be predicted by the behavioural parameters; however it was shown that a larger sample size was needed for this investigation. Therefore increasing the sample size may lead to better predictive power of the behavioural parameters.

CHAPTER 9

MULTISPECIES ANALYSIS

CHAPTER 9

MULTISPECIES ASSAYS

Introduction

The aim of this chapter is to show that further development of the technique could lead to a commercially acceptable bioassay for the investigations of the efficiency of non-biocidal antifouling coatings.

Although individual species were shown to predict fouling found in the field, to make the bioassays more robust and generically applicable, further analysis was carried out. The analysis of settlement behaviour presented previously within the thesis has related single species to field fouling at the target locations. However, it maybe the case that a function of certain individual or small suites of behaviour of one species, and a function of behaviour of one or more species might be able to predict field fouling more robustly. Consequently, a multispecies assortment of behaviours might better predict field fouling than the behaviours of a single species. Regression analysis was therefore performed predicting the field fouling at the three sites from the behavioural parameters from all the tested species to allow a suite of the best predictors to be established. The behavioural parameter data were used as the independent variables and total percent fouling or PCA axis one scores were used as dependent variables. All analysis was carried out using SPSS v11.

In order to carry out the multiple linear regression coating 615 was excluded from the analysis as no data was available for *B. improvisus*. Ten samples from the *S. borealis* and *B. improvisus* data sets were also randomly taken out so that data were in the

necessary balanced format (n = 30 for all included coatings). The mean of remaining data, after the 10 samples were removed, was checked to ensure it lay within the 95% confidence intervals of the complete data set. The results of this regression are presented below.

UK

MULTIPLE REGRESSION WITH TOTAL PERCENT FOULING VALUES.

Six behavioural parameters displayed by both *S. borealis* and *B. amphitrite* could be used to predict the total percent fouling burden found at Newton Ferrers ($R^2_{adj} = 0.326$, $F = 13.023$, $p < 0.001$) (Table 9.1). No behavioural parameters from the *B. improvisus* data set could be used to predict the percent fouling found at this site (Table 9.1).

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	56.575		6.860	<0.001
Sb turn rate	2.403	.370	3.145	0.002
Ba turn angle	$9.925x10^{-3}$	1.844	5.414	<0.001
Ba Meander	$-2.916x10^{-4}$	-1.564	-4.573	<0.001
Sb turn angle	-9.312	-.679	-5.132	<0.001
Sb Meander	2.595	.483	2.838	0.005
Sb Movement	-.124	-.198	-2.313	0.022

Table 9.1 The coefficients calculated for all the parameters used in the regression model of community fouling found at the UK site. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown. *B. amphitrite* (Ba), *S. borealis* (Sb).

MULTIPLE REGRESSION WITH PCA SCORES (UK)

Some behavioral parameters can be used to predict the community fouling found at Newton Ferrers, UK ($R^2_{adj} = 0.178$, $F = 17.187$, $p<0.001$). The best predictors found by the stepwise method were turn angle and meander of *B. amphitrite*, the significant relationship being:

$$\text{Community fouling} = 6.833 - (2.8 \times 10^{-3})\text{Ba turn angle} + (9.19 \times 10^{-5})\text{Ba meander}$$

Singapore

MULTIPLE REGRESSION WITH TOTAL PERCENT FOULING VALUES.

Some behavioural parameters can be related to the total fouling data at the Singapore site by linear regression ($R^2_{adj} = 0.368$, $F = 29.93$, $p<0.001$). Meander, mean distance and turn angle of *B. amphitrite* were found to be the best predictors of the total fouling burden found at the Singapore site (Table 9.2).

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	82.550		19.496	<0.001
Ba turn angle	6.097×10^{-03}	2.228	6.797	<0.001
Ba Meander	-1.850×10^{-04}	-1.952	-5.908	<0.001
Ba Mean distance	-317.292	-.260	-3.387	0.001

Table 9.2 The coefficients calculated for all the parameters used in the regression model of total fouling found at the Singapore site. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown. *B. amphitrite* (Ba).

MULTIPLE REGRESSION WITH PCA SCORES (SINGAPORE)

When using all the data set, 5 parameters can be used to predict community fouling in Singapore (Table 9.3). Total percent fouling at this site was significantly related to behaviour shown mainly by *S. borealis* ($R^2_{adj} = 0.417$, $F = 22.290$, $p<0.001$).

	Unstandardized Coefficients	Standardized Coefficients		
Model	**Beta**	**B**	**t**	**Sig.**
(Constant)	28.167		7.878	<0.001
Sb Turn rate	.774	.281	3.867	<0.001
Sb Mean distance	-211.658	-.563	-6.744	<0.001
Sb Total distance	3.099	1.009	6.182	<0.001
Sb Movement	-.189	-.713	-4.845	<0.001
Bi Velocity	-12.273	-.182	-2.858	0.005

Table 9.3 The coefficients calculated for all the parameters used in the regression model of community fouling found at the Singapore site. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown. B. *improvisus* (Bi), *S. borealis* (Sb)

Sweden

MULTIPLE REGRESSION WITH TOTAL PERCENT FOULING VALUES.

When using the total percent fouling found at the Swedish site a significant relationship ($R^2_{adj} = 0.259$, $F = 14.046$, $p<0.001$) was found for behavioural parameters from all three species (Table 9.4).

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	85.752		35.938	<0.001
Ba turn angle	$2.617E^{-3}$	1.483	4.177	<0.001
Sb turn rate	0.473	.222	3.096	0.002
Ba Meander	$-6.951E^{-5}$	-1.138	-3.194	0.002
Bi Total distance	-1.574	-.210	-2.944	0.004

Table 9.4 The coefficients calculated for all the parameters used in the regression model of total fouling found at the Swedish site. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown. *B. amphitrite* (Ba), *S. borealis* (Sb), *B. improvisus* (Bi).

MULTIPLE REGRESSION WITH PCA SCORES (SWEDEN)

Some behavioral parameters could be used to predict the community fouling as described by the PCA score, found at Swedish site ($R^2_{adj} = 0.262$, $F = 27.44$, $p<0.001$). The best predictors found by the stepwise method were turn angle and meander of *B. amphitrite* (Ba), the significant relationship being:

$$PCA = 38.719 + (6.69 \times 10^{-3})\text{Ba turn angle} - (1.95 \times 10^{-5})\text{Ba meander}$$

Using multispecies data it can be seen that at all three sites using both fouling indices, PCA scores and total percent fouling, laboratory gained behavioural parameters could be used to predict the fouling observed in the field. These results are more successful than the single species data, where at some sites none of the parameters were able to predict field fouling.

Assessment of multispecies behaviour as field fouling predictors.

The aim of the multispecies analysis was to investigate whether an array of behaviours of different species would lead to a more robust and generically applicable bioassay. It can be seen from the results that the variation of fouling explained by the behaviours (R^2_{adj}) has been greatly increased by this method of analysis. It is therefore realised that a multispecies bioassay provides a more robust prediction of field fouling.

It is vital as a commercial bioassay that the behavioural technique can provide a rapid assessment of the likely field fouling burden at a range of climatic areas. This is because most of the non-biocidal coatings are destined for global use, commonly transgressing climatic areas as part of the sea passages of the world fleet. Assuming that the 3 field sites represent the major climatic areas of the world (tropical, Singapore; temperate, UK and high latitudes, Sweden), then a similarity in the behavioural indicators sèlected in the regression analysis would suggest that the behavioural bioassay had global applicability.

The results in the above section in were summarised in Table 9.5 to enable generic trends to be identified in these sites of results.

	Fouling					
Site	**% field fouling**			**Field fouling PCA scores**		
UK	• TurnR	◎ TurnA	◎ Meander	◎ TurnA	◎ Meander	-
Singapore	◎ TurnA	◎ Meander	◎ MeanD	• TurnR	• MeanD	• TotalD
Sweden	◎ TurnA	• TurnR	◎ Meander	◎ TurnA	◎ Meander	-

Table 9.5. Summary table of multispecies analysis of field % fouling and PCA scores of field fouling. The best three predictors selected from each regression analysis. (• = *S. borealis*, ◎ = *B. amphitrite,* TurnA – Turn angle, TurnR – Turn rate, MeanD – Mean distance, TotalD- Total distance).

A number of features can be identified from Table 9.5: Firstly, there are notable absences from the table of both species and behaviours, compared to what would be expected by random. *B. improvisus*, one of the three tested species, does not appear anywhere in the table, suggesting that either its settlement behaviour is different to the other species and not as related to field fouling as the other two tested species, or, its behaviour is similar to one or both of the other tested species and thus masked in the regression as it is not as good a predictor as other behaviours from other species.

The second notable absence, is of some of the tested behavioural parameters; velocity and moving time do not appear in the table at all, whereas total distance appears only once in the summary table as the third predictor for PCA scores at the Singapore field site. This suggests that these behavioural indices are less important, or unimportant, at predicting field fouling.

It can also be seen that Table 9.5 does show a number of features of interest to the development of a commercial bioassay technique. It has already been stated that two behavioural parameters do not feature in the table, however, within the behavioural parameters included in the table there is not a consistent pattern. It can be seen that certain variables have greater representation; in terms of frequency of inclusion in the table; the ranking sequence is Turn Angle (5 inclusions) = Meander (5) > Turn Rate (3) > Mean Distance (2) > Total Distance (1). These results suggest that both Meander and Turn angle are the key variables at predicting field fouling.

The removal of coating 615 in this multispecies analysis, changes the second and third behavioural predictors of percent fouling in Singapore (see Chapter 7) moving time in the single species analysis becomes an insignificant predictor and meander and mean distance can be use to predict the percent fouling found at this site. Meander is also included as a significant predictor for community fouling in Sweden when excluding coatings 615 (Chapter 7). This suggests that coating 615 is not a suitable control and without it more parameters can be used as field fouling predictors.

The main aim of this work was to assess larval settlement behaviour for use as a technique for testing non-biocidal coatings. The multispecies analyses and subsequent summary assessment (Table 9.5) presented in this section indicate that this aim has been achieved. The results show that it is possible to use larval behaviour as a bioassay; the information from this bioassay gives a significant indication of the potential of the non-biocidal coating in the field in the major climatic areas of the world. Evidence for this global applicability is a key issue for the commercial development of the bioassay due to the global market and climatic area transgression of the coated ships and structures.

Moreover, the analysis has started to identify possibilities for development of the test into a rapid and efficient screening bioassay. These initial results suggest that a bioassay protocol could concentrate on the use of only two species (*B. amphitrite* and *S. borealis*) and need only to assess a limited suite of behavioural indices.

If only one species had to be used to get the same predictive power, the bioassay would be less time consuming and more simplistic. Therefore in an attempt to achieve this, turn rate of *S. borealis* was excluded from the analyses of percent fouling in the UK and Sweden. The regression was re-run and a summary of the results for these sites are shown below (Tables 9.6 and 9.7).

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	77.005		10.636	<0.001
Ba turn angle	$9.54x10^{-3}$	1.821	4.852	<0.001
Ba Meander	$-3.02x10^{-4}$	-1.621	--4.323	<0.001
Sb Mean distance	-203.92	-.230	-3.030	0.003
$R^2_{adj} = 0.179$, F=11.42, p<0.001				

Table 9.6 The coefficients calculated for all the parameters used in the regression model of total fouling found at the UK site excluding turn rate of *S. borealis*. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown as well as the R^2_{adj} F and p of the final model. *B. amphitrite* (Ba), *S. borealis* (Sb).

Model	Unstandardized Coefficients Beta	Standardized Coefficients B	t	Sig.
(Constant)	94.219		37.372	<0.001
Ba turn angle	2.752×10^{-3}	1.560	4.319	<0.001
Ba Meander	-7.479×10^{-5}	-1.224	-3.390	0.001
Bi Total distance	-1.614	-.215	-2.970	0.003
Sb Mean distance	-45.195	-.155	-2.128	0.035
$R^2_{adj} = 0.244$, F=12.401, $p<0.001$				

Table 9.7 The coefficients calculated for all the parameters used in the regression model of total fouling found at the Sweden site excluding turn rate of *S. borealis*. Standardized coefficients (adding up to zero), the t value for the parameter and the significance for that t value are also shown as well as the R^2_{adj} F and p of the final model. *B. amphitrite* (Ba), *S. borealis* (Sb), *B. improvisus* (Bi)

Table 9.6 shows that *B. amphitrite* was selected as the best predictor in the UK, and this is identical to the data from Sweden and Singapore (Table 9.5). The loss of explained variance (R^2_{adj}) in field fouling in the UK was only 0.02 (i.e 2%) in the 3 component model (using all 3 species) than from the regression omitting *S. borealis* turn angle. In the Swedish case (Table 9.7), with *S. borealis* turn rate excluded, *B. amphitrite* meander was selected as the second variable, this led to a loss of R^2_{adj} of 0.004 (< 0.5%). This speculative analysis therefore shows that, except for the third and fourth components of the model in Sweden (Table 9.7) and the third component of the model in the UK (Table 9.6), prediction of the field fouling data from behavioural parameters can be achieved through the use of *B. amphitrite* and record only turn angle, meander and mean distance.

This analysis has shown that the removal of coating 615 in the *B. amphitrite* data has allowed behavioural parameters (turn angle and meander) to become significant

predictors of the percent fouling found in the UK (Chapter 7) suggesting again that coating 615 is not a suitable control for the bioassay.

Using this research to develop a commercially acceptable bioassay for non-biocidal coatings.

The statistics used in the thesis to this point have been used to try to assess the potential for the bioassay technique. Moving on from this development work, to the development of statistics for a bioassay in a commercial setting requires a degree of reassessment; in a commercial environment the key question would be whether a test coating would be better than a control, or more formally stated as a null hypothesis "there is no difference in the behavioural indicators on a test surface compared to a control". Consequently, the test is a test between two variables; the test and the control. The "control" would be determined by the bioassay manager, but for screening bioassays this could be the companies' best commercial non-biocidal surface available (be it in the market place or at a pre-marketing stage). This would mean that only coatings which are significantly different to the control would be selected. The test surface would be the newly formulated coating which is at the initial screening stage.

As with all statistical analyses, comparisons of behavioural indices for these two test coating (control and test) could potentially lead to errors. These errors have been defined as Type 1 and Type 2 errors (Zar 1999). Type 1 error is when the null hypothesis is not accepted but there is in fact no difference (i.e. the coatings are not different but the statistics suggest that they are). Type 1 errors can be manipulated and controlled through the modification of the significance level of the test, for example through Bonferroni adaptation to the P-value (Zar 1999). Type 2 error is when the null

hypotheses should be rejected but is not (i.e. the coatings are different but the statistics suggest they are not). A Type 2 error is related to statistical power which can be manipulated through sample size, variance and the mean differences between the test and control coatings. The mathematical expression of power is a numeric ranging from 0 to 1.

A power value of, for example 0.1, means that, with the supplied mean difference, sample size and standard deviation, there is a 10% probability that the statistical test will find a significant difference (i.e. null hypothesis not rejected, when it should be, or alternatively a 90% chance of a Type 2 error). Whereas, a power of 0.9, means that there is a 90% probability that the statistical test would find a significant difference (i.e. a 10% probability of a Type 2 error). Theoretically the possibility of Type 2 errors can be decreased through increasing the mean difference, increasing sample size and decreasing standard deviation. Generally, the standard deviation of the population is not changeable (though this is potentially possible in some situations by closer delimitation of the sample "population", e.g. from barnacle shell size on rocky shores of the UK, to barnacle shell size on the mid-intertidal rocky shores of Northumbria). Consequently, and especially in this case, the remaining aspects which the operator can manipulate to increase power is sample size and mean difference.

If the bioassay is used as a pre-screening test, with only those surfaces which show a significant difference to the control being selected for subsequent field trials, then it is important to guard against Type 2 errors. Type 2 errors will lead to potentially effective coatings being rejected at the bioassay stage, which is commercially unacceptable. Whereas, Type 1 errors will lead to the selection of surfaces which will subsequently be found in field test to be ineffective, this is more acceptable, as it does not risk the de-

selection of potentially effective biofouling surfaces, but only creates a small extra field testing burden. Consequently, Type 2 errors need to be managed in a bioassay screening protocol.

A case example, presented below, indicates the possibility of Type 2 error manipulations that should be taking into account with a commercial bioassay screening test. The exact details of the manipulation of power will be related to the details of the commercial test, in terms of required power, sample variance, accepted mean difference between test and control and standard deviation. As this data is not available, then estimates from the work presented here will be used to give indicative answers.

Case example

If we select the test variable *B. amphitrite* turn angle as the best predictor of field fouling, (Table 9.5). Then for the mean value and range for the 5 coatings tested is 150 and the standard deviation of the mean is 22. If we use this population standard deviation and then manipulate the mean difference at a sample size up to 200 we can model the trends of power. The example presented below (Figure 9.1) uses samples size from 5 to 200, and mean difference of 5 to 25 *B. amphitrite* turn angle degrees. Power for each sample size and differences were calculated using Minitab v12.

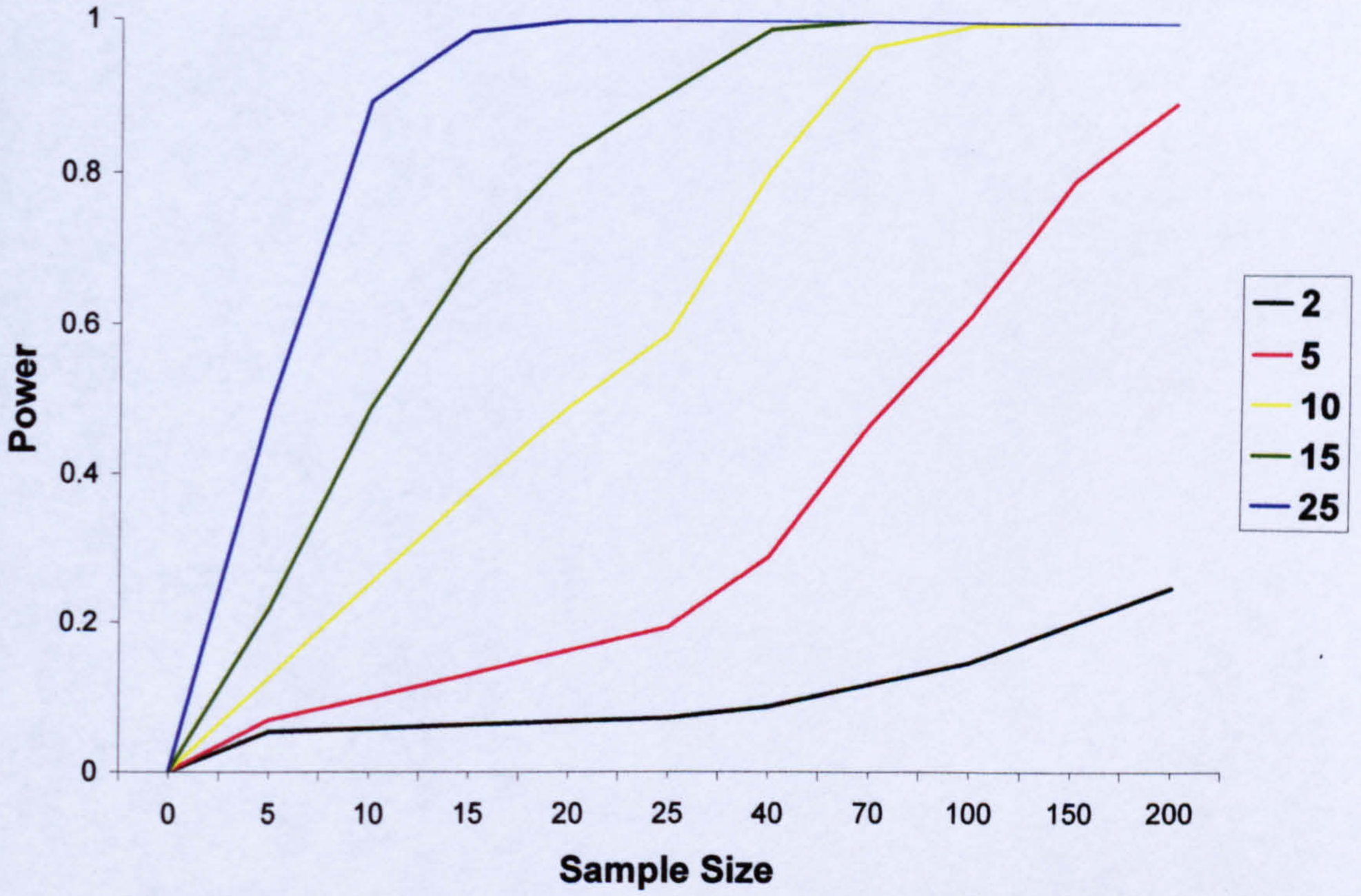


Figure 9.1 Theoretical plot showing the relationship between power and sample size at varying degrees of differences of degrees of turn angle for *B. amphitrite*.

A number of features are apparent from the theoretical plot (Figure 9.1):

1. As the mean difference between the test and the control that is required to be detected is increased, the power of the statistic increases.
2. As sample size increases power also increases.

The trends typified in this plot are clearly generic, but they exemplify the statistical considerations required for a commercial bioassay. In addition, it also gives a good indication of the required sample size for set differences to be detected to ensure minimisation of Type 2 errors and the consequent rejection of potentially effective non-biocidal coatings.

Further work is required to determine the details for the specific nature of the power relationships in a commercial bioassay. This would be in terms of the standard deviation of the behavioural parameter of the test species on the selected control surface. The mean difference also needs to be closely considered and an acceptable difference decided upon, within the commercial context. Using field data for the control surface, and a variety of different non-biocidal coatings, it would be possible, through regression found from the previous analysis (assuming that other non-biocidal coatings can be interpolated from the results collected in this investigation), to relate field percent fouling to behavioural indicators (see Tables 9.2, 9.6, 9.7). Thus, the mean difference required to be detected could be expressed in terms of percent fouling rather than behavioural indicators. Consequently, mean differences required to be detected could be expressed as "a mean difference in the control and test coating of at least 10 percent fouling cover". Additional considerations would also been needed to permit the use of multiple behavioural characteristics in prediction of field fouling and more complex statistical power analysis for a multi-species bioassay would be required. However, the principles presented above would remain the same.

Suggested protocol for a commercial screening bioassay for testing non-biocidal coatings.

The above case example has shown the statistical background work required for a reliable screening bioassay technique, which protects against Type 2 errors, and the consequent loss of effective coatings. Work is required to determine the statistical summary for the control surface that Akzo Nobel select to use, however, an identical approach can be used as above, substituting appropriate data and required commercial standards.

If suitable statistical background data are collected to allow power to be manipulated within the protocol of a commercial style test, then the requirement for further statistical testing could possibly be negated. This is because of the limited importance, or even irrelevance, of a Type 1 error. In a commercial screening test it is not the "significance" between coatings that is important, but it is the magnitude of difference that is vital. On the assumption of consistent standard deviation of behavioural parameters and sample sizes of suitable power to detect the required degree of difference between test and control coatings, then the information required by a commercial test becomes the size of the mean difference in behavioural indices itself.

Consequently, a simple three-stage analytical approach can be used:

Stage 1 – confirm that the standard deviations of the selected behavioural indice/s are within a set range. If they are not then the power assumptions are incorrect and then doubt must be cast on the power of the sample size used and consequently the possibility of Type 2 error. A set range could be for example that the mean:standard deviation ratio is between 5 and 10 (the example presented above had a ratio of 6.8). If this requirement is upheld then the analysis can move onto stage 2. If the requirement is

not upheld then reason for the greater, or less, standard deviation then expected should be considered, and if necessary the power curves to be reconsidered in this case.

Stage 2 – Use the regressions equations from the relationships between field fouling and behavioural indices found in this thesis using a range of non-biocidal coatings to predict percent fouling.

Stage 3 – Visually present this data on what will be termed a "fouling ruler" (Figure 9.2). These fouling rulers would have a scale of percent predicted fouling on the scale. Three rulers could be used for tropical, temperate and high latitude areas (or these data could be amalgamated into one overall global ruler).

In this way the behavioural bioassay can be used to visually present information which shows the predicted fouling level of the tested coating in three climatic areas, if an immersion trial would be carried out. Although, no significance testing is carried out in a "classical" statistical way, due to an awareness of power, the differences displayed on the ruler between control and test coating are statistically robust as well as being relevant in a commercial context.

The guidelines for a provisional protocol can be developed for a commercial application of a behavioural bioassay screening test. The protocol would use the same experimental technique outlined in the body of the thesis; filming, digitizing larval movement (either by Method 1 or 2, (Chapter 5), although larger sample sizes would require automated digitization) and calculating behavioural parameters (Chapter 5). However the data presented here suggests that for a parsimonious approach, the only species that needs to be included is *B. amphitrite* and the only parameters required would be turn angle, meander and mean distance. A pre-testing stage needs to be carried out to determine the required sensitivity and reliability of the bioassay (Figure 9.3). Following this a

statistically robust repeatable testing stage of novel surfaces can be used to screen potential surfaces (Figure 9.3). It is postulated that through this protocol an efficient and reliable screening test, with statistical power, can be developed for extensive and repetitive testing of novel non-biocidal coatings on a commercial scale.

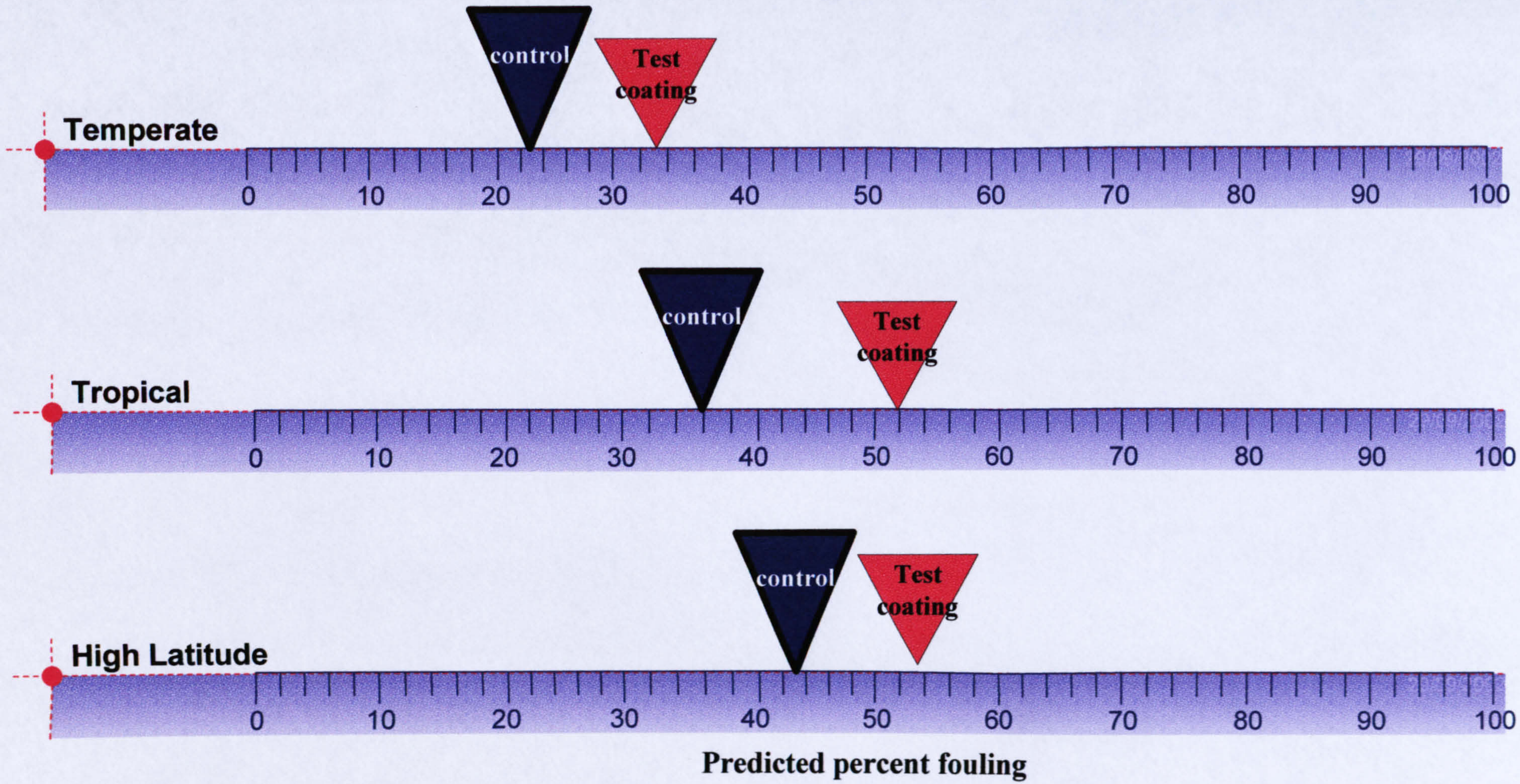


Figure 9.2 Example of suggested Fouling rulers to visually assess fouling of test coatings in comparison with control surface

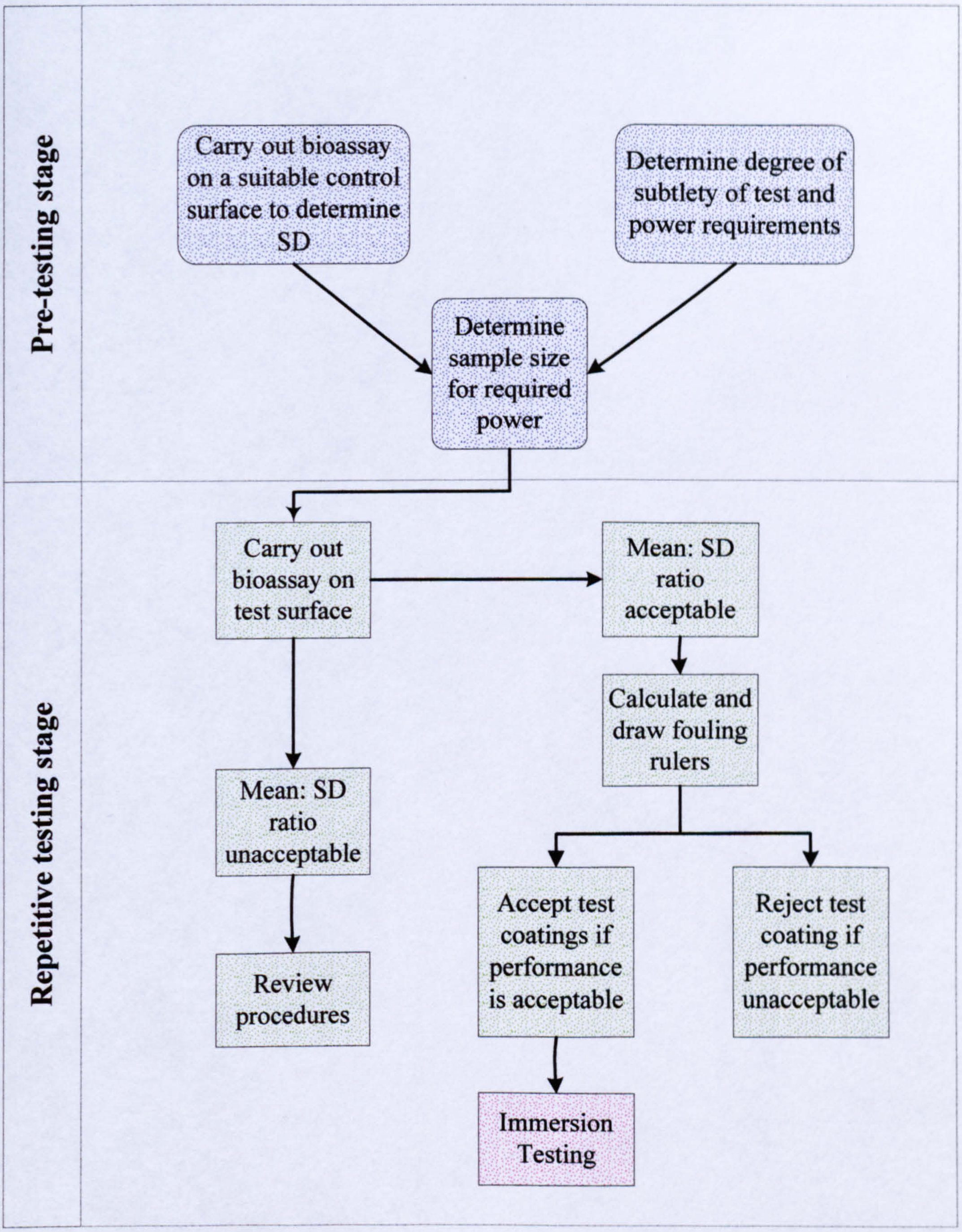


Figure 9.3 Proposed protocol procedure for a pre-screening bioassay for non-biocidal coatings, SD - standard deviation.

CHAPTER 10

OVERVIEW OF RESEARCH

CHAPTER 10

OVERVIEW OF RESEARCH

Introduction

It was stated in Chapter 1 that the aim of this investigation was "to study and analyse the exploratory behaviour of a selection of marine invertebrate fouling species and to use these behaviours to develop a commercial laboratory based screening bioassay which could be used to predict the performance of novel non-biocidal coatings prior to field trials". This chapter critically assesses the extent to which this investigation has achieved this stated aim. To achieve this, the aim is broken down into the three sequential parts which constitute it; each part is then examined in turn in the following sections.

The three parts of the aim are as follows:

1. To study and analyse the exploratory behaviour of a collection of marine invertebrate fouling species.
2. To use behavioural data to develop a commercial laboratory based screening bioassay.
3. To assess how the bioassay can be used to predict the performance of novel non-biocidal coatings prior to field trials.

Part 1 - Analysis of exploratory behaviour of marine invertebrates

This work has been successful at analysing the behaviour of a collection of marine invertebrate larvae. In this work two techniques have been used to determine behaviour. One technique used non-biocidal coatings and assessed the position of the larvae on the coatings after a set period of time, this was carried out using different specific methodologies; a novel approach using *M. edulis* (Chapter 3; choice test), and a routinely used method using *B. amphitrite* (Chapter 4; settlement assay). The second technique used an array of larval behaviour filmed over real-time, this was carried out on *S. borealis* (Chapter 6), *B. amphitrite* (Chapter 7) and *B. improvisus* (Chapter 8).

Results from the first technique, assessing the position of the larvae in terms of their choice between two test surfaces using *M. edulis*, were encouraging. However, problems of mortality from an unknown factor were encountered during the work with *B. amphitrite*. It was suggested that the *M. edulis* bioassay could be developed further for commercial use, however, it was apparent that the results were not very subtle. The antifouling efficiency of coatings that were quite different could be detected; however, differences between coatings that were quite similar could not. Ways in which the bioassay could be improved were given and with minor refinements this novel bioassay could be employed as a pre-screening test for non-biocidal antifouling coatings.

The settlement assay using *B. amphitrite* used a standard technique used in antifouling research. The aim of this was to establish a performance rating of the coatings and compare this to field data, however this aim was not met. The results of this method did however show that although this is a technique routinely used in some laboratories it is not suitable for all types of coatings.

Both these two bioassay techniques used for assessing the position of larvae after a set period of time, do not actually study larval behaviour itself, but the consequence of the behaviour (i.e. choice of surface or choice to settle). There is a difference between assessing the consequences of behaviour in the past, and actually assessing the behaviour as it happens. The second technique used in this thesis actually measures the behaviour itself. It was developed because this direct approach of measuring behaviour could potentially be more sensitive that just assessing the consequences of past behaviour. Although a number of technical problems were encountered through the development of this work, it has shown that the exploratory behaviour of a number of larval species can be studied and analysed in a robust and replicated manner. Consequently, the first part of the aim of this investigation has been achieved.

Part 2 - Development of a screening bioassay

The second part of the aim was to develop a laboratory based screening bioassay that could be used in a commercial context. Through the literature review, analysis of the behaviour of a collection of marine larvae and assessment of the statistical considerations required for a commercial test, a protocol for a laboratory-based bioassay has been proposed (Chapter 9). In addition, a method for graphically summarising and presenting the results has been devised, which would be understandable to both commercial scientists, but also to other staff in a coating manufacturer's workplace.

A number of features regarding this protocol need to be considered in more depth to help assess the potential value of the bioassay technique; these are tracking software,

larval quality and coating colour. In addition, a comparison of the proposed method with other bioassays is required:

Tracking software

The automated tracking software, Ethovision has been invaluable for this research however it does have its draw backs: Firstly the cost, such a program will cost in the region of £7,000-£9,000 depending on which version. This is however an initial set up cost and if considering employing the bioassay for continuous and high volume testing, it is really a pre-requisite in order to carry out the replication needed. Manually digitizing the movement as in Method 1 (Chapter 2) proved too laborious and time consuming.

Another draw back of using an automated system is the possible scope of error. The tracking device is only as good as the image given to it; any outside movements not made by the larvae, such as water movements, will be picked up and recorded. Although the software has various controls to minimize this, this invariably happens from time to time while tracking such small organisms. Therefore the tracks have to be edited before the behavioural parameters calculated which may lead to slight tracking errors. This problem although realised, was minimal in comparison to the benefits the software provided.

The small scale movement documented for pre-settling larvae (Knight-Jones 1951, Knight-Jones and Crisp 1953b, Crisp 1961, Nott 1973) may also have been missed due to the magnification used for tracking the larvae (as discussed in Chapters 6 and 7). This however was setup dependent, and may be reviewed with a smaller arena size which would increases the magnification and therefore minimize this effect.

Larval quality

A large part of research involved methodological advances; therefore many considerations were taken into account when designing the bioassay. One such consideration was concern of minimizing affects of larval quality and age which is known to affect settlement; both diet and large salinity changes can affect behaviour and settlement of *B. amphitrite* (Rittschoff *et al.* 1984, Rittschoff *et al.* 1992), and age of the cyprid can lead to decreased discrimination of settlement (Knight-Jones 1953a, Branscomb and Rittschof 1984, Satuito *et al.* 1996, Jarrett 1997). Although these effects were minimized in this research by replicating the same amount of coatings within a batch, and using larvae of the same age, these effects must be recognised if the bioassay is to be used on a large scale and for coatings produced at different times. A larger sample size may be needed if these potential effects cannot be minimised.

Coating colour

The research presented here was restricted to white coatings. The general commercially available tie coat is grey and therefore as most of the coatings are clear, the overall coating colour is usually grey. Staining was investigated for *B. amphitrite* and the results show that neutral red could be used as an aid to increase visibility. This experiment would have to be repeated for other species before use, but is believed to increase the contrast of the larvae sufficiently enough to allow for a darker back ground. A further technique could be to decrease the arena size and thus increase the magnification of the larvae which also may allow for a darker coating. This was beyond the scope of the research and further work is needed if coatings if various pigmentation are to be used in the bioassays.

Comparison with other available testing methods

It is of no doubt that immersion trials have proved a useful testing technique for non-biocidal antifouling coatings, however they are quite lengthy, requiring months rather than weeks (Swain *et al.* 2000, Terlizzi *et al.* 2000, Wood *et al.* 2000, Holm *et al* 2000a), can be restricted by deployment time (Nandakumur 1996 Thomason *et al.* 2000), can be limited by space (Thomason *et al.* 2002) and used in isolation do not always differentiate between antifouling performance of coatings (Swain *et al.* 2000).

Evaluation of the adhesion of organisms are also available to assess the antifouling performance of foul release coatings (Swain *et al.* 1992, Swain *et al.* 1994, Swain and Schultz 1996, Darkangelo Wood *et al.* 2000, Swain *et al.* 2000). These tests include calibrated jet washes (Griffith and Butman 1980, Swain and Schultz 1996) and the barnacle adhesion test, using both living barnacles (Swain *et al.* 1992, Swain and Schultz 1996, Swain *et al.* 1998, Kavanagh *et al.* 2001) and more recently pseudo-barnacles, made from epoxy glue (Swain *et al.* 1997, Singer *et al.* 2000). This method involves measuring the force required to remove the barnacle or pseudo-barnacle with a hand-held force gauge (Swain *et al.* 2000). However, although this method has proved useful for a comparison of foul release coatings (Swain and Schultz 1996), it does not reveal information on how organisms are responding to certain aspects of the surface, and when used on living organisms requires several weeks for pre-growth and is limited to the fouling season.

Settlement assays are quick and relatively easy to perform and can be informative in as much as a surface is liked/disliked by a species, however it does not reveal any further information and may not detect subtle differences between non-biocidal coatings. Also the problems of such coatings leaching detrimental substances during the duration of

the assays, shown in part of this research (see Chapter 4) has demonstrated that they can not be performed on all types of coatings, even non-biocidal variants.

The bioassays developed in this research have shown that non-biocidal antifouling coatings of varying efficiency can be discriminated between using the method proposed, and have been used to predict field data despite concerns of laboratory work being unrelated to finding in the field (O' Connor and Richardson 1996). It was not designed to replace field trials merely to complement them and be used as a pre-screening test to indicate if such immersion trials are warranted.

Biofouling is a multifaceted problem and thus, requires non-biocidal coatings should be tested in a multitude of ways. The tests available at present have proved beneficial to the understanding of the antifouling efficiency of the coatings and it is therefore proposed that a suite of tests become standard.

This section has been assessing the extent to which the aim of developing a laboratory based commercial bioassay has been achieved. It seems apparent that the background work required for the development of bioassay procedures in a commercial laboratory has been successfully carried out. It is clear that there are a number of features which need to be carefully considered and controlled, such as coating colour and larval age. However, this is maybe not surprising as the tests are attempting to be far more subtle than simply assessing the position of larvae after a set period of time. If the behavioural bioassay is carried out in a carefully controlled environment, then the probability of the commercial assay technique being successful is high. Moreover, the developed bioassay procedure compares strongly to other procedures available for the testing of non-

biocidal coatings. Through this assessment, it is suggested that the second part of the aim, to develop a commercial behavioural bioassay, has been achieved.

Part 3 - Prediction of field performance of coatings from the behavioural bioassay.

The third part of the aim was to assess how the bioassay can be used to predict the performance of novel non-biocidal coatings prior to field trial. This is a key issue, separate from the technical practicalities identified in the previous section. The role of the bioassay is envisaged as a screening test used prior to long-term and costly field-testing, and is explicitly required to make a prediction about the antifouling efficiency of coatings in the field.

It is clear from the single-species bioassay (Chapters 6, 7 and 8) that there are significant relationships between behavioural parameters of the larvae and the performance of the non-biocidal coating in the field. Although these relationships were significant in the statistical sense, the percentage of variation explained in field fouling by the behavioural parameters was relatively small (i.e. R^2_{adj}). For predictions to be accurate concerning the value of the antifouling coating, what is required is relatively high values of R^2_{adj}. However, a considerable increase in R^2_{adj} was experienced in Chapter 9, when multiple stepwise regressions were carried out linking multispecies behavioural parameters to fouling from field trials. This suggests that, from these data, to obtain the best possible prediction of field fouling, multispecies analysis is required.

These results provide an insight into the diversity of exploratory behaviour within and between species. It is apparent from the species tested that the same behavioural

parameter in different species is affected differently by a range of non-biocidal coatings, as no one single parameter was always selected by the single species stepwise regressions. Maybe this would be expected from the literature, (e.g. Rittschof and Costlow 1997, Roberts *et al.* 1991), however, it is clear from this the benefit of using a bioassay with a multispecies assemblage to predict field fouling.

The improvement in R^2_{adj} experienced between the single and multi-species analysis suggests that the predictive power of the bioassay can be improved through the use of more species, or possibly more behavioural parameters. Although it cannot be predicted which behavioural indices in a bioassay might provide explanatory power of field fouling rates, increasing the array of behavioural indices would be expected to lead to an increased in R^2_{adj} in stepwise multiple regressions beyond that provided by extra random data alone. On the other hand though, it appears from the similarity in behavioural parameters selected in the multispecies analyses presented in Chapter 9, that certain behavioural parameters are commonly better predictors of field fouling (e.g. turn angle, meander and mean distance) than others (e.g. moving time and velocity). The reasons for this are not clear.

In summary, it can be seen that significant amounts of variation in field fouling can be predicted from the behavioural bioassay. The predictive power is considerably improved by the addition of a multispecies assemblage of behavioural parameters. Determining the predictive power of the bioassay is a key component of the aim of the bioassay and this has been carried out and provides support for the use of the bioassay technique as a screening test.

Further work

The work has provided the basis for a commercial non-biocidal coating bioassay which has been shown to explain significant amounts of the variation in antifouling performance of these coatings in field trials. To develop this bioassay in a commercial laboratory requires an assisted transfer of knowledge and experience to ensure correct and controlled bioassay procedures are carried out. Although the bioassay is readily transferable to the commercial context, there are a number of areas which could be developed further which might potentially enhance the commercial value of the bioassay:

1. Identification of a suitable control surface on which to base the proposed protocol.
2. Analysis the behaviour of a wider array of species, and in addition a wider array of behavioural parameters.
3. Further immersion trials on the coatings to determine a global fouling quotient.

Conclusions

The requirement for bioassay techniques for screening potential non-biocidal antifouling coatings has long been recognised. However, no established technique has become apparent in the scientific literature or from the grey literature. In response to this perceived need, the investigations documented above were carried out. The research has robustly demonstrated, for the first time, that behavioural bioassays have potential as a bioassay technique to investigate non-biocidal coatings. It has shown:

- Larval behaviour can be used to discriminate between a suite of antifouling coatings with varying efficiency.

- Laboratory behaviour of some marine fouling species can be used to predict long term fouling on non-biocidal coatings at three different geographical locations.
- The development of the protocol, can lead to a rapid commercial screening test for the evaluation of non-biocidal antifouling coatings.

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