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The development and settlement of certain marine tubeworm (Serpulidae and Spirorbidae) larvae in response to biofilms

Hamer, John

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THE DEVELOPMENT AND SETTLEMENT OF CERTAIN MARINE TUBEWORM (SERPULIDAE AND SPIRORBIDAE) LARVAE IN RESPONSE TO BIOFILMS

A thesis submitted to the University of Wales

by

JOHN PAUL HAMER, B. Sc. (Hons), M. Sc.

in candidature for the degree of
Philosophiae Doctor

University of Wales, Bangor
School of Ocean Sciences
Menai Bridge
Anglesey
LL59 5EY

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Summary

The development, settlement behaviour and settlement preferences of various marine tubeworm larvae were investigated in the laboratory and in the field. Scanning electron microscope observations of laboratory reared *Pomatoceros lamarckii* revealed several morphological features such as gland pores and ciliary tufts not reported previously. In still water laboratory assays using experimental slate surfaces and laboratory developed biofilms, *P. lamarckii* larvae required a biofilm for settlement and settled preferentially on older biofilms; settlement intensity was closely correlated to bacterial density suggesting that bacteria and/or their extracellular products are the inductive cue. The way in which experimental surfaces are presented to tubeworm larvae, either in single- or multi-treatment laboratory assays, significantly affected the settlement patterns of the spirorbids *Spirorbis spirorbis* and *Spirorbis tridentatus* but not *P. lamarckii*, the larvae of which do not seem to become 'desperate' if not presented with a suitable surface for settlement. Interestingly, aerial drying a biofilm during simulated tidal emersion negates the settlement-inducing effect of a biofilm. *S. spirorbis*, *S. tridentatus* and *Flustrellidra hispida* (bryozoa) larvae also avoid recently dried biofilmed surfaces. Combined laboratory and field experiments showed that biofilms are an important settlement cue to *P. lamarkii* larvae, enabling them to settle selectively in their appropriate intertidal habitat. In the laboratory, monospecific bacterial films induced, had no effect upon or inhibited settlement of *Spirorbis spirorbis* larvae and experimental manipulation of biofilms suggested that the inductive biofilm cue is a commonly produced non-specific polysaccharide that is present either on the surface of the bacterial cell or in the extracellular polysaccharide matrix of the biofilm. The larvae of the tubeworms studied here display highly selective settlement preferences in response to biofilms and will continue to be useful subjects for further laboratory and field based studies that investigate how biofilms mediate the settlement of marine invertebrate larvae.

1. General introduction: Settlement of benthic marine invertebrate larvae, with particular emphasis on sedentary marine polychaetes.

The vast majority of benthic marine invertebrates possess a planktonic stage in their life history (Thorson, 1964), with the time spent in the water column by an individual larva varying from minutes (e.g. the larvae of spirorbid polychaetes (Gee and Knight-Jones, 1962; Gee, 1963)) to many months in extreme cases (e.g. the teleplanic larvae of some echinoderms and crustaceans (Scheltema, 1964)). During this time, larvae may feed (planktotrophy) or not feed (lecithotrophy) before settling and metamorphosing into the benthic juvenile.

Here, factors important to the settlement of marine invertebrate larvae are reviewed, particularly the factors known to be important to the settlement of sedentary polychaetes (Qian, 1999; Kupriyanova *et al.*, 2001). For various reasons, previous studies on polychaete larval settlement have been restricted primarily to species with aggregated distribution patterns and those that settle on hard substrata or seaweed; fewer studies have been conducted for infaunal polychaetes. Among polychaete families, sabellariid, spirorbid and serpulid polychaetes are the three groups that have been studied most extensively (Qian, 1999). For the purpose of this review, an emphasis is placed on biological settlement cues, particularly those from conspecifics and biofilms.

The relative importance of larval supply and post-settlement factors

It is now recognised that differential larval supply, i.e. the rate and timing of larval settlement or input to the benthos (Todd, 1998), can be a major factor influencing recruitment patterns and hence community structure in sessile and semi-sessile benthic marine communities (Strathmann *et al.*, 1981; Grosberg, 1982; Keough and Downes, 1982; Underwood and Denley, 1984; Connell, 1985; Gaines and Roughgarden, 1985; Gaines *et al.*, 1985; Raimondi, 1988, 1990, 1991; Chia, 1989; Sutherland, 1990; Menge, 1991; Miron *et al.*, 1995). This emphasis has been termed ‘supply side ecology’ (Lewin, 1986; Young, 1987; Underwood and Fairweather, 1989). Todd (1998) considers the relative importance of larval supply versus post-settlement processes in structuring benthic communities and suggests that among rocky intertidal assemblages, larval supply processes appear especially important in circumstances where recruitment levels are low whereas at higher recruitment levels, post-settlement processes take primacy. In soft sediment assemblages, post-settlement processes are considered of prime importance and in epifaunal assemblages, he suggests that both pre- and post-settlement factors are important.

Larval behaviour in the water column

Observations of the behavioural responses of planktonic larvae to environmental variables such as light and gravity have led to the suggestion that the uneven distribution of larvae frequently observed in the sea may be a consequence of both passive transport and direct movement by larvae in response to physico-chemical cues (Chia *et al.*, 1981; Chia and Koss, 1983; Mackas *et al.*, 1985; Banse, 1986; Jackson, 1986) such that larvae of certain species do vary their vertical distribution in the water column (Grosberg, 1982; Sulkin, 1984). Planktonic larvae may respond behaviourally to light (Groom and Loeb, 1890; Crisp and Ghobashy, 1971; Forward and Costlow, 1974; Ennis, 1975; Cronin and Forward, 1979; Young and Chia, 1982; Chia and Koss, 1983; Sebens, 1983; Forward *et al.*, 1984; Marsden, 1984, 1986, 1988, 1990), gravity (Sulkin *et al.*, 1980; Chia *et al.*, 1981), hydrostatic pressure (Knight-Jones and Qasim, 1955; Bayne, 1963; Rice, 1964; Knight-Jones and Morgan, 1966; Ennis, 1975; Sulkin *et al.*, 1980), temperature (Bolton and Havenhand, 1997), viscosity (Podolsky and Emlet, 1993; Bolton and Havenhand, 1997) and salinity (Latz and Forward, 1977; O'Connor and Epifanu, 1985; Forward, 1989). In some cases such behaviour has been shown to directly influence the distribution of adult organisms, for example Grosberg (1982) found that the differential stratification of *Balanus glandula* larvae in the water column can predetermine the environment in which they will eventually settle; the restriction of *B. glandula* to the intertidal zone may thus be primarily a consequence of pre-settlement events. Similarly, Gaines and Bertness (1993) found a close correlation between larval abundance and settlement and concluded that the colonisation of a particular site was primarily a function of larval density in the water column and water flow.

Many marine invertebrate larvae display phototactic behaviour (Thorson, 1964), including those of serpulimorph polychaetes. Marsden studied the light responses of three species of serpulids, *Spirobranchus giganteus* (Marsden, 1984, 1986), *Galeolaria caespitosa* (Marsden, 1988) and *Spirobranchus polycerus* (Marsden, 1990). Trochophore larvae of *S. giganteus* were attracted to visible light of wavelengths shorter than 590nm with the positive photoresponse developing gradually following the appearance of the eyespot (Marsden, 1984); larvae became photo-neutral at the late metatrochophore stage (Marsden, 1986). Contrastingly, larvae of *G. caespitosa* and *S. polycerus* are initially photonegative, becoming photopositive only at the metatrochophore stage (Marsden, 1988, 1990). Segrove (1941) noted that the larvae of *Pomatoceros triqueter* were photopositive and Moat (1985) found *Pomatoceros triqueter* larvae to be strongly photopositive by day 4 – 6 of development with the phototaxis then diminishing with increasing larval age, the larvae eventually becoming photonegative prior to settlement. Both reports were based upon observations made of larval behaviour in culture vessels. More recently, Roscoe (1993) studied the response of *Pomatoceros lamarckii* larvae to horizontal light both in culture and in a purpose-built experimental chamber; the results were somewhat ambiguous although they showed that as with *P. triqueter*, *P. lamarckii* metatrochophore larvae are predominantly photonegative. Laboratory settlement studies have shown that light is an important factor in *Spirorbis spirorbis* larval settlement. Interestingly, Doyle (1974) documented both inter- and intra-specific variability in the response to directional light in populations of *Spirorbis borealis* (= *S. spirorbis*). Larvae produced by individuals taken from within a tidal pool were 20% more photonegative than those produced by individuals collected from outside the pool with the degree of larval photoresponse being to some extent genetically determined. De Silva (1962) studied larval behaviour of spirorbids in response to light and found that in the laboratory, the planktonic larvae of *Spirorbis tridentatus* and *S. borealis* (= *S. spirorbis*) have different responses to light with concominant settlement site differences and that this larval behaviour could contribute to the natural distribution of the adult organisms. Larvae of *S. borealis* (= *S. spirorbis*) responded positively to both light and gravity, whereas those of *S. tridentatus* responded positively to gravity in the absence of light, but were overpoweringly photonegative. Knight-Jones (1951) found that in darkness in the laboratory, settlement of *S. borealis* (= *S. spirorbis*) occurred much less readily than in the light and Knight-Jones *et al.* (1971) note that in the laboratory, *S. borealis* (= *S. spirorbis*) larvae are only released when the light is fairly bright. In the field, Whitlatch and Osman (1998) reported that spirorbid polychaetes were only found on substrata exposed during the middle of the day and that no settlement occurred during the night. It was not clear whether this pattern was due to variations in larval supply (probable) or larval selectivity (less likely).

Larval settlement and metamorphosis

After some time in the water column, larvae become competent to settle, i.e. acquire the ability to respond to the appropriate stimuli and become physically capable of settling and undergoing metamorphosis (Coon *et al.*, 1990). Pawlik (1992) drew attention to the array of definitions in the literature relating to the settlement and metamorphosis of benthic marine invertebrate larvae. Some authors view settlement as the termination of planktonic life (Scheltema, 1974; Pawlik, 1992; Rodriguez *et al.*, 1993) whereas others consider the process as reversible whereby the larvae, although temporarily attached whilst exploring the substratum, may return to the water column

(Burke, 1983; Crisp, 1984). Metamorphosis was considered strictly in the morphological sense by Scheltema (1974) and involves the transition from larva to benthic juvenile. Crisp (1974) viewed fixation separately, as a process of irreversible attachment directly preceding metamorphosis and considered that the overall settlement process could be subdivided into recognisable larval behaviours - attachment, exploration, inspection, settlement and orientation. Pawlik (1992) and Rodriguez *et al.* (1993) define settlement as representing the entire process of transition from planktonic larvae to benthic juvenile, with metamorphosis, including fixation, being considered part of the process of settlement. However, settlement (a behavioural response) and metamorphosis (a morphogenetic response) may be triggered by different inducers or occur at different times (Chia, 1989; Weiner *et al.*, 1989; Bonar *et al.*, 1990; Rodriguez *et al.*, 1993; Holmström and Kjelleberg, 1994; Morse and Morse, 1996). Wilson (1932) documented situations where polychaete larvae even undergo metamorphosis in the water column prior to settlement. Crisp (1984) distinguishes between settlement and metamorphosis-inducing substances in cirripedes. The settlement inducer, called arthropodin, is a species-specific set of macromolecules which initiate the settlement routine when recognised in the adsorbed state on surfaces (Knight-Jones, 1953b; Crisp and Meadows, 1962, 1963), whereas metamorphosis can be induced artificially in unattached larvae by exposure to insect juvenile hormones and their analogues (Gomez *et al.*, 1973). The process of settlement and metamorphosis has different implications for sessile and non-sessile organisms which may explain why the various definitions have been used. Settlement of a sessile benthic invertebrate is a discrete and relatively easily identifiable event in an individual's life history and involves the irreversible fixation of an individual to the substratum, whereas many non-sessile organisms such as the polychaete *Ophelia bicornis* (Wilson, 1948) do not permanently attach and may not undergo dramatic metamorphosis. For serpulid and spirorbid tube-building polychaetes, settlement is a dramatic and final event which involves the larvae anchoring itself to the substratum and producing a mucous tube which acts as a template for the calcareous tube (Segrove, 1941; Wisely, 1958; Nott, 1973; Moat, 1985; Roscoe, 1993); at the same time, the larva loses most of its locomotory ciliation and begins to develop a rudimentary branchial crown. If the individual leaves this mucous tube for any reason, then it is unable to return to the water column or build another tube (Roscoe, 1993). Although there are valid reasons for separating larval settlement and metamorphosis, both processes tend to be coupled for the larvae of sessile tubeworms, therefore the term settlement will be used here in the same context as used by Pawlik (1992) and Rodriguez *et al.* (1993) to mean the entire process of transition from planktonic larvae to benthic juvenile.

Although the physiological mechanisms by which larvae become competent are poorly understood, some studies implicate the larval nervous systems (Burke, 1983; Hirata and Hadfiled, 1986; Pechenik and Heyman, 1987). Upon attaining competence larvae often display behaviours that bring them into contact with potential settlement sites (Crisp, 1974). This period is a critical point in the life history of a benthic marine invertebrate, particularly for the larvae of sessile organisms (Bourget, 1988; Chia, 1989) which are highly adapted to this role (Crisp, 1974, 1976). Competent larvae are usually equipped with a spectrum of sense organs (chemosensory, photosensory, geosensory, etc.) enabling them to respond to a wide range of environmental cues and to identify a suitable habitat for settlement and metamorphosis (Chia and Koss, 1979; Chia and Koss, 1982; Chia *et al.*, 1986; Amieva *et al.*, 1987; 1987; Chia, 1989). Such physiological adaptations are usually at a peak during competence (Crisp, 1974; Burke, 1983). Associated with these physiological adaptations are behavioural responses that are also at a peak of complexity during the competent period. When competent larvae contact a surface they undergo a period of exploration to determine whether it is suitable for settlement. If it is so, the larvae settle and metamorphose (Crisp, 1974).

Metamorphosis in response to bioactive compounds

An alternative approach to investigating larval settlement bypasses the study of external chemoreceptors and natural cues and directly stimulates internal pathways with compounds that act on the larval nervous system or affect membrane permeability, typically resulting in metamorphosis of larvae in the absence of any natural cue. In contrast to the few naturally occurring settlement cues which have been fully identified, a range of compounds have been found to induce metamorphosis in certain benthic marine invertebrate larvae (see Woolacott and Hadfield, 1996). Although some of these compounds induce metamorphosis for certain species, they may have no effect or cause abnormal metamorphosis and death for other species (Yool *et al.*, 1986; Pawlik, 1990; Ilan *et al.*, 1993; Okamato *et al.*, 1995; Bryan *et al.*, 1997). Such an ability to induce metamorphosis of larvae in the laboratory is useful because it enables workers to accurately determine the onset and between individual variations in metamorphic competence within a batch of larvae without the need to induce settlement.

Many of the pharmacological compounds that induce metamorphosis of various marine invertebrate larave have also been tested on polychaete larvae. Okamato *et al.* (1995) studied the action of eight neuro-active compounds for metamorphosis-inducing activity in the larvae of three species of serpulids, *Hydroides ezoensis*, *Pomatoleios kraussii* and *Ficopomatus enigmaticus*. In *H. ezoensis* and *P. kraussii* larvae, L-/D-DOPA, epinephrine and norepinephrine induced metamorphosis, while dopamine did not. In *F. enigmaticus* larvae, metamorphosis was induced by L-/D-DOPA with again dopamine having no effect. In all cases, L-DOPA induced metamorphosis in a dose-dependent manner. The authors suggest that the mechanism of metamorphosis in these three species is likely to be similar and because of a delayed reaction and the absence of tube formation, it was suggested that L-DOPA does not act on epithelial receptors but affects other internal pathways that lead to metamorphosis. Isobutyl methylxanthine (IBMX) induced normal metamorphosis of *Phragmatopoma lapidosa* larvae (Jensen and Morse, 1990; Pawlik, 1990). Bryan *et al.* (1997) investigated the induction of settlement and metamorphosis in *Hydroides elegans* larvae by five compounds and also found that IBMX induced a high percentage (81%) of normal metamorphosis; the other compounds either induced a low percentage of abnormal metamorphosis (L-DOPA, GABA and choline chloride), or had no effect (ammonia). Choline chloride induced metamorphosis of *P. lapidosa* and *H. elegans* larvae (Pawlik, 1990; Bryan *et al.*, 1997) but was also toxic to *Hydroides elegans* larvae within 24 hours of exposure (Bryan *et al.*, 1997). Carpizo-Ituarte and Hadfield (1998) found that both Cs^+ and K^+ induced metamorphosis of *H. elegans* larvae but the response was much slower than that to a biofilm. Maximum levels of metamorphosis were obtained at different ionic concentrations and after different exposure times to the two inducers.

Delayed metamorphosis

It was thought for a long time that marine invertebrate larvae settled randomly and that development proceeded according to a fixed timetable (see review by Pechenik, 1990). However, observations of differential settlement of such larvae (Mortensen, 1921; Mazzarelli, 1922) stimulated the idea that larvae may be able to exert some control over their final place of settlement (the original suggestion of Thompson, 1830) and laboratory studies have now demonstrated the selective settlement of a wide range of marine invertebrate larvae (Meadows and Campbell, 1972; Crisp, 1974; Scheltema, 1974). An ability to discriminate between and reject potential settlement sites before finally settling means that competent larvae must be capable of delaying metamorphosis for at least some time, whilst maintaining the capacity to metamorphose (Pechenik, 1990). Recognition that larvae are capable of discriminating between substrata and hence delaying metamorphosis was considered by Crisp (1974) to be "by far the most important influence in the progress of larval biology since pre-war days", because of the implications in terms of larval control over the settlement process and hence adult distribution. In the laboratory, the larvae of several species of marine invertebrates have been shown to delay settlement and metamorphosis in the absence of appropriate stimuli (Mortensen, 1921; Mazzarelli, 1922; Wilson, 1932; Thorson, 1950; Knight-Jones, 1953; Crisp, 1974; Jensen and Morse, 1984; Pechenik, 1985; Highsmith and Emlet, 1986; Coon *et al.*, 1990; Qian and Pechenik, 1998), with the ability to delay metamorphosis varying widely between species (Pechenik, 1990). Competent larvae may also delay metamorphosis and remain in the water column despite having encountered a favourable settlement cue due to additional factors preventing the normal settlement response. For example, Young and Chia (1981) found that competent larvae of the bryozoan *Bugula pacifica* delay metamorphosis in the presence of extracts of a dominant spatial competitor, the ascidian *Diplosoma macdonaldi*. Pechenik (1990) reviewed the phenomenon of delayed metamorphosis in marine invertebrate larvae and considered the limited field evidence for its occurrence under natural conditions. He concluded that the limited data suggest that at least a few species do delay metamorphosis in the field. Despite the ability of planktotrophic larvae to continuously replenish energy reserves, they are unable to postpone metamorphosis indefinitely and eventually they either become moribund or undergo spontaneous metamorphosis (Knight-Jones, 1953a; Highsmith and Emlet, 1986; Pechenik and Eyster, 1989; Pechenik, 1990). Also, even though larvae are capable of postponing metamorphosis, recent evidence suggests that a delay of metamorphosis may incur substantial sub-lethal costs to the individual in early post-larval life (Woolacott *et al.*, 1989; Pechenik, 1990; Pechenik and Cerulli, 1991; Pechenik *et al.*, 1993; Wendt, 1996). Several authors have reported that polychaete larvae appear capable of delaying metamorphosis in the absence of a suitable settlement cue (Roscoe, 1993; Toonen and Pawlik, 1994; Unabia and Hadfield, 1999). However, Qian and Pechenik (1998) conducted a detailed study on the ability of the larvae of *Hydroides elegans* to delay metamorphosis; their results suggested that the larvae are unable to delay metamorphosis for a significant amount of time without seriously compromising post-larval life. In contrast, Unabia and Hadfield (1999) report that larvae of *H. elegans* are able to delay metamorphosis for up to 24 days after attaining competency. However, the authors did not investigate whether factors such as post-larval growth and survival rates were adversely affected. In addition to delaying metamorphosis, Toonen and Pawlik (1994) found that starved larvae of the serpulid polychaete *Hydroides dianthus* revert to a pre-competent state but that upon feeding competency is regained.

The desperate larva hypothesis

Larvae of *Spirorbis borealis* (= *S. spirorbis*) become less selective when denied a suitable settlement surface for three hours, settling more readily in the absence of conspecifics compared to freshly liberated larvae which are highly gregarious in their settlement (Knight-Jones, 1953a). This decreased selectivity resulting from an extended planktonic life has been termed the 'desperate larva hypothesis' (Toonen and Pawlik, 2001b; see also Knight-Jones, 1953a; Wilson, 1953). Subsequently, Crisp (1974) showed that older barnacle cyprids settled more rapidly compared to younger larvae when both were presented with a standard surface and suggested that this observation supported the hypothesis. Similarly, Ritschof *et al.* (1984) found that older cypris larvae were less discriminating than younger ones, settling with greater frequency on less favourable surfaces. The species used in the studies that proposed the desperate larva hypothesis, *Spirorbis borealis* (Knight-Jones, 1951b, 1953a) and *Ophelia bicornis* (Wilson, 1953), both produce lecithotrophic larvae; the barnacle cyprid is also a non-feeding larval stage that is reliant upon previously accumulated energy reserves (Lucas *et al.*, 1979). Recently, Toonen and Pawlik (2001b) have tested the desperate larva hypothesis for planktotrophic larvae of the serpulid polychaete *Hydroides dianthus* in an attempt to understand how the larvae of this highly gregarious species found new aggregations; do some larvae simply become 'desperate' in the absence of a conspecific settlement cue and settle in the absence of adults? They suggest that because the desperate larva hypothesis is essentially an energetic model, its validity can be tested by subjecting larvae to energetic stress (starvation) and then comparing the settlement preferences of starved and non-starved larvae. Interestingly, they found that the desperate larva hypothesis does not apply to *H. dianthus* and speculate that probably the planktotrophic larvae of other marine invertebrate species may also not comply. They consider that because lecithotrophic larvae have finite energy reserves, they are more likely to encounter the scenario where they are presented with a 'settle or perish' situation and have evolved accordingly. Because planktotrophic larvae are generally capable of postponing settlement for a more substantial period of time after attaining competence, they have not evolved in the same way (Toonen and Pawlik, 2001b). However, if the desperate larva hypothesis does apply to lecithotrophic larvae, why cannot close to 100% settlement be obtained in laboratory assays for certain highly specialised non-feeding larvae, such as *Semibalanus balanoides* cyprids (Walker, pers. comm.)?

Settlement cues

Recognition that larvae are capable of rejecting one site and selecting another has stimulated research aimed at identifying the characteristic(s) of a particular substratum that elicits the settlement response. Laboratory experiments have shown that for the larvae of many species, active site selection is mediated by various physical or biological cues, with inductive cues frequently being associated with the preferred adult habitat (see reviews by Meadows and Campbell, 1972; Crisp, 1974; Scheltema, 1974; Chia and Rice, 1978; Chia, 1989; Pawlik, 1992). Some species will settle in response to very specific cues from a unique source, whilst others settle in response to cues originating from a variety of sources. Either way, it is the recognition of specific cues by exploring larvae that activates the genetically scheduled sequence of behavioural, anatomical and physiological processes leading to settlement (Morse, 1990).

Physical settlement cues

Marine invertebrate larvae are capable of responding to a wide range of physical variables associated with potential settlement sites (Crisp, 1974). Exploring larvae have been shown to discriminate between variations in surface contour (Knight-Jones, 1951b; Crisp and Barnes, 1954; Ryland, 1959; Wisely, 1960; Crisp, 1961; Wethey, 1986), texture (Barnes and Powell, 1950; Crisp and Ryland, 1960; Wethey, 1986; Raimondi, 1988; Hills and Thomason, 1998), rock type (Holmes *et al.*, 1997), grain size of sediment (Wilson, 1932, 1937, 1977; Day and Wilson, 1934; Gray, 1966a, 1967), sediment cover (Hodgson, 1990), water flow (Crisp, 1955; Ryland, 1977; Ritschof *et al.*, 1984; Wethey 1986; Butman, 1987; Wethey *et al.*, 1988; Judge and Craig, 1997), surface chemistry (Maki *et al.*, 1989, 1992; Ritschof and Costlow, 1989; Roberts *et al.*, 1991; Gerhart *et al.*, 1992; Becker, 1993; Holm *et al.*, 1997), surface colour (Gregg, 1945; Yule and Walker, 1984) illumination (Crisp and Ritz, 1973; Doyle, 1974; Sebens, 1983) and surface orientation (Cole and Knight-Jones, 1939; Crisp and Ghobashy, 1971). Generally, only one or a small subset of these factors has been studied for a particular species; nevertheless, some generalities are apparent. Larvae tend to settle preferentially on rougher surfaces and frequently locate in concavities (termed "rugotropism" by Crisp and Barnes (1954)) that may be much larger than the dimensions of the larvae (Ryland, 1959). Crisp (1974) reviewed the available literature on responses of invertebrate larvae to light and gravity and found that newly formed or recently released larvae are predominantly photopositive but that at the time of settlement, larvae exhibit a variety of responses. Nevertheless, he highlights the fact that "the great majority of animal larvae do in fact settle on the under sides [of surfaces] and in the shade". Such settlement patterns probably come about as a result of larval responses to physical factors such as light regime and surface orientation.

The early work of Wilson (1968) showed that larvae of *Sabellaria alveolata* settled in response to turbulence; he suggested that turbulence may represent a physical cue that larvae use as an indication that they were in the intertidal zone, the preferred adult habitat. *Spirorbis spirorbis* larvae settle preferentially in concavities and may use this behaviour to locate preferentially on either side of the midrib of *Fucus serratus* plants (Wisely, 1960). Crisp and Ryland (1960) investigated the settlement response of *Spirorbis spirorbis* larvae to surfaces of different roughness and found that they avoided rough surfaces and settled on glossy or smooth surfaces, in direct contrast to many other larvae which have been studied. Wisely (1958, 1960) reported that dark coloured substrata attracted more larvae of *Hydroides* sp. and *Spirorbis spirorbis* and James and Underwood (1994) found that spirorbids (mainly *Janua pseudocorrugata* and *Pileolaria pesudomilitaris*) recruited preferentially on dark grey boulders compared to yellow boulders, regardless of rock type. Other surface associated physical factors known to mediate settlement include surface charge, wettability, substratum type and degree of tidal exposure (Eckelbarger, 1978; Qian, 1999).

Biological settlement cues

Marine invertebrate larvae have been shown to settle in response to chemical cues originating from conspecific individuals (Knight-Jones, 1951b; Burke, 1984; Jensen and Morse, 1990; Pearce and Scheibling, 1990; Toonen and Pawlik, 1996), species associated with the adult habitat (de Silva, 1962; Bryan *et al.*, 1998), future prey species (Barnes and Gonor, 1973; Morse and Morse, 1984a; Boettcher and Targett, 1996) and biofilms (Knight-Jones, 1951b; Kirchman *et al.*, 1982a; Maki *et al.*, 1989; Hadfield *et al.*, 1994; Wieczorek and Todd, 1998).

Many species of serpulid and spirorbid polychaetes are aggregated in nature; certain species of spirorbids are specifically associated with particular substrata including foliose algae, encrusting coralline algae, erect coralline algae, seagrass blades and crustacean carapaces (Knight-Jones and Knight-Jones, 1977) and certain serpulids occur in dense aggregations on hard substrata such as rock and shells and in extreme cases may form dense serpulid 'reefs' (ten Hove and van den Hurk, 1993). Accordingly, in laboratory assays, the larvae of serpulid and spirorbid species have frequently been found to display highly selective associative and / or gregarious settlement behaviour (Qian, 1999), the two most common types of settlement behaviour exhibited by marine invertebrate larvae that settle in response to a chemical cue (Clare *et al.*, 1993).

Associative settlement

The phrase 'associative settlement' was proposed by Crisp (1974) to describe the specific settlement of one species in response to the presence of another species. The behaviour can be subdivided into herbivorous and predatory associations, parasitic associations and non-parasitic associations. Herbivorous and predatory associations occur when larvae settle in response to the presence of the future food organism (e.g. Hadfield and Karlson, 1969; Barnes and Gonor, 1973; Todd, 1979; Todd and Doyle, 1981; Morse and Morse, 1984a; Hadfield and Scheuer, 1985; Bahamondes – Rojas and Derbomez, 1990; Hadfield and Pennington, 1990; Marsden and Meeuwig, 1990). Perhaps the best studied example of herbivorous associative settlement is the preferential settlement of larvae of the red abalone *Haliotis rufescens* on crustose red algae *Lithophyllum* spp. and *Hildenbrandia* spp. (Morse *et al.*, 1980), where settlement is induced by a component of the macromolecular fraction isolated from the surface of the encrusting red algae (Morse and Morse, 1984a, b). Pawlik (1992) pointed out that settlement cues are particularly likely to be involved whenever the food organisms have a restricted distribution in time or space. For example, the nudibranch *Onchidoris bilamellata* feeds on barnacles (Todd, 1979) and metamorphosis of the planktonic veliger larvae only occurs upon contact with a live barnacle (Todd, 1981; Chia, 1989). Todd and Doyle (1981) suggest that the larval development and settlement of this species may represent a particularly well adapted example of a predatory association because the time spent in the plankton by *O. bilamellata* larvae enables individuals to reach a critical size capable of feeding upon newly settled barnacles and that larval competence coincides with periods of maximal abundance of such recently settled juvenile barnacles. However, it should be noted that such highly specific associative settlement behaviour may not always occur. For example, Hubbard (1988) studying the larval settlement preferences of the nudibranch *Hypselodoris infucata*, which preys upon the sponge *Dysidea* sp., found that larvae would settle in response to several species of sponges, including *Dysidea* sp. and also the presence of a microbial film. Similarly, Pawlik (1989) demonstrated that larvae of the sea hare *Aplysia californica* settled on a variety of macroalgae, despite the specific juvenile and adult diet being red algae of the genus *Laurencia*. In addition, larvae may avoid settling in response to certain cues, for example Grosberg (1981) found that certain sessile species avoid settling on surfaces that support superior competitors and Johnson and Strathmann (1989) reported that barnacle cyprids avoid settling on surfaces previously occupied by a mobile predator.

The often very specific nature of parasitic associations suggests that planktonic larvae of marine invertebrate parasites should also display strong associative settlement behaviour (Anderson and Dale, 1989; Blower and Roughgarden, 1989; Høeg and Lützen, 1995) that Pawlik (1992) notes is likely to involve chemical settlement cues. Rhizocephalan barnacles, which mostly parasitise decapod crustaceans, are a good example of parasitic species with larvae that appear to be well adapted to locating a specific and somewhat ephemeral settlement site. Clare *et al.* (1993) suggest that rhizocephalan larvae may be a useful model system for studying the chemical basis for the settlement response of barnacle cyprids. Non-parasitic associations, or symbioses include mutualism, commensalism and epibiotic associations (Pawlik, 1992). Such associations are common in marine communities (Crisp and Ryland, 1960; Wilson, 1968; Donaldson, 1974; Van Syoc, 1988) with many of the species involved in these symbioses having species-specific settlement behaviour. As a group, barnacles exhibit a remarkably wide range of epizoic settlement specificity (see Lewis, 1978 for review). Epibiotic associations are particularly common amongst certain groups of marine organisms with certain brown algae the preferred settlement substratum for some hydroids (Nishihira, 1965), spirorbid polychaetes (Gross and Knight-Jones, 1957; Wilson, 1968) and bryozoans (Ryland, 1958, 1959; Crisp and Ryland, 1960). These algae (or their exudates) have been shown to induce settlement of various epibiota, again suggesting the importance of chemically mediated settlement behaviour (Crisp and Williams, 1960; Pawlik, 1992).

Certain spirorbids have restricted distributions with the adults of different species occurring almost exclusively on a particular type of substratum (Knight-Jones and Knight-Jones, 1977). Laboratory studies have shown that their larvae are highly selective and settle preferentially upon the preferred adult habitat such as brown algae (Garbarini, 1936; Knight-Jones, 1951b; Gross and Knight-Jones, 1957; Williams, 1964; Al-Ogily, 1985), coralline algae (De Silva, 1962; Gee and Knight-Jones, 1962; Gee, 1965), seagrass blades (Nelson, 1979; Dirnberger, 1990) and on stones and shells (De Silva, 1962) suggesting that larval selectivity is a primary factor controlling adult distributions. This high settlement specificity may even vary between populations of the same species, for example, Knight-Jones *et al.* (1971) found that the larvae of an isolated race of *Spirorbis spirorbis* that live primarily on *Fucus vesiculosus* settle preferentially on *F. vesiculosus,* compared to the normal preference for *Fucus serratus* or a combination of both *F. serratus* and *F. vesiculosus,* depending upon the origin of the larvae. Al-Ogily (1985) reported that there are many ecologically isolated populations of *Spirorbis inornatus*, each settling preferentially on the alga that supports their parents. Williams (1964) found that extracts obtained by macerating *F. serratus* plants induced settlement of *S. spirorbis* larvae and showed that the inducing factor was a mucopolysaccharide. Not only do certain larvae settle preferentially on a particular species but some settle preferentially on a particular place on the host organism. For example, larvae of *Spirorbis spirillum* settle preferentially on the base of growing blades of the seagrass *Thalassia testudinum,* whilst avoiding the epiphytic algae which tend to be associated with the distal regions of the seagrass blades (Dirnberger, 1990); *Janua pagenstecheri* and *Spirorbis corallinae* settle preferentially on younger parts of *Laminaria* fronds (Stebbing, 1972). Al-Ogily (1985) also found that for some of the algae that induce settlement of *Spirorbis inornatus*, certain parts of the thallus were particularly favoured for settlement.

There are fewer examples of associative settlement in serpulid polychaetes. Marsden (1987) reported the preferential settlement of *Spirobranchus giganteus* on corals, with larvae responding positively to exudates of certain species of corals (Marsden, 1987; Marsden and Meeuwig, 1990). Bryan *et al.* (1998) found that in the laboratory, *Hydroides elegans* larvae settle in high numbers on the branches of the arborescent bryozoan *Bugula neritina* and on the bottom of containers containing *B. neritina* leachate and that in the field, *H. elegans* larvae settled preferentially on Phytagel discs imbued with extracts of *B. neritina*. In the laboratory, bioassay-guided isolation procedures suggested that free amino acids may be the inductive cue (Harder and Qian, 1999) although subsequent findings (Beckman *et al.*, 1999) suggest that this may not be the case (see following section).

Gregarious settlement

The selection by larvae of a site already inhabited by members of its own species is termed gregariousness (Knight-Jones and Stevenson, 1950; Scheltema, 1974) and was originally demonstrated for the larvae of oysters, barnacles and spirorbids (Cole and Knight-Jones 1949; Knight-Jones and Stevenson 1950; Knight-Jones, 1953a,b). Since this time, gregarious settlement has been demonstrated for at least 35 species representing 8 phyla (reviewed by Burke, 1986). In addition to settlement in response to adult conspecifics, the larvae of some species have been reported to also settle at a higher rate in response to conspecific larvae or newly-settled juveniles (Grave, 1944; Cole and Knight-Jones, 1949; Knight-Jones, 1953a; Keough, 1984; Wethey, 1984;

Yule and Walker, 1985; Grosberg and Quinn, 1986; Clare *et al.*, 1994; Bryan *et al.*, 1997); such behaviour continues to reinforce gregarious settlement even in the absence of adult conspecifics.

That there is a chemical basis to gregarious settlement has long been accepted. However, despite the widespread occurrence of gregarious settlement behaviour, we still know relatively little about the molecular structure of the inductive cues. The nature of the gregarious cue that stimulates *Semibalanus balanoides* cyprids to settle has been particularly well studied but its exact chemical nature and mode of action has so far escaped detection (Gabbott and Larman, 1987; Clare, 1995; Matsumura *et al.*, 1998; Clare and Matsumura, 2000). Partially characterised inducers of gregarious settlement have been reported for a number of species, including the oysters *Crassostrea virginica* (Crisp, 1967; Hidu, 1969; Keck *et al.*, 1971; Veitch and Hidu, 1971) and *Ostrea edulis* (Bayne, 1969), the gastropod mollusc *Crepidula fornicata* (McGee and Targett, 1989), the echiuran *Urechis caupo* (Suer and Phillips, 1983), the sipunculan *Golfingia misakiana* (Rice, 1986) and the echinoderms *Dendraster excentricus* (Highsmith, 1982; Burke, 1984) and *Echinarachinus parma* (Pearce and Scheibling, 1990) as well as certain polychaetes (see below).

Gregarious settlement of polychaete larvae was first described for *Spirorbis borealis* (= *S. spirorbis*) by Knight-Jones (1951b). Larvae were reported to settle preferentially on pieces of *Fucus serratus* which held previously settled individuals. The ability of larvae of *S. borealis and S. pagenstecheri* to distinguish conspecific adults was also investigated; larvae of each species settled preferentially on surfaces supporting adult conspecifics (Knight-Jones, 1951b). In the laboratory, gregarious settlement behaviour has been demonstrated for many species of tube-building marine polychaetes, including sabellariids (Wilson, 1968, 1970a; Jensen and Morse, 1984; Pawlik, 1986, 1988a,b; Jensen, 1992), spirorbids (Knight-Jones, 1951b; Wisely, 1960; Stebbing, 1972; Nelson, 1979) and serpulids (Crisp, 1977; Scheltema *et al.*, 1981; Marsden, 1991; Roscoe, 1993; Hadfield *et al.*, 1994; Toonen and Pawlik, 1994, 1996; Okamoto *et al.*, 1995; Bryan *et al.*, 1997; Chan and Walker, 1998). Knight-Jones (1951b) also reported that a higher percentage of larvae settled in assays with a large number of larvae compared to isolated larvae, suggesting larva-larva interactions and / or the induction of settlement by recently metamorphosed juveniles. Nott (1973) found that *S. spirorbis* larvae leave mucous threads behind whilst exploring a surface and suggested that these mucous threads may induce other larvae to settle.

Despite the evidence that chemical cues induce gregarious settlement of tube-building polychaetes, the exact nature of the cue has only been determined for the sabellariids *Phragmatopoma lapidosa californica* and *Phragmatopoma lapidosa lapidosa* (Pawlik, 1986; Jensen, 1992). Species of *Phragmatopoma* and *Sabellaria* have been shown to settle with a high degree of specificity on the sand tubes of adult conspecifics (Wilson, 1968, 1970a, b; Jensen and Morse, 1984; Pawlik, 1986, 1988a,b). Wilson (1968, 1970a, b) reported that *Sabellaria alveolata* larvae settle when they contact adult tubes or their remains, or the mucoid tubes of recently settled juveniles. Larval contact with the adult cement (used to bind sand grains for the adult tube) was found to be essential for induction of settlement, suggesting that the conspecific cue is insoluble in water. Interestingly, *S. alveolata* larvae were unable to discriminate between the cement of conspecifics and the closely related *S. spinulosa*. However, larvae of *S. spinulosa* were not induced to settle by *S. alveolata* conditioned sand. Jensen and Morse (1984) suggested that quinone tanned proteins (or a precursor or enzyme involved in their formation) were responsible for inducing gregarious settlement in *Phragmatopoma lapidosa californica*, an hypothesis previously advanced by Wilson (1968). Analogues of L-3,4-dihydroxyphenylalanine (L-DOPA) were reported to stimulate larval settlement. However, Pawlik (1998a) considers that larvae of *Phragmatopoma lapidosa californica* and *P. l. lapidosa* settle and metamorphose in response to specific free fatty acids (FFAs) isolated from the sand/cement matrix of adult tubes (Pawlik, 1986, 1988b), whereas larvae of *Sabellaria alveolata*, an ecologically equivalent species did not settle in response to FFAs (Pawlik, 1998a).

Serpulid larvae settle gregariously in the laboratory, particularly the larvae of colony-forming and reef-building species (ten Hove and van den Hurk, 1993). Crisp (1977) found that in the laboratory, tubes of conspecific animals enhanced settlement of the reef-building serpulid *Pomatoleios kraussii*. Scheltema *et al.* (1981) conducted laboratory experiments with the serpulid *Hydroides dianthus* and demonstrated that resident adults provide a strong settlement cue to competent larvae, with settlement of 90% or greater occurring in response to the presence of adult *H. dianthus*; as with Crisp (1977), no attempt was made to characterise the chemical cue. Marsden (1991) studied responses of larvae of the tropical serpulid *Spirobranchus polycerus* var. *augeneri* in an attempt to elucidate the mechanism of gregarious settlement. Responses of larvae to *Ulva lactuca* (common in the adult habitat), adult tubes, conspecific larvae and glass beads (control) were investigated but the larvae were prevented from contacting the material being tested, hence the behaviour of planktonic larvae to water-borne exudates only was investigated. Metatrochophore larvae showed a preference for exudates of adult tubes and to conspecific larvae of the same age. Again, no attempt was made to characterise the chemical cue and the amounts of material tested were arbitrary. Roscoe (1993) studied the settlement response of the intertidal serpulid *Pomatoceros lamarckii* and found that larvae settle preferentially on adult tubes and on slate surfaces biofilmed in the presence of adults. An experiment similar to that of Marsden (1991) demonstrated that planktonic *P. lamarckii* larvae were not attracted to adults removed from their tubes or empty tubes, suggesting that the settlement cue is effective in the adsorbed state (Roscoe, 1993). Attempts to isolate the chemical cue from conspecific adults and tubes were unsuccessful. Chan and Walker (1998) also working with *P. lamarckii* investigated the combined effects of a biofilm and the presence of conspecific adults upon settlement of *P. lamarckii* larvae. The gregarious settlement cue was found to be a water-soluble compound originating from the body of intact adult worms. This leachate induced greater than 75% settlement when adsorbed onto a biofilm, but was non-stimulatory in the absence of a biofilm; again no attempt was made to characterise the chemical nature of this cue. Toonen and Pawlik (1996) found that *Hydroides dianthus* larvae also settle in response to a water-soluble cue associated with the body of the adult worm. In the laboratory assays, settlement was significantly greater on the anterior section of adult tubes and could be induced by entire live adult worms removed from their tubes or by their amputated tentacular crowns, whereas dead worms, empty tubes and biofilmed slides induced only a low level of settlement; newly settled juveniles ($\geq$ 96 hours) also evoked a gregarious settlement response. Extractions made from adult worms with organic solvents removed the inductive capacity of the tissue and transferred it to both polar and non-polar fractions of an extraction series (Toonen and Pawlik, 1996). The authors suggest that in contrast to *P. lamarckii*, *H. dianthus* larvae may be able to perceive soluble chemical cues in the water column but only at very short range. Field observations confirmed aggregated distributions of the adult worms (Toonen and Pawlik, 2001a). Okamoto *et al.* (1998) investigated the gregarious settlement cue for *Hydroides ezoensis*. The conspecific cue was found to be contained in tube clumps and a partially purified fraction was obtained from a methanol extract that induced 50% larval metamorphosis. Further work (Watanabe *et al.*, 1998) identified a new monoacyl glycerol (isolated from the methanol fraction) as a metamorphosis-inducing substance. The compound induced greater than 50% metamorphosis at $3x10^{-6}$M. However, the authors note that since acyl glycerols are common primary metabolites that act as secondary messengers, this compound probably does not represent the primary cue for metamorphosis. Bryan *et al.* (1997) investigated induction of settlement and metamorphosis of *Hydroides elegans* larvae by cues emanating from adult conspecifics. Larval density was shown to affect percentage metamorphosis significantly with increased settlement densities occurring when larval concentrations reached 60 or more larvae per 5ml of seawater. Homogenates of the adult worm both in and removed from the tube evoked high percentage metamorphosis of 68 and 32 percent respectively, whereas the calcareous tube alone did not. The cue was found to induce higher percentage metamorphosis at specific concentrations. Attempts were made to characterise the cue which was found to be smaller than 10000 daltons and could be bound to amberlite XAD, suggesting that the substance may be a peptide or a compound of similar size and polarity. Bioassay guided chromatographic isolation of the larval settlement cue (Harder and Qian, 1999) using homogenates of adult *H. elegans* revealed that free amino acids were the inductive compounds that trigger settlement of *H. elegans* larvae; similar compounds in similar concentrations were isolated from the bryozoan *Bugula neritina* which also induces *H. elegans* larval settlement (Bryan *et al.*, 1998). However, Beckman *et al.* (1999) subsequently found that the metamorphic response was triggered by an inductive bacterial film rather than direct larval perception of amino acids. Despite extensive sterile measures, bacteria were found to be inadvertently introduced into assay dishes with larvae, the result suggests a systematic error in this established bioassay procedure whenever the cue being investigated may serve as a nutrition source for larval settlement-inducing bacteria.

In contrast to the findings of Bryan *et al.* (1997) which show that in Hong Kong, *Hydroides elegans* larvae are highly gregarious and settle in response to a conspecific cue, Walters *et al.* (1997) found that larvae from a Hawaiian population of *H. elegans* did not settle faster (in the laboratory) or in greater numbers (in the field) on surfaces supporting conspecifics. They suggest that hydrodynamics play an important role in creating the dense aggregations of *H. elegans* that are found on hard surfaces and propose that these aggregations most likely result from the passive deposition of larvae into the spaces between tubes of conspecific adults, followed by selective fixation in these locations. Hydrodynamics certainly play an important role in the settlement of marine invertebrate larvae (Eckman, 1983, 1996; Abelson and Denny, 1997; Eckman and Duggins, 1998) and a series of laboratory-based flume studies have demonstrated that for the larvae of *Phragmatopoma lapidosa californica*, flow conditions may play an important role in facilitating aggregations of conspecifics (Pawlik *et al.*, 1991). However, as with *H. elegans*, once larvae are delivered to the substratum, behavioural responses to chemical cues were considered ultimately responsible for selecting the final settlement site (Pawlik and Butman, 1993).

Studies on the settlement preferences of larvae of the gregarious serpulid *Hydroides dianthus* led Toonen and Pawlik (1994) to propose an interesting hypothesis; they suggest that adult *H. dianthus* produce larvae of two different behavioural classes termed founders and aggregators. The majority of larvae are the latter, exhibiting strong gregarious settlement behaviour and delaying metamorphosis in the absence of adult conspecifics. Founders will settle readily on a biofilm in the absence of conspecific cue(s). Subsequent work has shown that the proportion of founders produced by a female varies between individuals and that this variability appears to be genetically based (Toonen and Pawlik, 2001c).

A possible disadvantage of gregarious settlement behaviour is that individuals may become so crowded that they compete for space or food. Some species are adapted to minimise this problem by displaying spacing-out behaviour. Spacing-out behaviour has been documented for the larvae of spirorbids (Wisely, 1960) and barnacles (Crisp, 1961; Knight-Jones and Moyse, 1961), both of which are gregarious. Crisp (1961) showed that whilst cyprids are attracted to conspecifics, the final settlement process involves a slight movement away from the established individual. In contrast, spacing-out behaviour does not occur for the larvae of other gregarious species such as serpulids (Roscoe, 1993; Toonen and Pawlik, 1994).

Settlement in response to biofilms

Any solid surface submersed in the marine environment will develop a surface biofilm (an accumulation of organic and inorganic components and microorganisms including bacteria, diatoms and protozoans embedded in a matrix of extracellular polymeric substances (Wahl, 1989; Flemming *et al.*, 2000). Zobell and Allen (1935) first suggested that biofilms may play an important role in the settlement of marine invertebrate larvae and laboratory studies have demonstrated that biofilms or one or more of their constituent components, e.g. bacteria, diatoms, extracellular products may enhance (Knight-Jones, 1951b; Wilson, 1952; Neumann, 1979; Kirchman *et al.*, 1982a), inhibit (Crisp and Ryland, 1960; Maki *et al.*, 1988, 1990, 1992; Wieczorek *et al.*, 1995), or have no effect (Brancato and Woolacott, 1982; Maki *et al.*, 1988) upon the settlement preferences of a range of marine invertebrate larvae (see review by Wieczorek and Todd (1998)). These settlement responses are perhaps unsurprising given that any surface submerged in the sea develops a surface biofilm (Wahl, 1989; Chan and Walker, 1998). Although biofilms may inhibit or have no effect upon settlement, induction of settlement is common and has been demonstrated for polychaetes (Gray, 1966a,b; Kirchman *et al.*, 1982a; Roscoe, 1993; Hadfield *et al.*, 1994; Toonen and Pawlik 1994; Wieczorek, 1995; Unabia and Hadfield 1998), barnacles (LeTourneux and Bourget, 1988), hydroids (Müller, 1969, Freeman and Ridgway, 1987; Leitz and Wagner, 1993), scyphozoans (Schmahl, 1985; Hofmann and Brand, 1987), bivalves (Cole and Knight-Jones, 1949; Weiner *et al.*, 1985, 1989; Fitt *et al.*, 1989, 1990; Satuito *et al.*, 1995 Fusetani, 1998), gastropods (Scheltema, 1961, Morse *et al.*, 1984), bryozoans (Mihm *et al.*, 1981; Brancato and Woolacott, 1982; Mitchell and

Maki, 1988), echinoderms (Cameron and Hinegardner, 1974; Chen and Run, 1989; Johnson *et al.*, 1991; Pearce and Scheibling, 1991) and ascidians (Szerzyk *et al.*, 1991; Wieczorek and Todd, 1997).

In nature, biofilms have the capacity to vary in composition, growth phase and metabolic activity in response to the conditions pertaining at a particular site (Baier, 1984). Consequently, biofilms may be expected to vary considerably between different locations which have differing physical, biological and chemical regimes operating (Keough and Raimondi, 1995) and it has been proposed that such parameters are interpreted by marine invertebrate larvae in selecting an appropriate settlement site (Neumann, 1979; Wieczorek *et al.*, 1995). Certainly biofilms have the capacity to provide larvae with an integrated impression of environmental conditions at a particular site (Strathmann *et al.*, 1981; Neal and Yule, 1994) and studies have demonstrated that the larvae of certain species are capable of differentiating between biofilms of varying bacterial species composition (Kirchman *et al.*, 1982a; Unabia and Hadfield, 1999), ages (Maki *et al.*, 1988, 1990, 1992; Pearce and Scheibling, 1991; Szewzyk *et al.*, 1992; Wieczorek *et al.*, 1995; Keough and Raimondi, 1995; Tsurumi and Fusetani, 1998), volume (Tsurumi and Fusetani, 1998), bacterial densities (Hadfield *et al.*, 1994; Hamer *et al.*, 2001) and origins (Strathmann *et al.*, 1981; Keough and Raimondi, 1996) as well as between biofilms developed under different shear regimes (Neal and Yule, 1994) during the early settling process.

Wilson (1951, 1952, 1955) first demonstrated the ability of polychaete larvae to respond to biofilms when he found that larvae of *Ophelia bicornis* settle preferentially on sand of the kind in which adults are found. He considered that "The factors most active in inducing metamorphosis and settlement of *O. bicornis* is the presence on sand grains of microorganisms, such as bacteria…", he also speculated that "…certain species [of microorganisms] are more effective in promoting settlement than others, though there is at present no clue to the identities of the species concerned". Similar selective settlement on sediments obtained from the adult habitat has been reported for the larvae of many polychaete species, including *Owenia fusiformis* (Wilson, 1932), *Notomastus laticerus* (Wilson, 1937), *Pygopsio elegans* (Smidt, 1951), *Chaetopterus variopedatus* (Werner, 1953), *Phragmatopoma lapidosa californica* (Pawlik e*t al.*, 1991; Pawlik and Butman 1993), *Capitella* sp. I (Grassle *et al.*, 1992) and *Arenicola marina* (Hardege *et al.*, 1998); both surface biofilms and gregarious cues may be involved in these settlement preferences.

The larvae of many species of calcareous tube-building polychaetes (spirorbids and serpulids) discriminate between and settle in response to biofilms in the laboratory (Knight-Jones, 1951b; Wilson, 1952, 1955; Meadows and Williams 1963; Kirchman *et al.*, 1982a; Miura and

Kajihara, 1984; Roscoe 1993; Toonen, 1993; Hadfield *et al.*, 1994; Toonen and Pawlik, 1996; Lau and Qian, 1997; Chan and Walker, 1998; also, see Hamer *et al.*, 2001). Knight-Jones (1951b) first noted that larvae of *Spirorbis borealis* (= *S. spirorbis*) settled preferentially on surfaces supporting a biofilm and delayed metamorphosis in the absence of a biofilm; he also noted that the exploratory behaviour of the larvae was different when they contacted a biofilm rather than an unfilmed surface. A biofilm has now been shown to be a prerequisite for the settlement of larvae of the serpulids *Hydroides dianthus* (Scheltema *et al.*, 1981; Toonen and Pawlik, 1994), *Hydroides elegans* (Unabia and Hadfield, 1999) and *Pomatoceros lamarckii* (Roscoe, 1993; Chan and Walker, 1998), and some species have been shown to settle preferentially on older biofilms (Hadfield *et al.*, 1994; Miura and Kajihara, 1984; Chan and Walker, 1998; Hamer *et al.*, 2001). To date, there are no reports of serpulid or spirorbid larval settlement inhibition by natural, multispecies biofilms (Hamer *et al.*, 2001).

Despite the numerous laboratory studies on the effects of biofilms upon marine invertebrate larval settlement, relatively little is known about the ecological significance of such behaviour. For various reasons, most of the field studies carried out that investigate the importance of biofilms upon marine invertebrate larval settlement have focussed on sessile organisms which colonise hard substrata. Some of the benefits of using sessile invertebrate larvae are that the settlement response of such species has been comparatively well studied in the laboratory, recruits can be identified relatively easily and experimental panels are reasonably easy to prepare, deploy, retrieve and process (number of settled individuals are counted). To date, only a small number of these studies have been carried out (Wieczorek and Todd, 1998) with only a few including observations on the recruitment of more than one species (Todd and Keough, 1994; Keough and Raimondi, 1995; Keough and Raimondi, 1996). These studies have shown that biofilms influence the recruitment of certain invertebrate species under field conditions (Strathmann and Branscomb, 1979; Strathmann *et al.*, 1981; Bourget, 1988; Raimondi, 1988; Wieczorek and Todd, 1998; Miron *et al.*, 1999; Olivier *et al.*, 2000). Strathmann *et al.* (1981) demonstrated a facilitatory effect of a microbial film on the cypris larvae of the barnacles *Balanus glandula* and *Semibalanus cariosus* in the field. Cyprids settled preferentially on plates with biota characteristics of the lower intertidal shore suggesting that some component of the microflora provides a settlement cue to the larvae which allows them to locate and settle at the optimum tidal height. Wieczorek *et al.* (1996) studied temporal variations in larval settlement in the field by recording settlement responses to biofilming throughout the year. Interestingly, a reversal of the effect of biofilming on larval settlement response was noted with season for a number of bryozoan species including *Electra pilosa*, *Tubulipora* sp./ *Plagioecia* sp.. Raimondi (1988) showed that removal of the cyanobacterium *Calothrix anisopoma* from the natural hard substrata induced settlement of *Chthamalus anisopoma* above the normal vertical intertidal limit for this barnacle species. The recruitment of serpulid and spirorbid polychaetes in field studies seems to be particularly consistent with the findings of laboratory studies, i.e., a preference for a biofilm and selective settlement on older biofilms. During field experiments, Todd and Keough (1994) found that although the response of different species to the presence of a biofilm varied considerably, unidentified serpulids and spirorbids generally responded positively to the presence of a biofilm; total recruitment summed across all taxa also increased with the presence of a biofilm. Following on from this work, Keough and Raimondi (1995) investigated the effect of biofilm age upon recruitment of sessile taxa in the field. For all taxa, total recruitment increased with age of biofilm, almost doubling from unfilmed surfaces to those with 6-day old biofilms. Among the species examined, two species of polychaetes showed a positive relationship with age of biofilm (0, 1, 3 and 6 day old biofilms). Keough and Raimondi (1996) also investigated the effects of natural large scale variations of biofilms; at all sites tested, the most attractive surface for larval settlement was the densest biofilm. In this series of experiments, serpulid recruitment varied significantly between large temporal and spatial variations in biofilms; the effect of these parameters upon spirorbid recruitment was less apparent. It is unclear why larvae of serpulids and spirorbids settle preferentially on well developed biofilms but from an adaptive perspective it has been suggested that a biofilm may indicate a relatively stable underlying substratum with a good water flow regime over it (Wieczorek and Todd, 1998). Also, Keough and Raimondi (1996) note that spirorbids and serpulids are poor spatial competitors that are easily overgrown. Older biofilms may therefore indicate a habitat where settled individuals are likely to become fecund before being overgrown, an hypothesis also advanced by Wieczorek & Todd (1998) and Unabia and Hadfield (1999).

The mechanism(s) by which larvae are able to differentiate between certain microbial films and sense settlement cues are little understood. It has been suggested that microbial films may mediate settlement via physical properties such as surface wettability (Mihm *et al.*, 1981; Maki *et al.*, 1989, 1992; Roberts *et al.*, 1991) or directly by providing chemical cues, either surface bound or water borne, which stimulate settlement (see Pawlik, 1992). For example, Maki *et al.* (1989) suggested that bacteria could effect larval settlement by altering the environment of a surface by projection of extracellular domains which change the chemistry, charge and free energy characteristics of a surface. Alternatively, Szewsky *et al.* (1991) proposed that exopolysaccharides produced by the bacterium *Pseudomonas* sp. strain S9, facilitates settlement of larvae of *Ciona intestinalis via* simple physical entrapment. Attempts have been made in the laboratory to correlate biofilm characteristics with larval settlement preferences. It has been suggested that larvae may be capable of responding to individual constituents of biofilms including diatoms (Meadows and Williams, 1963; Harder *et al.*, 2002), bacterial numbers (Hadfield *et al.*, 1994), bacterial exopolymers (Maki *et al.*, 1988; Holmström *et al.*, 1992; Maki *et al.*, 1990), bacterial species composition (Lau and Qian, 1997), specific molecular components of bacterial exopolymers (Maki *et al.*, 1990, 1992) and general biofilm characteristics such as thickness and volume (Tsunami and Fusetani, 1998).

Settlement in response to bacteria and bacterial products

By selectively altering biofilms, or by assaying larvae against specific biofilm components, workers have established that the bacteria present in biofilms are an important settlement cue to many marine invertebrate larvae (see review by Wieczorek and Todd, 1998). For example, the treatment of bacteria with antibiotics has been shown to inhibit metamorphosis in the sand dollar *Arachnoides placenta*, inferring that bacteria play a role in inducing larval metamorphosis (Chen and Run, 1989). Avelin Mary *et al.* (1993) suggested that further work with bioactive natural products such as antibiotics and juncellins would help to probe bacterial-macrofouler interactions. Numerous studies have shown that marine invertebrate larvae are capable of sensing and responding to monospecific bacterial films and it is now apparent that different bacterial strains elicit various settlement responses for a wide range of species (Hoffman and Brand, 1987; Neumann, 1979; Maki *et al* 1988, 1990, 1992; Kirchman *et al.*, 1982a; Leitz and Wagner, 1993; O'Connor and Richardson, 1996; Lau and Qian, 1997, 2001; Unabia and Hadfield, 1999; Lau *et al.*, 2002). Several workers have now isolated and screened several bacterial strains for their settlement-inducing ability. Typically, some of the strains tested induce settlement, whilst others do not. It is interesting that variable settlement responses have been reported for different marine invertebrate larvae in response to a single bacterium species. Maki *et al.* (1988, 1990) have shown that the bacterium *Deleya marina* inhibits the settlement of *Balanus amphitrite*, whereas a *D. marina* film facilitated settlement of the bryozoan *Bugula neritina* (Maki *et al.*, 1989). The same bacterial strain stimulates settlement of the spirorbid polychaete *Janua brasiliensis* (Kirchman *et al.*, 1982a). Neal and Yule (1994) found that extra-cellular material of *D. marina* decreased the temporary adhesion (a surrogate measure of surface attractiveness) of *Elminius modestus* cyprids. However, a young biofilm (4 days old) increased temporary adhesion and older films (1 month) decreased adhesion in 3 out of 5 cases. The authors suggest separate stimulatory and inhibitory active agents which vary within the biofilm in close association with the development stage of the microbial film. Unfortunately, to date no study has investigated the settlement-inducing properties of various bacterial strains to a range of marine invertebrate larvae simultaneously; without such research it is premature to conclude that marine invertebrate larvae respond differently to particular bacterial strains.

Investigations into the settlement preferences of larvae of the polychaete *Ophelia bicornis* were the first to suggest that the different biofilm microorganisms might vary in their ability to promote larval settlement (Wilson, 1955). Meadows and Williams (1963) found that larvae of *Spirorbis borealis* (= *S. spirorbis*) settled readily on surfaces conditioned with certain diatoms together with their associated bacteria and suggested that variations in the constituents of such biofilms may affect settlement. Kirchman *et al.* (1982a) showed that larvae of the spirorbid *Janua brasiliensis* settle preferentially on biofilms and favoured mixed biofilms (composed predominantly of bacteria) over diatom biofilms, suggesting that bacteria induce settlement. Furthermore, only certain bacterial strains isolated from the surface of *Ulva lobata* (the adult habitat) induced settlement. Subsequent experiments showed that larvae would not settle in the presence of glucose (a constituent of bacterial exopolymers) or on surfaces treated with the lectin concanavalin A (binds to glucose and mannose) (Graham *et al.*, 1980; Kirchman *et al.*, 1982b; Mitchell and Kirchman, 1984; Maki and Mitchell, 1985) leading the authors to suggest that larval lectins mediate larval settlement by binding to specific extracellular bacterial polysaccharides, probably a glucose polymer (Maki and Mitchell, 1985). They also suggested that lectins might play a widespread role in the recognition of suitable microbial films for settlement by larvae of other sessile marine organisms (Mitchell and Kirchman, 1984); this suggestion remains untested. Working with *Pomatoceros lamarckii* larvae, Roscoe (1993) noted that bacterial number and species composition of films varied between films developed under static and running seawater and in the presence and absence of adult *P. lamarckii.* He found that larvae settled preferentially on surfaces filmed in the presence of adults which had the highest bacterial density; no larvae settled on the film developed in still water in the absence of adults (very low numbers of bacteria) or on clean control surfaces. Following on from this work, Hamer *et al.* (2001) found a close correlation between percentage settlement and bacterial density for biofilms of 1, 2 and 3 weeks old and suggested that bacteria or their extracellular components may be the settlement inductive cue in biofilms. Similarly, Hadfield *et al.* (1994) showed that larvae of *Hydroides elegans* were able to distinguish between microbial films ranging in age from 1 to 3 days and found that larval settlement densities correlated with the density of short rod-shaped bacteria in the biofilms. The authors suggested that larvae are able to assess the density of bacteria and respond to this factor at settlement. Further work (Unabia and Hadfield, 1999) demonstrated that *H. elegans* larvae discriminate between monospecific bacterial films and that different bacterial strains induce, did not induce, or had no effect upon settlement compared to the natural, multispecies biofilm control. Lau and Qian (1997) also found that *H. elegans* larvae settle in response to certain monospecific and mixed species bacterial films but not others. In addition, settlement in response to films composed of more than one bacterial strain gave a settlement response that was intermediate and dependent upon the relative ratio of the two strains compared to the settlement response for the respective individual strains. Despite the observation that serpulid and spirorbid larave settle in response to certain bacterial strains and not others, no obvious correlation between bacterial strain and inductive effect has been reported. Unabia and Hadfield (1999) found that of the strains tested, 13 induced settlement, 11 gave moderate or mixed results and 10 strains did not induce settlement. They concluded that the larvae are responding to either multiple cues or that the cue is common to many bacteria because such a wide diversity of bacterial strains induced settlement. Lau *et al.* (2002) tested the effect upon settlement of *H. elegans* of 38 bacterial strains; settlement was induced by bacteria from a wide range of taxa.

Subjecting natural biofilms or monospecific bacterial films to various chemical or physical treatments and then conducting larval assays has proven useful in revealing the nature of the inductive biofilm cue for various species of polychaetes (Kirchman *et al.*, 1982a; Kirchman and Mitchell, 1985; Unabia and Hadfield, 1999; Hamer and Walker 2001; Lau and Qian 2001). The rationale behind this method being that if a particular treatment reduces settlement then the particular biofilm component(s) affected by the treatment may represent the settlement cue. Investigations have shown that the settlement of *Hydroides elegans* larvae is reduced on metabolically inactive biofilms (Unabia and Hadfield, 1999; Lau and Qian, 2001) whereas, *Spirorbis spirorbis*, *Janua brasiliensis* and *Pomatoceros lamarckii* larvae settle in comparable numbers on such metabolically inactive biofilms (Kirchman *et al.*, 1982b; Hamer *et al.*, 2001). Unabia and Hadfield (1999) found that all physical and chemical treatments used to kill bacteria either eliminated or severely reduced the settlement-inducing effects of natural, multi-species biofilms to *H. elegans* larvae. Similarly, Lau and Qian (1997) reported that treating mono-specific bacterial films with formalin or antibiotics stopped the normal *H. elegans* larval settlement response. Formaldehyde kills bacteria by denaturing protein whereas the antibiotics streptomycin and kanamycin kill bacteria by disrupting protein synthesis (Lau and Qian, 2001). Therefore, as suggested by Lau and Qian (2001), since the bacterial cell surface is mainly comprised of a matrix of polysaccharides with <1% protein (Decho, 1990), these formalin and antibiotic treatments should cause little disruption to the surface chemistry of the bacterial cells. Lau and Qian (2001) therefore propose that for *Hydroides elegans* larvae, the bacterial settlement cue may be proteinaceous in nature and be derived from a metabolic pathway of the bacteria , being exported to and concentrated in the extracellular polymer layer outside the bacterial cells.

Despite the obvious importance of marine bacteria in the settlement of marine invertebrate larvae, the exact nature of the inductive cue has not been determined for any of the species studied to date. Bacterial components such as exopolysaccharides, proteins and fatty acids have been isolated and demonstrated to either increase or decrease the settlement of larvae (Kirchman *et al.*, 1982a; Maki and Mitchell, 1985, Maki *et al.*, 1990; Szewzyk *et al.*, 1991; Goto *et al.*, 1992; Holmström *et al.*, 1992, 1996; Kitamura *et al.*, 1993; Leitz and Wagner, 1993). Kirchman *et al.* (1982b) provide the most detailed account of the bacterial cue, suggesting that a glucose polymer contained in extracelluler bacterial polysaccharides may induce settlement of the spirorbid *Janua brasiliensis* (Maki and Mitchell, 1985). Bacterial exopolymer production is growth phase dependent and influenced by environmental conditions (Fletcher and Marshall, 1982). It may therefore be the component of biofilms that provides information relating to factors such as biofilm age, composition and condition to the larvae that are indicative of the ambient environmental conditions. Indeed, specific growth stages and / or nutrient conditions have been reported to be necessary for some bacteria to produce stimulatory factors for larval settlement (Müller, 1973; Weiner *et al.*, 1985). Bacterial extracellular polymers are also considered by Unabia and Hadfield (1999) to be likely candidates for inducing the settlement of the serpulid *H. elegans.*

Settlement in response to surface-bound or water-borne cues

Chemical cues were originally thought to be insoluble surface-bound chemicals that were recognised by larvae in the adsorbed state and many reports confirmed this hypothesis (Crisp and Meadows, 1963; Suer and Phillips, 1983). However, the chemical cue which elicits a settlement response may, in some instances, be a soluble, water-borne compound (Tamburri *et al.*, 1992; Coon *et al.*, 1985, 1990; Turner *et al.*, 1994; Zimmer-Faust and Tamburri, 1994; Lau and Qian, 2001). Bonar *et al.* (1990) demonstrated that the larvae of the oyster *Crassostrea gigas* respond to unknown dissolved chemical inducers found in supernants of cultures of bacteria and following size exclusion chromatography, the inductive activity was retained in a fraction with molecular weight ≤300 daltons (Fitt *et al.*, 1990). A number of researchers have investigated the ability of sedentary polychaete larvae to respond to water-borne cues and have shown that there are between-species differences in the ability to sense such cues. Kirchman *et al.* (1982b) considered that the inductive biofilm cue for *Janua brasiliensis* is extracellular polysaccharide found on the bacterial cell surface. Roscoe (1993) showed that planktonic larvae of *P. lamarckii* were not attracted to water-borne leachates from intact adults or their tubes and Chan and Walker (1998) found that although the gregarious settlement cue is a water soluble leachate emanating from the body of intact adult worms, it only induced larval settlement when adsorbed onto a biofilm. Toonen and Pawlik (1994, 1996) had earlier reported a similar situation for *Hydroides dianthus* larvae. Unabia and Hadfield (1999) found that settlement of larvae of *Hydroides elegans* was stimulated by the soluble products of all three bacterial strains tested, whereas Lau and Qian (2001) found that neither seawater conditioned by bacteria attached to Petri dishes nor the seawater conditioned by bacteria in suspension was inductive to larval settlement. Despite the ability of some species to respond to water-borne cues, Pawlik (1992) notes that in general, contact with the substratum is required for recognition of a naturally occurring settlement inducing chemical cue (Knight-Jones, 1953b; Morse and Morse, 1984a; Pawlik, 1986; Raimondi, 1988; Chan and Walker 1998). Other settlement cues may also be active in the water-borne state. For example, Marsden (1991) found that metatrochophore larvae of *Spirobranchus polycerus* var. *augeneri* swam towards adult tubes and to conspecific larvae, suggesting that they were responding to water-borne exudates. However, for the majority of sedentary polychaetes, settlement requires larval contact with a surface-bound cue.

Multiple settlement cues and extrapolation of results to the field

Studies in the laboratory are artificial and contrived since they are specifically designed to test hypotheses (Walker, 1995), with the majority of studies designed to investigate the influence of a single or occasionally a few factors upon larval settlement. Newell (1979) notes that there appears to be a hierarchy of factors involved with the location of a suitable settlement site by a marine invertebrate larva. This hierarchy has been viewed in terms of an instinctive sequence with a graduation of physical and chemical 'releasers' for behavioural responses which culminate in settlement (Crisp and Meadows, 1963). In the laboratory, sedentary polychaete larval settlement is influenced by a suite of factors, including water flow, turbulence, illumination, surface rugosity, surface contour, surface colour, surface orientation, surface biofilm, presence of conspecifics, presence of other species (Knight-Jones, 1951b; Wisely, 1958, 1960; De Silva, 1962; Wilson, 1968; Kirchman *et al.*, 1982a; Roscoe, 1993; Toonen and Pawlik, 1994, 1996; Bryan *et al.*, 1997; Chan and Walker, 1998; Unabia and Hadfield, 1999; Hamer *et al.*, 2000), whereas in nature none of these factors will be experienced in isolation by larvae, because the factors influencing settlement act simultaneously, with their degrees of influence operating at different spatial and temporal scales (Hudon *et al.*, 1983; Wethey, 1986; Bourget, 1988; LeTourneux and Bourget, 1988). The current challenge is to investigate the relative importance and hierarchies of the various cues important to larval settlement either in the laboratory under more realistic conditions and / or by testing the interaction of different variables under field conditions. More realistic laboratory studies have already been carried out for certain species, for example, Pawlik *et al.* (1991) investigated the interaction of water flow and larval choice in the settlement of *Phragmatopoma lapidosa californica* larvae and suggested that hydrodynamics play an important role in larval supply but that larval selectivity was responsible for the ultimate distribution of larvae; more studies of this nature are needed. As pointed out by Wieczorek and Todd (1998), 'major difficulties are perceived when extrapolating from the laboratory to the field'. Combined laboratory and field studies can be achieved (Wethey, 1986; O'Connor and Richardson, 1996; Thompson *et al.*, 1998), and are important as they should provide information on how the results from laboratory studies may be extrapolated to field conditions (Walker, 1995). Such studies to date have demonstrated that contrasting results may be obtained between the laboratory and the field, for example, Thompson *et al.* (1998) found that although biofilms were an important cue to barnacle cyprid settlement in the laboratory, settlement in the field was predominantly influenced by conspecifics which seemed to supersede the biofilm cue. Field based investigations which test multiple hypotheses simultaneously have also begun and are providing an important insight into the relative importance of factors controlling settlement, recruitment and the distributions of the adult. Miron *et al.* (1999) showed that the vertical distribution of *Semibalanus balanoides* on the shore results from complex relationships including larval supply, selection of habitat, availability of space and early post-settlement mortality. In a similar study, Olivier *et al.* (2000) investigated the influence of larval supply, tidal level, biofilm quality and biofilm age upon the recruitment of *Balanus amphitrite*. They also found that the degree of microbial pre-colonisation was the main variable affecting settlement, with cyprids settling preferentially on cleaner versus more heavily biofilmed surfaces. For this species, free space (minimal biofilm) availability is therefore a major factor controlling recruitment. Similar studies with sedentary polychaetes should now be attempted.

Aims of this study

The current study investigates the settlement preferences of serpulid (*Pomatoceros lamarckii*) and spirorbid (*Spirorbis spirorbis* and *S. tridentatus*) larvae in response to biofilms or their individual components through laboratory-based settlement assays and field settlement experiments. In the laboratory, biofilms induce larval settlement of all serpulids and spirorbids studied to date, including those of *P. lamarckii* (Roscoe, 1993; Chan and Walker, 1998), *S. spirorbis* (Knight-Jones, 1951b) and *S. tridentatus* (De Silva, 1962). Having considered the literature on the role of biofilms in serpulid and spirorbid larval settlement, the following work was undertaken:

i. The first experimental chapter investigates the larval development and settling behaviour of *Pomatoceros lamarckii* larvae.

ii. Given the intertidal zonation pattern of *Pomatoceros lamarckii* and the potential for intertidal biofilms to act as a cue to the adult habitat, the second experimental chapter investigates the effect of aerial drying of a biofilm upon larval settlement preferences.

iii. Because biofilm aerial drying was found to negate the previously settlement inducing effect of a biofilm to *Pomatoceros lamarckii* larvae, the following experimental chapter explores the effect of drying a biofilm upon the settlement preferences of the larvae of other species (spirorbids and bryozoa).

iv. *Pomatoceros lamarckii, Spirorbis spirorbis* and *S. tridentatus* larvae are useful subjects for use in laboratory settlement assays. This chapter investigates how the way in which assays are conducted (single-treatment versus multiple-treatment assays) may influence the perception of larval settlement preferences.

v. Because *Spirorbis spirorbis* larvae settle preferentially on biofilmed surfaces and because bacteria have been implicated in the settlement of the spirorbid *Janua brasiliensis*, the settlement response of *S. spirorbis* larvae to mono-specific bacterial films isolated from the field is investigated.

vi. The previous chapter demonstrates that bacteria may induce settlement of *Spirorbis spirorbis* larvae. An alternative approach was adopted in this chapter and attempts were made to identify the nature of the inductive settlement cue by chemical manipulation of natural biofilms.

vii. In laboratory assays, biofilms are an important settlement cue to *Pomatoceros lamarckii* larvae. Observations made during the second experimental chapter showed that drying a biofilm negates the previously settlement-inducing effect. The final experimental chapter investigates the potential for intertidal biofilms to act as a settlement cue to *P. lamarckii* larvae in both the laboratory and the field.

2. Developmental morphology, settlement behaviour and artificial induction of metamorphosis of laboratory reared *Pomatoceros lamarckii* (Annelida: Polychaeta: Serpulidae) larvae.

Abstract

Scanning electron microscope (SEM) observations of laboratory-reared *Pomatoceros lamarckii* larvae have confirmed the general observations made by previous workers. In addition, the SEM has revealed several morphological features such as gland pores and ciliary tufts not reported previously for the larvae of *Pomatoceros* sp. Larval behaviour during settlement also closely follows the sequence described for other tubeworm species. Excess K^+ ions in seawater were found to induce metamorphosis of *P. lamarckii* larvae.

Introduction

Most serpulids are broadcast spawners, releasing their gametes into the water column where fertilisation takes place to give planktonic 'trochophore' larvae (Kupriyanova *et al.*, 2001). This larval type represents the prototype polychaete larval form (Heimler, 1988). Various aspects of the development and morphology of serpulid larvae have been studied at the light, scanning and transmission electron microscope levels (Heimler, 1988) and studies to date have shown that serpulids are conservative in their larval morphology (Kupriyanova *et al.*, 2001). Larvae develop from a ciliated ball of cells to a free-swimming trochophore stage. After some time feeding, larvae develop additional annuli of cilia, the body begins to lengthen and the neurotroch develops. Finally, the ciliated bands begin to regress and a 'neck' is apparent in the 3 setiger 'metatrochophore' larva. The metatrochophore larva is capable of substratum exploration and when a suitable surface is encountered it attaches itself, undergoes a rapid, irreversible metamorphosis and becomes permanently attached to the substratum by the production of a calcareous tube around the body.

Segrove's (1941) description of the larval development of *P. triqueter* based upon observations at the light microscope level remains the classical account of serpulid larval development. Descriptions of serpulid larval developmental morphology have also been given for *Galeolaria caespitosa* (Andrews and Anderson, 1962; Marsden and Anderson, 1981), *Hydroides ezoensis* (Miura and Kajihara, 1981), *H. norvegica* (Wisely, 1958), *H. uncinatus* (Shearer, 1911), *Pomatoleios kraussii* (Crisp, 1977), *Protula tubularia* (Tampi, 1960), *Rhodopsis pusilla* (Nishi and Yamasu, 1992a) and *Spirobranchus giganteus* (Smith, 1984).

Grant (1981) was the first to describe the development of serpulid larvae, in this case *G. caespitosa*, based upon scanning electron microscopic (SEM) observations. Since this time, the external morphology of serpulid larvae has only been described for a few species including *Pomatoceros triqueter* (Moat, 1985), *P. lamarckii* (Roscoe, 1993) and *Salmacina dysteri* (Nishi and Yamasu, 1992b).

Pomatoceros lamarckii is a common intertidal and shallow subtidal serpulid (Hayward and Ryland, 1995) that displays the typical serpulid developmental mode described above (Roscoe, 1993; Giangrande, 1997). Gametes are released into the water column where fertilisation takes place, the planktotrophic larvae spend several weeks in the water column before undergoing settlement and metamorphosis into the sessile juvenile tubeworm form. Von Drasche (1884) first described the larval metatrochophore stage of *Pomatoceros* from the plankton. Segrove (1941) gave a detailed account of the development of *Pomatoceros triqueter*, although Crisp and Ekaratne (1984) considered that it is probable that the species described was *P. lamarckii*. Moat (1985), studying *P. triqueter*, largely repeated and confirmed the work of Segrove (1941) and gave an account of the external morphology of *P. triqueter* larvae based upon SEM observations. Roscoe (1993) described the development of *P. lamarckii* (light and SEM observations), but added little additional detail to the earlier observations of the larvae of *Pomatoceros* spp. made by Segrove (1941) and Moat (1985). The following account describes the development, settlement and metamorphosis of *P. lamarckii* larvae at the SEM level, paying particular attention to gland openings and sensory structures. The artificial induction of metamorphosis by K^+ ions is also investigated.

Materials and methods

Gamete collection and larval culture

Collections of adult *Pomatoceros lamarckii* on small rocks were made between May and October from shores of the Menai Strait, Anglesey, North Wales, U.K.. Gamete collection and larval culture were based upon methods described in detail by Roscoe (1993). Briefly, the rear end of a tube was broken open using a carpenter's bradawl and the worm pushed backwards by inserting a thin wire into the opening at the front of the tube. Eventually the worm is forced free of the tube. Entire worms were transferred to a watch glass containing approximately 10 ml of 0.2 μm filtered, u.v.-sterilised seawater (FSW). Adult *P. lamarckii* are dioecious and the sex of mature worms can be discriminated by the colour of the abdomen; females are pink and males are white / cream. For each fertilisation, the gametes liberated from several females and males were used. Gametes that were released from the body of the worm were collected using a Pasteur pipette. Eggs and a dilute sperm suspension were mixed in a 50 ml glass beaker and left for 30 minutes to allow fertilisation. After this time, most of the eggs have settled to the bottom of the beaker, allowing the overlying sperm suspension to be decanted off and discarded. Fresh FSW was added and the decanting process repeated twice to remove excess sperm.

Larvae were cultured in 2 l Pyrex glass beakers and fed on the microalgae *Rhinomonas reticulata* and *Tetraselmis chui* at a ratio of 1:1 and overall concentration of 200 cells ml $^{-1}$. Cultures were incubated at 19°C in a temperature-controlled cabinet with 12:12 hour light:dark cycle provided by a single 8 W fluorescent tube. All glassware was routinely cleaned in a 5% final concentration Chloros solution, rinsed in tap water followed by distilled water and allowed to dry.

Larval development - light microscopy

Fixed and live larvae were observed at various magnifications under compound and dissecting microscopes. Photomicrographs were taken with a Leitz Orthoplan photomicroscope using Agfa black and white 200 ASA film.

For sectioning, larvae were fixed in 2.5 % glutaraldehyde in 0.1 % phosphate buffer for 1 hour followed by post-fixation in 1 % osmium tetroxide in 0.1 % phosphate buffer for 1 hour. Following fixation, specimens were briefly rinsed in distilled water then dehydrated in an ascending ethanol series (30, 50, 70, 90, 100 %; 30 minutes each) and routinely embedded in Spurrs resin. Thick (1 μm) longitudinal larval sections were cut with a diamond knife using an LKB Ultratome III ultramicrotome. Thick sections were transferred to glass slides and stained with a solution of 1 % toluidine blue in 1 % aqueous sodium tetraborate on a hot plate and rinsed off thoroughly with distilled water before viewing with a compound microscope. Dry sections were mounted in DPX under a glass coverslip for photomicrography.

Larval development - scanning electron microscopy

Larvae of varying ages were relaxed for 1 hour in either 0.05 % MS222 or a 7 % Mg_2Cl in FSW solution, rinsed quickly in FSW and fixed for a minimum of 30 minutes and a maximum of 2 days in 2.5% glutaraldehyde in FSW. After fixation, samples were rinsed quickly in distilled water, dehydrated in an ascending ethanol series (30, 50, 70, 90, 100 %; 30 minutes each) and transferred to 100% acetone for 30 minutes before critical point drying from liquid carbon dioxide in a Polaron E3000 critical point drying apparatus. The dehydration process was carried out in a number of ways depending upon the size of the larvae. Small larvae (1-3 days) were placed in wells (4 mm diameter) drilled in a brass container with the ends covered by 5 μm Millipore filters (Figure 1). Larger larvae were held in small tubes (14 x 8 mm) with removable end caps; the end caps had their central sections removed and were replaced by 45 μm mesh plankton net as described by Roscoe (1993). Once critically point dried, larvae were transferred onto aluminium stubs covered with double-sided Sellotape and coated with gold using a Polaron E5000 Sputter Coater before examination in a Cambridge Stereoscan S120 scanning electron microscope operated at 10 KeV. Photomicrographs were taken with a Canon camera using Agfa black and white 100 ASA film. Larvae of *Spirorbis spirorbis* and *S. tridentatus*, prepared using the same protocol, were used for comparative purposes.

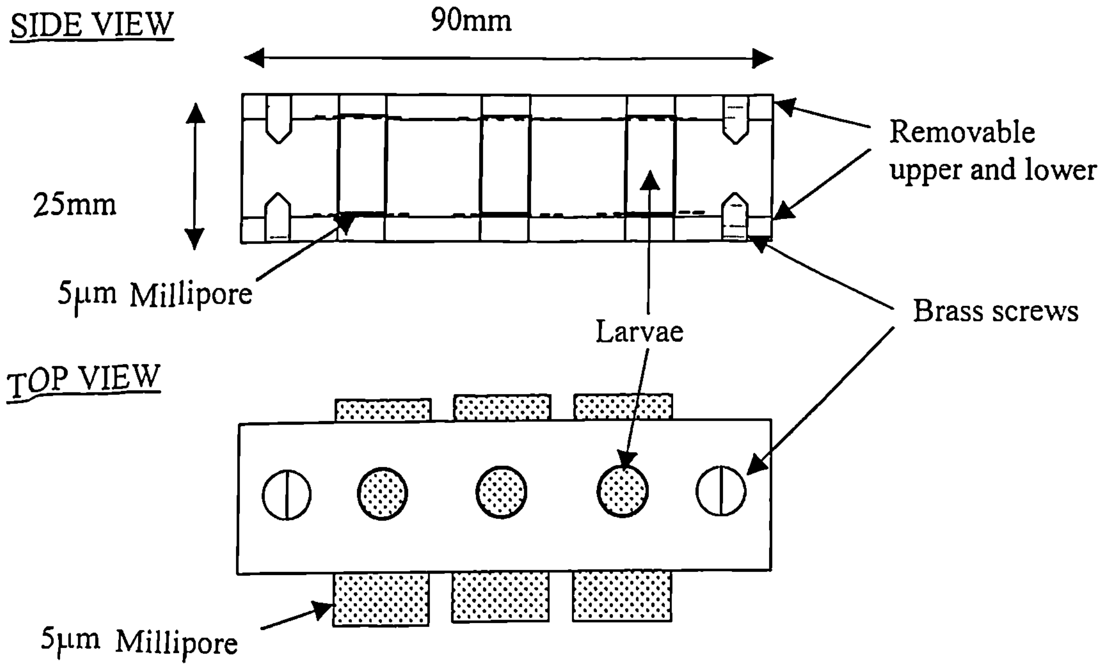


Figure 1. Lateral and top views of brass container used for the dehydration and critical point drying of small biological specimens. Larvae were retained in larval containers with 5 μm Millipore filters.

Exploration and settling behaviour

Larval exploratory and settling behaviour was observed under a dissecting microscope at various magnifications. Settlement was also recorded using a video camera on a trinocular head dissecting microscope linked to a video recorder. A small Petri dish (38 mm diameter) was allowed to develop a biofilm for 3 months in the laboratory running seawater system. The dish was wiped clean underneath, allowing larval exploration and settlement to be recorded at various magnifications. Some larval exploration behaviour was also captured directly to PC using the same microscope and camera set-up.

Artificial induction of metamorphosis

Metatrochophore larvae were exposed to KCl, a known inducer of marine invertebrate larval metamorphosis. KCl was assayed at 10, 50 and 100 mM above normal seawater levels, a concentration known to elicit a response for larvae of the serpulid *Hydroides elegans* (Bryan *et al.*, 1997; Carpizo-Ituarte and Hadfield, 1998) Solutions were prepared by adding an appropriate amount of a 0.5 M KCl stock solution (in FSW) to attain the desired concentration. Changes in osmolarity as a result of KCL addition were not controlled for. The control was FSW.

Results

Larval morphology

At 18 °C, *Pomatoceros lamarckii* larvae become free-swimming approximately 12 hours after fertilisation. At this stage each is little more than a ball of cells with a rudimentary prototroch (Figure 2). The amount of ciliation steadily increases in the young trochophore larvae, particularly around the mouth and after 48 hours, the prototroch, metatroch and feeding cilia are well developed (Figures 3 - 5). The apical tuft is a prominent feature of the *P. lamarckii* trochophore and is composed of a tight bunch of long, stiff cilia which project out in front of the larvae as it swims (Figure 6). At 48 hours the neurotroch has begun to extend from the metatroch on the ventral surface towards the terminally-located anus (Figures 3 & 5). Once the larvae have begun to feed, the body extends posteriorly and the neurotroch becomes much more prominent. The larva now begins to resemble the 3 setiger metatrochophore larva with an elongated trunk and well developed feeding and locomotory cilia (Figure 7).

Given optimum culture conditions in the laboratory, the metatrochophore stage is normally attained in 9-12 days. Metatrochophore larvae are characterised by the appearance of 3 setigerous segments and a fourth, terminal non-setigerous segment (Figures 8 - 11). Also, the anal vesicle and the associated mucous glands, which displace the anus onto the dorsal surface, are clearly visible as a rounded bulge immediately posterior to the anus (Figures 8 & 10). From this stage onwards, larvae are competent to settle when provided with a suitable surface. As development continues, larvae slowly begin to lose their locomotory ciliation. The neurotroch is first to recede and is lost completely from an area immediately posterior to the mouth (Figures 9 & 15). At the same time, the head becomes slightly flattened and the region between the head and the prototroch constricts forming a pronounced 'neck'. The collar fold becomes a conspicuous bulge and the thoracic membrane is everted (Figure 14). The prototroch slowly degenerates and swimming becomes erratic as the larvae become predominantly demersal. Eventually, swimming is not possible and the larvae are restricted to crawling or gliding on the bottom of the culture vessel by utilising their remaining ciliation. Such larvae were termed the 'creeping' stage larvae by Segrove (1941). The apical tuft also degenerates and is replaced by shorter apical cilia distributed more widely on the anterior region of the head (Figures 12, 19 and 22). If larvae are not presented with a suitable settlement surface, the process of metamorphosis slowly proceeds without attachment. Ultimately, all locomotory ciliation is lost and the collar is fully exposed (Figures 34 & 35). Larvae at this stage are incapable of successful settlement, even when presented with a suitable surface. Settlement competence has been lost.

Internally, a metatrochophore larva has numerous glands of different types. Mucous glands are a conspicuous feature (Figures 36-39 and 42). There are well-developed mucous glands and a prominent vesicle in the ventral region of the head. In the thorax, there are two types of glands of various sizes situated laterally and along the ventral surface (Figures 36 and 38). The type I glands are composed of irregularly shaped patches which stain to varying intensities with toluidine blue. The type II glands contain spherical vesicles (1 – 2 µm) that stain darkly with toluidine blue. Both types of glands are closely associated with the anus and the anal vesicle. A single, large type II gland is situated below the anal vesicle and a smaller type I gland occurs above the vesicle and forms much of the bulge that is visible externally (Figures 39 and 44). The anal vesicle itself opens to the outside through one or more anal vesicle pores (Figure 39). In the posterior of the larvae, there are also a pair of type I glands situated laterally which are also associated with the anal vesicle. A small number of much smaller glands also occur on the dorsal surface.

Externally, there are numerous openings on the surface of the metatrochophore larva (Figure 43). The ciliated anus opens on the dorsal surface anterior to the terminal segment, there is an anal vesicle pore 5-10 µm across which occurs at the posterior end of the raised bulge of the anal vesicle and a terminal anal vesicle pore (also 5-10 µm across) located at the tip of the terminal segment (Figures 20 and 21). Laterally, the uncini can be seen through vertical slits on each side of the larva. There are numerous gland pores on the ventral surface of the trunk, predominantly towards the anterior of the larvae. On the ventral surface of the head, there is a row of prominent gland pores (2 µm diameter) (Figures 12 and 13). A pair of similar pores occurs between the regressing neurotroch and the collar fold and these are surrounded by numerous smaller openings clustered around the site of the lost neurotroch (Figures 15 and 43). These small pores are of two types, one type is surrounded by a raised area of cuticle, the other is simply a hole in the larval cuticle; both are ~ 1 µm in diameter. A pair of small gland pores also occur close to the anus on either side of the mid-line. Similar gland pores are not found on the dorsal surface.

On the metatrochophore larva, four pairs of ciliary tufts consisting of a group of 6-10 blunt-tipped cilia (2-3 µm long) are present on either side of the anterior neurotroch region (Figures 16,17 and 19). Similar ciliary tufts consisting of a group of 4-5 blunt tipped cilia (5-6 µm long) are located laterally next to the second and third setigerous bulges (Figures 18 and 19).

Description of figures

Figures 2-7 are scanning electron micrographs of 1 to 6 day old *Pomatoceros lamarckii* trochophore larvae.

Figure 2. 12 hours post fertilisation trochophore with rudimentary prototroch (p. = prototroch). Scale bar = 20 µm.

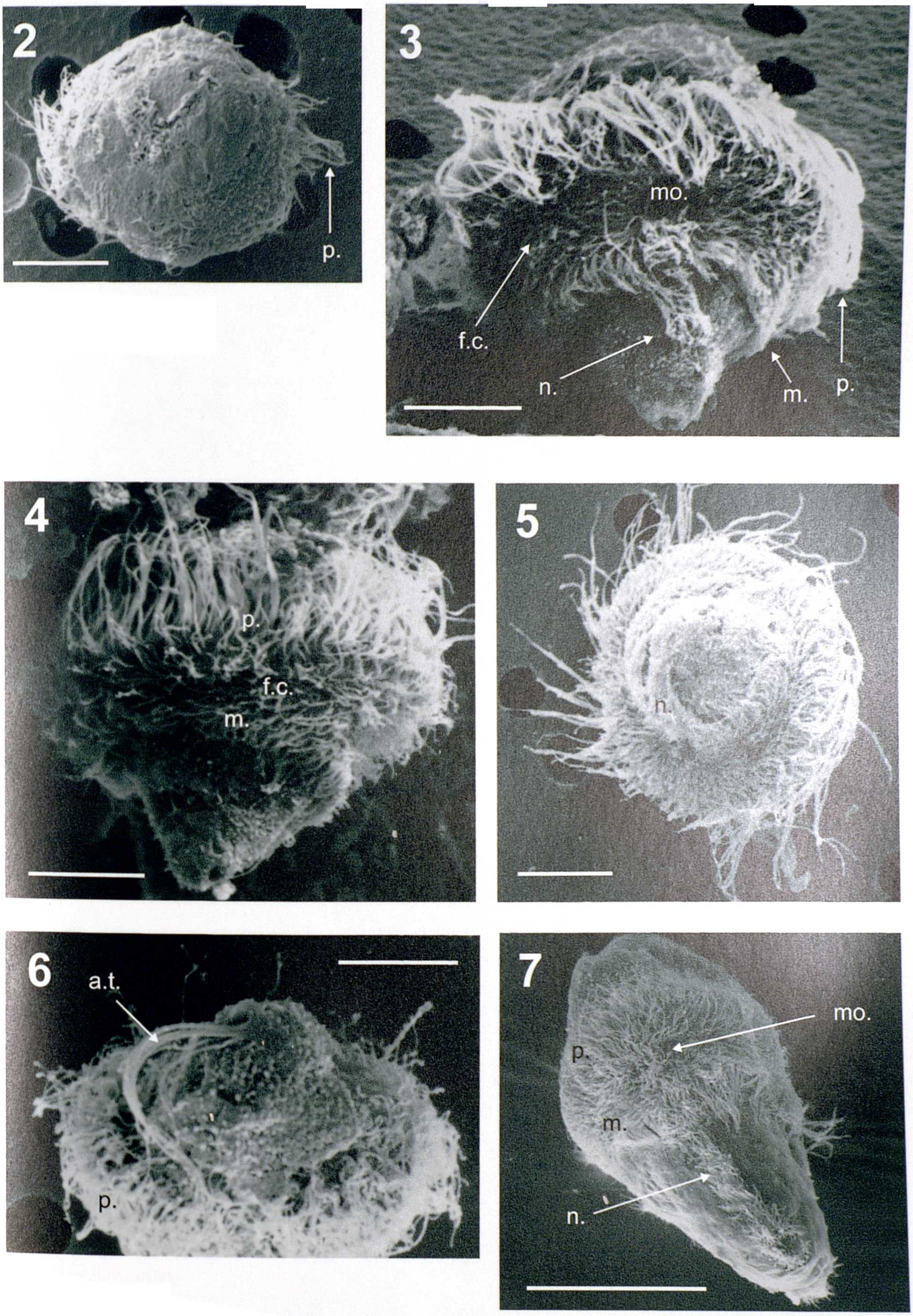
Figure 3. Ventral view of 2 day old trochophore with well developed ciliation (f.c. = feeding cilia; m. = metatroch; mo. = mouth; n. = neurotroch; p. = prototroch;). Scale bar = 20 µm.

Figure 4. Lateral view of 2 day old trochophore (f.c. = feeding cilia; m. = metatroch; p. = prototroch). Scale bar = 20 µm.

Figure 5. Posterior view of 2 day old trochophore larva (n. = neurotroch). Scale bar = 20 µm.

Figure 6. Anterior view of 2 day old trochophore larva with prominent apical tuft (p. = prototroch; a.t. = apical tuft). Scale bar = 20 µm.

Figure 7. Ventral view of 6 day old trochophore larva (m. = metatroch; mo. = mouth; n. = neurotroch; p. = prototroch). Scale bar = 50 µm.



2
p.
3
mo.
f.c.
n.
m.
p.
4
p.
f.c.
m.
5
n.
6
a.t.
p.
7
mo.
p.
m.
n.

Description of figures

Figures 8-15 are scanning electron micrographs of 10-14 day old *Pomatoceros lamarckii* metatrochophore larvae.

Figure 8. Dorsal view of 10 day old metatrochophore larva. Note the bulge of the anal vesicle between the anus and the dorsal anal vesicle pore (a. = anus; a.v.p. = anal vesicle pore; f.c. = feeding cilia; m. = metatroch; p. = prototroch s. = setae). Scale bar = 100 µm.

Figure 9. Ventral view of 10 day old metatrochophore larva (mo. = mouth; n. = neurotroch; p. = prototroch). Scale bar = 100 µm.

Figure 10. Dorsal view of 10 day old metatrochophore larva damaged during specimen preparation. The broken cuticle reveals the substantial anal vesicle (a.v. = anal vesicle). Scale bar = 100 µm.

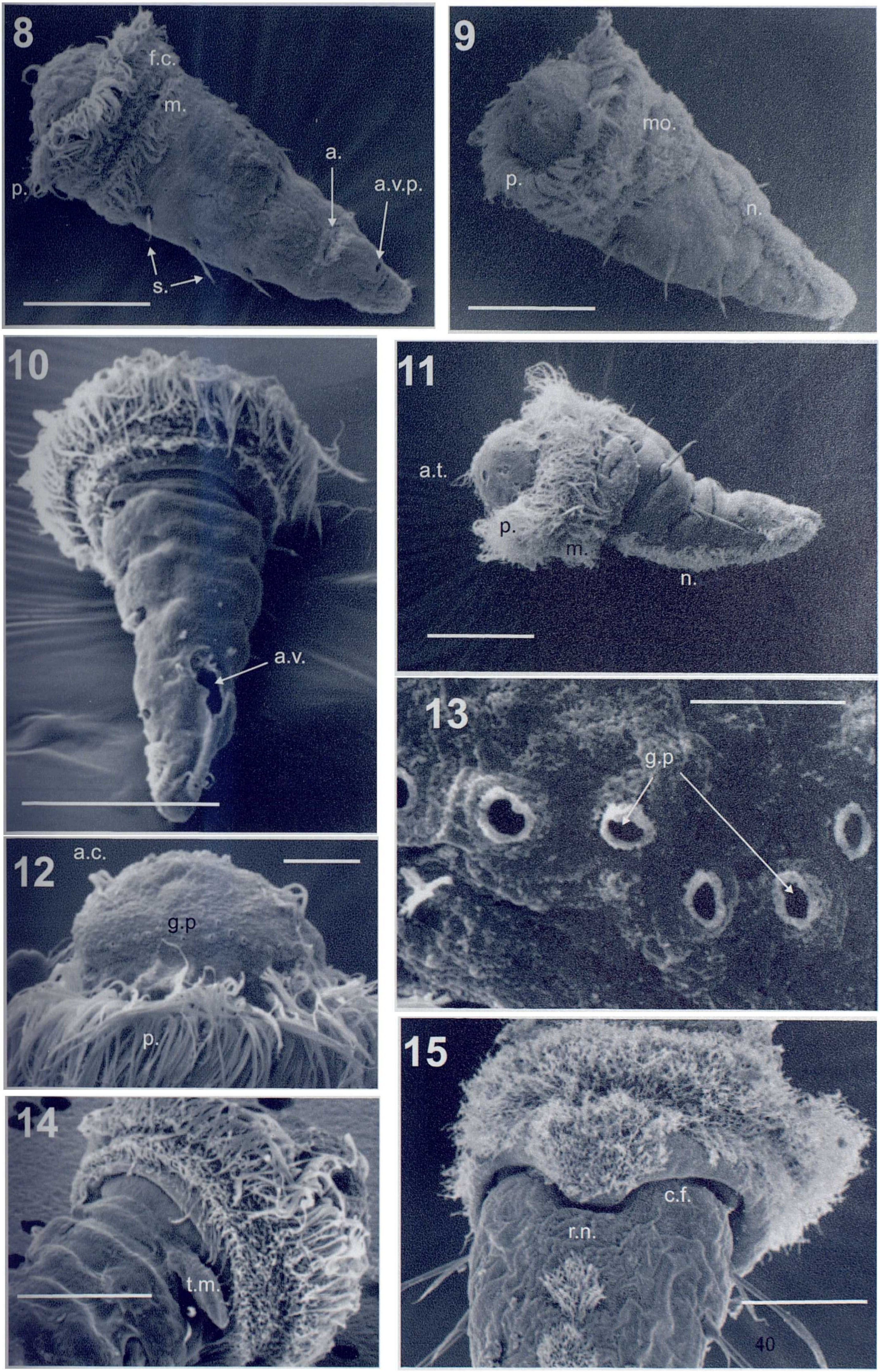
Figure 11. Lateral view of 10 day old metatrochophore larva with well developed locomotory and feeding cilia. Note the reduction in the apical tuft compared to Fig. 5 (a.t. = apical tuft; m. = metatroch; n. = neurotroch; p. = prototroch). Scale bar = 100 µm.

Figure 12. Head detail of 11 day old metatrochophore larva. Gland pores on the ventral surface are clearly visible immediately anterior to the prototroch. The change in the distribution of apical ciliation can be seen compared to younger larvae e.g. Fig. 5 (a.c. = apical cilia; g.p. = gland pore; p = prototroch). Scale bar = 20 µm.

Figure 13. Detail of ventral head gland pores shown in Fig. 11 (g.p. = gland pore). Scale bar = 5 µm.

Figure 14. Dorso-lateral view of 13 day old metatrochophore larva showing everted thoracic membrane (t.m. = thoracic membrane). Scale bar = 50 µm.

Figure 15. Ventral view of 14 day old metatrochophore larva with regressing neurotroch and well developed collar fold (c.f. = collar fold; r.n. = regressing neurotroch). Scale bar = 50 µm.



8
f.c.
m.
a.
a.v.p.
p.
s.
s.
9
mo.
p.
n.
10
a.v.
11
a.t.
p.
m.
n.
12
a.c.
g.p
p.
13
g.p
14
t.m.
15
c.f.
r.n.

Description of figures

Figures 16-22 are scanning electron micrographs of 12 – 14 day old *Pomatoceros lamarckii* metatrochophore larvae showing detail of sensory ciliation and cuticular pores.

Figure 16. Ventral view of the trunk region of 12 day old metatrochophore larva showing the 4 ciliary tufts and two types of pores that occur on the trunk (c.t. = ciliary tufts; p. I = type 1 pore; p.II = type 2 pore). Scale bar = 10 µm.

Figure 17. Detail of ciliarly tuft and type II pores (c.t. = ciliary tuft; p. II = type 2 pore). Scale bar = 5 µm.

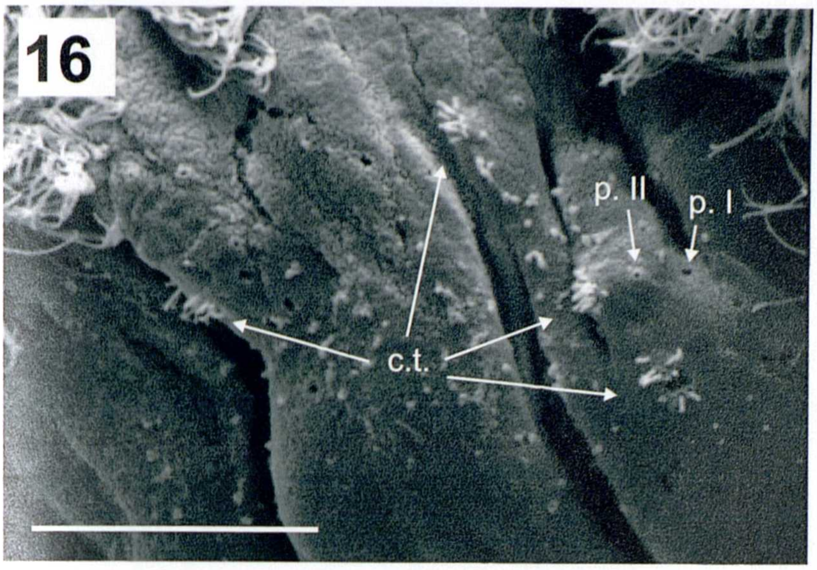
Figure 18. Detail of sensory ciliary tuft associated with the setal bulge. Note the bulbous ends to the cilia (c.t. = ciliary tuft; s. = seta; s.b. = setal bulge). Scale bar = 5 µm.

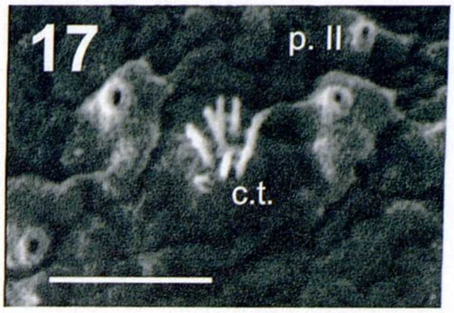
Figure 19. Ventral view of 14 day old metatrochophore larva showing position of sensory ciliation (a.c. = apical cilia; * = approximate position of ventral ciliary tufts; + = approximate position of lateral ciliary tufts associated with the setae). Scale bar = 50 µm.

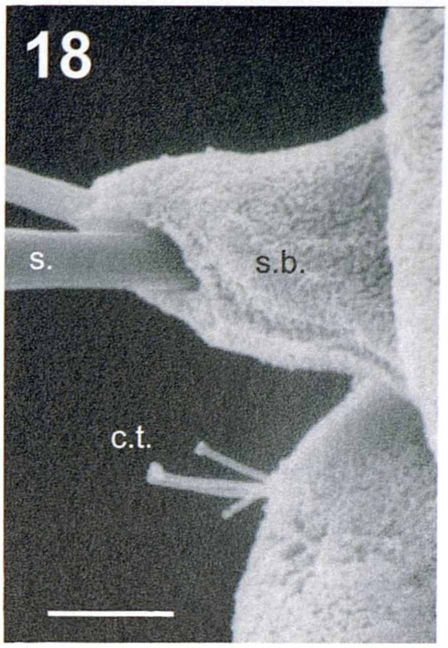
Figure 20. Dorsal view of terminal anal vesicle pore and ciliation of the posterior end of a 14 day old metatrochophore larva (a.v.p. = anal vesicle pore). Scale bar = 10 µm.

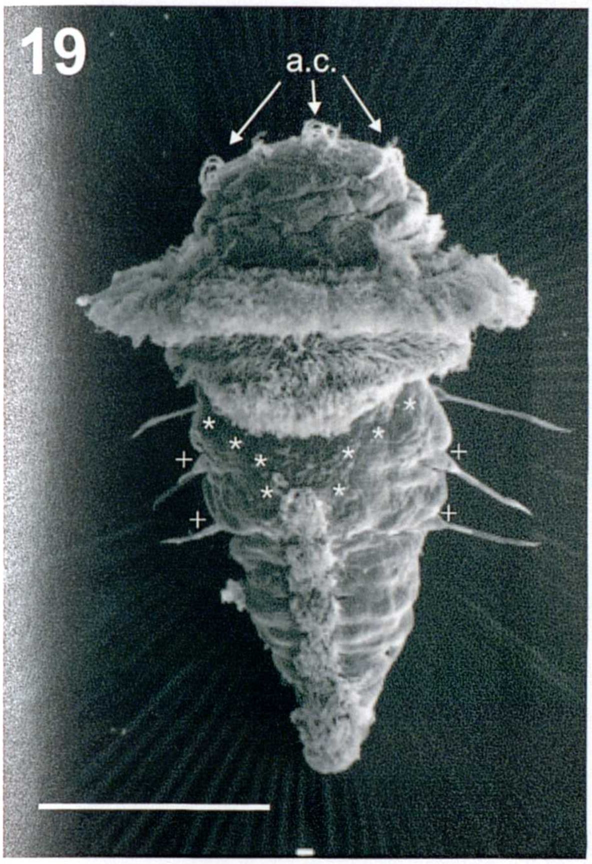
Figure 21. Lateral view of both anal vesicle pores (a.v.p. = anal vesicle pore; n. = neurotroch). Scale bar = 10 µm.

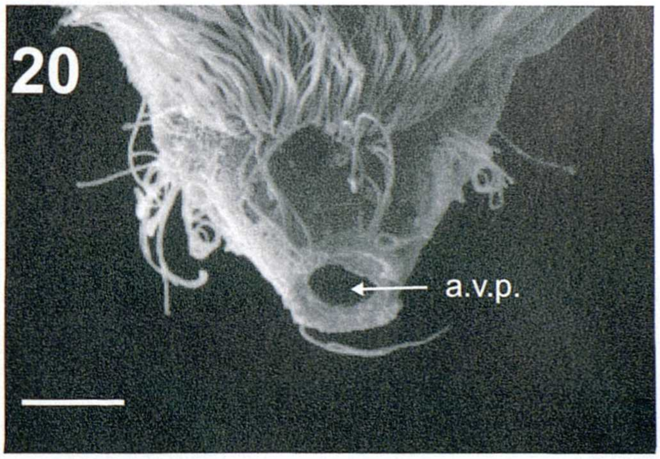
Figure 22. Anterior view of 12 day old metatrochophore larva with reduced apical ciliation compared to younger larvae (a.c. = apical cilia). Scale bar = 50 µm.

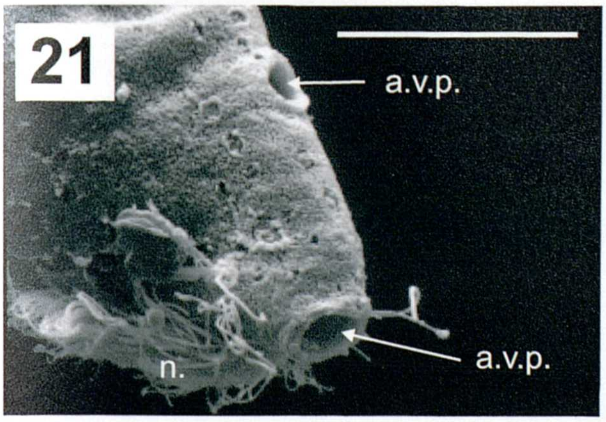

16
c.t.
p. II
p. I

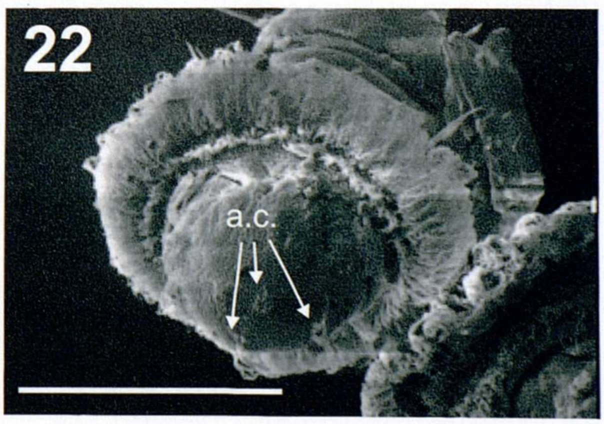

17
p. II
c.t.


18
s.
s.b.
c.t.


19
a.c.


20
a.v.p.


21
a.v.p.
a.v.p.
n.


22
a.c.

Description of figures

Figures 23-30 are scanning electron micrographs of *Pomatoceros lamarckii* juveniles at various stages of settlement and metamorphosis.

Figure 23. View of newly-settled juvenile (< 1 hour) on a biofilmed slate surface. The larva has rotated so that the ventral side is facing upwards. Note the mucous threads anchoring the juvenile to the substratum (m.t. = mucous thread). Scale bar = 100 μm.

Figure 24. View of newly-settled juvenile (< 1 hour) on a biofilmed slate surface. Note again mucous threads anchoring juvenile to the substratum (m.t. = mucous thread). Scale bar = 100 μm.

Figure 25. View of newly-settled juvenile (1-2 hours) anchored to the substratum by mucous threads. The trunk of the juvenile is covered by the primary mucoid tube (c. = collar; m.t. = mucous threads; p.m.t. = primary mucoid tube). Scale bar = 50 μm.

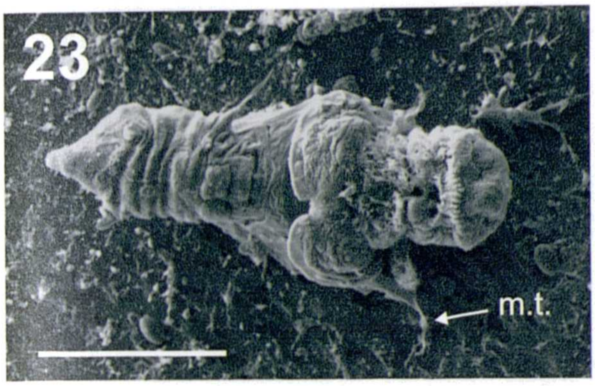
Figure 26. Detail of mucous threads shown in Fig. 30 anchoring larva to substratum (m.t. = mucous threads). Scale bar = 20 ? μm.

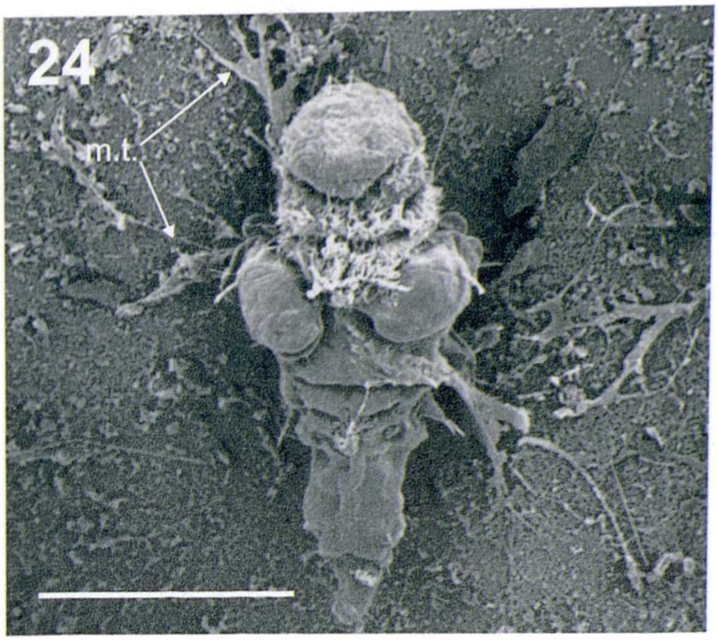
Figure 27. View of newly-settled juvenile (1-2 hours) on a biofilmed slate surface. The juvenile has rotated so that the ventral side is facing upwards. Note the formation of the primary mucoid tube (p.m.t. = primary mucoid tube). Scale bar = 100 μm.

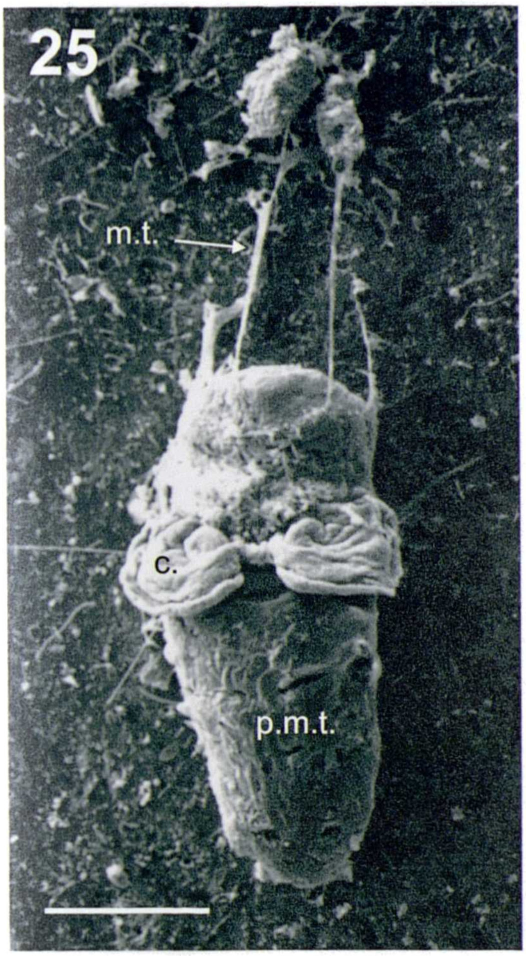
Figure 28. Detailed head view of newly-settled juvenile (1-2 hours). Note the almost complete loss of apical ciliation and the prototroch and the conservation of the gland pores (g.p. = gland pore; p. = prototroch). Scale bar = 20 μm.

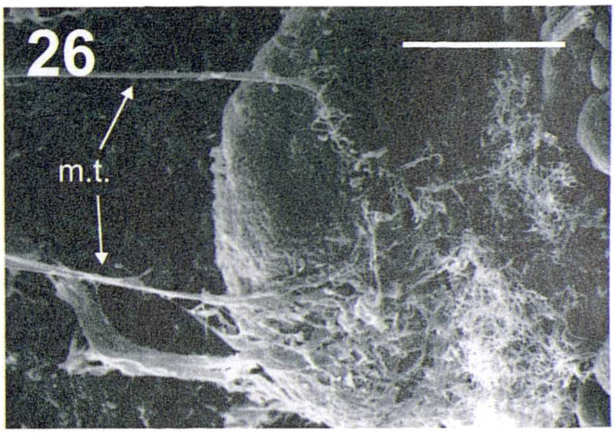
Figure 29. Lateral view of newly-settled larva (1-2 hours) that has backed into the primary mucoid tube during fixation (c. = collar; mo. = mouth; p.m.t. = primary mucoid tube). Scale bar = 100 μm.

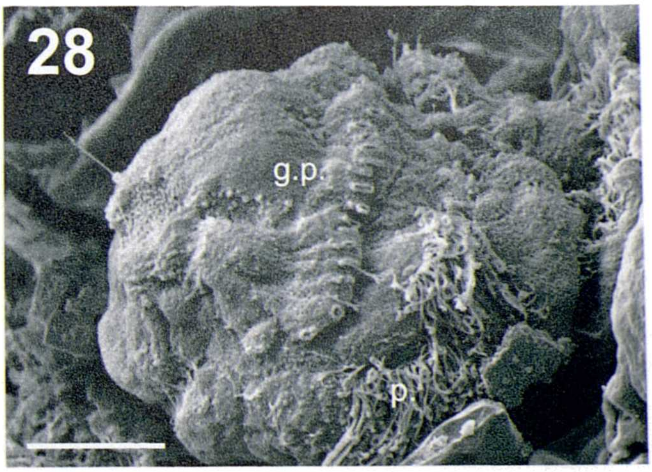
Figure 30. Rear view of primary mucoid tube (p.m.t.) attached to an adult calcareous tube. Note the open-ended rear of the tube (c.a.t. = calcareous adult tube; p.m.t. primary mucoid tube). Scale bar = 50 μm.

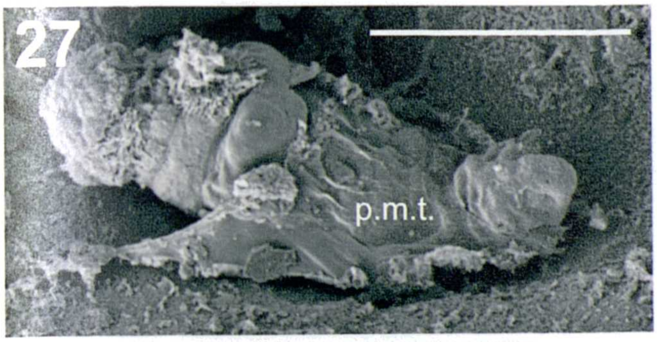

23
m.t.

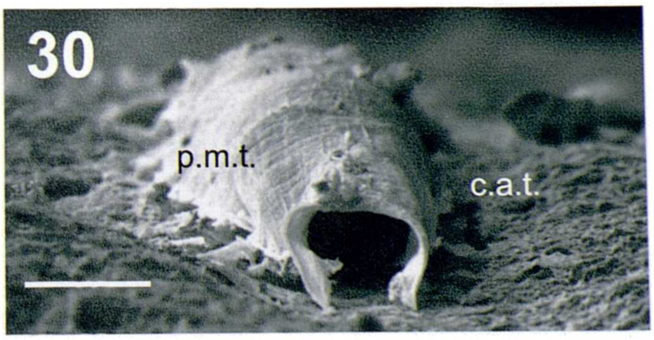

24
m.t.

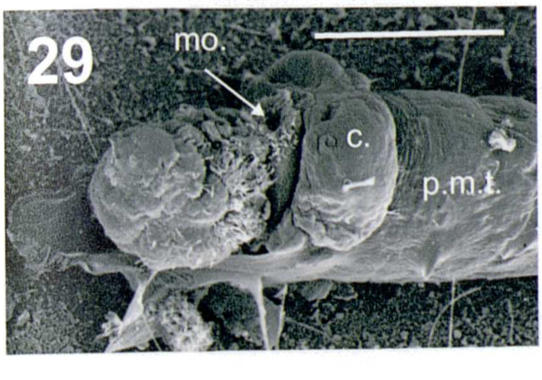

25
m.t.
c.
p.m.t.


26
m.t.


28
g.p.
p.


27
p.m.t.


30
p.m.t.
c.a.t.


29
mo.
c.
p.m.t.

Description of figures

Figures 31-35 are scanning electron micrographs of *Pomatoceros lamarckii* larvae at various stages of metamorphosis.

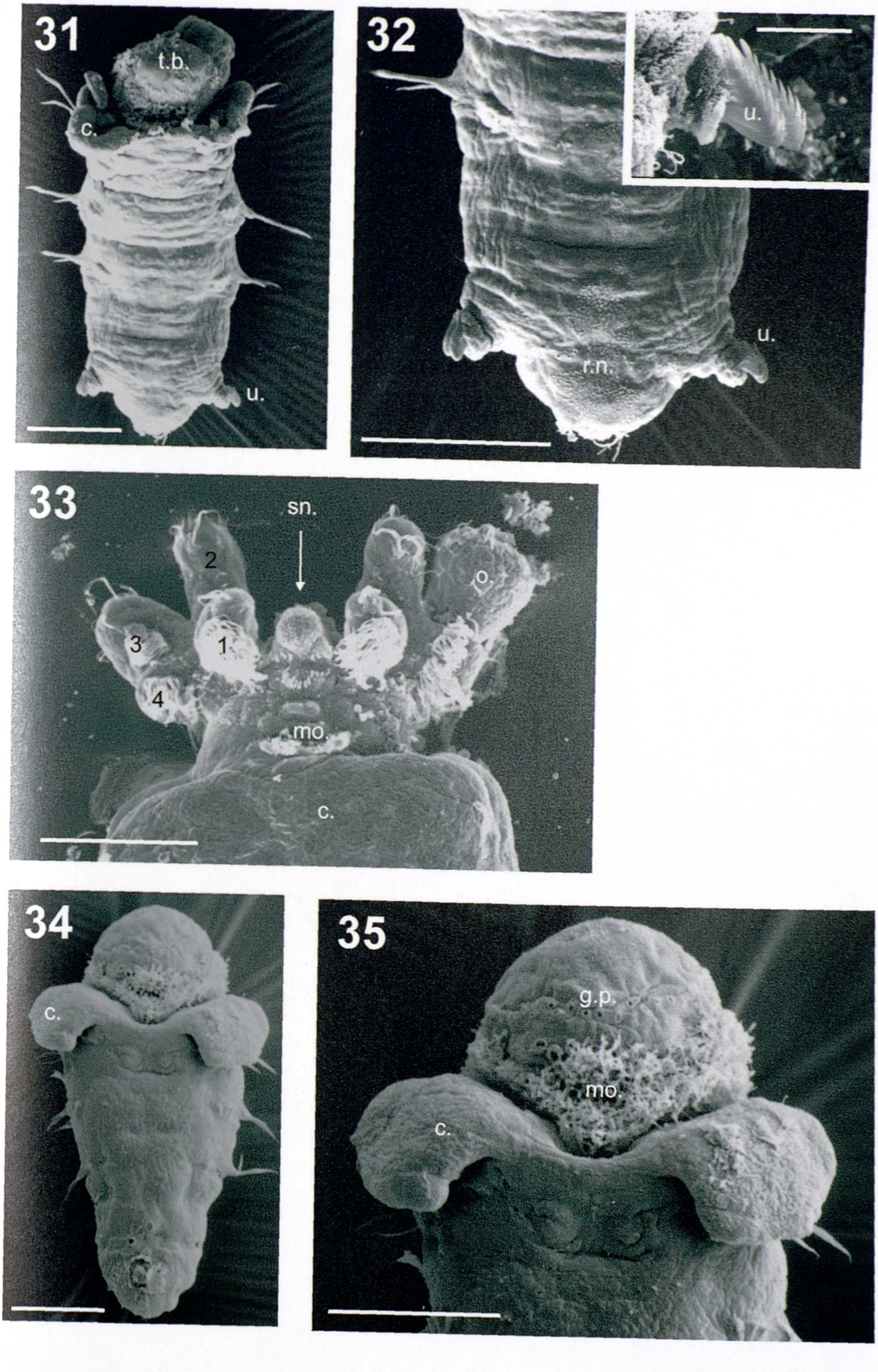
Figure 31. Ventral view of settled (6 hours post-settlement) juvenile after removal from primary mucoid tube. All locomotory ciliation has been lost, the uncini are visible, the collar is fully everted and tentacle buds are beginning to form (c. = collar; t.b. = tentacle buds; u. = uncini). Scale bar = 50 μm.

Figure 32. Detail of partly metamorphosed larva shown in Fig. 25. Note the remnants of the regressed neurotroch. The inset shows details of the uncini that are used by juveniles to move within the calcareous tube (r.n. = regressed neurotroch; u. = uncini). Scale bar = 50 μm.

Figure 33. Ventral view of metamorphosed (24 hours post-settlement) juvenile after removal from tube. The 4 primary tentacles are well developed with rudimentary ciliation and one tentacle has begun to develop into the operculum. The head has reduced in size and a small snout is apparent between the tentacles. Note that at this stage the larva has rotated so that the ventral side lies uppermost within the tube (1-4 = primary tentacles; c. = collar; mo. = mouth; o. = operculum; sn. = snout). Scale bar = 20 μm.

Figure 34. Ventral view of a larva kept for several weeks in culture. The larva has lost the ability to swim and has begun to metamorphose without settling. All locomotory ciliation has been lost and the collar is fully everted (c. = collar). Scale bar = 50 μm.

Figure 35. Head detail of the non-settled, partly metamorphosed larva shown in Fig. 23 (c. = collar; g.p. = gland pore; mo. = mouth). Scale bar = 20 μm.


31
t.b.
c.
u.
32
u.
u.
r.n.
33
sn.
2
o.
3
1
4
mo.
c.
34
c.
35
g.p.
mo.
c.

Description of figures

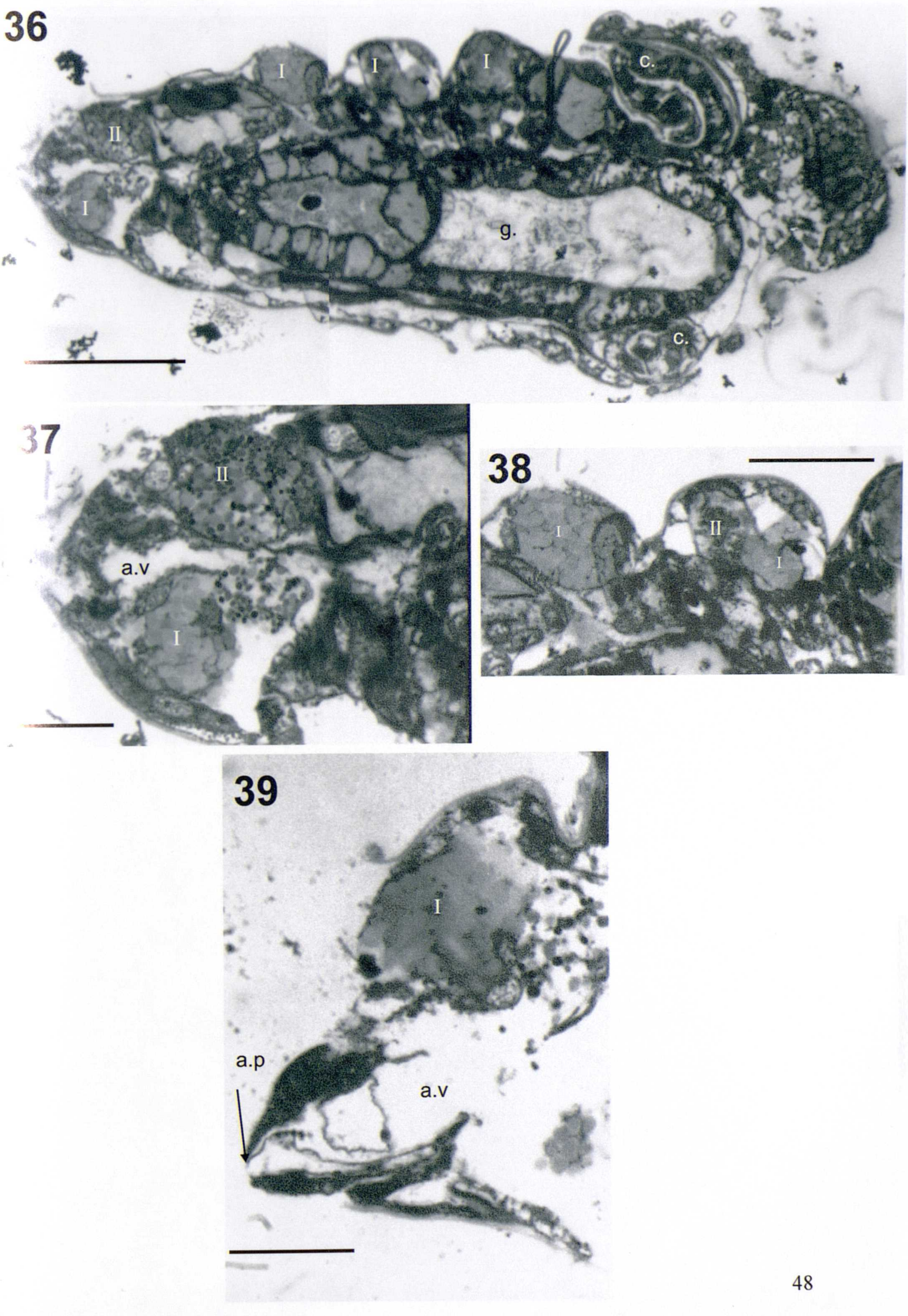
Figures 36-39 are photomicrographs showing the internal structure of a 12 day old, *Pomatoceros lamarckii* metatrochophore larvae.

Figure 36. Photomicrograph montage of longitudinal section of larva. The gut and surrounding cells are clearly visible in the centre of the larvae and the internal folding of the collar can be seen. Laterally and terminally located mucous glands are indicated (c. = collar; g. = gut; I = type 1 gland cells; II = type 2 gland cells). Scale bar = 100 μm.

Figure 37. Detail of posterior section of larva shown in Figure 36. The close association of type 1 and 2 glands with the anal vesicle can be clearly seen (a.v. = anal vesicle; I = type 1 gland; II = type 2 gland). Scale bar = 20 μm.

Figure 38. Detail of lateral section of larva shown in Figure 36. The large type 1 glands are shown (I = type 1 gland; II = type 2 gland). Scale bar = 20 μm.

Figure 39. Detail of posterior section of larva showing anal vesicle, associated glands and the terminal anal vesicle pore connecting the vesicle to the exterior of the larva (a.v. = anal vesicle; a.p. = anal pore; I = type 1 gland). Scale bar = 20 μm.


36
I
I
I
c.
II
I
g.
c.
37
II
a.v
I
38
I
II
I
39
I
a.p
a.v

Description of figures

Figures 40-43 are diagrammatic composites summarising the internal organisation and external morphology of *Pomatoceros lamarckii* trochophore and metatrochophore larvae. All scale bars = 50 µm.

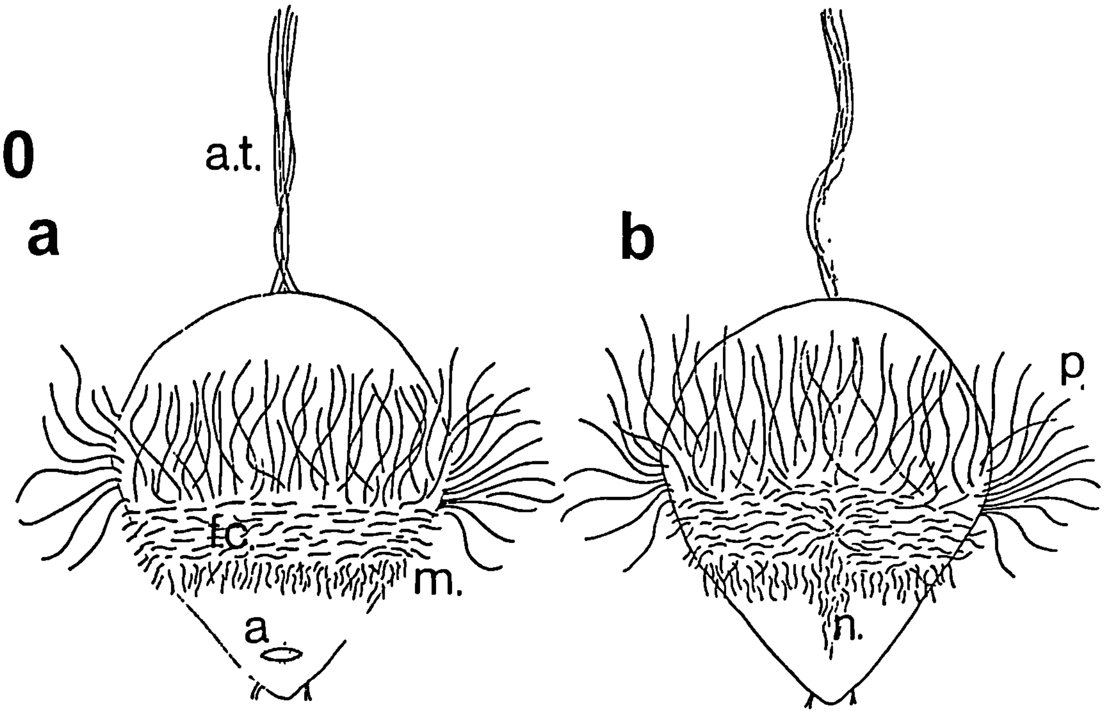
Figure 40. Ventral (a) and dorsal (b) views showing external morphology of an early stage trochophore larva (a. = anus: a.t. = apical tuft; f.c. = feeding cilia; m. = metatroch; n. = neurotroch; p. = prototroch).

Figure 41. Ventral (a) and dorsal (b) views showing external morphology of a metatrochophore larva (a. = anus; a.t. = apical tuft; c.f. = collar fold; f.c. = feeding cilia; g.p. = gland pore; m. = metatroch; mo. = mouth; n. = neurotroch; p. = prototroch; s. = setae; s.c. = sensory cilia; t.m. = thoracic membrane; u. = uncini).

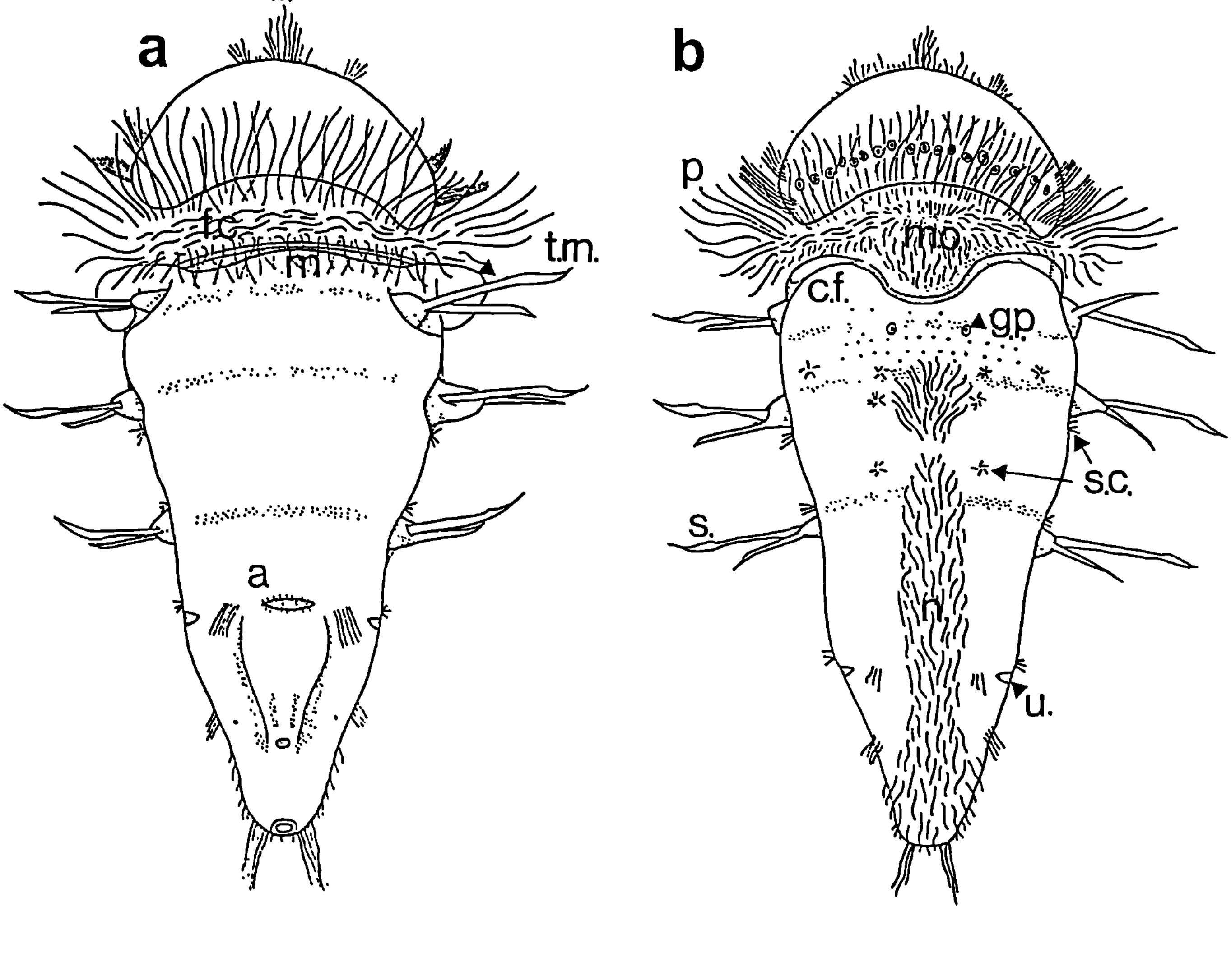
Figure 42. Longitudinal (a) and sagittal (b) section of a metatrochophore larva (a.v. = anal vesicle; g. = gut; h.v. = head vesicle; I = type 1 gland; II = type 2 gland).

Figure 43. Camera lucida drawing of *P. lamarckii* metatrochophore larva showing the anal vesicle, anal vesicle pore and the two glands closely associated with the anal vesicle (a.v. = anal vesicle; a.v.p. = anal vesicle pore; g = gut; I = type 1 gland; II = type 2).

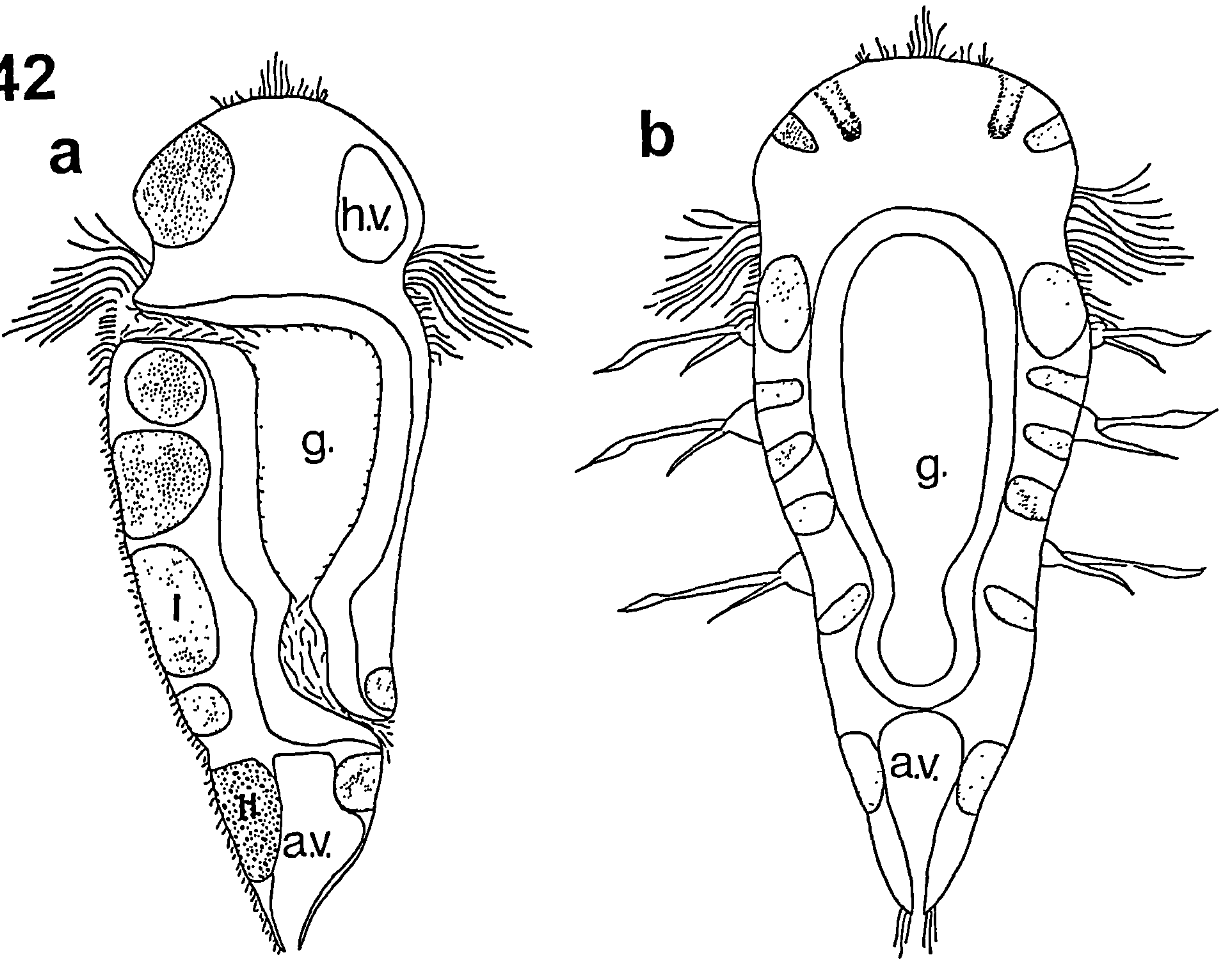
40



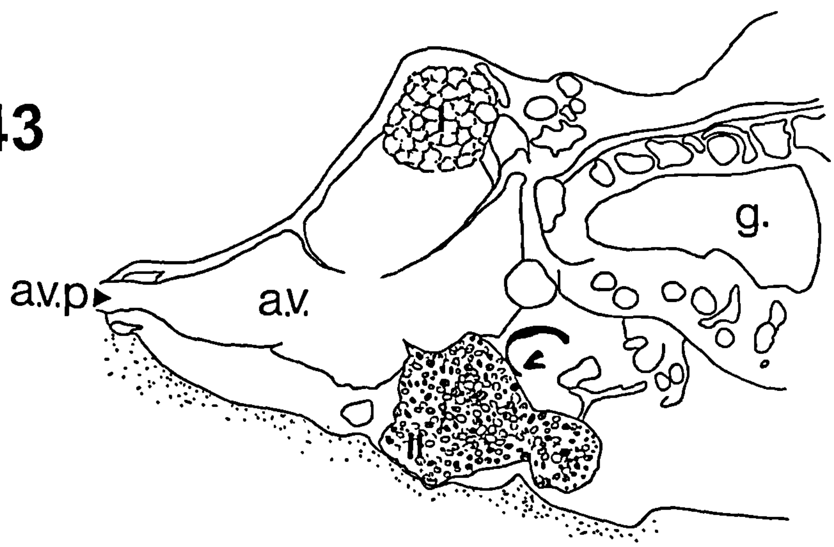
41



42



43



Swimming

In the laboratory, metatrochophore larvae swim in an undulating path that brings them into contact with the bottom of the culture container and returns to the water column. At this stage, larvae swim horizontally at a speed of 28 ± 0.15 mm sec^{-1} at 18 °C (1.12 ± 0.06 body lengths sec^{-1}) (n=30).

Competent larvae spend much of their time 'exploring' the bottom of the culture container or experimental substrata by slowly gliding over the surface. After a protracted planktonic period (> 14 days in culture) the larvae begin to lose the ability to swim as the prototroch and neurotroch begin to degenerate. If the larvae are not presented with a suitable surface for settlement, they eventually lose the ability to swim.

Larval behaviour during substratum exploration

During swimming and substratum exploration, a thin mucous thread can occasionally be seen trailing behind the metatrochophore larvae of *Pomatoceros lamarckii*. This thread often anchors the larvae to the substratum whilst the larvae attempt to swim away from the substratum. The mucous thread may be several times the length of the larvae. When anchored to the substratum by one of these mucous threads, larvae prove difficult to dislodge with a jet of water from a pipette.

Pomatoceros lamarckii larval exploratory behaviour follows the general marine invertebrate larval exploration pattern described by Crisp (1974) with three distinct sequential phases of exploration being clearly identifiable. These are the 'broad exploration', 'close exploration' and 'inspection' phases. In the laboratory, 'broad exploration' occurs as metatrochophore larvae swim in an undulating path taking them repeatedly into contact with the substratum before returning them to the water column. Initial contact with a surface is made by the ventral region of the head at such an angle that the apical cilia make contact with the substratum. If an 'attractive' surface is encountered, larvae stop swimming and begin the 'close exploration' phase. When exploring a surface, larvae use their cilia for propulsion and frequently roll their head from side to side over the surface as they move forwards. During exploration, larvae move at a speed of 0.45 ± 0.04 mm sec^{-1} (= 0.54 ± 0.04 body lengths sec^{-1}) (n=30), halting and changing direction frequently. During surface exploration larvae frequently halt and quiver. After some time exploring a surface, larvae either return to the water column or progress to the 'inspection' phase. The majority of exploring larvae will return to the water column repeatedly after conducting the 'close exploration' phase and may do so even after progressing to the 'inspection' phase, which may last in excess of 30 minutes.

Finally, usually after a protracted period of 'inspection', a suitable settlement site is chosen; the anterior part of the body is raised sharply at right angles to the substratum and fixation occurs. From this point onwards, the animal is irreversibly attached. Although the series of events described here represents the typical process of events leading to settlement, it is also not uncommon for some larvae to settle within minutes of their first contact with a suitable surface.

Settlement

The process of settlement begins when a larva arches its body at 90° away from the substratum. From this point onwards, the larva is fixed to the substratum by numerous mucous threads (Figures 23 - 26) emanating from the head, anal and thoracic gland pores. Once attached, the prototroch rapidly disappears, the head becomes clearly defined and the collar is unfolded (Figure 23). The larva then begins to roll from side to side and backwards and forwards, moulding a primary mucous tube around itself (Figure 27). At this stage, the larva also begins to rotate and spends more of its time with the ventral surface uppermost. After 30-60 minutes, the larva is fully covered by the primary mucoid tube, the collar is fully formed and the larva lies permanently inverted on the substratum (Figures 27 and 29). The mucoid tube appears to be formed by the secretions from the mucous gland pores, primarily those that are associated with the ventral glands of the head and anterior trunk area which are now uppermost. At this stage, larvae spend much of their time inactive, interspersed with brief periods of activity associated with construction and elongation of the primary mucoid tube (Figures 29 and 30).

Metamorphosis

Once the larva has fixed itself to the substratum, major metamorphic changes begin. The head gland pores become more prominent and elongate (Figure 28). Some minor ciliation remains on the head and around the mouth but the remaining ciliation of the apical tuft is quickly lost and the tentacle buds begin to develop (Figure 28). The uncini are everted and are used by the larva to move within the calcifying tube (Figures 31-32). Four ciliated primary tentacles and an operculum quickly develop and begin to increase in size. A small 'snout' is apparent ventrally, between the branchial crown (Figure 33). The tube now becomes increasingly calcified and is continually extended to accommodate the growing juvenile worm.

Artificial induction of metamorphosis

Larvae of *Pomatoceros lamarckii* underwent partial metamorphosis in response to 10 mM or greater excess K^+ ions in seawater. After 24 hours of exposure to K^+, three distinct categories of metamorphosis were observed:

1) thoracic membrane everted, collar unfolded, tentacle buds apparent and larvae swimming slowly and spending more time on the bottom of the container.
2) larvae attached to the substratum with primary tentacles forming but no tube.
3) larvae attached to the substratum with tentacles and tube.

The majority of larvae underwent partial or abnormal metamorphosis (types 1 and 2; Table 1). Many of the larvae that had undergone metamorphosis were attached to each other and to the substratum by mucous secretions.

Table 1. Metamorphic response of *Pomatoceros lamarckii* larvae after 24 hours exposure to various concentrations of KCl. Data shown are mean percentage (n=6).

Response	**Treatment (µM KCl)**			
	0	**10**	**50**	**100**
Normal swimming	90	70	0	0
1. Slow or no swimming + collar everted	10	30	80	0
2. Attached + collar everted	0	0	10	100
3. Attached with primary tentacles and tube	0	0	10	0

Discussion

Growth rate

In this study, with larvae cultured at 19-20 °C, *Pomatoceros lamarckii* larvae became competent to settle after 8-10 days post fertilisation and occasionally in as little as 7 days. Segrove (1941) reported that larval development of *Pomatoceros triqueter* (=*Pomatoceros lamarckii ?* – see introduction) took about 3 weeks for larvae cultured in still water conditions and fed on a diet of *Nitzschia*. At cooler temperatures during winter, larval development took several months, however, this period could be reduced to the normal 3 week time span by raising the temperature of the cultures to 18 °C. Føyn and Gjøen (1954) found that at a temperature of 18 -20 °C, larvae of *P. triqueter* began to feed about two days after fertilisation and that larval development to settlement took from 10 – 15 days. Roscoe (1993) found that under optimal conditions in the laboratory (18 °C; 12:12 hours light:dark), the larvae of *P. lamarckii* usually reached competency between 9 to 12 days post-fertilisation and exceptionally in as little as 8 days.

Larval morphology

Larvae of *P. lamarckii* follow a developmental pattern typical of most serpulids (Giangrande, 1997). The development of *P. lamarckii* proceeds very much as described by Segrove (1941). During this study a number of observations were made, primarily on the external structure of competent and metamorphosing larvae, that have not been reported previously. These were the presence of numerous gland pores on the larval head and the trunk, the presence of sensory ciliary tufts on the ventral surface of the larval trunk and also the occurrence of a 'snout' between the branchial rudiments of the metamorphosing larva.

Føyn and Gjøen (1954) disputed some of the metamorphic stages described by Segrove (1941) and considered that some of the specimens described represented abnormal larvae. It is plausible that Segrove (1941) and Føyn and Gjøen (1954) were working with different animals; Segrove studying *Pomatoceros lamarckii* and Føyn and Gjøen studying *Pomatoceros triqueter* in Norway, which is known to become more abundant with increasing latitude (Crisp and Ekaratne, 1984). Nevertheless, observations made by Moat (1985), Roscoe (1993) and during this study largely confirm the work of Segrove (1941) in that 'creeping stage' larvae do occur naturally in older larvae as a result of partial metamorphosis and these stages do not necessarily represent larvae that have settled and subsequently crawled out of their tubes as suggested by Føyn and Gjøen (1954).

The apical tuft is a feature of many invertebrate larvae, including the trochophore larvae of polychaetes and is a prominent feature of *Pomatoceros lamarckii* trochophore larvae. In *P. lamarckii*, the apical tuft degenerates as larvae reach the late metatrochophore stage and the long, stiff cilia of the apical tuft are replaced by shorter, more dispersed apical cilia. Degeneration of the apical tuft has also been reported in larvae of other serpulids including *Pomatoceros triqueter* (Moat, 1985), *Galeolaria caespitosa* (Grant, 1981; Marsden & Anderson, 1981), *Spirobranchus* and *Phyllodoce* (Lacalli, 1981). Moat (1985) observed that in *P. triqueter*, the apical tuft remained a prominent feature of the metatrochophore larvae right up to the appearance of the 'creeping stage' larvae but noted that in addition, a number of other, smaller cilia in groups or singly were also present.

The function of the apical tuft is uncertain but it is frequently supposed to function as a sensory organ (Lacalli, 1981) and Moat (1985) speculated that for the metatrochophore larvae of *Pomatoceros triqueter*, the apical tuft may be involved in the selection of suitable settlement sites. Likewise, Nott (1973) described the apical tuft in *Spirorbis spirorbis* as a possible chemo-sensory structure used in the selection of a suitable adult habitat. Marsden and Anderson (1981) showed that in the young trochophore larvae of *Galeolaria caespitosa*, stimulation of the apical tuft by mechanical or chemical means resulted in an instantaneous interruption of metatrochal beating of the prototrochal cilia. In addition, morphological observations provided evidence of neural activity in prototrochal and neurotrochal cells of the trochophore larvae. During this study it was observed that initial contact with a surface is made by the ventral region of the head at such an angle that the apical cilia are in contact with the substratum. Also, during substratum exploration the larvae of *Pomatoceros lamarckii* move the head from side to side during which time the ventral section of the head is in contact with the substratum. Because the apical cilia present on the head of *P. lamarckii* larvae are lost completely upon metamorphosis, as also described for *Spirorbis moerchi* (Potswald, 1978), it is possible that they play an important role in the selection of a suitable settlement site.

Several workers have noted an abundance of large mucous glands in the larvae of serpulid and spirorbid polychaetes (Segrove, 1941; Nott, 1973; Moat, 1985). Nott (1973) found numerous openings on the dorsal and ventral surface of *Spirorbis spirorbis* and identified three distinct gland types associated with these gland pores. Potswald (1977) also studied the ultrastructure of *Spirorbis* larvae and reported a complex of mucous glands in the ventral and lateral ectoderm of the thorax of *Spirorbis moerchi*, similar to *Spirorbis spirorbis*. Moat (1985) noted an abundance of mucous cells in the metatrochophore larvae of *Pomatoceros triqueter*, many of which were associated with the ventral region and suggested that because the cells were at various stages of maturity, mucus may be continually added to the surface of the larvae of *P. triqueter*. Similarly, Nott (1973) suggested that because the type A and B glands present in the chaetigerous segments of *S. spirorbis* were at different stages of maturity, they may be contributing to the mucus that covers the body of the animal.

The distribution of mucous glands in *Pomatoceros lamarckii* that was observed during this study agrees well with the observations of Segrove (1941) and Moat (1985). Segrove (1941) reported that most of the glands are densely and uniformly stained with Gelafield's haematoxylin but noted that some have a coarsely reticulate appearance possibly indicating a different phase in their secretory activity. Moat (1985) also reported that some glands stained to varying intensities and suggested that these glands were continually producing mucous. Moat (1985) found that all of the glands of the metatrochophore contained a mucopolysaccharide (MPS) secretion, whilst proteins were found in only the ventral mucous glands. The distribution of glands and vesicles of *P. lamarckii* is similar to that described for *Spirorbis spirorbis,* but with some important differences. *P. lamarckii* larvae are planktotrophic so therefore lack the 'attachment gland' of the lecithotrophic larvae of *S. spirorbis* that is formed within the posterior section of the non-functional gut (Nott, 1973). Therefore, *P. lamarckii* larvae must effect initial attachment in a different manner from that of *S. spirorbis*.

In the metatrochophore larvae of *Pomatoceros lamarckii*, gland pores are restricted to the ventral surface and are closely associated with the prototroch and neurotroch, the most prominent gland pores being those on the head. These are similar to those on the head of *Spirorbis spirorbis* which Nott (1973) suggested were connected directly to the head vesicle. The gland pores on the trunk of *P. lamarckii* are considerably smaller than those of *S. spirorbis* and there are none of the large, domed gland pores that occur on *S. spirorbis* (personal observation). The gland pores of *P. lamarckii* appear to be involved in the production of mucus used for both initial attachment and subsequent primary tube production.

During exploration, *Pomatoceros lamarckii* larvae were occasionally seen to be trailing a mucous thread which often acted as a temporary attachment to the substratum. Temporary attachments of this sort have been described previously for *P. lamarckii* (Roscoe, 1985) and for other serpulid larvae including *Pomatoceros triqueter* (Segrove, 1941; Fjøyn and Gjøen, 1954; Moat, 1985), *Ficopomatus uschakovi* (Straughan 1972), *Pomatoleios kraussii* (Crisp, 1977), *Hydroides norvegica* (Wisely, 1958) and *Galeolaria caespitosa* (Grant, 1981; Marsden and Anderson, 1981) and for the spirorbid larvae of *Spirorbis spirorbis* (Knight-Jones, 1951b; Nott, 1973; Nott and Parks, 1975), *Spirorbis rupestris* (Gee and Knight-Jones, 1962), *Spirorbis* *moerchi*, *Spirorbis spirillum* and *Spirorbis vitreus* (Potswald, 1978). Moat (1985) reported that mucous secretions were frequently observed emanating from the anal pore of *P. triqueter* metatrochophore larvae when viewed with the SEM and suggested that the large, paired mucous glands that are associated with the anal vesicle and open to the surface via one (or more) of the terminal pores may provide the bulk of the mucous material which forms the mucous threads. Similar pores are present on the terminal segment of *P. lamarckii*. However, during this study, at no time were mucous secretions observed emanating from this pore, but light microscope observations of exploring larvae suggest that the mucous threads originate from the terminal anal vesicle pore.

The anal vesicle is a prominent feature of *Pomatoceros lamarckii* metatrochophore larvae. Segrove (1941) considered that in *Pomatoceros triqueter*, the anal vesicle functions architecturally streamlining the body of the larva. Whereas Nott (1973) reported that in *Spirorbis spirorbis*, the group of type A glands that are in close association with the anal vesicle are in a transitory state between gland and vesicle and suggested that the anal vesicle is a void produced by discharged gland cells. In *P. lamarckii*, the anal vesicle and pores and the associated gland cells appear to function in the same way as in *P. triqueter* (Moat, 1985) and *S. spirorbis* (Nott, 1973) and are responsible for mucus production which leaves the body via the anal vesicle pores and contributes to the formation of mucous threads.

Although a common feature of serpulid and spirorbid behaviour, the function of the temporary attachment threads is not clear. Moat (1985) suggested that the mucus used for temporary attachment may also play a major role in anchoring the larvae to the substratum during primary tube formation. Roscoe (1993) considered that in *Pomatoceros lamarckii*, the role of the mucous threads may be to stabilise the larva and to allow it to flex the body sharply at right angles during the moment of attachment. Interestingly, Williams (1964) found that the addition of *Fucus* conditioned seawater to water containing the mucus from the larvae of Polyzoa epiphytic on *Fucus* resulted in a solidification of the mucus into long fibres. Williams (1964) suggested that such a change in the mucus may prove to be important in stimulating or making possible the fixation of the larvae. It is conceivable that in *P. lamarckii*, the mucous threads produced by the larvae may be used as a physical 'test' of the suitability of a surface for initial settlement (i.e. adhesion) although the infrequent observation of the threads questions their importance in the exploration and settlement process.

The four pairs of ciliary tufts observed on the trunk of *Pomatoceros lamarckii* metatrochophore larvae are similar to those described for the larvae of the serpulids *Salmacina dysteri* (Nishi & Yamasu, 1992b), *Hydroides ezoensis* (Miura and Kajihara, 1984) and

Pomatoleios kraussii (Miura and Kajihara, 1984), *Rhodopsis pusilla* (Nishi, unpublished) and *Galeolaria caespitosa* (Grant, 1981). Moat (1985) also reported single cilia and small groups of fine cilia present on the trunk of *P. triqueter,* but gave no information on their size, location or number. The ciliary tufts on the trunk of *Pomatoceros lamarckii* occupy a similar position as those described for *S. dysteri* on the ventral trunk of the larvae (Nishi & Yamasu, 1992b). Aside from serpulid larvae, similar ciliary tufts have also been described on the larvae of the sabellids *Phragmatopoma lapidosa* (Eckelbarger, 1978) *Sabellaria cementarium* (Smith and Chia, 1985), *Phragmatopoma californica* (Amizu & Reed, 1987; Amieva *et al.*, 1987) and the terebellid larvae of *Lanice conchilega* (Heimler, 1981). Eckelbarger (1978) described in detail the position of the numerous ciliary tufts on the larvae of the sabellid *Phragmatopoma lapidosa*. The circularly arranged, stiff, radiating cilia were shown in section to emanate from a single, small supportive cell and project through the larval cuticle. Synaptic contacts with sensory nerve fibres were observed at their base, suggesting a sensory function (Eckelbarger, 1978). The cilia were characterised by their stiff posture and blunt tips similar to those observed for *P. lamarckii* (Fig. 18; this study). Miura and Kajihara (1984) also considered that the ciliary tufts present on *Pomatoleios kraussii* have a sensory function. Amizua & Reed (1987) and Amieva *et al.* (1987) suggested that during substratum exploration by *Phragmatopoma californica* larvae, the ciliary tufts would be in contact with the substratum and may represent sense organs involved in substratum selection. Grant (1981) also suggested that the cilia on the head and trunk of *G. caespitosa* larvae may play a sensory role in substratum selection. The ciliary tufts of *P. lamarckii* seem likely to play a sensory role (chemo- or mechano-receptors) and may be involved, in combination with the apical cilia, in substratum selection.

Larval behaviour

Roscoe (1993) studied the light response of *P. lamarckii* larvae. Despite ambiguous results for 3 to 8 day old larvae, metatrochophore larvae were strongly photopositive when tested in a light chamber under laboratory conditions. Observations made during the present study largely confirm the work of Roscoe (1993). In addition, the undulating swimming behaviour observed for *P. lamarckii* has also been described previously by Roscoe (1993). Such behaviour may be an adaption to ensure that larvae 'test' numerous surfaces.

As with other marine invertebrate larvae, serpulid larvae have been shown to be highly selective during settlement and are capable of responding to a variety of physical and chemical cues (Qian, 1999; Hamer *et al.*, 2001). Accounts of the larval exploration and settlement behaviour have been given for *Pomatoceros* spp. by Segrove (1941), Føyn and Gjøen (1954), Moat (1985) and Roscoe (1993). In laboratory settlement assays, *P. lamarckii* larvae are highly selective during settlement (Chan and Walker, 1998; Hamer *et al.*, 2001) and are capable of postponing settlement and metamorphosis for several weeks in the absence of a suitable surface (Roscoe, 1993). This selectivity is reflected by the time that larvae spend exploring potential settlement surfaces under laboratory conditions.

The swimming speed of *Pomatoceros lamarckii* metatrochophore larvae is comparable with the reported swimming speeds of other annelid trochophore larvae (5 – 33 mm sec^{-1}), falling at the upper end of the range of swimming speeds reported for annelid metatrochophore larvae (5 – 30 mm sec^{-1}) (Knight-Jones, 1951b; Konstantinova, 1969; Chia and Buckland-Nicks, 1984). The swimming speed of serpulid larvae has been reported previously only by Roscoe (1993) who also studied *P. lamarckii* and quoted a mean swimming speed of 1.03 mm sec^{-1} for 12 day old metatrochophore larvae, a figure which is extremely low compared to the speed recorded during this study. There are fewer studies of the speed at which larvae explore surfaces. *P. lamarckii* explore surfaces during the broad exploration phase at a speed of 0.45 ± 0.04 mm sec^{-1} and have been observed to conduct this exploration phase repeatedly for substantial periods of time. In a 30 minutes period of such exploration, a larva would therefore be capable of traversing a distance of approximately 0.81 m. Given the selectivity of *P. lamarckii* larvae and their ability to delay metamorphosis unless presented with a suitable settlement surface under laboratory conditions, it is conceivable that in the natural environment, larvae explore a considerable number of different surfaces before settling.

Settlement

The mucus released by *Pomatoceros lamarckii* larvae at the time of settlement is a sulphomuco-polysaccharide (Hedley, 1956). Once released, the rotational and longitudinal movements of the larva shape this mucus into a primary mucous tube as described for *Spirorbis spirorbis* (Nott, 1973). Roscoe (1993) suggested the presence of a mucous 'pad' attaching the larva to the substratum at the beginning of the settlement process. However, no evidence of a mucous 'pad' was found during this study and attachment was considered to be effected by the numerous mucous threads which anchor the larva to the substratum.

Nott (1973) reported that the substantial glands present in *Spirorbis spirorbis* larvae are used sequentially at settlement to effect attachment of the larvae and to form the primary mucoid tube. However, planktotrophic species such as *Pomatoceros lamarckii* do not possess the large 'attachment gland' of *S. spirorbis* which is largely responsible for initial attachment

(Nott, 1973). Potswald (1978) studied larval metamorphosis of several spirorbid species. As with *S. spirorbis*, the initial settlement of *Spirorbis moerchi* is largely effected by the emptying of the 'shell gland' (= attachment gland) *via* the anus. However, for spirorbid larvae that lack a fully formed shell gland, Potswald (1978) found that the pair of anal glands played a much more important role in attachment and initial tube formation. Moat (1985) considered that in *Pomatoceros triqueter*, both permanent attachment and mucoid tube production are brought about by the array of mucous glands and did not consider that any single gland was particularly responsible for attachment. As in *P. triqueter*, the attachment of *P. lamarckii* appears to be effected by the secretion of numerous mucous threads from the array of mucous glands *via* the respective gland pores followed by the formation of the primary mucoid tube by mucus emanating from the ventral thoracic mucous glands in a manner similar to that described for *Spirorbis spirorbis* by Nott (1973) and *Spirorbis spirilum* and *Spirorbis vitreus* by Potswald (1978).

Metamorphosis

Segrove (1941) reports that for *Pomatoceros triqueter* (=*Pomatoceros lamarckii* ?) larvae at no stage was a 'snout' found as described by Wilson (1936) in *Branchiomma*, or a proboscis as in *Filograna* (Faulkner, 1930). However, during this study, a small 'snout' was observed on the ventral surface between the newly formed tentacles of recently metamorphosed *P. lamarckii* larvae. Potswald (1978) described a 'proboscis'-like structure that develops 24 hours after settlement in *Spirorbis moerchi*. The proboscis was similar to that observed during this study, developing centrally, ventral to the developing branchial crown. Ultimately, the constriction at the base of this 'proboscis'-like structure became so tight that it is eventually cast off from the *S. moerchi* juvenile. Therefore, in *S. moerchi*, the ventral prostomial glands together with the apical tuft and various trochs are lost during metamorphosis. The 'snout' observed for *P. lamarckii* may represent a similar process of casting off those head organs that are no longer required after settlement.

Artificial induction of metamorphosis

Potassium ions have been used to induce metamorphosis in a variety of invertebrate larvae (Hadfield and Pennington, 1990). However, during this study potassium ions induced abnormal metamorphosis of *Pomatoceros lamarckii* larvae. Larvae were stimulated to undergo partial metamorphosis and large quantities of mucus were secreted but few larvae successfully constructed a primary mucoid tube. Bryan *et al.* (1997) found that IBMX (isobutyl-methylxanthine) was the only artificial inducer to induce significant levels of normal metamorphosis of *Hydroides elegans* larvae. Whereas Carpizo-Ituarte and Hadfield (1998) reported that excess potassium ions in seawater did induce metamorphosis of *Hydroides elegans*, the response was dependent upon both duration and concentration. During their study, up to 77% of competent larvae metamorphosed within 72 hours when exposed to 50 mM excess potassium ions for 24 hours. It was also noted that this metamorphic response was much slower than the response to biofilms. Elevated concentrations of potassium ions clearly have an effect upon metamorphosis of *P. lamarckii* larvae but the pathway and reason for only partial metamorphosis is not clear at this stage. KCl may prove to be a useful tool for investigating the relationship between larval competence and natural settlement induction.

LIST OF ABBREVIATIONS AND SYMBOLS USED IN FIGURES

a.	anus
a.c.	apical cilia
a.t.	apical tuft
a.v.	anal vesicle
a.v.p.	anal vesicle pore
c.	collar
c.a.t.	calcareous adult tube
c.f.	collar fold
c.t.	ciliary tuft
f.c.	feeding cilia
g.	gut
g.p.	gland pore
h.v.	head vesicle
m.	metatroch
mo.	mouth
m.t.	mucous threads
n.	neurotroch
o.	operculum
p.	prototroch
p. I	type 1 pore
p. II	type 2 pore
p.m.t.	primary mucous tube
r.n.	regressing neurotroch
s.	setae
s.c.	sensory cilia
sn.	snout
t.b.	tentacle buds
t.m.	thoracic membrane
u.	uncini
1 – 4	Primary tentacles 1 - 4
I	type 1 gland cell
II	type 2 gland cell
*	approximate position of ventral ciliary tufts
+	approximate position of lateral ciliary tufts

3.1 Settlement of *Pomatoceros lamarckii* (Serpulidae) larvae on biofilmed surfaces and the effect of aerial drying

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Abstract

The settlement responses of *Pomatoceros lamarckii* (Polychaeta: Serpulidae) larvae to biofilms of varying age on slate surfaces and to dried biofilms on slate surfaces were investigated in the laboratory. Settlement experiments were performed as multi-treatment, still water assays. Larvae did not settle on clean, non-biofilmed slates but settled on biofilms up to 28 d old. Settlement intensity was closely related to the bacterial density of a biofilm. Drying a biofilmed surface for 1 to 2 h at 20°C to simulate a single tidal emersion completely negated the former inductive effect of the biofilm. Drying also negated the larval settlement-inducing effect that normally results from the presence of conspecific adults. The settlement inhibition lasted for approximately 5 d following a single drying event. Larvae settled readily on biofilms exposed to formalin and antibiotics. Treating biofilms with formalin or antibiotics before or after drying had no effect upon larval avoidance of dried biofilms. Freeze-drying a biofilm had the same effect as aerial drying. The biofilm drying effect could not be mimicked by exposing biofilms to hyper-saline seawater. The finding that *P. lamarckii* larvae do not settle on dried biofilms could have significance in explaining the natural distribution of this species in the intertidal.

Introduction

Benthic marine invertebrate larvae are capable of responding to a variety of cues during substratum exploration and settlement (see reviews by Meadows and Campbell, 1972; Crisp, 1974; Scheltema, 1974). Cues may be physical or biological and settlement-enhancing cues are frequently associated with the preferred adult habitat of the organism (Bonar *et al.*, 1986; Pawlik, 1992). Larvae have been shown to settle preferentially on rough surfaces (Crisp and Ryland, 1960) and in response to chemical cues originating from conspecific individuals (Knight-Jones, 1951b; Burke, 1984; Jensen and Morse, 1990; Pearce and Scheibling, 1990; Toonen and Pawlik, 1996), species associated with the adult habitat (de Silva, 1962; Bryan *et al.*, 1998), future prey species (Barnes and Gonor, 1973; Morse and Morse, 1984a) and biofilms (Knight-Jones, 1951b; Kirchman *et al.*, 1982a; Maki *et al.*, 1989; Hadfield *et al.*, 1994; Wieczorek and Todd, 1998).

Zobell and Allen (1935) first suggested that biofilms play an important role in the settlement of marine invertebrate larvae. Subsequently, biofilms and their individual components (bacteria, diatoms, protozoa, extracellular products) have been shown to enhance (Knight-Jones, 1951b; Neumann, 1979; Kirchman *et al.*, 1982a), inhibit (Crisp and Ryland, 1960; Maki *et al.*, 1988, 1990, 1992; Wieczorek *et al.*, 1995), or have no effect (Brancato and Woolacott, 1982; Maki *et al.*, 1988) upon the settlement preferences of a range of marine invertebrate larvae (see review by Wieczorek and Todd (1998)). Larvae are capable of differentiating between biofilms of varying bacterial species composition (Kirchman *et al.*, 1982a; Unabia and Hadfield, 1999), ages (Maki *et al.*, 1988, 1990, 1992), densities (Hadfield *et al.*, 1994; Tsurumi and Fusetani, 1998) and origins (Keough and Raimondi, 1996) during the settling process.

The intertidal zonation of sessile marine invertebrates is classically regarded as the product of post-settlement mortality resulting from biological interactions and physical conditions (Stephenson and Stephenson, 1949; Lewis, 1961). However, the distribution of sessile marine organisms may be strongly influenced by recruitment patterns (Gaines and Roughgarden, 1985) which may be a function of habitat selection by settling larvae (Underwood and Denley, 1984). For sessile, intertidal organisms there must be strong selective pressure for the larvae to avoid settling outside the zone capable of supporting post-settlement survival. Strathmann and Branscomb (1979) reported that cyprids of *Balanus* (= *Semibalanus*) *cariosus* avoided sites in the upper intertidal which dry out at low tide. Subsequently, Strathmann *et al.* (1981) showed that cyprids settled preferentially on plates with biota characteristics of the lower intertidal shore and concluded that some component of the microflora provides the settlement cue. Similarly, Thompson *et al.* (1998) showed that in the laboratory cyprids of *Semibalanus balanoides* preferentially selected rock chips taken from the mid-shore, the natural zone of this species. Alternatively, Grosberg (1982) suggested that cyprid pre-settlement behaviour i.e., stratification of cyprids in the water column, may play an important role in determining adult distribution. The possibility that biofilms in the intertidal zone may have a direct influence on sessile invertebrate distributions, other than for barnacles (see above), has not yet been widely recognised or investigated.

Pomatoceros lamarckii is an intertidal and shallow subtidal tube-dwelling polychaete (Serpulidae) common around the British coastline (Hayward and Ryland, 1995). Previous studies (Roscoe, 1993; Chan and Walker, 1998) have demonstrated that larvae of *P. lamarckii* settle preferentially on biofilms which have been exposed to a seawater-soluble leachate emanating from the body of intact adult worms. Furthermore, these studies showed that the larvae will not settle on surfaces without a biofilm and preferentially settle on older biofilms, up to 21 d old. A similar larval settlement preference has now been demonstrated for several serpulid species (see references in Table 1). Whilst collecting adult *P. lamarckii* from shores of the Menai Strait at low tide, it was noted that recent recruits (< 1 month old) occurred almost exclusively in the lower intertidal zone and higher on the shore, but only in damp areas. Clearly, such a distribution is common for low shore organisms and may be the result of larval selectivity during settlement and / or post-settlement mortality. Considering the importance of a biofilm during substratum exploration and settlement to *P. lamarckii* larvae, this laboratory study investigates the effect of biofilm age and aerial drying of a biofilmed surface upon the settlement of *P. lamarckii* larvae.

Materials and methods

Larval culture

Collections of adult *Pomatoceros lamarckii* (Zibrowius) attached to small rocks were made during the summer and autumn of 1998 and 1999 from the lower intertidal zone of the Menai Strait, Anglesey, North Wales, U.K.. Larval culture followed the methods of Roscoe (1993) with the exception that the cultures were maintained at the elevated temperature of 19°C, so that larvae typically became competent to settle in as little as 7 to 11 d after the initial fertilisation. Briefly, male and female worms were induced to release their gametes by removing them from their tubes. Eggs were fertilised with a dilute sperm suspension. Larvae were cultured in 2 l Pyrex glass beakers in 0.2 µm filtered, u.v.-irradiated seawater and fed on a 1:1 mixture of the flagellates *Rhinomonas reticulata* and *Tetraselmis chui* at approximately 200 cells μl^{-1}. All larval culture and settlement experiments were carried out in a constant environment cabinet at 19°C with a 12:12h light:dark cycle. All glassware was routinely cleaned in 5 % Chloros (sodium hypochlorite), rinsed thoroughly in hot tap water followed by distilled water, before drying at 60°C in a drying cabinet.

Biofilm development

Slates (Welsh slate cut to size (25 x 25 x 4 mm) by Inigo Jones & Co. Ltd., North Wales and polished uniformly with 600 grade wet and dry emery paper) were used in all laboratory settlement experiments. Biofilms were allowed to develop on slates submerged continuously in the laboratory running seawater system. This system uses seawater extracted from the Menai Strait and allowed to settle out in holding tanks before being pumped to a header tank from which it is gravity fed to aquaria and laboratories. Slates were held horizontally in plastic racks with the experimental surface face down,1 cm above the bottom of the tank. The tank was covered with a wooden lid so cutting out light and preventing microalgal growth on the slates.

Assay procedure

Assay procedures were based upon the methods described in Roscoe (1993) and Chan and Walker (1998). An initial experiment, testing the effect of larval density upon settlement, was conducted as detailed below but as a single treatment assay. All subsequent experiments were conducted as multi-treatment settlement assays with treatments and control surfaces placed together randomly in circular Pyrex glass dishes (250 ml capacity) filled with 100 ml of 0.2 µm filtered, u.v.-irradiated seawater. At the start of an assay, equal aliquots of larvae were added to each dish. Using this method, it was possible to keep the numbers of larvae reasonably constant (± 10 %) between replicate dishes for an experimental assay. The mean number of larvae per dish varied between experiments due to limitations in the number of larvae available. The mean number of larvae per dish ranged from 50 to 300 for these experiments.

Each experiment and any experimental repeat used larvae from a separate batch. At the end of each experiment and each experimental repeat, the numbers of settled individuals on the treated and control surfaces, as well as the non-settled larvae, were counted.

Effect of larval density

Because *Pomatoceros lamarckii* larvae are gregarious (Chan and Walker, 1998) there is the potential for larva-larva interactions to occur during settlement assays. To determine whether this is the case, the effect of larval concentration during an experimental assay upon percentage settlement was investigated. Different larval concentrations of 50, 100, 200 or 400 larvae were added to replicate assay dishes (n=6). Dishes contained 100ml of seawater and a single biofilmed slate (56 d biofilm) placed centrally. Counts were made after 24 h.

Effect of biofilm age

Biofilms were initiated weekly in the laboratory and allowed to develop for 7, 14, 21 or 28 d on slates and glass slides (36 x 12 x 1 mm). Biofilmed slates (7 – 28 d biofilms) and a clean, non-biofilmed control slate were placed together in replicate glass dishes (n = 6) and larvae added. Counts were made after 24 h. The biofilmed glass slides were fixed with glutaraldehyde (2.5 % in seawater), dehydrated in an ethanol series (30, 50, 70, 100 % ethanol for 20 min each), sputter-coated with gold and observed in a Cambridge S120 scanning electron microscope. Bacterial counts were made within 10 random fields (2400μm^2) per replicate slide (n = 3) for each of the biofilm ages.

Effect of drying

Experiment 1

Biofilmed slates were dried in the open air for 2 h under ambient conditions. During the drying process both treated and control surfaces were orientated so that the experimental surface faced upwards. Environmental conditions were noted. Control biofilmed slates were also placed outside the laboratory for 2 h but remained immersed in filtered seawater. After the drying period, control and dried slates were returned to the laboratory, placed together in replicate dishes (n = 4) and the settlement assay started immediately. The counts of settled individuals and non-settled larvae were made after 24, 48 and 72 h. This experiment was conducted four times.

Experiment 2

In the laboratory biofilmed slates (63 d biofilms) were dried under controlled conditions in an oven (EMScope Ltd.) at 20°C for 2 h. As a control, biofilmed slates (63 d biofilms) were placed in a 250 ml glass dish filled with filtered seawater which was also placed in the oven. Experimental and control slates were then placed together in replicate dishes (n = 4) and larvae added. The counts were made after 96 h. This experiment was conducted four times.

Experiment 3

The drying time required to markedly reduce percentage settlement of *P. lamarckii* larvae on a biofilm was investigated. Biofilmed slates (224 d biofilms) were allowed to dry at room temperature (20°C) for 5, 15, 30, 60 and 120 min immediately prior to the settlement assay. Each replicate dish (n = 8) contained the dried biofilmed surfaces and a non-dried biofilmed slate (also 224 d biofilm) as a control. The counts were made after 24 h. This experiment was conducted once.

Experiment 4

To investigate the duration of the drying effect, biofilmed slates were dried (2 h at 20°C) and returned to running seawater 0, 5, 10 or 15 d prior to a settlement assay (all biofilms were 57 d old at the start of the experiment). In addition clean, non-biofilmed slates were placed in the running seawater when the treated surfaces were returned after drying. These treatments were designed to investigate if drying a surface permanently negates the inductive properties of the biofilm and to show whether any subsequent inductive effect could simply be ascribed to the biofilm that developed post-drying. Each replicate (n = 6) consisted of the dried and newly developed biofilms and a non-dried (57 d biofilm) control. The counts were made after 24 h. This experiment was conducted twice.

Experiment 5

The effect of drying upon the gregarious settlement response of larvae was investigated. Slates bearing five *Pomatoceros lamarckii* individuals, which had settled 90 d prior to the assay and grown on in the laboratory running seawater system, were used as the conspecific treatment. Each replicate dish (n = 6) contained dried (1 h, 20°C) 90 d biofilmed slates, dried (1 h, 20°C) conspecific slates, non-dried conspecific slates and a non-dried 90 d biofilmed slates as a control. The counts were made after 24 h. This experiment was conducted twice.

Nature of the biofilm and dried biofilm cue

A series of experimental assays was carried out to investigate the nature of the biofilm cue and the drying effect on larval settlement. These experiments aimed to determine i) if larvae require a live biofilm for settlement, ii) if the negative effect of aerial drying could be mimicked by exposing the biofilm to osmotic stress and iii) the larval settlement response to freeze-dried biofilms. Each of these experiments was conducted as replicated (n=6), multi-treatment assays. Biofilms used for each of this series of experiments were all of the same age. The counts were made after 24 hours.

Experiment 1

This experiment was conducted to investigate whether larvae require a live biofilm for settlement and to determine if the aerial drying effect was a result of larvae responding to a product actively produced by bacteria (or other biofilm components) in response to aerial drying. In the first assay, biofilms were exposed to 4% formalin in seawater for 1 h, rinsed for 1 min in seawater then dried for 1 h at 20°C. Biofilms were treated with formalin immediately before or immediately after the drying process. Non-dried biofilms were also treated with formalin and other dried biofilms were not; the controls were non-dried biofilms. Because formalin is a preservative and has the potential to change the chemical nature of a biofilm, a similar protocol was carried out using antibiotics which inhibit protein synthesis by bacteria. Biofilms were exposed for 6 h to a 1:1 mixture of the antibiotics kanamycin and streptomycin (Sigma) at concentrations of 50 $\mu g\ ml^{-1}$ in the second assay and 200 $\mu g\ ml^{-1}$ in the third assay. Plating assays confirmed the effectiveness of the antibiotic treatment. Biofilms were treated with antibiotics immediately before or immediately after the drying process. Non-dried biofilms were also treated with antibiotics and other dried biofilms were not; the controls were non-dried biofilms.

Experiment 2

Biofilms were exposed to hyper-saline seawater to simulate osmotic stress during drying, but without the physical effect of drying. Hyper-saline seawaters (60 and 120 ‰) were prepared by gently heating at 50°C u.v.-irradiated, 0.2 µm filtered seawater until the respective salinities were reached. The hyper-saline seawaters were prepared the day prior to an assay and allowed to cool overnight. Biofilmed slates were immersed in the hyper-saline seawater for 1 h; control biofilmed slates were kept immersed in normal salinity seawater during this time and then assayed alongside the treated biofilms. This experiment was conducted twice; once with 60 ‰ and once with 120 ‰ seawaters.

Experiment 3

Biofilms were freeze-dried as an alternative to aerial drying. Biofilms were frozen by immersing them for 1 min in liquid nitrogen before being freeze-dried in a freeze-drier (Edwards) for 12 h at –50 °C. Biofilms were frozen in liquid nitrogen either directly from seawater or after being rinsed for 10 seconds in distilled water. A dried biofilm treatment was included in this experiment and the control was a non-dried biofilm.

Statistical analysis

The percentage larval settlement data in response to experimental treatments were arc-sine transformed before statistical analysis. Those treatments in which no larvae settled were given the value of 1/4n (n= no. of larvae) to improve the arc-sine transformation (Zar, 1996). Data were tested for normality and homogeneity of variances. Normal data without significantly heterogeneous variances were analysed by the appropriate parametric test; t-test for those experiments with two treatments and one-way ANOVA, followed by Tukeys test, for experiments with multiple treatments. Due to significant heterogeneity of variances most data were analysed by the appropriate non-parametric tests: Mann Whitney U test for experiments with two treatments and Kruskal-Wallis, followed by Dunns multiple comparisons test, for multiple-treatment assays. Further details of the tests used for each experiment are given in the text and figure legends.

Results

Larval density

There was no significant difference (One-way ANOVA, F=0.50, d.f. 5, p=0.685) in percentage larval settlement on biofilms between the various larval concentrations tested (Figure 1). Because of the time involved with accurately counting large numbers of larvae for an experimental assay and the lack of any apparent effect of different larval concentration upon percentage settlement, it was decided to add equal aliquots of larvae to each replicate dish in all subsequent assays.

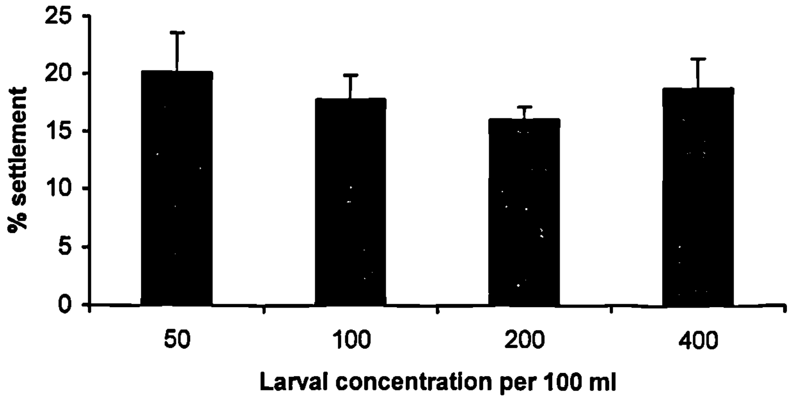


Figure 1. Mean percentage settlement of *Pomatoceros lamarckii* larvae after a 24 h no-choice settlement assay with a single biofilmed slate. Larval densities of 50, 100, 200 & 400 per 100 ml of seawater were used. Data plotted are the means + SE (n=6). There was no significant difference in percentage settlement between treatments.

Biofilm age

Biofilms were composed primarily of bacteria (1-2 μm long), most of which were rod-shaped. Other common biofilm components included fungal hyphae, choanoflagellates and detritus. Bacterial numbers increased with biofilm age from 8484 ± 1965 (SE) mm^{-2} after 7 d of biofilm growth to 21420 ± 2948 mm^{-2} after 28 d (Figure 2). Larvae settled in higher numbers on biofilms that had developed for the longer periods of time (Kruskal-Wallis; H=18.78; d.f. 5; p<0.001) and settlement intensity appeared to be closely related to bacterial density (Figure 2). Relatively high settlement of 32.15 ± 2.66 (SE) percent occurred on 28 d biofilms during this assay. No larvae settled on the clean, control slates. During other laboratory experiments, biofilms of various ages (7 – 224 d biofilms) were used. Although settlement was frequently variable and low both within and between experiments, no evidence emerged of the older biofilms becoming less attractive to larvae.

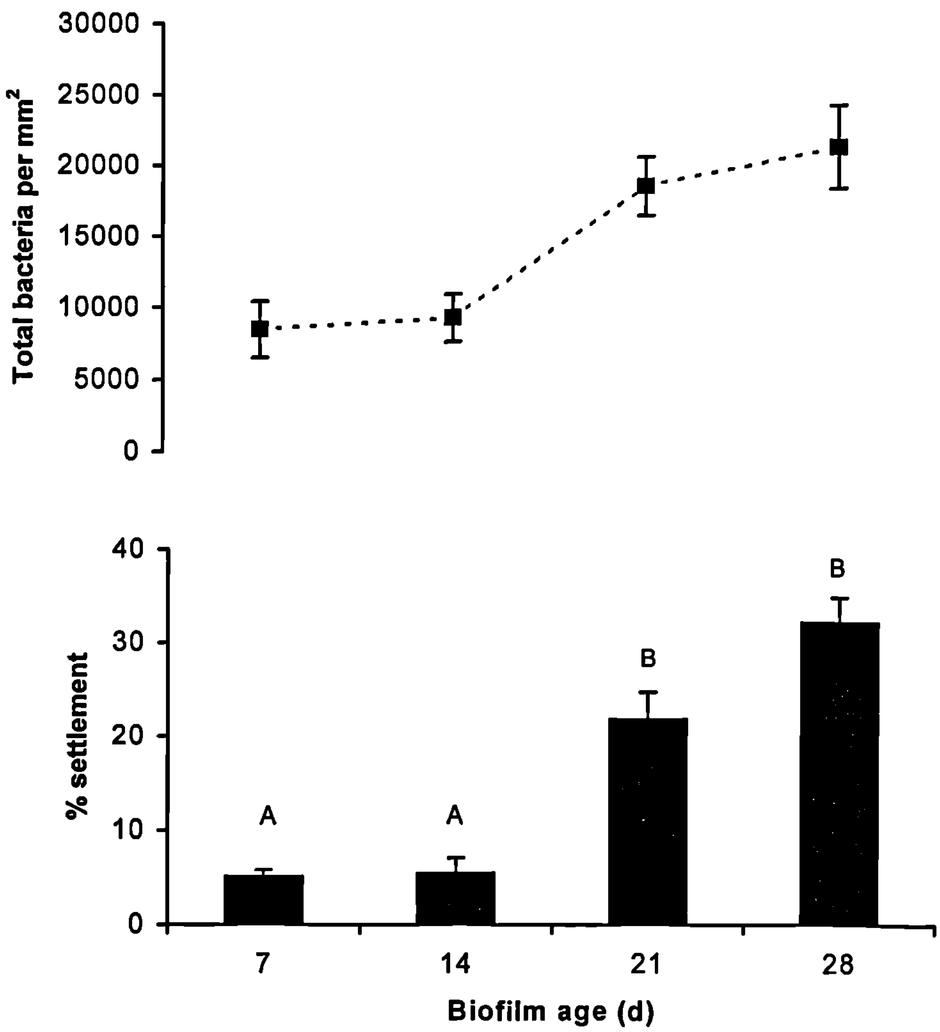


Figure 2. Line graph shows mean ± SE (n=3) bacteria per mm^2 for 7-28 d biofilms on glass slides. Bar chart shows mean percentage settlement of *Pomatoceros lamarckii* larvae after a 24 h settlement assay on control and biofilmed surfaces (7 – 28 d biofilms). No settlement occurred on the control surface (not shown) which was a clean, non-biofilmed slate. Data plotted are means + SE (n=6). Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$).

Drying

Experiments 1 and 2

Aerial drying a biofilmed surface outside dramatically reduced settlement of *Pomatoceros lamarckii* larvae on that surface (Figure 3). In all four assays, drying a biofilmed surface negated the former settlement inductive effect of the biofilm. Surfaces dried outside remained unattractive to larvae throughout the subsequent 72 h settlement assay. The least difference in percentage settlement on dried versus non-dried biofilms occurred in the second assay. The drying treatment for this assay was conducted on a cloudy day compared to the warmer, sunny days during the drying treatment of the other experimental assays (Figure 3). Large variations in overall percentage settlement occurred during these assays. Drying a biofilmed surface in the laboratory under controlled conditions also resulted in significantly reduced (Mann Whitney U test, $p<0.05$) percentage larval settlement on the dried surfaces in all four assays. The dried surfaces remained unattractive to larvae throughout the subsequent 96 h settlement assay.

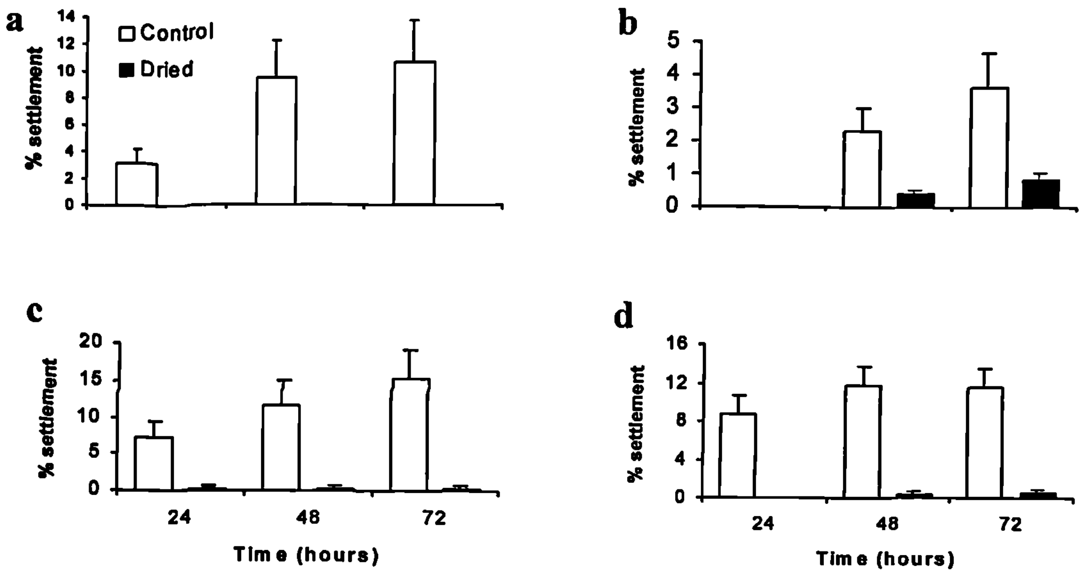


Figure 3 a-d. Mean percentage settlement of *Pomatoceros lamarckii* larvae on biofilmed slate surfaces (182 d biofilms) every 24 h over 72 h settlement assays. Slates were dried outside the laboratory (■), control (non-dried) surfaces (□) (182 d biofilms) remained immersed in seawater when placed outside for the 2 h period. Data plotted are the means + SE (n=4) for four experimental repeats. Ambient conditions during the drying process for each assay were **a)** 26°C, clear sky, slight breeze; **b)** 18°C, cloudy, slight breeze; **c)** 28°C, clear sky, no wind and **d)** 30°C, clear sky, breezy. Percentage settlement was significantly different (Kruskall-Wallis; d.f. 3, $p<0.05$) between treatment and control biofilms in all experimental repeats at each of the time periods (24, 48 & 72 h) apart from after 24 h in the second experimental repeat where no settlement occurred on either surface. Note the different y axis scales.

Experiment 3

Figure 4 shows the inverse relationship found between drying time (in an oven) of a biofilmed surface with percentage settlement of *Pomatoceros lamarckii* larvae. Larval settlement decreased significantly with increasing drying time (Kruskal-Wallis; H= 25.75; d.f. 5; $p<0.001$). Drying at 20°C for up to 30 min had no significant effect upon percentage settlement; drying for 1 hour significantly reduced percentage settlement and no larvae settled on biofilms that had been dried for 2 hours.

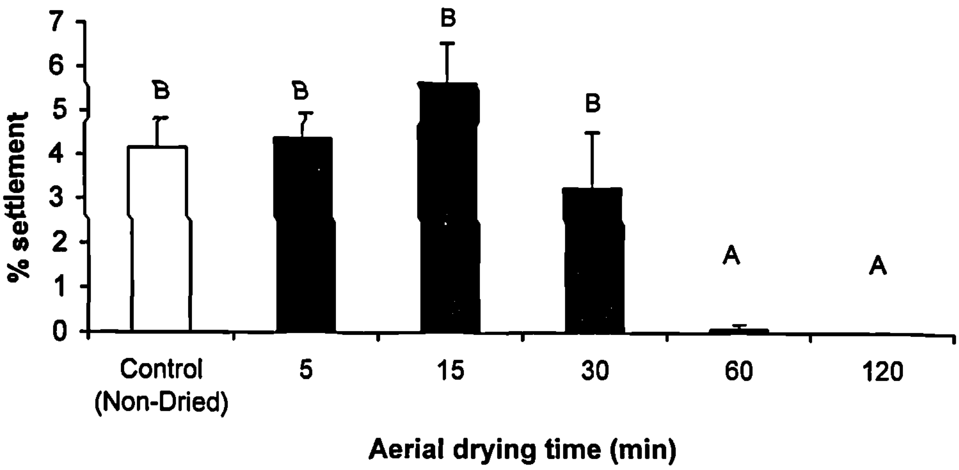


Figure 4. Mean percentage settlement of *Pomatoceros lamarckii* larvae after a 24 h settlement assay on biofilmed slate surfaces (224 d biofilms) after aerial drying at 20°C for 5, 15, 30, 60 & 120 min. The control surface is a non-dried 224 d biofilm (□). Data plotted are means + SE (n=6). Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$).

Experiment 4

In both experimental assays, settlement was less than the control on surfaces dried 0 and 5 d prior to the assay. Settlement was also lower on surfaces with 5 and 10 d old non-dried biofilms compared to the 57d control biofilm. Settlement on biofilms dried 10 and 15 d prior to the assay was not significantly different from that on the control, some settlement did occur on biofilms which were dried 5 d before the assay (Figure 5a,b). The effect of a single drying event therefore lasts approximately 5 d or more post-drying.

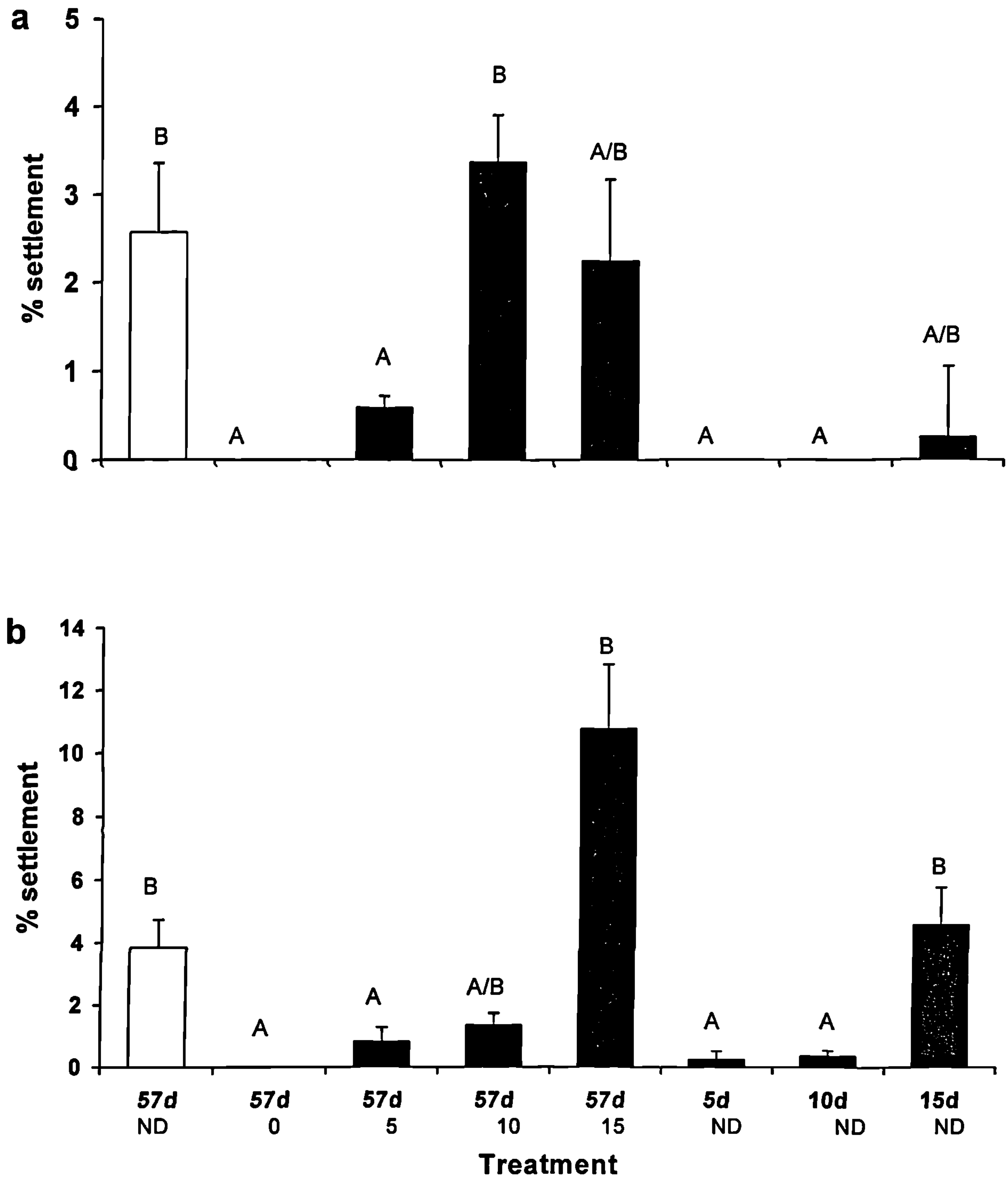


Figure 5 a & b. Mean percentage settlement of *Pomatoceros lamarckii* larvae after a 24 h settlement assay on dried and non-dried biofilms and on a non-dried biofilmed control slate (□). Numbers immediately below columns indicate biofilm age in days. Below these, the drying treatment is indicated: ND denotes non-dried, whereas 0, 5, 10 and 15 denote the number of days prior to the assay when the 2 h drying treatment took place. Data plotted are the means + SE (n=6) for the two experimental assays (a and b). Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$).

Experiment 5

In both experimental assays, larvae settled in greatest numbers on the non-dried conspecific treatment, percentage settlement was relatively high with 26 and 22 % settlement for the first and second assay respectively (Figure 6 a,b). Drying conspecific slates significantly reduced percentage settlement on these surfaces in both assays (Kruskal Wallis, H=12.07 & 12.37 respectively, d.f. 5, $p<0.05$); no larvae settled on the dried conspecific slates during the second assay.

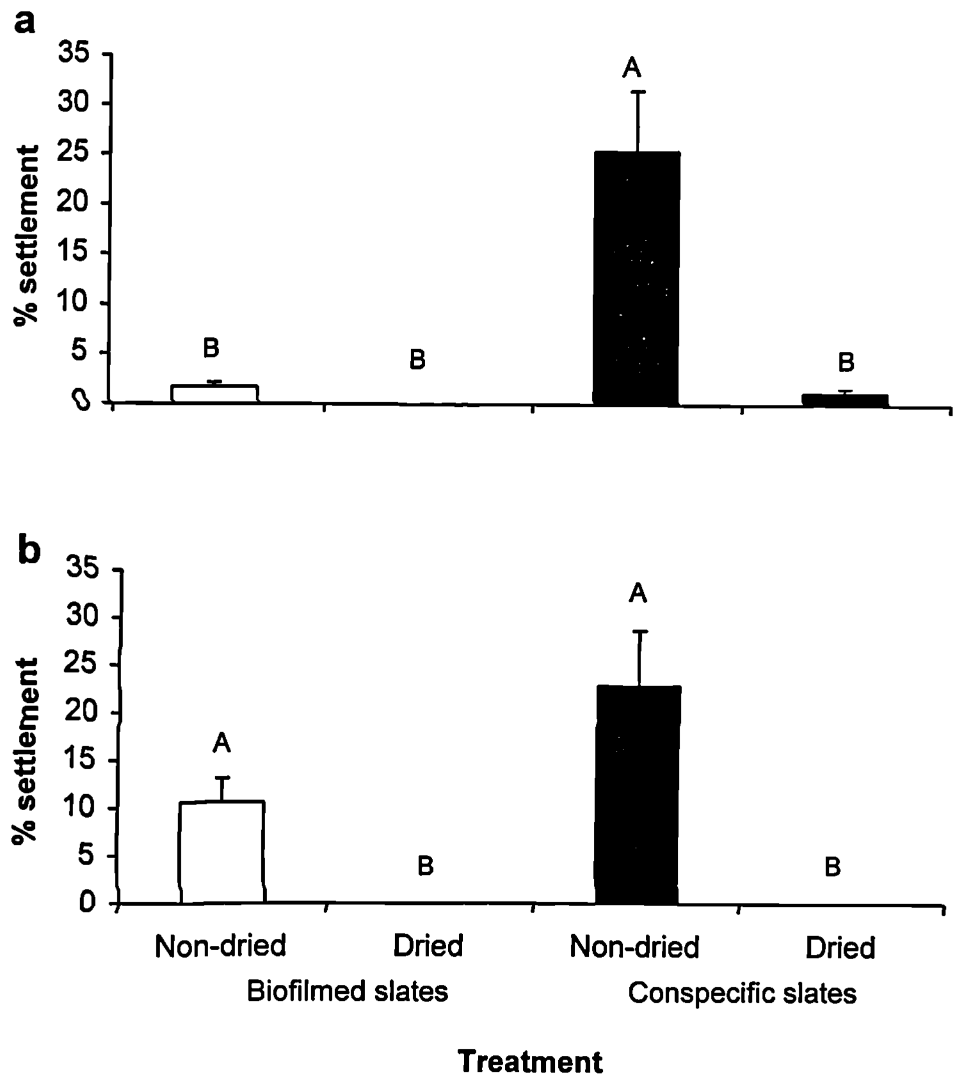


Figure 6 a & b. Mean percentage settlement of *Pomatoceros lamarckii* larvae after a 24 h settlement assay. Conspecific slates (five *P. lamarckii* individuals per slate grown on in laboratory for 90 d) and 90 d biofilmed slates were dried (1 h at 20 °C) or non-dried. The non-dried biofilmed slate is the control (□). Data plotted are the means + SE (n=6) for the two experimental assays. Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$).

Nature of the biofilm cue

Experiment 1

Treating a biofilm with formalin before or after aerial drying did not significantly alter the negative effect of drying (Kruskal Wallis, $H=27.11$, d.f. 5, $p<0.001$) (Figure 7a). Similarly, treating a biofilm with antibiotics either before or after drying had no apparent influence upon the larval settlement response to the drying effect (Figure 7b-c). Larvae settled in similar numbers on both the non-dried biofilm that had been treated with antibiotics and the control non-dried biofilm.

Experiments 2 & 3

Exposing a biofilm to 60 ‰ or 120 ‰ seawaters had no significant effect (t-test, $t = 0.38$ & -1.77 and $p = 0.72$ & 0.14 respectively, d.f. 5) upon percentage larval settlement. Larvae settled readily on biofilms exposed for 1 h to both seawaters. In Experiment 3, no settlement occurred on any of the biofilms that had been dried or freeze-dried. Relatively high settlement (29 ± 4 %) occurred on the non-dried control biofilm during this assay.

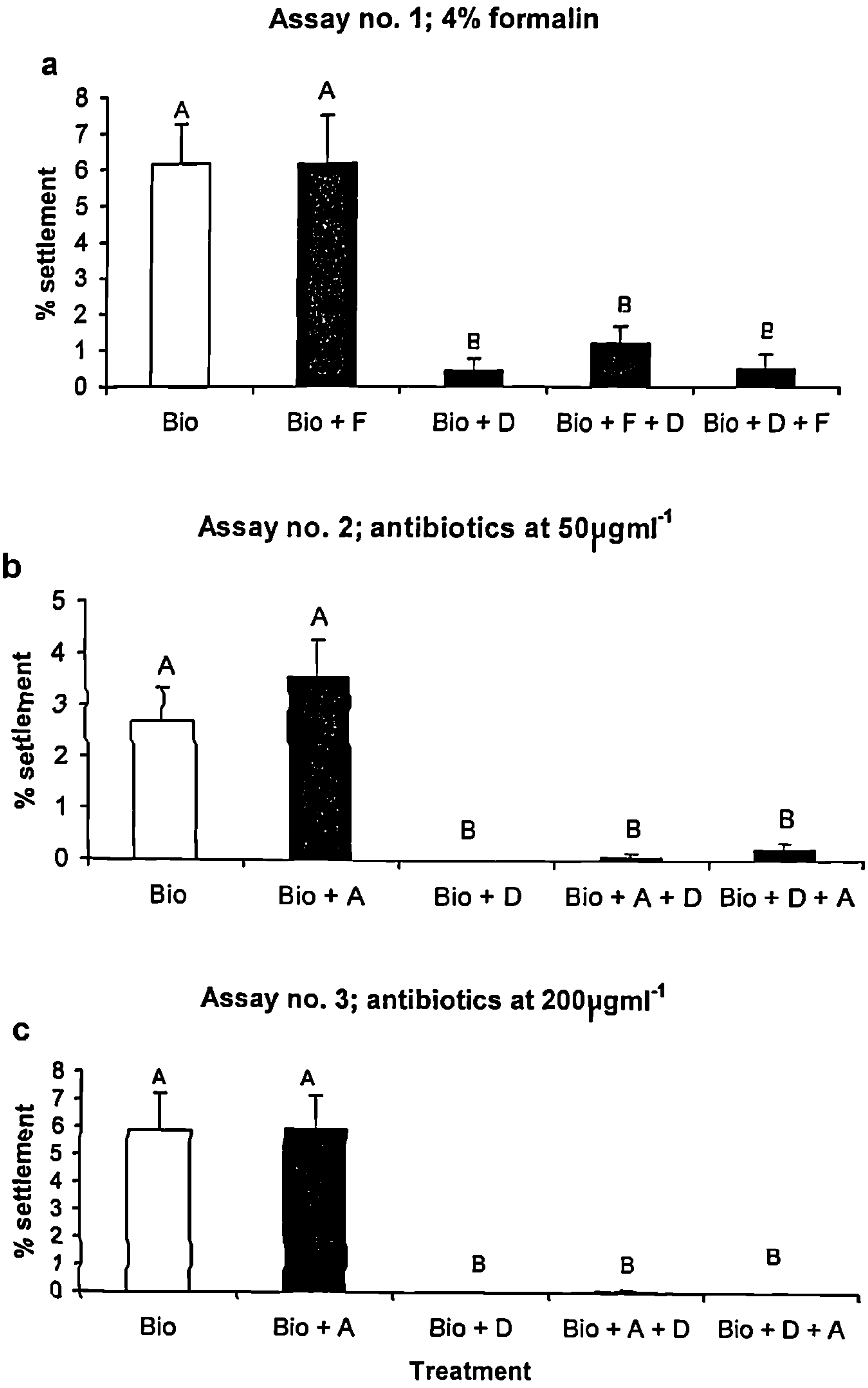


Figure 7 a-c. a) Mean percentage settlement of *Pomatoceros lamarckii* larvae after a 24 h settlement assay. Biofilms were exposed to 4% formalin for 1 h before (Bio+F+D) and after (Bio+D+F) drying. A non-dried biofilm was also exposed to formalin (Bio+F). A biofilm was dried but not exposed to formalin (Bio+D). The control (□) was a non-dried biofilm (Bio). **b)** Mean percentage settlement of *P. lamarckii* after a 24 h settlement assay. Biofilms were exposed for 6 h to the antibiotics kanamycin and streptomycin at a ratio of 1:1; two experiments were carried out with different antibiotic concentrations of 50 $ugml^{-1}$. Antibiotic treatments were administered before biofilm drying (Bio+A+D), after biofilm drying (Bio+D+F) and to non-dried biofilms (Bio+A). A dried biofilm treatment (Bio+D) was included and a non-dried biofilm (Bio) was used as a control (□). **c)** As previous experiment but a higher antibiotic concentration of 200 $ugml^{-1}$ was used. Data plotted are the means + SE (n=6). Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$).

Discussion

Pomatoceros lamarckii larvae do not settle on slate surfaces in the absence of a biofilm (Roscoe, 1993; Chan and Walker, 1998; this study, Figure 2). A preference for biofilmed surfaces also appears to be a common settlement requirement for the larvae of other serpulid and spirorbid species (see Table 1). Chan and Walker (1998) showed that larvae of *P. lamarckii* settle preferentially on surfaces that have been biofilmed for periods of up to 21 days. In this study surfaces were compared that had been allowed to develop biofilms for varying times (between 7 and 28 d biofilms); larvae consistently settled in higher numbers on the 'oldest' biofilm surfaces (Figure 2). In addition, surfaces with a 224 d biofilm were assayed in subsequent experiments and there was no obvious decrease in attractiveness to settlement on such biofilms developed over such a protracted period. Larvae of the serpulids *Hydroides ezoensis* and *Hydroides elegans* have also been shown to settle in higher numbers on older biofilms (Miura and Kajihari, 1984; Hadfield *et al.*, 1994). The larvae of several other marine invertebrates settle preferentially on biofilms (Crisp, 1974) and settle in higher numbers on older biofilms both in the laboratory (Pearce and Scheibling, 1990; Wieczorek *et al.*, 1995) and in the field (Wieczorek and Todd, 1998).

This present work leads to the belief that bacterial density is a major cue for the settlement response of *Pomatoceros lamarckii* (Figure 2). Useful comparisons may be drawn from studies on the settlement preferences of the larvae of other serpulid species. Hadfield *et al.* (1994) suggested that bacteria present in the biofilm were the most likely component that induced *Hydroides elegans* larvae to settle and reported that settlement was correlated to bacterial density, hence the attractiveness of the older biofilms. In addition, specific bacterial strains and microalgae have been shown to induce, have no effect or inhibit the settlement response of larvae of the tubeworms *Spirorbis spirorbis* (Meadows and Williams, 1963), *Janua brasiliensis* (Kirchman *et al.*, 1982a), *Hydroides ezoensis* (Miura and Kajihara, 1984) and *Hydroides elegans* (Lau and Qian, 1997; Unabia and Hadfield, 1999). Because of the nature of biofilms, it is likely that when larvae differentiate between and select certain biofilmed surfaces they are responding to a range of inductive and / or inhibitory components present (Neal and Yule, 1994b). Because the biofilms used in our experiments were developed in the dark and were composed primarily of bacteria, it is considered that *P. lamarckii* larvae are probably responding to the increased bacterial density and / or the presence of certain bacteria and / or components produced during the ageing process when they preferentially select the older biofilms.

It is unclear why the larvae of some marine invertebrates settle preferentially on well developed biofilms. Clearly, biofilms have the potential to provide information on the prevailing environmental conditions (Neal and Yule, 1994b). From an adaptive perspective it has been suggested that a biofilm may indicate a relatively stable underlying substratum with a good water flow regime over it (Wieczorek and Todd, 1998). Also, for the larvae of short-lived, ephemeral species that are easily overgrown, such as *Hydroides elegans,* biofilms under a certain age may indicate newly created space on which a settled individual would be at an advantage, being able to become fecund before being overgrown (Unabia and Hadfield, 1999). Compared to *H. elegans, Pomatoceros lamarckii* is a relatively slow-growing species (Segrove, 1941), its larvae most likely selecting those biofilmed surfaces which indicate a relatively stable substratum with adequate water movement operating over it.

Percentage settlement of *Pomatoceros lamarckii* during the experiments described here was very low compared with other serpulid species (Hadfield *et al.*, 1994; Toonen and Pawlik, 1994) but comparable to the levels previously reported by Roscoe (1993) and Chan and Walker (1998) for *P. lamarckii* (Table 1). For *Hydroides elegans*, very high settlement (in excess of 80%) may be obtained in a 24 h settlement assay on biofilmed surfaces only three days old (Hadfield *et al.*, 1994). Percentage settlement figures comparable to those of *Hydroides elegans* have not been recorded in any of the present assays using *P. lamarckii*, even on biofilms developed over 224 days (Figure 4). Only the presence of adult leachate adsorbed onto a biofilm consistently induce higher percentage settlement (see Chan and Walker, 1998; Figure 6, this study). *P. lamarckii* larvae therefore seem to have similar settlement requirements as *Hydroides dianthus* in that a certain proportion settles on a biofilm alone, but the majority will delay settlement in the absence of a biofilm-associated conspecific cue (Roscoe, 1993; Toonen and Pawlik, 1994; Chan and Walker, 1998). During this study, variability in percentage settlement between batches of larvae presented with biofilms of similar age was noted. Such variability suggests between-batch differences in the percentage of larvae at different stages of development at any given time. Although every effort was made to use only larvae that appeared to be at the same developmental stage, it is clear that between-batch differences did exist. However, although total percentage settlement did vary between experimental repeats, these assays gave consistent results in terms of larval settlement preferences.

Perhaps not surprisingly most studies of invertebrate larval settlement focus on inductive cues (Wieckzorek and Todd, 1998). Here an inhibitory cue in laboratory settlement experiments is reported which, when extrapolated to the intertidal environment, may have real ecological significance. When larvae of *Pomatoceros lamarckii* settle, they first secrete a primary mucoid tube, followed over the next several hours by the initiation of the juvenile calcareous tube using the mucoid tube as a template (Segrove, 1941). During the first hours of settlement, newly metamorphosed juveniles are therefore very susceptible to desiccation during tidal emersion and are unlikely to survive if they have settled on a surface which dries quickly. The present observations that larvae avoid surfaces which dry out suggests that their distribution on the shore may be strongly influenced by larval selectivity at the settling stage. Furthermore, throughout these laboratory experiments biofilmed slates were only submitted once to a relatively short drying event. On U.K. rocky shores in summer, surfaces are exposed to drying on a daily basis for longer periods of time and at higher temperatures than in the present experiments, presumably imposing an inhibitory effect on *P. lamarckii* settlement.

The reversal in attractiveness of the biofilmed surface to the settling larvae as a result of the drying process may be a purely physico-chemical effect or some component of the biofilm may be changed in response to drying. Little is known about the active components of a biofilm which stimulate or inhibit serpulid larval settlement, but bacteria and / or their extracellular products appear to be a common cue (Hadfield *et al.*, 1994; Lau and Qian, 1997, 1999, 2002; Unabia and Hadfield, 1999; this study). Interestingly, there is evidence that for *Hydroides elegans*, the bacteria have to be alive to induce the normal larval settlement response. Unabia and Hadfield (1999) found that all physical and chemical treatments used to kill bacteria either eliminated or severely reduced the settlement-inducing effects of natural multispecies biofilms to *H. elegans* larvae. Similarly, Qian (1999) reported that treating biofilms with antibiotics stopped the normal *H. elegans* larval settlement response. However, during this study it was found that larvae of *Pomatoceros lamarckii* will settle readily on biofilms treated with formalin or antibiotics (Figure 7). Similarly, Kirchman *et al.* (1982a) reported that killing a *Deleya marina* biofilm with formalin did not significantly alter its inductive effect to larvae of the spirorbid *Janua brasiliensis*. It is still not clear at this stage what effect(s) drying has upon a biofilm.

Clearly the distribution of *Pomatoceros lamarckii* in the intertidal zone (Nelson-Smith and Gee, 1966) may come about as a result of larval selectivity during settlement and / or post-settlement mortality. There is evidence that the larvae of certain barnacle species may be capable of differentiating and responding to differences between biofilms on different niche surfaces in the intertidal and hence settling in the appropriate zone (Strathmann and Branscomb, 1979; Strathmann *et al.*, 1981; Bourget, 1988; Thompson *et al.*, 1998). Strathmann and Branscomb (1979) and Strathmann *et al.* (1981) reported that cyprids of the barnacle *Balanus* (= *Semibalanus*) *cariosus*, a species found in the lower intertidal zone and in damp areas higher on the shore, are capable of discerning between rocks which remain damp and rocks which dry at low tide when the rocks are tidally immersed. The authors also concluded that the cyprids utilise the intertidal microflora as a cue to settlement at the optimum tidal height. Alternatively, Straughan (1969) studied the intertidal distribution of the serpulid *Pomatoleios kraussii* in Hawaii and concluded that its larval settlement zone is wider than that occupied by the adults. This adult zone was limited at the top by exposure to air and at the bottom by competition for space. Future work will investigate the nature of the drying effect on biofilms and the relative importance of larval selectivity and post-settlement mortality in determining the distribution of *P. lamarckii* in the intertidal zone by conducting simultaneous laboratory and field settlement studies with variously treated surfaces.

Pomatoceros lamarckii larvae avoid settling on recently dried biofilms under laboratory conditions. It is speculated that *P. lamarckii* larvae may use this inhibitory cue to avoid settling in inappropriate habitats in the intertidal zone and that other marine invertebrate larvae may also avoid settling on recently dried biofilms.

Table 1. Settlement preferences of polychaete tubeworm (Serpulidae and Spirorbidae) larvae in response to biofilmed surfaces.

Species	Prefer biofilmed surfaces ?	Prefer older biofilms ?	Minimum age (days) of inductive biofilm (% settlement)	Maximum % settlement (film age in days)	Assay period (hrs)	Notes	Reference
Pomatoceros lamarckii	Yes	n.r.				Larvae settle in higher numbers on surfaces biofilmed in the presence of adults.	Roscoe (1993)
	Yes	Yes	7 days (<1%)	3% (21)	n.r.	Stimulated to settle in higher numbers in response to a leachate from intact conspecific adult worms.	Chan and Walker (1998)
	Yes	Yes	21 days (0.5%)	15% (180)	72	Drying a biofilm makes it unattractive to larvae.	This study
Hydroides ezoensis	Yes	Yes	4 days (5%)	55% (10)	48	Settlement was variable in response to different biofilm components e.g diatoms and bacteria.	Miura and Kajihara (1984)
Hydroides dianthus	Yes	n.r.		20% (±5)	24	Stimulated to settle in higher numbers in response to the presence of conspecific adult worms.	Toonen and Pawlik (1994)
	Yes	n.r.		5 – 10% (±5)			Toonen and Pawlik (1996)
Hydroides elegans	Yes	Yes	1 day (3 – 13 %)	82 % (3)	72, 96	Settlement correlated to bacterial density.	Hadfield *et al.* (1994)
	Yes	n.r.		75% (5)	48	Killing biofilm reduces settlement. Different bacterial species have different effect.	Unabia and Hadfield (1998)
	Yes	n.r.		80% (3)	4	80% settlement in as little as 2 hours after start of assay.	Carpizo-Ituarte and Hadfield (1998)
	Yes	n.r.		25 – 50 % (1)	48	Different bacterial species have different effect.	Lau and Qian, (1997, 1999, 2001)
Spirorbis borealis (= spirorbis)	Yes	n.r.		91% (14)	24		Knight-Jones (1951b)
	Yes	n.r.		97 % (±1)	n.r.		Crisp and Ryland, (1960)
	Yes	n.r.					Williams (1964)
Spirorbis spirorbis	Yes	Yes	1 day			Settlement very variable.	Wieczorek (1995)
Janua brasiliensis	Yes	Yes	1 day (20 %)	70 – 80% (7)	24	Killing biofilm does not inhibit the settlement response. Single bacterial species films have different effect.	Kirchman *et al.* (1982a)
				75 - 80% (1)	24	Settlement on *Pseudomonas marina* film. Larvae may use lectins in detection of biofilmed surfaces.	Kirchman *et al.* (1982b)
Spirorbis tridentatus	Yes	n.r.					deSilva (1962)

n.r. = not reported

3.2 Avoidance of dried biofilms on slate and algal surfaces by certain spirorbid and bryozoan larvae

The contents of this chapter have been published as:

Hamer, J. P. and Walker, G. (2001). Avoidance of dried biofilms on slate and algal surfaces by certain spirorbid and bryozoan larvae. Journal of the Marine Biological Association of the United Kingdom, 81: 167-168.

Abstract

The effect of aerial drying of biofilmed surfaces to simulate a tidal emersion upon the settlement preferences of spirorbid and bryozoan larvae was investigated using choice experiments with still-water conditions carried out in the laboratory. Aerial drying of biofilmed slates and pieces of *Fucus serratus* for 1 h at 20°C negated their usual settlement-inducing properties to larvae of *Spirorbis spirorbis, S. tridentatus* and *Flustrellidra hispida*. Such larval settlement preference may contribute to observed variations in the natural distribution of these species in the intertidal.

Introduction

Benthic marine invertebrate larvae are capable of responding to a variety of physico–chemical cues during substratum exploration and settlement. Chemical cues are often of biological origin and may originate from adult, juvenile or larval conspecifics, prey organisms, or biofilms (Pawlik, 1992). Biofilms may induce, inhibit or have no apparent effect upon larval settlement (Wieczorek and Todd, 1998). Larvae frequently respond positively to cues that indicate that a substratum is likely to be suitable for settlement and post-settlement survival. It has been suggested that surface biofilms may provide this information to settling larvae because they reflect the physical regime which has acted on that surface over time (Wieczorek and Todd, 1998).

Aerial drying of biofilmed slate surfaces for as little as one hour, to simulate a tidal emersion, negates the previous settlement-inducing effect of the biofilms to larvae of the serpulid polychaete *Pomatoceros lamarkii* (Hamer *et al.*, 2001; Chapter 3.1). Furthermore, the negative settlement effect was shown to last for 5 to 10 d after a single drying event. It is speculated that such behaviour may have real ecological significance in explaining the distribution of this species which occurs at the low intertidal region. The purpose of the present laboratory study was to determine if the larvae of selected intertidal spirorbid and bryozoan species, already known to settle readily on either biofilmed slate surfaces or *Fucus serratus*, also avoid settling on dried biofilms under still-water laboratory conditions.

Materials and Methods

Spirorbis tridentatus (Polychaeta: Spirorbidae) larvae were obtained from adults collected from the Menai Strait, North Wales by inducing artificial liberation of larvae (see de Silva, 1962). *Spirorbis spirorbis* and *Flustrellidra hispida* (Bryozoa) larvae were obtained by inducing the liberation of larvae from adults on *Fucus serratus* plants also collected from the shores of the Menai Strait. *S. spirorbis* and *S. tridentatus* larvae are photopositive immediately after liberation and were collected by pipette at a point-light source. Larvae of *F. hispida* were collected by eye, using a pipette. The larvae of these species are competent to settle immediately after being released; larvae that were used in the settlement assays were collected for up to 1 hour after larval liberation was initiated.

All assays were conducted in 300 ml Pyrex glass dishes containing 100 ml of U.V. irradiated and filtered (0.2 μm) seawater; surfaces to be assayed were placed randomly together in the centre of each of 12 replicate dishes. Dishes were kept at 19°C in a constant environment cabinet in the dark during the 1 hour assay period. For each assay,10 larvae of the species being assayed were added to each of the 12 replicate dishes. An assay was repeated three times on different occasions for each of the larval species.

To determine the effect of drying biofilmed slates on the settlement of spirorbid larvae, they were dried for 1 h at 20°C immediately prior to an assay. The control in each dish was a non-dried biofilmed slate. All slates (25 x 25 x 4 mm; cut from Welsh slate by Inigo Jones, N. Wales; polished with 600 grade wet and dry paper) were submerged in the laboratory running seawater system and allowed to accumulate a biofilm for at least 8 weeks prior to an assay. The dried and non-dried slates were then placed together in each dish and larvae added. The number of settled individuals was counted on each slate after the 1 hour period allowed for settlement. This first experimental series was carried out separately for *Spirorbis spirorbis* and *Spirorbis tridentatus* larvae.

The second experimental series aimed to determine if the larvae of *S. spirorbis* and *Flustrellidra hispida* avoid settling on pieces of *Fucus serratus* that had been dried. Two cm long sections of *F. serratus* fronds were cut from the middle portion of plants freshly collected from the shore. Each section was cut down the midrib to give two pieces of equal area. Again, one of these was dried for 1 hour at 20°C and the other remained immersed in seawater. The two pieces were then placed in each replicate dish and larvae of the relevant species added. The number of settled larvae on each piece of alga was counted after the 1 hour settlement period allowed.

Results

The larvae of all three species strongly avoided biofilmed surfaces that had been dried prior to the assay and settled preferentially on non-dried biofilmed surfaces (Tables 1 and 2). No larvae of *Spirorbis spirorbis* or *Spirorbis tridentatus* settled on the dried slates in any of the experimental repeats (Table 1). Settlement of *S. spirorbis* and *Flustrellidra hispida* larvae was significantly higher on the non-dried pieces of *Fucus serratus*, although some settlement did occur on the dried *Fucus* (see Table 2).

Table 1. Percentage settlement (Mean (SE): n=12) of *Spirorbis spirorbis and S. tridentatus* larvae on non-dried and dried (1 h at 20 °C) biofilmed slates after 1 h still water, choice assay. Data for all three experimental repeats (A-C) are shown. Percentage settlement was significantly different between treatments for all assays for both species (X^2; n=12; $p<0.05$).

Species	Experimental repeat	Non-dried biofilmed slate	Dried biofilmed slate
Spirorbis spirorbis	A	**29.10** (5.41)	**0**
	B	**30.00** (6.40)	**0**
	C	**49.10** (4.96)	**0**
	A	**56.70** (5.23)	**0**
Spirorbis tridentatus	B	**58.30** (5.32)	**0**
	C	**52.50** (5.59)	**0**

Table 2. Percentage settlement (Mean (SE); n=12) of *Spirorbis spirorbis* and *Flustrellidra hispida* larvae on non-dried and dried (1 h at 20 °C) pieces of *Fucus serratus* after 1 h still-water, choice assay. T-test statistic (t) and level of significance at 11 d.f. are shown.

Species	Experimental repeat	Non-dried *Fucus*	Dried *Fucus*	t	Sig. level
Spirorbis spirorbis	A	**75.83** (4.17)	**0.80** (0.81)	17.65	p<0.001
	B	**71.74** (5.70)	**7.50** (2.50)	10.23	p<0.001
	C	**54.29** (5.00)	**5.06** (2.61)	8.72	p<0.001
	A	**28.04** (5.50)	**1.70** (1.70)	5.43	p<0.001
Flustrellidra hispida	B	**33.35** (4.71)	**2.50** (1.34)	6.37	p<0.001
	C	**25.03** (3.44)	**3.32** (1.46)	5.92	p<0.001

Discussion

The non-random distribution of adult *Spirorbis spirorbis* and *S. tridentatus,* on algal and rock surfaces respectively, is well documented. Seed *et al.* (1981) reported the non-random distribution of *S. spirorbis* (*=borealis*) on *Fucus serratus* plants in Strangford Lough, Northern Ireland and also that variations in recruitment of *Flustrellidra hispida* occurred between plants and even within individual plants (Seed and Wood, 1994). In the intertidal zone, *S. tridentatus* is most abundant on the undersides of rocks and in dark and shaded places (Nelson-Smith and Gee, 1966). Wood and Seed (1980) reported that in the Menai Strait, *F. hispida* is less abundant on higher shore *F. serratus* plants and attributed this observation to the increased desiccation higher on the shore. The larval behaviour described here may help to explain how larval settlement preferences may be important factors that account for the adult distributions of these species.

The nature of the settlement-inducing cue from a biofilm or the inhibitory effect of drying a biofilm to the larvae of these species is not known. Certainly, bacteria and/or their extra-cellular products are frequently reported to play an important role in inducing settlement of a wide range of marine invertebrate larvae (see Wiekzorek and Todd, 1998) and bacteria have already been shown to induce settlement of the spirorbid *Janua brasiliensis* (Kirchman *et al.*, 1982a).

3.3 Laboratory settlement assays with larvae of the polychaete tubeworms *Pomatoceros lamarckii, Spirorbis spirorbis* and *S. tridentatus* - the importance of choice?

Abstract

Simultaneous single- and multi-treatment laboratory assays were conducted with larvae of the tubeworms *Pomatoceros lamarckii* (Serpulidae), *Spirorbis tridentatus* and *Spirorbis spirorbis* (Spirorbidae). Larvae were presented with dried and non-dried biofilmed slate surfaces and clean control surfaces either singly 'no choice' or in combination 'choice'. When given a 'choice' in multi-treatment assays, larvae of *P. lamarckii, Spirorbis spirorbis* and *S. tridentatus* settled almost exclusively on the preferred surface (a biofilmed surface), whereas, in the absence of a 'choice' in single-treatment assays a significant number of *S. spirorbis* and *S. tridentatus* larvae settled on a less favourable surface (a dried biofilmed surface). Settlement on less favourable surfaces in the absence of a 'choice' is probably due to decreased larval selectivity because larvae become 'desperate'. In contrast, the *P. lamarckii* larval settlement response was not significantly different between the single and multi-treatment assays. *P. lamarckii* larvae are planktotrophic whereas *S. spirorbis* and *S. tridentatus* larvae are lecithotrophic. Consequently, their larvae have developed a different strategy to ensure settlement in a habitat that maximises the chances of post-larval survival; the desperate larva hypothesis does not seem to apply to *P. lamarckii* larvae. In the single-treatment assay, percentage settlement on dried biofilms was lower than on non-dried biofilmed surfaces and the larvae that did settle took longer to commit to settlement (1-2 h). When designing still-water, laboratory settlement assays for marine invertebrate larvae, careful consideration must be given as to the way in which experimental surfaces are presented to larvae. When the relevance of choice and no-choice assays to larval exploration and settlement behaviour in the field are considered, multi-treatment assays are considered to represent a more realistic approach.

Introduction

Since the early laboratory-based studies of larval settlement preferences of sessile marine invertebrates (Knight-Jones, 1951b,1953a; Wilson, 1952, 1954; Ryland, 1959; Crisp and Williams, 1960; Crisp and Ryland, 1960; Crisp, 1961), researchers have continued to rely largely upon laboratory settlement assays as a means of identifying the cues important to larvae in the settling process. This approach has shown that the larvae of benthic marine invertebrates are capable of perceiving and responding to a wide spectrum of physical and biological surface-associated settlement cues (see reviews by Meadows and Campbell, 1972; Crisp, 1974; Pawlik, 1992). Although this approach is now well established, there are differences in the experimental methodology adopted by different researchers (see review by Wieckzorek and Todd, 1998). Still-water assays comparing the settlement response of larvae to experimental surfaces are usually carried out as either multi-treatment assays (various experimental surfaces presented to larvae together; experimental containers replicated) or as single treatment assays (various experimental surfaces presented to larvae singly; each surface replicated in separate experimental containers). Traditionally, multi-treatment assays were the preferred experimental design with the numerous treatments placed in large dishes, that were sometimes rotated on a turntable, and relatively large numbers of larvae added (see Crisp, 1974). However, there has been a tendency in recent years for researchers to favour single treatment assays and a move towards the entire dish approach with higher replication, described by Wieczorek and Todd (1998), combined with a trend towards smaller numbers of larvae used per replicate. These changes have come about partly in recognition that density dependent larva-larva interactions may occur under laboratory conditions (Keough, 1984; Yule and Walker, 1985; Marsden, 1991; Clare *et al.*, 1994) and because of the high within-batch variability in settlement behaviour that exists for larvae (reviewed by Raimondi and Keough, 1990). The results of the multi-treatment or single-treatment larval settlement assays are taken as a measure of the relative 'attractiveness' of the surfaces being tested in relation to larval settlement 'preferences'. However, different experimental approaches may result in different settlement responses. Although both approaches have proven useful in laboratory settlement assays, there does not appear to be any study that directly compares the two experimental approaches.

This study investigates the settlement response of the larvae of three species of tube building polychaete, the serpulid *Pomatoceros lamarckii* and the spirorbids *Spirorbis spirorbis* and *Spirorbis tridentatus* to surfaces known to either induce or not induce settlement during simultaneous multi-treatment and single- treatment assays. Under laboratory conditions, the larvae of *P. lamarckii*, *S. spirorbis* and *S. tridentatus* are known to display highly discriminatory settlement behaviour (Knight-Jones, 1951b; Crisp and Ryland, 1960; de Silva, 1962; Williams, 1964; Roscoe, 1993; Chan and Walker 1998;

Hamer *et al.*, 2001). Earlier laboratory investigations based upon multi-treatment assays have shown that the larvae of all three species will settle preferentially on surfaces that have a biofilm (Knight-Jones, 1951b; Roscoe, 1993; Chan and Walker 1998; Hamer *et al.*, 2001) and that *P. lamarckii* larvae settle in higher numbers on older biofilms (Hamer *et al.*, 2001). Also, drying a biofilm completely negates the prior settlement-inducing effect of that biofilm for the larvae of all three species (Hamer *et al.*, 2001; Hamer and Walker, 2001). This study aims to determine whether presenting surfaces to these larvae in combination (multi-treatment assay) or in isolation (single-treatment assay) affects the settlement response.

Materials and Methods

Larval culture and liberation

Larvae of *Pomatoceors lamarckii* (Quatrefages), *Spirorbis spirorbis* (Linnaeus) and *Spirorbis tridentatus* (Levinsen) were obtained as described previously (Chapters 3.1 and 3.2).

Laboratory assay procedure

Slates (Welsh slate cut to size (25 x 25 x 4 mm) by Inigo Jones & Co. (North Wales) and polished uniformly with 600 grade wet and dry emery paper) were used in all laboratory settlement assays. Biofilms were allowed to develop on slates submerged continuously in the laboratory running seawater system. This system uses seawater extracted from the Menai Strait which is allowed to settle out in holding tanks before being pumped to a header tank from which it is gravity-fed to aquaria and laboratories. Slates were held horizontally in plastic racks with the experimental surface down, 1 cm above the bottom of the tank. The tank was covered with a wooden lid cutting out light and so preventing microalgal growth on the slates.

All assays were conducted in 300 ml Pyrex glass dishes (95 mm diameter) containing 100 ml of u.v.-sterilised, 0.2 µm filtered seawater (FSW). In multi-treatment assays, treatments were placed randomly in the centre of each replicate dish. In the single-treatment assays, treatments were placed individually in the centre of each replicate glass dish. Assays were conducted at 19°C in a constant environment cabinet in the dark (*S.*

spirorbis and *S. tridentatus*) or with a 12:12 light dark regime (*P. lamarckii*). All glassware was routinely cleaned in Chloros (sodium hypochlorite solution), rinsed thoroughly in tap water, followed by distilled water, before drying in a drying cabinet.

Multi versus single treatment assays

This experiment was conducted as simultaneous multi-treatment and single-treatment assays and was run independently for the larvae of *Pomatoceros lamarckii*, *Spirorbis tridentatus* and *Spirorbis spirorbis*. The treatments were clean, non-biofilmed slates (control), biofilmed slates and dried biofilmed slates (dried at room temperature for 1 hour immediately prior to the assay). These treatments were selected because they are known to either induce (biofilm) or not induce (dried biofilm and clean slate) larval settlement of the three species (Hamer *et al.*, 2001; Hamer and Walker, 2001). Each treatment was replicated eight times. Because of differences in the larval settlement behaviour (*S. spirorbis* and *S. tridentatus* are lecithotrophic and settle at a high rate in the laboratory, whereas *P. lamarckii* larvae are planktotrophic and settle at a much lower rate) of the three species used, the number of larvae per replicate and the duration of the assay varied accoringly. In the *Spirorbis* spp. assays, 10 larvae were used per replicate dish. In the *Pomatoceros* assays, equal aliquots (~100 larvae) of larvae were added to each dish; at the end of the assay, the number of settled individuals and non-settled larvae were counted to allow determination of percentage settlement. For both *S. spirorbis* and *S. tridentatus*, the assay was run for a 12-hour period and settlement counts were made hourly. Because of the slower rate of settlement that is obtained with *P. lamarckii* larvae, these assays were run for 72 hours with the number of settled individuals counted every six hours.

Larval swimming speed

The swimming speeds of freshly liberated larvae of *S. tridentatus* and *S. spirorbis* were measured (the swimming speeds of competent *P. lamarckii* larvae have been reported elsewhere; Chapter 2). A few larvae were added to one end of a Bogorov tray with a light source positioned at the other end. The time it took for a single larva to swim over a 1cm distance marked on the tray was recorded to the nearest 1/10th second using a stop watch.

Only larvae that were continuously swimming in a straight line were timed. The process was repeated until 30 larvae had been timed. To determine relative larval swimming speed (body lengths per second), a sub-sample of the same batch of larvae was relaxed in MS222 (0.1% w:v) in seawater and the body length (excluding apical tuft) of 30 larvae measured on a calibrated Nikon inverted microscope.

Exploratory behaviour

Spirorbis spirorbis larval exploratory behaviour in response to dried and non-dried biofilms was investigated. A small number (10 – 20) of freshly liberated larvae were pipetted into a small glass bowl containing the experimental surface (either a biofilmed slate or a biofilmed slate that had been dried for 1 hour at room temperature). Larvae contacting the surface were observed until one began the 'close exploration' crawling behaviour. The time such larvae spent crawling on the surface was recorded to the nearest $1/10^{th}$ second with a stopwatch. If a larva encountered the side of the experimental surface during exploration then its data were discarded. Once a larva had been timed, it was removed. Ten larvae were timed on 3 slates for each of the biofilmed and dried biofilmed treatments.

Statistical analysis

Final percentage settlement was compared statistically. The percentage larval settlement data were arc-sine transformed before statistical analysis. Those treatments in which no larvae settled were given the value of 1/4 n (n=no. of larvae) to improve the arc-sine transformation (Zar, 1996). Due to significant heterogeneity of variances, data were analysed by Kruskal-Wallis followed by Dunn's multiple comparisons test.

Results

Multi-treatment versus single-treatment assays

Larvae of *Pomatoceros lamarckii, Spirorbis tridentatus* and *S. spirorbis* showed similar settlement preferences in the multi-treatment assays that were consistent with previous studies (Hamer *et al*., 2001; Hamer and Walker, 2001). Larvae did not settle on the dried biofilm or non-biofilmed slates, even after 12 hours for *S. tridentatus* or 72 hours for *P. lamarckii*, but a small number (< 5%) of *S. spirorbis* did settle on the dried biofilm (Figures 1a, 2a and 3a). In the simultaneous single-treatment assays, the settlement pattern was significantly different (at p=0.05) for the spirorbids. Larvae settled on the dried ›filmed surface, whereas, as with the multi-treatment assay, no larvae settled on the clean, non-biofilmed slates (Figures 1b, 2b and 3b). Percentage settlement on these 'less attractive' surfaces was lower compared to the non-dried biofilmed slates. Larvae also took longer to settle on these surfaces, with no settlement of *S. tridentatus* and *S. spirorbis* larvae on dried slates after 1 hour compared to 40 – 50% settlement occurring on the non-dried biofilmed surfaces after the same time. *P. lamarckii* larvae did not settle on the dried biofilm during the assay (Figure 3a).

Larval swimming speed

Horizontal swimming speeds for the larvae of all three species are given in Table 1. Both *Spirorbis tridentatus* and *S. spirorbis* larvae, newly released, swim at very similar mean speeds of 0.42 and 0.40 cm.sec^{-1} respectively. The metatrochophore larvae of *Pomatoceros lamarckii* larvae swim at a slower mean speed of 0.28 cm.sec^{-1}. Relative swimming speeds in relation to larval body length are also presented in Table 1, larvae of all three species swim at between 1 and 2 body lengths per second.

Exploratory behaviour

The time that *Spirorbis spirorbis* larvae spent exploring surfaces was closely related to their relative 'attractiveness', as based on the results of the experimental assays (Table 2).

Table 1. Mean swimming speeds (cm.sec^{-1} ± SE; n = 30) of newly liberated *Spirorbis spirorbis* and *S. tridentatus* and competent *Pomatoceros lamarckii* metatrochophore larvae.

	Swimming speed	
	cm. sec^{-1}	body lengths / sec
Pomatoceros lamarckii	0.28 ± 0.015*	1.12 ± 0.06 *
Spirorbis tridentatus	0.42 ± 0.01	1.47 ± 0.05 [a]
Spirorbis spirorbis	0.40 ± 0.07	1.13 ± 0.16 [b]

*Data taken from Chapter 2
[a] based on a mean body length of 280.13 µm
[b] based on a mean body length of 351.25 µm

Table 2. Mean time in seconds (mean ± SE; n = 30) that larvae of *Spirorbis spirorbis* spend conducting the 'close exploration' behavioural phase during their first contact with biofilmed, dried biofilmed and non-biofilmed slate surfaces.

	Exploration time (sec)
Biofilm	13.87 ± 2.17
Dried biofilm	1.46 ± 0.19
No biofilm	1.13 ± 0.16

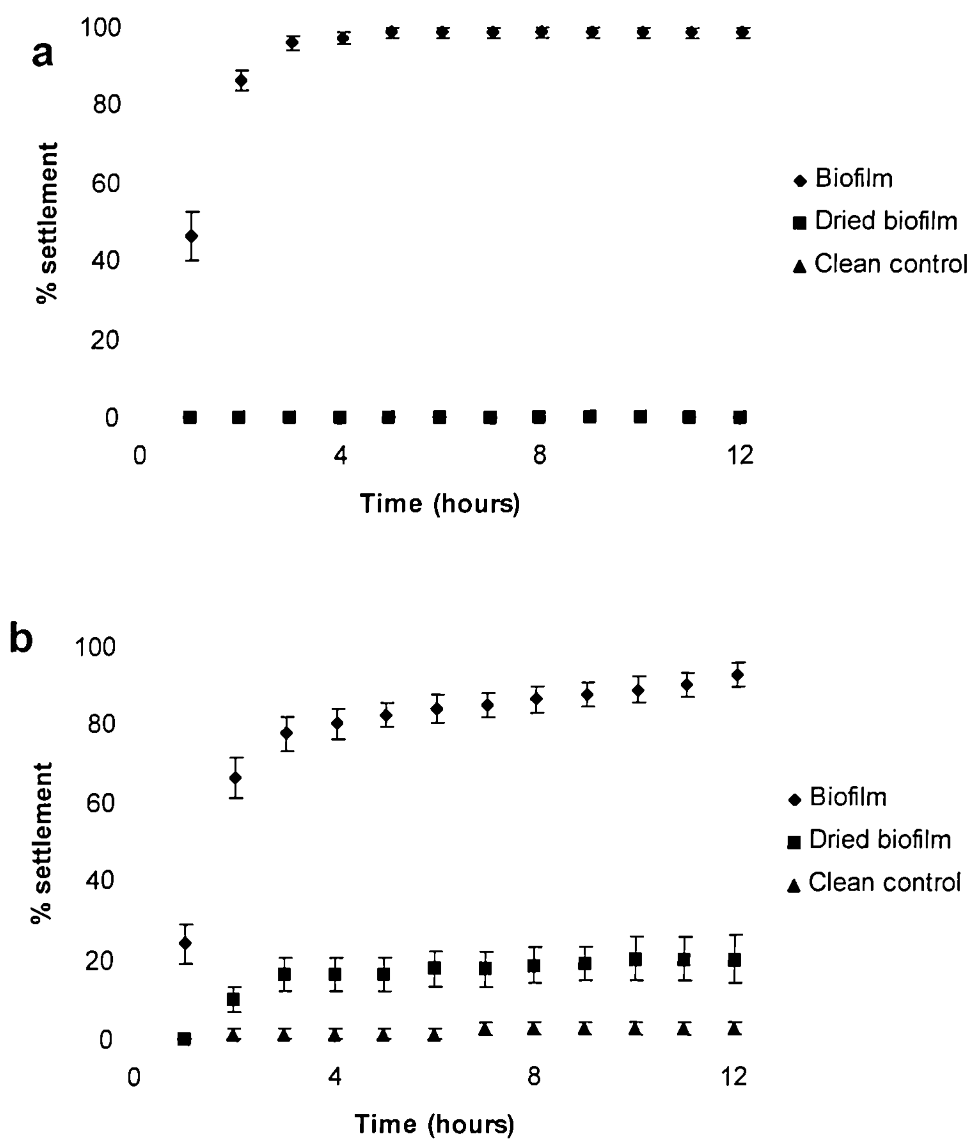


Figure 1 a & b. Mean percentage settlement with time of *S. tridentatus* larvae during 12 hour multi-treatment **(a)** and single-treatment **(b)** settlement assays. Treatments were biofilmed slates (◆), dried biofilmed slates (■) and clean, non-biofilmed slates (▲). Data plotted are the means ± S.E. (n=8). At 12 hours, percentage settlement was significantly different between all treatments ($p < 0.05$) for both assay types.

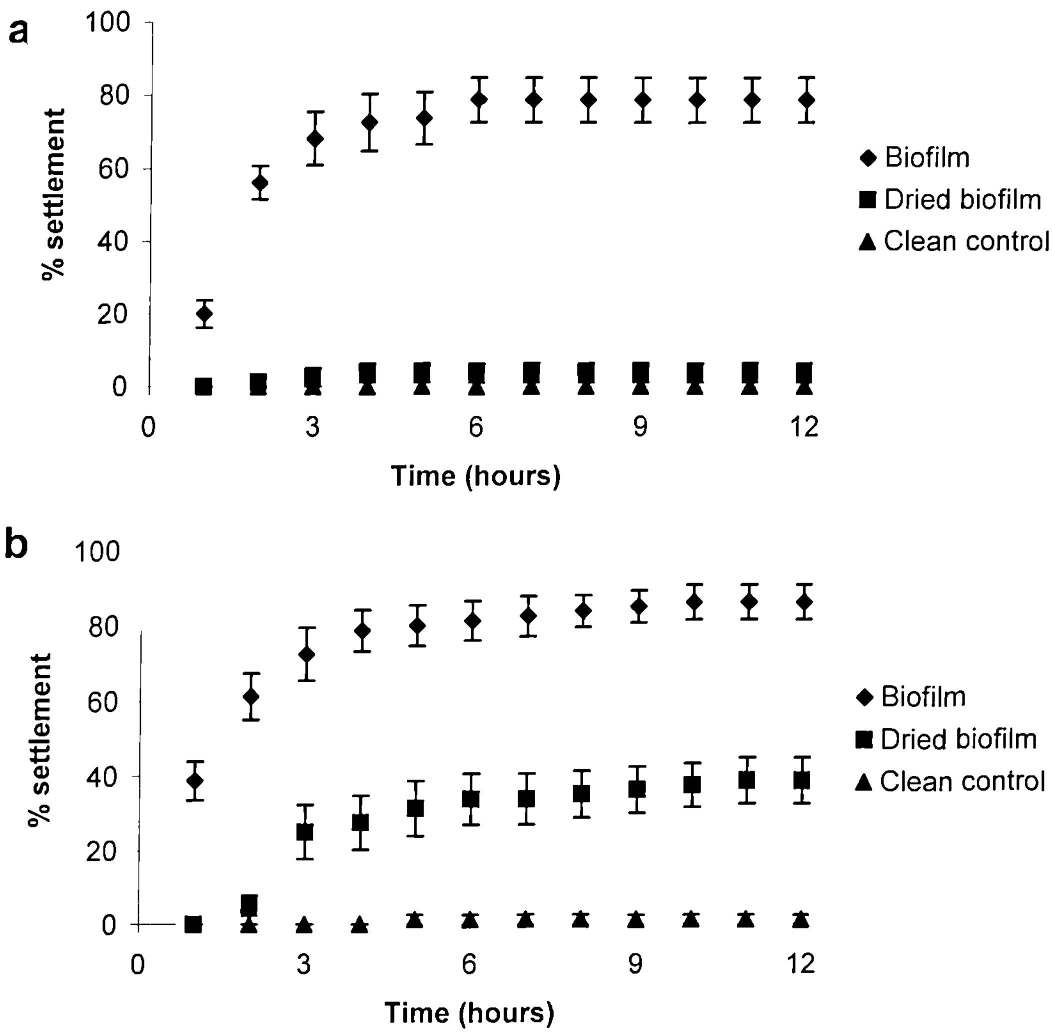
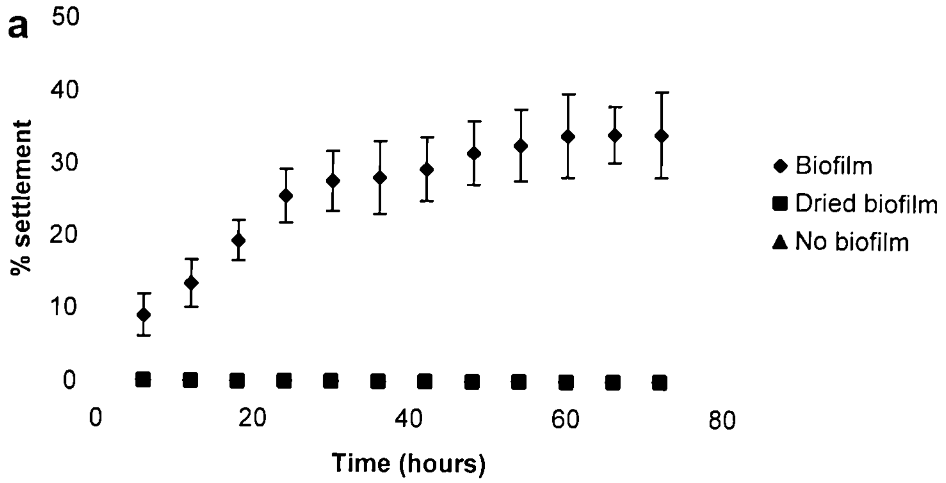


Figure 2a & b. Mean percentage settlement with time of *S. spirorbis* larvae during 12 hour multi-treatment **(a)** and single-treatment **(b)** settlement assays. Treatments were biofilmed slates (◆), dried biofilmed slates (■) and clean, non-biofilmed slates (▲). Data plotted are the means ± S.E. (n=8). At 12 hours, percentage settlement was significantly different between all treatments ($p < 0.05$) for both assay types.



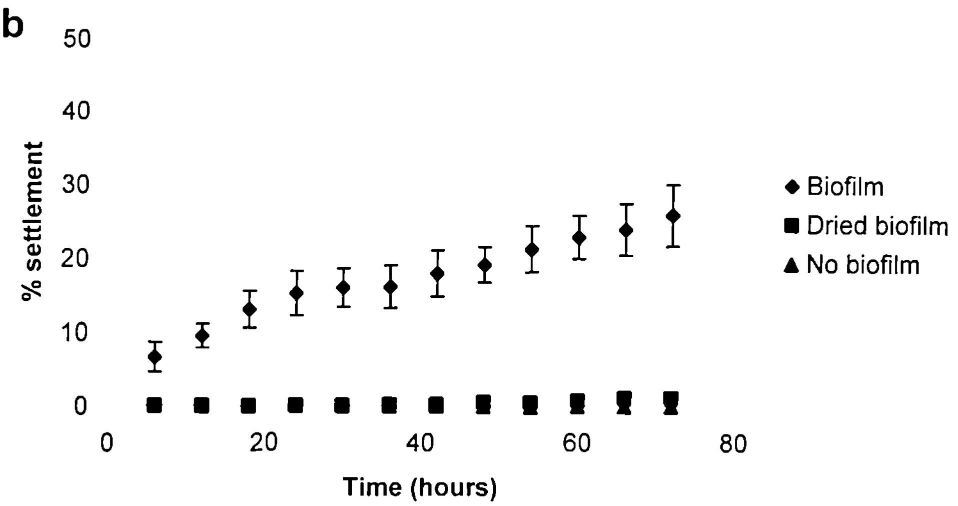


Figure 3a & b. Mean percentage settlement with time of *P. lamarckii* larvae during 72 hour multi-treatment **(a)** and single-treatment **(b)** settlement assays. Treatments were biofilmed slates (◆), dried biofilmed slates (■) and clean, non-biofilmed slates (▲). Data plotted are the means ± S.E. (n=8). At 72 hours, percentage settlement was significantly different between all treatments ($p < 0.05$) for both assay types.

Discussion

This study investigates one particular aspect of experimental design - the way in which different surfaces are made available, either singly or in combination, to tubeworm larvae during laboratory settlement assays. For two of the three species investigated (*Spirorbis spirorbis* and *Spirorbis tridentatus*), assay type (multi- or single-treatment) did significantly affect the outcome and hence the perceived larval settlement preferences. The result revealed that when given a 'choice', larvae of all three species settle almost exclusively on the preferred surface (in this case, a biofilmed slate surface), whereas, in the single-treatment assays a significant number of *S. spirorbis* and *S. tridentatus* larvae settled on a less favourable surface (a dried biofilmed slate surface), but still did not settle on the least attractive surface (a non-biofilmed slate). Interestingly, *Pomatoceros lamarckii* larval settlement results were similar for both the single and multi-treatment assays. Based upon the result from both assay types, larvae of *S. spirorbis* and *S. tridentatus* therefore appear to have the following settlement preferences: biofilm > dried biofilm > no biofilm, whereas those of *P. lamarckii* prefer: biofilm > dried biofilm ≈ no biofilm.

The laboratory settlement behaviour of *Spirorbis spirorbis*, *Spirorbis tridentatus* and *Pomatoceros lamarckii* has been studied previously (e.g. Knight-Jones, 1951b, Williams, 1964; Roscoe, 1993; Chan and Walker, 1998; Hamer and Walker, 2001; Hamer *et al.*, 2001). *S. spirorbis* and *S. tridentatus* larvae are brooded by the adult and are lecithotrophic so that once released they are only capable of undergoing successful settlement and metamorphosis within a finite time. Knight-Jones (1953a) found that *S. spirorbis* larvae subjected to an enforced period of planktonic life of 3 hours before being presented with potential settlement surfaces were less discriminatory (desperate) during settlement. Subjecting larvae to a planktonic life for 8 hours significantly decreased the rate of settlement due to depletion of larval energy reserves. In both of the *Spirorbis* spp. single-treatment assays carried out during this study the larvae settled on the less favourable dried biofilm, but such settlement was delayed by one to two hours compared with the biofilm treatment. Conducting both the single and multi-treatment assay provided information about the settlement preferences of *Spirorbis spirorbis* and *S. tridentatus* larvae that would not have been apparent from the multi-treatment assay alone. However, it is still not clear if this settlement response represents a true hierarchy of surface 'attractiveness' as settlement may have occurred because:

- the larvae become less discriminatory with time and a proportion will eventually settle on less attractive surfaces (the 'desperate larva' hypothesis proposed for *S. spirorbis* by Knight-Jones, 1953a),
- a small proportion of larvae will settle on less attractive surfaces if no other is available (termed 'founders' by Toonen and Pawlik, 1996) or
- the experimental surface becomes more attractive with time because it has been explored and altered by larvae or through larval induced changes in biofilm composition.

Pomatoceros lamarckii larval settlement preferences were similar in the single and multi-treatment assays. *P. lamarckii* larvae are planktotrophic and are capable of postponing settlement for a considerable length of time (Roscoe, 1993); these larvae are highly specific in their settlement requirements such that when left in the culture container for an extended period of time, the majority perish rather than settle on the glass of the culture container itself. Previous studies (Chapter 3.1; Roscoe, 1993; Chan and Walker, 1998; Hamer *et al.*, 2001) have shown *P. lamarckii* larvae to settle at a much lower rate than those of *Spirorbis spirorbis* and *Spirorbis tridentatus* in the laboratory, even when presented with a highly favourable settlement surface. As with *S. spirorbis*, *P. lamarckii* larvae are highly gregarious in response to adult conspecifics (Roscoe, 1993) but Hamer *et al.* (2001) found no evidence to suggest that high concentrations of larvae (up to 400 larvae per 100 ml) induce a higher rate of settlement. In addition, previous studies (Roscoe, 1993; Hamer *et al.*, 2001b) have found no evidence of an increased rate of settlement in response to recently settled (24-72 hour old) juveniles. This lack of evidence for larva-larva interactions, combined with the ability of *P. lamarckii* larvae to postpone settlement may explain why such larvae did not settle on the less favourable dried biofilm treatment in the single-treatment assay. Either the 'desperate larva' hypothesis does not apply to *P. lamarckii* larvae or the assay duration was not sufficient to invoke this state. The desperate larva hypothesis is essentially an energetic model whereby lecithotrophic (i.e. non-feeding) larvae continue to search for specific substrata as long as their energetic reserves allow, but eventually will begin to accept sub-optimal sites rather than fully exhaust their reserves. Recently, Toonen and Pawlik (2001b) have tested whether the desperate larva hypothesis applies to the planktotrophic (i.e. feeding) larvae of the tubeworm, *Hydroides dianthus*. Neither altering the feeding regime nor starving the larvae led to decreased substratum-specificity. In fact, starving the larvae led to a loss of competency rather than desperation. It seems likely that *P. lamarckii* larvae are similar to *H. dianthus* - the desperate larva hypothesis does not apply. As suggested by Toonen and Pawlik (2001b), there is a natural selection advantage for lecithotrophic larvae becoming less discriminatory as their energy reserves become depleted - they will die anyway, whereas planktotrophic larvae have evolved the ability to delay settlement / metamorphosis and are able to remain opportunistic.

In this study, the single-treatment assay consisted of a single test surface placed in the middle of a clean glass dish but the sides of the glass dish and free bottom area also represent a potential settlement site, therefore larvae were still presented with a 'choice' of surfaces. For this reason, the whole assay dish (container) approach used by many researchers may be seen as an advantage (see Wieckzorek and Todd, 1998), particularly when investigating larval settlement in response to biofilms. With this approach, the surface of the experimental container itself represents the test surface. Plastic petri dishes are frequently used for this purpose as they offer the advantages of being cheap, disposable and sterile whilst having a uniform experimental surface. However, the effect of plasticisers upon larval behaviour and physiology is not well understood (Wieckzorek and Todd, 1998) and has the potential to affect results. During this study, no larvae settled on the glass dishes during any of the assays. Knight-Jones (1953a) also found that *S. borealis* (– *spirorbis*) larvae rarely attach themselves to freshly cleaned (wiped) glass surfaces.

Marine invertebrate larvae display a surprising similarity in their surface exploration and settlement behaviour (Crisp, 1974). In the laboratory, larvae typically first undergo the 'broad exploration phase' upon initial contact with a surface and often quickly return to the water column, presumably because the surface is not suitable. During laboratory assays, larvae of all three species followed this typical behaviour pattern (Segrove, 1941; Wisely, 1960; Nott, 1973; Crisp, 1974; Roscoe, 1993). With a swimming speed of 0.28 cm s^{-1}, a *Pomatoceros lamarckii* larva (the slowest swimming of the three species investigated) would be capable of traversing the 90 mm diameter experimental dishes in a little over 32 seconds and exploring the experimental surfaces repeatedly during the assay. Larvae of all three species were consistently observed contacting, exploring and leaving surfaces repeatedly before committing to settlement. When larvae contact a suitable surface, why don't they settle there immediately? There are indeed reports of larvae settling immediately upon initial contact with a substratum. For example, de Silva (1962) reported that some *Spirorbis* larvae settled on favourable substrata within minutes of being liberated from the adults. Crisp (1974) considered the question of whether larvae automatically occupy suitable sites or whether they exercise a genuine power of choice. He explained apparently purposeful larval choice on the basis of 'a gradual lowering of the threshold stimulus necessary to release the "consummatory act" as the animal becomes older, or as it is stimulated by more prolonged exploration of the surface" and showed that barnacle cyprids of increasing age settled more rapidly on a standard test surface. The larvae studied here appear to be stimulated to settle with increasing time spent exploring a surface. Differences in the time that individuals take to settle may be due to inherent variability between larvae in the threshold needed for larvae to settle. Variability in the settlement behaviour of larvae in laboratory assays is widespread (see review by Raimondi and Keough, 1990).

The time larvae spent exploring each of the different surfaces is likely to be correlated with the attractiveness of the surface; larvae spending significantly longer exploring stimulatory surfaces (Table 2). In laboratory studies, measurement of barnacle cyprid temporary adhesion has been correlated with the settlement preferences of cyprids for some species of barnacles (Yule and Walker, 1984; Neal and Yule, 1992; Neal and Yule, 1994a). In a similar way, the time tubeworm larvae spend exploring a surface may prove a useful surrogate measure of that surface's attractiveness without necessarily invoking the need for post-settlement counts.

An important consideration relating to experimental design is its relevance to conditions in the field. Although many laboratory-based settlement studies have been carried out, there are few examples where direct comparisons have been made between laboratory and field settlement experiments (Wieczorek and Todd, 1998) and those studies that have been carried out have demonstrated that contrasting results may be obtained between the laboratory and the field (O'Connor and Richardson, 1996; Thompson *et al.*, 1998; also, see Chapter 4). Whilst acknowledging the importance of controlling the variables under laboratory conditions, it is important to consider how single and multi-treatment assays relate to natural conditions. The multi-choice laboratory assay represents a more realistic situation whereby larvae have the opportunity to explore and leave different surfaces before committing to settlement. Although currently less popular, the traditional multi-treatment assay is certainly responsible for many theories on larval settlement preferences that still persist today.

When designing still-water, laboratory-based, settlement assays for marine invertebrate larvae, consideration must be given to a wide range of practicalities, including size and nature of the experimental container, water depth, light regime, assay duration, replication, number of larvae per experimental container and assay type; Wiekzorek and Todd (1998) have discussed some of these factors. Clearly, the selection of a suitable experimental protocol requires an understanding of the larval settlement behaviour of the species being investigated. For instance, during surface exploration and settlement, marine invertebrate larvae may differ in their swimming speed, exploratory behaviour, settlement rate or the ability to delay settlement and may also display settlement behaviour that has the potential to confound experimental results such as gregariousness and spacing-out. It has been shown that of particular relevance to the decision of whether to conduct a single or multi-treatment assay is the ability of the larvae being studied to 'delay metamorphosis' (see review by Pechenik, 1990) and the applicability of the 'desperate larva' hypothesis (Knight-Jones, 1953a; Wilson, 1953; Toonen and Pawlik, 2001b) which suggests that larvae will endeavour to find an optimum settlement site as long as their energetic reserves allow, but will eventually accept a sub-optimal settlement site rather than die without metamorphosing (Toonen and Pawlik, 2001b). This study demonstrates that the way in which experimental surfaces are presented to larvae during an experimental assay may significantly affect the results. Caution should be applied in designing and interpreting the results of laboratory-based settlement assays for marine invertebrate larvae. The findings of the present study signify and reinforce the need for much forethought in planning, designing and interpreting of results for laboratory-based settlement assays for those marine invertebrate larvae that settle on hard substrata.

3.4 The role of bacteria in the settlement of *Spirorbis spirorbis larvae*: a laboratory study.

Abstract

The settlement response of *Spirorbis spirorbis* L. (Polychaeta: Spirorbidae) larvae to monospecific bacterial films was investigated in the laboratory. Settlement experiments were performed on biofilmed glass slides in single-treatment, still-water assays. Sixteen distinct bacterial strains were isolated from natural biofilms and tested for their settlement-inducing properties. Larvae were induced to settle by certain bacterial strains and not by others. The settlement response was not correlated to bacterial density. Relatively high percentage settlement was induced by 3 of the 16 strains tested but in all cases the settlement response induced by the monospecific biofilms was much lower than that induced by a natural biofilm control treatment. The settlement cue was found to be associated with either the bacterial cells or their surface-associated extracellular products; water-borne bacterial products did not induce settlement.

Introduction

Biofilms are an important settlement cue to a wide range of marine invertebrate larvae (see review by Wieczorek and Todd, 1998). Although biofilms may induce, have no effect or inhibit settlement, they frequently induce settlement of larvae and studies have shown that the bacteria present in the biofilms often provide the inductive cue. Under laboratory conditions, monospecific bacterial films have been shown to influence the settlement of the larvae of various sessile marine invertebrates including barnacles (Maki *et al.*, 1988, 1990; Holmström *et al.*, 1992; Avelin Mary *et al.*, 1993), spirorbids (Kirchman *et al.*, 1982a), serpulids (Lau and Qian, 1997, 1999, 2001; Unabia and Hadfield, 1999; Hamer *et al*, 2001) and hydroids (Neumann, 1979; Müller, 1973). Furthermore, Maki *et al.* (1988) showed that different mono-specific bacterial films may stimulate, have no effect on, or inhibit the attachment of *Balanus amphitrite* cyprids and suggested that it is the bacterial extracellular material present in the biofilm that influences cyprid attachment.

The larvae of calcareous tube-building serpulid and spirorbid polychaetes are stimulated to settle in response to biofilms and, in some cases, settlement has been closely correlated with the density of the bacteria present in the biofilm (Hadfield *et al.*, 1994; Hamer *et al.*, 2001). Kirchman *et al.* (1982b) demonstrated that larvae of the spirorbid *Janua brasiliensis* were stimulated to settle in response to biofilms composed of the bacterium *Pseudomonas marina* and provided evidence to suggest that extracellular polysaccharides were the settlement cue. Subsequently, Lau and Qian (1997, 1999, 2001) and Unabia and Hadfield (1999) reported that *Hydroides elegans* larvae also settled in response to certain monospecific biofilms and found that different settlement responses were obtained to the different bacterial strains. In addition, Lau and Qian (1997) found that settlement on biofilms composed of more than one bacterial strain gave a settlement response that was intermediate compared to the settlement response to the respective individual strains.

This study investigates the settlement response of the calcareous tube-building polychaete *Spirorbis spirorbis* to monospecific biofilms composed of bacterial strains isolated from natural biofilms. *S. spirorbis* is a spirorbid polychaete locally abundant around the UK as an epifaunal representative on the brown macroalga *Fucus serratus* and less frequently *F. vesiculosus*. Brooded, lecithotrophic larvae are liberated during periods of neap tides and laboratory studies have shown the larvae to exhibit highly selective settlement behaviour. Under laboratory conditions, larvae settle readily on pieces of *F. serratus* and preferentially on plants with conspecific adults (Knight-Jones, 1951b). Larvae will also settle in high numbers on glass and slate surfaces that have been allowed to develop a natural biofilm (Knight-Jones, 1951b; Hamer and Walker, 2001) and on surfaces treated with an *F. serratus* extract obtained from macerated plants (Williams, 1964). In the absence of a biofilm cue, larvae settle much more slowly and in smaller numbers (see Chapter 3.3). Based upon the information above, this study aimed to test the following hypotheses; 1) larvae of *S. spirorbis* are stimulated to settle in response to bacteria or their extracellular products, 2) the settlement response is different for different bacterial strains and 3) larvae settle preferentially on bacterial strains isolated from the surface of the natural adult habitat (*F.serratus* plants).

Materials and Methods

Larval liberation

Fucus serratus or *F. vesiculosus* fronds bearing adult *Spirorbis spirorbis* (L.) were collected from the shores of the Menai Strait, North Wales, UK. Fronds were kept damp, but out of seawater in a covered container. When larvae were required, the fronds were placed in a tank containing fresh seawater. The released larvae were collected by pipette at a point light source.

Larval settlement assays

Bacterial biofilms and soluble bacterial products were tested for effects upon larval settlement in laboratory bioassays performed in glass dishes (250 ml, Pyrex), using either glass slides or the glass dish itself as the test surface. Each dish was filled with 100 ml of sterile (autoclaved) 0.2 μm filtered, u.v.-irradiated seawater (SFSW). Five or six replicate dishes were used for each treatment depending upon the experiment.

All glassware was routinely cleaned in a 5% final concentration Chloros (sodium hypochlorite) solution, rinsed in tap water, followed by distilled water before drying in a drying cabinet at 60°C.

For the assays, 10 freshly liberated larvae (< 1 hour post-liberation) were transferred to each replicate dish. The dishes were placed in the dark at room temperature and the number of larvae settled was counted after 1, 2, 4, 8, 12 and 24 hours. Details of the experimental treatments are given below.

Bacterial isolation, culture and characterization

Bacteria were isolated either from biofilms developed under dark conditions in the laboratory running seawater system or from natural biofilms present on intertidal rock and algal surfaces in the Menai Strait, North Wales, UK (Table 1). Nutrient agar plates (Marine Agar 2216, Difco) were inoculated from the respective biofilms using sterile cotton swabs. After 24 hours incubation at 30°C, the bacterial colonies from each source were screened according to colony morphology. Dominant or distinct bacterial colonies were selected, isolated and subsequently grown in nutrient broth (Marine Broth 2216, Difco) as pure cultures. Subsamples of the broth culture (2 ml from 48 h old cultures) were mixed with an equal volume of sterile 40% aqueous glycerol solution and stored at -20°C in 2 ml aliquots.

Bacterial counts

Bacteria attached to glass slides were fixed in 2.5% glutaraldehyde in 0.2 μm filtered, u.v.-irradiated seawater (FSW), stained with DAPI and the density determined by epifluorescence microscopy at 400x magnification. Ten random field counts (2400 μm^2) were carried out on each of three replicate slides for each treatment.

Monospecific biofilm assays

Isolated strains of biofilm bacteria were tested for their effect upon larval settlement of *Spirorbis spirorbis* in laboratory assays. Aseptic techniques were used throughout and all equipment and seawater was sterilised in an autoclave for 20 minutes at 121°C and 15 psi. To minimise the transfer of bacteria to the test dishes when larvae were added, larvae were rinsed thoroughly with SFSW whilst being retained on a 45 μm sieve before being transferred to the assay dishes.

Experiment 1

The settlement response of *Spirorbis spirorbis* larvae to the 16 isolated bacterial strains (Table 1) was investigated in laboratory assays. Bacterial strains were cultured in 200 ml of marine broth (Marine Broth 2216, Difco) at 30°C. After 48 hours incubation, cultures were transferred to separate glass dishes (500 ml capacity) containing sterile glass slides. The glass dishes were allowed to stand at room temperature for 6 hours to allow the bacteria to attach onto the slides. After this period, the slides were removed and dipped 10 times into 500 ml SFSW to remove unattached bacterial cells. The slides were then placed in the 250 ml glass assay dishes containing 100 ml SFSW and the larvae added. The positive control comprised glass slides that had been allowed to accumulate a natural biofilm during continuous immersion in the laboratory running seawater system for 1 week prior to the assay. The negative control comprised glass slides that had been treated with sterile marine broth alone, as outlined above.

Due to logistical constraints, not all 16 bacterial strains could be tested together. The experiment was therefore conducted as three separate, sequential assays, each comprising 5 or 6 of the monospecific bacterial treatments and the appropriate controls. The entire experiment was conducted three times.

Experiment 2

An experiment was carried out to determine whether the availability of nutrients influenced monospecific bacterial biofilm formation on glass slides and hence the settlement response of *S. spirorbis* larvae. Bacterial strain # 6 (Table 1) was cultured in 200 ml of marine broth at 30°C for 48 hours. After this time, 20 ml subsamples of the suspension were harvested from the broth culture by centrifugation (10,000 rpm, 15°C, 20 minutes), re-suspended in artificial seawater, centrifuged again and re-suspended in either artificial seawater (ASW) (Kent Seasalt; 34 ‰), sterile filtered seawater (SFSW) (autoclaved 0.2µm, u.v.-irradiated seawater from the Menai Strait; 34 ‰) or in the original marine broth suspension that was removed after the first centrifugation step. The control was sterile marine broth solution treated in the same way. After centrifugation, 5 ml of each bacterial suspension was added to autoclaved glass dishes (Pyrex, 250 ml) which were then covered and left for 24 hours. Dishes were then dipped 10 times in 2 litres of SFSW to remove any unattached bacterial cells and 100 ml of SFSW added. Larvae were then added to each dish at the start of an assay.

Experiment 3

The time required for a suitable biofilm to form which would induce settlement of *Spirorbis spirorbis* larvae was investigated. Bacterial strain # 6 (Table 1) was cultured in 200 ml of marine broth at 30°C for 48 hours. After this time, the bacterial suspension was transferred to a 1 litre glass dish. Sterile glass microscope slides (38 x 12 x 1 mm; n = 5) were introduced into the culture periodically (8, 6, 4 and 2 hours before the assay was started). Slides were removed, dipped 10 times in SFSW to remove unattached bacterial cells and placed in 100 ml of SFSW in 250 ml glass dishes to which larvae were added.

Experiment 4

The effects of altering the bacterial community composition upon *Spirorbis spirorbis* larval settlement were investigated by assaying biofilms composed of a 1:1 mixture of two strains alongside the individual strains. Since strain # 2 did not induce any settlement during Experiment 1 and strains # 4 and # 6 consistently induced comparatively high settlement, these strains were assayed in combination. Glass slides were allowed to develop a biofilm (as described above) in bacterial cultures of

i) either strain # 2 or # 4 or in a 1:1 mixture of strains # 2 and # 4 and ii) either strain # 2 or # 6 or in a 1:1 mixture of strains # 2 and # 6. Slides were removed, dipped 10 times in SFSW to remove unattached bacterial cells and placed in 100 ml of SFSW in 250 ml glass dishes to which larvae were added.

Soluble bacterial products assays

This experiment was carried out to investigate whether the bacterial cue is a water soluble bacterial product or a surface bound bacterial product. Bacterial strains # 4 and # 6 were grown for 48 hours in 200 ml marine broth (Marine Broth 2216, Difco), centrifuged (10, 000 rpm at 15 °C for 20 minutes) and re-suspended in 100 ml FSW. After 6 hours, their soluble metabolites were obtained by centrifuging and filtering the supernatant through a sterile 0.22 μm Millipore filter. The bacterial pellet was re-suspended in 100 ml FSW. Twenty ml aliquots of the bacterial suspension and the filtered bacterial supernatant were transferred to glass dishes. After 6 hours, a sufficient time for bacteria and bacterial products to become attached to the glass dish, the bacterial suspension and bacterial supernatant were poured off and 100 ml of SFSW added to each dish. The bacterial supernatant treatment aimed to test the inductive capacity of surface-bound bacterial products (SB). The filtered bacterial supernant removed from these dishes was transferred to clean glass dishes and the volume made up to 100 ml with SFSW; this treatment was to test the inductive capacity of water-borne bacterial products (WB). The dish treated with the bacterial cell suspension represented the positive control (B). Clean dishes were used as the negative control (C).

Statistical analysis

The percentage larval settlement data in response to experimental treatments were arc-sine transformed before statistical analysis. Those treatments in which no larvae settled were given the value of 1/4n (n=no. of larvae) to improve the arc-sine transformation (Zar, 1996). Data were tested for normality and homogeneity of variances. Normal data without significantly heterogeneous variances were analysed by one-way ANOVA followed by Tukey's test. Where there was significant heterogeneity of variances, data were analysed by Kruskal-Wallis followed by Dunn's multiple comparisons test. Further details of the tests used for each experiment are given in the text and figure legends.

Results

Isolated baterial strains

A total of 16 bacterial strains were isolated; the strains varied in terms of colony morphology, growth rate and cell morphology (Table 1). Most were rod-shaped, ranging in size from 1 to 4 μm. Interestingly, on the several occasions that agar plates were inoculated with swabs taken from the surface of *F. serratus* and *F. vesiculosus* plants, only one colony type (based upon colony morphology) was found.

Monospecific biofilm assays

Experiment 1

Spirorbis spirorbis larvae were induced to settle by certain bacterial strains and not others. The inductive effect varied significantly between the different strains (Figure 1 a-c; Table 1). The effects of larval induction of the 16 strains tested were divided into 2 categories: (1) settlement induction, where settlement was significantly greater (at $p<0.05$) from that on the clean glass slide control treatment and (2) no inductive effect, where settlement was not significantly different (at $p<0.05$) from that on the clean glass slide control treatment (neutral).

There was no obvious relationship between the ability of a bacterial strain to induce settlement and bacterial or colony morphology. Highest percentage settlement was consistently induced by strains 4, 6 and 8 (Figure 1 and Table 1) whereas strains 1, 2, 9, 10, 11,13 and 14 remained neutral in all trials. The strain isolated from the surface of *Fucus* plants (strain # 16) induced significantly greater settlement compared to the clean control (C) in two of the three experimental trials, but only induced relatively low settlement compared to some of the other more inductive bacterial strains. Natural, multi-species biofilms consistently induced the highest settlement of 60-80%. These biofilms were dominated by small rod-shaped bacteria and coccoid bacteria. There was no significant correlation between bacterial density and percentage settlement for the different strains tested (at $p = 0.05$; Figure 2).

Table 1. Details of morphological characteristics of bacterial strains isolated. Bacteria were isolated from natural biofilms in laboratory running seawater system (*LAB*), on intertidal rock surfaces (*INT*) and on algal surfaces (*ALG*). Diameters of colonies were measured after 24 hours growth on marine nutrient agar at 30°C. Flocculent (Floc.) form of growth refers to bacteria that aggregate to produce a woolly appearance in the broth, while cloudy refers to the formation of a cloudy broth. A summary of the settlement response of *Spirorbis spirorbis* larvae to the different bacterial strains in settlement assays is also given in the final column.

Bacterial strain #	Origin	Colour	Shape of colony	Diameter (mm)	Appearance in marine broth	Cell shape	Cell size (μm)	Effect on settlement
1	INT	Orange	Circular	2-3	Cloudy	Rod	3-4	+
2	INT	White / Orange	Circular	2-3	Cloudy	Rod	1-2	+
3	INT	Cream	Circular	2-3	Cloudy	Rod	~1	++
4	INT	White	Irregular	>5	Cloudy	Rod	1-2	+++
5	INT	Orange	Circular	1-2	Cloudy	Rod	1-2	+
6	INT	Creamy / Orange	Circular	2-3	Cloudy	Rod	~2	+++
7	LAB	White	Circular	2-3	Floc.	Coccoid	1	++
8	LAB	Green	Circular	2-3	Cloudy	Coccoid	1	+++
9	LAB	Cream	Circular	2-3	Floc.	Rod	~1	+
10	LAB	Colourless	Circular	2-3	Cloudy	Rod	~1	+
11	LAB	Creamy/ Orange	Circular	2-3	Cloudy	Rod	2-3	+
12	LAB	Yellow	Circular	1-2	Cloudy	Rod	1-2	+
13	LAB	White	Circular	2-3	Cloudy	Rod	3-4	+
14	LAB	Creamy / Orange	Circular	< 1	Cloudy	Coccoid	1-2	+
15	LAB	Orange	Circular	2-3	Cloudy	Rod	1-2	+
16	ALG	White	Irregular	4-5	Cloudy	Rod	3-4	++

+ = no significant effect on settlement compared to clean control; ++ = variable effect on settlement compared to the clean control; +++ = settlement consistently significantly greater than the clean control

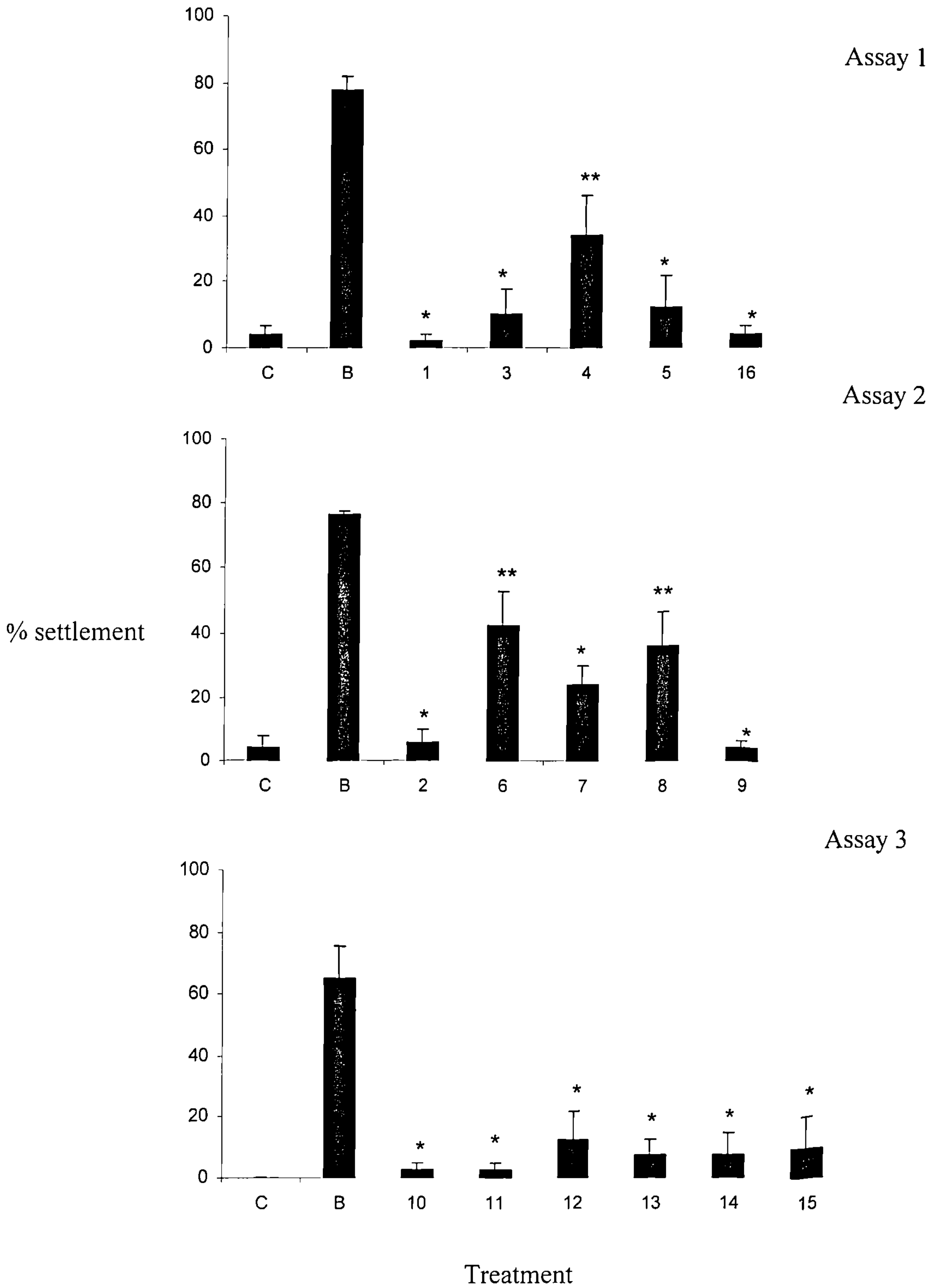


Figure 1a. Mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assays on glass slides biofilmed with different bacterial strains (treatment numbers) isolated from natural biofilms (see Table 1). The controls were a glass slide treated with sterile marine broth (C) and glass slides with 7 day-old natural biofilms developed in the laboratory running seawater system (B). Data plotted are means + S.E. (n = 5) of three separate assays for the first of three experimental repeats. Results of Tukey's pairwise comparisons test are given; bars with * are not significantly different from clean control (C), bars with ** are significantly greater than clean control but significantly less than the natural biofilm (B) treatment (at $p < 0.05$).

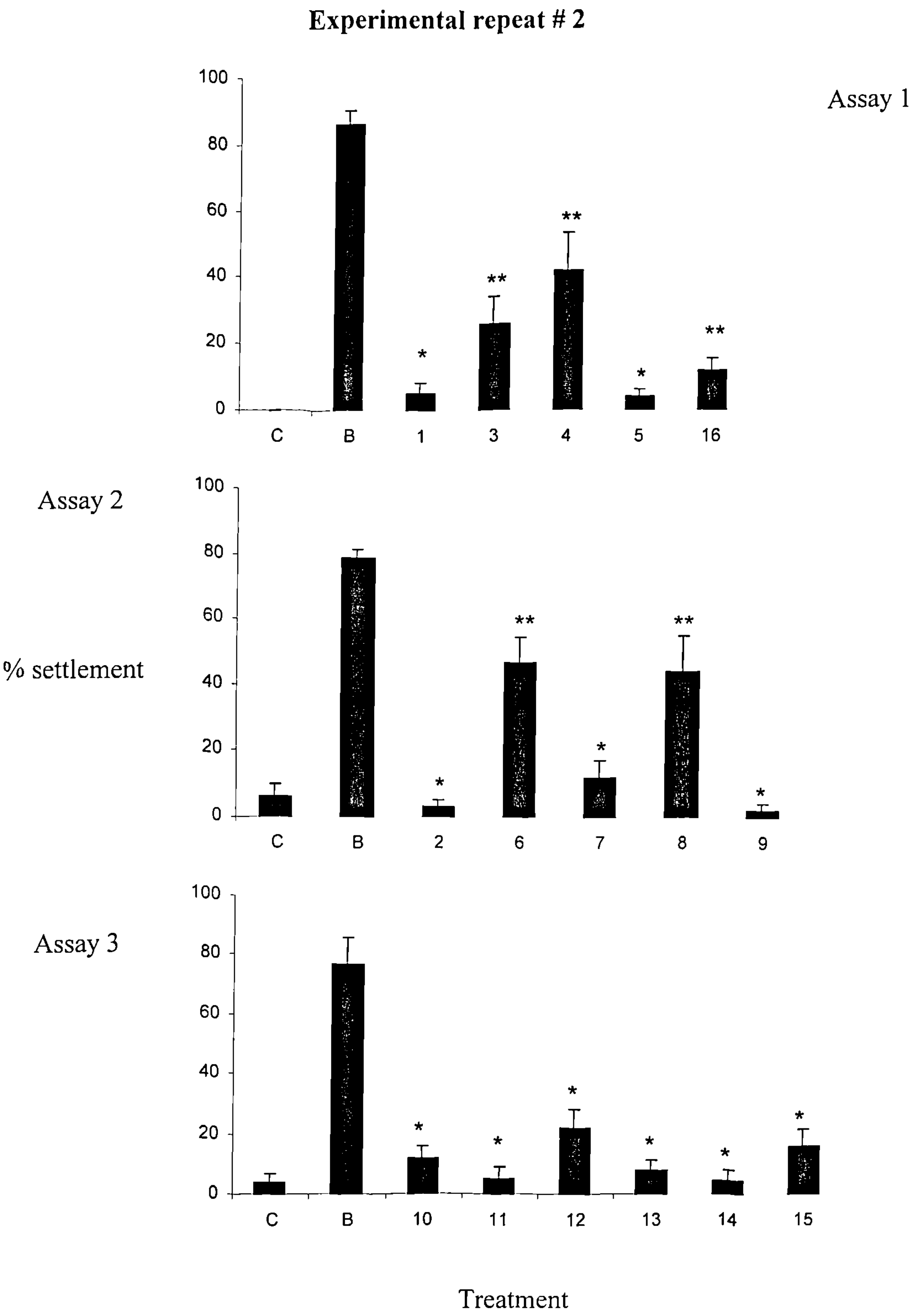


Figure 1 b. Mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assays on glass slides biofilmed with different bacterial strains (treatment numbers) isolated from natural biofilms (see Table 1). The controls were a glass slide treated with sterile marine broth (C) and glass slides with 7 day-old natural biofilms developed in the laboratory running seawater system (B). Data plotted are means + S.E. ($n = 5$) of three separate assays for the second of three experimental repeats. Results of Tukey's pairwise comparisons test are given; bars with * are not significantly different from clean control (C), bars with ** are significantly greater than clean control but significantly less than the natural biofilm (B) treatment (at $p < 0.05$).

Experimental repeat # 3

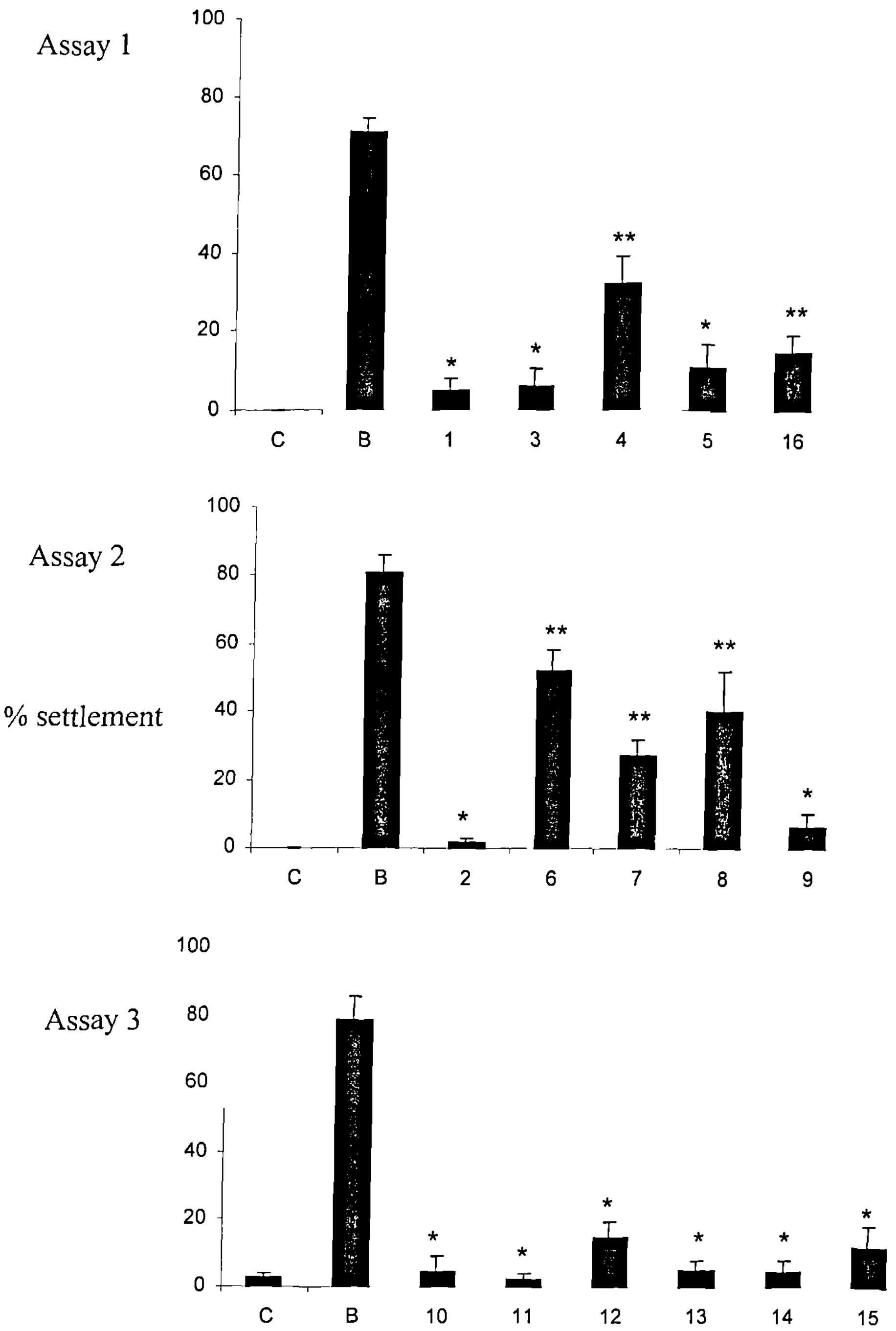


Figure 1 c. Mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assays on glass slides biofilmed with different bacterial strains (treatment numbers) isolated from natural biofilms (see Table 1). The controls were a glass slide treated with sterile marine broth (C) and glass slides with 7 day-old natural biofilms developed in the laboratory running seawater system (B). Data plotted are means + S.E. (n = 5) of three separate assays for the third of three experimental repeats. Results of Tukey's pairwise comparisons test are given; bars with * are not significantly different from clean control (C), bars with ** are significantly greater than clean control but significantly less than the natural biofilm (B) treatment (at $p < 0.05$).

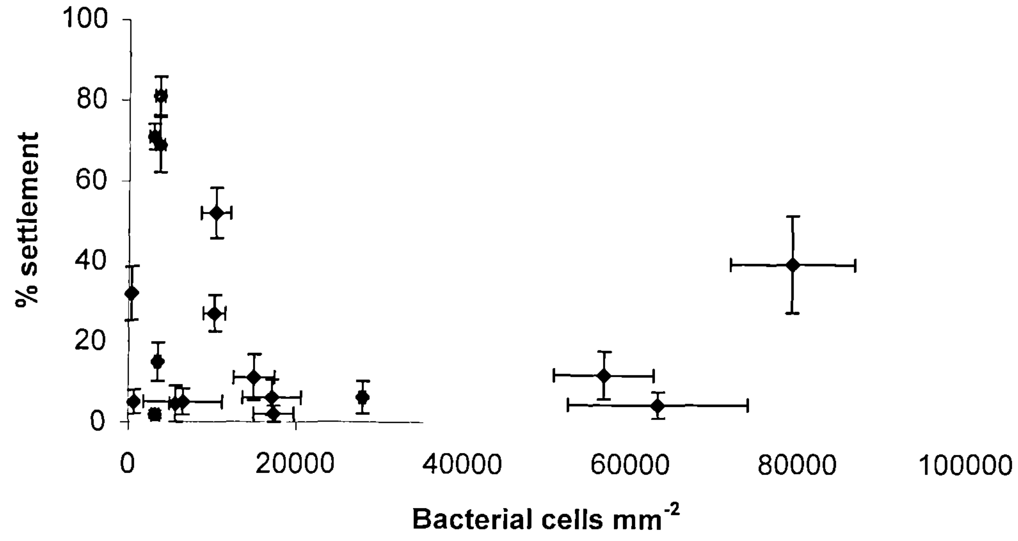


Figure 2. Mean percentage settlement of *Spirorbis spirorbis* larvae in relation to density of bacterial cells for all bacterial strains and natural biofilms assayed during the first of three repeats of Experiment 1. Data plotted are means ± S.E. (n=6 for percentage settlement counts; n = 10 for three replicates for the bacterial counts).

Experiment 2

There was no significant difference between percentage settlement of *S. spirorbis* larvae on the marine broth, FSW or ASW treatments ($F = 41.52$; $p<0.001$; d.f. = 5; one-way ANOVA) but significantly fewer larvae settled on the control treatments (Figure 3). There was no significant difference between the number of attached bacterial cells for the various treatments ($F = 0.83$; $p = 0.438$; one-way ANOVA).

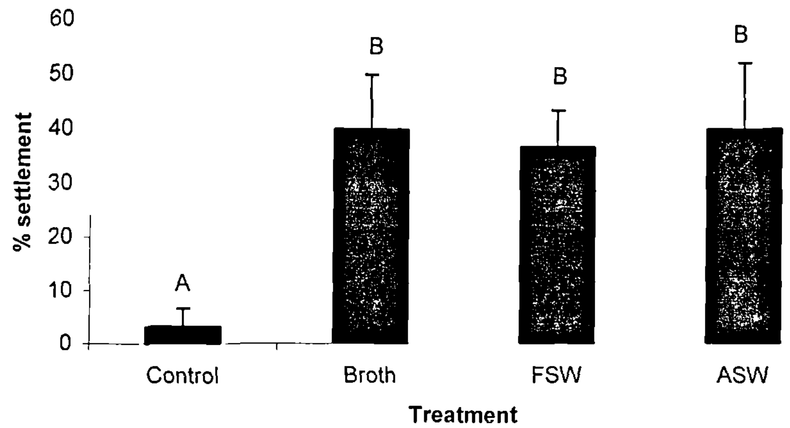


Figure 3. Mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assay on glass dishes biofilmed for 24 hours with bacterial strain # 6 (see Table 1) in marine broth (= Broth), 0.2 μm filtered, u.v.-irradiated seawater (FSW) and artificial seawater (ASW). The control was a glass dish treated with sterile marine broth for 24 hours. Data plotted are means + S.E. (n = 6). Results of Tukey's pairwise comparisons test are given; bars with the same letter are not significantly different (at p<0.05).

Experiment 3

Larvae settled in significantly greater numbers on the 8 hour treatment compared to the 2 hour treatment (Figure 4; $p<0.05$; $F=3.35$, one-way ANOVA). The density of attached bacterial cells increased with the increasing time of exposure to the bacterial cell suspension (Figure 4).

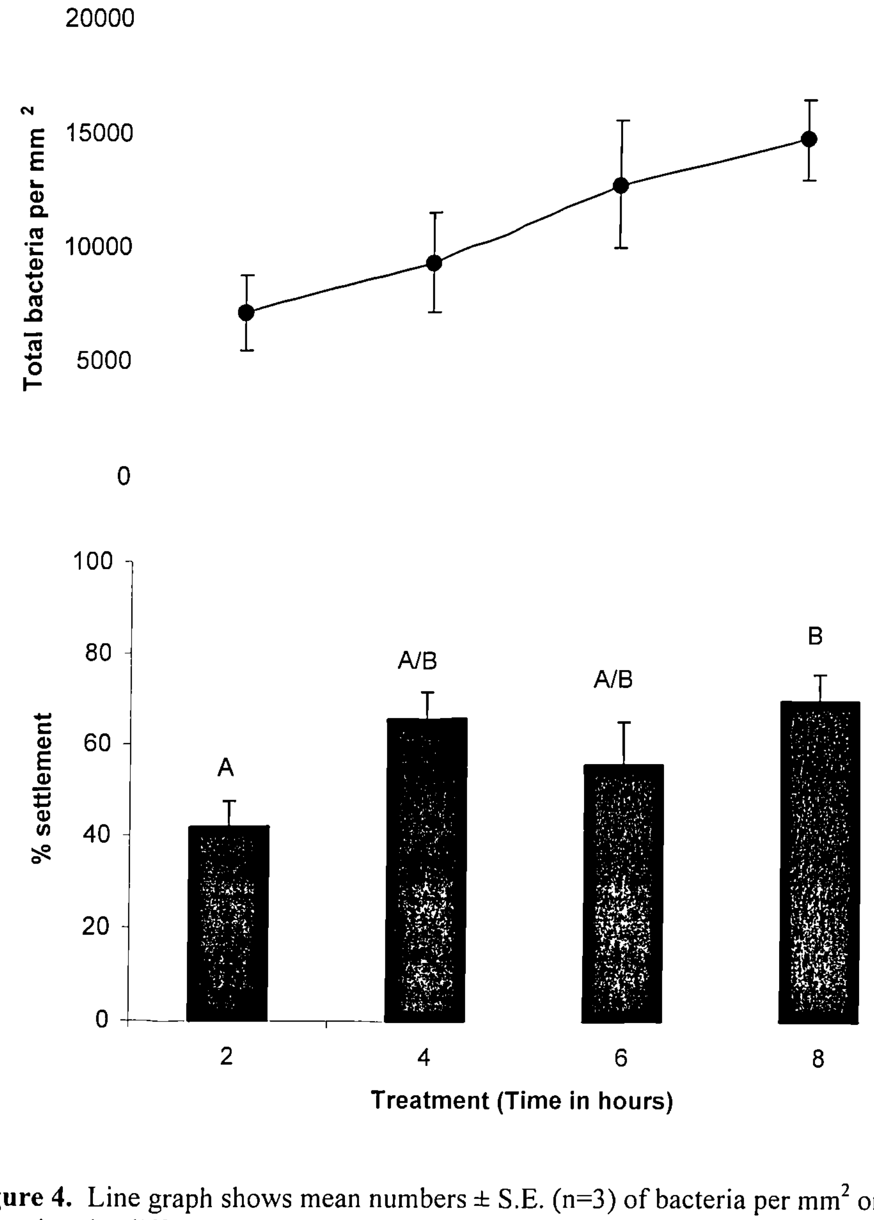


Figure 4. Line graph shows mean numbers ± S.E. (n=3) of bacteria per mm^2 on glass slides following the different treatments. Bar chart shows mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assay on glass slides biofilmed for 2, 4, 6 and 8 hours with bacterial strain # 6 (see Table 1) in marine broth. Data plotted are means + S.E. (n=6). Results of Tukey's test are given; bars with the same letter are not significantly different (at p<0.05).

Experiment 4

For both assays, the percentage larval settlement induced by the mixed biofilm was intermediate between the significantly different percentage settlement that was induced by the respective strains in isolation (Figure 5 a & b; Kruskal Wallis, H = 8.21; $p<0.05$ and H = 13.23; $p < 0.05$ for the two experiments respectively).

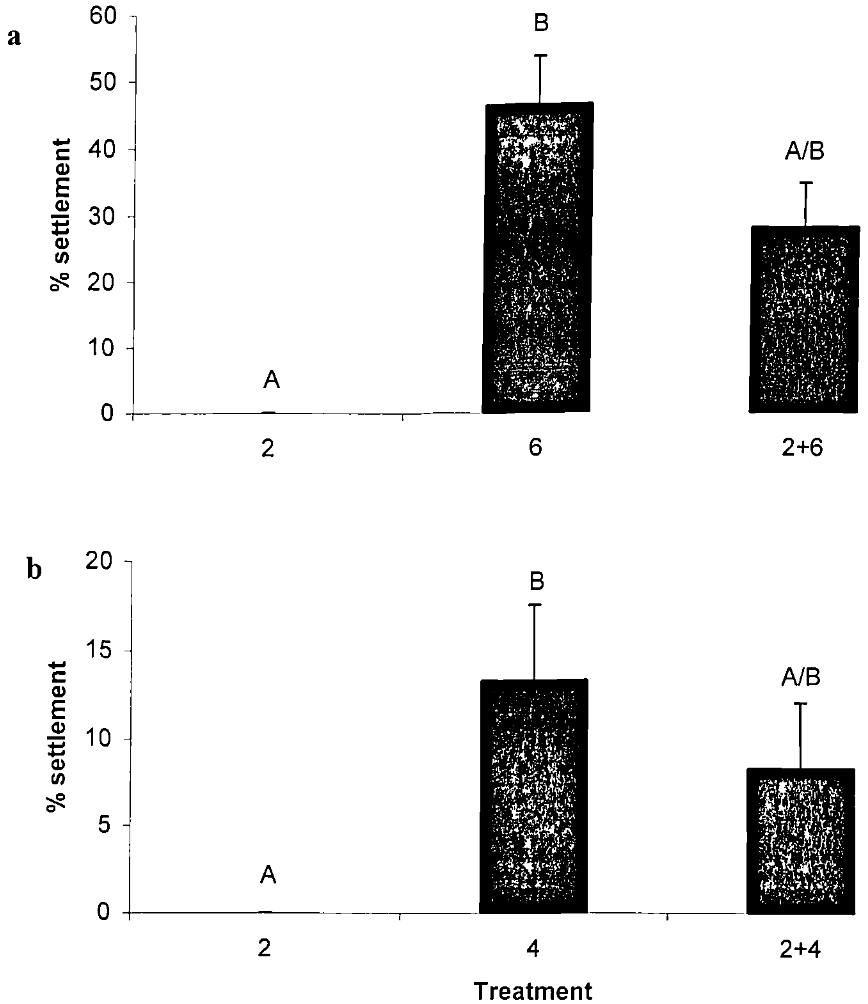


Figure 5a & b. Mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assay on glass slides biofilmed with single and mixed bacterial strains. Slides were biofilmed with **a)** bacterial strains 2, 6 or a 1:1 mixture of 2 and 6 and **b)** bacterial strains 2, 4 or a 1:1 mixture of 2 and 4. Data plotted are means (+SE) (n=6). Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$; Kruskal Wallis).

Soluble bacterial products assays

Water-borne bacterial products were obtained in the form of seawater conditioned with bacteria. Larval settlement was not induced by water-borne or surface associated soluble bacterial products for either of the bacterial strains tested (Fig 6; Kruskal Wallis, $H = 21.14$; $p<0.05$).

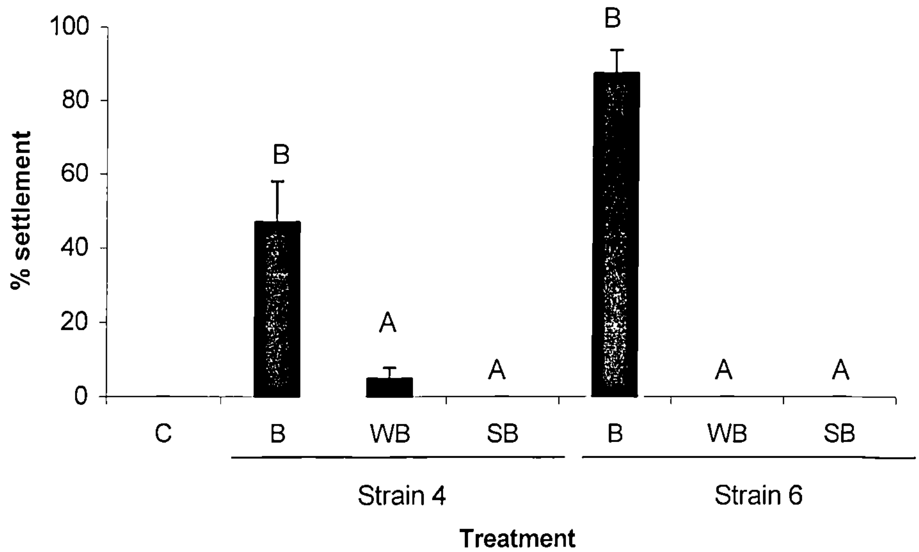


Figure 6. Mean percentage settlement of *Spirorbis spirorbis* larvae after 24 hour settlement assay on glass dishes treated with bacterial cells (B), with water-borne bacterial products (WB), or with surface-bound bacterial products (SB) from bacterial strains 4 and 6 (Table 1). The control (C) was a clean, non-biofilmed dish. Data plotted are means (+SE) (n=6). Results of Dunn's multiple comparisons test are given; bars with the same letter are not significantly different (at $p<0.05$; Kruskal Wallis).

Discussion

During the present study, certain monospecific bacterial films induced settlement of *Spirorbis spirorbis* larvae whilst others did not, suggesting that at least some of the bacteria present in a biofilm are important as a larval settlement cue. Individual bacterial strains have also been shown to induce, have no effect upon or inhibit the settlement of various other marine invertebrate larvae (see review by Wieczorek and Todd, 1998) including the larvae of the spirorbid *Janua brasiliensis* (Kirchman *et al.*, 1982a) and the serpulid *Hydroides elegans* (Lau and Qian, 1997; Unabia and Hadfield, 1999). There was no obvious correlation between, for example, bacterial morphology and inductive effect of the various bacterial strains tested during this study. The diversity of the bacterial strains which induced settlement of *S. spirorbis* larvae during this study suggests that the inductive cue may be a substance commonly produced by or associated with a wide range of bacteria but may be produced in different quantities or be present in different 'active' and 'non-active' forms for the different bacterial strains tested. Wieczorek and Todd (1998) suggested that invertebrate species which are wide-ranging on geographical or ecological scales are likely to be affected by a relatively simple common biofilm signal; this seems to be the case for the bacterial induction of settlement of *S. spirorbis* larvae.

There is considerable diversity of behaviour towards biofilms and their individual components between different marine invertebrate larvae (Wieczorek and Todd, 1998). Few general patterns have emerged other than the observation that generally, biofilms tend to induce settlement of larvae and the bacteria present in the biofilms appear to be the principal source of the settlement cue.

Wilson (1955) first suggested that the different biofilm forming microorganisms might vary in their ability to promote larval settlement. Meadows and Williams (1963) found that larvae of *Spirorbis borealis* (= *S. spirorbis*) settled readily on surfaces conditioned with certain diatoms together with their associated bacteria and suggested that variations in the constituents of such films may affect the settlement of *S. borealis*. In the experimental assays carried out here, presenting larvae of *S. spirorbis* with a mixed bacterial biofilm resulted in an intermediate settlement response compared to the individual bacterial strains when assayed in isolation, suggesting that variations in bacterial composition of biofilms do affect the settlement of *S. spirorbis* larvae. Natural marine biofilms are composed of aggregations of microorganisms embedded in extracellular polymeric substances (EPS) and are normally composed of a wide range of bacterial strains as well as other microorganisms (Flemming *et al.*, 2000). Therefore, when exploring a surface biofilm, *S. spirorbis* larvae are most likely to respond to a mixture of inductive and inhibitory chemical cues in addition to a range of physical cues such as surface roughness, orientation and reflected light. Presumably, natural biofilms with a high proportion of inductive bacterial strains are more likely to induce settlement and biofilms with a high proportion of bacterial strains which either inhibit or have little effect upon settlement will be unlikely to induce settlement, i.e. neutral.

Marine invertebrate larvae have been reported to respond to either water-borne products derived from bacteria (Neuman, 1979; Fitt *et al.*, 1990; Zimmer-Faust and Tamburri, 1994) or substances associated with the biofilm surface (Kirchman *et al.*, 1982b; Maki *et al.*, 1990; Szewzyk *et al.*, 1991; Lau and Qian, 2001) as a cue to settlement. In this study, larvae of *Spirorbis spirorbis* settled in response to mono-specific biofilms but not in response to water-borne products, suggesting that the inductive effect is a component of either the bacterial cell wall or the surface-associated extracellular products. Kirchman *et al.* (1982b) suggested that the inductive biofilm cue for *J. brasiliensis* was extracellular polysaccharides found on the bacterial cell surface. *Hydroides elegans* larvae have also been shown to settle in response to individual bacterial strains but it still is not clear whether the inductive cue is surface-bound or water-borne (Unabia and Hadfield, 1999; Lau and Qian, 2001). Unabia and Hadfield (1999) found that settlement of larvae of *H. elegans* was stimulated by the soluble products of all three bacterial strains tested, whereas Lau and Qian (2001) found that neither seawater conditioned by bacteria attached to Petri dishes nor the seawater conditioned by bacteria in suspension was inductive to larval settlement. Given the potential for the rapid dilution of a soluble, water-borne product emanating from a biofilm in the marine environment, it seems more intuitive to expect that the bacterially derived inductive cue is likely to be a surface associated phenomenon. Larvae of *S. spirorbis* probably rely largely upon geo-, photo- and baro-sensitive behavioural responses whilst in the water column which act to bring them into contact with a potential settlement site; selection of a settlement site is then based upon a combination of physical and chemical cues, including the presence of inductive bacterial strains within the surface biofilm (see Crisp, 1974).

Lau and Qian (1997) found that bacterial strains isolated from the surface of the alga *Sargassum tenerrimum* only gave relatively low percentage settlement of *Hydroides elegans* larvae compared to that induced by strains isolated from other surfaces. The authors infer that this finding may represent an antifouling strategy evolved by the alga. Here the opposite hypothesis was tested since *Spirorbis spirorbis* occurs predominantly on *Fucus serratus* fronds and its larvae are known to settle in response to biofilms and preferentially on *F. serratus*. It was hypothesised that the bacterial strains occurring on the fronds would induce high settlement of *S. spirorbis* larvae. However, the bacterial strain isolated from the surface of *F. serratus* fronds, did not induce high levels of settlement compared to some of the other isolated strains that originated from biofilms on intertidal rock surfaces. Clearly both a bacterial cue and the associative cue which emanates from *Fucus* plants (Williams, 1964) are important to *S. spirorbis* larvae but the *Fucus* cue appears to act as the overriding chemical cue at settlement.

Spirorbis spirorbis occurs primarily on the fronds of the alga *Fucus serratus* and less frequently on *Fucus vesiculosus*. This distribution is thought to come about principally because the larvae display highly selective associative settlement behaviour and gregariousness (Knight-Jones, 1951b, 1953a). Adult *S. spirorbis* also occur naturally on rock surfaces which may explain previous laboratory observations that the larvae settle in high numbers in response to natural biofilms on rock surfaces and settle in low numbers in the absence of a suitably biofilmed surface. During this study, it has been demonstrated that individual bacterial strains are capable of inducing settlement of *S. spirorbis* larvae and that different strains differ in their inductive effect. The significance of the inductive strains is not clear at present, as they do not seem to be associated with the natural adult habitat.

The finding that certain bacterial strains induce settlement whilst others do not and that larvae respond to changes in the relative proportion of strains in a mixed bacterial species biofilm confirms that bacteria present in a biofilm represent an important settlement cue to *Spirorbis spirorbis* larvae.

3.5 Settlement of *Spirorbis spirorbis* L. larvae in response to biofilms: nature of the inductive cue.

Abstract

The settlement response of *Spirorbis spirorbis* L. (Polychaeta: Spirorbidae) larvae to variously treated biofilms and in the presence of simple sugars was investigated in the laboratory. Settlement experiments were performed as single or multi-treatment, still-water assays. Larvae settled readily on biofilms exposed to formalin, antibiotics and subjected to osmotic stress. Treating biofilms with formalin or antibiotics before or after drying had no effect upon larval avoidance of dried biofilms. Exposing larvae to artificially high concentrations of glucose and fructose in solution significantly reduced settlement, whereas exposure to similar concentrations of the sugars lactose, sucrose, galactose and maltose had no apparent effect. If larvae were exposed to the glucose solution before an assay or the glucose solution was replaced with normal seawater during the assay, settlement proceeded as normal. The importance of sugars in the settlement process was investigated further, yielding somewhat conflicting results. Treating biofilms with the lectin concanavalin A, which binds specifically to glucose, did not significantly reduce larval settlement, whereas treating biofilms with sodium periodate and periodic acid did significantly reduce larval settlement, particularly for the periodic acid treatment. The chemical cue does not appear to be a glycoprotein and is not proteinaceous. It is concluded that larvae do not require a metabolically active biofilm for settlement and that the biofilm settlement cue is probably one of the commonly produced non-specific polysaccharides produced by a wide range of bacterial strains. More than one chemical cue may be involved in *S. spirorbis* larval perception of a suitable biofilm.

Introduction

Biofilms are an important settlement cue to a wide range of marine invertebrate larvae (see review by Wieczorek and Todd, 1998). Although biofilms may induce, have no effect or inhibit settlement, they frequently induce settlement of larvae and studies have shown that the bacteria present in the biofilms often provide the inductive cue. Although bacteria are now known to be important in the settlement of a wide range of marine invertebrate larvae, including barnacles (Maki *et al.*, 1988, 1990; Holmström *et al.*, 1992; Avelin Mary *et al.*, 1993), spirorbids (Kirchman *et al.*, 1982a), serpulids (Lau and Qian, 1997, 1999, 2002; Unabia and Hadfield, 1999; Hamer *et al.*, 2001) and hydroids (Neumann, 1979; Müller, 1973; Leitz and Wagner, 1993), the exact nature of the settlement cue from bacteria has been fully characterised for a very limited number of species (see Pawlik, 1992).

Kirchman *et al.* (1982a) demonstrated that the spirorbid *Janua brasiliensis* was induced to settle when presented with monospecific bacterial films and subsequently presented evidence suggesting that a glucose moiety within the extracellular polysaccharides present on the bacterial cell wall of *Deleya marina* provided the settlement cue (Kirchman *et al.*, 1982b). Larval lectins were considered to mediate the process (Kirchman *et al.*, 1982b; Maki and Mitchell, 1985). Other spirorbid and serpulid larvae also settle in response to biofilms and a number of species have been shown to settle in response to certain bacterial strains (see Hamer *et al.*, 2001; Chapter 3.1) but there is only limited information on the nature of the inductive bacterial cue for these species.

Bacterial exopolymers consist mainly of polysaccharides and proteins (Flemming *et al.*, 2000) with the three neutral sugars (D-mannose, D-glucose and D-galactose) occurring most frequently amongst marine bacterial exopolysaccharides (Sutherland, 1980). Given the occurrence and abundance of polysaccharides in bacterial films, it is plausible that, as suggested by Kirchman *et al.* (1982b), bacterial polysaccharides are an important settlement cue for other marine invertebrate larvae. For example, Neal and Yule (1994a) demonstrated that temporary adhesion (a surrogate measure for surface attractiveness) of barnacle cyprids (several species) was significantly decreased by exposure to sugar-enriched seawater and suggested that the chemical identity of bacterial exopolymers, particularly in respect to the ratios of certain sugars, may be an important factor in determining the strength of temporary adhesion by cyprids.

Subjecting natural multispecies or monospecific bacterial films to various chemical or physical treatments and then conducting larval assays has proven useful in revealing some general features of the nature of the biofilms and more specifically the settlement cues of bacteria (Kirchman *et al.*, 1982a; Unabia and Hadfield, 1999; Lau and Qian, 2001; Hamer and Walker, 2001). The rationale behind this method being that if a particular treatment reduces settlement then the biofilm component(s) affected by the treatment may represent the settlement cue. To date, such investigations have been carried out on larvae for only a small number of species. There is therefore the need to conduct similar laboratory studies, using the larvae of other species, as a means of investigating the generality of these observations. In addition, the 'lectin model' proposed by Kirchman *et al.* (1982b) has not yet been tested more widely for the larvae of other species.

Spirorbis spirorbis is a common intertidal tubeworm locally abundant on the brown macroalga *Fucus serratus*. Brooded, lecithotrophic larvae are liberated during neap tides and are competent to settle immediately upon release; laboratory studies have shown these larvae to exhibit highly selective settlement behaviour (see de Silva, 1962 and Hamer *et al.*, 2001). They settle preferentially on *F. serratus* plants or on biofilmed surfaces compared to clean surfaces (Knight-Jones, 1951b; Crisp and Ryland, 1960; Meadows and Williams, 1963; Hamer and Walker, 2001) and are stimulated to settle in high numbers by treating inert surfaces with a *F. serratus* extract obtained from macerated plants (Williams, 1964). Drying biofilmed slate and *F. serratus* surfaces for 1 hour at room temperature negates their previously inductive effect (Hamer and Walker, 2000; Chapter 3.2), but the nature of this effect has not been investigated. Meadows and Williams (1963) demonstrated that *S. spirorbis* larvae settle in response to biofilms composed of certain diatoms and their associated bacteria, but not others. Following on from this finding, studies have demonstrated that *S. spirorbis* larvae settle in response to certain mono-specific bacterial films, but not others and that the bacterial cue is surface-bound and suggest that it is either associated with the bacterial cell wall or the associated extracellular products (Chapter 3.4). Because of similarities between the larval settlement behaviour of *Janua brasiliensis* and *S. spirorbis*, it is plausible that as with *J. brasiliensis*, certain sugars within the exopolysaccharides represent the inductive biofilm cue. This study investigates the nature of the biofilm settlement cue for *Spirorbis spirorbis* larvae by subjecting biofilms to various chemical and physical treatments. It also tests the 'lectin model' proposed by Kirchman *et al.* (1982b) by exposing competent larvae to simple sugars in solution. Specifically, the following hypotheses are tested: (1) larvae require a metabolically active biofilm for settlement, (2) larval settlement is inhibited by certain low molecular weight sugars and (3) larval settlement is inhibited following exposure of the biofilm to certain lectins.

Materials and Methods

The settlement of *Spirorbis spirorbis* L. (= *S. borealis* Daudin) (Polychaeta: Spirorbidae) was examined in laboratory assays. A series of experiments was designed to investigate the nature of the settlement-inducing biofilm and bacterial cue. The experiments are based upon those of Hamer *et al.* (2001) for the general biofilm cue and Kirchman *et al.* (1982b) for more specific investigations on the possible role of sugars in *S. spirorbis* larval settlement.

Collection of larvae

Larvae were obtained as described previously (Chapter 3.2). Briefly, *Fucus serratus* and *F. vesiculosus* plants with large numbers of adult *Spirorbis spirorbis* tubes were collected between June and September from intertidal shores of the Menai Strait, North Wales, U.K and stored in air overnight in the laboratory in covered tanks. When larvae were required, the cover was removed and seawater from the laboratory running seawater system was passed through the tank. Seawater leaving the tank was passed through a 63 μm mesh sieve. Larvae retained on the sieve were re-suspended in 0.2 μm filtered, u.v.-irradiated seawater (FSW) and counted into assay dishes. For all assays, the larvae used were liberated within 1 hour of the start of the assay.

Assay procedure

The assay procedure varied depending upon the treatment used (see below). All assays were conducted with biofilmed (minimum 6 month old biofilm developed in the laboratory as described previously) slate surfaces (25 x 25x 4 mm Welsh slate cut to size by Inigo Jones, North Wales and polished to a uniform roughness using 600 grade emery paper). Each assay dish (Pyrex 250 ml capacity glass dishes) contained the test surface(s) and 100 ml of 0.2 μm filtered, u.v. irradated seawater (FSW), in some cases enriched with various low molecular weight sugars; 10 freshly liberated larvae were also added to each dish. All assays were carried out at room temperature in the dark. Assay duration and the number of replicates used are detailed where appropriate. At various stages throughout the assays, the number of larvae that had settled was counted.

Because of the additional information on larval settlement preferences that may be gained by conducting simultaneous single and multi-treatment assays ('no-choice' and 'choice' assays respectively; see chapter 3.3), the first series of experiments was conducted in this manner. However, the nature of the subsequent experiments required them to be conducted as single treatment assays.

Biofilm treatments

This first series of experiments was conducted as simultaneous single and multi-treatment 6 hour assays (n=6).

Experiment 1: biofilm exposure to formalin and aerial drying

This experiment was conducted to investigate whether larvae require a live biofilm for settlement and to determine if the aerial drying effect reported by Hamer and Walker (2000) was a result of larvae responding to a product actively produced by bacteria (or other biofilm components) in response to the drying. In the first experiment, biofilms were exposed to 4% formalin in seawater for 30 minutes then rinsed thoroughly in running seawater for 1 minute to remove the free formalin from the biofilm. Biofilms were treated with formalin immediately before or immediately after the drying process (1 hour at 20°C). Non-dried biofilms were also treated with formalin and other dried biofilms were not; the controls were non-dried biofilms.

Experiment 2: biofilm exposure to antibiotics and aerial drying

Because formalin is a preservative and has the potential to change the chemical nature of a biofilm, a similar protocol was carried out using antibiotics which specifically inhibit protein synthesis by bacteria. Biofilms were exposed for 6 hours to a 1:1 mixture of the antibiotics kanamycin and streptomycin (Sigma) each at a concentration of 200 $\mu g\ ml^{-1}$. Plating assays confirmed the effectiveness of the antibiotic treatment. Biofilms were treated with the antibiotics immediately before or immediately after the drying process. Non-dried biofilms were also treated with antibiotics and other dried biofilms were not; the controls were non-dried biofilms.

Experiment 3: biofilms subjected to osmotic stress

Biofilms were exposed to hypersaline seawater and distilled water to simulate extreme osmotic stress during drying, but without the physical effect of drying. Hypersaline seawater (60 ‰) was prepared by gently heating u.v.-irradiated, 0.2 µm filtered seawater at 50°C until the respective salinity was reached. The hypersaline seawater was prepared the day prior to an assay and allowed to cool overnight to room temperature.

a) Biofilmed slates were immersed in the hypersaline seawater for 1 hour; control biofilmed slates were kept immersed in normal salinity FSW during this time and then assayed alongside the treated biofilms.

b) Biofilmed slates were immersed in distilled water for 1 hour; control biofilmed slates were kept immersed in normal salinity FSW during this time and then assayed alongside the treated biofilms.

Biofilm and larval treatments to investigate the role of sugars

The second series of experiments was aimed at investigating the possible role of sugars upon larval settlement. These experiments were conducted as single treatment (= no choice) 24 hour assays with individual test surfaces placed in separate glass dishes (replicated).

Experiment 1: larval exposure to sugars

The settlement of *Spirorbis spirorbis* larvae in response to different levels of sugar-enriched seawater was investigated. The sugars used were the monosaccharides D(-) fructose, D-(+)-glucose and D-(+)-galactose and the disaccharides lactose, sucrose and maltose (Sigma) at concentrations of 0.1, 0.05, 0.01, 0.001 M. Individual sugars were assayed during separate experiments, each with a control treatment. The control was FSW only (no added sugar). Settled individuals were counted after 1, 2, 4, 12 and 24 hours.

Experiment 2: further glucose assays

In order to further investigate the longevity of the settlement inhibition effect of glucose, two experiments were conducted:

i) Freshly liberated larvae were placed into glucose-enriched (0.1 M) seawater for 1 hour. Larvae were then either rinsed in filtered seawater and assayed in filtered seawater or not rinsed and assayed in glucose-enriched (0.1 M) seawater. The control involved larvae that were placed in FSW for the 1 hour period. Settlement counts were made after 1, 2, 4, 12 and 24 hours.

ii) An assay was started with a glucose-enriched seawater treatment and a FSW control. After six hours, the larvae from half of the glucose treatment dishes were removed and rinsed in filtered seawater, the glucose-seawater was replaced with FSW and the larvae returned to the same assay dishes. Hourly settlement counts were made throughout the 24 hour assay.

Experiment 3: biofilm exposure to lectins

The effect of treating biofilms with lectins was investigated. Biofilmed slates were exposed to concanavalin A (Jack Bean (*Canavalis ensiformis*) lectin; Type III; Sigma) which binds specifically to glucose, or peanut lectin (*Arachis hypogaea*; Sigma) which binds specifically to galactose. Biofilms were exposed to these lectins for 1 hour before rinsing for 1 min in FSW. The lectin concentrations used were based upon the methodology of Kirchman *et al.* (1982b); a concentration of 2 mg / ml of concanavalinA and 0.4 mg / ml of peanut lectin dissolved in 3 % (w/ v) NaCl were used. A glucose-enriched seawater treatment of 1.0M D-(+)-glucose was also assayed. The control was a biofilm that was exposed for 1 hour to 3 % (w / v) NaCl. Settlement counts were carried out at 1, 2, 4, 12 and 24 hours.

Two further lectin assays were carried out using the methodology described above. However, because of limitations in availability, the peanut lectin treatment was omitted. The second assay was a direct repeat of the first assay but without the peanut lectin treatment. For the third assay, the concanavalin A 3 % NaCl solution was enriched with Mn^{2+} and Ca^{2+} ions from the respective chloride salts $MnCl_2{\bullet}4H_20$ and $CaCl_2$ at a final ionic concentration of 1 mM. This treatment was tested because the effectiveness of Concanavalin A is enhanced by the presence of these ions (Sigma).

Experiment 4: biofim exposure to sodium periodate and periodic acid

In the first assay, biofilms were exposed to 10mM sodium periodate dissolved in either distilled water or 3.0 % NaCl for 1 hour, rinsed in FSW and an assay started. Periodate oxidises 1,2-dihydroxyl groups in carbohydrates (Kirchman *et al.*, 1982b). The controls were biofilms exposed to distilled water or 3% NaCl for the same period and an unadulterated biofilm. Settlement was counted after 1, 2, 4, 12 and 24 hours.

In the second assay, biofilms were exposed to a 10 % aqueous periodic acid (BDH) solution for 1 hour, rinsed in FSW and an assay started. Periodic acid also oxidises 1,2-dihydroxyl groups that make up sugars (the reaction gives units containing 2 aldehyde groups as shown in Figure 1). The controls for this experiment were a biofilm exposed to distilled water for the same time period and an unadulterated biofilm. Settlement was counted after 1, 2, 4, 12 and 24 hours.

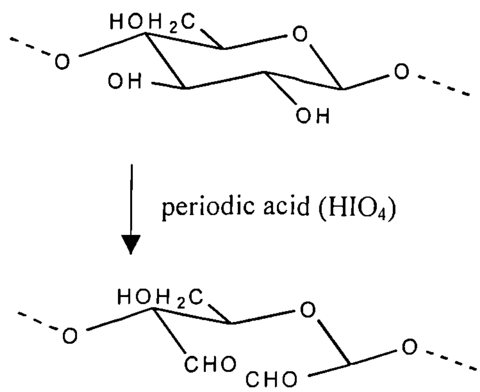


Figure 1. The reaction of periodic acid with the glucose unit of a polysaccharide.

Experiment 5: biofilm exposure to trypsin

Biofilms were exposed for 1 hour at 35 °C to 100 ml of 1.0 mg / ml of trypsin (Type III; Sigma) dissolved in 0.1 M phosphate buffer (pH = 7.0) or in the buffer with 3 % (w/ v) NaCl added. As controls, biofilms were treated with phosphate buffer, 3 % NaCl or both phosphate buffer and 3 % NaCl for 1 hour at 35 °C. The films were rinsed with FSW for 1 minute after the treatments described. Settlement was counted at 1, 2, 4, 12 and 24 hours.

Experiment 6: gum arabic and gum xanthan

Two similar experiments were carried out to test the effect of exposure of larvae and biofilms to i) gum arabic and ii) gum xanthan (Sigma). Gum arabic and gum xanthan are naturally occurring, water soluble polysaccharides. Gum arabic is a branched polymer containing the sugars galactose, rhamnose, arabinose, and glucoaronic acid and has a molecular weight of 250, 000. Gum xanthan contains the sugars glucose and mannose and forms a hydrophilic colloid (Sigma). Biofilms were exposed for 1 hour at room temperature to 0.001 g / ml of gum dissolved in FSW. Clean, non-biofilmed slates were also exposed for 1 hour to the gum xanthan solution and 1ml of the solution was dried (1 hour at room temperature) onto clean and dry slates. Biofilms and clean slates exposed to the gums were then rinsed quickly in FSW and the assay started. The controls were a biofilmed and clean, non-biofilmed slates that had not been exposed to the gums.

Experiment 7: Settlement on agar plates

Agar is a naturally occurring polypeptide extracted from marine algae. Because of the potential use of agar for incorporating normally soluble biofilm components as a means of isolating the biofilm settlement cue, the settlement response of larvae to treated agar plates was investigated. Preliminary experiments showed that nutrient broth agar (Difco) rapidly induced metamorphosis, but not settlement of *S. spirorbis* larvae. The larval metamorphic and non-settlement response was measured during this experiment (larvae that had lost the ability to swim, had a pronounced 'neck' region and were beginning to develop tentacle buds and an operculum, were considered to have metamorphosed). Agar (Sigma) plates (15 g Agar to 1 l distilled water) were made up in 90 mm sterile petri dishes (Sterilin). Sugar-enriched agar solutions (glucose and sucrose) were made at a concentraion of 0.1 M. Petri dishes were allowed to stand as the agar cooled and solidified. Larvae (n=10) were then added to each Petri dish (n=4 for each treatment). The control was non-enriched agar. The number of metamorphosed individuals was counted after 1, 2 and 4 hours.

Statistical analysis

The percentage larval settlement data in response to experimental treatments were arc-sine transformed before statistical analysis. Those treatments in which no larvae settled were given the value of 1/4n (n=no. of larvae) to improve the arc-sine transformation (Zar, 1996). Data were tested for normality and homogeneity of variances. Normal data without significantly heterogeneous variances were analysed by one-way ANOVA followed by Tukey's test. Where there was significant heterogeneity of variances, data were analysed by Kruskal-Wallis followed by Dunn's multiple comparisons test. Further details of the tests used for each experiment are given in the text and figure legends.

Results

Biofilm treatments

Experiment 1: biofilm exposure to formalin

Exposing biofilms to formalin had no significant effect upon larval settlement compared to the biofilm control in either of the assays (One-way ANOVA; $F=89.68$; df 3; $p<0.001$; $F=34.07$; df 3; $p<0.001$ respectively for the multi-treatment and single treatment assays) (Figure 2 a & b). No larvae settled on either of the treatments which involved drying in the multi-treatment assay, whereas a small percentage (~ 15-20%) settled in significantly lower numbers ($p = 0.05$) on these treatments in the single treatment assay.

Experiment 2: biofilm exposure to antibiotics

Similarly, exposing biofilms to antibiotics had no significant effect upon larval settlement compared to the biofilm control in either of the assays (One-way ANOVA; $F = 26.55$; df 3; $p<0.001$; $F = 13.80$ df 3; $p<0.001$) (Figure 3 a & b). No larvae settled on the dried biofilm treatment or the dried biofilm and antibiotic treatment in the multi-treatment assay. In the single treatment assay, larvae settled in significantly lower numbers ($p = 0.05$) on the dried treatments than on the biofilm control.

Experiment 3: biofilms subjected to osmotic stress

Exposing biofilms to hypersaline seawater or distilled water had no significant effect upon their ability to induce settlement of *S. spirorbis* larvae, with larvae settling readily on such treated biofilms (Figure 4 a & b).

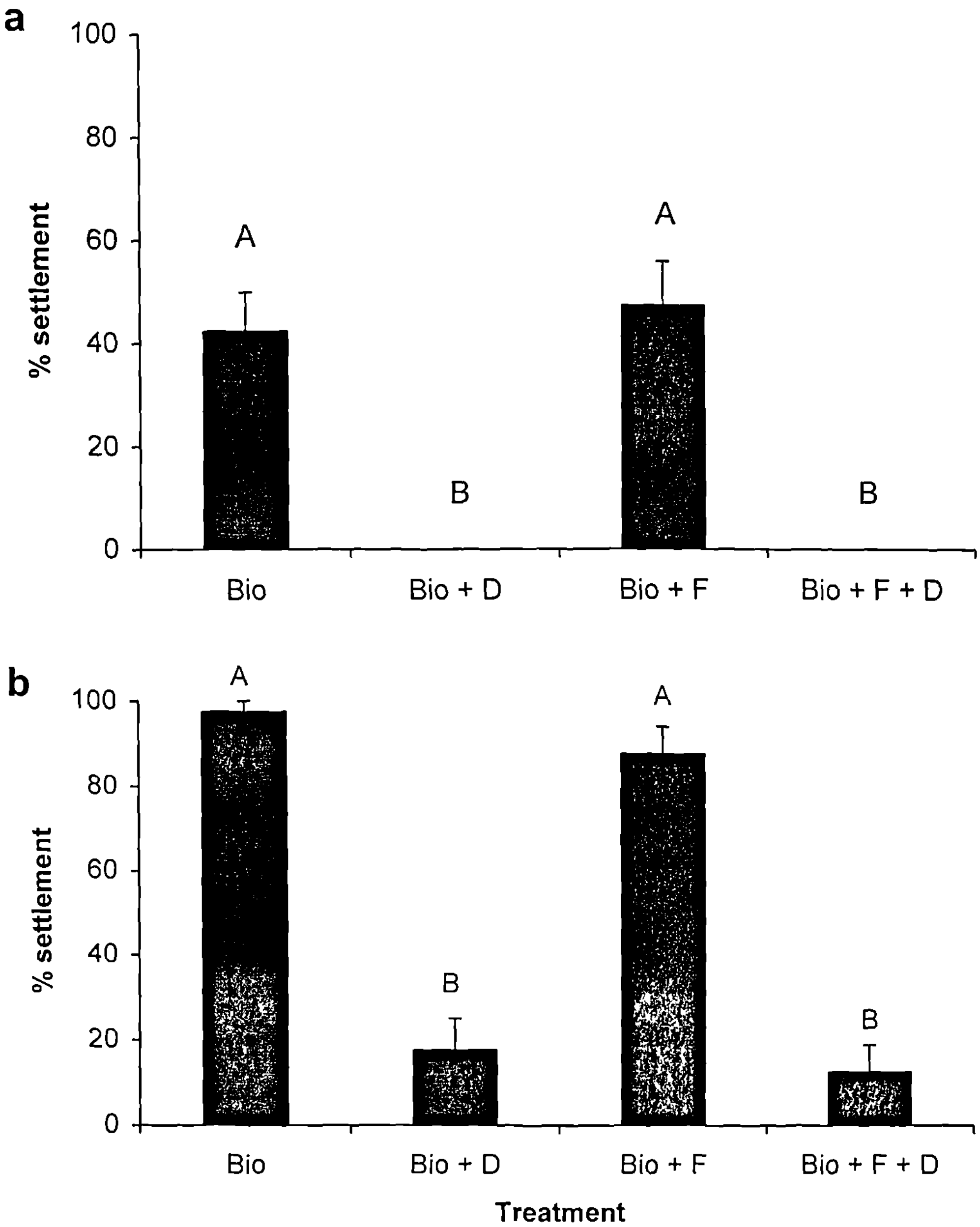


Figure 2a & b. Mean percentage settlement of *S. spirorbis* larvae after 6 hour 'multi-treatment **(a)** and 'single-treatment' **(b)** settlement assays. Biofilms were exposed to 4% formalin for 1 hour before drying at 20 °C (Bio + F + D). A non-dried biofilm was also exposed to formalin (Bio + F). A biofilm was dried but not exposed to formalin (Bio + D). The control was an unadulterated biofilm (Bio). Data plotted are the means + S.E. (n=6). Results of Tukey's test are given; bars with the same letter are not significantly different (at $p < 0.05$).

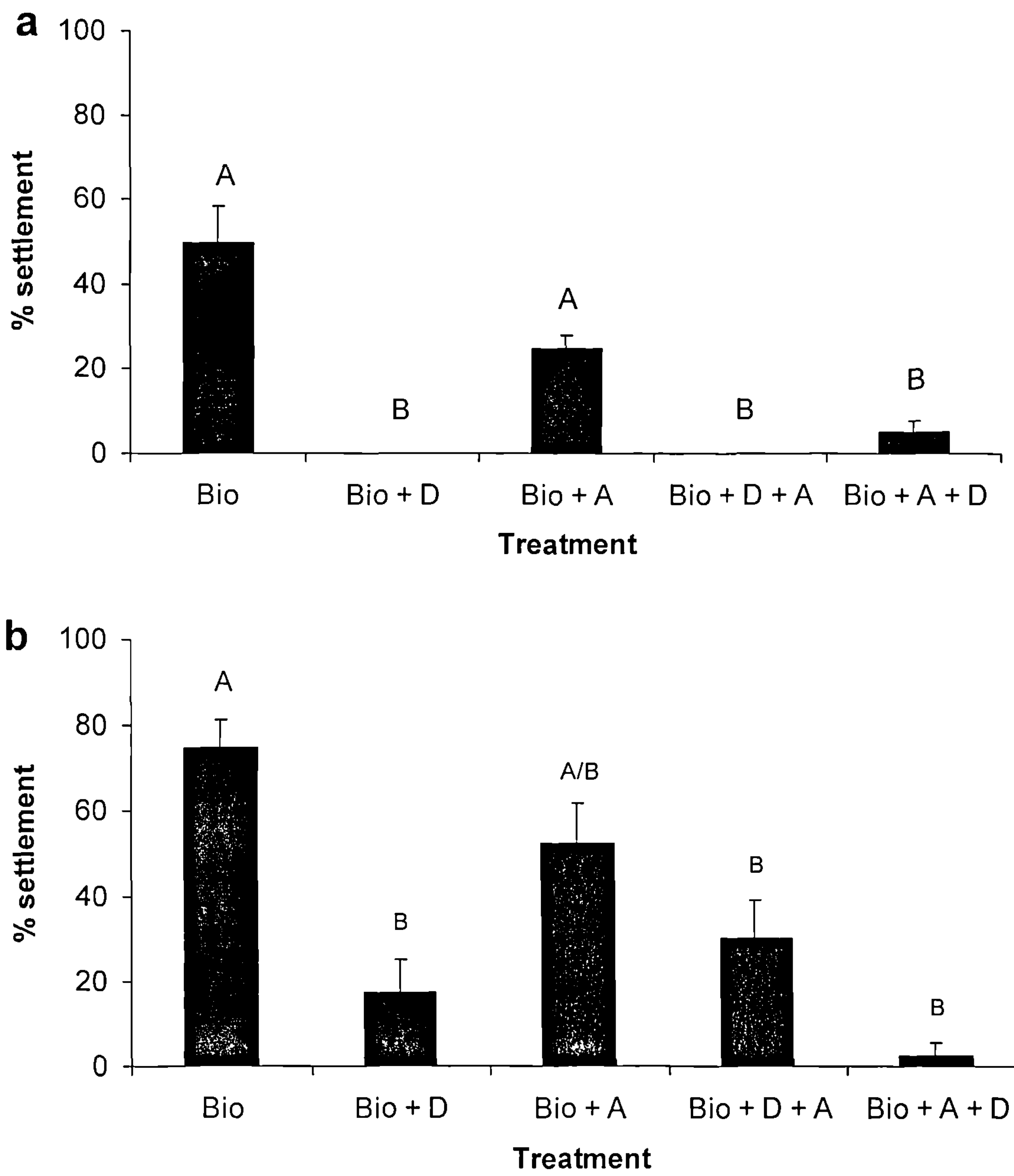


Figure 3a& b. Mean percentage settlement of *S. spirorbis* larvae after 6 hour 'multi-treatment' **(a)** and 'single-treatment' **(b)** settlement assays. Biofilms were exposed to 200μgml^{-1} of a 1:1 mixture of the antibiotics kanamycin and streptomycin for 4 hours before (Bio + A + D) or after (Bio + D + A) drying at 20°C. A non-dried biofilm was also exposed to antibiotics (Bio + A). A biofilm was dried but not exposed to formalin (Bio + D). The control was an unadulterated biofilm (Bio). Data plotted are the means + S.E. (n=6). Results of Tukey's test are given; bars with the same letter are not significantly different (at $p<0.05$).

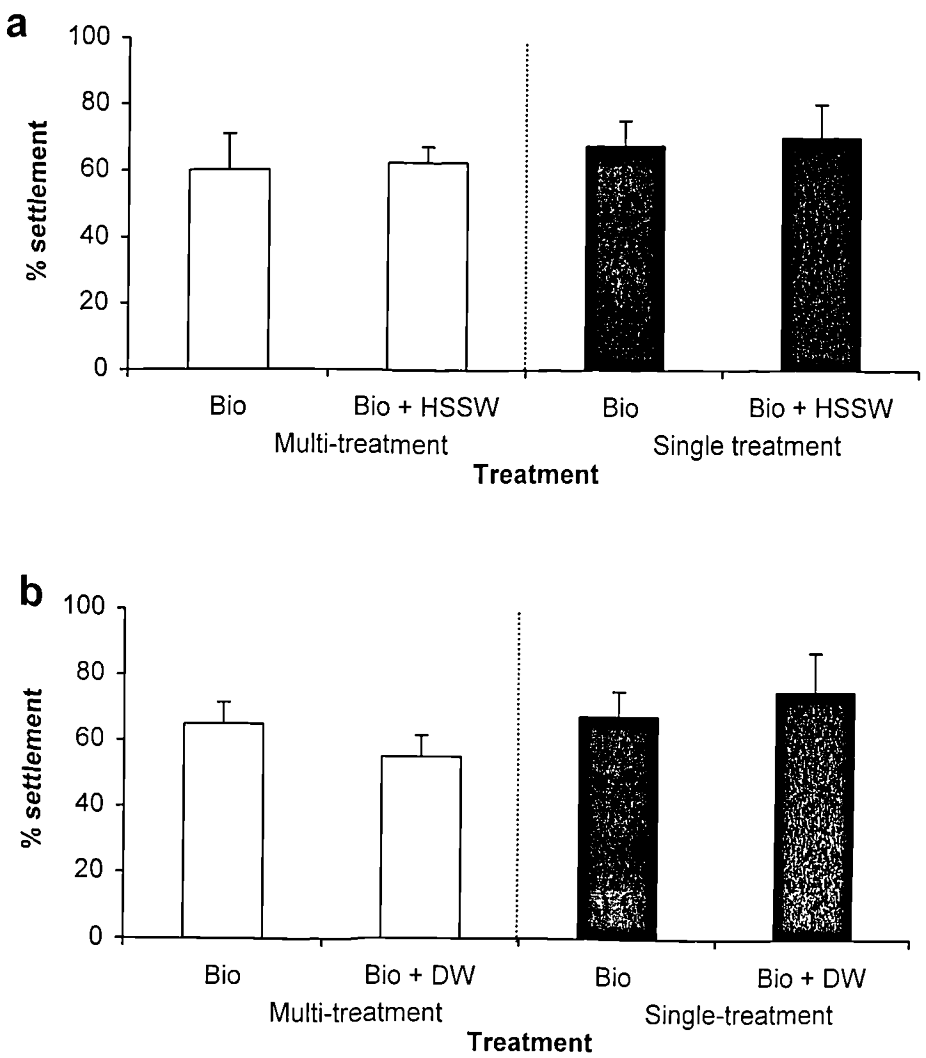


Figure 4a & b. Mean percentage settlement of *Spirorbis spirorbis* larvae after 6 hour 'multi-treatment' and 'single-treatment' settlement assays which were conducted simultaneously. **a)** Biofilms were immersed in 60 ‰ hypersaline seawater (HSSW) for 1 hour. The control was an unadulterated biofilm (Bio). Data plotted are the means + S.E. (n=4). There was no significant difference between treatments (t-test; T = -0.21; P = 0.84; df 4; T = -0.63; P = 0.55; df 4 for multi-treatment and single treatment assays respectively). **b)** Biofilms were immersed in distilled water (DW) for 1 hour. The control was an unadulterated biofilm (Bio). Data plotted are the means + S.E. (n=4). There was no significant difference between treatments (t-test; T = 1.10; P = 0.32; df 4; t = -0.38; P = 0.72; df 4 respectively for multi-treatment and single treatment assays respectively).

Biofilm and larval treatments to investigate the role of sugars

All of the following experiments were carried out as single treatment, 'no-choice' assays.

Experiment 1: larval exposure to sugars

Generally, percentage settlement was reduced at high concentrations for all sugars (Figure 5a-f), with larvae taking longer to settle. At the highest concentrations, there was some abnormality in tube formation with a small proportion of larvae having constructed tubes that were vertical, only being anchored to the substratum at their base. Larvae that did not settle appeared to be swimming and exploring the surfaces normally even after 24 hours at the highest sugar concentrations. After 24 hours of exposure, total settlement was not significantly different (at $p<0.05$) between the various sugar concentrations tested and the control for lactose, sucrose, galactose and maltose. Settlement was significantly different (at $p<0.05$) however, between the control and the fructose and glucose treatments at the higher concentrations (0.05 and 0.1M). Settlement was approximately 20 – 25 % at 0.05 and 0.1M fructose after 24 hours and 5 – 15% at 0.05 and 0.1M glucose after 24 hours.

Experiment 2: further glucose assays.

Exposing larvae to 0.1 M glucose solution prior to an assay did not significantly affect percentage settlement compared to the control treatment (Figure 6).

Larvae exposed to 0.1 M glucose solution for the first 6 hours of an assay and then placed in FSW for the remaining time began to settle once removed from the glucose solution so that at the end of the 24 hour assay settlement was significantly greater than the glucose treatment but significantly lower than the control (at $p<0.05$) (Figure 7).

Experiment 3: biofilm exposure to lectins

Treating a biofilm with both concanavalin A and peanut lectin had no significant effect upon percentage settlement (Figure 8a) compared to the control at the end of the 24 hour assay. However, there was a significant difference after 1 hour where > 40% of larvae settled on the control biofilm compared to < 20% on both lectin-treated biofilms. Because of this significant difference, further lectin assays were carried out.

In the second and third lectin assays, there was no significant difference between settlement on the Concanavalin A treated biofilm and the biofilm control (Figure 8b shows the results of the second lectin assay).

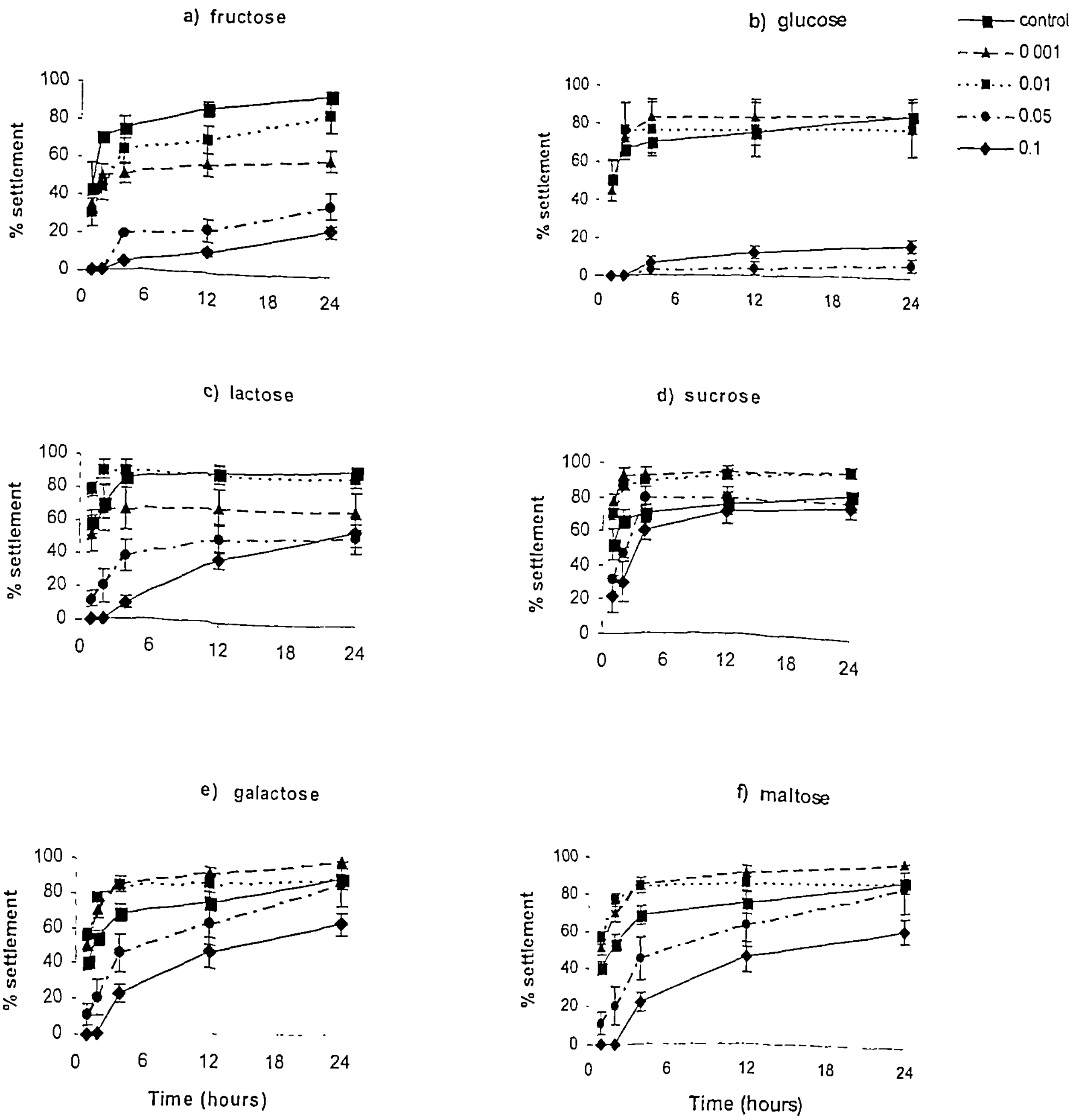


Figure 5 a-f. Mean percentage settlement of *Spirorbis spirorbis* larvae on biofilmed slate surfaces in the presence of various concentrations (0.1, 0.05, 0.01, 0.001 M in filtered seawater) of the sugars fructose (a), glucose (b), lactose (c), sucrose (d), galactose (e) and maltose (f), during a 24 hour settlement assay. The control for each assay was FSW. Data plotted are means ± SE (n = 5).

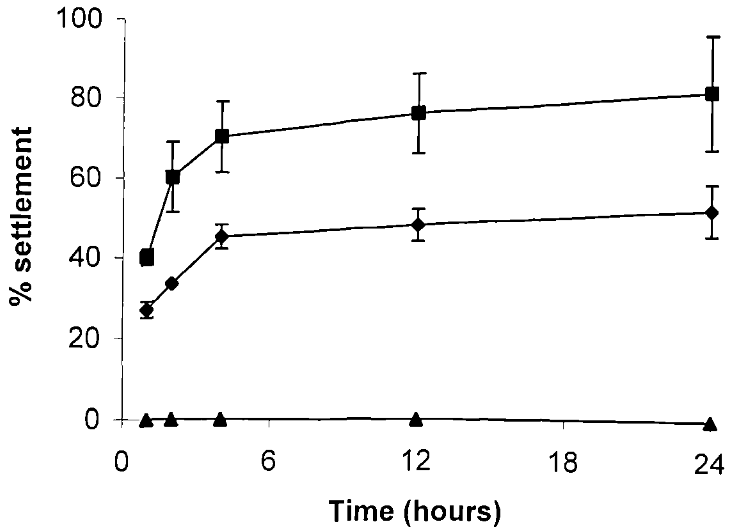


Figure 6. Mean percentage settlement of *Spirorbis spirorbis* larvae on biofilmed slate surfaces during a 24 h settlement assay. Larvae were exposed to 0.1 M glucose for 1 hour immediately prior to the assay and then assayed in FSW (■) or 0.1 M glucose (▲). The control treatment consisted of larvae not exposed to the glucose-enriched seawater (♦). Data plotted are means ± SE (n = 4).

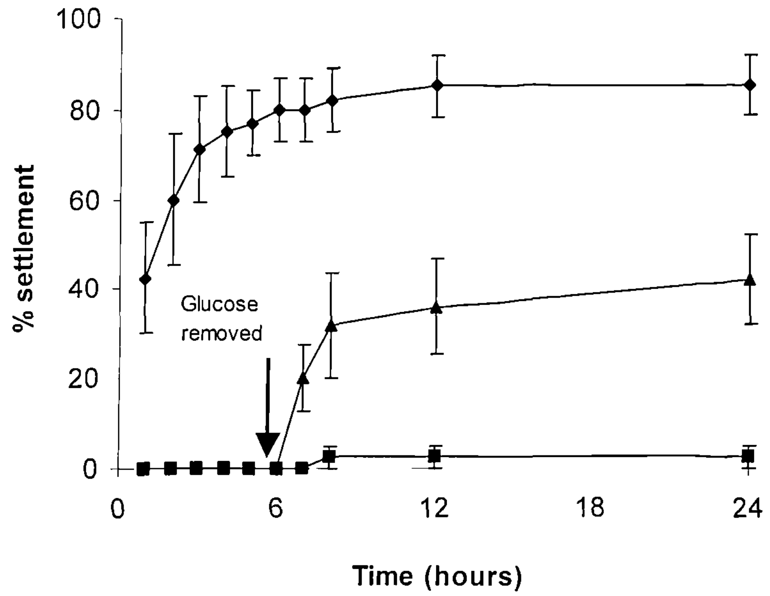


Figure 7. Mean percentage settlement of *Spirorbis spirorbis* larvae on biofilmed slate surfaces during a 24 h settlement assay. During the assay, larvae were exposed to 0.1M glucose for 24 hours (■), 6 hours and then replaced with FSW (▲) and in FSW for the duration of the 24 hour assay (♦) (control). Data plotted are means ± SE (n = 4).

Experiment 4: biofilm exposure to sodium periodate and periodic acid

Percentage larval settlement was significantly different between biofilms treated with sodium periodate and unadulterated control biofilms (Figure 9). Control biofilms treated with the NaCl solution and distilled water gave an intermediate settlement response but were not significantly different from the sodium periodate treatments (at $p=0.05$).

Periodic acid completely negated the settlement-inducing effect of the biofilms; no larvae settled on such treated biofilms in any of the replicates (68.9 ± 6.2 % settlement on the control).

Experiment 5: biofilm exposure to trypsin

Exposing biofilms to trypsin had no significant effect upon larval settlement preferences (Figure 10).

Experiment 6: gum arabic and gum xanthan

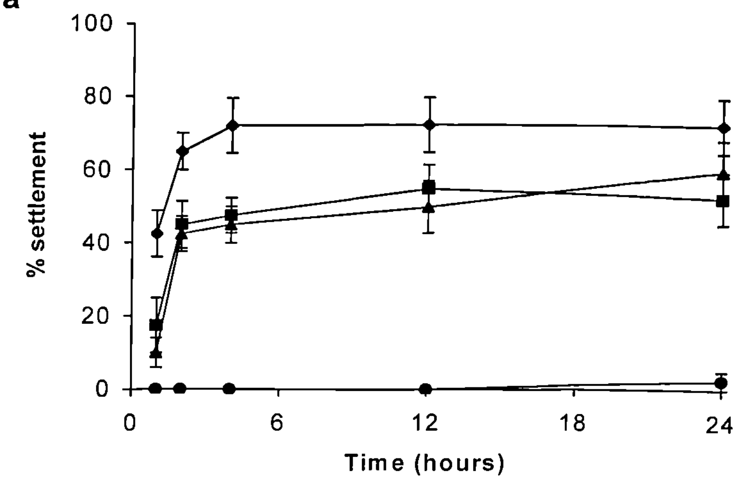
Gum arabic and gum xanthan failed to induce any larval settlement.

Experiment 7: settlement on agar plates

One hundred ± 0 % larval metamorphosis was obtained in the nutrient broth plate treatment, whereas no metamorphosis or settlement occurred for any of the other treatments. Larvae that metamorphosed in the nutrient broth cultures were unable to construct calcareous tubes in the normal manner and remained unattached after the 24 hour period.

During these assays, some larvae were placed in clean Petri-dishes containing filtered seawater only. All of these larvae immediately (<30 seconds) appeared to lose the ability to swim and began to undergo metamorphosis. After a 6 hour period, many of them had begun to construct a calcareous tube which attached them to the dish surface. This observation was made many times with different batches of larvae.

a



b

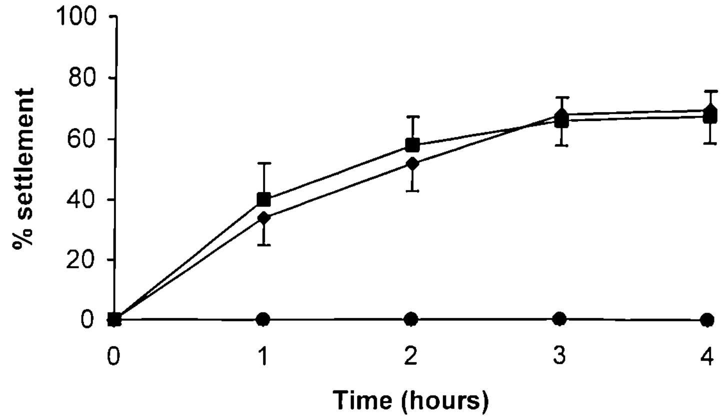


Figure 8a & b. (a) Mean percentage settlement of *Spirorbis spirorbis* larvae on biofilmed slate surfaces during a 24 h settlement assay. Biofilms were exposed to the lectins concanavalin A (■) or peanut lectin (▲) for 1 hour immediately prior to the assay. Biofilms not exposed to lectins were assayed in 0.1M glucose (•) or in FSW (♦) (control). Data plotted are means ± SE (n = 4). **(b)** repeat of (a) without peanut lectin.

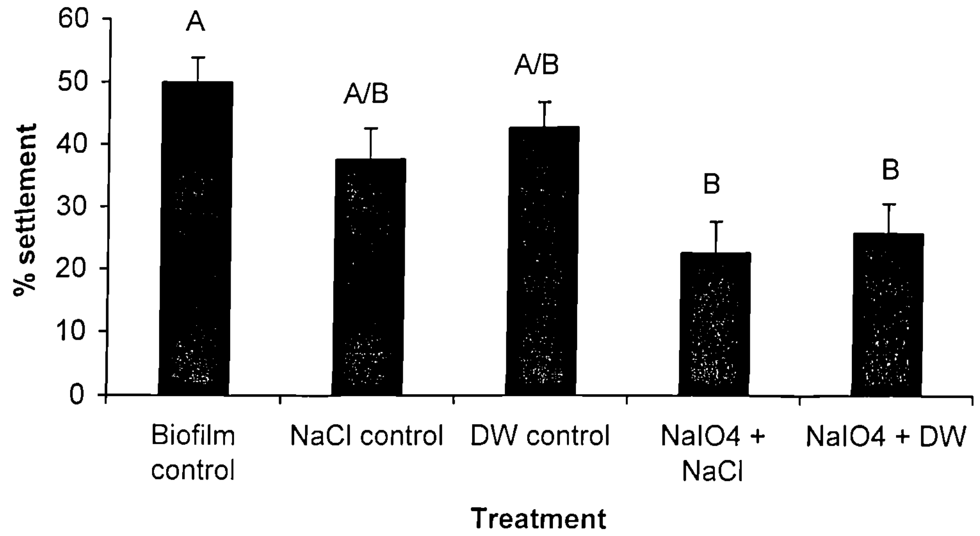


Figure 9. Mean percentage settlement of *Spirorbis spirorbis* larvae on biofilmed slate surfaces after 24 h settlement assay. Biofilms were treated with sodium periodate (NaIO4) in either NaCl-enriched seawater ($NaIO_4$ + NaCl) or distilled water ($NaIO^4$ + DW). Controls were unadulterated biofilms, and biofilms treated with NaCl-enriched seawater (NaCl control) or distilled water (DW control). Data plotted are means + SE (n =4) after 24 hours. Results of Tukey's test are given; bars with the same letter are not significantly different (at $p<0.05$).

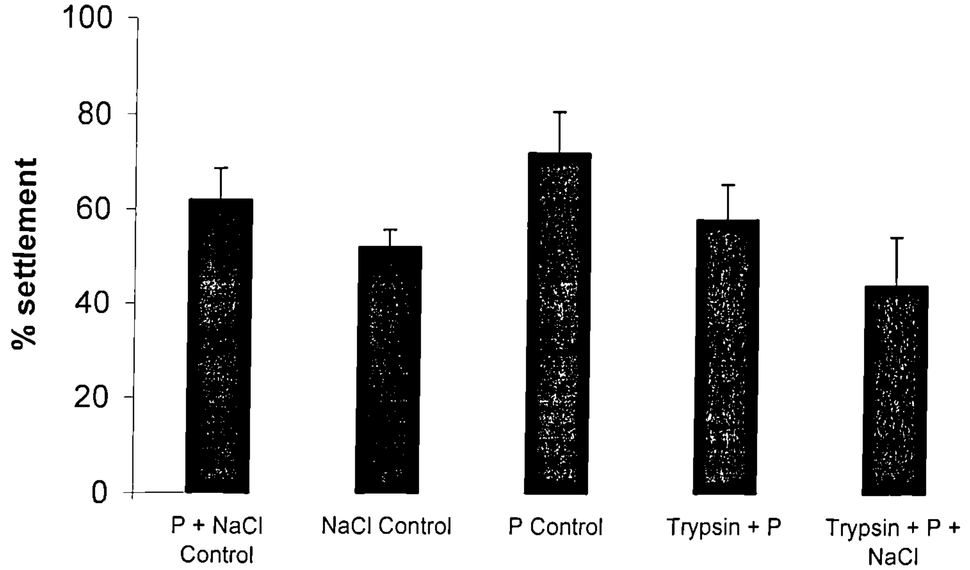


Figure 10. Mean percentage settlement of *Spirorbis spirorbis* larvae on biofilmed slate surfaces after 24 h settlement assay. Biofilms were treated with trypsin in phosphate buffer (P) and trypsin with phosphate buffer and 3 % NaCl at 35 °C for 1 hour. Controls were biofilmed slates treated with phosphate buffer alone, 3 % NaCl alone or both phosphate buffer and 3 % NaCl at 35 °C for 1 hour. Data plotted are means + SE (n=5) after 24 hours. There was no significant difference between treatments (One-way ANOVA; $F = 1.90$, df 4; $p = 0.150$).

Discussion

This series of experiments has shown that *Spirorbis spirorbis* larvae readily settle on metabolically inactive biofilms, but do not settle on biofilms when exposed to elevated concentrations of the simple sugars glucose and fructose. Treating a biofilm with sodium periodate and periodic acid significantly reduced larval settlement, whereas treatment with the glucose-specific lectin concanavalin A and trypsin had no effect. These findings provide a useful insight into the nature of the biofilm settlement cue, particularly when comparisons are made with similar studies which have been carried out using the larvae of different serpulid and spirorbid species (see Table 1).

Although the identity of the inductive biofilm settlement cue has not yet been fully characterised for any of the serpulid or spirorbid species studied, some general features of the nature of the biofilm and bacterial settlement cues have been revealed. For example, all of the species studied to date settle in response to a biofilm and for some of these species, bacteria are the inductive cue (see Hamer *et al.*, 2001; Chapter 3.4). In addition to similarities, there are also significant differences in the nature of the settlement cue between the various serpulid and spirorbid species so far studied, for example, the inconsistency between species in their requirement for a metabolically active biofilm for settlement induction (Table 1). The settlement of *Hydroides elegans* larvae is reduced on metabolically inactive biofilms (Unabia and Hadfield, 1999; Lau and Qian, 2001) whereas, *Spirorbis spirorbis*, *Janua brasiliensis* and *Pomatoceros lamarckii* larvae settle in comparable numbers on such metabolically inactive biofilms (Kirchman *et al.*, 1982b; Hamer *et al.*, 2001; this study). Unabia and Hadfield (1999) found that all physical and chemical treatments used to kill bacteria either eliminated or severely reduced the settlement-inducing effects of natural multi-species biofilms to *H. elegans* larvae. Similarly, Lau and Qian (1999) reported that treating mono-specific bacterial films with formalin or antibiotics stopped the normal *H. elegans* larval settlement response. Formaldehyde kills bacteria by denaturing protein whereas the antibiotics streptomycin and kanamycin kill bacteria by disrupting protein synthesis (Franklin and Snow, 1989). Therefore, as suggested by Lau and Qian (2001), since the bacterial cell surface is mainly comprised of a matrix of polysaccharides with <1% protein (Decho, 1990), these formalin and antibiotic treatments should cause little disruption to the surface chemistry of the bacterial cells. Lau and Qian (2001) therefore propose that for *Hydroides elegans* larvae, the bacterial settlement cue may be proteinaceous in nature and be derived from a metabolic pathway of the bacteria and exported to and concentrated in the extracellular polymer layer. However, *S. spirorbis* larvae will settle on biofilms that have been treated with formalin and antibiotics, suggesting that the inductive cue for *S. spirorbis* larvae is not proteinaceous in nature and that it is a relatively stable and persistent compound.

Exposing *Spirorbis spirorbis* larvae to excess glucose and fructose reduced settlement. This settlement inhibition may be because the larval receptors normally involved in the recognition of the biofilm cue had been saturated. Glucose and fructose are chemically similar structures and may therefore stimulate a common larval receptor. The monosaccharides did not induce metamorphosis by themselves, otherwise they would do so in solution, suggesting that a larger molecule may be involved. Treating biofilms with sodium periodate and periodic acid reduced and completely negated settlement respectively, further suggesting that sugars are somehow involved in the perception of a biofilm by *S. spirorbis* larvae.

The experiments carried out here with *Spirorbis spirorbis* larvae suggest that as with *Janua brasiliensis* (Kirchman *et al.*, 1982b), polysaccharides produced by bacteria are the settlement-inducing cue (glucose and fructose inhibit settlement and treating a biofilm with periodate reduces its attractiveness). Previously, it has been shown that larvae of *S. spirorbis* settle in response to certain bacterial strains (but not others) and it was proposed that surface associated bacterial products common to a variety of bacterial strains are the major biofilm settlement cue (Chapter 3.4). The findings reported here suggest that the cue is associated with bacterial extracellular polysaccharides. Polysaccharides are complex carbohydrates made up of sugar molecules linked into a branched or chain structure. The most common sugar found in polysaccharides is glucose, which may explain the pronounced effect upon settlement when larvae are exposed to excess glucose. Specific polysaccharides (e.g. xanthan) are produced only by individual bacterial strains whereas non-specific polysaccharides (e.g. dextran, alginate) are found in a variety of bacterial strains (Christensen and Characklis, 1990). The inductive cue for *S. spirorbis* larvae is probably one of the commonly produced, non-specific polysaccharides. The variable settlement of *S. spirorbis* larvae in response to different bacterial strains (Chapter 3.4) may be related to the ability of the various bacterial strains to produce the inductive polysaccharide(s). Lau and Qian (2001) also suggested that the larval settlement cues provided by the bacteria *Roseobacter* and *Proteobacter* may share common characteristics. Kirchman *et al.* (1982b) also considered that the inductive biofilm cue for *J. brasiliensis* was associated with the bacterial extracellular polysaccharides and reported that treating films with both periodate and trypsin inhibited settlement, suggesting that glycoproteins may be involved (Maki and Mitchell, 1985). However, in this study, treating biofilms with trypsin or formalin had no significant effect upon larval settlement, indicating that glycoproteins are not involved in the settlement of *S. spirorbis* larvae.

In this study, exposing biofilms to the glucose-specific lectin concanavalin A before the settlement assay did not reduce larval settlement, indicating that either (1) the lectin model as proposed by Kirchman *et al.* (1982b) does not apply to *Spirorbis spirorbis* larvae, or, (2) the biofilm cue which binds to the same larval receptor as glucose and fructose in solution is not the only inductive cue. In this study, natural, multispecies biofilms were used, whereas Kirchman *et al.* (1982b) chose to conduct experimental assays with monospecific bacterial biofilms. Furthermore the bacterial species used had been relatively well characterised and it is known that glucose is the major simple sugar present in the exopolysaccharides of *Pseudomonas marina* (Fazio *et al.*, 1982). The failure to block settlement using concanavalin A in this study may be because a natural, multi-species biofilm was used and other settlement cues induced the settlement of larvae. Therefore, the lectin model suggested by Kirchman *et al.* (1982b) cannot be ruled out as the mechanism by which *S. spirorbis* larvae respond to biofilms. Certainly sugars are somehow involved in the *S. spirorbis* settlement response and larval lectins appear likely candidates for mediating this response.

In summary, *Spirorbis spirorbis* larvae do not require a metabolically active biofilm for settlement. The biofilm settlement cue may be a bacterially produced polysaccharide containing glucose or fructose or a structurally similar compound which is present either on the surface of the bacterial cell or in the extracellular polysaccharide matrix of the biofilm. The cue is not proteinaceous and does not appear to be a glycoprotein. The bacterially derived cue is thought to be commonly produced by a wide range of bacterial strains. More than one chemical cue may be involved in *S. spirorbis* larval perception of a suitable biofilm.

It is not clear whether larval lectins mediate the settlement of *Spirorbis spirorbis*. Recognition of certain sugar containing compounds does seem to be involved in larval perception of a biofilmed surface.

Table 1. Settlement responses of polychaete tubeworm (Serpulidae and Spirorbidae) larvae in response to biofilms and monospecific bacterial films subjected to various physical and chemical treatments. Bacterial film = monospecific bacterial film.

Species	Biofilm / bacterial film tested	Treatment	Response	Reference
Janua brasiliensis	Bacterial film	Formalin	No effect	Kirchman *et al.* (1982b)
	Bacterial film	Antibiotics	No effect	Kirchman *et al.* (1982b)
	Bacterial film	Sodium Periodate	Settlement reduced	Kirchman *et al.* (1982b)
	Bacterial film	Concanavalin A	Settlement reduced	Kirchman *et al.* (1982b)
	Bacterial film	Peanut lectin	No effect	Kirchman *et al.* (1982b)
	Bacterial film	Trypsin	Settlement reduced	Maki and Mitchell (1985)
Spirorbis spirorbis	Biofilm	Aerial drying for 1 hour at 20 °C.	Settlement inhibited	Hamer and Walker, (2000); this study
	Biofilm	Formalin	No effect	This study
	Biofilm	Antibiotics	No effect	This study
	Biofilm	Periodic acid	Settlement inhibited	This study
	Biofilm	Sodium periodate	Settlement reduced	This study
	Biofilm	Distilled water	No effect	This study
	Biofilm	Hypersaline seawater	No effect	This study
	Biofilm	Concanavalin A	No effect	This study
	Biofilm	Peanut lectin	No effect	This study
Spirorbis tridentatus	Biofilm	Aerial drying for 1 hour at 20 °C.	Settlement inhibited	Hamer and Walker, (2001); Chapter 3.2
	Biofilm	Formalin	No effect	Unpublished data
	Biofilm	Antibiotics	No effect	Unpublished data
	Biofilm	Distilled water	No effect	Unpublished data
Pomatoceros lamarckii	Biofilm	Aerial drying for 1 hour at 20 °C.	Settlement inhibited	Hamer *et al.* (2001); Chapter 3.1
	Biofilm	Formalin	No effect	Hamer *et al.* (2001); Chapter 3.1
	Biofilm	Antibiotics	No effect	Hamer *et al.* (2001); Chapter 3.1
	Biofilm	Hypersaline seawater	No effect	Hamer *et al.* (2001); Chapter 3.1
	Biofilm	Freeze-drying	Settlement inhibited	Hamer *et al.* (2001); Chapter 3.1
Hydroides elegans	Biofilm	Ultra-violet	Settlement reduced	Unabia and Hadfield (1999)
	Biofilm	Heat	Settlement reduced	Unabia and Hadfield (1999)
	Biofilm	Ethanol	Settlement reduced	Unabia and Hadfield (1999)
	Biofilm	Methanol	Settlement reduced	Unabia and Hadfield (1999)
	Biofilm	Formalin	Settlement reduced	Unabia and Hadfield (1999)
	Biofilm	Glutaraldehyde	Settlement reduced	Unabia and Hadfield (1999)
	Bacterial film	Formalin	Settlement reduced	Lau and Qian (2001)
	Bacterial film	Antibiotics	Settlement reduced	Lau and Qian (2001)

4. Intertidal epilithic biofilms, a primary settlement cue for *Pomatoceros lamarckii* larvae: laboratory and field evidence.

Abstract

This study investigates the intertidal recruitment and settlement of *Pomatoceros* spp. in response to various biofilm treatments during laboratory and field settlement experiments. Recruitment of *Pomatoceros* spp. in the intertidal zone of the Menai Strait corresponded closely with the distribution of adult organisms. Recruitment occurred throughout the study period (April – October 2000) with peaks during June and August. In the laboratory, *Pomatoceros lamarckii* larvae settled preferentially on biofilms developed in intertidal pools and avoided surfaces developed out of pools. The biofilms that developed on glass slides in and out of pools were significantly different; percentage settlement of *P. lamarckii* was positively correlated to the number of bacterial cells in the biofilms and negatively correlated to percentage cover of cyanobacteria. During field settlement experiments, *Pomatoceros* spp. settled in higher numbers on non-dried biofilms compared to recently dried biofilms. In simultaneous laboratory assays with *P. lamarckii* larvae and similar biofilm treatment, larvae settled almost exclusively on the non-dried biofilm treatment. In a second experiment, *Pomatoceros* spp. settled in higher numbers on biofilms developed outside the laboratory under shaded conditions compared to biofilms developed in full light conditions. In a simultaneous laboratory assay, *P. lamarckii* larval settlement followed the same pattern. *Pomatoceros* spp. settlement preferences were again positively related to the number of bacterial cells in the biofilms but negatively related to the number of diatoms and cyanobacterial cells in the biofilm. For an intertidal species to survive, it must not settle where conditions exceed its thermal or desiccation tolerances. The results of this study suggest that intertidal epilithic biofilms are a primary settlement cue for *P. lamarckii* larvae enabling them to settle in appropriate habitats in the intertidal. Larvae settle preferentially on biofilms indicative of the adult habitat (non-dried biofilm with high bacterial density) and avoid settling on biofilms that have been recently dried or that are dominated by microalgae (cyanobacteria and diatoms).

Introduction

The intertidal zonation of marine organisms is a well documented feature of rocky shores (Stephenson and Stephenson, 1949; Lewis, 1961). The mechanisms responsible for the maintenance of such patterns were classically regarded as a combination of both physical (desiccation and heat stress) and biological (primarily competition and predation) factors.

More recently it has become clear that larval behaviour and settlement preferences can result in non-random settlement and recruitment of sessile marine invertebrates and that this may be a major factor influencing recruitment patterns and hence community structure in intertidal communities (Strathmann *et al.*, 1981; Grosberg *et al.*, 1982; Keough and Downes, 1982; Keough, 1983; Underwood and Denley, 1984; Connell, 1985; Gaines and Roughgarden, 1985; Lewin, 1986; Grosberg, 1987; Young, 1987; Raimondi, 1988, 1990, 1991; Young and Gotelli, 1988; Chia, 1989; Underwood and Fairweather, 1989; Stoner, 1990; Sutherland, 1990; Menge, 1991; Miron *et al.*, 1995). For sessile intertidal organisms, the selection of a suitable habitat is a particularly crucial stage in an individual's life history; failure to settle in the correct intertidal zone could prove lethal during subsequent tidal emersion.

There is considerable laboratory evidence suggesting that biofilms provide an important settlement cue to a range of sessile marine invertebrate larvae (see Wieczorek and Todd, 1998). In nature, biofilms vary in composition, growth phase and metabolic activity in response to the conditions at a particular site (Baier, 1984) and so have the capacity to provide larvae with an integrated impression of environmental conditions, relating to factors such as water flow, shading and tidal elevation (Strathmann *et al.*, 1981; Neal and Yule, 1994). Laboratory studies have demonstrated that the larvae of certain marine invertebrates are capable of differentiating between biofilms of varying ages (Maki *et al.*, 1988, 1990, 1992; Pearce and Scheibling, 1991; Szewzyk *et al.*, 1992; Wieczorek *et al.*, 1995; Keough and Raimondi, 1995; Tsurumi and Fusetani, 1998), volume (Tsurumi and Fusetani, 1998), bacterial densities (Hadfield *et al.*, 1994; Hamer *et al.*, 2001), bacterial species composition (Kirchman *et al.*, 1982a; Unabia and Hadfield, 1999) and origins (Strathmann *et al.*, 1981; Keough and Raimondi, 1996) and between biofilms developed under different shear regimes (Neal and Yule, 1994) during the settling process. Such biofilm parameters may be used by marine invertebrate larvae in the selection of an appropriate settlement site (Neumann, 1979; Wieczorek *et al.*, 1995).

Although numerous studies have shown that larvae are capable of discriminating between biofilms under laboratory conditions, there are few field studies on the effect of biofilms upon marine invertebrate settlement. Despite the logistical and practical difficulties of conducting such field settlement experiments, a few studies have successfully investigated the ability of larvae to discriminate between biofilms in the field and have demonstrated that in some cases, biofilms may be an important cue to larval settlement under field conditions (Strathmann *et al.*, 1981; Todd and Keough, 1994; Keough and Raimondi, 1995; Keough and Raimondi, 1996; Wieczorek and Todd, 1998; Olivier *et al.*, 2000). Todd and Keough (1994) studied the larval settlement response of a range of taxa from hard substratum, subtidal epifaunal assemblages to biofilmed surfaces in the field. They reported that the initial biofilm that develops on a surface in the field acted as a positive cue for the settlement of some species, while other species were unaffected. Following on from this study, Keough and Raimondi (1995, 1996) showed that in the field recruitment rates of larvae varied significantly in response to biofilm age for 5 of the 13 taxa studied and in response to biofilms developed over greater temporal and spatial scales (Keough and Raimondi, 1996). Although these studies have shown that biofilms are important to the settlement of various marine invertebrate larvae in the field, simultaneous laboratory and field studies have shown that considerable care should be taken when extrapolating the results of laboratory experiments to the field situation (Todd and Keough, 1994; O'Connor and Richardson, 1996; Wiekzorek and Todd, 1998). For example, Thompson *et al.* (1998) conducted both laboratory and field experiments on the settlement of *Semibalanus balanoides* cyprids and found that the results of laboratory studies were not consistent with those of field experiments. In the laboratory, cyprids preferentially selected rock chips taken from the mid-shore, the natural zone of this species, whereas in the field, cyprids settled primarily in response to conspecifics.

In the intertidal zone, an ability to discriminate between surfaces located at different tidal heights has obvious adaptive value in terms of post-settlement survival for sessile species and it has been suggested that biofilms enable the larvae of some species to discriminate between surfaces from different tidal heights. Strathmann and Branscomb (1979) reported that cyprids of *Balanus* (= *Semibalanus*) *cariosus* avoided sites in the upper intertidal which dry out at low tide. Subsequently, Strathmann *et al.* (1981) showed that cyprids settled preferentially on plates with biota characteristics of the lower intertidal shore and concluded that some component of the microflora provides the settlement cue. Raimondi (1988) also studied the factors responsible for the intertidal zonation of barnacles and demonstrated that cyprids were attracted to surfaces coated with extracts made from other species typical of the tidal height where the adult barnacle population was most abundant. Olivier *et al.* (2000) found that the presence of a biofilm was the main factor affecting the intertidal recruitment of *Balanus amphitrite*, with settlers exhibiting a strong preference for surfaces with low bacterial density.

Previous laboratory studies have shown that *Pomatoceros lamarckii* larvae require a biofilm for settlement (Roscoe, 1993) and settle in higher numbers on older biofilms (Chan and Walker, 1998; Hamer *et al.*, 2001) but that aerial drying of a biofilm during simulated tidal emersion negates the previous settlement inducing effect of a biofilm (Hamer *et al.*, 2001; Chapter 3.1). It is possible that avoidance of recently dried biofilms may enable *P. lamarckii* larvae to avoid settling above their appropriate intertidal zone. This study investigates: 1) intertidal recruitment of *Pomatoceros* spp.; 2) whether *P. lamarckii* larvae settle in higher numbers on biofilms developed in the adult habitat under laboratory conditions and 3) the ability of *Pomatoceros spp. and P. lamarckii* larvae to discriminate between various biofilms during simultaneous laboratory assays and field settlement experiments.

Materials and Methods

Larval culture

Collections of adult *Pomatoceros lamarckii* (Quatrafages) attached to small rocks were made during the spring and summer of 2000 and 2001 from the lower intertidal zone of the Menai Strait, Anglesey, North Wales, U.K.. Larval culture followed the methods of Hamer *et al.* (2001). Briefly, male and female worms were induced to release their gametes by removing them from their tubes. Eggs were fertilised with a dilute sperm suspension. Larvae were cultured in 2 l Pyrex glass beakers in 0.2 µm filtered, u.v.-irradiated seawater and fed on a 1:1 mixture of the flagellates *Rhinomonas reticulata* and *Tetraselmis chui* at approximately 200 cells μl^{-1}. All larval culture and laboratory settlement assays were carried out in a constant environment cabinet at 19°C with a 12:12h light:dark cycle. All glassware was routinely cleaned in 5 % Chloros (sodium hypochlorite), rinsed thoroughly in hot tap water followed by distilled water, before drying at 60°C in a drying cabinet.

Experimental surfaces

Slate was used as the settlement surface in all laboratory assays and field settlement experiments. Slate was chosen because *Pomatoceros lamarckii* larvae settle readily on slate surfaces that have been biofilmed (Roscoe, 1993; Chan and Walker 1998; Hamer *et al.*, 2001) and because slate is a natural substratum that is common in the intertidal zone of the study area. Slates were cut to size by Inigo Jones & Co. Ltd., North Wales and polished uniformly with 600 grade wet and dry emery paper before use. All field settlement experiments were conducted with 120 x 75 x 10 mm slates (hereafter referred to as 'slate tiles'). All laboratory assays were conducted with 25 x 25 x 4 mm slates (hereafter referred to as 'assay slates'). All biofilm observations were made on biofilms developed on glass microscope slides (38 x 12 x 1 mm). Glass slides were cleaned (warming for 30 minutes in chromic acid followed by thorough rinsing in tap water followed by distilled water) before use.

Study site

All field work was carried out in the intertidal zone of Ynys Gorad Goch, a small island in the middle of the Menai Strait, North Wales, U.K. (Figure 1). Recruitment observations and experimental work were carried out at high, mid and low shore sites, corresponding to approximately 1, 3 and 5 m respectively above chart datum. Large pools (roughly 3 x 2 x 0.3 – 0.5 m deep) are present at each of the tidal heights and were used during recruitment observations and settlement experiments (see Figure 1 and 3c).

General laboratory settlement assay procedures

Laboratory settlement assay procedures were based upon the methods described in Hamer *et al.* (2001). All settlement experiments were conducted as multi-treatment settlement assays with treatment and control assay slate surfaces placed together randomly in circular Pyrex glass dishes (250 ml capacity) filled with 100 ml of 0.2 μm filtered, u.v.-irradiated seawater (FSW). At the start of an assay, equal aliquots of metatrochophore stage *Pomatoceros lamarckii* larvae were added to replicate assay dishes. Using this method, it was possible to keep the numbers of larvae reasonably constant (± 10 %) between replicate dishes for an experimental assay. The mean number of larvae per dish ranged from 50 to 300 between assays. The number of settled individuals and remaining free-swimming larvae were counted after 24 hours.

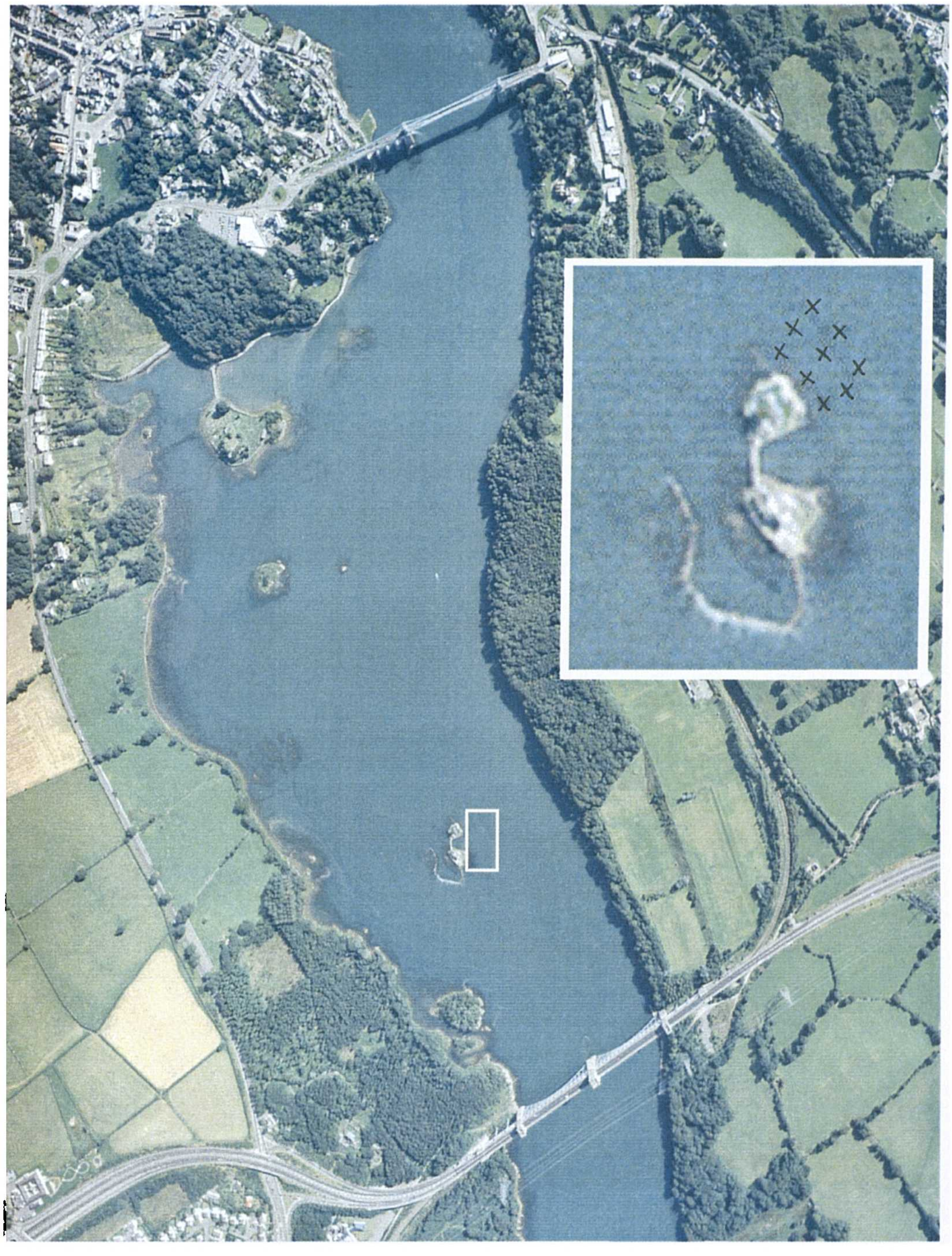


Figure 1. Aerial photograph showing 'swellies' area of Menai Strait, North Wales and Gorad Goch island. The location of the study site for recruitment experiment is shown by the white box. Crosses in the inset indicate the approximate location of intertidal pools used during settlement studies. Scale bar = 0.2 km.

Biofilm composition

Glass slides were fixed in 2.5 % glutaraldehyde in FSW and stored in the dark in preparation for diatom and bacterial counts using scanning electron microcopy (SEM) and epi-fluorescence microscopy respectively. For SEM, slides were rinsed quickly in distilled water, dehydrated in an ethanol series (30, 50, 70, 90, 100 % ethanol for 20 min each), air dried slowly in partially covered Petri-dishes, sputter coated with gold and observed in a Cambridge S120 scanning electron microscope operated at 10 KeV. Diatom counts were made for 10 random fields per replicate slide (n = 3). For epi-fluorescence microscopy, slides were stained by immersion in DAPI (200μg ml^{-1}) for 5 mins in the dark and observed under blue excitation light (385 nm). Bacterial counts were made for 10 random fields (at x 400) per replicate slide (n = 3). Cyanobacterial cells were counted in 10 random fields using epi-fluorescence microscopy (intertidal biofilms) and SEM (laboratory developed biofilms) (n=3); cyanobacteria were quantified by direct counts with SEM or by estimating percentage cover (16 random points) with epi-fluorescence microscopy.

Intertidal recruitment

Large slate slabs (approximately 45 x 45 x 10 cm, with two flat surfaces) were placed in the intertidal zone of Gorad Goch island between April and October 2000. Three slabs were placed at high, mid and low shore sites both in and out of pools. Every two weeks, recruitment of *Pomatoceros* spp. was counted within a central 15 x 15 cm square (225 cm^2) on the upper and lower surfaces of each slab. Photographs of the surfaces were taken regularly with a Ricoh 500 digital camera.

Laboratory settlement assay with intertidal biofilms

Laboratory settlement assays were carried out to investigate the settlement response of *Pomatoceros lamarckii* larvae to biofilms developed at different shore heights (high, mid and low), in and out of pools (Figure 3c). The hypothesis under test was that larvae would settle preferentially on biofilms developed in the adult habitat. Observations on the composition of biofilms that developed at each of the sites were made with SEM and epi-fluorescence microscopy.

Laboratory settlement assay with intertidal biofilms - biofilm development

Assay slates and glass slides were biofilmed for three months between April and June 1999 at different tidal heights (high, mid and low) in the Menai Strait and allowed to develop natural biofilms. At each site (high, mid and low shore; in and out of rockpools), ten assay slates and three glass slides were attached, experimental surface facing upwards, to plywood panels (45 x 45 x 1 cm) (Figure 3c). In the field, the plywood panels were then secured, experimental surface facing upwards, to concrete paving slabs (45 x 45 x 5 cm) using plastic cable ties. After the three month deployment period, slates were collected and returned to the laboratory for the settlement assay. Any assay slates or glass slides with natural recruits were discarded. Glass slides were also returned to the laboratory and fixed for biofilm analysis using SEM and epi-fluorescence microscopy.

Laboratory settlement assay with intertidal biofilms - laboratory assays

Multi-choice laboratory assays were carried out in June 2000 to compare the *Pomatoceros lamarckii* larval settlement response to the biofilms from different tidal heights in the intertidal zone. Biofilmed assay slates from each tidal height both in and out of rockpools (six biofilm treatments) were placed in nine replicate circular assay dishes (250 ml capacity) and an aliquot larvae added. Settlement was counted after 24 hours.

Simultaneous laboratory and field settlement experiments

Simultaneous laboratory assays and field settlement experiments were carried out to compare the settlement response of *Pomatoceros lamarckii* (laboratory) and *Pomatoceros* spp. (field) larvae to laboratory-developed biofilms. The biofilm treatments were designed to simulate intertidal biofilms and to test the hypotheses that under both laboratory and field conditions, larvae of *Pomatoceros* spp. discriminate between biofilms that have / have not been subjected to aerial drying during simulated tidal emersion or biofilms with different compositions as a result of variable light regime. Laboratory biofilms were used as opposed to natural intertidal films to avoid confounding effects of uncontrollable factors (e.g. conspecific adults, conspecific recruits, other recruits and grazing effects,).

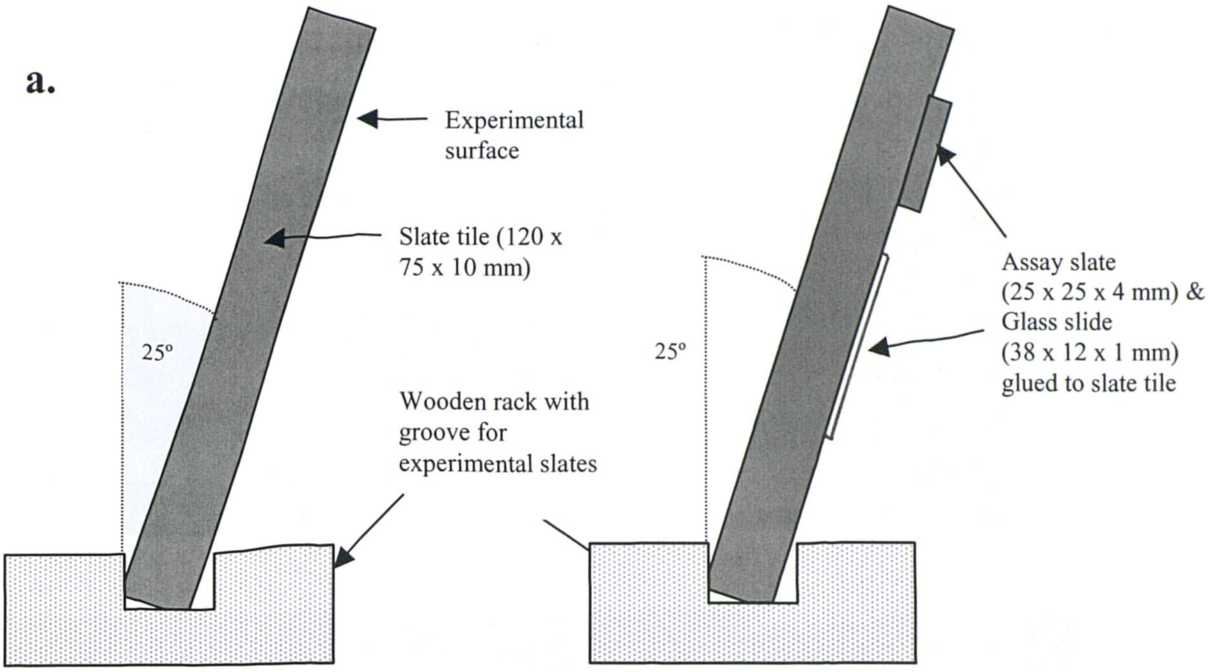
Simultaneous laboratory and field settlement experiment 1 - biofilm development

Slate tiles were used as test substrata for the field settlement experiment, assay slates that had been treated in the same way were used to conduct simultaneous laboratory settlement assays and glass slides were used for subsequent biofilm analysis. Biofilms were developed on the slate tiles, assay slates and glass slides in tanks outside the laboratory (Figure 2a-c). The tanks were supplied with seawater from the laboratory running seawater system at a rate of 2 l / min. In each of the three treatment tanks (55 x 40 x 22 cm), slate tiles were held in grooved wooden racks at an angle of 25° from vertical with the experimental surface as the overhanging surface; this approach was used to minimise sediment accumulation. Laboratory assay slates and glass slides were attached to duplicate slate tiles with a small blob of aquarium silicone sealant and were allowed to develop biofilms alongside the experimental slate tiles. Experimental surfaces were allowed to develop a biofilm for one month before the start of an experiment. During this time, biofilms were either a) developed continuously submerged, b) submerged, but dried under ambient conditions outside the laboratory for 3-4 hours daily in order to simulate tidal emersion, c) developed submerged and allowed to dry only once for 1 hour immediately prior to a settlement experiment. The control was a clean, non-biofilmed slate tile. After the one month period experimental surfaces were then used for: 1) the field settlement experiment (slate tiles), 2) the simultaneous laboratory settlement assays with *Pomatoeros lamarckii* larvae (assay slates) or 3) fixed in 2.5 % glutaraldehyde in FSW for subsequent biofilm analysis (glass slides) using microscopes.

Simultaneous laboratory and field settlement experiment 1 - field settlement experiment

The variously treated slate tiles were deployed for 7 days in three large pools at mid-tide level of the Menai Strait. Six experimental runs were carried out, one every other week from May - July 2000 (May 11th - 18th; May 25th - June 1st; June 8th -15th; June 21st - 28th; July 6th - July 13th and July 21st - 28th). Each experimental run consisted of attaching the treated and control slate tiles to three plywood panels, with three replicates of each treatment located in the same random order on each panel (n = 9) (Figure 3a & b). The panels were then attached (experimental surface facing downwards) to paving slabs using plastic cable ties and deployed in each of the three pools. There was a 1 cm gap between the experimental surfaces and the underlying concrete paving slabs which was open to water flow at opposite ends of the panel. These end gaps were covered with plastic mesh (Nytex mesh, 6 mm hole diameter supplied by Netlon) to prevent grazing of the surface biofilm and possible removal of newly settled juveniles (Figure 3b). After 7 days deployment, panels were returned to the laboratory and examined under a dissecting microscope for settled juveniles. Because of difficulties in identifying settled individuals to species level, settlers were identified as far as possible with the majority of settlers being either *Pomatoceros* spp., *Spirorbis* spp. or barnacle juveniles. At the time of the initial deployment of the experimental panels at the start of each experimental run, glass slides were removed from the tanks, returned to the laboratory and fixed for biofilm analysis using SEM and epi-fluorescence microscopy. Biofilm analysis therefore represents biofilm composition at the start of each experimental run.

a.

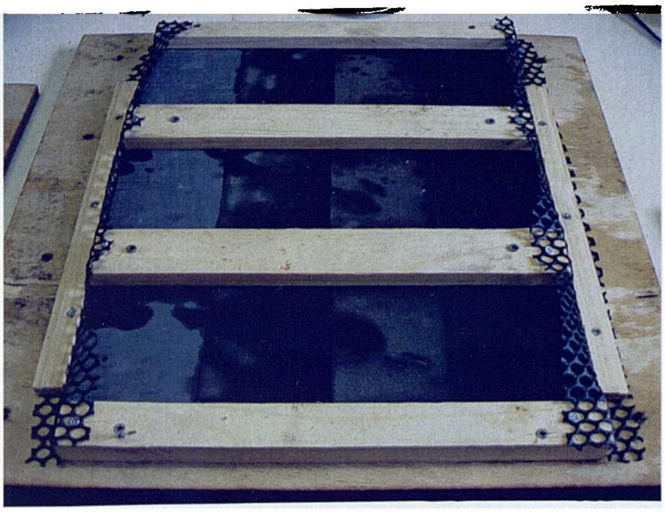


b.

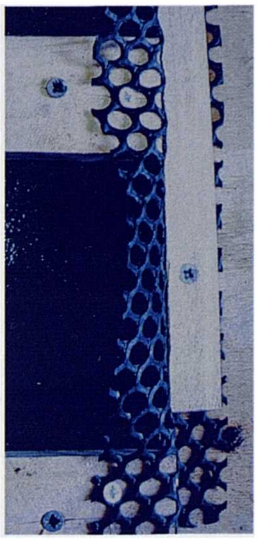
c.

Figure 2 a-c. **a)** Diagrams showing the procedure used for the biofilming of experimental surfaces slate tiles and assay slates and glass microscope slides. **b)** Tanks with running seawater used for biofilming of slates outside laboratory; two tanks were fed constantly with running seawater, the other (middle tank) was drained for 3-4 hours daily **c)** slates held in grooved wooden racks within tank. Seawater (gravity fed from the laboratory system at 2 l / min) was fed into the bottom of the tank at one end and drained from the other end.

a.



b.



c.

Figure 3 a-c. a) Plywood panel with experimental slate tiles used during field settlement experiments. **b)** detail of plastic mesh (Netlon) used on sides of panels to prevent entry by macrograzers but allow water flow over experimental tiles. **c)** one of the large mid-shore rock pools on Gorad Goch island, Menai Strait with experimental panels deployed in and out of intertidal pool.

Simultaneous laboratory and field settlement experiment 1 - laboratory assay

Within four hours of the slate tiles being deployed for the field experiment, laboratory settlement assays were initiated to compare the settlement response of *Pomatoceros lamarckii* larvae to the various biofilm treatments under still-water laboratory conditions. Laboratory assays were conducted on four occasions corresponding to the second, third, fifth and sixth field settlement experimental run (insufficient numbers of competent larvae were available for the other experimental runs). Biofilmed assay slates and control assay slates were placed in nine replicate assay dishes and larvae added. Settlement was counted after 24 hours.

Simultaneous laboratory and field settlement experiment 2

A second field experiment was conducted during 2001, the aim of which was to investigate the settlement response of *Pomatoceros* spp. in the laboratory and the field in response to biofilms developed under different light regimes, i.e. biofilms characteristic of upper (top) and under surfaces of intertidal substrata. As with the previous experiment, slate tiles were biofilmed in tanks outside the laboratory before deployment in the field (Figure 2 b-c). Three biofilm treatments were tested, by exposing experimental surfaces to different light regimes. One tank was completely covered with a wooden lid, the second tank was partially shaded by covering it with Netlon and the third tank was left uncovered. Experimental tiles were biofilmed in this manner from May to June before being deployed for a single experimental run that lasted 7 days. Slate tiles were attached to three replicate plywood panels (see Figure 3a) with three of each treatment plus control slate tiles per panel (12 tiles per panel; $n = 9$). Control surfaces were clean, non-biofilmed slate tiles. After 7 days tiles were retrieved, returned to the laboratory and screened for settled individuals.

Statistical analysis

Count data were transformed with square root transformation ($\sqrt{(n+1)}$) and checked for normality and significant heterogeneity of variances. Data were analysed by t-test or ANOVA, followed by Tukey's test. Percentage data from the laboratory settlement assays were arc-sine transformed ($x' = \text{arcsine} \sqrt{x / 100}$) and checked for normality and significant heterogeneity of variances before analysis by ANOVA followed by Tukey's test. Due to significant heterogeneity of variances in some of the data, the non-parametric Kruskal-Wallis test followed by Dunns multiple comparisons test were carried out. Data from the field experiment was analysed with a balanced ANOVA with treatment, date and pool as factors. Date was treated as a fixed factor and pools as a random factor.

Results

Intertidal Recruitment

Recruitment of *Pomatoceros* spp. on slate slabs occurred from April to October with peaks occurring in June and August (Figure 4). Almost all recruitment of *Pomatoceros* spp. was restricted to the under surface of the slate slabs (Figures 4-6). At each tidal level, recruitment was greatest on surfaces that were permanently submerged in pools compared to those out of pools (Figures 4 & 6). No recruitment occurred on the surfaces out of pools at either the high or mid shore level but a significant number of *Pomatoceros* spp. individuals did recruit onto the under surfaces of slabs placed at the low shore. Highest recruitment occurred on the undersides of surfaces placed in pools at the mid-shore level. Recruitment of other species on the slate slabs was limited primarily to barnacles and spirorbids (Figure 5). Most barnacles that recruited were *Semibalanus balanoides* but some recruitment of *Elminius modestus* did also occur. The spirorbids were primarily *Spirorbis tridentatus* but *Spirorbis spirorbis* was also common.

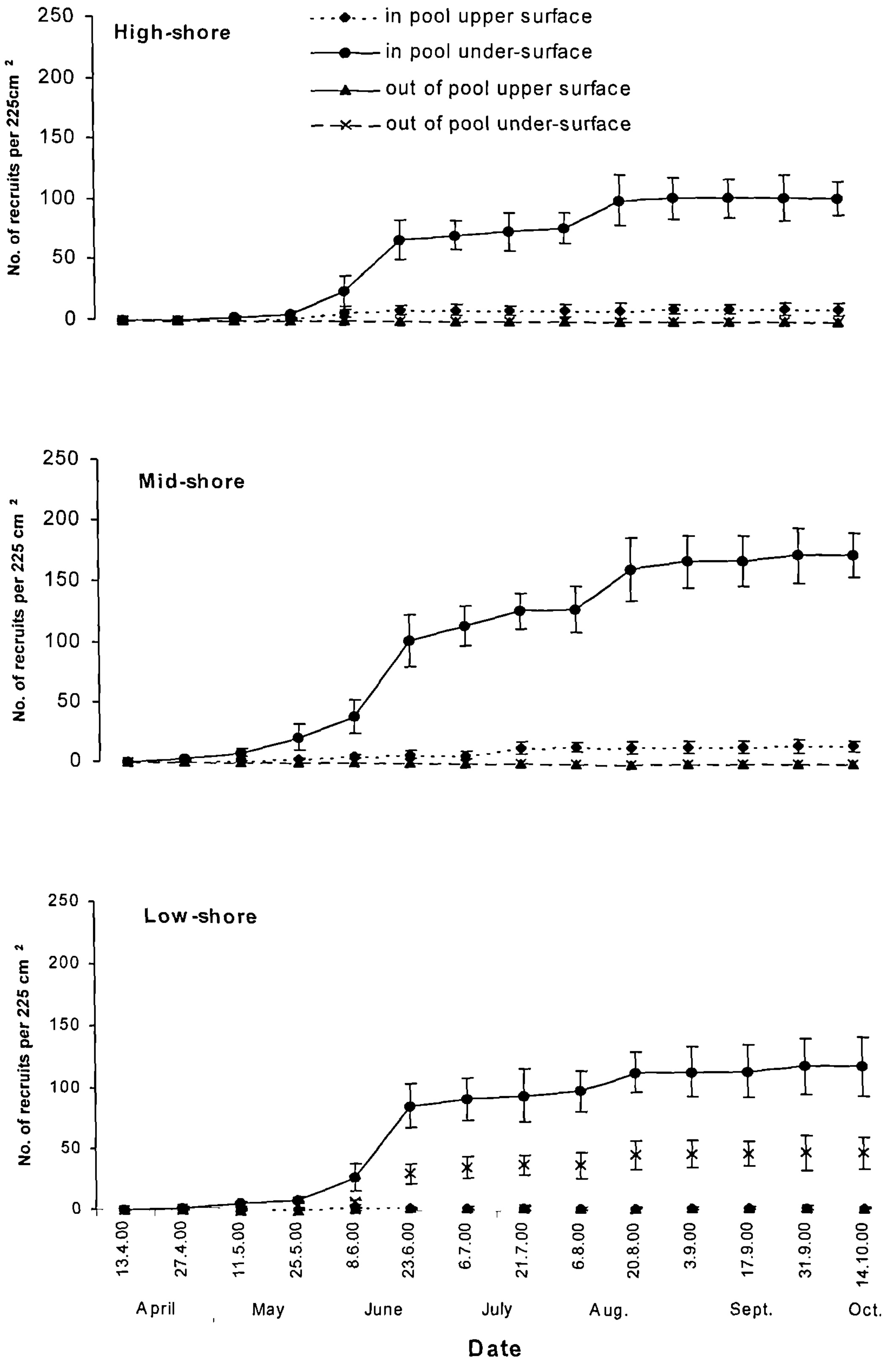


Figure 4. Mean (± S.E.) (n=3) cumulative recruitment of *Pomatoceros* spp. on upper and under-surfaces of slate slabs deployed in and out of pools at high, mid and low shore levels of Gorad Goch island, Menai Strait, between April and October 2000. Recruitment within a central area (225 cm^2) was counted every 2 weeks.

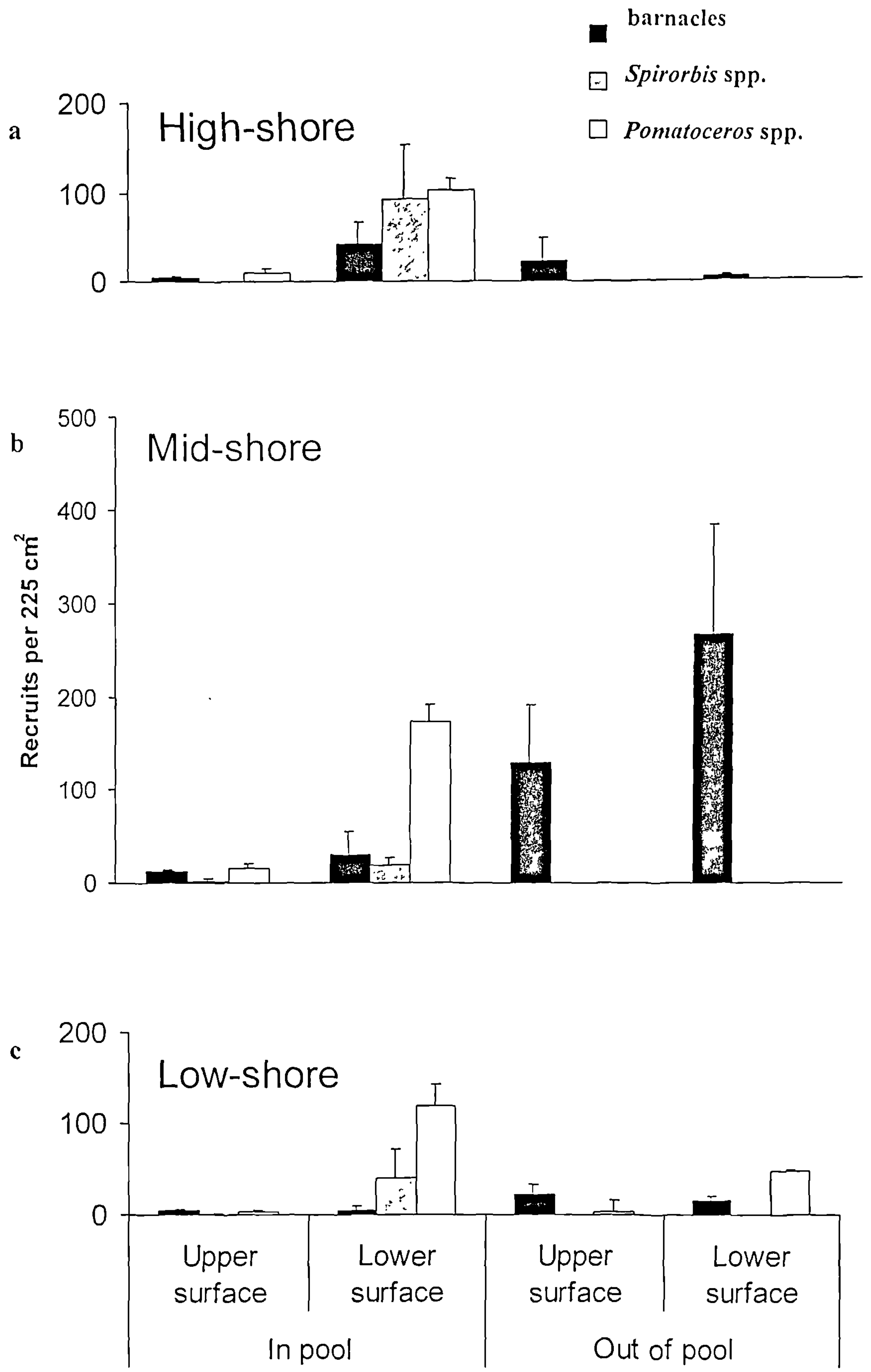


Figure 5a-c. Total recruitment of *Pomatoceros* spp. (□), *Spirorbis* spp. ( ) and barnacles (■) onto upper and under surfaces of slate slabs deployed at high (a), mid (b) and low (c) shore, in and out of rock pools of Gorad Goch island, Menai Strait from April to October 2000. Data plotted are means (+ S.E.) (n=3).

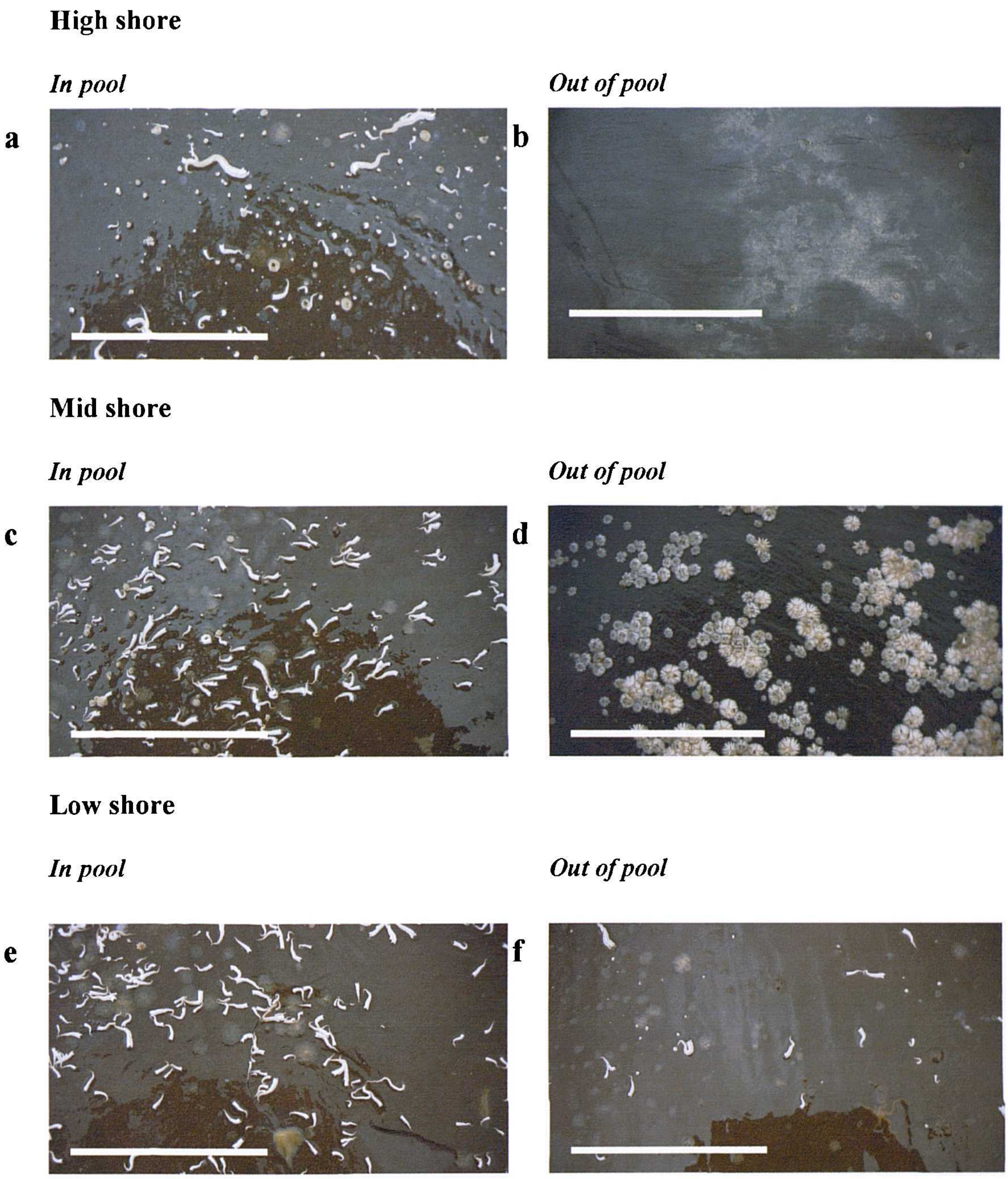


Figure 6 a – f. Digital photographs showing recruitment of *Pomatoceros* spp. and other species onto under surfaces of slate slabs placed in and out of rock pools at high **(a & b)**, mid **(c & d)** and low **(e & f)** shore on Gorad Goch Island, Menai Strait. Photographs were taken on August 4th 2000 after 16 weeks deployment. Scale bar = 10 cm.

Laboratory settlement assays with intertidal biofilms - biofilm composition

Biofilms were composed of a mixture of diatoms, cyanobacterial colonies and bacteria (Figures 7a-d & 8a-f). Total diatom, bacterial and cyanobacterial densities varied significantly between the various biofilms (one-way ANOVA; $p<0.05$; Figure 7 a-d & 8 a-f). Bacterial and diatom densities were significantly greater in biofilms developed in pools at each shore level compared to biofilms developed out of pools at the same shore level, whereas percentage cover of cyanobacteria showed the opposite pattern.

For the biofilms developed at the different shore levels, bacterial densities were significantly greater at mid-shore level compared to high-shore. Diatom densities increased down the shore. The most obvious difference between biofilms developed in and out of rock pools, particularly at the mid and high shore level was the high numbers of chain forming cyanobacterial colonies in biofilms developed out of pools (Figure 7a & 8d). Diatom species diversity was greater in the biofilms developed in rock pools; the most abundant type of diatom in biofilms developed in pools were relatively small and thin pennate forms (15-20 μm) (Figure 8c), whereas those that dominated the biofilms developed out of pools were slightly larger (~20 μm), and curved (Figure 8d).

Laboratory settlement assays with intertidal biofilms - settlement assay

In laboratory assays, settlement of *Pomatoceros lamarckii* larvae was significantly different between biofilms developed at different intertidal locations (one-way ANOVA; 5 d.f.; $F = 18.58$; $p<0.001$) (Figure 7d). Larvae settled exclusively on biofilms developed in pools. Settlement was significantly higher on biofilms from mid shore pools compared to those from high and low shore pools which were not significantly different from each other (at $p=0.05$).

No clear correlation between *P. lamarckii* larval settlement and biofilm composition was apparent (Figure 7 a-d). Percentage settlement of larvae followed the same general pattern as bacterial density and the opposite pattern to that of cyanobacterial percentage cover.

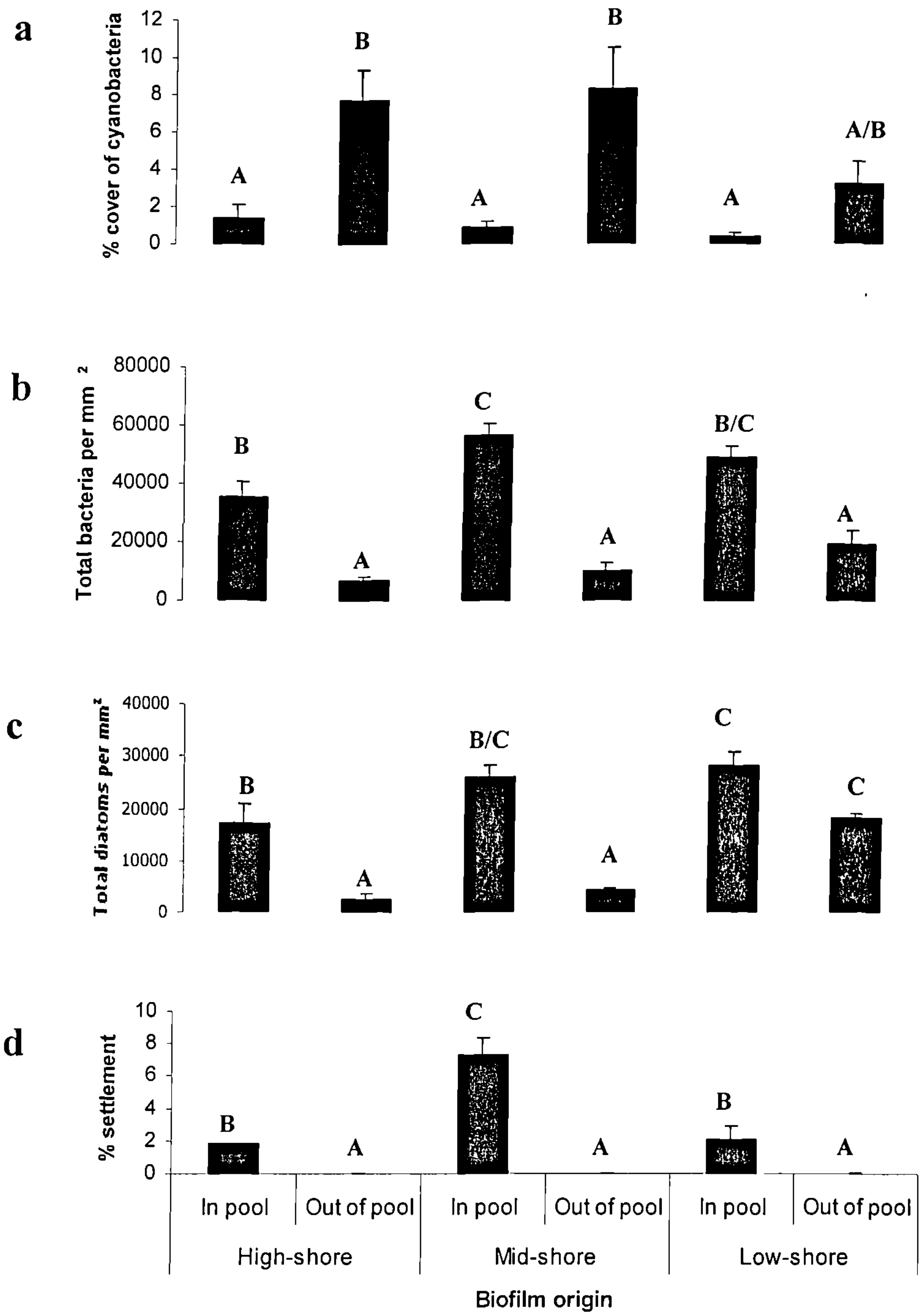


Figure 7 a-d. **a)** % cover of cyanobacteria in biofilms developed on glass slides alongside biofilmed assay slates. **b)** Total bacteria in biofilms developed on glass slides alongside biofilmed assay slates. **c)** Total diatoms in biofilms developed on glass slides alongside biofilmed assay slates. Data plotted for diatom, bacteria and cyanobacterial cell counts are means (+S.E.) (n=3). **d)** Mean percentage settlement of *Pomatoceros lamarckii* larvae on biofilmed slates after a 24 hour laboratory settlement assay. Biofilms were developed for three months in the intertidal zone at high, mid and low shore levels both in and out of rock pools between April - June 1999. Data plotted are means (+ S.E.) (n = 9). Results of Tukey's test are given; bars with the same letter are not significantly different (at $p<0.05$).

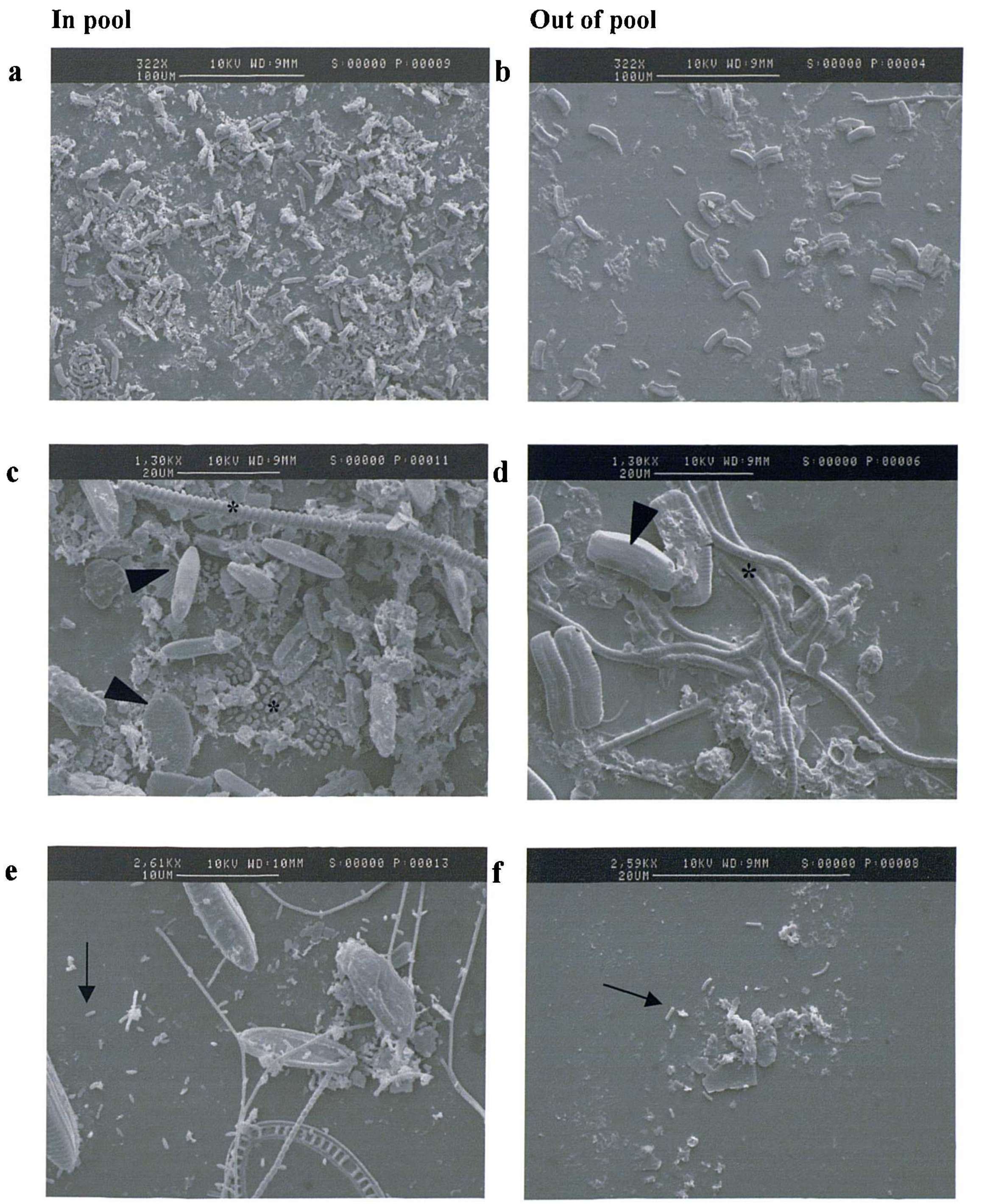


Figure 8 a-f SEM micrographs showing biofilms developed on glass slides deployed in and out of pools at mid shore level of the intertidal zone of Gorad Goch island, Menai Strait between April – June 1999. **a & b)** biofilms shown at lower magnification. **c & d)** biofilms at medium magnification, note diatoms (arrowhead) and cyanobacteria (*). **e & f)** biofilms at higher magnification showing surface colonising bacteria (arrows).

Simultaneous laboratory assays and field settlement experiment 1 - field settlement experiment.

Pomatoceros spp. settled on experimental tiles during all experimental runs apart from the fifth run (Figure 9) when no *Pomatoceros* spp. settlement occurred on any of the experimental tiles. Total settlement of *Pomatoceros* spp. varied significantly (Table 1) between experimental runs during the study period and followed the recruitment patterns observed on the experimental slate slabs (Figure 4). Settlement intensity was greatest, by an order of magnitude, during the third experimental run (starting May 11th). This peak in settlement occurred three weeks before the peak in recruitment was recorded on the slate slabs that were monitored throughout the settlement season. The maximum number of settlers recorded on the biofilm treatment during this time was approximately four times greater than the number of recruits recorded for an equivalent area on the under surfaces of the slate slabs at this time. Settlement of *Pomatoceros* spp. varied significantly (two-factor ANOVA; $p<0.001$) between the different biofilm treatments and between the experimental runs (Table 1). There were also significant interactions between Treatment and Time and Pool and Time.

Table 1. Results of two-factor ANOVA for field settlement results for *Pomatoceros* spp. with the factor pool as the random factor. n.s. = not significant.

Factor	DF	SS	MS	F	P
Treatment (Tr)	3	32.9733	10.9911	129.97	0.001
Pool (P)	2	2.5463	1.2731	0.82	n.s.
Time (T)	5	406.1717	81.2343	49.98	0.001
Tr x P	6	0.5074	0.0846	0.54	n.s.
Tr x T	15	39.1694	2.6113	16.53	0.001
P x T	10	16.2532	1.6253	10.29	0.001
Tr x P x T	30	4.7389	0.1580	0.87	n.s.
Error	144	26.0372	0.1808		
Total	215	528.3975			

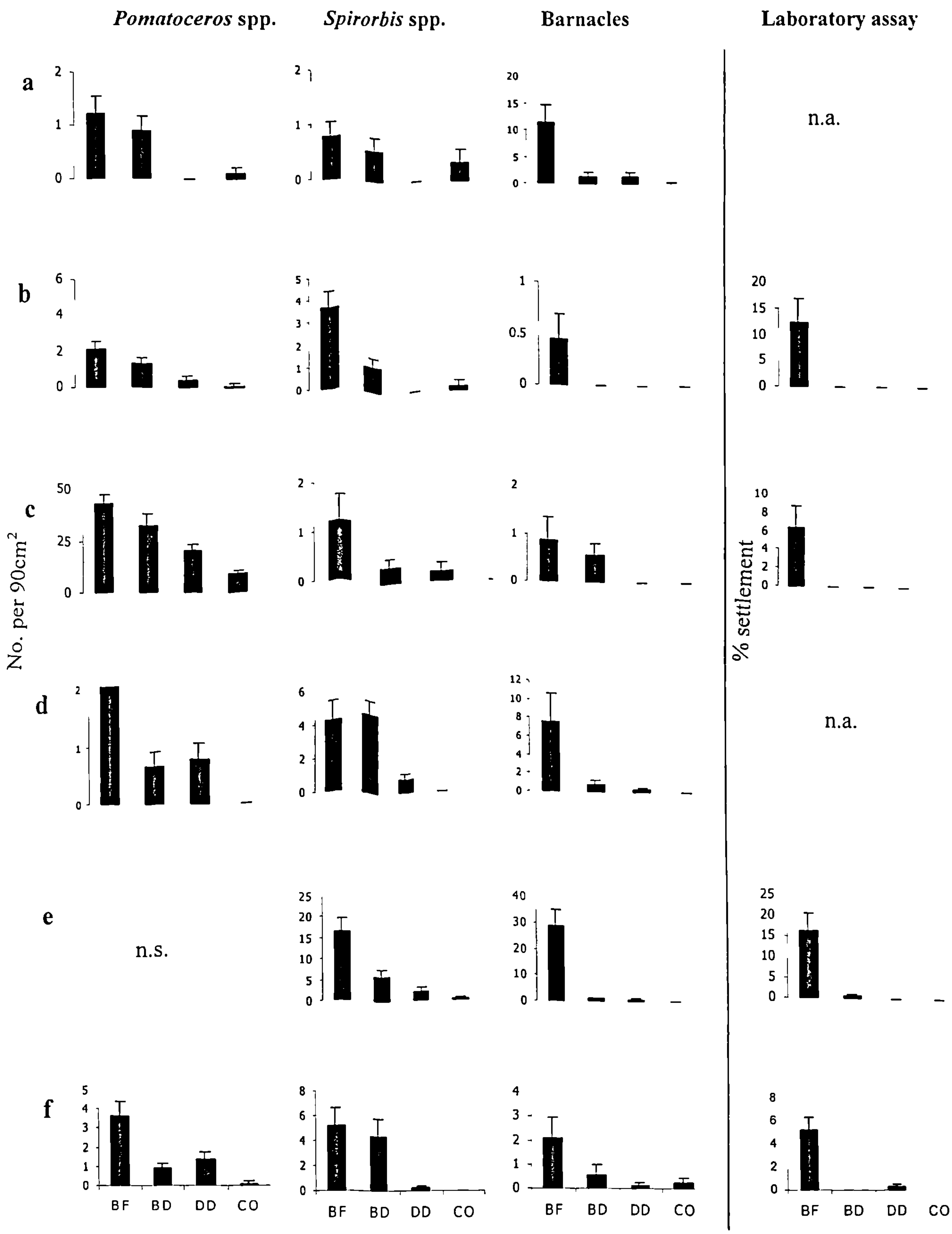


Figure 9 a-f. Histograms showing settlement of *Pomatoceros* spp., *Spirorbis* spp. and barnacles on variously treated experimental tiles (BF = biofilm; BD = biofilm dried once immediately prior to deployment; DD = biofilm dried daily; CO = clean control slate with no biofilm at deployment) after being deployed for seven days in pools at mid shore level of Gorad Goch island, Menai Strait. Dates of deployment for the six experimental runs between May and July, 2000 were: a) May 11th, b) May 25th, c) June 8th, d) June 21st, e) July 6th, f) July 21st. Bars represent mean number (+S.E.) (n=9) of settlers per $90cm^2$ for the field settlement experiment. Percentage settlement of *Pomatoceros lamarkii* during simultaneous laboratory settlement assays are shown on the right hand side of the figure. Bars in these histograms represent mean percentage settlement (+S.E.) (n=9) of *Pomatoceros lamarkii* larvae. n.s. = no settlement. n.a. = no data available.

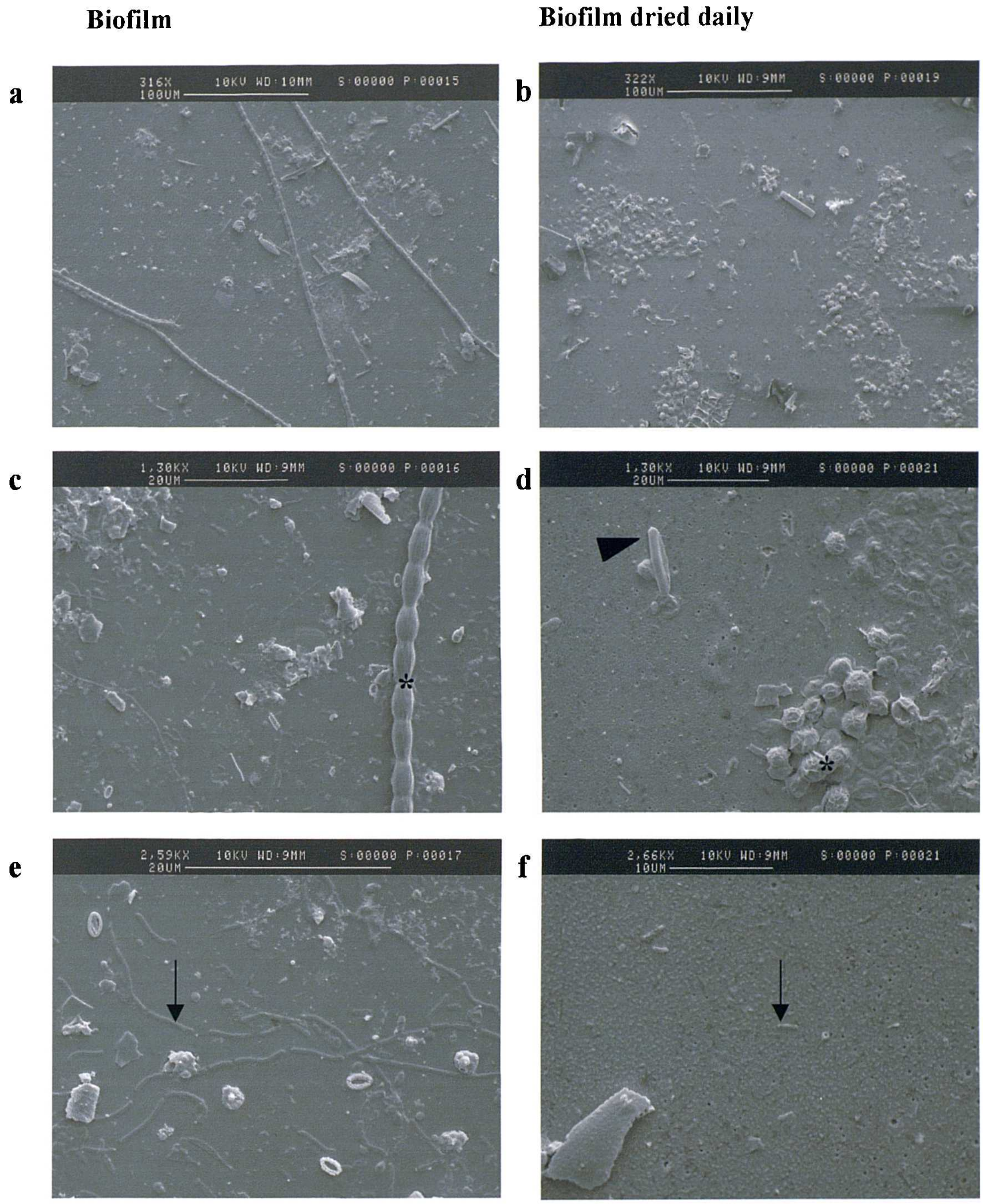


Figure10 a-f. SEM micrographs showing biofilms developed on glass slides in tanks outside the laboratory for one month. **a & b)** biofilms shown at lower magnification. **c & d)** biofilms at medium magnification, note diatoms (arrowhead) and cyanobacteria (*). **e & f)** biofilm at higher maginification showimg surface colonising bacteria (arrows).

Settlement of *Pomatoceros* spp. was always greatest on the non-dried biofilm treatment for all six experimental runs but some settlement did occur on all of the experimental surfaces, including the non-biofilmed clean control. Overall, *Pomatoceros* spp. settlement occurred in the order of preference: biofilm > biofilm dried once > biofilm dried daily > clean control slate. In addition to *Pomatoceros* spp., *Spirorbis* spp. and barnacles settled in considerable numbers on the slate tiles during all of the experimental runs. As with *Pomatoceros* spp., settlement of *Spirorbis* spp. and barnacles varied between the experimental runs with maximum settlement of both taxa occurring between July 6^{th} and July 13^{th} (Figure 9e). Both species showed broadly a similar settlement preference pattern to *Pomatoceros* spp. although the preference for a non-dried biofilm was most pronounced for barnacles.

Simultaneous laboratory assays and field settlement experiment 1 - laboratory assay

In the simultaneous laboratory assays, *Pomatoceros lamarckii* larval settlement was significantly different (Kruskal-Wallis, d.f. = 3; $p<0.001$) between the various treatments for all four laboratory assays with *P. lamarckii* larvae settling almost exclusively on the non-dried biofilm treatment. A small number of larvae settled on the dried biofilm treatments, but no larvae settled on the clean control slate.

Simultaneous laboratory assays and field settlement experiment 1 - biofilm composition

Biofilms were developed outside the laboratory for one month and either dried daily for 3 – 4 hours, dried once immediately prior to deployment or not dried before deployment in the field. Biofilm composition was studied for all experimental runs for the non-dried biofilms and dried daily biofilm treatments. Surprisingly, both treatments had a considerable amount of free space with relatively low densities of diatoms and bacteria compared to biofilms developed for three months in the intertidal zone (Figure 10a-f; Table 2). At the time of deployment, biofilms from both treatments were composed of diatoms, cyanobacteria and bacteria but varied in terms of numbers (diatoms, cyanobacteria and bacteria) and species diversity (diatoms). Biofilms dried daily had significantly fewer bacteria per mm^2 compared to non-dried biofilms (one-way t-test; $p<0.05$) whereas there were significantly greater numbers of cyanobacteria per mm^2 on the dried biofilm compared to the non-dried biofilms (one-way t-test; $p<0.05$); these differences were consistent for all experimental runs although total numbers varied between experimental runs. Total number of diatoms was significantly greater on the non-dried biofilm but the difference was less pronounced compared to total numbers of bacteria and cyanobacteria. There was no obvious difference in diatom species diversity between the two treatments. Cyanobacterial colonies were common on the dried daily biofilm treatment but were of a type different from those that were abundant within the biofilms developed out of pools in the intertidal zone. Cyanobacterial colonies present in the biofilms that were dried daily were primarily cluster-type colonies (Figure 10d), whereas those from the intertidal were predominantly chain-forming colonies (Figures 8d).

Table 2. Mean (± S.E.) numbers of diatom, cyanobacterial and bacterial cells recorded per mm^2 (10 replicate counts per glass slide; n = 3) for 1 month old biofilms (non-dried and dried daily biofilm treatments) developed outside the laboratory alongside the biofilmed slate tiles and assay slates. Biofilms were either kept continually immersed (biofilm non-dried) or were dried for 3-4 hours daily (biofilm dried daily) to simulate tidal emersion.

	Non-dried biofilm			**Biofilm dried daily**		
Exp. run (start date)	Diatoms	Cyanobacteria	Bacteria	Diatoms	Cyanobacteria	Bacteria
1 (11.5.00)	463.3 ± 56.3	101.8 ± 46.3	26321 ± 2216	95.1 ± 31.3	210.7 ± 63.3	6788 ± 1642
2 (25.5.00)	192.6 ± 111.3	63.1 ± 23.6	36671 ± 4126	56.2 ± 26.5	194..2 ± 39.1	5663 ± 1132
3 (8.6.00)	161.0 ± 43.6	48.8 ± 19.4	42618 ± 6143	76.3 ± 29.4	362.5 ± 90.1	8426 ± 1561
4 (23.6.00)	159.4 ± 32.6	30.99 ± 9.41	34397 ± 4340	66.4 ± 20.9	489.4 ± 108.9	8848 ± 1068
5 (6.7.00)	113.0 ± 27.4	40.3 ± 16.4	29614 ± 4007	57.3 ± 16.3	411.3 ± 69.4	7316 ± 904
6 (21.7.00)	136.2 ± 41.4	31.6 ± 22.4	37816 ± 2630	91.6 ± 14.1	301.6 ± 97.3	9643 ± 1421

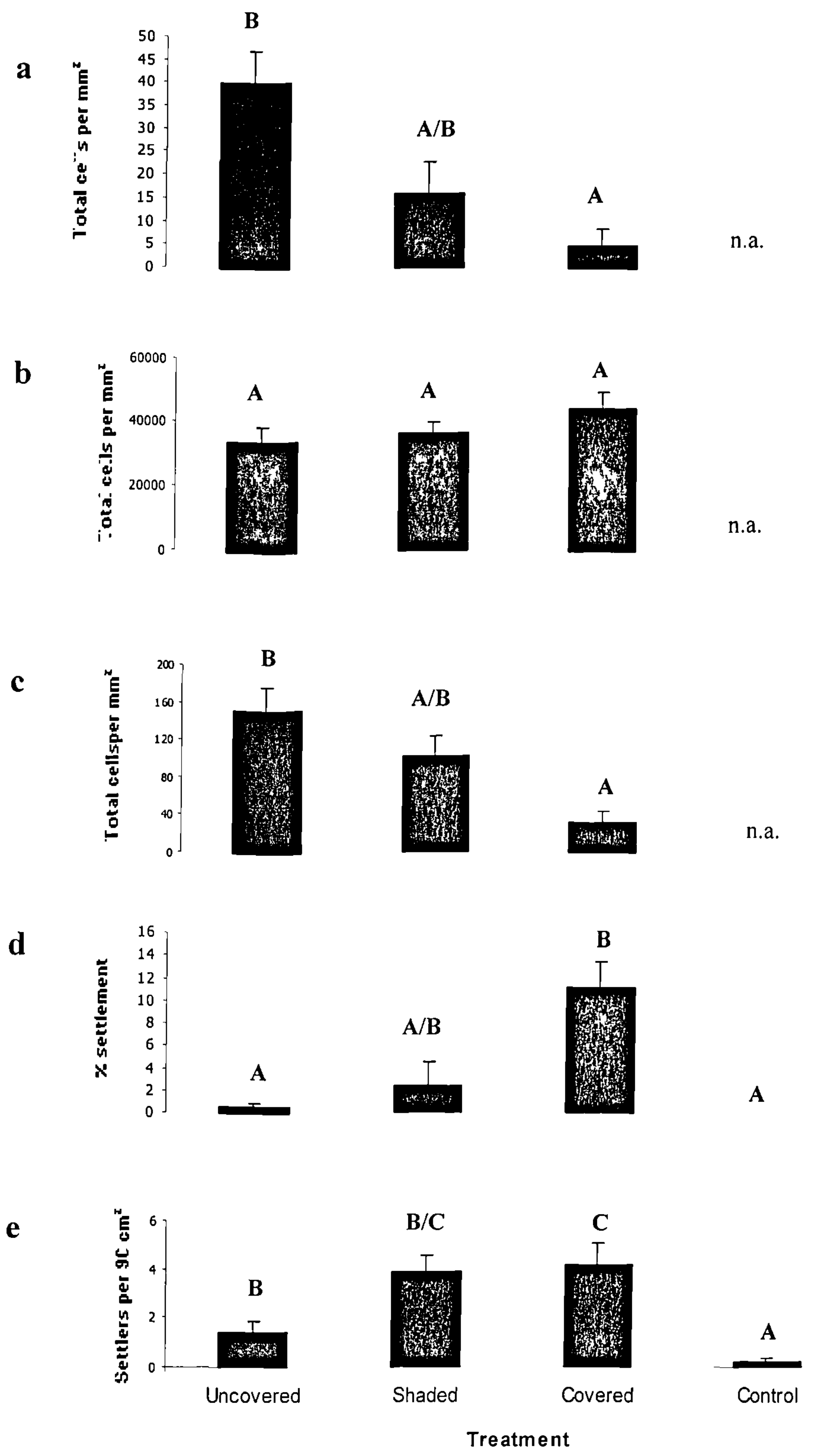


Figure 11 a-e. Histograms showing biofilm composition (diatoms, bacteria and cyanobacteria) of the different biofilm treatments and results of field settlement experiment. **a)** Total cyanobacterial cells per mm^2. **b)** Total bacterial cells per mm^2. **c)** Total diatom cells per mm^2. **d)** Settlement of *Pomatoceros* spp. on various biofilm treatments in laboratory assay. **e)** Settlement of *Pomatoceros* spp. on various biofilm treatments in field settlement experiments. Slate tiles were deployed for seven days in pools at mid-shore of Gorad Goch island, Menai Strait. The control was a clean, non-biofilmed slate (no biofilm data for control slate). Data plotted are means (+ SE) (n=3 for biofilms counts, n 9 for both field and laboratory settlement experiments). Results of Tukey's pairwise comparison are given; bars with the same letter are not significantly different (at $p<0.05$). n.a. = not applicable.

Simultaneous laboratory assays and field settlement experiment 2- biofilm composition

Increased shading significantly reduced (one-way ANOVA; $p<0.05$) total numbers of diatoms and cyanobacterial cells present in biofilms developed in the experimental tanks (Figure 11a & c). Total numbers of bacterial cells were not significantly different (at $p=0.05$) between the different biofilm treatments (Figure 11b).

Simultaneous laboratory assays and field settlement experiment 2- settlement experiment

Settlement of *Pomatoceros* spp. varied significantly (one-way ANOVA; $p<0.05$) between the different biofilm treatments (Figure 11d) with significantly greater settlement on the shaded and covered biofilms compared to the uncovered biofilm treatment. Least settlement occurred on the clean control surfaces. This pattern of settlement was negatively correlated with the numbers of diatoms and cyanobacteria present in the biofilms (c.f. Figure 11e with 11 a and c).

Simultaneous laboratory assays and field settlement experiment 2 - laboratory assay

Percentage settlement of *Pomatoceros lamarckii* larvae varied significantly between the different biofilm treatments in laboratory assays (one-way Anova; $p<0.05$). Larvae settled in significantly greater numbers on the covered biofilm treatment compared to the other biofilm treatments. Few larvae settled on the uncovered biofilm treatment and no larvae settled on the clean control (Figure 11d).

Discussion

Recruitment

Throughout this study, a subjective but significant distinction is made between *Pomatoceros* spp. recruitment onto the experimental tiles compared to recruitment onto the slate slabs by referring to them as settlement and recruitment respectively. The term settlement was used in recognition that routine field based observations would not have detected these recently settled individuals. However, the settlement of *Pomatoceros* spp. on the experimental tiles represents a more accurate measure of recruitment because post-settlement mortality would be limited (see Keough and Downes, 1982).

Although no attempt was made to discriminate between settled *Pomatoceros lamarckii* and *Pomatoceros triqueter* individuals during these studies, Ekaratne *et al.* (1982) note that 97% of all *Pomatoceros* spp. individuals found in the littoral region of the Menai Strait were *P. lamarckii* and that 99% of all individuals collected subtidally were *P. triqueter*. Observations made during these studies have confirmed these observations, therefore it seems reasonable to assume that *Pomatoceros lamarckii* settlement was being observed during the field studies.

Recruitment of *Pomatoceros* spp. varied with height on the shore, between surfaces in and out of pools and between upper and lower surfaces of experimental slate slabs and corresponded with the distribution of adult *Pomatoceros lamarckii* in the intertidal zone around the U.K. (Nelson-Smith and Gee, 1966; Ekaratne *et al.*, 1992; Crisp and Ekaratne, 1984). Variable recruitment of sessile marine invertebrates may come about as a result of differential larval supply, larval selectivity at settlement and post-settlement mortality (Olivier *et al.*, 2000). It is not clear at this stage how these different factors contribute to the observed recruitment patterns of *Pomatoceros* spp.. It is now recognised that differential larval supply, both spatially and temporally, can be a major factor influencing recruitment patterns and hence community structure in sessile and semi-sessile benthic marine communities (Strathmann *et al.*, 1981; Grosberg, 1982; Keough and Downes, 1982; Underwood and Denley, 1984; Connell, 1985; Gaines and Roughgarden, 1985; Gaines *et al.*, 1985; Raimondi, 1988, 1990, 1991; Chia, 1989; Sutherland, 1990; Menge, 1991; Miron *et al.*, 1995). Variations may come about at a larger-scale through species biogeography and hydrodynamic processes, whereas at a smaller scale variations may be the result of larval behaviour in the water column. Grosberg (1982) suggested that cyprid pre-settlement behaviour i.e., stratification of cyprids in the water column, may play an important role in determining adult distribution. Around Gorad Goch island, the waters of the Menai Strait are fast flowing and turbulent. It is therefore unlikely that larval distribution in the water column can alone account for the observed differences in recruitment intensity of *Pomatoceros* spp. with tidal height and would not explain differential recruitment onto upper and lower surfaces of slate slabs and between slabs placed in and out of pools. Variations in recruitment are therefore most likely due to a combination of larval selectivity at settlement and post-settlement mortality but the relative importance of these factors is unclear. *Pomatoceros lamarckii* larvae are highly selective at settlement under laboratory conditions, favouring older biofilms and avoiding settlement on recently dried biofilms (Roscoe, 1993; Chan and Walker, 1998; Hamer *et al.*, 2001; Chapter 3.1). The series of experiments reported here have shown that *Pomatoceros* spp. settle preferentially on certain biofilms in the field, settling preferentially on non-dried biofilms with high numbers of bacteria and fewer diatoms and cyanbacteria compared to biofilms that have been recently dried or that have lower numbers of bacteria or higher numbers of diatoms or cyanobacteria. These observations suggest that the recruitment patterns observed were largely a result of larval selectivity in response to natural variations in intertidal epilithic biofilms. However, it was noted that the number of *Pomatoceros* spp. individuals recorded during the field based settlement experiments was considerably greater per unit area than the number of recruits that were recorded on slate slabs present in similar habitats, suggesting that early post-settlement juvenile mortality may be high. In a review of juvenile mortality of benthic marine invertebrates, Gosselin and Qian (1997) found that high juvenile mortality is widespread with mortality being particularly high during the first few months of benthic life. Early post-settlement mortality of *Pomatoceros* spp. remains largely an unknown quantity. O'Donnell (1984) found that in Australia, grazing by gastropods in the intertidal zone significantly reduced the survival of newly recruited *Galeolaria caespitosa* on bare rock surfaces but that survival was not significantly reduced for individuals that settled in adult tube clumps. Grazers are certainly capable of removing newly settled *P. lamarckii* individuals (pers. obs.) and may represent a significant source of mortality to newly settled juveniles. Nevertheless, larval selectivity of *Pomatoceros* spp. in response to biofilms and other cues (e.g. gregariousness, photo-response) probably represents the primary mechanism responsible for determining observed recruitment patterns and hence adult distribution of *Pomatoceros* spp. in the intertidal zone of the Menai Strait.

Differential recruitment onto biofilms

In laboratory assays, a wide range of marine invertebrate larvae discriminates at settlement between different biofilms (see review by Wieczorek and Todd, 1998). The adaptive significance of such behaviour has not been clearly demonstrated but it has been suggested that biofilms may provide information to exploring larvae relating to the ambient environmental conditions operating at a particular site and may therefore indicate that a surface is suitable for settlement and subsequent survival (Neal and Yule, 1994b; Wieczorek and Todd, 1998). Despite the large number of studies demonstrating the ability of larvae to discriminate between different biofilmed surfaces in still-water conditions, relatively few such studies have compared settlement in response to different natural biofilms or have been carried out under field conditions.

This study is the first to compare the settlement of *Pomatoceros* spp. in response to various biofilms under field conditions. *Pomatoceros* spp. do discriminate between and settle preferentially in response to certain biofilms under field conditions. In addition, individuals settled in maximum numbers on biofilms indicative of the adult habitats (well developed biofilms dominated by bacteria) and settled in lower numbers on biofilms that were indicative of the high shore (recently dried, higher number of cyanobacteria) and of upper rock surfaces (higher numbers of microalgae). During earlier work, Hamer *et al.* (2001; Chapter 3.1) speculated that such behaviour may have significance in the field and that the negative cue of a dried biofilm may help to explain how the intertidal distribution comes about. The observations made here further support this suggestion. There is evidence that the larvae of certain barnacle species may also be capable of differentiating and responding to differences between biofilms on different niche surfaces in the intertidal and hence settling in the appropriate zone (Strathmann and Branscomb, 1979; Strathmann *et al.*, 1981; Bourget, 1988; Thompson *et al.*, 1998; Raimondi, 1988; this study).

Few studies have investigated whether the settlement response of tubeworm larvae is influenced by the surface biofilms under field conditions and none have been conducted intertidally. Notable exceptions are the work of Todd and Keough (1994) and Keough and Raimondi (1995,1996), in which field based settlement experiments were carried out designed to test whether the larvae of a range of marine invertebrates settled selectively between different biofilms developed under natural conditions in the field. Although the settlement response was highly variable between taxa, they found that the recruitment of serpulids was particularly affected by variations in biofilms.

Seasonality of recruitment

During this study, *Pomatoceros* spp. recruited throughout the study period (April - October), with a major peak in recruitment occurring during June and a second, smaller peak in the Autumn. Few other studies have documented the seasonal recruitment of *Pomatoceros* spp. in the intertidal zone. Most studies of *Pomatoceros* spp. settlement in the field have been made through recruitment on settlement panels held subtidally; nevertheless, these are useful for comparative purposes (Table 3). *Pomatoceros lamarckii* is capable of producing gametes throughout the year and low levels of recruitment may occur even during winter (Castric-Fey, 1983). In the U.K. adult worms reach their reproductive peak between late March and April (Segrove, 1941). The peaks in recruitment observed during this study suggest that around the U.K., *P. lamarckii* may also have a certain degree of synchrony at spawning, with peaks in recruitment occurring due to large cohorts of larvae reaching competence simultaneously at particular times of the year. Castric-Fey (1984) also notes that recruitment of *P. lamarckii* occurred in distinct peaks. Castric-Fey (1984) also reported that *Pomatoceros lamarckii* oocyte diameter exhibits rhythmic fluctuations that have a certain degree of synchrony. Maximum oocyte diameters (regarded to represent spawning periods) were recorded for *P. lamarckii* in January, May and August but it was reported that the timing varied between years depending upon water temperatures. As with the majority of marine polychaetes, it is not known whether *Pomatoceros* spp. spawn in response to a particular environmental or physiological cue (Bentley and Pacey, 1992).

Table 3. Summary of recruitment times reported for *Pomatoceros* spp. onto settlement panels.

Species studied	Main settlement period	Location	Reference
Pomatoceros spp.*	June, Autumn	Menai Strait, U.K.	This study
P. triqueter		Southampton, U.K.	Moat (1985)
P. lamarckii & *P. triqueter*	April, June, August and September - October	Concarneau, France	Castric-Fey (1984)
Pomatoceros spp.*	March-April	Naples, Italy	Lo Bianco (1909)
Pomatoceros spp.**	July - October	Trondheim, Norway	Dons (1943)
Pomatoceros spp.	Late spring-summer	Marseilles, France	Berner (1994)
Pomatoceros spp.*	May – July & September - October	Mediterranean	Senz-Braconnot (1968)
P. triqueter	Late summer-autumn	Helgoland, Germany	Klöckner (1976)
Pomatoceros spp.	June - July	West coast of Ireland	Purcell and Cross (1994)

* probably mostly *P. lamarckii;* **probably mostly *P. triqueter*

Intertidal biofilms

As with other intertidal organisms, bacteria and diatoms vary in their ability to withstand various environmental extremes, consequently the composition of epilithic biofilms may be expected to vary in response to environmental gradients (e.g. desiccation, temperature and exposure to ultraviolet radiation) associated with the intertidal zone. During this study, diatom, bacteria and cyanobacteria densities and species diversity within biofilms developed on glass surface in the intertidal zone of the Menai Strait were found to vary considerably with tidal height. Biofilms developed in rock pools were also different from those developed out of rock pools. Relatively few studies have so far documented the composition of epilithic biofilms from the intertidal zone of temperate rocky shores. Those that have tend to focus on the microalgae, primarily diatoms, and have been conducted either by direct counts or indirectly through chlorophyll measurements (Castenholz, 1963; Underwood, 1984; MacLulich, 1986, 1987; Hill and Hawkins, 1990, 1991). Albin (2000) compared bacterial colonisation of glass surfaces deployed for up to five weeks (July - August) at various locations along the Menai Strait. She found that bacterial densities were lower on intertidally-developed biofilms compared to subtidally-developed biofilms which were structurally more complex with greater species diversity. Bacterial densities of 160,000 – 231,175 cells per mm^2 were reported for biofilms developed on glass surfaces from the lower intertidal, significantly more than were found in this study (maximum bacterial cell density of ~ 60,000 per mm^2). In contrast, Cistoldi (1997) found a maximum bacterial density of only 6726.5 cells mm^2 on 28-day old biofilms developed on glass coverslips in the Menai Strait. Clearly, as for diatoms (Hill and Hawkins, 1991), there are large variations in densities of bacteria in intertidal biofilms.

Epilithic biofilms are difficult to study quantitatively because of their highly heterogeneous nature, they may also be complex three-dimensional structures making the direc counting of cells problematic. Accurate enumeration of bacterial cells is particularly problematic due to their small size. However, these problems can be overcome by the use of the recently developed confocal scanning laser microscopy (CSLM) as a means of optically sectioning a fully hydrated biofilm (see Lawrence *et al,*. 1991; Thompson *et al.*, 1998). Another major obstacle in conducting field based studies into the effect of different biofilms upon settlement is the fact that as soon as experimental biofilms are deployed in the field, they will begin to change to reflect ambient environmental conditions; the species composition and structure of what may initially have been very different biofilms may quickly converge under field conditions. Consequently, the duration of field experiments must be time limited. Less time in the field means that fewer larvae will settle, hence those studies that have been carried out have tended to focus on species that normally recruit in large numbers at certain times of the year.

Biofilm induction of settlement

It is still not clear what components(s) of the biofilm are responsible for inducing settlement of

Pomatoceros spp. but useful comparisons may be drawn with studies on the larval settlement of other serpulid species. Laboratory studies have demonstrated that bacteria (or their extracellular products) in biofilms induce the settlement of various serpulids and it has been suggested that bacteria may be the biofilm component that induces *Pomatoceros lamarckii* larvae (Hamer *et al.*, 2001; Chapter 2). However, other biofilm components, particularly microalgae, vary with tidal height and may also represent inhibitatory of facilitatory settlement cues. Raimondi (1988) reported that the cyanobacterium *Calothrix crustacea* inhibited settlement of *Chthamalus anisopoma* and suggested that this may prevent the cyprids settling above their appropriate zone in the intertidal. Recently, Harder *et al.* (2002) investigated the settlement response of *Hydroides elegans* larvae to monospecific diatom films under still-water laboratory conditions. They found that as with monospecific bacterial strains (Lau *et al.*, 2002), the settlement effect varied from inductive to inhibitory with the different diatoms. The minimal settlement on biofilms with high number of diatoms and cyanobacteria in the second laboratory and field experiment suggests that bacteria represent the positive settlement cue and that diatoms and cyanobacteria may provide a negative cue in the field.

Simultaneous laboratory and field studies

Relatively little is known about how the results of laboratory settlement assays can be applied to the field situation. O'Connor and Richardson (1996) and Thompson *et al.* (1998) demonstrated that the result of laboratory assays may contrast with those obtained in the field for barnacles. Conducting simultaneous still-water laboratory assays alongside field settlement experiments during this study has shown that although the overall patterns of *Pomatoceros* spp. settlement in response to different biofilms in the field were less clear than those obtained from laboratory assays with *Pomatoceros lamarckii* larvae, there was general similarity. *Pomatoceros* larvae have proven to be ideal for both laboratory and field based studies on the effects of biofilms upon settlement preferences and recruitment patterns.

5. General discussion: Towards an understanding of the settlement behaviour of *Pomatoceros lamarckii* larvae: habitat location and ranking of settlement cues.

The principal purpose of any living organism besides survival is to perpetuate the species. For sessile marine invertebrates with planktonic larvae, the selection of a suitable surface for settlement represents a particularly crucial point in the life-history of an individual. Consequently, the larval stage that undergoes settlement is normally equipped with well developed sensory structures enabling it to respond to a range of physical and chemical variables initially in the water column and subsequently during substratum / substrate exploration. Such exploration exposes larvae to multiple surface-associated physical and chemical settlement cues, many of which are of biological origin, that may induce, inhibit or have no effect upon settlement. Settlement is normally induced by cues indicative of the adult habitat which act to maximize an individual's potential for post-settlement survival, growth and reproduction (Meadows and Campbell, 1972; Crisp, 1974; Pawlik, 1992). This present study has focussed on the settlement preferences of the larvae of the marine tubeworms *Pomatoceros lamarckii* (Serpulidae), *Spirorbis spirorbis* and *Spirorbis tridentatus* (Spirorbidae) in response to natural and manipulated biofilms under laboratory and field conditions.

Larval development of *Pomatoceros lamarckii*

Laboratory observations revealed that the *Pomatoceros lamarckii* larval development, surface exploration and settlement behaviour and metamorphosis are similar to other planktotrophic serpulid larvae (Shearer, 1911; Segrove, 1941; Fjøyn and Gjøen, 1954; Wisely, 1958; Tampi, 1960; Andrews and Anderson, 1962; Straughan, 1972; Crisp, M., 1977; Grant, 1981; Marsden and Anderson, 1981; Miura and Kajihara, 1981; Smith, 1984; Moat, 1985; Chapter 2), with development proceeding much as described for *Pomatoceros triqueter* (Segrove, 1941; Fjøyn and Gjøen, 1954). As with other marine invertebrate larvae, the *P. lamarckii* metatrochophore is highly adapted both morphologically and behaviourally to respond to a range of environmental variables, particularly during substratum exploration. In the present study various larval sense organs have been described, some of which have not been reported previously for *P. lamarckii* larvae, such as the presence of sensory ciliary tufts on the ventral surface of the trunk and isolated sensory cilia on the lateral surface of the trunk. The ciliary tufts (chemo or mechano-receptors) are likely to be involved, in combination with the apical cilia, in site selection during surface exploration.

Pomatoceros lamarckii metatrochophores were occasionally observed trailing a mucous thread(s) that acted as a temporary attachment to the substratum (Chapter 2); temporary attachments of this sort have been described previously for *P. lamarckii* (Roscoe, 1993) and other serpulid (Segrove, 1941; Fjoyn and Gjoen, 1954; Wisely, 1958; Straughan 1972; Crisp, 1977; Grant, 1981; Marsden and Anderson, 1981; Moat, 1985) and spirorbid (Knight-Jones, 1951b; Gee and Knight-Jones, 1962; Nott, 1973; Nott and Parks, 1975; Potswald, 1978) larvae. Although a common feature of exploring serpulid and spirorbid larvae, the true function of these threads remains unclear. It has been suggested that they may play a role in stabilising larvae during the first stages of settlement and in anchoring them to the substratum during primary tube formation (Moat, 1985; Roscoe, 1993), or, that they may in some way be used as a physical 'test' of the suitability of a surface for attachment (Williams, 1964). The role of the mucous threads produced by *P. lamarckii* larvae merits further study; are they simply a consequence of the large quantities of mucus produced by these larvae or do they play a crucial role during substratum exploration and settlement? More sensitive fixation and preparation procedures for SEM, combined with the use of an Environmental Scanning Electron Microscopy to study the process of temporary attachment, permanent attachment and metamorphosis, may reveal more about these threads.

Artificial induction of metamorphosis of *Pomatoceros lamarckii* larvae

Potassium ions are known to be a potent inducer of metamorphosis for a range of marine invertebrate larvae (Woolacott and Hadfield, 1996) including *Pomatoceros lamarckii* larvae (Chapter 2). An ability to induce metamorphosis is a useful experimental tool that has the potential to contribute more to our understanding of the way in which *P. lamarckii* larvae attain and maintain competence under various conditions. For example, what is the within- and between-batch variability in larvae attaining competence and does this account for the differences in percentage settlement between larval batches in laboratory assays? Also, can *P. lamarckii* larvae postpone settlement after attaining competence without incurring a substantial 'cost' to post-larval life? A better understanding of post-settlement pressures will perhaps enable a more thorough appreciation of the constraints to which competent larvae are subjected.

Settlement of tubeworm larvae in laboratory assays

The time that *Spirorbis spirorbis* larvae spend exploring various test surfaces corresponded closely to the 'attractiveness' of the surface inferred from settlement assays. Time spent exploring a surface may prove to be a useful surrogate measure of surface attractiveness for *S. spirorbis* larvae and perhaps other tubeworm larvae in the same way that barnacle cyprid temporary adhesion can be used as a proxy for their settlement preferences (Neal and Yule, 1992).

When given a 'choice' in multi-treatment assays, larvae of *Pomatoceros lamarckii, Spirorbis spirorbis* and *Spirorbis tridentatus* settled almost exclusively on the predicted preferred surfaces (biofilmed surfaces), whereas, in the absence of a 'choice' in single-treatment assays a significant number of *S. spirorbis* and *S. tridentatus* larvae settled on less favourable surfaces (dried biofilmed surfaces) (Chapter 3.3). Settlement of *Spirorbis* larvae on less favourable surfaces in the absence of a 'choice' probably represents decreased larval selectivity as larvae become 'desperate', a behaviour originally described for *S. borealis* (= *S. spirorbis*) by Knight-Jones (1953a). In contrast, the *P. lamarckii* larval settlement response was not significantly different between single and multi-treatment assays. *P. lamarckii* larvae are planktotrophic and are capable of postponing settlement (Roscoe, 1993), whereas *S. spirorbis* and *S. tridentatus* larvae are lecithotrophic and have a finite time path. The 'desperate larva' hypothesis suggests that larvae will become less discriminatory at settlement with increasing time spent in the plankton and will settle more readily on less inductive surfaces rather than senesce and die (Knight-Jones, 1953a). The hypothesis is essentially an energetic model that was based upon the laboratory settlement behaviour of non-feeding larvae whose planktonic existence is effectively constrained by finite energy reserves (Knight-Jones, 1951b; Wilson, 1953). Toonen and Pawlik (2001b) tested the hypothesis for the planktotrophic larvae of the tubeworm *Hydroides dianthus* and proposed that because the larvae are not reliant upon finite energy reserves, they may not behave in the same way (i.e. become desperate) as lecithotrophic larvae. Neither altering the feeding regime nor starving larvae led to decreased substratum specificity, in fact, starving larvae actually led to a loss of competency rather than desperation. The difference in the settlement response of *S. spirorbis* and *S. tridentatus* larvae compared to *P. lamarckii* larvae between single- and multi-treatment assays suggests that as with *H. dianthus*, *P. lamarckii* larvae do not become desperate. The lecithotrophic larvae of *S. spirorbis* and *S. tridentatus* have likely evolved to become less discriminatory as their energy reserves rapidly deplete, whereas planktotrophic *P. lamarckii* larvae can act differently because this acute selective pressure does not apply.

If *Pomatoceros lamarckii* larvae do not become desperate, why do some larvae settle on less attractive surfaces in laboratory assays? Relatively high percentage settlement of *P.*

lamarckii larvae occurs only in the presence of adult conspecifics or on biofilms that have been exposed to an adult leachate, but a certain low proportion of larvae do settle on biofilms without the conspecific cue (Roscoe, 1993; Chan and Walker, 1998). The percentage of larvae that settle in response to a biofilm is low compared to some serpulids, e.g. *Hydroides elegans* (Hadfield *et al.*, 1994), but comparable to the levels previously reported for *P. lamarckii* (Roscoe, 1993; Chan and Walker, 1998; Hamer *et al.*, 2001) and *Hydroides dianthus* (Toonen and Pawlik, 1994, 2001a, 2001b, 2001c). The observation that a low proportion of *H. dianthus* larvae consistently settles on a biofilm whereas the majority of larvae require a conspecific cue led Toonen and Pawlik (1994) to suggest that *Hydroides dianthus* produces two types of larvae, founders and aggregators. They also proposed that the production of founder larvae (larvae that will settle in response to a biofilm) may enable the colonisation of new habitats by species with gregarious larvae (Toonen and Pawlik, 2002b). Significant variation in the proportion of founders spawned by an individual worm suggested that there is an heritable genetic component to larval settlement preferences of *H. dianthus* (Toonen and Pawlik, 2001c). They further propose that this genetically based behavioural variation may act to provide dispersal polymorphism which is advantageous for species inhabiting spatially or temporally variable niches. Observations of the settlement behaviour of *P. lamarckii* indicate that it may also have a 'bet-hedging' strategy and produce the two types of larvae, with the majority representing the 'safe' option (aggregators) and a small proportion of representing the more 'risky' option (founders), which would be able to exploit a suitable but previously uncolonised habitat.

Accepting that there may be genetically based variability in larval settlement preferences, why do larvae with the same settlement preferences immediately settle when they contact what is to them an 'attractive' surface? It is becoming increasingly apparent that between-larval variability in settlement preferences is the norm rather than the exception. Raimondi and Keough (1990) reviewed the literature on variability of larval settlement behaviour and concluded that 'Whatever the mechanisms, variable behaviour exists for the larvae of most species; even under controlled laboratory conditions, most parents produce offspring that exhibit a range of behaviours in response to clear, simple stimuli...'. In laboratory assays, most larvae contacted, explored and rejected surfaces repeatedly before committing to settlement, which occurred at a relatively steady rate, i.e. individuals varied considerably in the time they took to commit to settlement. Variation in larval behaviour may reflect individual variation in settlement ability at a particular moment in time (Rittschof *et al.*, 1984), thus variations in the time at which *Pomatoceros lamarckii* larvae attain competence may partly explain such settlement variability. However, in these studies, variability in settlement occurred despite the use of larvae of the same age that were morphologically similar and displayed the same photo-tactic behaviour. The sensory specificity of larvae may also change with age and thereby alter behaviour (Rittschof *et al.*, 1984), however, no change in substratum specificity of *P. lamarckii* larvae was noted during any of the assays. If *P. lamarckii* does produce two types of larvae (founders and aggregators), the proportion of each may vary between larval batches as described for *Hydroides dianthus* (Toonen and Pawlik, 2001c). Crisp (1974) explained apparently purposeful larval choice on the basis of appetitive behaviour 'a gradual lowering of the threshold stimulus necessary to release the "consummatory act" as the larva becomes older, or as it is stimulated by prolonged exploration of the surface". It seems likely that the larvae studied here are stimulated to settle with increasing time spent exploring a surface and that the time differences that individuals take to settle on a particular surface are due to individual variation in the threshold required (either the potency of inductive settlement cue or time spent exploring) to release the consummatory act of fixation. The high between-batch variability observed during this study is probably due to a combination of these factors.

When designing still-water, laboratory settlement assays for marine invertebrate larvae, careful consideration must be given as to the way in which experimental surfaces are presented. Selection of a suitable assay requires some prior knowledge of the larval settlement behaviour of the species being investigated. Of particular relevance to this decision is the ability of the larvae being studied to delay metamorphosis and whether the desperate larva hypothesis (Knight-Jones, 1953; Wilson, 1953; Pechenik, 1990; Toonen and Pawlik, 2001b) is applicable. Based upon the results obtained during this study, single-treatment assays may be more appropriate for tubeworm species with planktotrophic larvae, whereas multi-treatment assays may be more appropriate for lecithotrophic tubeworm larvae because they will become desperate and begin to settle on less attractive surfaces if there is no alternative. Experimental laboratory studies are necessarily contrived yet represent a useful means of reducing and controlling environmental variables (Sulkin, 1990). Whilst acknowledging the importance of controlling variables, it is nevertheless important to consider how single and multi-treatment laboratory assays relate to natural conditions. Multi-choice assays represent the more realistic situation, whereby larvae have the opportunity to explore and leave different surfaces before committing to settlement. Although currently less popular, the traditional multi-treatment assays were certainly responsible in helping to understand many of the parameters important to settling larvae.

Settlement of *Pomatoceros lamarckii* larvae in response to biofilms: laboratory and field observations

Marine invertebrate larvae are capable of differentiating between biofilms and respond at settlement both in the laboratory (Pearce and Scheibling, 1990; Wieczorek *et al.*, 1995 and in the field (Wieczorek and Todd, 1998; Olivier *et al.*, 2000). There is considerable diversity between marine invertebrate larval behaviour in response to biofilms (Crisp, 1974; Wieczorek and Todd, 1998) and few general patterns have emerged other than their generally inductive effect. *Pomatoceros lamarckii* larvae require a biofilm for settlement and settle preferentially on older biofilms (Roscoe, 1993; Chan and Walker, 1998; Hamer *et al.*, 2 01; Chapter 3.1). A preference for biofilmed surfaces, and in some cases older biofilms, also appears to be a widespread settlement response for the larvae of other serpulids and spirorbids (Hamer *et al.*, 2001), but the adaptive significance of this behaviour (if any) is unclear. Biofilms have the potential to provide considerable information to exploring larvae relating to prevailing environmental conditions (Neal and Yule, 1994b; Wieczorek and Todd, 1998); perhaps an older biofilm indicates a relatively stable substratum with adequate water movement operating over it (Wiekzorek and Todd, 1998) or it may indicate a surface free from superior spatial competitors (Unabia and Hadfield, 1999). Alternatively, older biofilms may simply contain greater quantities of the biofilm cue(s) that induce settlement, and are consequently more attractive to larvae.

Pomatoceros lamarckii larvae are capable of discriminating between different biofilms both in the laboratory (Chapter 3.1) and in the field (Chapter 4) and settle preferentially on older biofilms, whilst avoiding recently dried biofilms (Hamer *et al.*, 2001). Perhaps not surprisingly, most studies of invertebrate larval settlement focus on inductive cues (Wieckzorek and Todd, 1998). In this study, an inhibitory cue has been found that may play a significant role in *Pomatoceros lamarckii* larvae avoiding settlement in inappropriate habitats in the intertidal zone. Recruitment in the field was found to be highest on surfaces placed in the normal adult habitat of the intertidal zone and laboratory assays confirmed that *P. lamarckii* larvae settle preferentially on biofilms developed in these habitats (Chapter 4). Furthermore, recruitment on surfaces deployed in the adult habitat was greatest on biofilm>biofilm dried once> biofilm dried daily> no biofilm (at deployment), suggesting that *P. lamarckii* larvae settle at the appropriate tidal height in response to variations in epilithic biofilms. Gregariousness probably acts to both reinforce settlement in the correct adult habitat and to ensure the development of conspecific aggregations within this habitat. Given the importance of biofilms to the settlement of other serpulid and spirorbid species Hamer *et al.*, 2001), their larvae may also locate the appropriate adult habitat with the aid of biofilm cues coupled with conspecific cues. There is evidence that the larvae of certain barnacle species are also capable of differentiating and responding to differences between biofilms on different niche surfaces in the intertidal and hence settling in the appropriate zone (Strathmann and Branscomb, 1979; Strathmann *et al.*, 1981; Bourget, 1988; Thompson *et al.*, 1998; Olivier *et al.*, 2000).

Only a limited number of studies have investigated the settlement of marine invertebrate larvae in combined laboratory and field experiments (O'Connor and Richardson, 1996; Thompson *et al.*, 1998). These studies have proven useful in demonstrating that the result of laboratory assays should be interpreted with caution when extrapolating to the field and that the results of laboratory assays may actually be in direct contrast to those obtained in the field (O'Connor and Richardson, 1996). The *Pomatoceros lamarckii* larval settlement preferences obtained in simultaneous laboratory assays did broadly correspond to settlement preferences obtained in the field study, confirming that the biofilm is also an important cue to the settlement of *P. lamarckii* larvae in the field.

Settlement in response to bacteria

During laboratory assays, certain monospecific bacterial films induced settlement of *Spirorbis spirorbis* larvae whilst others did not, suggesting that bacteria present in a biofilm represent an important larval settlement cue (Chapter 3.4). Wilson (1958 first suggested that the different biofilm-forming microorganisms might vary in their ability to promote larval settlement and Meadows and Williams (1963) suggested that variations in the constituents of biofilms may affect the settlement of *S. borealis* (= *S. spirorbis*) larvae. Presenting such larvae with a mixed bacterial biofilm resulted in an intermediate settlement response compared to the individual bacterial strains when assayed in isolation, suggesting that when *S. spirorbis* larvae discriminate between biofilms, they are responding to variations in bacterial composition. Natural biofilms will contain a variety of inductive and inhibitory cues, presumably natural biofilms with a high proportion of inductive bacterial strains are more likely to stimulate larval settlement and biofilms with a high proportion of bacterial strains which either inhibit or have little effect upon settlement will be less likely to induce settlement. Individual bacterial strains have now been shown to have different effects upon settlement, either inducing, having no effect upon or inhibiting the settlement of various marine invertebrate larvae (see review by Wieczorek and Todd, 1998) including serpulids and spirorbids (Kirchman *et al.*, 1982a; Lau and Qian, 1997, 2001, 2002; Unabia and Hadfield, 1999; Chapter 3.4). The wide diversity of the bacterial types which induce larval settlement of *S. spirorbis* and other serpulid and spirorbid species suggests that the inductive cue may be a substance commonly produced by or associated with a wide range of bacteria and that it may be produced in different quantities or be present in different 'active' and 'non-active' forms for different bacterial strains.

Efforts have been made to characterise the various bacterial species isolated from natural biofilms and used in assays in an attempt to determine whether certain bacterial types tend to induce whilst others tend to inhibit settlement. No clear patterns have yet emerged, with a wide range of bacterial types inducing the settlement of serpulid and spirorbid larvae (Lau and Qian, 1997; Unabia and Hadfield, 1999; Lau *et al.*, 2002; Chapter 3.4). Surprisingly, relatively little emphasis has been placed on determining the ecological significance of larval settlement induction or inhibition by certain bacterial strains and not others but it seems intuitive to investigate whether those bacteria that are particularly inductive to settlement tend to be associated with the adult habitat. Although *Spirorbis spirorbis* larvae were found to be capable of discriminating between biofilms composed of various bacterial strains, the bacterial strains most abundant on *Fucus serratus* fronds (the natural adult habitat) did not induce high levels of *S. spirorbis* settlement compared to some of the strains isolated from epilithic biofilms (Chapter 3.2). Clearly both a bacterial cue and the associative chemical cue emanating from *Fucus* plants (Williams, 1964) are important to *S. spirorbis* larval settlement but the *Fucus* cue appears to act as the overriding cue at settlement. *Pomatoceros lamarckii* larvae also appear to settle in response to bacteria in biofilms (Hamer *et al.*, 2001) and may also be expected to discriminate between different bacterial strains. The selection of biofilms from certain intertidal habitats by *P. lamarckii* larvae (Chapter 4) may represent a response to variations in bacterial species diversity in the epilithic biofilms at different tidal heights. Further work should investigate whether *P lamarckii* settlement on intertidal biofilms correlates with the distribution and abundance of inductive and inhibitory bacterial strains.

Numerous studies have now demonstrated that the settlement of certain marine invertebrate larvae varies in response to different bacterial strains, but there is little information on the settlement response of more than one species of larvae to the same bacteria. Although studies have investigated the settlement response of various larvae to the bacterium *Deleya marina* (Mihm *et al.*, 1981; Maki *et al.*, 1990; Neal and Yule, 1994a; O'Connor and Richardson, 1998) and have reported a range of effects, these studies have been conducted independently and are not directly comparable given that the bacterial films may vary significantly depending upon factors such as age and nutrient availability. Therefore the rather broad question remains - do certain bacterial strains always induce settlement of all or most marine invertebrate larvae, whereas others inhibit settlement?

Identification of the bacterial settlement cue to *Spirorbis spirorbis* larvae

Although bacteria appear to be a common settlement cue, the identity of the exact inductive cue has not yet been fully characterised for any of the larvae of serpulid or spirorbid species. The available data suggest that the larvae of the different species may be responding to different bacterially-associated settlement cues. For example, the settlement of *Hydroides elegans* larvae is reduced on metabolically inactive (formalin and antibiotic treated) biofilms (Unabia and Hadfield, 1999; Lau and Qian, 2001), whereas *Spirorbis spirorbis*, *Janua brasiliensis* and *Pomatoceros lamarckii* larvae will settle readily on biofilms treated in this way (Kirchman *et al.*, 1982b; Hamer *et al.*, 2001; Chapter 3.5). Furthermore, some tubeworm larvae are capable of responding to water-borne cues whereas the larvae of the majority of species appear to require contact with a surface-associated cue for settlement (Marsden, 1991).

The experimental manipulation of biofilms using various chemical treatments provided a useful insight into the possible nature of the *Spirorbis spirorbis* larval biofilm settlement cue (Chapter 3.5). Settlement of *S. spirorbis* larvae on biofilms that have been treated with formalin and antibiotics was not significantly different from non-treated biofilms suggesting that the inductive cue is not proteinaceous in nature but that it is a relatively stable and persistent compound. *S. spirorbis* larvae do not settle on biofilms when exposed to elevated concentrations of the simple sugars glucose and fructose and treating a biofilm with sodium periodate and periodic acid significantly reduced larval settlement, suggesting that certain bacterially-derived polysaccharides may represent the inductive cue. However, the failure to reduce settlement by treating biofilms with the glucose-specific lectin concanavalin A suggests that either the lectin model as proposed by Kirchman *et al.* (1982b) does not apply to *S. spirorbis* larvae or that the biofilm cue which binds to the same larval receptor as glucose and fructose in solution is not the only inductive biofilm cue. In the present study, natural, multispecies biofilms were used, whereas Kirchman *et al.* (1982b) chose to conduct experimental assays with monospecific bacterial films. Similar settlement assays using similarly treated monospecific biofilms are now needed for *S. spirorbis*. Current information suggests that the *S. spirorbis* settlement cue is a commonly produced bacterial polysaccharide that contains glucose or fructose (or a structurally similar compound) that is present either on the surface of the bacterial cell or in the extracellular polysaccharide matrix of the biofilm. More than one biofilm or bacterially derived chemical cue may be recognised by *S. spirorbis* larvae at settlement.

Towards an understanding of the ecological basis of the settlement preferences of *Pomatoceros lamarckii* larvae

Whilst in the water column *Pomatoceros lamarckii* larvae rely upon behavioural responses to environmental gradients (e.g. light), which act over time to bring them into contact with a potential settlement site; selection of a suitable settlement site is then based upon responses to a combination of surface-associated physical and chemical cues. Previous laboratory studies have demonstrated that *P. lamarckii* larvae settle in response to biofilms, settle preferentially on older biofilms and settle at a high rate in response to adult conspecifics or a water-soluble adult leachate adsorbed onto a biofilm (Roscoe, 1993; Chan and Walker, 1998). This study has demonstrated that *P. lamarckii* larvae avoid recently dried biofilms and that settlement intensity on biofilms is closely correlated with the bacterial density of the biofilm (Chapter 3.1; Hamer *et al.*, 2001). Laboratory and field experiments demonstrated that biofilms are an important cue to *P. lamarckii* settlement in the field but that care must be taken when extrapolating laboratory settlement results to the natural environment. In order to gain a better understanding of the relative hierarchy of cues that are important to the settlement of *P. lamarckii* larvae it will be necessary to conduct laboratory settlement assays that combine more than one factor; such protocols should be the focus of future research. At the same time, multi-factorial field experiments that are capable of teasing out the relative importance of the various factors upon recruitment patterns (see Olivier *et al.*, 2001) are also needed.

Upon contacting a surface and exploring it whilst temporarily attached the competent larvae of sessile marine invertebrates are exposed to and respond to a multitude of facilitatory, inhibitory and neutral settlement cues. Settlement occurs in response to a hierarchy of physical and chemical cues which serve to locate these larvae in the adult habitat (Crisp, 1976; Newell, 1979). The cues may be considered as an instinctive sequence of physical and chemical factors acting as 'releasers' for behavioural responses which culminate in fixation (Crisp and Meadows, 1963). Newell (1979) proposed a simplistic but useful model describing the sequence of behavioural responses leading to the settlement of *Spirorbis spirorbis* larvae and a similar model is used here. The main cues thought to play a role in the selection of a suitable site for settlement by *Pomatoceros lamarckii* larvae are summarised in Figure 1; some of these factors are known to influence *P. lamarckii* larval settlement behaviour, whereas others are inferred from the larval settlement behaviour of other serpulid and spirorbid species. Although delivery of larvae from the water column to the substratum is considered to be a largely hydrodynamic process, *P. lamarckii* larval selectivity probably results in most of these larvae returning to the water column from surfaces repeatedly before finally settling. A few larvae will be stimulated to settle in response to a biofilm, whilst more larvae will be stimulated to settle in response to a well developed biofilm, particularly if it contains a high proportion of inductive bacterial strains. The vast majority of larvae will reject these surfaces until either contacting a surface with a biofilm characteristic of the adult habitat (i.e. a biofilm with an adsorbed conspecific chemical cue, particularly a biofilm on the anterior section of a conspecific tube containing a live worm which is made more potent with its high concentration of conspecific chemical cue) at which point they are most likely to settle, or, they die if no surface contact is made. Polychaete larval mortality remains largely an unknown quantity (Kupriyanova *et al.*, 2001). Although this sequence of events represents a rather crude view of the settlement process, it allows more realistic predictions to be made that can be tested in future settlement studies of the larvae of this tubeworm which is common on lower shores and in the shallow sublittoral around the British coast.

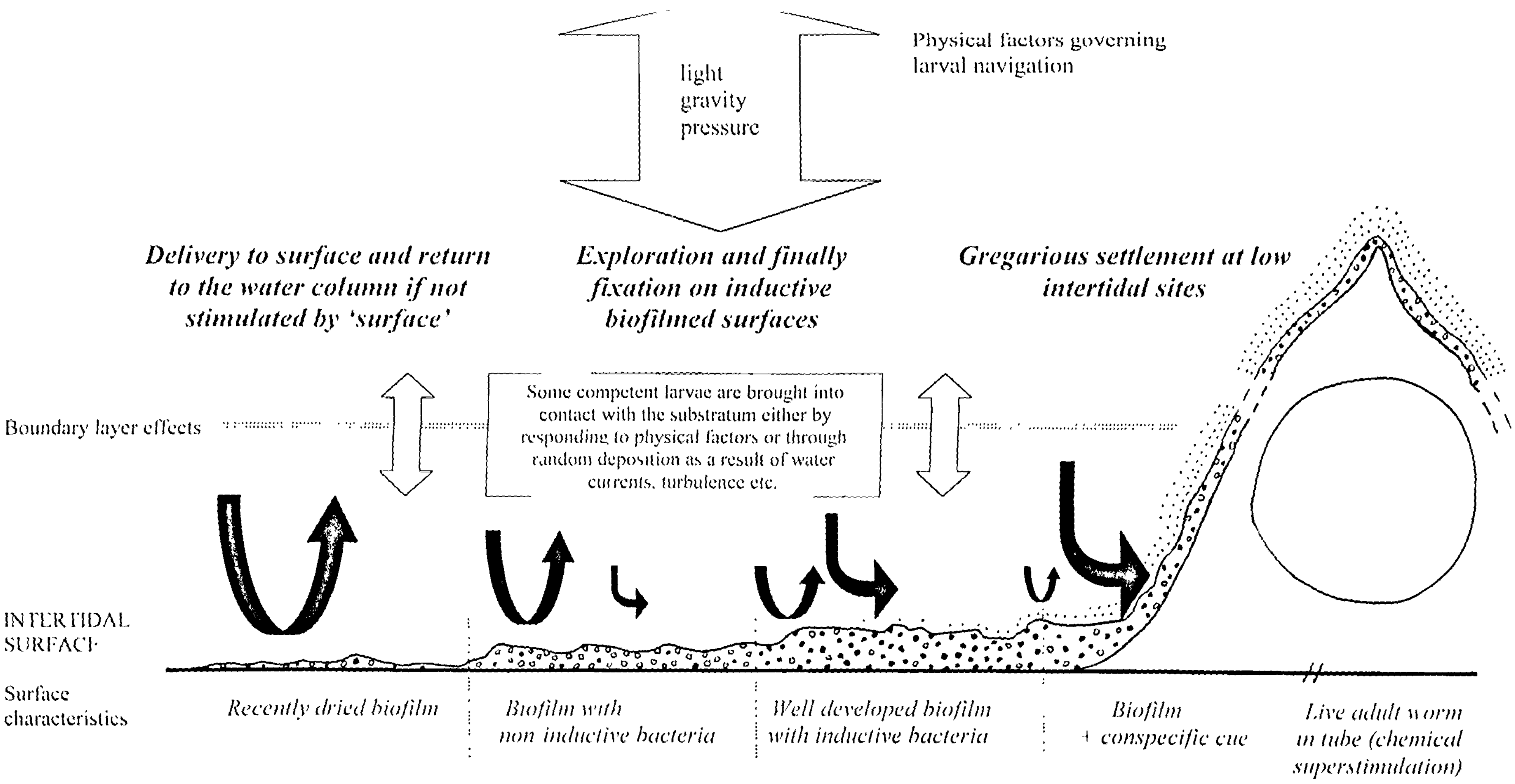


Figure 1. *Diagram summarising factors thought to be important in the selection of a suitable habitat in the intertidal zone by Pomatoceros lamarkii* larvae (not to scale). Black dots represent inductive bacteria; open circles represent non inductive bacteria dotted line represents adsorbed conspecific cue.

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