EFFECTS OF MARINE MICROFOULING ON THE ESTABLISHMENT OF SUBTIDAL HARD SUBSTRATUM COMMUNITIES

Sabine Katharina Wieczorek

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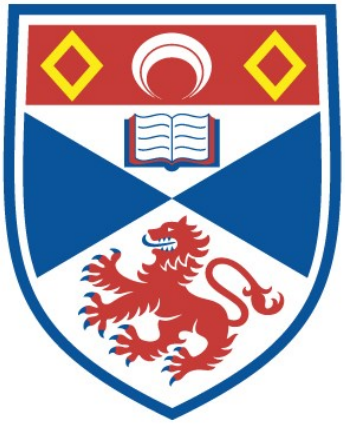

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Effects of marine microfouling on the establishment of subtidal hard substratum communities

by

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being a thesis submitted to the University of St. Andrews in candidature for the degree of Doctor of Philosophy.

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ABSTRACT

Sessile marine invertebrate larvae can recognize suitable settlement substrata by using various environmental cues, including organic/microbial "biofilms". In laboratory assays the effect of biofilm age on the settlement of a range of fouling species was assessed: the species included barnacle, bryozoan, spirorbin and ascidian larvae. The larvae of most species responded differentially to films of varying ages. A general trend of reversal of the effect of filming on the settlement response of the barnacle *Balanus amphitrite amphitrite*— from inhibitory to facilitatory — was noted with increasing film age. The settlement of the solitary ascidian *Ciona intestinalis* clearly was facilitated on filmed substrata of all ages. The larvae of the arborescent bryozoan *Bugula flabellata* generally were inhibited by biofilming (irrespective of film age), and the settlement of the polychaete *Spirorbis spirorbis* was found to be enhanced on "older" (12d) films only. In a series of manipulative panel experiments in a tidal rapid on the west coast of Scotland the effects of biofilming on subsequent larval settlement in the field were quantified. Inhibitory and facilitatory responses to biofilming were noted and these varied markedly between species and between two local habitats of contrasting flow regimes. The larvae of certain year-round settling species were found to vary in their response to biofilmed substrata depending on the season. Quantitative data were obtained for biofilms on artificial substrata by enumerating bacteria, diatoms, protozoans, fungi and the percent cover of microbial exopolymeric substances. These permitted comparisons of biofilms both in field and laboratory experiments. The results of the present study emphasize the importance of microbial surface film cues in explaining recruitment patterns of macrofouling assemblages during the first stages of substratum colonization.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 SETTLEMENT CUES AND SUBSTRATUM SELECTION BY MARINE INVERTEBRATE LARVAE

The majority of marine invertebrates produce planktonic larvae which persist in the water column for varying periods of time. In the cases of forms with planktotrophic larval development, such as barnacles, echinoderms, mussels, bivalves and certain encrusting bryozoans (e.g. *Electra pilosa, Membranipora membranacea*), the free-swimming period can last up to several weeks. In contrast, forms with lecithotrophic larval development (e.g. species which brood their larvae, such as ascidians, the polychaete *Spirorbis spirorbis* or the cheilostome bryozoans *Celleporella hyalina* and *Bugula flabellata*) metamorphose and settle within hours after larval release. Pelagic dispersal potential thus differs widely among epibenthic organisms (Scheltema & Carlton, 1984; Keough & Chernoff, 1987; Barnes & Hughes, 1988; Strathmann, 1990), and reproductive strategy has been considered to be of particular importance with respect to the colonizing ability of a species (Sutherland, 1977; Seed & Hughes, 1992). Because most macrofouling invertebrates of epibenthic assemblages are either permanently fixed to their substratum or have only very limited mobility during adult life, the location of settlement with respect to food availability, predation pressure, the presence of mates for reproduction, and physical disturbances is of obvious adaptive importance.

It has been much argued whether initial recruitment patterns (preceding early post-larval mortality) observed in the field are predominantly determined by the larval supply to a substratum from a pool of propagules available in the plankton (Gaines & Roughgarden, 1985; Underwood & Fairweather, 1989; Grosberg & Levitan, 1992; Hurlbut, 1992), or due to larval habitat selection (e.g. Meadows & Campbell, 1972; Scheltema, 1974; Keough & Downes, 1982; Burke, 1983). The first process may involve the passive deposition of larvae (e.g. Hannan, 1984; Eckman, 1990) and is thought to be ruled

largely by hydrodynamic processes (Eckman, 1983; Butman, 1987; Havenhand & Svane, 1991; Gross *et al.*, 1992; Hardin *et al.*, 1994; Snelgrove, 1994), whereas larval choice of a substratum includes an active behavioural response. The widely accepted contemporary opinion is that both processes are likely to contribute importantly to the observed settlement patterns, albeit at different spatial and temporal scales (Doyle, 1975; Butman, 1987; Bourget, 1988; Butman *et al.*, 1988; LeTourneux & Bourget, 1988; Morse, 1990; Walters, 1992; Pawlik & Butman, 1993; but see also Moore, 1975).

It is well established that marine invertebrate larvae are able to utilize a variety of cues from their environment to detect suitable sites for settlement and metamorphosis. These include surface wettability, texture and colour (e.g. Wethey, 1986; Raimondi, 1988a; Henschel *et al.*, 1990; Gerhart *et al.*, 1992; Walters, 1992; James & Underwood, 1994; O'Connor & Richardson, 1994), light (e.g. Lynch, 1949; Ryland, 1960, 1962a; 1977; Svane & Dolmer, 1995), flow regime (e.g. Crisp, 1955; Bushek, 1988; Dolmer & Svane, 1993; McKinney & McKinney, 1994), orientation of substratum (e.g. Harris & Irons, 1982; Vandermeulen & DeWreede, 1982), the presence of primary algal host species (e.g. Ryland, 1959, 1962b; Sebens, 1983; Al-Ogily, 1985; Kitamura *et al.*, 1993), conspecifics (e.g. Knight-Jones, 1951; Crisp & Meadows, 1963; Larman & Gabott, 1975; Pearce & Scheibling, 1991; Toonen & Pawlik, 1995), predators (Johnson & Strathmann, 1989; McGee & Targett, 1989; Young, 1989; Hurlbut, 1993), dominant competitors (e.g. Goodbody, 1961; Young & Chia, 1981), or food organisms (e.g. Barnes & Gonor, 1973; Hadfield, 1977, 1978; Hadfield & Pennington, 1990; Lambert & Todd, 1994), as well as microbial surface films (see Chapter 1, Section 1.3). Extensive reviews on larval habitat selection are given elsewhere: general discussions are provided by Meadows & Campbell (1972) and Scheltema (1974). The articles by Thorson (1966) and Butman (1987) focus on settlement cues of soft sediment substratum organisms, whereas Crisp's reviews (1974, 1976, 1984) emphasize hard substratum species. Burke (1983) concentrates on the induction of metamorphosis with respect to sessile and mobile invertebrates, and the recent article by Rodriguez *et al.* (1993) makes an attempt at integrating ecological, physiological and molecular aspects of the settlement process, and includes reference to biotechnological applicabilities.

In the past, reported studies of larval settlement behaviour have been biased towards positive cues. With the increasing importance of antifouling technology, however, inhibitory cues have recently featured more in the focus of research interests (e.g. Bonar *et al.*, 1986; Rittschof *et al.*, 1985, 1986, 1988, 1992; Davis & Wright, 1990; Gerhart *et al.*, 1988, 1992; Clare *et al.*, 1992; Holmström *et al.*, 1992; Holmström & Kjelleberg, 1994; Young, 1994; Clare, 1995). There are few accounts of adaptive larval responses related to negative cues associated with sources of mortality: Young (1989) reported the selective settlement of larvae of the solitary ascidian *Boltenia villosa* at predator-free refuge sites on the tunic of adult ascidians (see also Young & Chia, 1981; Hurlbut, 1993), and Johnson & Strathmann (1989) described how settlement of the barnacle *Balanus glandula* is reduced on substrata that previously had been occupied by the predatory whelk *Nucella lamellosa*.

There is still only a limited understanding of the adaptive significance of responses to particular cues. Although the field study by Strathmann *et al.* (1981) related avoidance of settlement sites of certain tidal heights in the barnacles *Balanus glandula* and *Semibalanus (= Balanus) cariosus* to increased chances of survival, for most cues a direct impact of larval settlement response on future growth rate, mortality or reproductive output has yet to be established experimentally. Nevertheless, in some examples the adaptive value of larval behaviour is obvious: gregarious behaviour at settlement is assumed to enhance success of subsequent sexual reproduction, and possibly is advantageous by lowering the risk of predation (Meadows & Campbell, 1972). Certain sessile (and non-sessile) sedentary species have been found to settle and metamorphose in response to the presence of their major prey: for example the opisthobranch mollusc *Adalaria proxima* is induced to metamorphose by the bryozoan *Electra pilosa* (Thompson, 1958), and *Tritonia hombergi* responds to its soft-coral prey species *Alcyonium digitatum* (Thompson, 1962). Selective advantages are, however, less easy to identify in the case of the highly specific settlement of many *Spirorbis* species onto certain algal substrata (Williams, 1964; Gee, 1963, 1965; Knight-Jones *et al.*, 1971; Al-Ogily, 1985).

The exact (morphological) larval structures and processes involved in cue recognition and response still remain unknown in many cases. Information on larval

sensory receptors is very limited (Bonar, 1978; Lindner, 1984; Clare *et al.*, 1994; Clare & Nott, 1994, and references therein), and yet it is possible that the ability to utilize settlement cues from the environment is constrained by the resolution of these receptors (Young, 1989). Their development is thought to coincide generally with the attainment of competence in a larva (Chia, 1978). The underlying mechanisms and signal transduction pathways of cue reception also are largely unknown, and conclusions about their involving electrical impulses, neurotransmitters or hormones remain largely speculative (Pawlik, 1992). It is further possible that settlement (a behavioural response) and metamorphosis (a morphogenetic process) (Holmström & Kjelleberg, 1994) may be triggered by different inducers (Rodriguez *et al.*, 1993). Crisp & Meadows (1963) proposed that the recognition of the substance (arthropodin) triggering gregarious settlement in barnacle larvae is mediated via a tactile chemical sense (compared by Crisp & Meadows (1962) with the antibody-antigen reaction), because arthropodin in solution was shown to be ineffective in inducing cyprid attachment. There is, however, recent evidence emerging which suggests that processes similar to olfaction in mammals might play a role in the settlement behaviour of cirripedes (Clare *et al.*, 1994, 1995; A.S. Clare pers. comm.). Water-borne cues have, for example, been shown to be involved in the substratum choice of oyster larvae (Zimmer-Faust & Tamburri, 1994) and larval metamorphosis of the nudibranch *Adalaria proxima* (Lambert & Todd, 1994). Some recent progress has been made in characterizing the molecular and chemical structures of settlement-inducing cues (see reviews by Morse, 1984, 1990, and Pawlik, 1992); these include both free fatty acids (e.g. Pawlik, 1986; Pawlik & Faulkner, 1986; Kitamura *et al.*, 1993, but see also Jensen & Morse, 1990) and peptides (e.g. Tegtmeyer & Rittschof, 1989; Pearce & Scheibling, 1990; Tamburri, 1992; Zimmer-Faust & Tamburri *et al.*, 1994). A lectin-mediated induction of settlement has been proposed in the response of the larvae of the polychaete *Janua (Dexiospira) brasiliensis* to bacterial surface films, and it has been suggested that lectins located on the larval surface bind specifically to bacterial exopolymer glycoconjugates (Kirchman *et al.*, 1982a, 1982b; Kirchman & Mitchell, 1983; Maki & Mitchell, 1985). Several authors are in favour of the hypothesis that chemical cues associated with the substratum are mediated via the larval nervous system

and may be substances which mimic the actions of neurotransmitters (Morse *et al.*, 1984; Coon & Bonar, 1985; Morse, 1988; Coon *et al.*, 1990; Jensen & Morse, 1990, see also Burke, 1983; Bonar *et al.*, 1990; Leitz, 1993, and references therein).

However elusive the underlying molecular mechanisms of settlement cue transduction may be, the ecological and economic significance of substratum selection by marine invertebrate larvae as a determining factor in recruitment processes cannot be denied. It has been shown that for many species substratum specificity at settlement plays an important rôle in determining the distributional patterns of macrofouling organisms both on natural and man-made substrata in the field (e.g. Keough & Downes, 1982; Connell, 1985; Davis, 1987; Bourget, 1988; LeTourneux & Bourget, 1988; Raimondi, 1988; Todd & Keough, 1994). "Fouling" of surfaces by epifaunal marine invertebrates remains a major applied and commercial problem, with costs running into hundreds of millions of pounds worldwide. Antifouling protocols and technology continue to focus on attempts to prevent and inhibit larval settlement or to interfere with the further development of settled post-larvae (Bonar *et al.*, 1986; Gerhart *et al.*, 1988; Clare *et al.*, 1992; Rittschof *et al.*, 1992; Holmström & Kjelleberg, 1993, 1994; Young, 1994; Clare, 1995). Knowledge of settlement cues and mechanisms of larval attachment will provide important ecological information on the general processes involved in larval perception and choice of settlement site. With the rapid increase in aquaculture of molluscan and crustacean species throughout the world's economies it is evident that a clearer understanding of the effects of certain cues on larval settlement and metamorphosis can readily enhance the efficiency, viability and profitability of such enterprises.

The present study focuses on the utilization of surface-associated biofilms as settlement cues by the larvae of marine epibenthic invertebrates. The emphasis lies on the scale of the effects of biofilming on the larval settlement responses, ranging from inhibitory to facilitatory, and on the importance of film "type" — assessed in terms of age, metabolic activity, composition and cell density. The following two Sections are brief reviews of the current knowledge of the microbial ecology of biofilms and of previous studies investigating the rôles of microbial cues on substratum selection by the larvae of hard substratum marine invertebrates.

1.2. BIOFILMS

Almost any surface submerged in the marine environment will be colonized by microorganisms within minutes or in many cases "instantaneously" (Characklis & Cooksey, 1983). Preceding the attachment of microorganisms is the adsorption of organic molecules ("molecular fouling"), resulting in a "conditioned" surface (Baier, 1981; Characklis & Cooksey, 1983), which might alter the wettability and other physical and chemical characteristics of the surface (Dexter *et al.*, 1975; Marszalek *et al.*, 1979). Microbial cells are thought to be transported to the wetted surface by various mechanisms, including diffusion (Brownian), electrostatic forces, fluid dynamic forces and taxis.

Bacterial attachment is mediated mainly by extracellular polymeric substances (EPS) (Costerton *et al.*, 1978; Fletcher, 1979; Marshall, 1980; Wardell *et al.*, 1980; Allison & Gilbert, 1992), which often form a tangled filamentous mass lending structure to the microbial assemblage. These microbial exopolymers are high-molecular weight mucous secretions, and largely polysaccharide in composition (Decho, 1990). Bacterial cells can be found frequently embedded in this EPS matrix (Marshall *et al.*, 1971; Costerton *et al.*, 1978; Fletcher, 1979; Cooksey, 1992a). EPS present a means by which microbial cells (algal and bacterial) maintain an association with other cells, although not all cells produce EPS obligatorily; rather, the secretion of EPS depends on the bacterial strain and the physiological condition of the biofilm (Fletcher, 1977; Decho, 1990; Wrangstadh *et al.*, 1990). The production of EPS and their functional rôles are reviewed in Geesey (1982), Decho (1990), Cooksey (1992a) and Meyer-Reil (1994).

In addition to bacteria, a wide variety of microalgae, fungi, protozoans, organic debris and inorganic particles will contribute to the formation of the biofilm. The attached microorganisms grow and multiply, resulting in more surface bound cells. Bacterial cells are reported to benefit from attachment to solid surfaces (especially at low environmental nutrient concentrations) by utilization of the nutrients accumulated at the liquid-surface interface (Marshall, 1980; 1981, 1989; Marshall & Bitton, 1980; Fletcher & Marshall, 1982; Medlin *et al.*, 1985). Attachment to a surface was found to also stimulate EPS synthesis in some microorganisms (Vandevivere & Kirchman, 1993). Thus, as for

macrofouling assemblages, there is a distinct succession in the development of microfouling assemblages, leading to a complex fouling layer (Marszalek *et al.*, 1979; Characklis, 1981; Dempsey, 1981; White 1988); biofilms therefore should not to be thought of as surface accumulations which are static in time or space. Just as for the members of a macrofouling community, the abiotic and biotic components of biofilms interact in a multitude of ways. These include bacterial predation by protozoans (Pedersen, 1982), the clustered distribution of bacteria around inorganic and organic particles, and metabolic interactions between phototrophs and heterotrophs (Cooksey, 1992b); hence, the removal of one component may create other changes in biofilms beyond merely the absence of that group (Haack & McFeters, 1982; Cooksey, 1992b).

Substratum type is known to affect the physiology, metabolism and organisation of a biofilm (Marszalek *et al.*, 1979; Baier, 1980; Allison, 1992), and surface characteristics such as surface free energy are of importance to bacterial attachment (Fletcher & Marshall, 1982; Gantzer *et al.*, 1989). Due to abrasion and other physical impacts at wave and current exposed sites, or even simply as a result of film maturation and the related cell deaths, parts of the biofilm are sloughed off, resulting in a net loss of film materials (Rittmann, 1989). Further, the composition and density of biofilms are affected also by current regime and shear stress (Korte & Blinn, 1983; Characklis & Cooksey, 1983; Nowell & Jumars, 1984; Rittmann, 1989; Munteanu & Maly, 1991; Allison & Gilbert, 1992; Wolfaardt & Cloete, 1992; Lau & Liu, 1993), and microbial assemblages in even adjacent habitats can segregate into discrete populations in response to physical, chemical and biological factors (Davis *et al.*, 1983). Castenholz (1963) and Hudon & Bourget (1981), for example, describe compositional differences between intertidal and sublittoral biofilms (see also Underwood, 1984). Seasonal changes in the composition, density and/or physiological activity of epibenthic microfilms have been reported also (e.g. Hudon & Bourget, 1981; Munteanu & Maly, 1981; Carson & Allsopp, 1983; Underwood, 1984; MacLulich, 1987). Thus, microbial distributions on submerged surfaces can be extremely patchy, and there are distinct local and temporal variations in marine biofilms.

1.3. MICROBIAL CUES AND THE SETTLEMENT OF HARD SUBSTRATUM MARINE INVERTEBRATE LARVAE

There has been a considerable recent increase in the number of studies reporting the important rôle of microbial filming of substrata in triggering settlement responses in marine macrofouling species (see Todd & Keough, 1994, and references therein). We know today that the presence of certain microbial cues induces metamorphosis and settlement in the larvae of many marine invertebrate taxa, including barnacles (Strathmann *et al.*, 1981; Hudon *et al.*, 1983; Raimondi, 1988; Maki *et al.*, 1988, 1990, 1992; Avelin Mary *et al.*, 1993; Neal & Yule, 1994a, 1994b; and see also Chapter 2, Section 2.2), echinoderms (Cameron & Hindgardner, 1974; Barker, 1977; Pearce & Scheibling, 1991; Johnson & Sutton, 1994), polychaetes (Meadows & Williams, 1963; Kirchman *et al.*, 1982; Roscoe, 1993; Toonen & Pawlik, 1994), bryozoans (Mihm *et al.*, 1981; Brancato & Woollacott, 1982; Maki *et al.*, 1989), bivalves (Fitt *et al.*, 1990; Tamburri *et al.*, 1992; Parsons *et al.*, 1993), ascidians (Szewzyk *et al.*, 1991; Holmström *et al.*, 1992), gastropods (Morse *et al.*, 1984; Slattery, 1992), scyphozoans (Brewer, 1976; Neumann, 1979), actinians (Leitz & Wagner, 1993), and sponges (Keough & Raimondi, 1995, see also Chapter 3). The effects of biofilming can be either facilitatory or inhibitory, depending on larval species, biofilm "type" and assay used. Whilst the settlement of many macrofouling species is affected by biofilming, the presence of films is not a prerequisite for successful attachment and metamorphosis in all cases, and some invertebrate larvae are indifferent to the microbial preconditioning of substrata (Crisp & Ryland, 1960; Mihm *et al.*, 1981; Kirchman *et al.*, 1982; Mitchell & Kirchman, 1984; Roberts *et al.*, 1991; McGrath *et al.*, 1994; Todd & Keough, 1994). Nevertheless, it should be emphasized that the use of microbial cues has been shown to be of importance in affecting the behaviour of larvae representing all major macrofouling phyla of temperate regions (see above). It largely remains to be experimentally proven whether many of the settlement cues attributed to algal hosts or surface characteristics of abiotic substrata are not indeed the result of surface-associated heterogeneities of microbial cues (Holmström & Kjelleberg, 1994; Mountfort & Pybus, 1992). Several authors have raised the

possibility that both the stimulatory and inhibitory components extracted from marine eukaryotes could be due to associated bacteria (Holmström & Kjelleberg, 1994, see also references therein: Standing *et al.*, 1984; Johnson *et al.*, 1991; Davis & Wright, 1990; Mountfort & Pybus, 1992).

The majority of the studies concerning microbial cues attribute the effects on macroinvertebrate settlement responses to a bacterial rather than a microalgal source (recent examples are Maki *et al.*, 1988, 1989, 1990, 1992; Fitt *et al.*, 1990; Johnson *et al.*, 1991; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992; Leitz, 1993; Leitz & Wagner, 1993; Roscoe, 1993; Neil & Yule, 1994a, 1994b; Johnson & Sutton, 1994). However, the use of positive and negative microalgal and cyanobacterial cues has been shown for the larvae of the barnacle species *Semibalanus balanoides* (LeTourneux & Bourget, 1988) and *Chthalamus anisopoma* (Raimondi, 1988), and for the mollusc *Haliotis rufescens* (Morse *et al.*, 1984; Slattery, 1992). The larvae of the polychaete *Spirorbis spirorbis* were found to settle preferentially on surfaces filmed with the green algae *Chlamydomonas* and *Prasinocladus*, but avoided surfaces bearing the diatom *Navicula* and the green alga *Dunaliella* (Knight-Jones, 1951; Williams, 1964; Meadows & Campbell, 1972). Marine microalagae are known to also produce EPS (Characklis & Cooksey, 1983; Decho, 1990; Cooksey, 1992a), which have been implicated in the specific response to bacterial film components (see below). Rare evidence of the influence of marine fungi on larval settlement is provided by shipworms of the genus *Teredo*, whose larvae are attracted to wooden substrata decomposed by fungi (Meadows & Campbell, 1972, and references therein).

It has been proposed by several authors (e.g. Kirchman *et al.*, 1982a, 1982b; Maki & Mitchell, 1985; Decho, 1990; Maki *et al.*, 1990; Szewzyk *et al.*, 1991) that the production of particular EPS components of biofilms is the key to the use of biofilms as settlement cues by marine invertebrate larvae (see below). However, the involvement of substances of microbial origin in larval settlement processes is not a new idea, and Zobell & Allen (1935) were among the first authors to describe enhanced attachment of marine organisms in response to "bacterial slimes" (another early account is the study by Miller *et al.*, 1948). The mechanisms by which biofilm EPS promote larval settlement have not

been elucidated in most cases, but possible processes include passive "entrapment", metabolite and inducer amplification, microbial modulation, and direct attraction and induction (Holmström & Kjelleberg, 1994, see also Chapter 1, Section 1.1). One possible mechanism for signal transduction is the lectin model described by Kirchman *et al.* (1982a, 1982b, see Chapter 1, Section 1.1) for the larvae of *Janua (Dexiospira) brasiliensis* (see also Kirchman & Mitchell, 1983; Maki & Mitchell, 1985). First attempts have been made to fluorescently label lectins associated with molecular biofilms (Michael & Smith, 1995) and this new technique, potentially allowing the definition and localization of microbial cues, appears to be very promising for future routine use in bioassays.

Other authors suggest that the effects of microbial films are less specific (Brewer, 1976), and due mainly to physico-chemical changes of the substratum surface in their presence. Such changes have been proposed to affect larval settlement either by passive "entrapment" of larvae onto the film (Decho, 1990; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992), or through modifications of the adhesive properties of the filmed surface due to changes in its surface free energy/wettability (e.g. Eiben, 1976; Gerhart *et al.*, 1992; Maki *et al.*, 1992; Neil & Yule, 1994a, 1994b).

Evidence has been presented that biofilm "type" — an expression of film composition (e.g. Meadows & Williams, 1963; Kirchman et al., 1982a; Holmström *et al.*, 1992; Maki *et al.*, 1988, 1990), density (Szewzyk *et al.*, 1991; Neal & Yule, 1994b), growth phase (Neumann, 1979; Schmahl, 1985a, 1985b; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992) and age (e.g. Kirchman *et al.*, 1982a, Maki *et al.*, 1988, 1990, 1992; Holmström *et al.*, 1992; Pearce & Scheibling, 1991; Keough & Raimondi; 1995, see also Chapter 2) — is of significance to its ability to induce or inhibit settlement. The latter findings imply a high specificity of the biofilm cues, and no broad generalizations can be justifiably drawn (see also Barker, 1977; Weiner, 1985).

Few studies have assessed the effects of natural multi-species biofilms on invertebrate settlement under field conditions (Strathmann *et al.*, 1981; Roberts *et al.*, 1991; Todd & Keough, 1994; Keough & Raimondi, 1995), and most of the current knowledge about microbial cues derives from laboratory experiments which were mainly

carried out with monospecific biofilms and a small selection of invertebrate taxa (Neumann, 1979; Weiner *et al.*, 1985; Maki et al., 1988, 1990, 1992; Fitt *et al.*, 1990; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992, Avelin Mary *et al.*, 1993; Leitz & Wagner, 1993; Neal & Yule, 1994a). Because experimental protocols from these studies vary, direct comparisons among larvae of different species are often impossible. Whilst controlled field experiments are subject to certain constraints in design and replication, they are essential in order to test the applicability of conclusions drawn from laboratory studies to the natural environment. Furthermore, *in situ* studies allow the simultaneous examination of the cues for a broad range of taxa, thus enhancing comparability between species. Virtually nothing is known about how spatial (site specific) and temporal (seasonal) heterogeneities in natural microbial assemblages affect larval settlement (but see Neal & Yule, 1994b, and Chapter 3).

Our present understanding of the underlying processes of larval facilitation or inhibition by biofilms is still hampered by several constraints: (i) difficulties in readily identifying bacterial strains and microalgae, (ii) the complexity of microfilms, which might contain more than one active assemblage constituent and exert either facilitatory or inhibitory influence, leading to potential overlapping effects, (iii) spatial and temporal heterogeneities of biofilm cues, and (iv) logistic problems in undertaking controlled field experiments. However, the identification of particular microbial strains or species exerting inhibitory effects on macrofoulers could open a new era of antifouling technology (Gatenholm *et al.*, 1994, see also reviews by Bonar *et al.*, 1986, and Holmström & Kjelleberg, 1994). Whilst past research into naturally produced antifouling agents has concentrated mainly on eukaryotic organisms (Targett *et al.*, 1983; Standing *et al.*, 1984; Rittschof 1985, 1986; Gerhart *et al.*, 1988), the advantages of using bacterially derived products in antifouling technology are obvious. Bacteria generally are easy to culture, have extremely short generation times, and can readily be manipulated by genetic engineering; thus they are capable of producing large biomass with minimal culturing efforts and expenditure.

The objective of the present study was to assess specific differences in the responses of a wide range of taxa to biofilms under field conditions, to distinguish

between facilitatory and inhibitory effects of biofilming, and to further quantify and characterize the parameter biofilm "type" (with respect to film age, composition, density and metabolic activity). Some of the laboratory results of previous authors on microbial settlement induction/inhibition were re-examined. Furthermore the effects of contrasting flow regimes and season on the settlement response of marine invertebrate larvae to biofilming were investigated *in situ*.

CHAPTER 2

THE INFLUENCE OF BIOFILM AGE ON THE SETTLEMENT RESPONSES OF MARINE INVERTEBRATE LARVAE

2.1 INTRODUCTION

Many previous detailed analyses of the effects of microfouling on the settlement and metamorphosis of invertebrate larvae have focused primarily on overall physicochemical properties of microfilms, such as surface free energy (Eiben, 1976, Mihm *et al.*., 1981; Kirchman *et al.* ,1982; Maki & Mitchell, 1985; Maki *et al.* , 1989, 1992; Roberts *et al.* , 1991; Neal & Yule, 1994b). In the past little emphasis has been placed on the composition, growth phase or metabolic activity of the microbial film assemblages involved, and most studies generally considered only single species films (e.g. Neumann, 1979; Weiner *et al.* , 1985; Fitt *et al.* , 1990; Szewzyk *et al.* , 1991; Holmström *et al.* , 1992; Leitz & Wagner, 1993; Neal & Yule, 1994a). Although single species studies enhance analytical tractability they cannot mimic the complexity of natural systems (Allison & Gilbert, 1992).

Kirchman *et al.* (1982) postulated that lectins produced by bacterial films may mediate the settlement of the polychaete *Janua brasiliensis* (see also Maki & Mitchell, 1985). With particular reference to the inhibition of barnacle settlement by microbial films, Maki *et al.* (1990, 1992) deduced that specific molecular components of bacterial extracellular polymers were more important than overall physical properties of the microfilms. Szewzyk *et al.* (1991) describe how exopolysaccharides produced by bacteria for attachment could be altered by changing their growth conditions (see also Fletcher, 1977; Fletcher & Marshall, 1982; Characklis, 1981; Allison & Gilbert, 1992) and changes in the successional composition, physiological condition and growth phase of the microfilm assemblage may well alter their effects on the settlement of macrofouling organisms (Neumann, 1979; Schmahl, 1985a, 1985b). Some evidence has been

presented that such changes are indeed of great importance in the cueing of invertebrate settlement. Thus, for example, larvae of the echinoid *Strongylocentrotus droebachiensis* prefer to settle onto "older" (75d) rather than "young" (40d) films of similar biomass (Pearce & Scheibling, 1991). Other biofilm age-related behavioural variability in larvae in response to biofilming has been reported for barnacles (Maki *et al.*, 1988, 1990, 1992, see below) and spirorbid polychaetes (Kirchman *et al.*, 1982a; Keough & Raimondi, 1995). In a recent field study Keough & Raimondi (1995) detected significant film age-related effects on the settlement of a number of marine invertebrate species in southeastern Australia, including slime sponges, and the bryozoan species *Bugula stolonifera* and *Bugula neritina*. They found that the effects of biofilm age could be either facilitatory (e.g. for a *Serpula* species and spirorbid polychaetes) or inhibitory (e.g. for the barnacle *Balanus variegatus*); the groups and taxa for which no response to variations in film-age were detected included an orange sponge species, *Ciona intestinalis*, *Botryllus schlosseri*, didemnid ascidians and *Ascidia* sp.. Keough & Raimondi (1995) also observed an increase in the total numbers of settlers with biofilm age. However, Turner (1988) concluded that the factor film age was of minor significance in the settlement response of macrofouling organisms to microbial surface cues under field conditions.

Here, laboratory assays on the effects of biofilms of varying ages were carried out using the larvae of four selected species representing major macrofouling groups of temperate regions: the barnacle *Balanus amphitrite amphitrite* Darwin, the solitary ascidian *Ciona intestinalis* (L.), the polychaete *Spirorbis spirorbis* (L.) and the arborescent bryozoan *Bugula flabellata* (Thompson). The objective of the following experiments was to assess whether short term changes of the biofilm "cues" during the early phases of exposure of newly available substrata affect the choice of settlement site in the larvae of these taxa. The present controlled laboratory experiments allowed the isolation of the effect of changes in the biofilm "cues" from the potentially confounding influence of contemporary larval settlers of other species.

2.2 INHIBITORY AND FACILITATORY EFFECTS OF BIOFILMS ON SETTLEMENT OF *BALANUS AMPHITRITE AMPHITRITE* (CRUSTACEA : CIRRIPEDIA) LARVAE

2.2.1 Introduction

Maki *et al.* (1988, 1990, 1992) found that 4d old cyprids of *Balanus amphitrite* were inhibited by seven of the 18 bacterial species tested in their pure culture assays. They further noted age-related effects of films of the bacterium *Deleya marina* on the inhibition of attachment of the cyprids when compared to unfilmed polystyrene control dishes (see also Holmström *et al.* , 1992): "older" films were more inhibitory than were "young" films. Inhibition of cyprid attachment also has been reported for natural multi-species microfilms on polystyrene petri dishes preconditioned in seawater (Maki *et al.* , 1988, 1990, 1992), but those observations were confined to relatively young films, with substrata being preconditioned for only 24-120h. The use of varying, and often large, numbers of larvae in their assays (15-200 per dish, Maki *et al.*, 1988, 1990, 1992) renders their data difficult to interpret in view of possible density-related behavioural effects. Yule & Walker (1985) and Clare *et al.* (1994) have raised the possibility of cyprid-cyprid interactions at the time of settlement having important implications for the design of barnacle settlement assays, and the latter recommend utilization of low cyprid densities or even single larvae wherever possible.

The effects of film age on the settlement response of *Balanus amphitrite amphitrite* cyprids to natural multi-species biofilms developed over longer preconditioning periods than for previous studies were examined. Data are presented also on settlement choice assays involving either a fixed small number (20) of cyprids per dish, as recommended by Rittschof *et al.* (1992), or one larva per dish only (see Clare *et al.*, 1994) to preclude any possible larva-larva interactions. Comparative quantitative analyses of film composition also were carried out, in addition to assessments of their metabolic activity, in order to more clearly define the film age parameter.

2.2.2 Material and Methods

Larvae

All cypris larvae of *Balanus amphitrite amphitrite* were obtained from Duke University Marine Laboratory (Beaufort, North Carolina, USA), where they had been reared in batch culture from stage 1 nauplii on a diet of *Skeletonema costatum* (Greville) (CCMP 1332) as described by Rittschof *et al.* (1992). Larvae were stored in the dark at 6°C until used, and only 3- and 4d old cyprids (day 0 = day of collection of newly transformed cyprids) were included in the settlement assays (Rittschof *et al.* , 1984). For each of Experiments 1 and 2 cyprids from separate single mass spawnings of a large number of adults were used.

Settlement assays

The water used in all settlement assays was 0.2 µm filtered Eddystone seawater (FSW). For the initial assay (Experiment 1, with five repeats denoted A-E) natural microbial films of different ages (1, 3, 6, 12 and 18d) were obtained by submerging polystyrene petri dishes (Falcon 1006, 50 x 9 mm; basal area 19.6 cm^2) in a roofed outdoor flow-through tank at the Citadel Hill Laboratory in Plymouth for the appropriate period of time. Water is pumped directly from Plymouth Hoe into the laboratory and enters the outdoor tank after being circulated through the laboratory's aquarium system. During the experimental period (April/May 1994) the water temperature in the tank was approximately 22°C. Following filming, the dishes were emptied and any detritus was carefully washed off by dipping each dish three times in FSW. After filling with 5 ml of FSW, 20 larvae were added to each dish. The control dishes (new, initially sterile = film age 0, Fig. 2.1) were unconditioned and contained 5 ml of FSW only. Cyprid settlement was counted after an incubation period of 24h at room temperature (24°C). The terms

"inhibition" and "facilitation" are here applied to those cases in which settlement was, respectively, significantly lower and higher than settlement for the (unfilmed) control. Larval "settlement" as used in this study refers to permanent attachment (following expulsion of cement) of a cyprid.

Experiment 2 concerned assays with single larvae (ten replicates of each treatment) in dishes with 4, 8 and 12d old films, but here the dishes were tilted during film conditioning by placing a support under one edge. Water from the flow-through tank was added until the meniscus had spread half way across the bottom of each dish (matching a prescored diagonal on the underside), so that one half (the "left hand side") of each dish could be preconditioned whereas the other half remained untreated. The designation of the left hand side for the sterile control was arbitrary. Dishes were preconditioned in the dark and the conditioning water was changed daily until commencement of the experiments. For the assays, each dish was washed as above, placed horizontally and filled with 5 ml of FSW. Controls were provided by (a) unconditioned (new, sterile) dishes and (b) dishes which had been half filled with FSW only for 12d. Orientation of dishes was randomized and, to preclude any light effects on settlement position, this experiment was run in the dark (at 28°C); attached cyprids were counted after 26h. The three repeats of this experiment all were initiated within 24h, using larvae from the same batch; there was, therefore, the possibility of cyprid age effects between the repeats of this experiment which had to be allowed for in the analysis.

Analysis of microbial films

Following repeat E (Experiment 1) all petri dishes from that experiment were preserved in formalin for acridine orange direct count epifluorescence analysis (AODC) (Parsons *et al.*, 1984) and SEM (JEOL JSM-35CF) preparation. For AODC estimates, film bacteria for four of the replicates from each of the 1, 3, 6, 12 and 18d old film treatments were counted directly from subsamples of whole dish filtrations. For SEM,

1 cm discs were cut with a heated metal cork borer from four other replicate petri dishes; these then were dehydrated through an alcohol series, critical point dried in liquid CO_2 and sputter coated with gold. The various categories of microorganisms (i.e. cocci, short rods, long rods, spirilla, vibrios, diatoms, protozoans) then were enumerated by counting ten separate fields (600 μm^2) along four randomly selected diameter transects for each disc. Activity of the microbial films was determined from the SEM counts by the frequency of dividing cells method (Hagström *et al.*, 1979).

Data analysis

Experiment 1:

All percentage data were arc-sine transformed prior to analysis. The pooled data for all five repeats were compared using the GLM procedure in MINITAB (version 8.2, 1991), with film age as the explanatory variable, number of attached cyprids as the response variable, and repeat number and cyprid age as the covariates. Since the probability values of both covariates were not significant at the 5% level, it was justifiable to analyze these as pooled data, but results for the separate repeats also are given. Analysis was by ANOVA, followed by Tukey's Multiple Comparisons for the factor film age.

Experiment 2:

There was no significant interaction between the factor film age and either of the covariates cyprid age and repeat number ($\alpha_{[2]} = 0.05$, GLM procedure, MINITAB, version 8.2, 1991). Accordingly, the analysis was carried out for the pooled data from the three repeats using log likelihood ratio *G*-tests (with Williams' correction) for contingency tables (Zar, 1984), followed by unplanned tests of the heterogeneity of replicates tested for goodness of fit (Sokal & Rohlf, 1981). The critical value for *G* was $\chi^2_{0.05\,[4]} = 9.488$. ANCOVA was carried out with the number of attached cyprids as the

response variable, Experiment repeat number as the explanatory variable and film age as the covariate.

Epifluorescence and SEM direct counts:

Comparisons between film treatments of varying ages were undertaken for the total counts of diatoms, protozoans and bacteria·cm^{-2}, and for percentages of the dividing cell counts and the seven individual film categories. Because the percentage data were not normally distributed, even after angular transformation, the analysis was carried out by Kruskal-Wallis test, followed by Tukey-type Nonparametric Multiple Comparisons (Zar, 1984). An overall general measure of film composition was obtained for the film age treatments with the Shannon-Wiener diversity index (H', computed using $\log_2$) and the derivative measure of dominance, or evenness (J, computed as H'/H'_{max}): these indices were obtained using the enumerated proportional abundances for the seven components (five bacterial types, diatoms, protozoa).

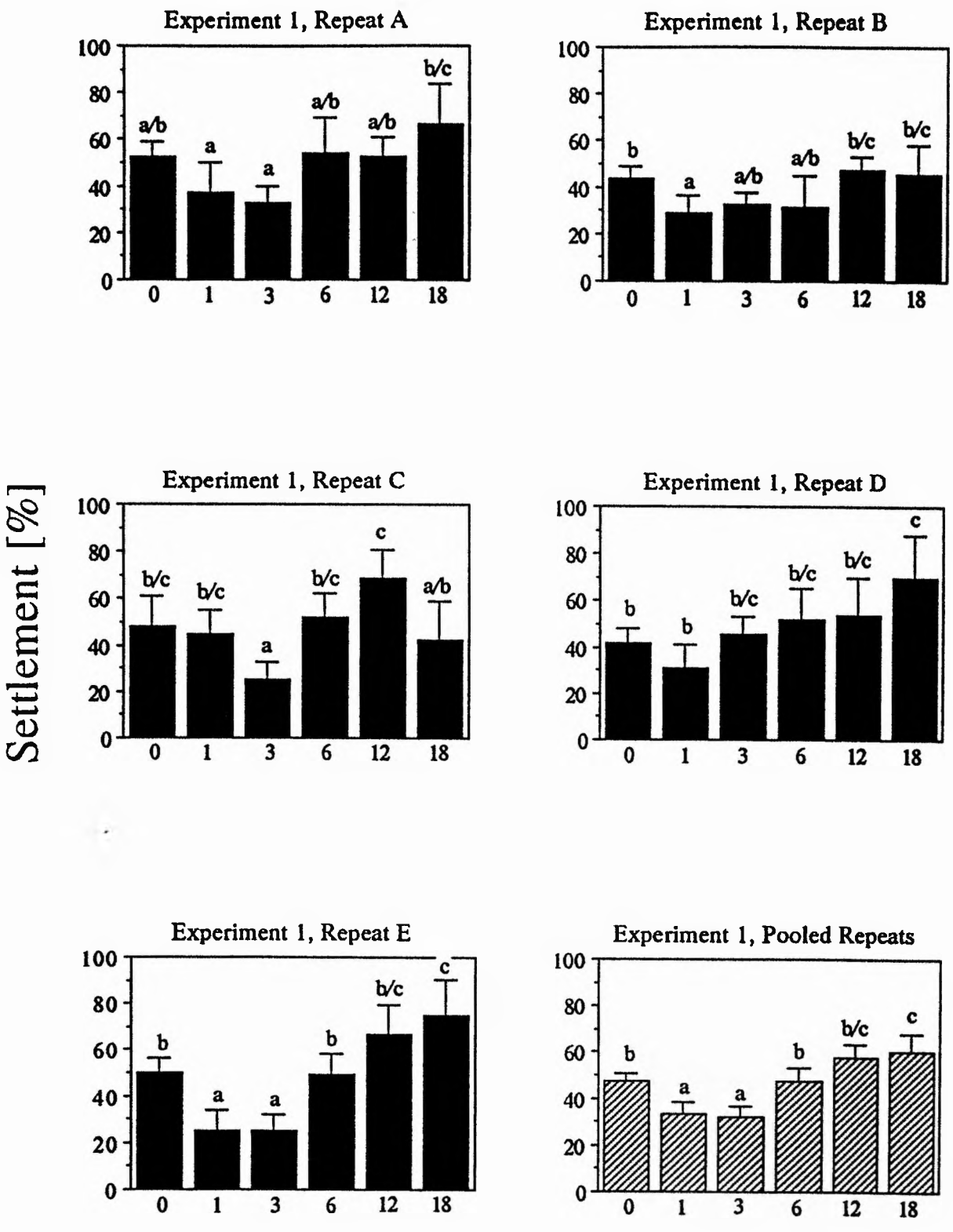
2.2.3 Results

Experiment 1

Figure 2.1 includes separate histograms for repeats A-E of Experiment 1, in addition to that for the entire pooled data set. For each of the five repeats, as well as the pooled data, the general pattern was one of inhibition by 1 and 3d old films and facilitation by 12 or 18d old films; but there were between-repeat variations in outcome (Fig. 2.1). Despite the clear overall effect of inhibition by both 1 and 3d old films (Fig. 2.1, pooled data set), among the separate repeats it was only for B, C and E that there was significantly less cyprid settlement than for the control on 1 and/or 3d old films: of these only for repeat E did both 1 and 3d old films give identical results. Similarly, it was only for D and E that there was significant facilitation by "older" films; but in all repeats except C there was significantly more cyprid settlement on 18d than 1d old films.

FIGURE 2.1

Balanus amphitrite amphitrite, Experiment 1. The histograms show mean percentages of cyprid settlers (+ 95% confidence limits). The ANOVA Tukey groups are designated a, b and c, where a indicates the grouping with percentage settlement significantly less than the unfilmed (film age 0) control, and c indicates the grouping with percentage settlement significantly greater than the control ($\alpha_{[2]} = 0.05$).


Experiment 1, Repeat A
Experiment 1, Repeat B
Experiment 1, Repeat C
Experiment 1, Repeat D
Experiment 1, Repeat E
Experiment 1, Pooled Repeats
Settlement [%]
Film Age [days]
0
20
40
60
80
100
0
1
3
6
12
18
a/b
a
a
a/b
a/b
b/c
b
a
a/b
a/b
b/c
b/c
b/c
b/c
a
b/c
c
a/b
b
b
b/c
b/c
c
b
a
a
b
b/c
c
b
a
a
b
b/c
c

Experiment 2

There were no significant between-repeat differences, thereby indicating that the unavoidable small differences in cyprid age (≤ 24h) had no effect on the outcome of the experiment. The results for the pooled data incorporating all three repeats for Experiment 2 are given in Table 2.1. Cyprid settlement on the treatment (= "left hand") side of experimental dishes bearing "old" (12d) films was significantly higher than expected for a 50:50 outcome. Conversely, settlement on the treatment side for "young" (4d) films was significantly less than the expected 50%. The additive tests of heterogeneity did, however, show that although cyprid settlement was significantly different between the above two treatments, they were not heterogeneous from the observed numbers of cyprids attaching on the "intermediate" (8d) films and for the controls. The overall outcome for this experiment thus complements that for Experiment 1 in showing that "young" films are unattractive to larvae (9/30 on film) whereas "old" films are very attractive (24/30 on film). Importantly, the outcomes of these two sets of experiments indicate also that there are no behavioural differences in responses to films for isolated individuals *versus* groups of 20 larvae.

Analysis of microbial films

Total bacterial cell counts:

In contrast to those obtained by SEM direct counting, total cell numbers·cm^{-2} estimated by AODC (Table 2.2) were significantly lower for 0- and 1d old films and significantly higher for 3, 12 and 18d old films (Mann-Whitney U tests, $\alpha = 0.05$). There were no differences between the total cell number estimates for the 6d old films ($P = 0.117$). Although quantitatively discrepant, the two methods do both show progressive and significant age-related increases in total bacterial cells·cm^{-2} (Fig. 2.2), as indicated by the letters of the Tukey groups in Table 2.2. Of interest is the observation that the 12- and 18d counts were not significantly different from one another, indicating that although cells

Table 2.1. *Balanus amphitrite amphitrite*, Experiment 2. Unplanned tests of heterogeneity of replicates tested for goodness of fit. Observed frequency distributions of cyprid settlers (given in descending order) are considered heterogeneous for additive tests with *P*-values ≤ 0.05 (n = number of replicates, FSW = 0.2 µm filtered seawater, G_{adj} = *G* adjusted by Williams' correction).

Experimental substratum	Observed frequency of settlers on treatment side*	Expected frequency of settlers on treatment side*	n	G_{adj} for additive tests of heterogeneity (highest observed frequencies first)	G_{adj} for additive tests of heterogeneity (smallest observed frequencies first)	*P*
12d old film	24	15	30		15.711	< 0.01
sterile (control)	19	15	30	1.957	7.292	> 0.05
12d exposure to FSW (control)	17	15	30	3.834	4.909	> 0.05
8d old film	16	15	30	5.430	3.204	> 0.05
4d old film	9	15	30	15.713		< 0.01

critical value for $G = X^2_{0.05[4]} = 9.488$, H_0 = 50:50 distribution

critical value for $G = X^2_{0.01[4]} = 13.277$, H_0 = 50:50 distribution

* treatment side = "left hand side" (see Materials and Methods)

Table 2.2. Experiment 1. Average counts of total bacterial cells, diatoms and protozoansof all film treatments and the control obtained by Acridine Orange direct counts (= AODC) and scanning electron microscopy counts (= SEM). Initial analysis was done by Kruskal-Wallis tests (*P*) followed by Tukey-type analysis. The letters a, b and c indicate homogeneous (α = 0.05) groups from the Tukey-type analysis (n = number of observations, SE = standard error of the means).

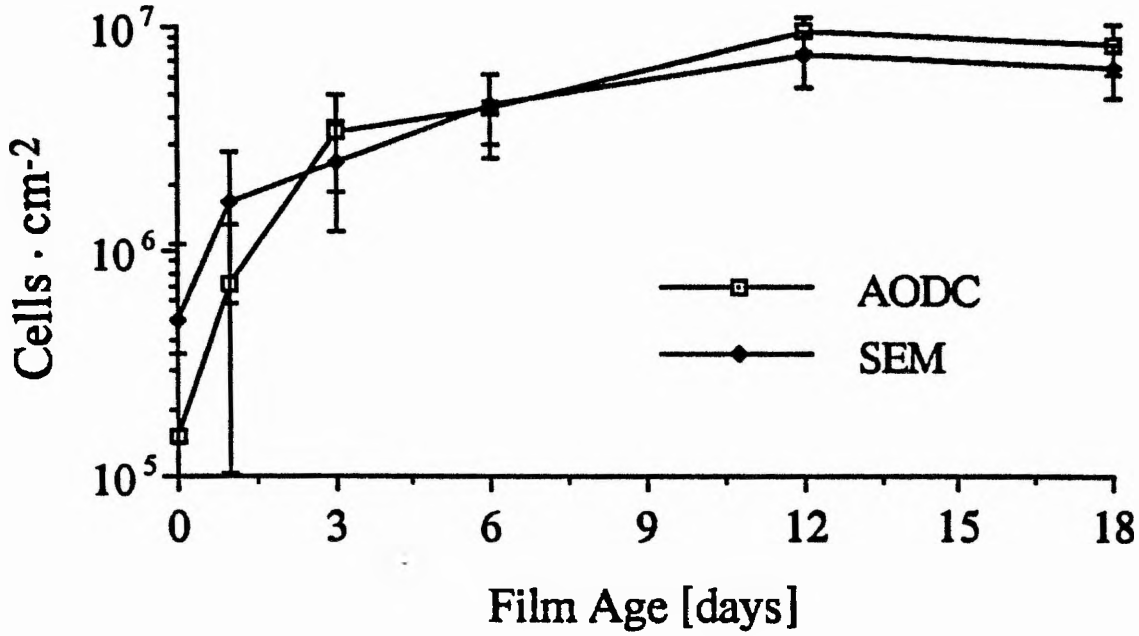
Film age [days]	Mean counts·cm^{-2}									
	AODC			SEM						
	n*	total bacteria (SE)		n**	total bacteria (SE)		diatoms (SE)		protozoans (SE)	
0	80	1.5×10^5 (2.3×10^4)	a	160	4.9×10^5 (4.7×10^4)	a	1.0×10^3 (1.0×10^3)	a	0.0 (0.0)	a
1	80	7.2×10^5 (6.9×10^4)	b	160	1.7×10^6 (8.8×10^4)	b	4.2×10^3 (2.1×10^3)	a	3.1×10^3 (1.8×10^3)	a/b
3	80	3.5×10^6 (1.8×10^5)	c	160	2.6×10^6 (1.0×10^5)	c	6.3×10^3 (2.9×10^3)	a	1.0×10^4 (3.5×10^3)	a/b
6	80	4.3×10^6 (1.9×10^5)	c	160	4.5×10^6 (1.2×10^5)	d	1.1×10^4 (3.7×10^3)	a	1.9×10^4 (5.1×10^3)	a/b/c
12	80	9.4×10^6 (1.8×10^5)	d	160	7.4×10^6 (1.6×10^5)	e	2.7×10^4 (5.7×10^3)	a	6.1×10^4 (8.9×10^3)	c
18	80	8.2×10^6 (2.3×10^5)	d	160	6.7×10^6 (1.4×10^5)	e	1.9×10^4 (4.7×10^3)	a	3.9×10^4 (7.0×10^3)	b/c
P		< 0.001			< 0.001		0.302		< 0.001	

* 4 replicates, 20 counts per treatment

** 4 replicates, 40 counts per treatment

FIGURE 2.2

Experiment 1. Mean counts (± standard deviation) of bacteria from epifluorescence (AODC) and scanning electron microscopy (SEM) for all film treatments and the control (see also Table 2.2).


10^7
10^6
10^5
Cells · cm^{-2}
AODC
SEM
0
3
6
9
12
15
18
Film Age [days]

were still dividing some must be lost in some way. It is possible that after reaching a certain bacterial density, these microfilms on polished plastic substrata undergo sloughing (Rittmann, 1989; Allison & Gilbert, 1992), either as a natural process or perhaps as a result of the preparatory rinsing of the dish. The increasing numbers of protozoans for the older film treatments also may be implicated in reducing those bacterial numbers.

Film activity:

The estimate by SEM direct counting of the frequency of bacterial cell division showed an apparent trend of decreasing activity over the investigated age range (Table 2.3), as indicated by the two overlapping Tukey groups (Fig. 2.3).

Diatoms:

Diatom counts·cm^{-2} were low (Table 2.2) and although numbers apparently increased with film age, there was no significant between-treatment difference (P= 0.302).

Protozoans:

Protozoans were absent from 0d controls but where present on other biofilms they were predominantly attributable to an *Acanthoeca*-like colonial choanoflagellate. The numbers of protozoans·cm^{-2} varied between treatments but generally increased with film age (Table 2.2).

Bacterial film composition and overall diversity:

With the exception of spirilla (P= 0.122), all other recognized bacterial categories showed significant age-related changes (Table 2.3). Short rods dominated the film composition throughout, but their average proportion declined significantly with film age, and was inversely related to the relative abundances of other categories (Fig. 2.4). The overall pattern of a general increase in complexity of the biofilms (bacteria, diatoms and protozoa) with time is reflected both by the diversity and evenness values in Figure 2.4.

FIGURE 2.3

Experiment 1. The histogram shows the mean percentages of dividing cell counts (+ 95% confidence limits) for all film treatments and the control. The Tukey-type groups are designated by the letters a, b and c above each column (Details as for Fig. 2.1).

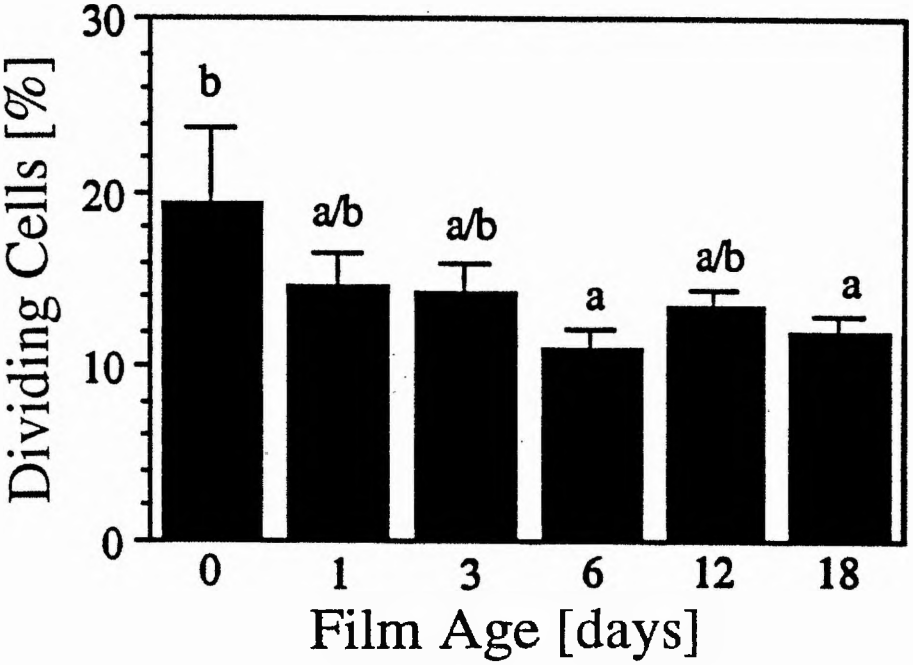
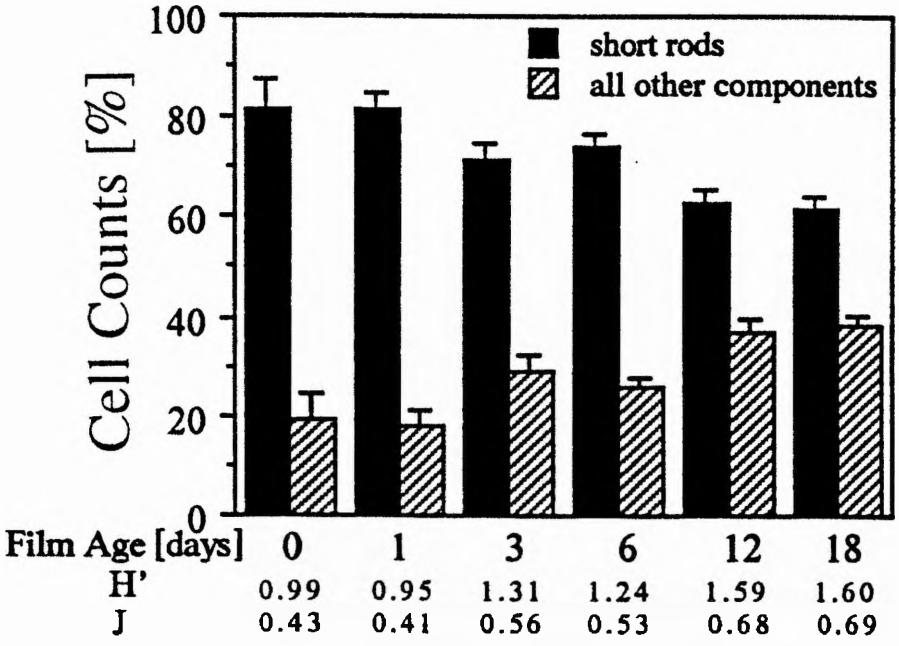

Dividing Cells [%]
30
20
10
0
b
a/b
a/b
a
a/b
a
0
1
3
6
12
18
Film Age [days]

Table 2.3. Experiment 1. Average percentages of dividing cells and recognized bacterial components of all film treatments obtained by scanning electron microscopy counts. Initial analysis was done by Kruskal-Wallis tests (*P*) followed by Tukey-type analysis. The letters a, b and c indicate homogeneous ($\alpha = 0.05$) groups from the Tukey-type analysis (n = number of observations, SE = standard error of the means).

Film age [days]	n	dividing cells (SE)	short rods (SE)	long rods (SE)	spirilla (SE)	vibrios (SE)	cocci (SE)
0	160	19.5 b (3.9)	81.7 c (2.8)	2.1 a (1.0)	0.8 a (0.5)	6.9 a (2.0)	9.0 a (2.0)
1	160	13.4 a/b (1.0)	81.5 c (1.7)	1.1 a (0.3)	1.1 a (0.3)	6.3 a (0.8)	9.2 a/b (1.2)
3	160	13.9 a/b (0.8)	71.3 b (1.8)	2.9 a/b (0.5)	0.9 a (0.2)	12.8 b (1.3)	12.3 b/c (1.2)
6	160	10.8 a (0.6)	74.2 b/c (1.2)	4.9 b (0.5)	0.7 a (0.2)	10.9 b (0.6)	9.0 a/b/c (0.6)
12	160	12.9 a/b (0.5)	63.2 a (1.3)	10.7 c (0.7)	1.1 a (0.2)	12.3 b (0.5)	12.7 c (0.6)
18	160	11.2 a (0.5)	61.9 a (1.2)	9.6 c (0.6)	0.9 a (0.2)	14.6 b (0.6)	13.1 c (0.6)
P		0.001	< 0.001	< 0.001	0.122	< 0.001	< 0.001

FIGURE 2.4

Experiment 1. The histogram shows mean percentages of bacterial cell counts for short rods and the pooled remaining four bacterial film components (+ 95% confidence limits) for all film treatments and the control. the overall diversity (H', Shannon-Wiener index) and evenness (J) computed for all seven microbial components enumerated are included also.


100
80
60
40
20
0
Cell Counts [%]
short rods
all other components
Film Age [days] 0 1 3 6 12 18
H' 0.99 0.95 1.31 1.24 1.59 1.60
J 0.43 0.41 0.56 0.53 0.68 0.69

2.2.4 Discussion

Maki *et al.* (1990, 1992) showed that components of the bacterial extracellular polymers are involved in determining barnacle cyprid electivity and the present data show clearly that the effect of a natural biofilm on the settlement response of *Balanus amphitrite amphitrite* larvae in the laboratory can change from inhibitory (less than unfilmed control) to facilitatory (greater than unfilmed control) as the film ages.

It is intuitive that larvae of intertidal invertebrates such as barnacles might, therefore, be able to obtain indirect information about the tidal height of a given substratum from components of surface biofilms alone. An apparently adaptive response was shown by Strathmann *et al.* (1981) from field experiments on the barnacles *Balanus glandula* and *Semibalanus (= Balanus) cariosus*. Those species seem able to utilize biofilms to identify their optimal tidal height for settlement on the shore (but see also Grosberg, 1982).

The present analysis of biofilms of different ages showed an increase in bacterial density and overall microbial diversity, as well as a change in metabolic activity; these shifts in film composition and physiological state are reflected in significant changes in the "attractiveness" of films to settling cyprids of *Balanus amphitrite amphitrite*. Because numbers of microalgae were very low, and did not vary significantly between film age treatments, it seems unlikely that they are implicated in overall attractiveness of films to the larvae, although they might still to some extent affect larval behaviour at settlement.That previous investigators (Maki *et al.* , 1988, 1990, 1992; Holmström *et al.* , 1992; Avelin Mary *et al.* , 1993) also found *Balanus amphitrite* larvae to respond to bacterial films, supports the suggestion that bacteria rather than microalgae are used as a settlement cue.

Avelin Mary *et al.* (1993) tested the effects on *Balanus amphitrite* settlement of films of individual bacterial strains isolated from natural biofilms associated with the adult barnacles. They found that all *Vibrio* films, and most other isolates, were inhibitory and no film facilitated cyprid settlement; this is in contrast to the present study, in which "older" films (comprising larger proportions of the shape category "vibrios") clearly

facilitated settlement. Maki *et al.* (1988, 1990, 1992) also reported the majority of their monoculture films to be either inhibitory or to have no effect on the behaviour of *Balanus amphitrite* larvae at settlement. However, Maki *et al.* (1990) did show facilitation of *Balanus amphitrite* settlement on polystyrene petri dishes bearing 1d old natural multi-species biofilms in comparison to unfilmed control dishes. Moreover, they found significantly fewer attached cyprids on dishes with 3d old films. They concluded that the predominant effect both of individual bacterial species and natural multi-species films on *Balanus amphitrite* settlement is inhibitory. Those results ascribing inhibitory effects of microbial films are not necessarily contradictory to the present findings, since facilitation was noted only for the "older" (12 and 18d) films. Significant temporal changes in film effects on the attraction of larvae to a substratum are not unusual. For example, a recent study on *Elminius modestus* by Neal and Yule (1994a) showed that the production of stimulatory and inhibitory factors within the biofilm is dependent upon biofilm age and is manifest by a reversal in cyprid responses to *Deleya marina* films. Those larvae which had temporarily attached to a film-bearing surface adhered more strongly to 4d old films than to unfilmed surfaces, but less strongly to 1 month old films.

Results of settlement assays for invertebrate larvae often are difficult to replicate, even when the same species and an identical methodology are used (see Raimondi & Keough, 1990, for review of variation in larval behaviour). Irrespective of the inevitable between-batch variation in larval responses, experimental variation is perhaps compounded when different ages or numbers and densities of larvae are utilized by different investigators. This was assessed here by undertaking assays both with groups of 20 larvae, as well as isolated individuals. Previous work specifically on *Balanus amphitrite* settlement (Maki *et al.* , 1988, 1990, 1992) has shown that cyprid age and batch of larvae do indeed affect larval settlement behaviour (e.g. Holm, 1990; but see also O'Connor & Richardson, 1994), but in a quantitative rather than qualitative way: cyprids become less discriminating with age, but still display the same response in settlement assays (Rittschof *et al.* , 1984; Mitchell & Maki, 1988). In the present study only larvae from single batches were utilized in the two experiments, and cyprid age was restricted to 3 and 4d old larvae; moreover, isolated larvae responded similarly to groups of 20 larvae

(at four larvae·ml^{-1}; approximately one larva·cm^{-2} of basal dish area), indicating that there were no density-related changes in larval behaviour at least at that larval concentration. With specific reference to barnacle cyprids it is likely that cueing of larval settlement by microbial surface films is a highly complex process, in which even slight local, seasonal and short-term temporal changes in film composition, density and physiology play an important rôle. Such is apparent from the present results which show significant changes from inhibition to facilitation as the films age.

2.3 FACILITATED SETTLEMENT OF *CIONA INTESTINALIS* (ASCIDIACEA: ENTEROGONA) LARVAE ON SUBSTRATA BEARING NATURAL MULTI - SPECIES BIOFILMS

2.3.1 Introduction

The solitary ascidian *Ciona intestinalis* (L.) is a major component of subtidal fouling communities on many natural and man-made submerged surfaces throughout most temperate regions of the world (Millar, 1953; Havenhand & Svane, 1991). Although the species occurs in aggregations in the field, this probably is the result of limited dispersal and hydrodynamic processes rather than gregarious larval settlement (Havenhand & Svane, 1991; Svane & Havenhand, 1993). Like the larvae of many other ascidian species, the tadpoles of *Ciona intestinalis* can recognize the character of a surface and, depending on the conditions found, either attach or delay settlement until a more suitable substratum is found (Torrence & Cloney, 1983; Svane & Young, 1989). Its use of surface-colonizing marine bacteria as settlement cues has recently been under investigation (Szewzyk *et al.* , 1991; Holmström *et al.* , 1992). Szewzyk *et al.* (1991) reported increased attachment of *Ciona intestinalis* larvae to surfaces coated with extracellular polymeric substances isolated from the slime-forming marine bacterial strain *Pseudomonas* sp. S9. They suggested that exopolysaccharides produced by surface bound marine bacteria non-specifically trap larvae, and thus increase recruitment onto a substratum (for further discussion of the involvement of bacterial extracellular polymers in triggering larval settlement see also Kirchman *et al.*, 1982a, 1982b; Maki & Mitchell, 1985; Maki *et al.* 1990, 1992). Specifically for *Ciona intestinalis*, Holmström *et al.* (1992) attempted to isolate settlement inhibiting bacteria from the adult ascidian tunic and the surrounding natural environment. Five of their 40 marine bacterial isolates displayed inhibitory effects against the larvae.

While the above two studies showed that *Ciona intestinalis* larvae respond to microbial cues at settlement, and that physiological/metabolic changes of the cue can be detected and responded to by the larvae, they did not test for the effects of natural multi-species biofilms. However, if any general inferences about the species' settlement

behaviour with respect to microbial cues are to be drawn it is necessary to test the effects of the intact natural biofilm community. Also, in neither of the previous studies on the effects of biofilming on *Ciona intestinalis* settlement were the exact numbers of larvae used in the experiments known. However, larval density might be of importance in settlement assays (see for example Knight-Jones (1951) for "crowding" effects in *Spirorbis* larvae), and larva-larva behavioural interactions at settlement have been reported to be important in other macrofouling species (Yule & Walker, 1985; Clare *et al.*, 1994).

In the previous experiments with the cyprid of *Balanus amphitrite amphitrite* (Chapter 2, Section 2.2) it was shown that the larvae were able to detect biofilm-age related differences in the composition, metabolic activity and density of natural multi-species biofilms. In this context it seems important to investigate whether the larvae of *Ciona intestinalis* also are capable of doing so. The objective of the present experiment was, therefore, to assess the effects of natural multi-species biofilms of varying ages on the settlement response of small known numbers (25 per treatment) of *Ciona intestinalis* larvae.

2.3.2 Materials and Methods

Larvae

Adult specimens of *Ciona intestinalis* were collected between June and August 1994 from the floating pontoons at Craobh Haven (56°13'N, 05°33'W), Argyll, western Scotland at depths between 0.5 and 6 m, using SCUBA. Larvae were obtained by artificial cross-fertilisation (Havenhand & Svane, 1991; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992). This is readily achieved throughout most of the year, since reproduction of *Ciona intestinalis* is seasonal only in so far as maturity is growth dependent (Berrill, 1947; Dybern, 1965). Eggs and sperm were removed from several freshly collected sexually mature individuals, recognizable by their full reddish oviducts

and white sperm ducts, and mixed in a finger bowl containing a small volume of 2 µm filtered seawater (FSW) at 10°C. After 1h the zygotes, which had sunk to the bottom of the bowl, were carefully separated from the excess sperm and oviductal fluid by repeatedly rinsing with FSW. The zygotes then were transferred to aerated 500 ml glass beakers of FSW. The culture water was supplemented with the antibiotics Streptomycin sulphate and Penicillin-G at final concentrations of 50 and 60 $mg \cdot l^{-1}$ respectively to reduce bacterial infestation. The embryonic/larval cultures were maintained at 10°C and the water was changed twice daily. The tadpole larvae hatched after 2d, after which they were immediately harvested for use in the experimental repeats. Eggs and larvae of *Ciona intestinalis* tend to remain trapped and clustered, in the eggs' mucus (Svane & Havenhand, 1993; Petersen & Svane, 1995), and care was therefore taken to collect free-swimming tadpoles only. A different "batch" of larvae was used for each experimental repeat.

Settlement assay

The water used throughout this experiment was FSW. For the settlement assay of *Ciona intestinalis* a similar protocol to that for *Balanus amphitrite amphitrite* Experiment 1 was adopted (Chapter 2, Section 2.2). In preliminary experiments with *Ciona intestinalis* larvae it was found that polystyrene petri dishes (Falcon 1006, 50 x 9 mm; basal area 19.6 cm^2) were equally suitable, if not better, substrata for use in settlement assays as were small roughened perspex panels. Polystyrene petri dishes also have successfully been used in experiments with *Ciona intestinalis* by Szewzyk *et al.* (1991) and Holmström *et al.* (1992).

The assay used here was repeated six times (experimental repeats denoted A-F). Petri dishes were preconditioned with natural multi-species biofilms of different ages (1, 3, 6 and 12d) by submerging them in the aquarium header tanks of the Gatty Marine Laboratory for the appropriate periods of time. The header tanks feed the laboratory's aquaria with seawater pumped from St. Andrews Bay, and the header tank reservoirs

(whose water level never drops below 50 cm) are refilled at 15 minute intervals. Water temperatures in the tanks during June-August, when preconditioning of the dishes took place, were about 14-16°C. Following preconditioning, the dishes were emptied and any detritus washed off by dipping each dish three times in FSW. After filling with 5 ml of FSW seawater, 25 larvae were added to each dish by carefully pipetting of the counted tadpoles. The control dishes (new, initially sterile = film age 0, Fig. 2.5) were unfilmed and contained 5 ml of FSW only. There were four replicate dishes of each treatment per experimental repeat (= 20 dishes), which were randomly positioned in a 4 x 5 array on a tray. All experimental repeats were carried out in the dark at a constant air temperature of 10°C. Exclusion of light was achieved by wrapping aluminium foil tightly around the tray holding the dishes and by sealing all gaps with masking tape. Larvae which (i) had attached to the biofilmed substrata by the adhesive papillae located at the anterior end of the trunk ("attached"), (ii) were attached by other body parts or obviously trapped on the biofilms ("trapped"), and (iii) were free swimming/"unattached" (see Holmström *et al.*, 1991) were distinguished and counted after 8h, and the data then were converted to percentages. Larvae which became trapped and metamorphosed at the water surface after 8h were not counted. The sequence in which the dishes were monitored for settlers was the same as that for the addition of larvae at the onset of the experiment. The terms inhibition and facilitation are here applied to those cases in which settlement was, respectively, significantly lower and higher than the settlement for the (unfilmed) control.

Data analysis

All percentage data were arc-sine transformed prior to analysis. The pooled data for the enumerated larval categories "attached", "trapped" and "settled total" (= larvae "attached" + larvae "trapped") for all six experimental repeats were initially compared individually by ANOVA using the GLM procedure in MINITAB (version 8.2., 1991), with Film Age and Experimental Repeat as the crossed factors and Number of "attached"/"trapped"/"settled total" tadpoles as the response variable(s). This initial

analysis indicated significant interactions between the factors Film Age and Experimental Repeat for all of the larval categories ($P < 0.05$). Therefore it was not justified to carry out the analysis on the pooled data, but separate analyses were done for the individual repeats. Analysis was by ANOVA for each of the categories of larvae, followed by Tukey's Multiple Comparisons for the factor Film Age. The significance criterion for all tests was $\alpha_{[2]} = 0.05$.

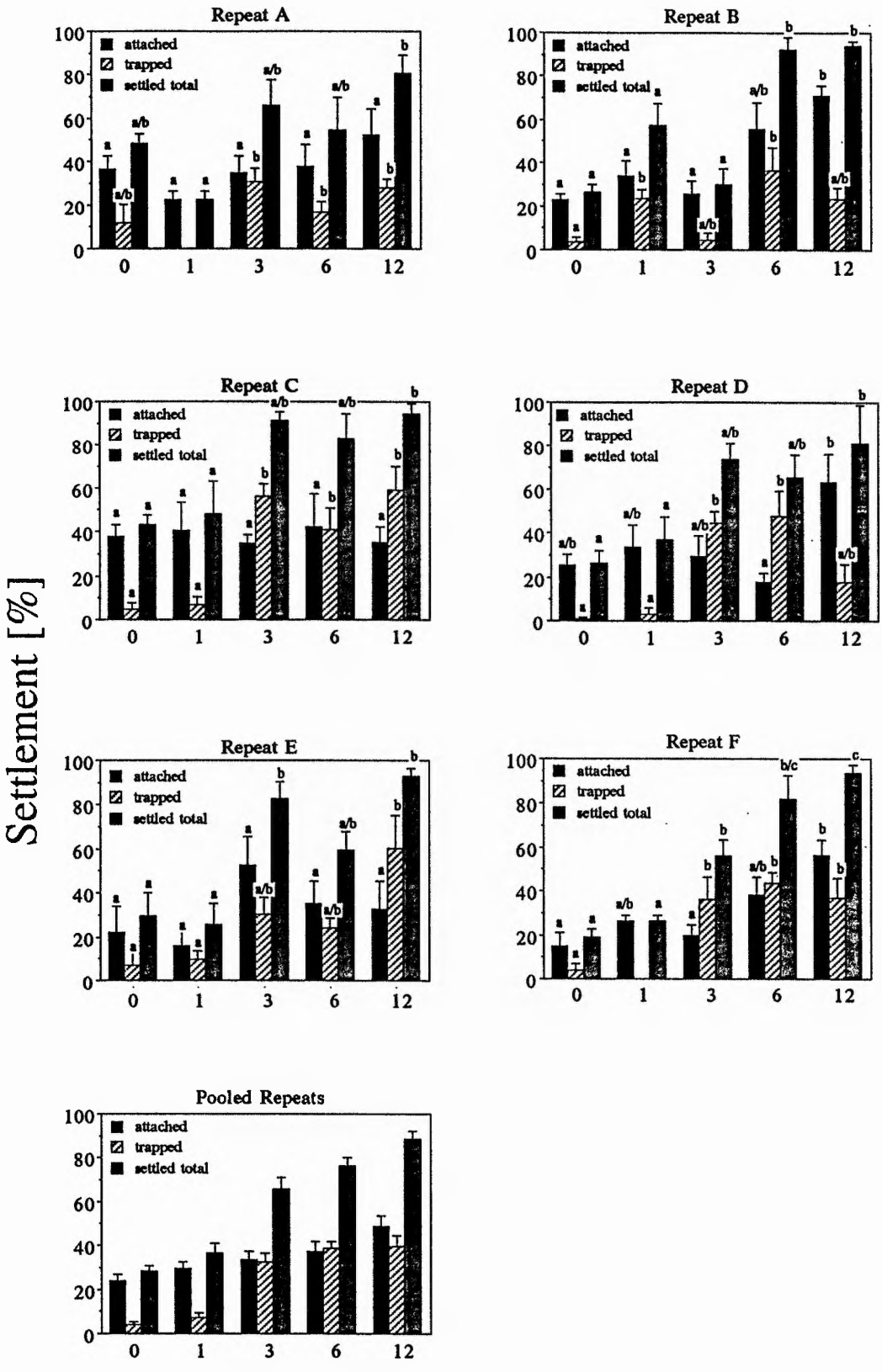
2.3.3 Results

The overall settlement success for this assay was 59%. The numbers of "attached" tadpole larvae were in general higher than those of "trapped" larvae for all biofilm age treatments. Overall numbers of larvae "attached" to the substrata increased with biofilm age, with the highest mean numbers in general counted on the 12d old biofilms (in four of the six repeats and on the controls; Fig. 2.5). However, only for Repeats B, D and F were these between-treatment differences significant: in Repeat B the substrata bearing 12d old biofilms had a markedly higher number of larvae "attached" to them, than the unfilmed control, and dishes bearing 1 and 3d old biofilms; the only significant difference in the numbers of "attached" tadpoles in Repeat D was between the 3 and 12d old biofilm treatments. Repeat F revealed significantly higher numbers of "attached" larvae on dishes bearing 12d old biofilm, than for the 1d biofilm dishes and the control (Fig. 2.5).

Between-treatment differences in the numbers of "trapped" larvae were highly significant in all of the six experimental repeats, with overall numbers of "trapped" tadpoles being lowest on the control and 1d old biofilm. The numbers of larvae settling by "entrapment" increased with biofilm age, as generally did the numbers of "attached" larvae, but here the between-treatment differences were more marked, especially in the comparisons for the controls or the 1d biofilms and the "older" biofilms (3, 6 and 12d old biofilms) (Fig. 2.5).

FIGURE 2.5

Ciona intestinalis. The histograms show the data for the separate repeats (A-F) and the pooled results. Each bar represents the mean percentages (+ standard error) of larvae "attached", "trapped" and "settled total" for all experimental treatments and the controls (film age 0). The ANOVA Tukey groups are designated a and b. Settlement onto film age treatments which do not belong to the same Tukey groups is considered significantly different at $\alpha_{[2]} = 0.05$.


Repeat A
Repeat B
Repeat C
Repeat D
Repeat E
Repeat F
Pooled Repeats
attached
trapped
settled total
Settlement [%]
Film Age [days]
0
1
3
6
12

As expected, the analysis of the two pooled categories (= "attached" + "trapped" larvae; in the Figures and above referred to as "settled total") also showed a clear overall increase in the numbers of settled tadpoles with biofilm age (Fig. 2.5). As for the analysis of the category "trapped" larvae, all experimental repeats revealed highly significant between-treatment differences. For this pooled category of settlers, significant differences were not only revealed between the treatments of the greatest age difference (e.g. between the control, or the 1 or 3d old biofilm treatments and the 12d old biofilm treatment), but also between "intermediate" aged biofilms (e.g. between the 2 or 3d old biofilm treatments and the 6d old biofilm treatment), which is also reflected by the occurrence of three rather than two Tukey groups in Repeats D and F (Fig. 2.5).

2.3.4 Discussion

The results of the present experiment showed that the settlement of *Ciona intestinalis* is facilitated onto substrata bearing natural multi-species biofilms The numbers of both "attached" and "trapped" larvae increased with biofilm age. Assuming that "attachment" essentially is a process involving a larval behavioural response to environmental cues (Szewzyk *et al.*, 1991), it seems that the facilitated settlement of *Ciona intestinalis* tadpoles onto biofilmed substrata is due to the combined effect of active habitat selection and passive deposition and "entrapment" of larvae onto the "sticky" biofilmed surface. It is interesting to note that, for the settlement category "trapped" between-treatment differences were more marked (all six experimental repeats showed significant Treatment effects) than for the category "attached" (only three out of six repeats showed significant Treatment effects). This could indicate that "entrapment" of larvae by biofilms is of greater importance to recruitment than is active substratum choice. However, both processes eventually will contribute to recruitment into the population, since "trapped" larvae are reported to metamorphose and grow into juveniles as well as initially "attached" larvae (Szewzyk *et al.*, 1991). Another possible explanation for the observed increase in "attached" larvae on substrata bearing "old"

(12d) biofilms is that as the film ages the percentage of larvae becoming "trapped" in an "attached" position increases.

Szewzyk *et al.* (1991) proposed that exopolysaccharides produced by biofilm bacteria are involved in the "trapping" of larvae (see also Decho, 1990; Holmström *et al.*, 1992; Holmström & Kjelleberg, 1995). Other authors suggested active behavioural responses through direct attraction and induction by bacterial exopolymers in the case of larvae of other macrofouling species (Maki & Mitchell, 1985; Kirchman *et al.*, 1982b; Maki *et al.*, 1990; Holmström & Kjelleberg, 1995). It is known that bacterial exopolymer production is growth phase dependent (Wrangstadh *et al.*, 1990; Allison & Gilbert, 1992; Meyer-Reil, 1994), which could explain why the ability of biofilms of the present study to "attract" and "trap" larvae changed with film age. The quantitative microbial analysis of the natural multi-species biofilms from the previous *Balanus amphitrite amphitrite* experiments (Chapter 2, Section 2.2) revealed that density, composition and metabolic activity of the biofilm community changed with film age. These biofilm parameters, as well as the occurrence of exopolymeric substances at the film surface, also vary with respect to environmental factors, such as the habitat flow regime (Chapter 3). Larvae of many marine invertebrate taxa (including ascidians) are found to respond differentially to such variations at settlement (Chapters 3 and 4 and Introduction). In view of the strong evidence for an active selection of substrata bearing biofilms of a certain "type" in other species, it would seem likely that some behavioural responses in the larvae of *Ciona intestinalis* also are involved in creating the between-treatment differences in numbers of "settled" larvae revealed in the present study. There were no observations of passive "trapping" of larvae by biofilms in any of the present assays using larvae of other macrofouling species (but see also Knight-Jones, 1951, and Cook & Henschel, 1984).

2.4 AMBIGUOUS SETTLEMENT RESPONSES OF *SPIRORBIS SPIRORBIS* (POLYCHAETA : SPIRORBIDAE) LARVAE TO BIOFILMED SUBSTRATA

2.4.1 Introduction

One of the classic groups of organisms studied in settlement assays are the spirorbids, the extensive use of which in laboratory experiments results from the fact that spirorbid larvae are very easy to obtain (see below). The larvae also have only a short planktonic life and settlement takes place within hours of liberation (Knight-Jones, 1953; DeSilva, 1958). Another advantage of the group is the fact that embryos are brooded by the parents, and thus no laboratory rearing is required (Williams, 1964). *Spirorbis spirorbis* (L.), the species used in this study, is one of the most common British species of this group. It is highly periodic in its release of large masses of larvae, which are liberated during the first and last quarters of the moon, peaking between April and October, although low numbers of larvae are liberated throughout the year, both in the laboratory and the field (pers. obs.).

Previous studies revealed that spirorbid larvae respond to a variety of environmental cues at settlement, including light, gravity (DeSilva, 1962; Dirnberger, 1993), colour and surface texture of the substratum (James & Underwood, 1994), specific algal substrata and chemical extracts from algae (Williams, 1964; Gee, 1963, 1965; Knight-Jones *et al.*, 1971; Al-Ogily, 1985) and the presence of established conspecifics (Knight-Jones, 1951; Knight-Jones & Moyse, 1961). The high degree of selectivity displayed by spirorbids (in particular with respect to their preference for specific algal substrata) is thought to be important in the process of speciation in this group (DeSilva, 1962; Williams, 1964; Crisp, 1965; Knight-Jones *et al.*, 1971). In fact, substratum preferences have been described as being more diagnostic of a certain *Spirorbis* species than are morphological features (DeSilva, 1962). There also is some evidence that substratum selectivity varies between sub-populations of the same species (Knight-Jones *et al.*, 1971).

A number of studies have addressed the effects of microbial cues on spirorbid settlement (Knight-Jones, 1951; Crisp & Ryland, 1960; DeSilva, 1962; Meadows & Williams, 1963; Williams, 1964; Kirchman *et al.*, 1982a, 1982b), and all authors agree that settlement in general is facilitated by bacterial biofilms. However, many of the earlier work on the settlement responses of spirorbids failed to include independent replication (i.e. did not account for possible between-batch differences in larval behaviour) and appropriate controls (DeSilva, 1962; James & Underwood, 1994), or completely failed to carry out any statistical data analysis (Gee, 1963; Williams, 1964). These shortcomings make a re-assessment of the effects of microbial settlement cues on spirorbid larval behaviour desirable. Also, in previous studies, too little attention has been paid to larval densities used in the assays. Larva-larva interactions at settlement seem to play a rôle in barnacles (Yule & Walker, 1985; Clare *et al.*, 1994), and Gotelli (1990) points out that gregarious behaviour (larva-adult interactions) can confound treatment effects (see also Toonen & Pawlik, 1994), especially if replication is low. In addition, spacing-out behaviour further complicates matters (see Knight-Jones, 1951, and Knight-Jones & Moyse, 1961, for a discussion of spacing-out behaviour in spirorbids).

The majority of the early studies investigating microbial cues used by spirorbid larvae considered biofilms to be static and uniform, and failed to acknowledge the complex nature of surface-bound microbial communities. These are known to change in their composition, density, metabolic activity and production of exopolymeric substances depending on growth phase, film age and environmental conditions (Fletcher, 1977; Characklis, 1981; Wrangstadh *et al.*, 1990; Szewzyk *et al.*, 1991; Allison & Gilbert, 1992). The early *Spirorbis* settlement studies on the effects of biofilms either included "young" films (12-48h preconditioning time) only, or completely failed to mention the age of the biofilms used (Knight-Jones, 1951; Crisp & Ryland, 1960; Meadows & Williams, 1963; Williams, 1964). However, evidence is growing that larvae (including those of *Spirorbis spirorbis*) are able to detect such variabilities in biofilm "type" and respond to these at settlement in the field (Todd & Keough, 1994; Keough & Raimondi, 1995; see also Chapters 2 and 3).

Meadows & Williams (1963) made an attempt to further characterize the biofilm components responsible for the enhanced settlement of *Spirorbis borealis* (now *Spirorbis spirorbis*) onto the filmed surfaces by preconditioning substrata in the presence and absence of certain uni-cellular algae (using algal cultures which were kept in the dark as control inocula). They reported higher settlement onto films developed in the presence of diatoms and their "associated bacteria" than onto films developed in the presence of the green flagellate *Dunaliella galbana*. Kirchman *et al.* (1982a, 1982b) (see also Kirchman & Mitchell, 1983, and Maki & Mitchell, 1985) proposed that the facilitated settlement of *Janua (Dexiospira) brasiliensis* (and possibly the larvae of other marine invertebrates) onto biofilmed surfaces involves the binding of lectins on the larval surface to particular glycoconjugates in the bacterial exopolymer. However, their tests, involving the use of antibiotics, indicated that viability of the biofilm was not necessary in order to facilitate settlement of *Janua* (Kirchman *et al.*, 1982a). The only experimental evidence of the effect of biofilm age (substrata preconditioned with natural multi-species biofilms for 1, 3, 5 and 7d) on the settlement response of spirorbids was also reported by Kirchman *et al.* (1982a). They observed an increase in numbers of settlers with film age for *Janua (Dexiospira) brasiliensis*.

The objective of the following experiments was to re-examine the settlement response of *Spirorbis spirorbis* larvae to natural multi-species biofilms developed over longer preconditioning periods than for previous studies. The effects of biofilms of varying ages which were incubated under illumination and in the dark were assessed. In order to investigate the possibility of behavioural differences between sub-populations, comparisons were drawn for larvae released by parents collected from the east and west coast of Scotland. Repeating the experiments with larvae from separate collections of adult organisms (from the same habitat) allowed an assessment of between-batch differences in the larval response to biofilming. Potential confounding effects related to larva-larva interactions at settlement, larval density or gregarious behaviour were accounted for by carrying out different types of assay, including substratum choice experiments either with groups of approximately 1500 larvae per repeat experiment, fixed numbers of 50 larvae per dish, or one larva per dish only.

2.4.2 Materials and Methods

Larvae

Larvae of *Spirorbis spirorbis* were obtained from adult animals epizoic on *Fucus serratus* L., the species' predominant algal substratum, collected during the neap tide periods of larval liberation. *Spirorbis spirorbis* was identified according to Hayward & Ryland (1990). East coast specimens were collected from Kinkell Braes, an area of exposed rocky shore 1.5 km south-east of the Gatty Marine Laboratory (56°20'N, 02°47'W), St. Andrews, Fife, and west coast specimens at Clachan Seil, Argyll (56°20'N, 05°35'W). The two sites are quite different from each other, in that at Clachan Seil, a very sheltered habitat, the *Fucus serratus* "zone" and the *Spirorbis spirorbis* "zone" overlapped almost entirely, while at Kinkell Braes, an exposed site (Laverack & Blackler, 1974), larger numbers of *Spirorbis* were also found growing on *Laminaria digitata* (Hudson) in the lower intertidal region and on *Fucus vesiculosus* in the higher intertidal region. The collected algae were kept in aerated circulating seawater tank at ambient temperatures: fronds were tied together by the bases of their stipes and immersed in the tanks by hanging them from lines running across the tank just below the water surface. This ensured maximal water movement around the plants and prevented their decay. The algae were kept in the dark until liberation of the larvae was required (Williams, 1964). On the evening prior to the day when an experiment was carried out, algal fronds bearing several thousand adult spirorbids were placed in a separate glass aquarium. There they were held in the dark overnight with no aeration. To initiate larval liberation, the aquarium water was replaced, and the parent organisms exposed to the overhead room light (1.5-3 $\mu E \cdot m^{-2} \cdot s^{-1}$). Two additional swan-neck fibre optic light sources were positioned by the side of the aquarium. Because the larvae of *Spirorbis spirorbis* are initially photopositive, large numbers could be readily collected in the beams of the fibre optics. If larger quantities of larvae were required (i.e. for the turntable experiment, see below) the whole volume of aquarium water was run through a filter of 40 μm mesh size, onto which the larvae were retained. Care was taken to keep the filter

immersed in a water-filled beaker throughout this process. In this way large numbers of larvae could be obtained in a small volume of water. The larvae were used in experiments immediately after liberation and collection.

Settlement assays

The water used throughout all experiments was 2 μm filtered seawater (FSW). All glass dishes were carefully cleaned prior to their use in the experiment, to prevent larvae from settling onto the glass walls of the dishes rather than onto the settlement substrata provided. Larvae do not settle readily onto unfilmed smooth glass surfaces (Knight-Jones, 1953).

In Experiment 1 small black perspex panels (3 x 3 x 0.5 cm) were used as the substrata, and these were roughened with medium coarse sandpaper and soaked in fresh water overnight prior to their preconditioning period to leach any contaminants. Although Gee (1963) described perspex as an unfavourable substratum for spirorbid settlement, preliminary data (and the present results of both laboratory and field experiments (Chapters 3 and 4)) yielded relatively high settlement rates onto such substrata (see above).

The panels were allowed to acquire natural multi-species biofilms of different ages (1, 3, 6 and 12d) by submerging them on a tray held in the seawater header tanks of the Gatty Marine Laboratory for the appropriate periods of time at ambient temperature. Panels treated in the same way as above, but remaining unconditioned, were used as the controls. One panel of each film age treatment plus a parallel control (five panels in total), were arranged at random around the periphery of six flat-bottomed circular crystallizing dishes (20 cm diameter) containing 300 ml of FSW. 50 larvae per dish were added, and the numbers of settlers onto the individual treatments were counted under a dissecting microscope after an exposure time of 24h at a constant temperature of 10°C. The dishes were scored for settlement in the same sequence as larvae were added at the onset of the experiment to minimize differences in incubation periods. Although overall settlement

success has been found to be lower in the dark than under illumination (Knight-Jones, 1951; pers. obs.), Experiment 1 was carried out in the dark because an even illumination of all experimental treatments and dishes could not be achieved, and the larvae of *Spirorbis spirorbis* are known to react to even small differences in light at settlement (Knight-Jones, 1951; pers. obs.). The experiment was repeated six times (experimental repeats denoted A-F) using larvae from the same batch of adults in two consecutive repeats each (repeats A and B, C and D, and E and F, respectively) within two days of each other.

Experiment 2 followed the methodology of the *Balanus amphitrite amphitrite* Experiment 2 (Chapter 2, Section 2.2). Polystyrene petri dishes (Falcon 1006, 50 x 9 mm; basal area 19.6 cm^2) were used as the substrata. The assay was carried out with single larvae (ten replicates of each treatment) in dishes with 1, 3, 6 and 12d old films. The dishes were tilted during film conditioning by placing a support under one edge. Water from the header tank was added until the meniscus had spread half way across the bottom of each dish (matching a prescored diagonal on the underside), so that one half (the "left hand side") of each dish would be preconditioned whereas the other half remained untreated. The designation of the left hand side for the sterile control was arbitrary. Dishes were preconditioned in the dark and the conditioning water was changed daily until commencement of the experiments. For the assays, each dish was washed as above, placed horizontally and filled with 5 ml of FSW. Unconditioned (new, sterile) dishes served as the controls. Here, the "treatment" side was arbitrarily determined before the onset of the experiment. The dishes were set out in a 5 x 10 random array, and orientation of the dishes was also randomized: to preclude any light effects on settlement position, this experiment was run in the dark (at 10°C) for approximately 48h (until all larvae had either metamorphosed or died). Occasionally a larva did not settle onto the petri dish, but metamorphosed at the water/air interface. These cases were excluded from the analysis, because it was considered possible that such larvae had become entrapped and did not necessarily make an active "choice" to metamorphose at the water surface. The experiment was repeated three times.

In Experiment 3 large numbers of larvae (approximately 500·l^{-1} final density) were exposed to all replicate film age treatments in a single round glass trough (28 cm diameter) which was mounted on a slowly revolving turntable (approximately 1.5 revolutions per minute). A sheet of white paper was placed onto the turntable and beneath the trough, and a collar of white paper was taped around the trough to facilitate an even illumination of the substrata. The trough was filled with 3 l of FSW. Here, trapezium-shaped black perspex panels (0.5 cm height, 7 cm length, and 1 and 2 cm width respectively) which fitted together to form an annular array were used to maximize the number of substrata that could fit around the periphery of the trough. Six replicate panels per treatment or control (= 30 panels) were placed randomly in the annular array but leaving 2-5 mm gaps between panels. The perspex substrata were preconditioned at ambient temperature by threading them onto a line through a hole (0.5 mm diameter) in the wider end of the panel, placing spacers between panels, and submerging them in chains for the appropriate periods of time (1, 3, 6, and 12d) in the header tanks at the Gatty Marine Laboratory. Unfilmed panels served as the controls. After their release from adult worms, larval densities were estimated and adjusted as necessary to yield the final density by carefully stirring the contents of the collecting beaker, and counting the larvae present in 2 ml of water. This procedure was repeated five times, and the mean of the counts was used as the density estimate. The collected sample then was either concentrated by gathering more larvae into the beaker or diluted with FSW as required. Larvae were added by slowly pouring the contents of the collecting beaker into the centre of the trough. The numbers of settlers were counted after 24h exposure of larvae at a constant room temperature of 10°C. The experiment was repeated eight times; four repeats (denoted G-J) were carried out with larvae from separate batches from west coast *Spirorbis spirorbis* (collected at Clachan Seil), and another four repeats (denoted K-N) with larvae from separate batches of east coast parents (collected at Kinkell Braes, St. Andrews).

Experiment 4 followed the protocol of Experiment 3 with the exception that here, two replicate panels per treatment were preconditioned with biofilm in a flow-through tank under illuminated by overhead room lights, and another two replicate panels per

treatment were preconditioned in a covered and darkened tank. The control substrata were preconditioned for 12d in FSW only: two replicate panels were conditioned under illumination, whilst another two replicate panels were incubated in the dark. The control panels were preconditioned in small trays kept in the same room as the flow-through tank, and the FSW in the trays was replaced twice daily. The experiment was repeated four times (repeats denoted O-R), with larvae released from different batches of west coast adults.

Data analysis

Experiment 1

All percentage data were arc-sine transformed prior to analysis. Each dish was treated as a repeat. Initially the effect of biofilm age was tested by ANOVA for the pooled data of all six experimental repeats, using the GLM procedure in MINITAB (version 8.2, 1991), Number of Settled Larvae as the response variable, and Film Age, Experimental Repeat (nested within batch) and Replicate Dish as the explanatory variables. There were no marked differences between dishes within experimental repeats, but because the probability values of the factors Experimental Repeat and Batch were significant at $\alpha_{[2]} = 0.05$, seperate analyses were carried out for the individual repeats as well as for the pooled data. Analysis was by ANOVA, followed by Tukey's Multiple Comparisons for the factor Film Age. The significance criterion for all tests was $\alpha_{[2]} = 0.05$.

Experiment 2

Because there was no significant interaction between the factor Experimental Repeat and the covariate Film Age ($\alpha_{[2]} = 0.05$, GLM procedure, MINITAB version 8.2,

1991), the analysis was carried out for the pooled data from the three repeats using log likelihood ratio *G*-tests (with Williams' correction) for contingency tables (Zar, 1984). These were followed by unplanned tests of the heterogeneity of replicates tested for goodness of fit (Sokal & Rohlf, 1981). The critical value for G was $\chi^2_{0.05\,[4]} = 9.488$.

Experiment 3

The pooled data of all experimental repeats were initially compared by 3-factor crossed ANOVA using the GLM procedure in MINITAB (version 8.2, 1991), with Film Age, Adult Site and Experimental Repeat (nested within Adult Site) as the factors, and Numbers of Settled Larvae as the response variable. This initial comparison revealed significant Adult Site ($P = 0.039$) and Experimental Repeat effects ($P < 0.001$). Therefore it was not justified to carry out a pooled data analysis. Instead comparisons were drawn between treatments for each separate experimental repeat. Analyses were done by ANOVA, followed by Tukey's multiple comparisons of the factor Film Age. The significance criterion for all tests was $\alpha_{[2]} = 0.05$.

Experiment 4

As for Experiment 3, the pooled data of all experimental repeats were compared by 3-factor crossed ANOVA. Film Age, Experimental Repeat and Filming Condition (light *versus* dark) were the factors, and Settlement was the response variable. This initial analysis did not reveal any differences between the numbers of settlers on panels preconditioned in the light or in the dark ($\alpha_{[2]} > 0.05$). There were, however, marked differences in settlement between experimental repeats ($\alpha_{[2]} < 0.05$). Thus, each experimental repeat was analysed by separate ANOVA, and all between-treatment comparisons were then drawn using Tukey's test.

2.4.3 Results

Experiment 1

The average total proportions of settlers per dish in this assay ranged between 14% (Repeat A) and 38% (Repeat F) (Fig. 2.6). Neither of the covariates Experimental Repeat, Batch or Dish had a significant effect on the settlement of *Spirorbis spirorbis* larvae onto the test substrata. The analysis of the pooled data revealed a Film Age effect (P= 0.003), but only for the control and the 12d biofilm treatment was there a significant between-treatment difference (Fig. 2.6). The general trend was one of increased settlement with biofilm age. However, the analysis of the individual repeats showed that only in Repeats B and C were there marked between-treatment differences (Fig. 2.6). For Repeat C a closely similar pattern to that for the pooled data was observed: only for the 12d biofilms was settlement markedly higher than on the control substrata. For Repeat B, settlement onto 3, 6 and 12d old biofilms was significantly higher than the unfilmed controls, but there were no significant differences between these treatments for the factor Film Age.

Experiment 2

The results for the pooled data for all three experimental repeats of Experiment 2 are given in Table 2.4. Larval settlement on the treatment (= "left hand") side of experimental dishes bearing 12d old biofilms was markedly higher than expected for a 50:50 outcome. The additive tests of heterogeneity showed that settlement onto the 12d biofilm treatment was significantly different from that onto the unfilmed control substrata. This apparent heterogeneity was, however, due mostly to settlement on the arbitrarily pre-determined treatment ("left hand") side of the control dishes being lower than the expected 50%. The numbers of settlers onto 12d biofilms were not heterogeneous from those of any of the other biofilm treatments.

FIGURE 2.6

Spirorbis spirorbis, Experiment 1. The histograms show the data for the separate repeats (A-F) and the pooled results. Each bar represents the mean number of settlers (+ standard error) for the various film age treatments and controls (film age 0). The ANOVA Tukey groups are designated a and b. Settlement onto film age treatments which do not belong to the same Tukey group is considered significantly different at $\alpha_{[2]} = 0.05$. Note the differences in the abundance scales applied.

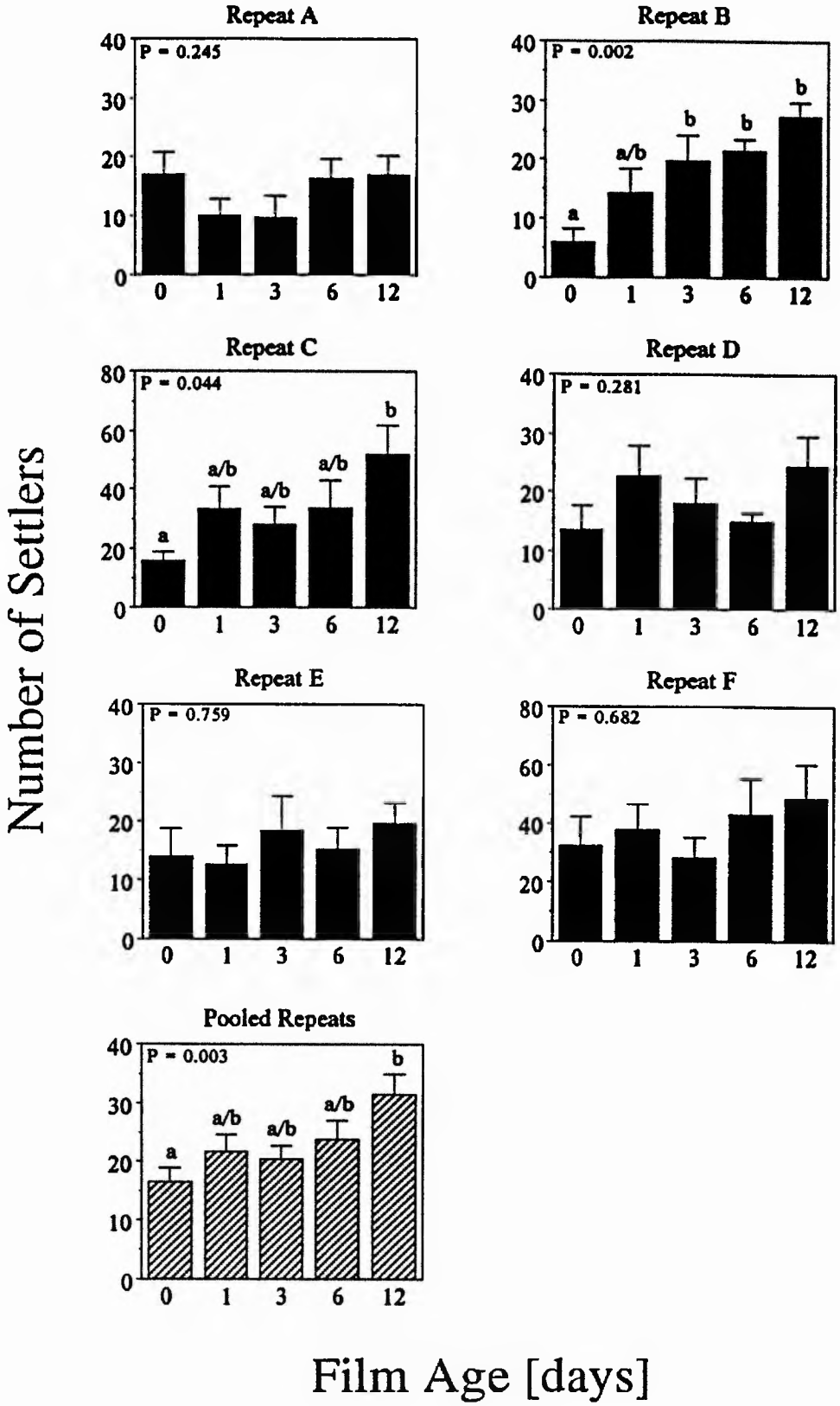

Repeat A
P = 0.245
Repeat B
P = 0.002
a
a/b
b
b
b
Repeat C
P = 0.044
a
a/b
a/b
a/b
b
Repeat D
P = 0.281
Repeat E
P = 0.759
Repeat F
P = 0.682
Pooled Repeats
P = 0.003
a
a/b
a/b
a/b
b
0
1
3
6
12
Number of Settlers
Film Age [days]

Table 2.4. *Spirorbis spirorbis,* Experiment 2. Unplanned tests of heterogeneity of replicates tested for goodness of fit. Observed frequency distributions of larval settlers (given in descending order) are considered heterogeneous for additive tests with *P*-values ≤ 0.05 (n = number of replicates, G_{adj} = *G* adjusted by Williams' correction).

Experimental substratum	Observed frequency of settlers on treatment side*	Expected frequency of settlers on treatment side*	n	G_{adj} for additive tests of heterogeneity (highest observed frequencies first)	G_{adj} for additive tests of heterogeneity (smallest observed frequencies first)	*P*
12d old film	23	14.5	29		12.444	< 0.05
3d old film	20	15	30	2.555	2.820	> 0.05
6d old film	14	14	28	7.623	0.091	> 0.05
1d old film	15	15	30	5.430	0.070	> 0.05
unfilmed control	13	14	28	10.173		< 0.05

critical value for $G = \chi^2_{0.05[4]} = 9.488$, H_0 = 50:50 distribution

critical value for $G = \chi^2_{0.01[4]} = 13.277$, H_0 = 50:50 distribution

* treatment side = "left hand side" (see Materials and Methods)

Experiment 3

The approximated spirorbid settlement per experimental repeat for this assay ranged between 26% (Repeat L) and 60% (Repeat H) The initial analysis failed to reveal any Treatment*Adult Site interactions ($P = 0.957$). Treatment effects also were found to be independent of the factor Experimental Repeat ($P = 0.742$), but there were marked between-treatment differences in the numbers of settlers ($P = 0.001$). Although there was a general trend of increased settlement with film age, only two of the eight experimental repeats, L and N (both carried out with larvae of west coast parents), showed marked Film Age effects (Fig. 2.7). However, despite of the outcome of the initial analysis, no between-treatment differences were detected for Repeat L in the Tukey test (Fig. 2.7). For Repeat N the only significant between-treatment difference revealed was a higher percentage of settlers on 6d biofilms in comparison to the control (Fig. 2.7).

Experiment 4

The overall percent settlement per experimental repeat was between 20% (Repeat Q) and 36% (Repeat P). No differences in larval settlement response were detected between substrata preconditioned with biofilm in the dark *versus* the light ($P = 0.475$), but the initial analysis revealed a significant effect of the factor Film Age on numbers of settlers ($P = 0.027$). Nonetheless, when analyzed separately, only one of the four repeats (Repeat R, Fig. 2.8) showed a marked Treatment effect; again, only the 12d biofilm treatment revealed significantly higher settlement than on the unfilmed controls. Analysis of the pooled data of all four repeats indicated significantly higher numbers of settlers on 12d biofilms than on the 1 and 6d biofilms (Fig. 2.8).

FIGURE 2.7

Spirorbis spirorbis, Experiment 3. The histograms show the data for the separate repeats G-J (larvae released from west coast parents) and K-N (larvae released from east coast parents). Each bar represents the mean number of settlers (+ standard error) for the various film age treatments and controls (film age 0). Note the differences in the abundance scales applied. Details as for Fig. 2.6.

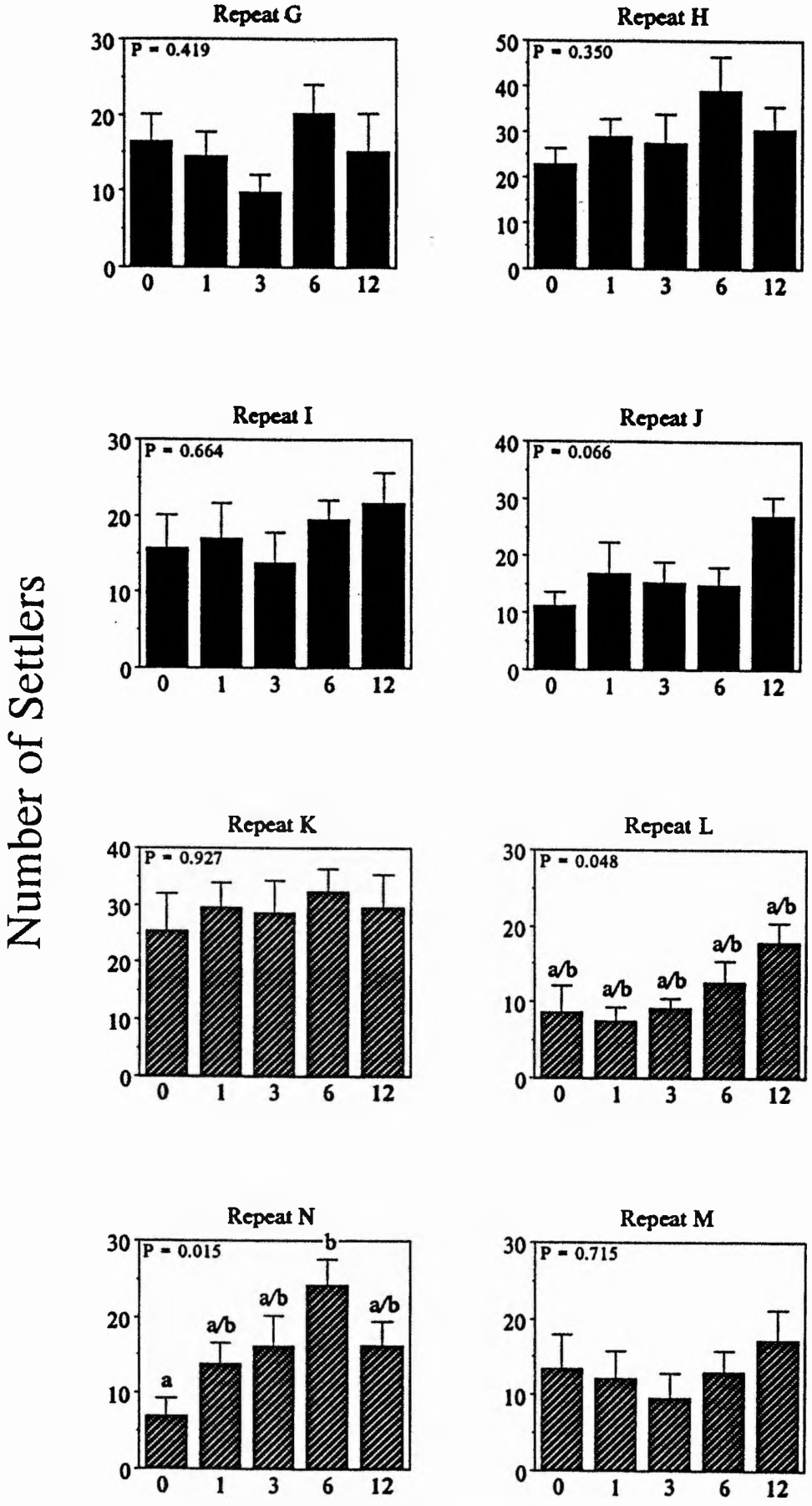
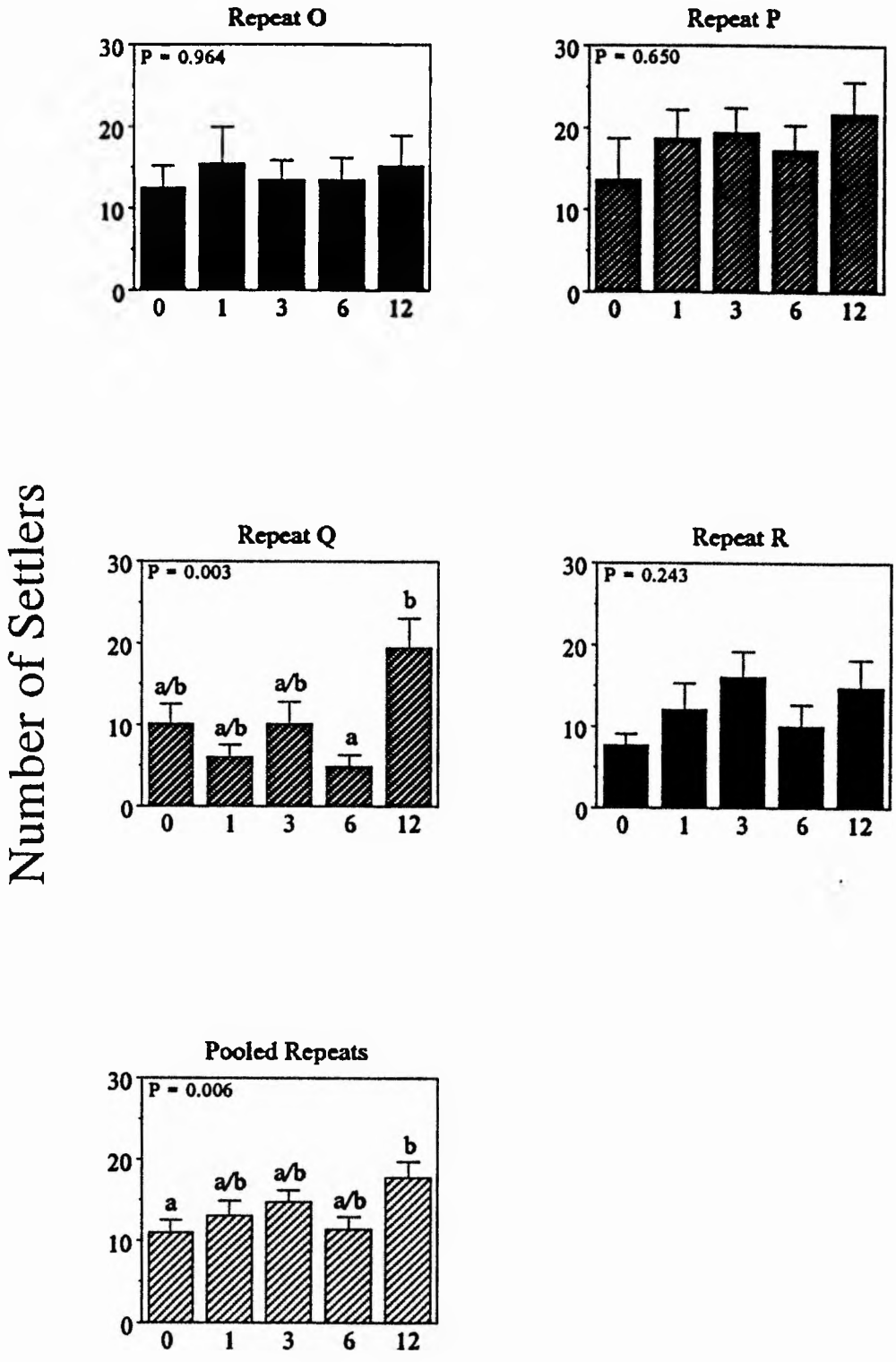

Repeat G
P = 0.419
Repeat H
P = 0.350
Repeat I
P = 0.664
Repeat J
P = 0.066
Repeat K
P = 0.927
Repeat L
P = 0.048
a/b
Repeat N
P = 0.015
a
b
Repeat M
P = 0.715
0 1 3 6 12
Number of Settlers
Film Age [days]

FIGURE 2.8

Spirorbis spirorbis, Experiment 4. The histograms show the data for the separate repeats O-R (= "dark" experiments), P-Q (= "light" experiments) and the pooled results. Details as for Fig. 2.6.


Repeat O
P = 0.964
Repeat P
P = 0.650
Repeat Q
P = 0.003
a/b
a/b
a/b
a
b
Repeat R
P = 0.243
Pooled Repeats
P = 0.006
a
a/b
a/b
a/b
b
0
10
20
30
0
1
3
6
12
Number of Settlers
Film Age [days]

2.4.4 Discussion

The results of the present study do not allow any general inferences about the settlement behaviour of *Spirorbis spirorbis* in response to microbial biofilming, other than one of filming appearing to have an enhancing rather than inhibiting effect. A general facilitatory effect of biofilming on the settlement of *Spirorbis spirorbis* was also indicated in the subsequent field experiments (see Chapters 3 and 4). The outcomes of Experiments 1 and 2 indicate significantly higher settlement onto substrata bearing 12d old biofilms than on the unfilmed controls (Fig. 2.6, Table 2.4). There was, however, no marked facilitation of settlement on any of the "younger" biofilms. The results of Experiments 3 and 4 also are ambiguous: although there was a general trend of an increase in the numbers of settlers with biofilm age (as was indicated also for Experiments 1 and 2), for only two of eight repeats in Experiment 3, and one of four repeats in Experiment 4, was the settlement response of *Spirorbis spirorbis* larvae significantly enhanced on "older" (12d) biofilms. It is possible that in the latter experiments the biofilming effects were masked by gregarious larval behaviour or pre-emption of settlement space (Gotelli, 1990; Minchinton & Scheibling, 1993). It is relevant to note here that in particular those experimental repeats with high overall settlement success rates (e.g. Repeats H and K in Experiment 3) did not display any between-treatment differences.

The above results are in clear contrast to previous studies, wherein substrata preconditioned with biofilms for only 12-24h exerted a positive (facilitatory) effect on settlement numbers in *Spirorbis spirorbis* (Crisp & Ryland, 1960; Meadows & Williams, 1963; Williams, 1964). The present study included more than one type of assay, and Experiment 3 was very similar to designs used in the past (Crisp & Ryland, 1960; Meadows & Williams, 1963; Williams, 1964). All four assays showed a similar trend of only "older" biofilms having facilitatory effects on *Spirorbis spirorbis* settlement. Accordingly, these deviations from previous results are unlikely to be due to variations in the experimental protocol. Irrespective of the number of larvae used in each assay, none of the experiments revealed significantly facilitated settlement on 1d biofilms. The assays

including a single larva per dish (Experiment 2) showed similar results to assays using numbers of larvae (50-1500) per dish (Experiments 1, 3 and 4). It is possible that some of the settlement patterns found in the latter have been amplified by gregariousness of the larvae (James & Underwood, 1994). In Experiment 1, the counting of 50 larvae per dish was time consuming and it cannot be ruled out that the results obtained in this assay are biased: thus, for example, larvae which were added to the dishes last might have been less discriminating in their substratum choice, than were freshly released individuals. The larvae of spirorbids are reported to become less discriminating in their choice of substratum, and more prone to settle, if the planktonic stage is prolonged and metamorphosis is delayed (Knight-Jones, 1953).

The possibility that more than one *Spirorbis* species was utilized in the experiments cannot be ruled out, and such might indeed account for some of the between-repeat variations observed. This possibility is discounted, however, because all larvae used in this study were liberated by adults encrusting *Fucus serratus*, the major natural substratum of *Spirorbis spirorbis* (Gee, 1963; Knight-Jones *et al.*, 1971; Hayward & Ryland, 1990). Although other spirorbid species also can be found on *Fucus serratus* (Hayward & Ryland, 1990) these are generally much more specialized in their choice of substratum, and thus sample contamination with larvae of other species can be regarded as minimal.

Experiment 4 was designed to compare settlement responses to substrata biofilmed under contrasting physical conditions (dark and light), but no significant differences were found. One should however be careful in concluding that the presence of photosynthetic microorganisms is irrelevant to the biofilming effect on settlement, especially since Experiment 4 did not account for any potential between-tank differences during biofilm preconditioning of the panels. There may also have been other differences, apart from microalgal or photosynthetic microbial abundances between light and dark conditioned panels (see Cooksey, 1992b, for discussion of interactions between bacterial and algal biofilm components).

No between-batch differences in larval behaviour were detected in the present experiments (but see Raimondi & Keough, 1990, and Toonen & Pawlik, 1994), although

there were significant between-repeat effects in Experiment 3. This result emphasizes that data of independent settlement experiments with spirorbid larvae cannot be pooled, despite this commonly having been done in the past (Kirchman *et al.*, 1982a, 1982b) and without testing for Repeat (or Batch) effects.

In view of the present results it seems that general conclusions derived from early studies of the settlement behaviour of *Spirorbis spirorbis* larvae may have been too easily drawn in the past (see also James & Underwood, 1994). Further careful re-examination and re-interpretation of some of the classical *Spirorbis* settlement assays seems necessary in order that ecologically realistic and valid conclusions might be drawn regarding spirorbid settlement in the field.

2.5 INHIBITION OF *BUGULA FLABELLATA* (BRYOZOA: CHEILOSTOMATA) LARVAE BY NATURAL MULTI-SPECIES BIOFILMS

2.5.1 Introduction

The utilization of microbial films as settlement cues has been reported for a range of bryozoan species both in the laboratory (Mihm *et al.*, 1981; Brancato & Woollacott, 1982; Mitchell & Maki, 1988; Maki *et al.*, 1989) and in the field (Todd & Keough, 1994; Keough & Raimondi, 1995). Most of the laboratory-based studies on bryozoan settlement responses have concerned the physico-chemical characteristics of substrata, such as surface free energy (Eiben, 1976; Mihm *et al.*, 1981; Roberts *et al.*, 1991; Gerhart et al., 1992), rather than the density, physiological activity and composition of the substratum-associated biofilms. However, the present laboratory experiments on larval settlement behaviour of the barnacle *Balanus amphitrite amphitrite* (Chapter 2, Section 2.2), as well as the field experiments (Chapters 3 and 4), suggest that larvae are capable of recognizing local, seasonal and film-age related differences in metabolic activity, density and/or composition of the microbial film community and of responding to these during settlement.

The model group of bryozoans used in settlement studies has been the anascan cheilostome genus *Bugula* (Lynch, 1949; 1961; Brancato & Woollacott, 1982; Roberts *et al.*, 1991; Gerhart *et al.*, 1992), and in particular the temperate/tropical species *Bugula neritina* (L.) (Ryland, 1960; Mihm *et al.*, 1981; Woollacott, 1984; Keough, 1986, 1989; Rittschof *et al.*, 1988; Mitchell & Maki, 1988; Maki *et al.*, 1989). *Bugula* larvae generally are large (300-400 μm in length, 200-300 μm in diameter) and easily released from natural colonies in the laboratory (see below).

The only experiments testing the response of *Bugula flabellata* (Thompson) to biofilming are those by Crisp & Ryland (1960), who found settlement to be facilitated on clean, unfilmed surfaces. There seems to have been some confusion in the past as to whether or not *Bugula flabellata* and *Bugula simplex* Hincks are conspecific (Ryland,

1958; 1974; Lynch, 1961), and this must lead to some confusion regarding specific behavioural responses. In contrast to the settlement responses of *Bugula flabellata* larvae — described by Crisp & Ryland (1960) — Brancato and Woollacott (1982) found the settlement of *Bugula simplex* (and that of *Bugula stolonifera* and *Bugula turrita*) to be facilitated by the presence of microbial films. The latter authors refer to *Bugula flabellata* and *Bugula simplex* as distinct species, and this seems to be the recognized opinion today (Ryland & Hayward, 1977). Identification of the species in the present study was made according to Ryland & Hayward (1977). Given the above results, indicating differential settlement of *Balanus amphitrite amphitrite* onto biofilms of varying ages (Chapter 2, Section 2.2), a re-examination of the response of *Bugula flabellata* seemed necessary. Crisp and Ryland (1960) did not test the effect of older (up to 21d old) biofilms on *Bugula flabellata* larvae in their turntable experiments (their films were said to be "24h and older"), and no mention of experimental replication was made in their report. It also is not clear whether the experiments were carried out with one or more batches of larvae (see for example Raimondi & Keough, 1990 and Toonen & Pawlik, 1995 for a discussion of behavioural differences between batches of larvae). As Crisp & Ryland (1960) state "considerable diversity of behaviour towards films exists even in these simple ciliated larvae". The present experiments therefore were designed to re-assess the effects of biofilmed substrata on *Bugula flabellata* settlement, and included a range of film ages.

2.5.2 Materials and Methods

Larvae

Colonies of *Bugula flabellata* containing mature and developing embryos in the brood sacs were collected in September 1994 from a sublittoral cliff on the mainland coast (56°16'N, 05°36'W) opposite the northern tip of the isle of Torsay, Argyll, western Scotland at depths between 6 and 15 m, using SCUBA. Colonies survived and

continued to release larvae for 2-3 weeks in the laboratory without the addition of supplementary food when kept in a circulating seawater tank at ambient temperatures. On the evening prior to the day when liberation of larvae was required, the colonies were transferred to a small glass aquarium where they were kept in the dark overnight in non-aerated seawater . To induce larval release the next morning, the water in the aquarium was changed and the colonies exposed to the overhead room light for 1h. This technique has successfully been used for *Bugula flabellata* (Grave, 1930) and a range of other *Bugula* species (Lynch, 1949; Ryland, 1960; Brancato & Woollacott, 1982; Rittschof *et al.*, 1988). The positive phototactic larvae gathered in the beam of two swan-neck optic light sources, and could be carefully captured with a small glass beaker. This method of collection is likely to cause the least disturbance or stress to the larvae, and was chosen because the behaviour of larvae of other bryozoan species has been found previously to be affected by pipetting (Ryland, 1962a). The larvae were free-swimming for about two hours after liberation.

Settlement assays

The water used in all settlement assays was 2 μm filtered seawater (FSW). All experiments were run at ambient temperature (approximately 15-16°C), and larvae released from four different collections of adult colonies were used. The first batch of larvae released by each collection of adult colonies was used in Experiment 1, and the second batches were used in Experiment 2 within a fortnight.

In one assay (Experiment 1) four repeats, denoted A-D, were carried out. Each repeat used larvae liberated by different batches of adult colonies (see above). A similar protocol to that of Crisp and Ryland (1960) was adopted: batches of larvae were added to a circular glass trough (27.5 cm diameter) containing unfilmed substrata (= controls) and substrata preconditioned with biofilms of varying ages. The trough was mounted on a slowly revolving turntable (approximately 1.5 revolutions per minute). A sheet of white paper was placed onto the turntable and beneath the trough, and a collar of white paper

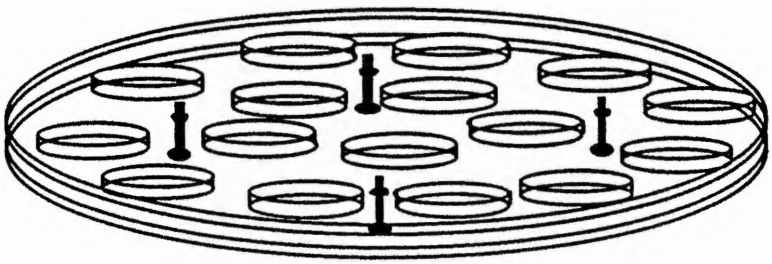
was taped around the trough to facilitate an even illumination of the substrata. Turning of the trough throughout the experiment ensures a constant change of the position of the individual substrata, and thus that all areas of the trough receive the same amount of light (Crisp & Meadows, 1963). Changes in light are known to influence the behaviour of bryozoan larvae at settlement (Ryland, 1960, 1977), and a turntable seems the most appropriate means of reducing this source of experimental error. Polystyrene petri dishes (Falcon 1006, 50 x 9 mm; basal area 19.6 cm^2) have been successfully used in other settlement studies with *Bugula* species (Mihm *et al.*, 1981; Rittschof *et al.*, 1988; Maki *et al.*, 1989) and were used here. Sixteen of these dishes, arrayed in two circles (11 in the outer and five in the inner circle), were held in place on the flat bottom of the turntable dish by means of two clear perspex discs (Fig. 2.9). The upper perspex disc had 16 holes, into which the rims of the polystyrene dishes fitted tightly from underneath. The dishes were "sandwiched" between these perspex discs, which were bolted together by plastic screws, and placed on the bottom of the circular trough. Preconditioning of the petri dishes with natural multi-species biofilms was achieved by submerging them in the seawater header tanks of the Gatty Marine Laboratory for the appropriate periods of time before an experiment. Water temperatures in the tanks in August/September, when preconditioning of the dishes took place, were 14-16°C.

Four replicate dishes, each bearing either 1, 6 or 12d old biofilms were randomly allocated to 12 of the 16 slots of the glass trough. Four replicate, new (initially sterile) dishes served as the unfilmed controls and were assigned to the remaining four slots in the perspex discs. Because conditions pertaining to the inner ring of dishes could not be assumed to be the same as for the outer ring of dishes, the two sets were treated as separate blocks in the data analysis (see below). It was therefore necessary that one replicate dish per treatment and control was randomly allocated to one of the five inner ring slots (the fifth inner ring slot was haphazardly filled with a replicate of any of the biofilm treatments or the control). The experiments were carried out under illumination provided by a 60 Watt white light bulb desk lamp positioned centrally 1 m above the trough (85 $\mu E \cdot m^{-2} \cdot s^{-1}$). Approximately 200 larvae per litre (final density 600 larvae) were added in each experimental repeat to the glass trough. Larval densities were

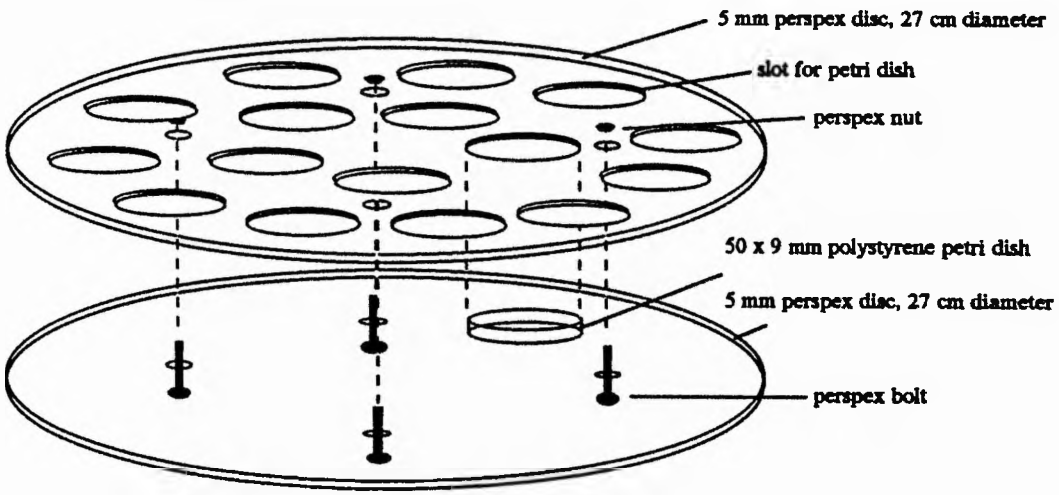
FIGURE 2.9

Bugula flabellata. Diagram of the glass trough and perspex disc arrangement for holding the experimental petri dish substrata used in settlement Experiments 1 and 2.

assembled:



dismantled:



estimated and adjusted as necessary to yield this final number (as described for *Spirorbis* larvae in Section 2.4.2, Experiment 3). Larvae were added by slowly pouring the contents of the collecting beaker into the centre of the trough. Experiments were left for 8h, after which settled and metamorphosed individuals on the dishes were counted under a dissecting microscope, and percentage settlement success onto the dish treatments were enumerated.

A second assay (Experiment 2) included four repeats denoted E-H. Each repeat was carried out with larvae liberated from a different batch of adult colonies (see above). The same protocol was applied as for Experiment 1, except that the glass trough (holding the larvae and settlement substrata) here was not revolved on a turntable. Instead the experiments were run in the dark to preclude any differences in light over the individual substrata might receive, by placing an inverted dark plastic bowl over the trough. Additionally, a black bin liner was pulled over the plastic bowl. Initial water temperatures in the glass trough were between 11 and 12°C. Treatments, controls and experimental times were as for Experiment 1.

Data analysis

The statistical analysis was the same for Experiments 1 and 2. The pooled data of all repeats of each Experiment were initially compared by 3-factor crossed ANOVA using the GLM procedure in MINITAB (version 8.2, 1991), with Film Age, Experimental Repeat and Block (position of dish treatment in the glass trough: block 1 = "outer ring", block 2 = "inner ring") as the factors and Settlement as the response variable. Since this initial comparison revealed significant Experimental Repeat (Experiment 1) and Block effects (Experiments 1 and 2) at the 5% level it was not justifiable to carry out pooled data analyses. Rather, comparisons between treatments were drawn for each separate experimental repeat by 2-factor ANOVA, with Film Age and Block as the crossed factors, and Settlement as the response variable. Because the replication of film age treatments within blocks was unbalanced, the GLM procedure in MINITAB was applied.

For those experimental repeats that showed no significant Block or Block*Film Age interaction effects, the initial ANOVA was followed by Tukey's multiple comparisons of the factor Film Age (for the data of Blocks 1 and 2 pooled). The significance criterion for all tests was $\alpha_{[2]} = 0.05$.

2.5.3 Results

Experiment 1

The experimental replicates of Experiment 1 showed total settlement onto experimental substrata at levels ranging between 25% (Repeat D) and 46% (Repeat C). There was an obvious trend of *Bugula flabellata* settlement being higher on the outer than on the central ring of substrata ($P < 0.001$ for analysis of the pooled data of all experimental repeats). The analysis of the pooled data also revealed highly significant differences in the numbers of settlers among experimental repeats.

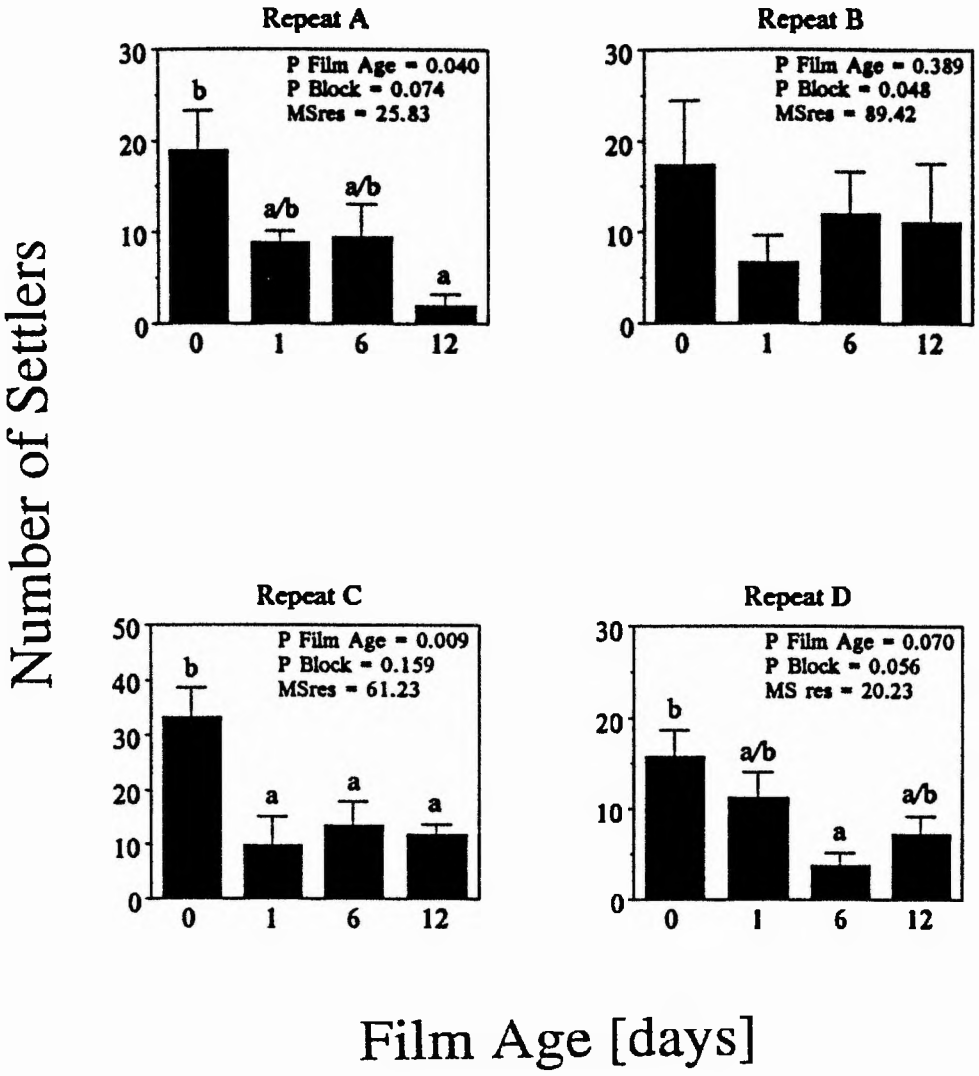
For three out of the four repeat experiments (Repeats A, C and D), *Bugula flabellata* settlement was significantly inhibited by one or all three biofilm treatments. Although in Repeat D film age did not appear to have an effect on settlement at the 5% level, the Tukey test revealed markedly lower numbers of settlers on 6d old biofilms than on the control (Fig. 2.10). No differences among treatments were detected for Repeat B; there was, however, a marginally significant difference between the blocks ($P = 0.048$).

Experiment 2

Total settlement levels here ranged between 10% (Repeat G) and 24% (Repeat E). The analysis of the data for all repeats pooled revealed marked between-block differences ($P = 0.001$), with *Bugula flabellata* settlement on the central ring of substrata being higher than on the outer ring (cf. Experiment 1, above). Significant differences in numbers of

FIGURE 2.10

Bugula flabellata, Experiment 1. The histograms show the data for the separate repeats (A-D). Each bar represents the mean number of settlers (+ standard error) for the various film age treatments and the controls (film age 0). The ANOVA Tukey groups are designated a and b. Settlement onto film age treatments which do not belong to the same Tukey group is considered significantly different at $\alpha_{[2]} = 0.05$.


Repeat A
P Film Age = 0.040
P Block = 0.074
MSres = 25.83
b
a/b
a/b
a
Repeat B
P Film Age = 0.389
P Block = 0.048
MSres = 89.42
Repeat C
P Film Age = 0.009
P Block = 0.159
MSres = 61.23
b
a
a
a
Repeat D
P Film Age = 0.070
P Block = 0.056
MS res = 20.23
b
a/b
a
a/b
0
1
6
12
Number of Settlers
Film Age [days]

settlers also were noted among experimental repeats ($P = 0.049$) and a significant Repeat *Film Age interaction ($P = 0.011$) was revealed.

Settlement was significantly inhibited by one or more biofilm treatments (Fig. 2.11) in two out of the four experimental repeats (Repeats E and H). However, there was no difference among the treatments in Repeat G, in which overall numbers of settlers were low. Although in Repeat F settlement was higher on the unfilmed controls and the 1d old biofilms than on the 6 and 12d old biofilms, only between the 1 and 6d old film treatments was this difference statistically significant. There was a marked Block effect ($P = 0.004$) for Repeat G.

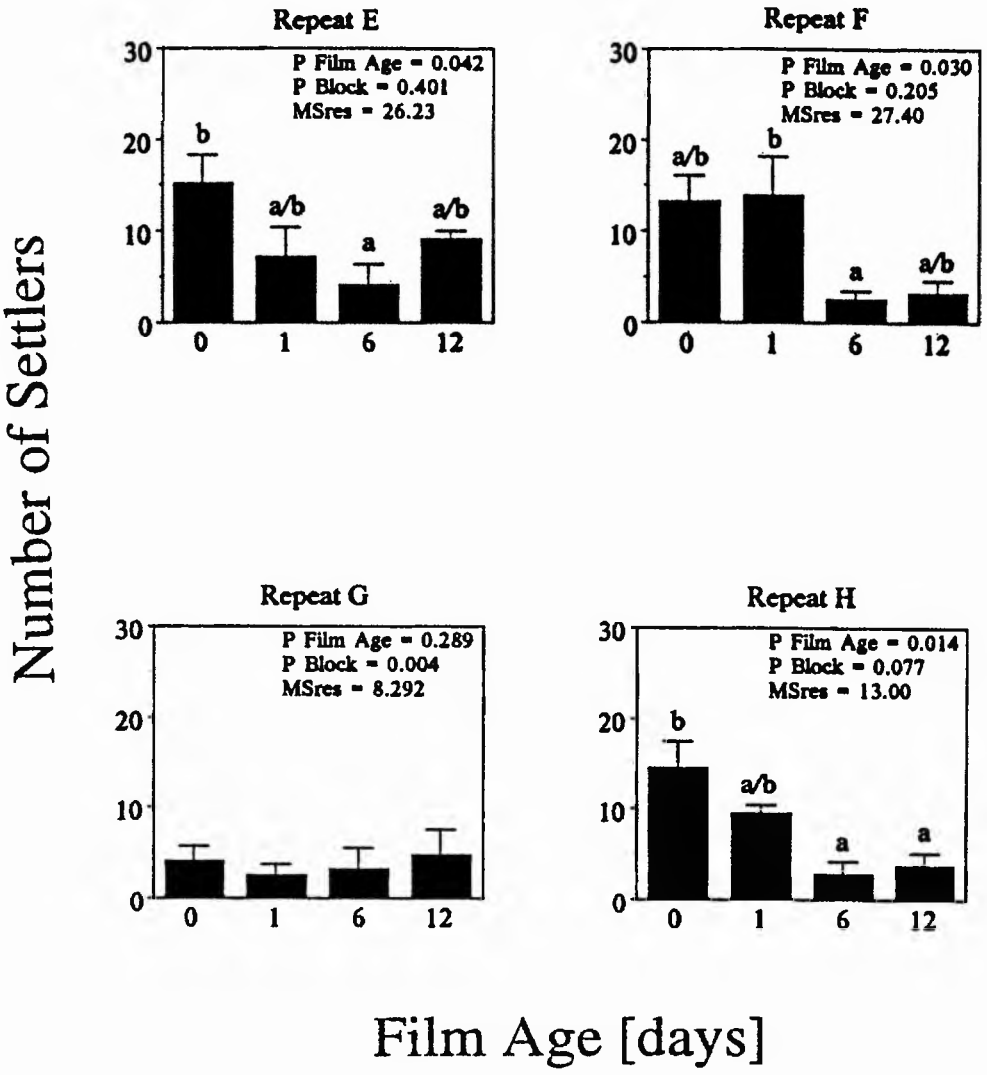
2.5.4 Discussion

The outcome of both of these experiments on *Bugula flabellata* support Crisp & Ryland's (1960) demonstration of an inhibitory effect of biofilmed substrata. Although inhibition by biofilms was not shown in all repeats for either of the experiments, all significant between-treatment effects revealed (with the exception of the Film Age effect in Experiment 2, Repeat F), were due to significant differences between one or more biofilm treatments and the unfilmed control substrata (in five out of a total of eight repeats).

Neither of the two experiments showed an obvious pattern of any gradual increase in the inhibitory effect of biofilmed surfaces with film age (Fig. 2.10 and 2.11). Thus, although *Bugula flabellata* larvae tend to "choose" unfilmed substrata over substrata bearing biofilms, film age does not seem to affect larval behaviour at settlement. It would be possible to speculate that the effect of biofilmed surfaces on *Bugula flabellata* larvae is due to a difference in physical properties (e.g. surface free energy) between unfilmed and filmed substrata, and that once a biofilm is established successional changes in density, composition or physiological activity of the microbial community have no impact on those physical properties, and thus on larval behaviour. Consequently, the complex biochemical cues provided, and possibly constantly changed, by the biofilm microflora

FIGURE 2.11

Bugula flabellata, Experiment 2. The histograms show the data for the separate repeats (E-H). Details as for Fig. 2.10.


Repeat E
P Film Age = 0.042
P Block = 0.401
MSres = 26.23
b
a/b
a
a/b
Repeat F
P Film Age = 0.030
P Block = 0.205
MSres = 27.40
a/b
b
a
a/b
Repeat G
P Film Age = 0.289
P Block = 0.004
MSres = 8.292
Repeat H
P Film Age = 0.014
P Block = 0.077
MSres = 13.00
b
a/b
a
a
0
10
20
30
0
1
6
12
Number of Settlers
Film Age [days]

may allow for a more finely tuned response repertoire in the larvae of other species (as suggested for *Balanus amphitrite amphitrite* above). However, there is as yet no evidence that *Bugula flabellata* larvae are inhibited by biofilm-associated physical cues rather than biochemical cues at settlement. It is interesting to note, however, that Kirchman & Mitchell (1983) and Maki & Mitchell (1985) mention preliminary results indicating a similar involvement of lectins in the settlement response of other *Bugula* species as has been found for the larvae of *Janua (Dexiospira) brasiliensis* (Kirchman *et al.*, 1982a, 1982b).

Whereas the turntable experiment (Experiment 1) showed significantly higher settlement on the outer ring of substrata, the general direction of the Block effect was reversed in the "dark" experiment (Experiment 2), for which the highest numbers of settlers were counted on the inner circle. It is possible that in spite of the very slow turning speed of the turntable motor (approximately 1.5 revolutions per minute), larvae in Experiment 1 were pushed to the edge of the glass trough through a centrifugal force. The elevated settlement levels for the inner ring of substrata in Experiment 2 were probably due to rapid larval settlement after larvae were added to the centre of the glass trough. It is possible that many of the larvae did not swim very far before permanent attachment and metamorphosis, and thus they did not reach the outer ring of substrata. This would imply that the larvae did not really "choose" between all possible substrata, but encountered only some of the substratum "types" (treatments). Stirring of larvae in the collecting beaker (although done as carefully as possible) to determine densities might have been responsible for some of the behavioural variability displayed in the experiments, and for the differences in settlement levels among repeats. None the less, it is likely that the variability in settlement responses among repeats is to some extent due to behavioural differences between batches of larvae (Raimondi & Keough, 1990). It is possible also that larvae did not settle independently of each other, which could have generated bias in the observed settlement patterns (Gotelli, 1990). Although there are no published accounts of gregarious behaviour in *Bugula flabellata*, the larvae of the related species *Bugula neritina* have been reported to respond differentially to siblings, whilst settling independently of unrelated larvae (Keough, 1984a, 1989). While this is not

gregariousness in the true sense, facilitated settlement in the presence of kin may have confounded the effects of biofilming to some extent.

The apparent preference of *Bugula flabellata* for clean unfilmed substrata is somewhat in contrast to the species' being reported as predominantly found attached to other bryozoans (Ryland & Hayward, 1977), since it seems much more likely that a newly disturbed (unfilmed) area on a hard substratum would be free of biofilming than the surface of an established bryozoan colony. Nevertheless, many sessile marine fouling organisms possess some means of anti-microfouling properties (e.g. Wahl, 1989). However, *Bugula flabellata* has been reported also to occur on stones and boulders (Ryland, 1962b), and was found to grow directly on vertical rock substrata (pers. obs.).

2.6 DISCUSSION

The larvae of all four species used in the present laboratory assays responded differentially to filmed and unfilmed substrata. Although the experimental protocols applied for the individual species varied, some comparative conclusions can be drawn: while the effect of natural biofilms on the settlement response of *Balanus amphitrite amphitrite* larvae was found to change from inhibitory to facilitatory as the films aged, settlement of *Ciona intestinalis* was clearly facilitated on filmed substrata of all ages. The larvae of *Bugula flabellata* generally were inhibited by filming (irrespective of film age), and the settlement of *Spirorbis spirorbis* was found to be enhanced on "older" (12d) films only. The above experiments revealed that differences in biofilm density, metabolic activity and composition related to film-age can be detected by the larvae of certain invertebrate taxa (e.g. *Balanus amphitrite amphitrite*) under laboratory conditions, resulting in differential responses to "young" *versus* "old" biofilms.

In their recent field study Keough & Raimondi (1995) found no major differences in the patterns of recruitment onto substrata bearing laboratory-derived films and substrata preconditioned with natural field microbial films. However, it should be borne in mind that all information obtained through laboratory assays in isolation are of only limited value to the assessment of settlement responses to natural biofilms in the field. For example, the present laboratory assays clearly showed facilitated settlement of *Ciona intestinalis* larvae onto biofilmed substrata, but Keough & Raimondi (1995) failed to detect any responses of this species to filming. Unfortunately *Spirorbis spirorbis* was the only one of the four taxa used in these present laboratory experiments which also recruited in the field experiments described below (Chapters 3 and 4). *Spirorbis spirorbis* larvae here, however, in general responded similarly (facilitated settlement onto filmed substrata) in both laboratory and field experiments.

The above experiments strongly emphasize the need for both replication and repetition in laboratory settlement assays. In the past conclusions about the settlement responses of entire species were too hastily based on too few experiments, with sometimes inadequate design (see Introductions and Discussions of the Sections 2.2-2.5

above). The present study also shows how variations in the experimental protocol can potentially lead to different conclusions about a species' behaviour at settlement, and points to the importance of employing more than one type of assay, if any general inferences about a species' settlement behaviour are to be made.

CHAPTER 3

THE EFFECTS OF VARIATION IN FLOW REGIME AND BIOFILMING OF HARD SUBSTRATA ON SETTLEMENT OF MARINE INVERTEBRATE LARVAE IN THE FIELD

3.1 INTRODUCTION

Microorganisms are the first colonizers of any surface submerged in the sea. Their attachment precedes that of macroinvertebrates and microbial attachment to surfaces can be detected in as little as a few hours after submersion (Mihm *et al.*, 1981; Characklis & Cooksey, 1983, Abarzua & Jakubowski, see also Chapter 1, Section 2). Much laboratory evidence has shown that microbial biofilms play an important rôle as one of the settlement cues used by marine invertebrate larvae, and thus in processes determining epifaunal assemblage dynamics (see Todd & Keough, 1994, and references therein, and Chapter 1, Section 3). Although an influence of biofilms on substratum selection is not found for all macrofouling species investigated (Roberts *et al.*, 1991; McGrath *et al.*, 1994), the response of larvae to microbial cues has been well established for a wide range of taxa (e.g. Neumann, 1979; Eckman, 1983; Hudon *et al.*, 1983; Maki *et al.*, 1988, 1989, 1990, 1992; Raimondi, 1988, Fitt *et al.*, 1990; Johnson *et al.*, 1991; Holmström *et al.*, 1992; Leitz & Wagner, 1993; Parsons *et al.*, 1993; Neal & Yule, 1994a, 1994b; Johnson & Sutton, 1994; Todd & Keough, 1994; Keough & Raimondi, 1995).

Depending on larval species, biofilm age, and the assay used, the effect of biofilming could be either facilitatory or inhibitory (see also Chapter 2). Brancato & Woollacott (1982) observed that three species of *Bugula* significantly preferred biofilmed over "non-filmed" substrata when offered a choice. Parsons *et al.* (1993) found settlement of the scallop *Placopecten magellanicus* to be enhanced by biofilming, whereas settlement of the solitary ascidian *Ciona intestinalis* was inhibited in the presence of a certain marine bacterial species (Holmström *et al.*, 1992). Maki *et al.* (1988) proposed a general inhibition of barnacle, *Balanus amphitrite*, cyprid settlement by bacterial surface

films. However, there is as yet a distinct lack of repeated field studies for an appropriately wide range of invertebrate taxa (see Rodriguez *et al.*, 1993; Todd & Keough, 1994; Keough & Raimondi, 1995). Furthermore, Raimondi & Keough (1990) have discussed the marked plasticity in the behavioural responses of larvae to settlement cues, and point to the possible importance of spatial and temporal variations in both physical and biological aspects of the environment.

If biofilms, or certain components thereof, serve as cues for settling larvae, then site-specific differences in film composition or physiological condition may enable larvae to identify and distinguish between sites of high and low survival value. Strathmann *et al.* (1981) showed a stimulatory effect of biofilm on the barnacles *Balanus glandula* and *Semibalanus cariosus* in the field, and described how microbial cues may be utilized by larvae to identify their optimal tidal height for settlement on the shore (but see also Grosberg, 1982). Raimondi (1988) was able to show that removal of the cyanobacterium *Calothrix crustacea* from natural hard substrata induced settlement of the barnacle, *Chthalamus anisopoma*, above this species' vertical distribution in the field. However, Todd & Keough (1994) found no inhibitory effect of an intertidal source of biofilming on subsequent sublittoral settlement for a wide array of taxa.

Other factors influencing successful recruitment and survival probability of a larva are surface shear stress characteristics and flow regime found at the site of settlement (e.g. Marszalek *et al.*, 1979; Rittschof *et al.*, 1984; Wethey, 1986; Butman, 1987; Butman *et al.*, 1988; Mullineaux & Butman, 1991; Pawlik *et al.*, 1991; Gross *et al.*, 1992; Dolmer & Svane, 1993; Mullineaux & Garland, 1993; Pawlik & Butman, 1993; Abelson *et al.*, 1994; McKinney & McKinney, 1994). It has been reported that bacterial and microalgal assemblages in even adjacent habitats segregate into discrete populations in response to physical, chemical and biological factors (Hudon & Bourget, 1981; Davis *et al.*, 1983; MacLulich, 1986, 1987). Shear stress is known to have a marked impact on surface-bound microbial communities (Korte & Blinn, 1983; Characklis & Cooksey, 1983; Nowell & Jumars, 1984; Rittmann, 1989; Allison & Gilbert, 1992; Wolfaardt & Cloete, 1992; Lau & Liu, 1993), and it would appear to be adaptive if larvae were able to utilize these differences in biofilm characteristics to find their optimal (adult) flow regime.

Neal & Yule (1994b) describe how multi-species biofilms developed under contrasting flow regimes affected the tenacity of settling barnacle cyprids (*Elminius modestus* and *Balanus perforatus*) under stillwater laboratory conditions. They found thin, dense biofilms, associated with high shear (83 s^{-1}), to produce increased larval tenacity, and thick, less dense films, associated with low shear (15 s^{-1}), to afford comparatively less tenacity in both species.

The present study concerned the settlement responses of marine invertebrate larvae to natural multi-species biofilming of hard substrata in the field. The objective was to document specific differences in the responses of a wide range of taxa to biofilming and the presence of "early" settlers. Furthermore, comparisons were drawn of the effects of contrasting flow regimes, within the same habitat (a tidal strait), both on biofilms and larval settlement. Black plastic (perspex) panels were used as artificial settlement substrata. Prior to their exposure to larval settlement, panels were biofilmed *in situ* by enclosing them in tight-fitting removable mesh screens which prevented larvae from reaching the panel surfaces. In order to assess the possible effects of netting on biofilm formation, and to compare biofilms between sites, total biofilm cell density, bacterial growth activity of whole biofilms, chlorophyll *a* content, and differences in the biofilm surface abundance of bacteria, protozoans, diatoms, fungi, extracellular polymeric substances (EPS), organic debris and sediment particles were measured.

3.2 MATERIALS AND METHODS

Study site

The field experimental site was Clachan Seil, west Scotland, which is a very sheltered tidal strait of approximately 30 m width and 1km length separating Seil Island (56°20'N, 05°35'W) from the Argyll mainland (Fig. 3.1; Plate 3.1.). There are tidal sills at both ends of the narrows, and as a result the spring tides ebb to the same level between the sills irrespective of open coast fluctuations; hence, the enclosed narrows are

FIGURE 3.1

Map showing the location of the study site, Clachan Seil, on the west coast of Scotland.

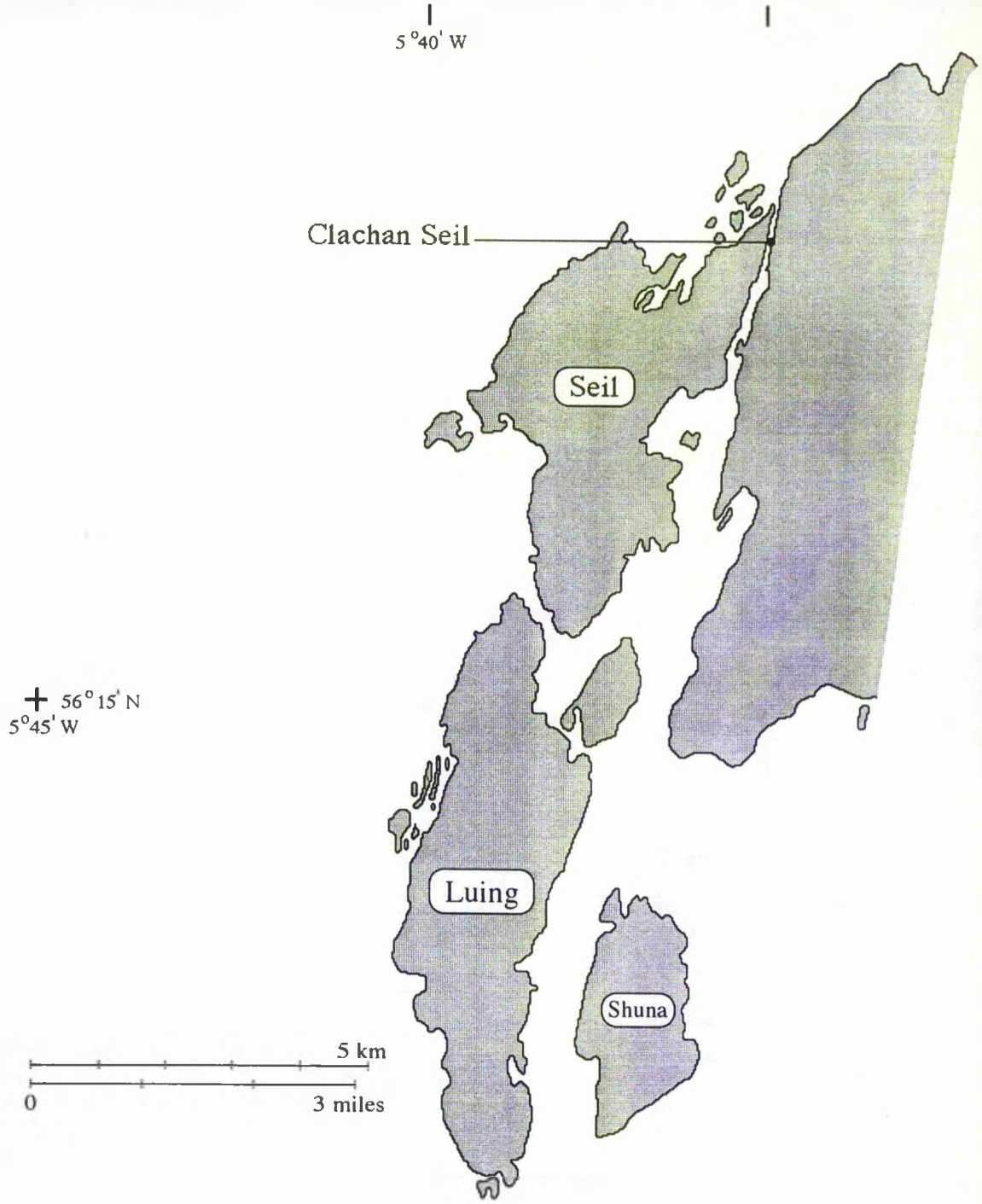

5°40' W
Clachan Seil
Seil
56°15' N
5°45' W
Luing
Shuna
5 km
0
3 miles

PLATE 3.1

Clachan Seil at low tide: looking north from Atlantic Bridge towards Firth of Lorne (top), and looking south at Atlantic Bridge and towards Seil Sound (bottom).

never emersed (see Todd & Turner, 1986). This feature, together with the fact that the narrows provide a very sheltered locality, renders the site ideal for sublittoral field experiments involving the deployment of unattached panel-frames and the placement and retrieval of experimental frames is possible without diving.

Field experiments

Black perspex panels (15 x 15 x 0.5 cm) served as settlement substrata. The downward facing side of the panels were used as the experimental surfaces to prevent any confounding effects of sediment deposition (see also Keough & Raimondi, 1995); these were abraded with medium coarse sandpaper to remove the surface sheen of the new material. The surfaces of the panels then were finely etched (groves < 1mm deep) with a 1x1 cm grid, to facilitate the subsequent location of established organisms (Plate 3.2), and soaked in freshwater for 2d to leach any contaminants. Each of the settlement panels was bolted horizontally to another panel of the same dimensions. The pairs of panels were separated by perspex spacers of 2.5 cm height (Plate 3.2). In preliminary experiments (at both flow regime sites) this "sandwich" design (Todd & Turner, 1986) was shown to collect larger numbers of settlers onto the downward facing site of the experimental (upper) panels than "free" (un-sandwiched) downward facing panels of the same material. It allowed also for the secure placement of the panels into the holding frames. The frames were of welded steel, treated with anti-corrosive paint. Each frame was designed to accommodate 15 sandwiched panels (in a 3 x 5 array), which were held in place by attaching stainless steel studs protruding upwards from spaced metal strips across the bottom of the frames. The panel sandwiches could, therefore, readily be slotted over the studs by means of holes running through the spacers and the corners of each panel (see Todd & Turner, 1986).

The experiments included four panel treatments (Fig. 3.2). The present lettering of treatments conforms to the scheme utilized by Todd & Keough (1994) in southeastern Australia. Treatment A panel substrata were un-netted and exposed to larval settlement

PLATE 3.2

Panel designs used in the flow regime experiments: un-netted "sandwiched" panel assembled (top), and dismantled (bottom).

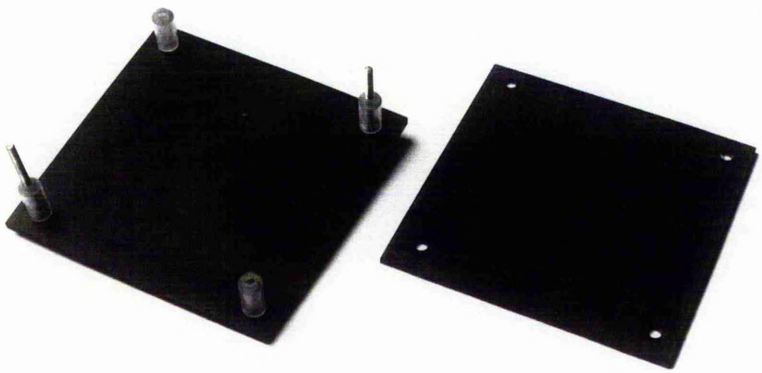
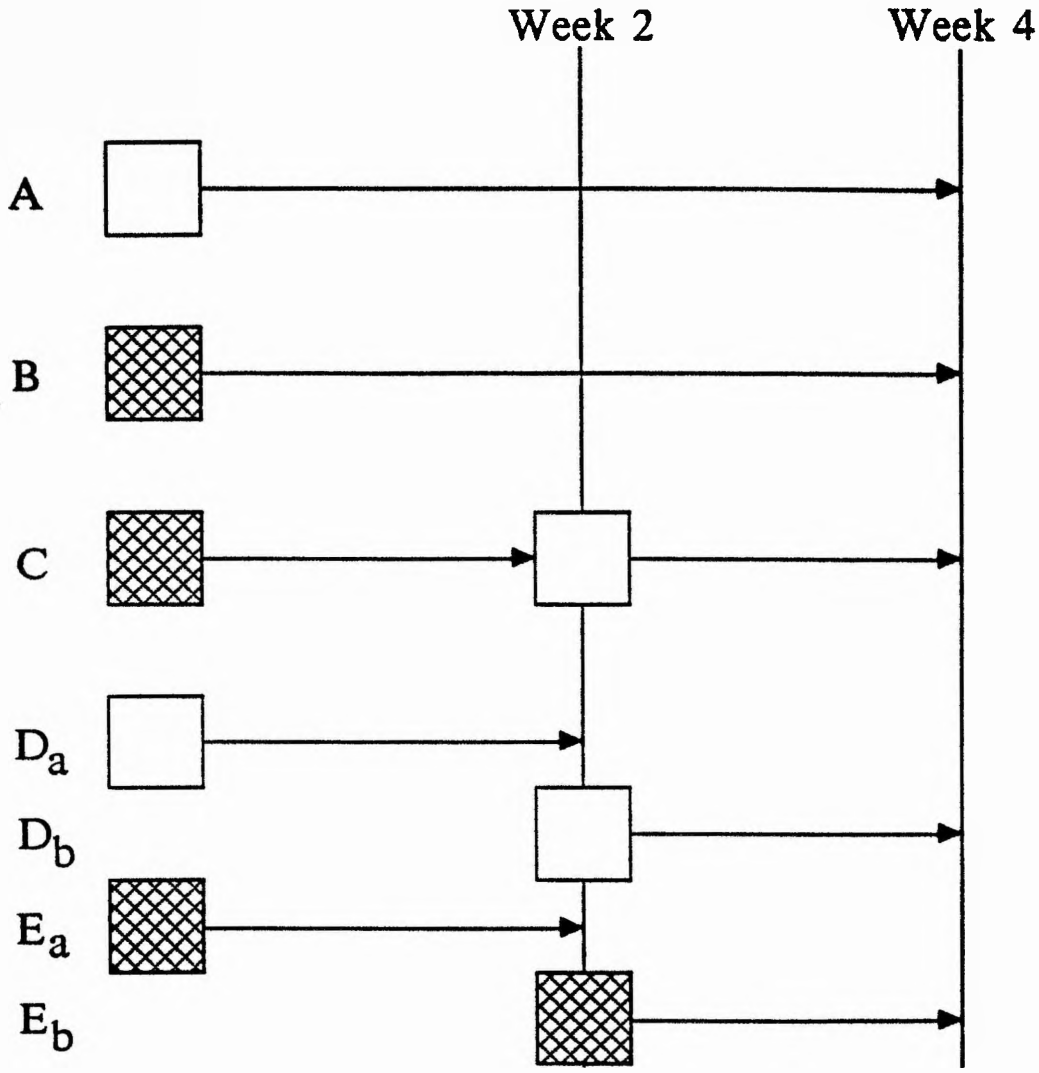


FIGURE 3.2

Schematic diagram of the experimental treatments and their codes for the first and second halves of the experiments. Netted and unnetted panels are represented by hatched and unhatched squares respectively.


Week 2
Week 4
A
B
C
D_a
D_b
E_a
E_b

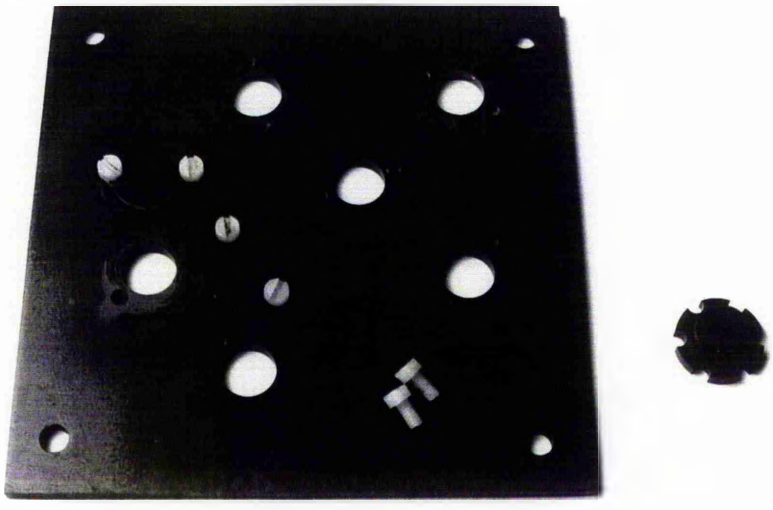
throughout the experimental time of one month. For treatment C, biofilming of the panel substrata, whilst excluding larval settlement, was achieved by the enclosure of experimental panels within tight-fitting, but removable, polyester mesh pouches of 100 µm pore size (Plate 3.3) (see Todd & Keough, 1994 and Keough & Raimondi, 1995 for the effects of mesh sizes). The upper experimental panel of each sandwich was sewn into its pouch before being bolted to its bottom panel. After preconditioning these netted panels for a period of 14d, the mesh pouches were removed, and the experimental surfaces exposed to larval settlement for a further 14d. Treatments D (D_a and D_b) and E (E_a and E_b) were the controls for the un-netted and netted treatments respectively and were included to provide separate data for the first (D_a, E_a) and the second (D_b, E_b) half of each experiment: each D_a and E_a panel was replaced by an appropriate D_b or E_b panel midway through each experiment.

Three replicate panels of treatments A, C, D and E were allocated randomly within each of the holding frames in Experiments 1 and 2 and scored for larval settlement at the conclusion of the experiment. The remaining slots on the frames were filled with netted panels which remained unmanipulated throughout the experiment (treatment B). These panels, originally designed to serve as an additional control for the effect of netting, were not included in the final analysis. At the end of each experiment the panels were transferred into holding containers filled with seawater and transported to St. Andrews, where the settlers were counted and identified to the lowest possible taxon. The species were grouped into categories, which were generally according to taxonomy, although bryozoans were grouped according to growth form (see also Jackson, 1979, and Chapter 5). This seemed appropriate because the phylum contains several quite distinct morphological groups (sheet-, erect- and mound bryozoans) with very different ecological requirements (see for example Walters & Wethey, 1991).

During removal and transport care was taken to prevent the panels from contacting the air water interface, which might have caused drying of biofilms or contamination with bacterioneuston (Marszalek *et al.*, 1979). Only the central areas (13 x 13 cm) of the panels were counted and considered in the analysis, leaving a 1 cm margin

PLATE 3.3

Panel designs used in the flow regime experiments: netted "sandwiched" panel assembled (top), and plugged panel (viewed from the back) used for microbiological analyses (bottom).



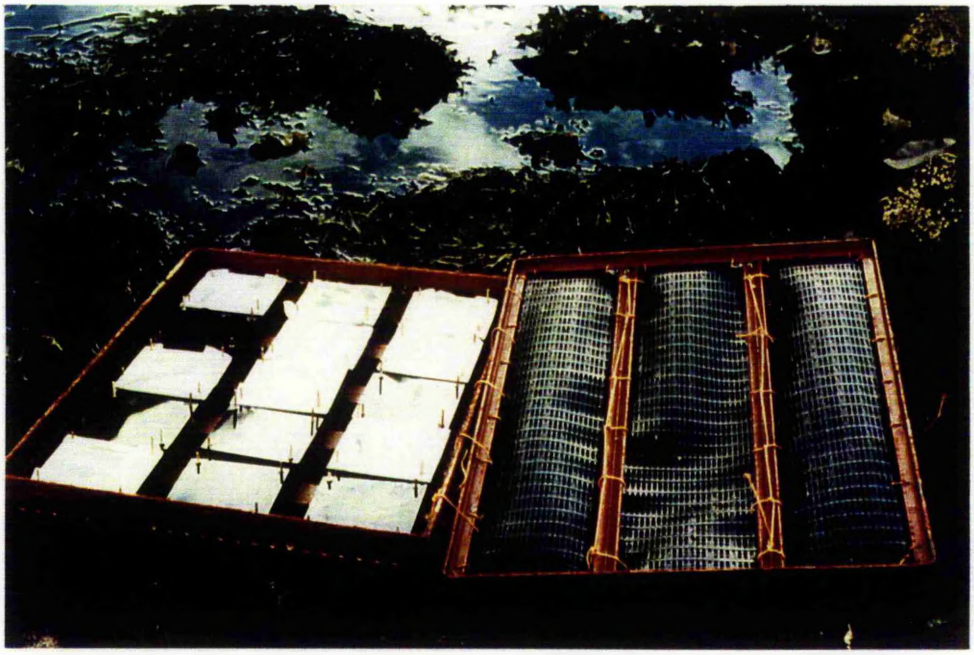
to allow for possible edge effects (see, e.g., Muntenau & Maly, 1981; Nowell & Jumars, 1984) and inadvertent handling during retrieval.

For Experiment 3, biofilm analyses were carried out to compare between netted and un-netted panel treatments and between sites. In this experiment only two replicate panels per frame were allocated to each treatment (A, C, D and E) for macrofaunal analysis but an additional three replicate panels per treatment were deployed at each site for biofilm analyses; the remaining slots were occupied by dummy panels. Biofilm samples, small enough in area for scanning electron microscopic (SEM) examination and the various microbiological analyses, were obtained from flush fitting removable perspex "plugs" of 1.5 cm diameter (see Turner & Todd, 1994) designed to tightly fit into holes drilled into these additional experimental panels (Plate 3.3). The plugs were cut from clear perspex rod and their position within the panels was randomized. The surfaces of the plugs were sanded flush with the panel surface and their upper sides (which protruded from the back of the panels) were spray-painted with black enamel, thus ensuring that the surfaces of the plugs matched the rest of the panel in texture and colour (Plate 3.3). The replicate plugged panels were allocated randomly within the two frames (using tables of random numbers). There were eight plugs per panel, which were randomly allocated to 16 possible locations on a grid placed on top of the panels. The plugs were held in place by two nylon screws which were driven through the edge of the enlarged plug bases and 2mm into the reverse side of the experimental panels. These plugs were randomly assigned to be used for one of three purposes: two were frozen in liquid nitrogen immediately following retrieval in the field for use in the SEM analysis, three were preserved in light-protected vials in a known volume of 90% acetone for chlorophyll *a* measurement, and three preserved in 2% formaldehyde for epifluorescence microscopy.

Two experimental frames (= "Blocks"; Plate 3.4) each were deployed within 20 cm from each other at Clachan Seil parallel to the direction of flow in two locations (= "Sites"): the sites differed significantly in their mean flow velocity above the frames. One site (hereafter referred to as LowFRS = low flow rate site) was situated on the mainland side of the narrows, with mean flow velocities 2h after low tide ranging between 0.07

PLATE 3. 4

The frames used in the flow regime experiments holding the experimental panel substrata.



and 0.16 m·sec^{-1}. The other site (hereafter referred to as HighFRS = high flow rate site) was located in the centre of the narrowest part of the Seil, with mean flow velocities 2h after low tide ranging between 0.19 and 0.44 m·sec^{-1}. Flow velocity above the frames was measured on 2-3 September 1993 and on 14 October 1993 with a propeller anemometer (A. Ott, type C2 "10.150" No. 45189, Prop. No. 3-45104).

Each experiment was run over the course of approximately one month, and was repeated at different times of the year — Experiment 1: 20 August 1993 - 17 September 1993; Experiment 2: 2 October 1993 - 2 November 1993; Experiment 3: 25 May 1994 - 26 June 1994.

Statistical analysis of larval settlement data

Initially 3-factor ANOVAs were carried out, including treatments A, C, D_a, D_b, E_a and E_b (with Site, Block within Site, and Treatment as the factors (and Site and Treatment crossed). Most important here were interactions between the factors Site and Treatment, suggesting site-related differences in the larval response to biofilmed substrata. The significance criterion was $\alpha_{[2]} \leq 0.05$. Each ANOVA then was followed by comparisons between the means of certain treatments and treatment combinations drawn for each site individually (analysis of the "simple" effects) to further elucidate the patterns underlying the "main" and interaction effects on settlement indicated by the ANOVA, and to separate the effects of biofilming from those of the presence of early incumbent settlers. The standard errors of the differences were calculated by dividing the difference between two means (or combinations of means in the case of the composite treatments) by the square root of the sum of the standard errors of those means. Those groups and taxa for which mean counts per panel consistently were < 1 (across treatments and at both sites) were excluded from the formal analysis.

Because a total of six comparisons (2 sites x 3 contrasts) were drawn for each experiment, the significance level for the tests was lowered from $\alpha_{[2]} \leq 0.05$ to $\alpha_{[2]} \leq 0.01$ using a Bonferroni approach (Brown & Rothery, 1993). Although the variances of

count data are generally stabilized by log-transformation (this is desirable in ANOVA which assumes homogeneity of variances), no transformation was carried out on the present data because the summation or subtraction of transformed data means would have a multiplicative rather than an additive effect in the case of the composite treatments ([E_a + D_b] and [C - E_a + D_a]). In acknowledgement of the fact that an analysis of the count data in their natural scale will introduce bias into the analysis, the conservative significance criterion of $\alpha_{[2]} = 0.01$ (see above) should provide some protection against the possibility of making a type I error (Sokal & Rohlf, 1981). However, care must be taken in the interpretation of the present results with regards to over-generalizing inferences. The results were compared with the appropriate critical values of the *t*-distribution. The following treatment contrasts were made:

1.) A *versus* [D_a + D_b]. This comparison allowed the assessment of the effect of biofilming on settlement (albeit confounded by the presence of early incumbent settlers). The D_b panels were essentially unfilmed and free of invertebrate settlers at the onset of the second half of the experiment, whereas settlement on A was onto a filmed surface in the presence of early incumbent post-larvae.

2.) C *versus* [E_a + D_b]. The combination [E_a + D_b] provided a comparable netted treatment to C. Whereas the surface of a treatment C panel was filmed at the onset of the second half of the experiment (after removal of the mesh screen), the composite variable [E_a + D_b] was unfilmed at the commencement of the second half of the experiment. This comparison therefore allowed an assessment of the effect of biofilming only on larval settlement.

3.) A *versus* [C - E_a+ D_a]. This comparison permitted the assessment of the effect of early incumbents on subsequent larval settlement in the presence of biofilming (see also Chapter 4, Materials and Methods).

Examination of biofilms

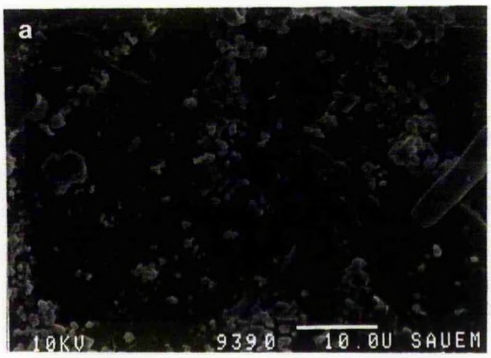
Plug samples preserved in liquid nitrogen were lyophilized in a Modulyo Vacuum Freeze Dryer (Edwards, No. 3988), in an especially designed brass chamber, which could hold 7 plugs at a time, and which prevented ice formation on the samples during the process. The plugs were then sputter coated with gold for SEM (JEOL JSM-35CF) observation. The biofilm surfaces of plugs were analysed "blind" by examining four SEM images (2000 μm^2) each along three randomly assigned transects per plug. The SEM images were viewed on the computer screen (22.5 x 18 cm) of an microanalysis system (Link Analytical, AN 10000/55S), which was overlaid with unique acetates bearing 50 randomly generated dots of 1mm diameter, and the frequency of occurrences of bacteria, diatoms, protozoans, EPS, organic debris, sediment particles and panel surface recorded (Plate 3.5).

Total bacterial cell counts were obtained by acridine orange direct count epifluorescence microscopy (AODC) (Parsons *et al.*, 1984). Estimates of relative total bacterial growth activity also were obtained by counting the total numbers of dividing bacteria (Hagström *et al.*, 1979) for the same AODC preparations. Biofilms were removed from plugs by gently rubbing the plug surface in sterile formaldehyde solution with the open end of sterile rubber tubing attached to a glass rod. In order to yield sufficient numbers of bacteria per filter area the biofilms of all three replicate plugs of a panel were combined for filtration. Bacteria were counted directly from subsamples of these plug filtrations. Membrane filters stained with Amido Black (Sigma, N-3005) were used to reduce background fluorescence, following the procedures in Hobbie *et al.* (1977).

For chlorophyll *a* analysis biofilms were removed from plugs by rubbing the plug surface in sterile acetone in the same way as described above for AODC analysis and extinctions were measured spectrophotometrically (Parsons *et al.*, 1984).

PLATE 3.5

SEM photoraphs of biofilm categories enumerated in the microbial analysis of panel surfaces of flow regime Experiment 3: a) bacteria held together by EPS structures, (b) individual bacteria, (c) diatom (bottom left quarter), (d) protozoan (choanoflagellate), (e) filamentous fungal structures,.(f) organic debris, diatoms and sediment particles, and (g) inorganic depositions.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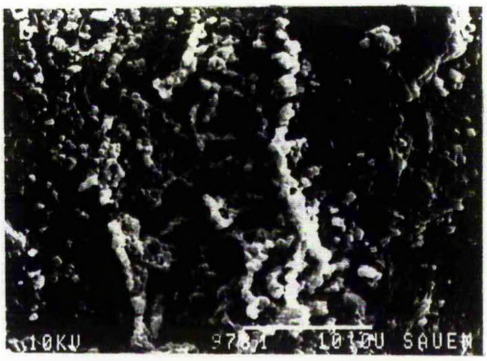
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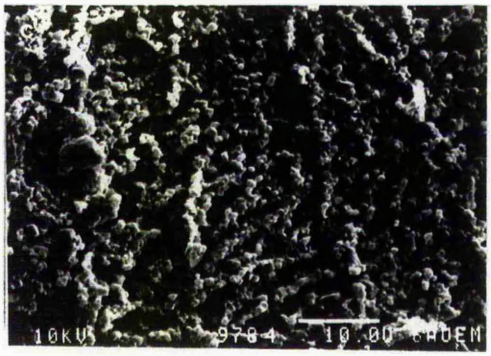
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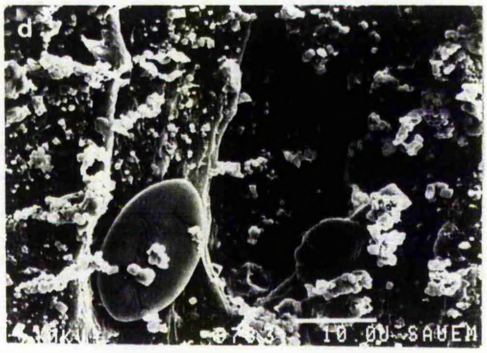
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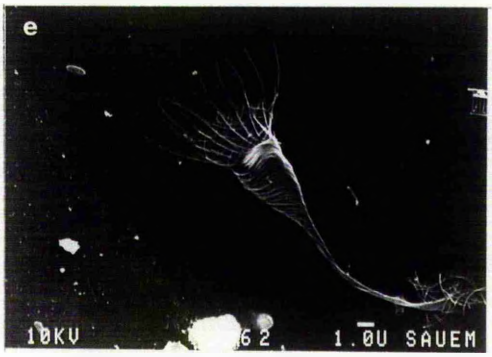
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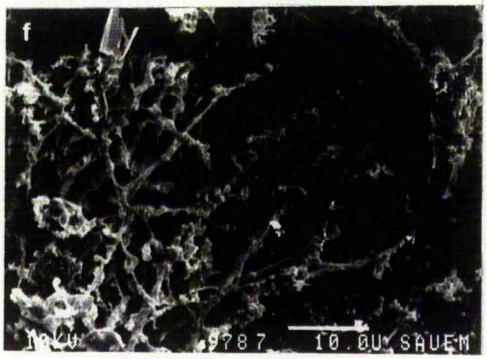
10KV 1.0U SAUEM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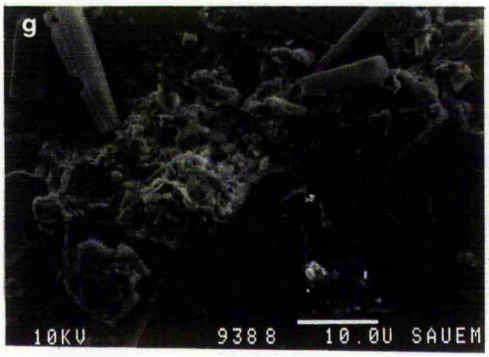
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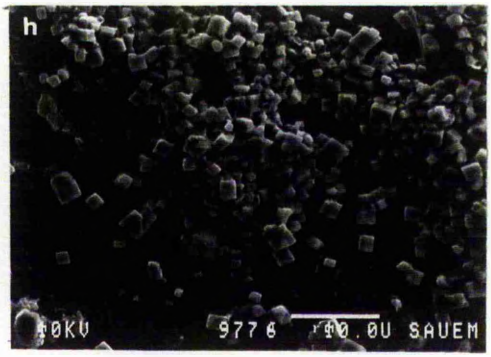
10KV 9388 10.0U SAUEM


h
10KV 9776 10.0U SAUEM

Statistical analysis of biofilm data

Comparisons between biofilm treatments were drawn for the total counts·cm^{-2} of bacteria and percentage dividing bacteria (determined by AODC), and for the frequency of occurrence of bacteria, diatoms, protozoans, fungi, organic debris, sediment particles, and EPS on the biofilm surface (determined by SEM). All percentage data and random dot SEM counts (which were also expressed in percentages) were arcsine transformed prior to analysis. Initially ANOVAs were carried out for each biofilm category including treatments A, C, D_a, D_b, E_a, and E_b. The significance criterion was $\alpha \leq 0.05$. The analysis of the biofilm data here was more concerned with the comparison of netted and un-netted panel treatments, and between-site differences, rather than differences between the first and second half of the experiment, or the differences between "old" (4 week old) and "young" (2 week old) biofilms. Thus the initial ANOVAs were followed by the between-mean comparisons A *versus* C, D_a *versus* E_a and D_b *versus* E_b. The null hypothesis was that there were no significant differences between biofilm components for netted and un-netted substrata. Standard errors of the differences were calculated as for the macrofaunal analysis; no adjustments were made to the significance level for the multiple comparisons, because using a less stringent significance criterion of $\alpha_{[2]} \leq 0.05$ in this case was the more conservative option. In accordance with the large sample sizes for the biofilm data (n = 72 for SEM counts and n = 90 for the AODC counts) the results were compared to the appropriate critical value for z(= 1.96). Because nesting of plugs and panels within blocks was unbalanced, preliminary analyses were carried out using GLIM (version 3.77, update 2, 1985), with Treatment, Site, Block (nested within Site), Panel (nested within Block and Treatment) and Plug (nested within Panel) as factors. Because the biofilms of replicate plugs from each panel had been combined for AODC, plug effects could not be determined for total bacterial cell counts and dividing cells. The full generalized linear model fitted was: (Treatment+Site/Block)/Panel/Plug. Because this full model could lead to an underestimation of the effects of the factors Treatment and Site, due to its over-parameterization, the terms Plug and Panel were deleted from the model wherever these proved non-significant in the full analysis (Crawley, 1993). In the

few cases where significant Plug or Panel effects were detected, Block effects could be fully confounded. Because the preliminary analysis in GLIM did not reveal any significant Plug effects, and only very few Panel effects, the further analysis of the biofilm data (see above) was carried out using the GLM procedure in MINITAB (version 8.2, 1991). Site, Block within Site and Treatment were the crossed factors, and the terms Plug and Panel were ignored.

3.3 RESULTS

Experiment 1 (20 August - 17 September 1993)

The average density of total larval settlers in Experiment 1 on un-netted panels exposed throughout the experiment (treatment A) was approximately 1.1·cm^{-2} at the HighFRS, and 1.6·cm^{-2} at the LowFRS. For both sites the predominating taxonomic groups were Spirorbin, Serpulin and Serpulid (*Pomatoceros* spp.) polychaetes, various species of Sheet Bryozoans (e.g. *Celleporella hyalina* (L.), *Electra pilosa* (L.), *Escharoides coccinea* (Abildgaard), *Schizoporella unicornis* (Johnston)) and Mound Bryozoans (*Tubulipora* sp., *Plagioecia* sp.), and colonial ascidians (e.g. *Botryllus schlosseri* (Pallas), *Botrylloides leachi* (Savigny), and several Didemnid species). Erect Bryozoans (*Crisia* sp. and *Scrupocellaria scruposa* (L.)), various slime sponge taxa and *Leucosolenia* sp. (an erect branching sponge species) were relatively abundant on panels from the LowFRS, but numerically unimportant at the HighFRS. Among the less frequent groups were the scyphistoma of *Aurelia aurita* (L.) and the bivalve *Heteranomia squamula* (L.). Spirorbins (*Spirorbis* spp.) were by far the most abundant settlers on all panel treatments for both sample sites. Figs. 3.3, 3.4 and 3.5 show abundances of taxonomic groups and species for all treatments at both flow regime sites. Overall it is obvious that the 100 µm mesh screens either excluded or markedly reduced, by up to an order of magnitude, the numbers of settlers of the respective species (see treatments D_a/D_b and treatments E_a/E_b in the histograms for comparison between netted and un-

FIGURE 3.3

Experiment 1 (20 August - 17 September 1993). Larval settlement of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/ *Pomatoceros* sp., Total Ascidians, *Botryllus schlosseri/Botrylloides leachi*, Didemnid Ascidians, Total Bryozoans and Erect Bryozoans. The histogram bars show the mean (+ standard error) numbers of settlers per panel for all experimental treatments. Note the differences in the abundance scales.

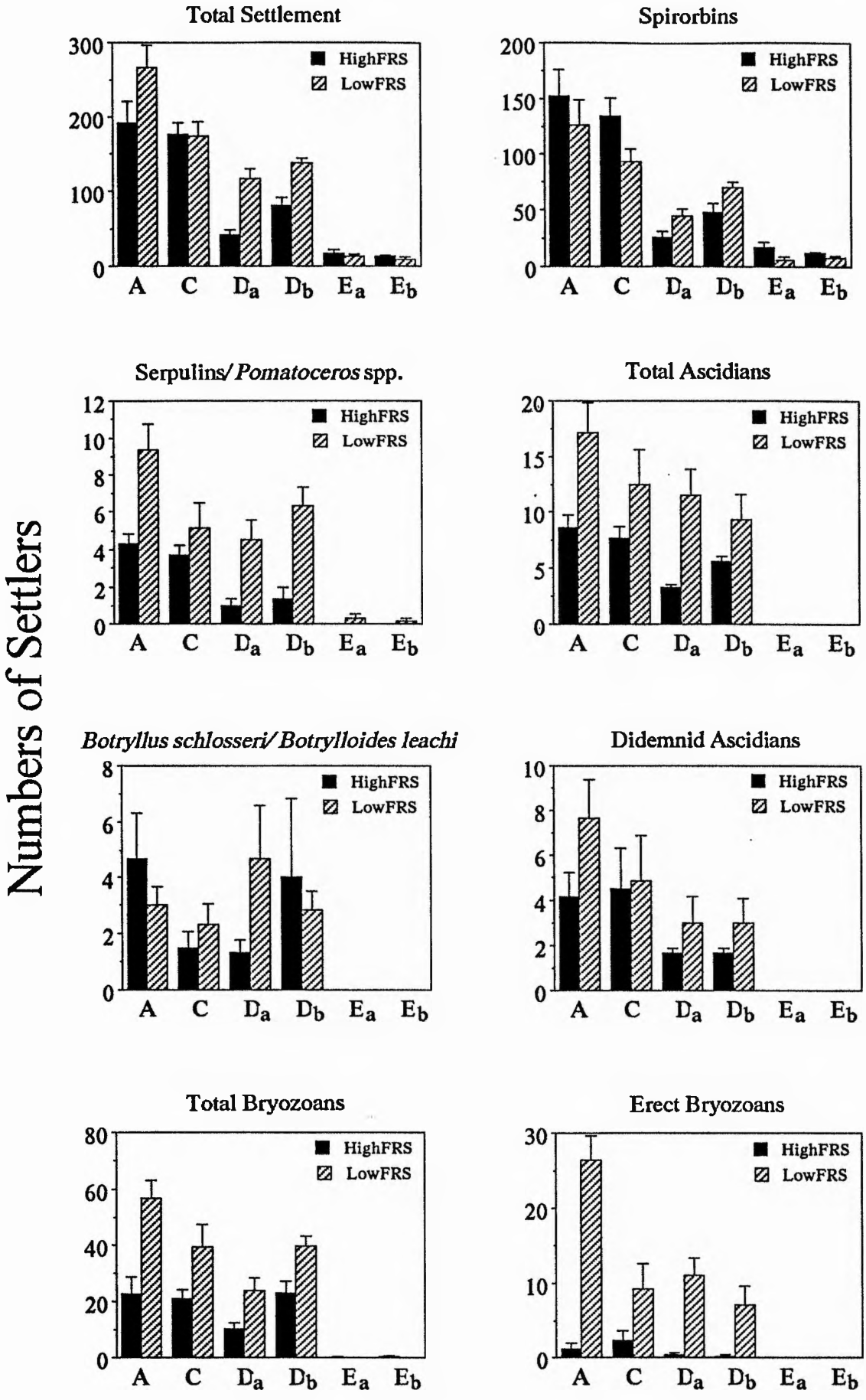

Total Settlement
Spirorbins
Serpulins/*Pomatoceros* spp.
Total Ascidians
Botryllus schlosseri/Botrylloides leachi
Didemnid Ascidians
Total Bryozoans
Erect Bryozoans
HighFRS
LowFRS
A
C
Da
Db
Ea
Eb
Numbers of Settlers
Treatment

FIGURE 3.4

Experiment 1. Larval settlement of *Scrupocellaria scruposa*, *Crisia* sp., Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.), Sheet Bryozoans, *Celleporella hyalina*, *Electra pilosa*, *Escharoides coccinea* and Slime Sponges. The histogram bars show the mean (+ standard error) numbers of settlers per panel for all experimental treatments. Details as in Fig. 3.2.

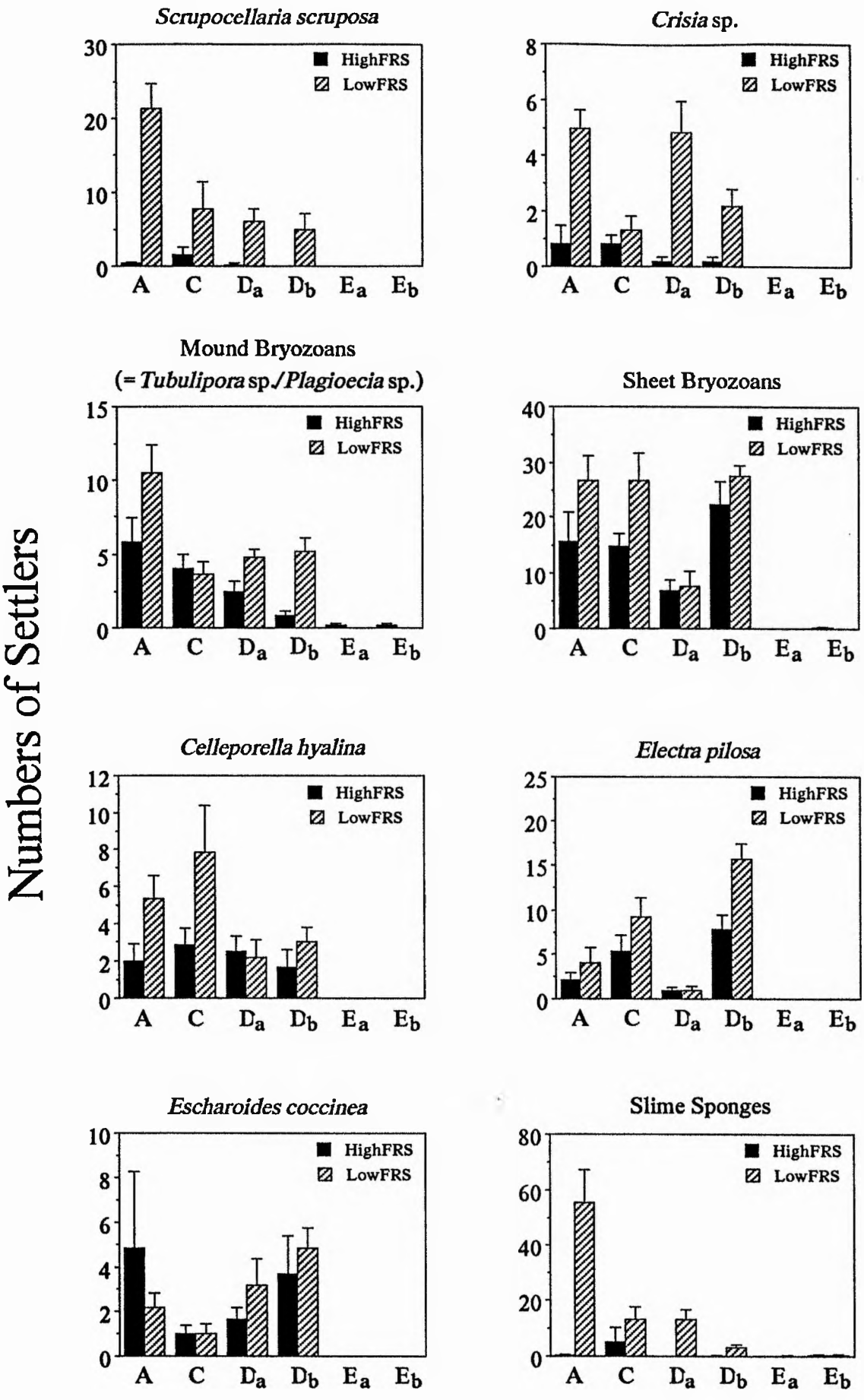
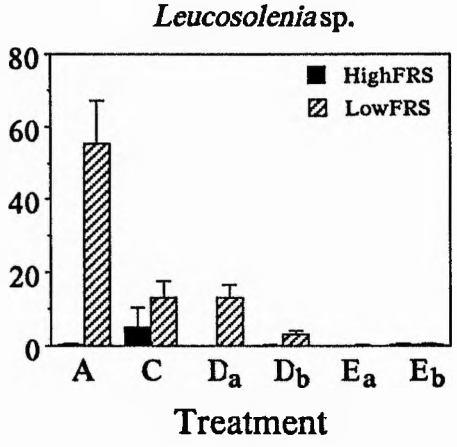

Scrupocellaria scruposa
Crisia sp.
Mound Bryozoans
(= Tubulipora sp./Plagioecia sp.)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
Escharoides coccinea
Slime Sponges
HighFRS
LowFRS
A
C
Da
Db
Ea
Eb
Numbers of Settlers
Treatment

FIGURE 3.5

Experiment 1. Larval settlement (mean + standard error) of *Leucosolenia* sp.. Details as in Fig. 3.2.


Leucosolenia sp.
80
60
40
20
0
HighFRS
LowFRS
A
C
D_a
D_b
E_a
E_b
Treatment

netted panels respectively). The majority of the few larvae that were able to penetrate the mesh successfully were Spirorbins; none the less, some Serpulins and *Pomatoceros* spp. also were found on netted panels at the HighFRS, and some Mound Bryozoans settled on netted panels at the LowFRS. Ascidians and Sheet Bryozoans were virtually excluded by the meshes at both sites. The low numbers of settlers on panels with mesh screens were accounted for in the analysis of putative biofilming effects on settlement (see above).

The initial ANOVAs (including treatments A, C, D_a, D_b, E_a and E_b) and the *t*-values of the between-mean comparisons for the particular taxonomic groups and species for Experiment 1 are shown in Table 3.1 (full ANOVA tables and lists of the treatment means for each group are presented in Appendix 1). As expected (since netted panel treatments were included in the analysis), treatment "main" effects were highly significant for the majority of the enumerated groups (the exception being *Leucosolenia* sp.). Only for the settlement of the Serpulins/*Pomatoceros* spp. group did the analysis reveal a significant between-frame difference (within-site Block effect), although the factor Block was only marginally non-significant for Total Bryozoans ($P = 0.034$) (Table 3.1). The differences in settlement densities between the HighFRS and LowFRS were highly significant for all groupings, except for Spirorbins, the *Botryllus schlosseri/Botrylloides leachi* grouping, Didemnid Ascidians, *Celleporella hyalina* and *Escharoides coccinea* (Table 3.1). Most important, there were many significant Site*Treatment interaction effects (Table 3.1), suggesting that differences in settlement patterns between sites did not arise solely from possible site-specific variations in larval supply and/or early mortality. It is likely that behavioural responses of larvae to biofilmed surfaces play an important rôle in creating those differences also, and that these responses may vary with the prevailing flow regime under which the biofilms were formed.

The comparison between means of treatments or treatment combinations separated by site (Table 3.1) elucidated some of the main effects patterns and allowed the assessment of the effects of biofilming and early incumbents on subsequent larval settlement:

1.) A *versus* [D_a + D_b] (Table 3.1). This comparison allowed assessment of the effect of biofilming of substrata on larval settlement, albeit confounded by the

Table 3.1. Experiment 1 (20 August 1993 - 17 September 1993). Analysis of larval settlement. The table shows, for each taxon or grouping, the results of the initial 3-factor ANOVA which included treatments A, C, D_a, D_b, E_a and E_b, and the t-values of the between-mean comparisons of biofilmed and unfilmed substrata (A *versus* [D_a + D_b] and C *versus* [E_a + D_b]) and of the effects of netting a substratum for the first two weeks (A *versus* [C - E_a + D_a]). The design of the experiment was blocked (4 blocks = 2 frames per site). Degrees of freedom: Site = 1; Block (nested within Site) = 2; Treatment = 5; Site*Treatment interaction = 5; Residual = 58. Significant effects of the ANOVA ($\alpha_{[2]} \leq 0.05$), and significant *t*-values for the comparisons between treatment means ($\alpha_{[2]} \leq 0.01$) are shown in bold face. Significant outcomes with *t*-values preceded by a '-' symbol are taxa for which the left hand term (C or A) was the smaller, '+' symbols identify taxa for which the right hand term was smaller. Full ANOVA tables and lists treatment means and standard errors for each taxonomic group and species are given in Appendix 1. (Treat. = Treatment; n = number of observations).

	ANOVA						A *vs.* [D_a + D_b]		C *vs.* [E_a + D_b]		A *vs.* [C - E_a + D_a]	
	MSres	Site	Block (Site)	Treat.	Interaction Site*Treat.		HighFRS	LowFRS	HighFRS	LowFRS	HighFRS	LowFRS
		P	*P*	*P*	*P*	n	*t*	*t*	*t*	*t*	*t*	*t*
Total Settlement	1369	**<0.001**	0.318	**<0.001**	**0.008**	6	2.265	0.342	**+ 4.155**	1.210	0.234	0.345
Spirorbins	782	0.300	0.229	**<0.001**	0.055	6	**+ 3.173**	0.486	**+ 3.762**	1.445	0.327	0.237
Serpulins/*Pomatoceros* spp.	3.623	**<0.001**	**0.003**	**<0.001**	**0.003**	6	1.678	0.737	2.056	0.888	0.218	0.000
Total Ascidians	25.57	**<0.001**	0.866	**<0.001**	0.162	6	0.093	0.876	0.469	0.826	0.619	1.565
Botryllus schlosseri **(Pallas)/ *Botrylloides leachi* (Savigny)**	7.886	0.738	0.156	**0.002**	0.325	6	0.207	2.097	0.870	0.516	1.025	1.847
Didemnid Ascidians	7.081	0.089	0.420	**<0.001**	0.588	6	0.764	0.719	1.561	0.773	0.954	0.057

critical value for $t_{0.01(2),\,58} = 2.392$

Table 3.1 contd.

	ANOVA						A *vs.* [D_a + D_b]		C *vs.* [E_a + D_b]		A *vs.* [C - E_a + D_a]	
	MSres	Site	Block (Site)	Treat.	Interaction Site*Treat.		HighFRS	LowFRS	HighFRS	LowFRS	HighFRS	LowFRS
		P	*P*	*P*	*P*	n	*t*	*t*	*t*	*t*	*t*	*t*
Total Bryozoans	92.4	**0.001**	**0.034**	**<0.001**	**<0.001**	6	1.452	0.838	0.460	0.038	1.221	0.616
Erect Bryozoans	18.54	**<0.001**	0.426	**<0.001**	**<0.001**	6	0.762	1.722	1.583	0.472	0.933	1.161
Scrupocellaria scruposa (L.)	16.49	**<0.001**	0.740	**<0.001**	**<0.001**	6	0.615	2.382	1.342	0.680	1.160	1.432
Crisia sp.	1.349	**<0.001**	0.057	**<0.001**	**<0.001**	6	0.717	1.395	1.908	1.017	0.225	0.852
Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)	5.012	**0.001**	0.836	**<0.001**	**0.011**	6	1.375	0.155	**+ 2.752**	1.209	0.241	0.865
Sheet Bryozoans	52.32	**0.008**	0.159	**<0.001**	0.142	6	1.952	1.545	1.580	0.154	1.017	1.063
Celleporella hyalina (L.)	6.487	**0.012**	0.335	**<0.001**	0.070	6	1.390	0.094	0.902	1.798	2.146	1.542
Electra pilosa (L.)	8.790	**0.002**	0.425	**0.010**	0.297	6	**- 3.657**	**- 5.153**	1.026	2.336	2.082	2.186
Escharoides coccinea (Abildgaard)	8.667	0.905	0.110	**0.001**	0.529	6	0.129	**- 3.853**	1.481	**- 4.240**	0.810	1.644
Slime Sponges	100.7	**<0.001**	0.237	**<0.001**	**<0.001**	6	0.615	**+ 3.113**	1.046	1.968	1.012	2.193
Leucosolenia sp.	79.87	**<0.001**	0.071	0.144	0.123	6	0.000	0.107	0.936	0.735	0.936	0.143

critical value for $t_{0.01(2),\,58} = 2.392$

presence of early incumbent post-larvae. While the D_b panels were unfilmed and without incumbents at the commencement of the second half of the experiment, panels of treatment A bore 2 week old biofilms, as well as settlers from the first half of the experiment. The comparison revealed significant facilitatory effects on treatment A for Spirorbins at the HighFRS, and for Slime Sponges at the LowFRS. *Electra pilosa* showed significant inhibition on treatment A at both sites, whereas *Escharoides coccinea* was inhibited at the LowFRS only (Table 3.1). The results of this treatment contrast, thus, explain some of the patterns of interaction between the factors Site and Treatment indicated by the initial ANOVA.

2.) C *versus* [E_a + D_b] (Table 3.1). This comparison allowed the assessment of the effects of substratum biofilming on larval settlement response for the second half of the experiment, with the virtual exclusion of the influence of settlers from the first half of the experiment (comparison of netted panels). Here facilitated settlement on filmed substrata (C > [E_a + D_b]) was revealed for all categories of settlers pooled (Total Settlement), Spirorbins and Mound Bryozoans at one of the sites only (HighFRS) (Table 3.1), again explaining some of the interactions between the factors Site and Treatment. Only *Escharoides coccinea* was shown to be inhibited by biofilming at the LowFRS (Table 3.1).

3.) A *versus* [C - E_a + D_a] (Table 3.1). This comparison was included to assess the influence of early incumbents (although confounded by the presence of biofilming) on subsequent larval settlement. However, none of the taxonomic groups and species were significantly influenced in their settlement response by the presence of incumbents in this experiment.

Experiment 2 (2 October - 2 November 1993)

Settlement densities overall were lower for Experiment 2 than for Experiments 1 and 3, but again higher at the LowFRS (1.2·cm^{-2}, treatment A) than the HighFRS (0.3·cm^{-2}, treatment A). Although different in their relative abundances from the

previous experiment, the predominant taxa and species were almost identical to those of Experiment 1, with Spirorbins the most frequent. Figs 3.6 and 3.7 show the mean abundances for taxonomic groups and species for all treatments of Experiment 2 at both sites. Settlement on netted panels was higher in this experiment than for Experiments 1 and 3: here the mesh screens had been penetrated by small numbers of larvae of almost all taxonomic groups at the LowFRS, and by many groups at the HighFRS (Figs. 3.6 and 3.7).

Table 3.2 presents the initial 3-factor ANOVAs and the *t*-values of the between-mean comparisons for the particular taxonomic groups and species for Experiment 1 (full ANOVA tables and lists of the treatment means for each group are presented in Appendix 1). No significant differences between frames (blocks) were found for any of the groups and species enumerated in this experiment. Significant overall between-site differences in settlement counts were indicated for the categories Total Settlement, Spirorbins, Erect Bryozoans and Slime Sponges (note, however, that the factor Site was only marginally non-significant for Total Bryozoans ($P = 0.029$ and Mound Bryozoans ($P = 0.012$)) (Table 3.2). Only for Total Settlement and Spirorbins did the initial analysis reveal significant Treatment "main" effects (with marginally non-significant probabilities for the Serpulin/*Pomatoceros* spp. grouping ($P = 0.039$), Total Bryozoans ($P = 0.043$) and Sheet Bryozoans ($P = 0.046$)) and significant interaction effects for the factors Site and Treatment (Table 3.2). The outcome for the interaction term was marginally non-significant for the sheet forming bryozoan species *Electra pilosa* ($P = 0.042$).

The comparisons between treatment means following the initial ANOVAs for Experiment 2 are shown in Table 3.2:

1.) A *versus* [D_a + D_b] (Table 3.2). Significant Treatment effects, indicating facilitation of settlement by biofilmed substrata and/or the presence of incumbents, were noted only for Total Settlement and Spirorbins at the LowFRS. However, significantly lower numbers of settlers on treatment A were indicated for the Serpulin/*Pomatoceros* spp. grouping, Total Bryozoans, Erect Bryozoans, Sheet Bryozoans, and *Electra pilosa* at the HighFRS.

FIGURE 3.6

Experiment 2 (2 October - 2 November, 1993). Larval settlement (mean + standard error) of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/*Pomatoceros* sp., Total Ascidians, Didemnid Ascidians, Total Bryozoans, Erect Bryozoans and *Scrupocellaria scruposa*. Details as in Fig. 3.2.

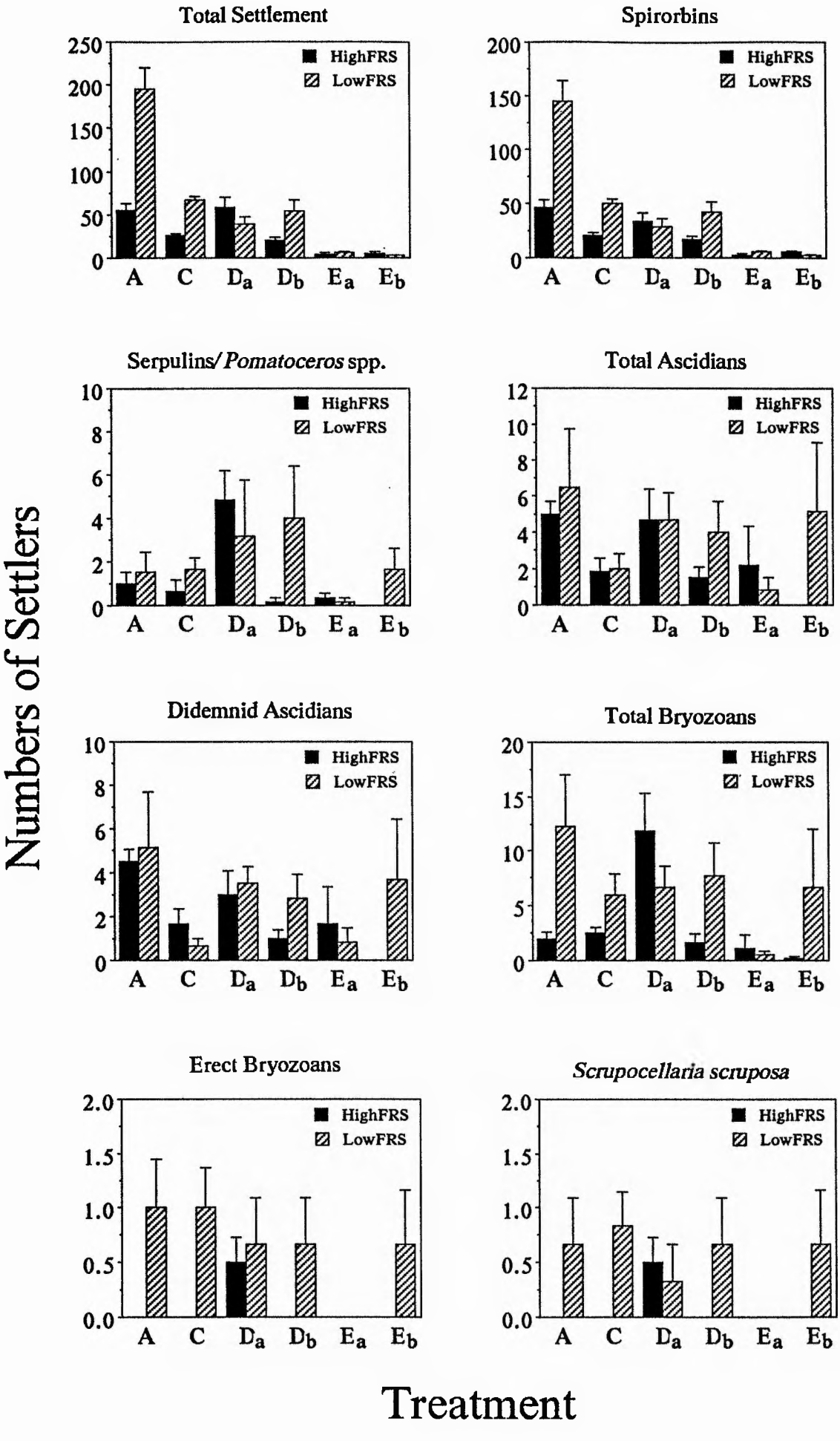

Total Settlement
Spirorbins
Serpulins/*Pomatoceros* spp.
Total Ascidians
Didemnid Ascidians
Total Bryozoans
Erect Bryozoans
Scrupocellaria scruposa
HighFRS
LowFRS
A
C
Da
Db
Ea
Eb
Numbers of Settlers
Treatment

FIGURE 3.7

Experiment 2. Larval settlement (mean + standard error) of Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.), Sheet Bryozoans, *Celleporella hyalina*, *Electra pilosa*, *Escharoides coccinea*, Slime Sponges and Leucosolenia sp.. Details as in Fig. 3.2.

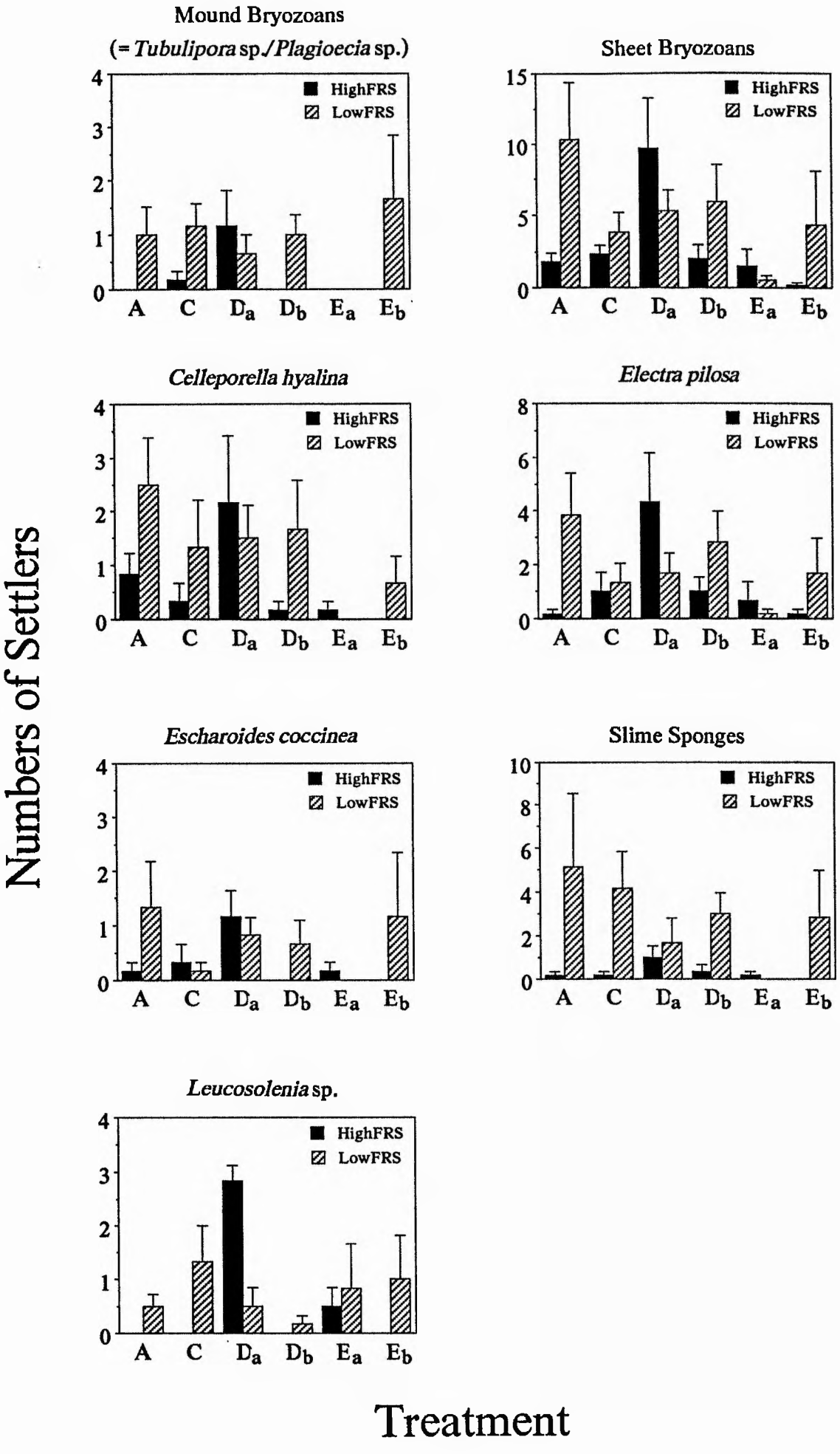

Mound Bryozoans
(= Tubulipora sp./Plagioecia sp.)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
Escharoides coccinea
Slime Sponges
Leucosolenia sp.
HighFRS
LowFRS
A
C
Da
Db
Ea
Eb
Numbers of Settlers
Treatment

Table 3.2. Experiment 2 (2 October 1993 - 2 November 1993). Analysis of larval settlement. Details as for Table 3.1.

	ANOVA						A *vs.* [D_a + D_b]		C *vs.* [E_a + D_b]		A *vs.* [C - E_a + D_a]	
	MSres	Site	Block (Site)	Treat.	Interaction Site*Treat.		HighFRS	LowFRS	HighFRS	LowFRS	HighFRS	LowFRS
		P	*P*	*P*	*P*	n	*t*	*t*	*t*	*t*	*t*	*t*
Total Settlement	551.0	**<0.001**	0.961	**<0.001**	**<0.001**	6	1.743	+ **3.469**	0.045	0.471	1.743	+ **3.541**
Spirorbins	334.8	**<0.001**	0.976	**<0.001**	**<0.001**	6	0.436	+ **3.220**	0.097	0.145	0.464	+ **3.383**
Serpulins/*Pomatoceros* spp.	8.284	0.209	0.197	0.039	0.304	6	- **2.745**	1.548	0.000	1.009	- **2.699**	1.133
Total Ascidians	20.18	0.213	0.520	0.164	0.566	6	0.627	0.552	0.803	1.424	0.227	0.180
Didemnid Ascidians	10.85	0.304	0.770	0.064	0.523	6	0.397	0.441	0.557	- **3.900**	0.694	0.670
Total Bryozoans	42.09	**0.029**	0.839	**0.043**	0.066	6	- **3.188**	0.340	0.222	0.603	- **2.973**	0.031
Erect Bryozoans	0.460	**0.001**	0.055	0.349	0.349	6	- **2.232**	0.448	0.000	0.597	2.232	0.933
Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)	1.290	**0.012**	0.870	0.430	0.218	6	1.784	0.934	1.000	0.308	1.976	1.138
Sheet Bryozoans	29.25	0.099	0.942	**0.046**	0.084	6	- **2.591**	0.202	0.720	0.920	2.235	0.374
Celleporella hyalina	2.536	0.081	0.896	0.056	0.419	6	1.135	0.470	0.002	0.262	1.100	0.239
Electra pilosa	5.727	0.223	0.791	0.129	**0.042**	6	- **2.696**	0.322	0.776	1.231	2.165	0.531

critical value for $t_{0.01(2),\,58}$ = 2.392

Table 3.2 contd.

	ANOVA						A *vs.* [D_a + D_b]		C *vs.* [E_a + D_b]		A *vs.* [C - E_a + D_a]	
	MSres	Site	Block (Site)	Treat.	Interaction Site*Treat.		HighFRS	LowFRS	HighFRS	LowFRS	HighFRS	LowFRS
		P	*P*	*P*	*P*	n	*t*	*t*	*t*	*t*	*t*	*t*
Escharoides coccinea	1.407	0.170	0.613	0.429	0.406	6	1.976	0.169	0.445	1.101	1.857	0.365
Slime Sponges	10.88	**0.002**	0.967	0.520	0.395	6	1.833	0.137	0.814	0.613	1.469	0.171
Leucosolenia sp.	3.582	0.710	0.182	0.409	0.233	6	1.239	0.378	1.462	0.308	1.010	0.437

critical value for $t_{0.01(2),\,58} = 2.392$

2.) C *versus* [E_a + D_b] (Table 3.2). The only significant between-mean difference for this contrast was indicated for Didemnid Ascidians, which were inhibited on biofilmed substrata (treatment C) at the LowFRS.

3.) A *versus* [C - E_a + D_a] (Table 3.2). Significant Inhibition of settlement by the presence of incumbents (A < [C - E_a + D_a]) was shown for the Serpulin/*Pomatoceros* spp. grouping and Total Bryozoans at the HighFRS only. Facilitation of settlement by early incumbent settlers ([C - E_a + D_a] < A) was indicated for the categories Total Settlement and Spirorbins at the LowFRS only. Note, that the contrast outcomes were only marginally non-significant (A < [C - E_a + D_a]) for Erect Bryozoans, Sheet Bryozoans and *Electra pilosa* at the HighFRS. Thus, it appears that most of the significant between-mean differences indicated by the comparison A *versus* [D_a + D_b] (see above) were due to incumbent rather than biofilming effects in this experiment.

Experiment 3 (25 May - 26 June 1993)

Of the three experiments, between-site differences in total numbers of settlers were greater for this experiment (Figs. 3.8 and 3.9), with an overall mean settlement density on treatment A at the LowFRS (1.8 cm^{-2}) alone being three times higher than at the HighFRS (0.6·cm^{-2}). This resulted in highly significant Site effects for many of the taxonomic groupings in the ANOVAs (Table 3.3). Although relative abundances of the enumerated categories differed from those of Experiments 1 and 2, overall the species composition did not: Spirorbins again were numerically dominant, and the same analytical groupings were used. Settling larvae were almost entirely excluded from netted panels at both sites, and were reduced to very few Spirorbins, Serpulins and Bryozoans.

The initial 3-factor ANOVAs for the macrofaunal groupings of Experiment 3 are shown in Table 3.3. There was a highly significant Block effects for Total Settlement (and marginally non-significant between-frame differences for the categories Spirorbins (P = 0.015) and Mound Bryozoans (P = 0.023)). Of the 12 macrofaunal groups

FIGURE 3.8

Experiment 3 (25 May, 1994 - 26 June 1994). Larval settlement (mean + standard error) of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/*Pomatoceros* sp., Total Ascidians, *Botryllus schlosseri/Botrylloides leachi*, Total Bryozoans, Erect Bryozoans and *Crisia* sp.. Details as in Fig. 3.2.

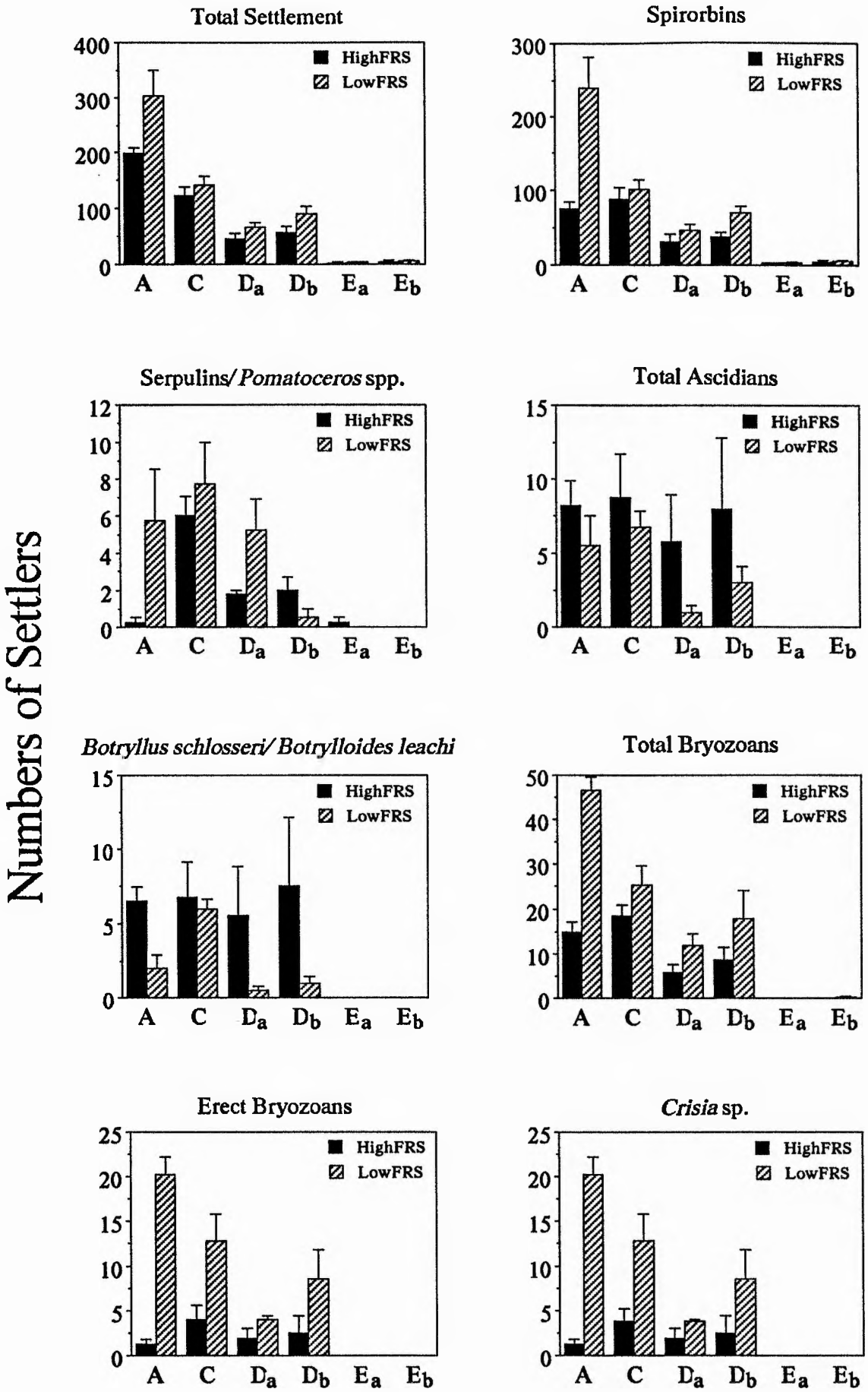

Total Settlement
Spirorbins
Serpulins/ *Pomatoceros* spp.
Total Ascidians
Botryllus schlosseri/ Botrylloides leachi
Total Bryozoans
Erect Bryozoans
Crisia sp.
HighFRS
LowFRS
A
C
Da
Db
Ea
Eb
Numbers of Settlers
Treatment

FIGURE 3.9

Experiment 3. Larval settlement (mean + standard error) of Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.), Sheet Bryozoans, *Celleporella hyalina* and *Electra pilosa*. Details as in Fig. 3.2.

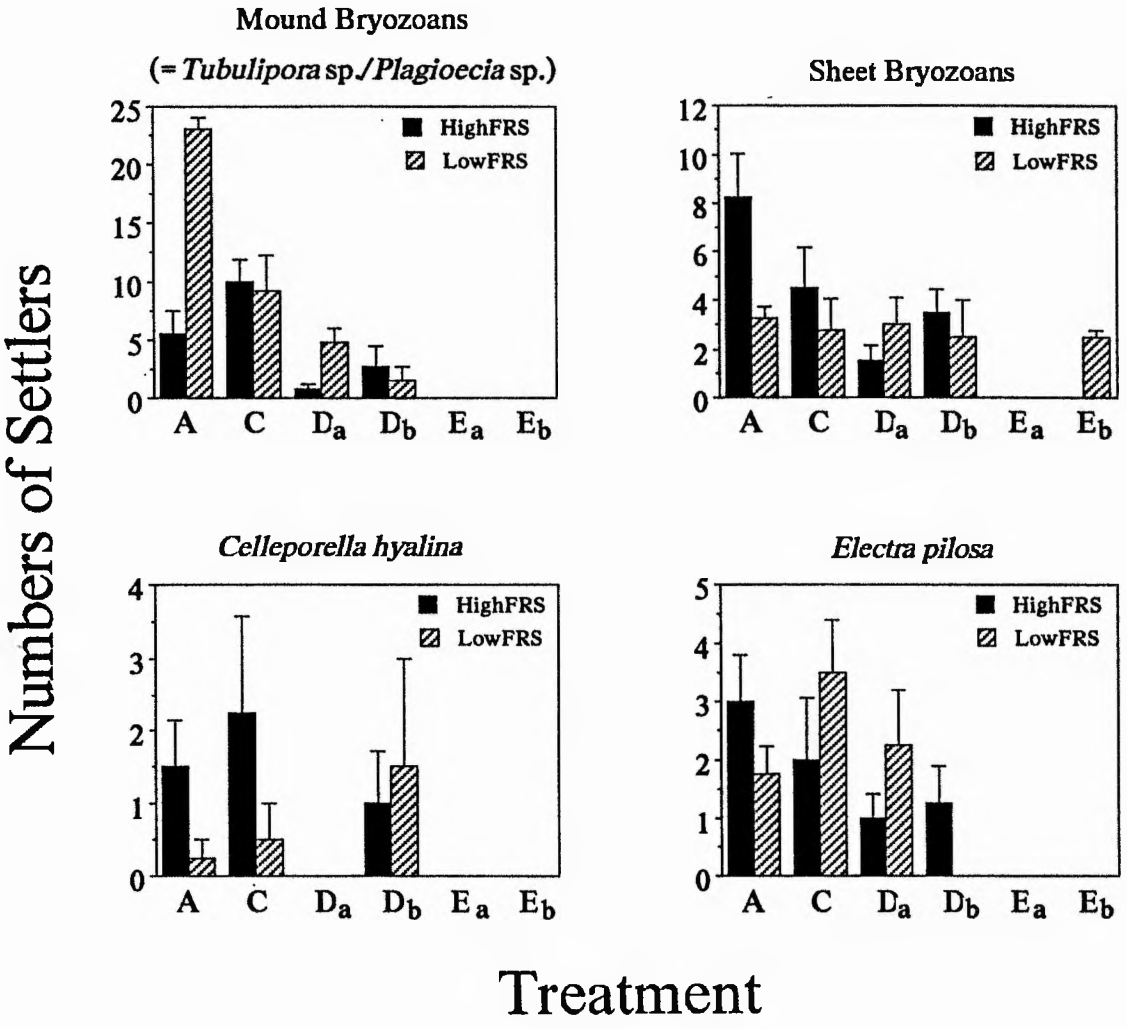

Mound Bryozoans
(= Tubulipora sp./Plagioecia sp.)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
HighFRS
LowFRS
A
C
Da
Db
Ea
Eb
Numbers of Settlers
Treatment

Table 3.3. Experiment 3 (25 May 1994 - 26 June 1994). Analysis of larval settlement. Table details as for Table 3.1, except that here Residual degrees of freedom were 34.

	ANOVA						A *vs.* [D_a + D_b]		C *vs.* [E_a + D_b]		A *vs.* [C - E_a + D_a]	
	MSres	Site	Block (Site)	Treat.	Interaction Site*Treat.		HighFRS	LowFRS	HighFRS	LowFRS	HighFRS	LowFRS
		P	*P*	*P*	*P*	n	*t*	*t*	*t*	*t*	*t*	*t*
Total Settlement	852.0	**<0.001**	**0.005**	**<0.001**	**<0.001**	4	0.224	**+ 2.924**	**+ 3.377**	2.392	**- 3.144**	1.893
Spirorbins	750.0	**<0.001**	**0.015**	**<0.001**	**<0.001**	4	0.392	2.684	**+ 2.924**	1.717	2.016	2.113
Serpulins/*Pomatoceros* spp.	5.944	**0.040**	0.461	**<0.001**	0.065	4	**- 4.425**	0.000	**+ 2.852**	**+ 3.145**	**- 6.229**	1.840
Total Ascidians	17.81	0.055	0.708	**<0.001**	0.744	4	0.917	0.635	0.133	2.422	1.347	0.947
Botryllus schlosseri / *Botrylloides leachi*	14.16	**0.013**	0.866	**0.010**	0.359	4	1.127	0.480	0.144	**+ 6.333**	1.372	**- 3.234**
Total Bryozoans	28.98	**<0.001**	0.068	**<0.001**	**<0.001**	4	0.127	2.245	2.723	0.970	2.584	1.549
Erect Bryozoans	10.68	**<0.001**	0.381	**<0.001**	**<0.001**	4	1.433	2.025	0.600	0.939	2.310	0.960
Crisia sp.	10.38	**<0.001**	0.347	**<0.001**	**<0.001**	4	1.433	2.098	0.526	0.939	2.363	1.032
Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)	8.960	**<0.001**	**0.023**	**<0.001**	**<0.001**	4	0.762	**+ 8.227**	**+ 2.867**	2.413	1.737	2.639
Sheet Bryozoans	4.213	0.101	0.326	**<0.001**	0.057	4	1.522	1.179	0.522	0.125	0.883	1.415
Celleporella hyalina	1.816	0.292	0.814	0.117	0.528	4	0.522	0.822	1.711	1.000	0.512	0.447
Electra pilosa	1.339	0.901	0.134	**<0.001**	0.094	4	0.676	0.471	0.600	**+ 4.042**	0.000	**- 2.920**

critical value for $t_{0.01(2),\,34} = 2.728$

enumerated, 11 showed highly significant Treatment effects, and there were marked Site*Treatment interactions for the settlement responses of more than half of the latter.

The planned comparisons for Experiment 3 are also presented in Table 3.3:

1.) A *versus* [D_a + D_b] (Table 3.3). Only the Serpulin/*Pomatoceros* spp. grouping was markedly inhibited by biofilming of panels and/or the presence of incumbents (at the HighFRS). Facilitatory effects of biofilming and/or incumbents were shown for Total Settlement, and Mound Bryozoans at the LowFRS only. Spirorbin settlement, too, was enhanced on treatment A at the LowFRS, and the outcome of the contrast was only marginally non-significant.

2.) C *versus* [E_a + D_b] (Table 3.3). All significant biofilming effects (which in this comparison were isolated from the effect of incumbent settlers) at both sites were facilitatory rather than inhibitory. Settlement on treatment C was markedly facilitated for Total Settlement, Spirorbins, the Serpulins/*Pomatoceros* spp. grouping and Mound Bryozoans at the HighFRS, and for the Serpulins/*Pomatoceros* spp. and *Botryllus schlosseri/Botrylloides leachi* groupings and *Electra pilosa* at the LowFRS.

3.) A *versus* [C - E_a + D_a] (Table 3.3). The presence of early post larval settlers had a marked inhibitory effect on Total Settlement and the Serpulins/*Pomatoceros* spp. grouping at the HighFRS, and on the *Botryllus schlosseri/Botrylloides leachi* grouping and *Electra pilosa* at the LowFRS.

The fact that all of the between-treatment differences for the contrast [A - D_a] *versus* [C - E_a] were due to the left hand terms being smaller, whilst biofilming in the absence of incumbents (contrast C *versus* [E_a + D_b]) had only facilitatory effects, suggests that biofilming effects in the comparison A *versus* [D_a + D_b] for this experiment were masked by the inhibitory effects of incumbents and *vice versa*.

Between-experiment comparison

Although Total Settlement varied significantly among the three experiments (P< 0.001), with the lowest counts for Experiment 2 (October - November 1993), the species

composition was very similar throughout (Tables 3.1-3.3). The experimental repeats were similar also in that they all showed significant differences between the two sites of contrasting flow regime for many of the enumerated taxa and categories (Tables 3.1-3.3). Overall, settlement was significantly higher at the LowFRS (Figs. 3.3-3.9). The large majority of the significant effects of biofilming on larval settlement (comparison C *versus* [E_a + D_b]) were facilitatory rather than inhibitory, whereas for the comparison A *versus* [D_a + D_b] inhibitory effects of biofilming and/or incumbents were predominating (particularly at the HighFRS) (Tables 3.1-3.3). The effects of early incumbent settlers (comparison A *versus* [C - E_a+ D_a]) generally were inhibitory, with the exception of facilitated settlement of Total Settlement and Spirorbins at the LowFRS in Experiment 2. Spirorbins are known to settle gregariously, and because this group made up the largest proportion of both the present incumbents and the total numbers of settlers, it is perhaps not surprising to find enhanced abundances of the categories Spirorbins and Total Settlement on treatment A substrata.

Biofilming overall either facilitated or had no effect on Total Settlement (Figs. 3.3, 3.6 and 3.8). However this facilitation was not always significant (Tables 3.1-3.3). The outcome of comparisons between biofilmed and unfilmed panels was not consistent between experiments for a variety of species groupings (Tables 3.2, 3.4 and 3.6). Groups that were facilitated by biofilming and/or the presence of incumbents in one experiment failed to show Treatment effects, or even were inhibited by biofilms in another (e.g. Spirorbins, *Electra pilosa*, Mound Bryozoans). Just as experiments varied in the settlement responses of particular groups to biofilming and the presence of incumbents, so too differences were seen in the groups for which the effects of Site and Treatment interacted significantly (Tables 3.1-3.3). Experiment 2 showed much fewer Site*Treatment interaction effects than did Experiments 1 and 3.

Analysis of biofilms

Mean percentage substratum cover of the identified surface biofilm components (estimated from SEM examinations) for all experimental treatments are presented in Table 3.4. The categories Bacteria (mean 27-40 %, treatment A, both sites) and EPS (mean 12-35 %, treatment A, both sites) accounted for the largest fractions of the biofilm surface composition, and Organic Debris was comparatively abundant on substrata at the LowFRS (mean 16 %, treatment A). Diatoms, Protozoans, Fungi and Sediment Particles, although present on all panel treatments, were numerically far less important, particularly on the younger biofilms (treatments D_a, D_b, E_a and E_b). Many of the protozoans observed were *Acanthoeca*-like choanoflagellates.

The plug biofilms were shown to be distinctly three-dimensional in structure when observed under SEM. The biofilms of substrata immersed for 4 weeks at the HighFRS were characterized by mesh-like strands of exopolymeric material. The biofilms of all panel treatments were very heterogeneous and large proportions of panel surface (especially in the younger biofilms) were free of microbes (Table 3.4). Photographic examples of biofilm categories enumerated are shown on Plate 3.5.

The results of the analyses of biofilm data (Table 3.4) are summarized in Table 3.5. There were several consistent patterns, with the HighFRS showing more free panel surface but less organic debris and sediment particles, more EPS and fewer bacteria than did the LowFRS. These between-site differences were more obvious for older biofilms (A *versus* C) than for the younger biofilms (D_a *versus* E_a; D_b *versus* E_b). Some Block effects for the less abundant biofilm components were apparent. Significant Site*Treatment interaction effects were indicated for the biofilm categories bacteria, EPS, organic debris and sediment particles; however, there were strikingly few significant between-means differences for the comparisons of netted and un-netted substrata within sites. For the younger biofilms alone (D_a/D_b *versus* E_a/E_b) it was apparent that netting of the substrata had little effect on the films (Table 3.5). This indicates that while counts could be higher on netted substrata than on un-netted substrata at one site, this numerical

Table 3.4. Experiment 3 (25 May 1994 - 26 June 1994). Mean percentage surface cover of all biofilm categories enumerated by SEM for all experimental treatments. All data are given as mean arcsine back-transformed percentages. Figures in brackets are the lower and upper back-transformed standard errors of the means (n = 72, EPS = extracellular polymeric substances, HighFRS = high flow rate site, LowFRS = low flow rate site, SE = standard error).

		BIOFILM SURFACE AREA [%]							
		Bacteria (SE)	EPS (SE)	Diatoms (SE)	Protozoans (SE)	Fungi (SE)	Organic Debris (SE)	Sediment Particles (SE)	Panel Surface (SE)
High FRS	A	26.89 (1.36, 1.38)	35.16 (1.57, 1.58)	0.22 (0.09, 0.11)	0.35 (0.23, 0.16)	0.13 (0.07, 0.07)	2.21 (0.38, 0.42)	1.17 (0.28, 0.31)	25.99 (2.13, 2.19)
	C	30.79 (1.47, 1.44)	31.35 (1.62, 1.65)	0.24 (0.09, 0.11)	0.22 (0.09, 0.11)	0.10 (0.05, 0.06)	1.37 (0.30, 0.34)	0.59 (0.18, 0.21)	27.29 (2.07, 2.12)
	D_a	17.32 (1.13, 1.16)	12.31 (0.99, 1.02)	0.06 (0.03, 0.04)	0.10 (0.05, 0.07)	0.05 (0.03, 0.04)	0.55 (0.16, 0.19)	0.79 (0.21, 0.24)	63.41 (1.53, 1.51)
	D_b	14.05 (0.87, 0.90)	15.72 (0.94, 0.96)	0.03 (0.02, 0.02)	0.09 (0.07, 0.07)	0.04 (0.02, 0.03)	0.66 (0.21, 0.24)	0.55 (0.17, 0.20)	64.15 (1.56, 1.55)
	E_a	18.09 (1.16, 1.19)	13.20 (0.80, 0.90)	0.06 (0.03, 0.04)	0.14 (0.05, 0.05)	0.12 (0.05, 0.07)	0.35 (0.12, 0.14)	0.72 (0.21, 0.24)	62.15 (1.56, 1.54)
	E_b	14.75 (0.61, 0.62)	12.42 (0.74, 0.76)	0.06 (0.03, 0.04)	0.06 (0.03, 0.04)	0.02 (0.01, 0.02)	0.57 (0.17, 0.20)	0.69 (0.19, 0.22)	67.63 (1.15, 1.14)

Table 3.4 contd.

		BIOFILM SURFACE AREA [%]							
		Bacteria (SE)	EPS (SE)	Diatoms (SE)	Protozoans (SE)	Fungi (SE)	Organic Debris (SE)	Sediment Particles (SE)	Panel Surface (SE)
Low FRS	A	39.08 (1.55, 1.56)	12.00 (1.14, 0.99)	0.28 (0.10, 0.12)	0.25 (0.09, 0.11)	0.30 (0.10, 0.12)	16.16 (0.90, 0.92)	2.83 (0.46, 0.50)	20.81 (1.80, 1.86)
	C	37.38 (1.75, 1.76)	11.02 (1.19, 1.25)	0.19 (0.07, 0.09)	0.38 (0.14, 0.18)	0.15 (0.07, 0.08)	12.85 (1.11, 1.16)	2.04 (0.33, 0.36)	26.33 (2.14, 2.20)
	D_a	21.35 (1.28, 1.31)	8.03 (0.87, 0.92)	0.08 (0.04, 0.05)	0.35 (0.12, 0.15)	0.03 (0.02, 0.03)	4.79 (0.62, 0.66)	0.61 (0.16, 0.19)	58.49 (1.67, 1.66)
	D_b	19.76 (1.44, 1.48)	7.69 (0.89, 0.94)	0.06 (0.03, 0.04)	0.19 (0.08, 0.10)	0.09 (0.04, 0.05)	4.30 (0.63, 0.68)	0.69 (0.19, 0.22)	60.31 (2.04, 2.02)
	E_a	19.30 (1.18, 1.21)	8.36 (0.86, 0.90)	0.04 (0.02, 0.03)	0.18 (0.08, 0.10)	0.06 (0.03, 0.05)	8.26 (0.80, 0.84)	0.67 (0.17, 0.19)	57.11 (1.40, 1.40)
	E_b	20.74 (1.48, 1.52)	7.43 (0.69, 0.72)	0.10 (0.04, 0.06)	0.08 (0.04, 0.05)	0.02 (0.01, 0.02)	5.29 (0.66, 0.70)	0.93 (0.21, 0.24)	59.79 (1.66, 1.65)

relationship could be reversed at the other site and yet within-site means did not differ significantly. The only significant between-mean differences were those of higher EPS counts on D_b than on E_b at the High FRS and lower counts of organic debris on D_a than on E_a at the LowFRS (Tables 3.4 and 3.5).

Mean total bacterial cell counts·cm^{-2} (obtained by AODC) for substrata exposed during the first and second half of the experiment ranged only from $2.5 \cdot 10^6$ (treatment C) to $2.7 \cdot 10^6$ (treatment A) (Fig. 3.10). Overall mean counts of bacteria were significantly higher on substrata from the LowFRS than on those from the HighFRS (Table 3.5). Mean bacterial counts were significantly higher for 4 week old panels (treatments A and C) than for 2 week old panels (D_a, D_b, E_a and E_b) resulting in a significant Treatment effect (Table 3.5). There was no marked interaction between the factors Site and Treatment on the mean numbers of bacteria. On average 2-4% of the bacterial cells from the AODC examinations were counted as dividing but there were no significant Site, Treatment or interaction effects (Table 3.5). It is possible that numbers of bacterial cells obtained by AODC were underestimates, due to cell loss during removal of the biofilms from the plugs (see also Schoener, 1984). None the less, the analysis, including substrata bearing both 2- and 4-week old biofilms, indicated significant Treatment effects for all other biofilm parameters examined.

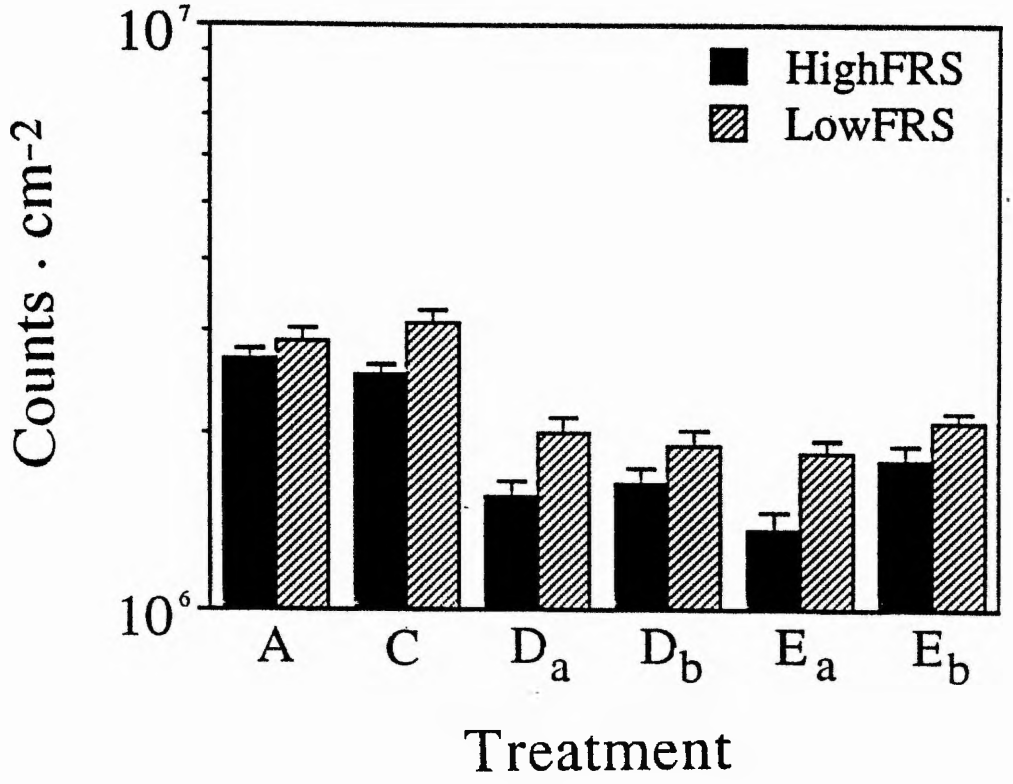
Chlorophyll *a* content of biofilm plug samples was either too low to be measured accurately or could not be detected at all by the spectrophotometer. The lack of detectable photosynthetic pigment corroborates the low overall numbers of diatoms estimated from SEM, and was expected from the downward and shaded orientation of panels.

3.4 DISCUSSION

In this series of manipulative experiments it was shown that the effect of microbial biofilming of substrata on larval settlement of marine invertebrate taxa is species-specific under field conditions. There were, however, many intra-specific variations in the settlement response between experimental repeats. Equivocal evidence

FIGURE 3.10

Experiment 3. Mean total bacterial cell counts (+ standard errors) from acridine orange direct count epifluorescence microscopy (AODC) preparations of biofilms from treatments A, C, D_a, D_b, E_a, and E_b at both sample sites (HighFRS = high flow rate site; LowFRS = low flow rate site; n = 90).


10^7
10^6
Counts · cm-2
HighFRS
LowFRS
A
C
D_a
D_b
E_a
E_b
Treatment

about the response of certain taxa to biofilms was reported also by Keough & Raimondi (1995). Some of the present variations can possibly be explained by the composite groups comprising several indistinguishable species (e.g. Spirorbins, Serpulins/*Pomatoceros* sp.). Low overall abundances and high variances for some of the groups in one or more of the experiments might have added to the apparent variations in larval response; but between-experiment differences are more likely a reflection of seasonal factors, including changes of biofilm cues, which may themselves result in alterations of larval settlement response. Such putatively important seasonal changes in composition, density and/or physiological activity of epibenthic biofilms have been reported elsewhere (e.g. Castenholz, 1963; Marszalek *et al.*, 1979; Hudon & Bourget, 1981; Munteanu & Maly, 1981; MacLulich, 1987, Anderson, 1995).

As in Todd & Keough's (1994) study of epifaunal assemblages in southeastern Australia, the present experiments revealed an influence of early incumbent settlers on subsequent settlement patterns (see also Dean & Hurd, 1980; Davis *et al.*, 1991). The incumbent effects varied from facilitatory to inhibitory (although the majority of the significant effects were inhibitory), depending on the taxon, and they varied intra-specifically between experiments. Again, this could be due to seasonality of certain larval species or perhaps in their production of allelochemical metabolites. The avoidance of future dominant competitors at settlement is likely to be of high adaptive value, and there are many examples of inter-specific inhibition among fouling species in the literature (e.g. Goodbody, 1961; Jackson & Buss, 1975; Grosberg, 1981; Young & Chia, 1981; Bingham & Young, 1991; Rittschof *et al.*, 1985, 1986; Davis & Wright, 1990; Martin & Uriz, 1993; Todd & Keough, 1994). The use of co-occurring species as indicators for favourable settlement sites has been reported for barnacles by Standing *et al.* (1984), Raimondi (1988) and LeTourneux & Bourget (1988), and other examples for enhanced settlement in the presence of other species are given in Todd & Keough (1994) (see also Scheltema, 1974; Osman & Whitlatch, 1995a, 1995b).

It is likely that some caging effects, such as alterations of hydrodynamic flow over netted panels, were caused by their enclosure in mesh bags (see also Marshall *et al.*, 1980). However, flow through the holding frames and across the panel surfaces will be

turbulent irrespective of the presence of mesh screens (see also Todd & Keough, 1994). Also, machine-sewing the panels into the mesh bags resulted in a tight fit, with the nets projecting from the panel surfaces by little more than a few millimetres. Keough & Raimondi (1995) failed to detect any strong effects of mesh-covering the substrata on the responses of larvae to biofilming. Although there were some differences in biofilm surface composition between the netted and un-netted panels (organic debris and inorganic particles were excluded from netted panels to some extent), overall the biofilm surface compositions were strikingly similar.

Despite there having been more reports of enhanced larval settlement onto biofilmed surfaces in the past, evidence of larval settlement inhibition by microbial organisms is increasing (see for example Maki *et al.*, 1988; Holmström *et al.*, 1992; Rodriguez *et al.*, 1993), and the data of the present study showed that the effect of natural biofilms on the settlement response of barnacle (*Balanus a. amphitrite*) larvae can change from inhibitory to facilitatory as the films age (Chapter 2, Section 2.2; see also Neal & Yule, 1994a). Therefore, unlike Todd & Keough (1994), no *a priori* assumption was made that all effects of biofilms on settlement would be either facilitatory or inhibitory, and two-tailed rather than one-tailed comparisons were drawn. The few significant Block effects found in the present experiments were expected, and are more or less inevitable in this type of field experiment. They were most likely due to within-site differences in larval supply (Miron *et al.*, 1995), gregariousness of certain taxa (e.g. Pawlik *et al.*, 1991; Toonen & Pawlik, 1994) and/or small scale habitat heterogeneities (see also Roberts *et al.*, 1991; Todd & Keough, 1994).

Although marked influences both of early incumbents and biofilming on epifaunal settlement were shown in the present experiments, the outcome of the between-means comparisons showed that other factors also must be responsible for the settlement patterns observed at Clachan Seil. For example, although settlement of Erect Bryozoans, Sheet Bryozoans and *Electra pilosa* in Experiments 2 was inhibited by biofilms in the presence of incumbents at the HighFRS (A *versus* [$D_a + D_b$]), neither the isolated effects of biofilming (C *versus* [$E_a + D_b$]) nor incumbents (A *versus* [$C - E_a + D_a$]) was significant at this site (Table 3.2).

Opinions vary as to whether settlement patterns in marine benthos are predominantly mediated through active habitat selection by the larvae (e.g. Meadows & Campbell, 1972; Burke, 1983), or passive deposition of larvae (e.g. Hannan, 1984; Eckman, 1990) and differential mortality of early recruits (e.g. Keough & Downes, 1982; Connell, 1985; Holm, 1990; Hurlbut, 1991a, 1991b; Dalby & Young, 1992). Settlement patterns caused by passive deposition of larvae are thought to be largely ruled by larval supply (Gaines & Roughgarden, 1985; Underwood & Fairweather, 1989; Grosberg & Levitan, 1992; Miron *et al.*, 1995), and hydrodynamic forces (Eckman, 1983; Butman, 1987; Havenhand & Svane, 1991; Gross *et al.*, 1992; Hardin *et al.*, 1994; Snelgrove, 1994). It presently is largely accepted that both processes (passive deposition of larvae and active habitat selection) play important rôles in creating the settlement patterns found in the field, although they might operate at different temporal and spatial scales (Butman, 1987; Butman *et al.*, 1988; Pawlik & Butman, 1993). To date, field studies of settlement patterns for a wide range of taxa in relation to hydrodynamic regime have not considered both flow regime and biofilming at the same time.

Flow regime is known to have a direct impact on community structure by altering fluxes of dissolved and particulate matter to benthic organisms (Nowell & Jumars, 1984). Lesser *et al.* (1994) showed how flow regime and seston availability markedly influenced the growth rates and population structure of hard substratum benthic suspension-feeders. They reported differential flow regime requirements for optimal growth for the sea anemone *Metridium senile*, a passive suspension feeder, and the northern horse mussel *Modiolus modiolus*, an active suspension feeder. Flow-modulated differences in metabolic rate and food capture have been described for a number of marine suspension feeding species (Best, 1988; Hughes, 1989; Wildish & Miyares, 1990; Patterson, 1991; Grizzle *et al.*, 1992; Eckman & Duggins, 1993), and active larval choice of a settlement site of appropriate flow conditions would seem to be highly adaptive to these species. The present results suggest that larvae of certain hard-substratum taxonomic groups and species (e.g. Spirorbins, Mound Bryozoans and

Electra pilosa) are capable of detecting differences in flow regime-associated biofilm patterns within a local habitat, and to respond to these during settlement.

The panels from sites of contrasting flow regimes differed markedly in their biofilm surface compositions. Because of the three dimensional structure of the biofilms (see also Marszalek *et al.*, 1979) it was impossible to obtain total counts of the film components by SEM. However, it is likely that putative biofilm cues triggering larval settlement are located at the film surface, where contact is made, rather than close to the epilithic/panel substratum. Biofilms from the LowFRS and the HighFRS also differed significantly in their total numbers of bacterial cells (estimated by AODC). Although other factors may influence the substratum-associated microfloral assemblages at the sample sites at Clachan Seil, the apparent differences in biofilm surface characteristics and overall bacterial cell numbers between sites are most likely attributable to contrasts in flow regime and shear stress (see also Korte & Blinn, 1983; Characklis & Cooksey, 1983; Nowell & Jumars, 1984; Rittmann, 1989; Allison & Gilbert, 1992; Wolfaardt & Cloete, 1992; Lau & Liu, 1993; Neal & Yule, 1994b). The highly significant Site*Treatment interactions found for various taxa in all three experiments suggest that between-site differences in macrofaunal settlement patterns cannot be explained by variations in larval supply alone, but that larval behaviour at the time of attachment also plays an important rôle. This result is in contrast to the observations by Keough & Raimondi (1995), who report similar effects on settlement of many taxa to biofilms developed under varying conditions, for similar periods of time.

Whereas the use of microbial cues by settling larvae has been reported for many taxa under laboratory conditions (often using mono-specific biofilm cues) and in the field (see Introduction), the present study shows how very subtle and finely tuned are invertebrate larvae in their response to these cues. It remains to be determined what these putative cues are, and how they are mediated. A number of authors (Kirchman *et al.*, 1982; Maki *et al.*, 1988, 1990, 1992; Decho, 1990; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992; Holmström & Kjelleberg, 1994) suggest the involvement of bacterial extracellular polymeric substances in such larval/biofilm interactions. Here, EPS was one of the biofilm surface categories for which there was a highly significant between-site

difference, but further experimental evidence is required to determine which biofilm components are responsible for site-related and seasonal differences in larval settlement response.

CHAPTER 4

SEASONAL VARIATION IN THE EFFECT OF HARD SUBSTRATUM BIOFILMING ON SETTLEMENT OF MARINE INVERTEBRATE LARVAE IN THE FIELD

4.1 INTRODUCTION

In a novel field experimental approach, in which biofilming of panel substrata under exclusion of larval settlers was achieved by enclosure of panels within tight-fitting, but removable, mesh screens (Todd & Keough, 1994; Keough & Raimondi, 1995, Chapter 3), it was revealed that *in situ* larval responses to microbial cues are highly species-specific, and can be either facilitatory or inhibitory. In Chapter 3 it was shown that larvae of certain hard-substratum species show flow regime related differences in their settlement response to biofilmed surfaces within a local habitat. It was concluded that such differential larval settlement is likely to be due to flow-regime associated differences in surface microcolonization patterns. Many species also are able to distinguish between microfilms of varying ages developed under similar conditions (Maki *et al.* 1988, 1990, 1992; Pearce & Scheibling, 1991; Holmström *et al.*, 1992; see also Chapter 2). Thus, there is growing evidence that larval responses to microbial cues are indeed very subtle and finely tuned, and that small-scale differences in successional composition, physiological condition and growth phase of the biofilm community may well alter the effect on larval settlement (Neumann, 1979; Szewzyk *et al.*, 1991; Anderson, 1995; Chapter 2, Section 2.2). In this context it has been proposed by several authors that microbial cues can help larvae to recognize settlement sites of high survival value (Strathmann & Branscomb, 1979; Strathmann *et al.*, 1981; Raimondi, 1988; see Chapter 3).

In view of the evidently highly complex interactions between microfilm dynamics, larval responses and environmental fluctuations, it seems futile to study microbial settlement cues as isolated parameters. Most research in the past has been

laboratory based, and often concerned only mono-specific films and a small selection of invertebrate taxa (Neumann, 1979; Weiner *et al.*, 1985; Fitt *et al.*, 1990; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992, Leitz & Wagner, 1993; Neal & Yule, 1994a). Whilst such controlled laboratory assays increase experimental tractability, they fail to consider the complexity of natural larva-biofilm interactions. Recent field studies examining larval settlement patterns in response to biofilming were short-term experiments and which did not allow the assessment of seasonal and annual changes in the effect of filming (Todd & Keough, 1994; Keough & Raimondi, 1995). Seasonal changes of the composition, density and/or physiological activity of epibenthic microfilms have been reported (e.g. Hudon & Bourget, 1981; Munteanu & Maly, 1981; Underwood, 1984; MacLulich, 1987; Anderson, 1995), and intuitively these will be important in explaining variations of settlement patterns for those species that reproduce throughout most parts of the year. Raimondi and Keough (1990), who discussed the marked plasticity in behavioural responses of larvae to settlement cues, also pointed to the possible impact of temporal variations in the environment. It is possible, for example, that the importance of biofilming as a positive or facilitatory settlement cue for the larvae of certain taxa might vary inversely with a seasonal increase in the effects of the presence of dominant competitor species.

The present series of experiments is the first to investigate the seasonality of the effects of biofilms on larval settlement in the field. The objective was to assess any temporal variation in the species-specific responses for a range of taxa, and to separate the effects of biofilming from those of the presence of other early incumbent post larvae (Chapter 3; see also Todd & Keough, 1994; Keough & Raimondi, 1995).

4.2 MATERIALS AND METHODS

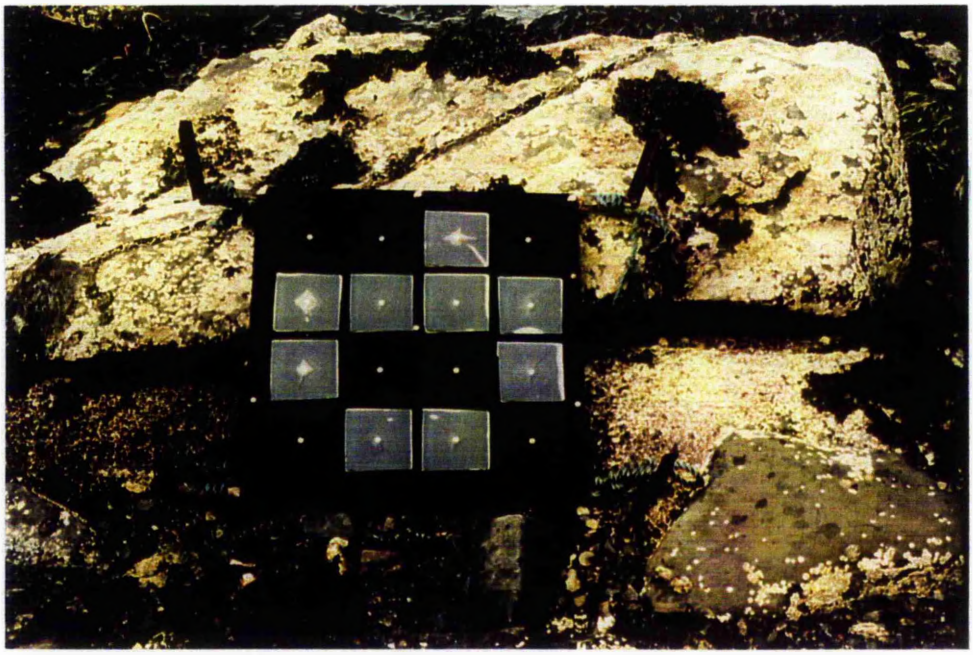
Field Experiments

The field experimental location was at Clachan Seil, western Scotland (see Chapter 3, and Todd & Turner, 1986; Turner, 1988). The general experimental protocols followed those of Chapter 3 (see also Todd & Keough, 1994, and Keough & Raimondi, 1995): black plastic (perspex) panels (0.5 mm thickness, 11 x 11 cm square) were used as settlement substrata. Preliminary field experiments that compared settlement rates onto different types of artificial substrata (perspex, natural slate, darkened slate) revealed black perspex to be a most suitable material, which has the advantage also of being easily cut to size and of light weight. Panels were bolted to square mounting plates (also made of black perspex) by means of plastic screws running through counter-sunk holes in the centres of the panels and retained by plastic wing nuts. Each frame could hold 16 panels in a 4 x 4 array on a mounting plate (Plate 4.1), leaving spaces of 3 cm between adjacent panels. The mounting plates were attached horizontally in an inverted position to four-legged steel frames which were coated in epoxy resin to prevent corrosion. The downward facing sides of the panels comprised the experimental substrata. This orientation of the panels was chosen to prevent any confounding effects of sediment deposition. Prior to their use in experiments, all panels were sanded with medium coarse sandpaper to remove the original surface-sheen of the material, and then soaked in freshwater for 2d to leach any potential contaminants. The surfaces of the panels then were lightly marked in a 1 x 1 cm grid to facilitate the scoring of established organisms at the end of the experiments.

The experiments included 5 panel treatments (Fig. 4.1) and these were letter coded as by Todd & Keough (1994). In treatment C initial biofilming of the panel substrata, whilst excluding larval settlement, was achieved by the enclosure of experimental panels within tight-fitting (but removable) polyester mesh screens of 100μm pore size (see Todd & Keough, 1994 for the effect of mesh sizes). These screens were folded tightly beneath the edges of the panels where they were securely held between the panel and the

PLATE 4.1

The frames used in the time series experiments holding the experimental panel substrata (viewed from below).



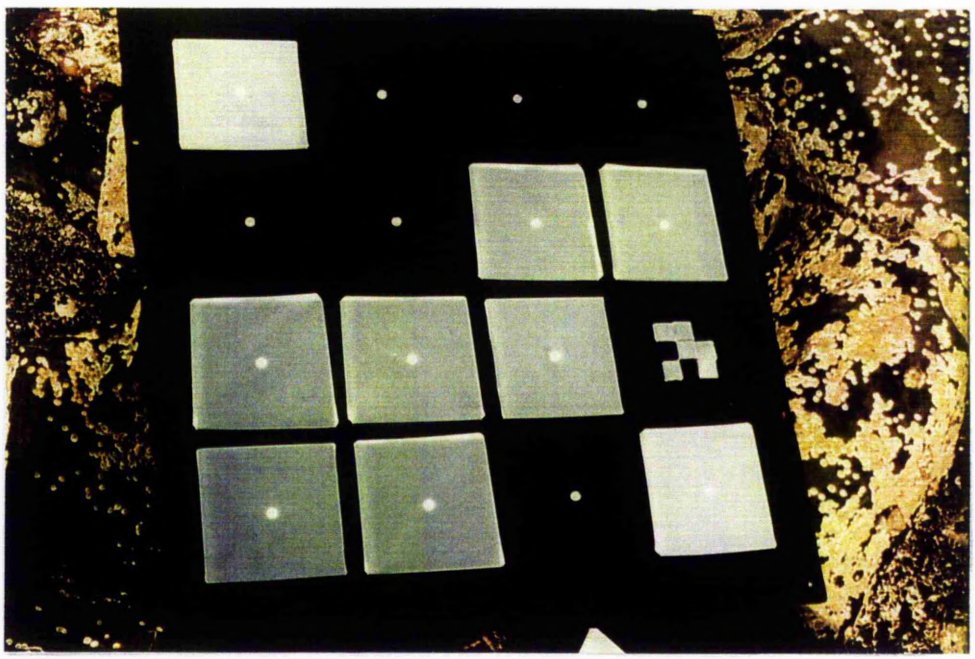
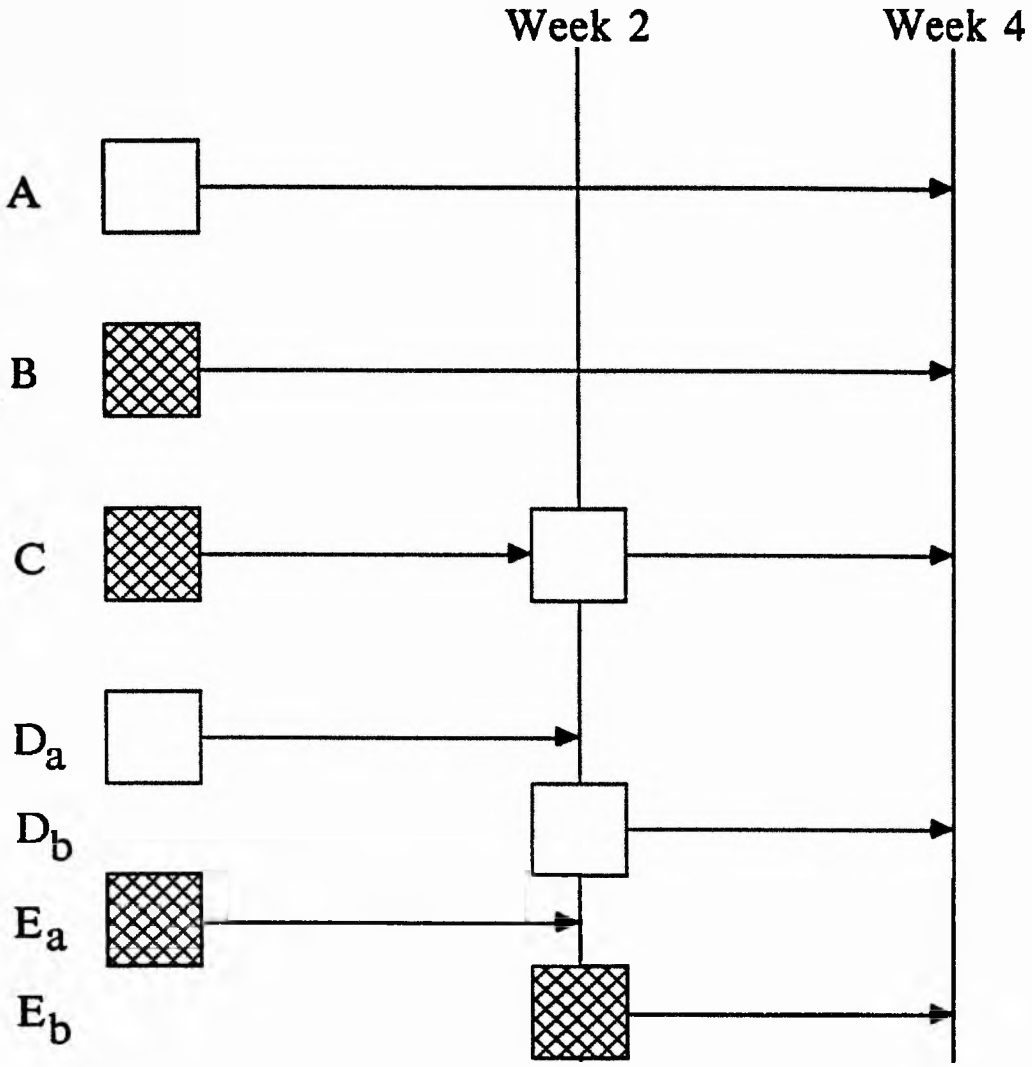


FIGURE 4.1

Schematic diagram of the experimental treatments and their codes for the first and second halves of the experiments. Netted and un-netted panels are represented by hatched and unhatched squares respectively.


Week 2
Week 4
A
B
C
D_a
D_b
E_a
E_b

mounting plate (Plate 4.1). After an initial 14d period of immersion, allowing the development of a biofilm but an essential exclusion of larval settlers, the meshes were removed and the experimental surfaces exposed to larval settlement for a further 14d. In Chapter 3 it was shown that netting did not have any major effects on the establishment of microbial films onto panel surfaces (Chapter 3, but see also Keough & Raimondi, 1995). Treatment A panel substrata were un-netted and exposed to larval settlement throughout the experimental time of one month. Treatments D (D_a and D_b) and E (E_a and E_b) were the respective controls for the un-netted and netted treatments and were included to provide separate data for the two halves of the experimental period: D_a/E_a was an un-netted/netted panel, which was replaced after 14d by another un-netted/netted panel, D_b/E_b. In a fifth treatment, B, netted panels remained deployed and unmanipulated throughout the experimental period of 28d. Although initially included in the experiments as an additional control treatment B was subsequently not used in the statistical analysis.

Following each experiment the panels were transferred into holding containers filled with seawater and transported to St. Andrews, where the settlers were counted under a dissecting microscope and identified to the lowest possible taxon (grouping of taxa into categories as described in Chapter 3). Except for the 1994 spring experiment, in which panels from the first half of the experiment were preserved for 1wk in a 4% formaldehyde seawater solution, all panels were scored within two days of return to the laboratory. During removal and transport of the panels care was taken to prevent them from contacting the air water interface. Only the central areas (10 x 10 cm) of the panels were counted and considered in the analysis, leaving a 1 cm wide margin to allow for potential edge effects and inadvertent handling during retrieval (see for example Keough & Raimondi, 1995).

The frames (= blocks in the statistical analysis) were anchored by four 12 kg concrete cylinders attached to the frame legs. There were three frames per experiment, with the exception of the 1992 autumn experiment, the 1993 winter experiment and the 1994 summer experiment, in which there were two frames only. Each frame included three replicate panels of experimental treatments A, B, C, D and E (= 15 panels) in a

randomized array; the 16th slot of each frame was filled with a combination of nine small Perspex panels (1.5 x 1.5 cm), which were attached to the mounting panel by means of silicon glue. The small panels were assembled in randomized positions in a 3 x 3 array, and three replicates each of treatments A, B and C were used. At the end of the experiments these panels were preserved in a 4% glutaraldehyde solution, because they were initially included for SEM comparison of biofilms. No quantitative examinations were carried out for these film samples, and only a number of them were used for visual comparisons between treatments (for a more detailed quantitative comparison of biofilms from netted and un-netted panel treatments see Chapter 3). No obvious treatment effects were noted between netted and un-netted substrata.

Experiments were repeated twice for each season: autumn (September/October 1992 and 1993), winter (January/February 1993 and 1994), spring (March/April 1993 and 1994) and summer (June/July 1993 and 1994).

Statistical Analysis

The Experiments 1 to 8 were treated as a time series and the pooled data for the individual taxonomic groups and species of all eight experiments were analysed by three-factor ANOVA, with Block, Experiment and Treatment as the factors (and Experiment and Treatment crossed). The frames were placed in the same locations (marked by the concrete anchors) for all of the eight experiments, and therefore the factor Block did not require to be nested within the factor Experiment. Block effects were assumed to be mostly due to positional variation in settlement between frames, thus to be of a fixed rather than a random nature, and had to be accounted for in the analysis. Block interaction terms with other factors were omitted from the model, because their contribution to the variance was considered to be largely random and of little interpretational value. Because the number of blocks varied between experiments (see above) the analytical design was unballanced and the GLM procedure in MINITAB (version 8.2, 1991) was used. In this initial analysis those treatments and experiments

were excluded for which mean counts per panel consistently were < 1 (across experiments and treatments respectively).

Following the ANOVAs, between-mean comparisons of settlers were drawn for the treatment contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a]. The standard errors of the differences were estimated as the square root of the sum of the standard errors of the treatment means to be compared. The use of mean counts per treatment across frames rather than counts per panel was chosen because the latter would have necessitated a random combination of individual panels (within a frame) into composite variables (i.e. [E_a + D_b] and [C - E_a + D_a]), which could potentially have confounding effects on the analysis.

Whilst the first of those contrasts, C *versus* [E_a + D_b], allowed an assessment of the effects of biofilming on larval settlement, the second, A *versus* [C - E_a + D_a], permitted to assess the effect of early incumbents on subsequent larval settlement (see Chapter 3). The combination A *versus* [C - E_a + D_a] was here used in preference to the original contrast [A - D_a] *versus* [C - E_a] because in the computations [C - E_a + D_a] generally resulted in positive terms, which could be plotted as mean numbers of settlers per panel (there can be no negative counts of settlers), whereas [A - D_a] *versus* [C - E_a] sometimes resulted in negative terms.

Although the variances of count data are generally stabilized by log-transformation (this is desirable in ANOVA which assumes homogeneity of variances), no transformation was carried out on the present data because the summation or subtraction of transformed data means would have a multiplicative rather than an additive effect in the case of the composite treatments ([E_a + D_b] and [C - E_a + D_a]). In acknowledgement of the fact that an analysis of the count data in their natural scale will introduce bias into the analysis, a highly conservative significance criterion of $\alpha_{[2]}$ = 0.001 was chosen for all tests to provide protection against the possibility of making a type I error (Sokal & Rohlf, 1981). However, care must be taken in the interpretation of the present results with regards to over-generalizing inferences.

For those groups and species for which the initial ANOVA revealed significant interactions of the factors Experiment and Treatment, between-treatment mean

comparisons were drawn for each experiment individually (analysis of the "simple" effects) to further elucidate the patterns underlying the "main" and interaction effects on larval settlement (indicated by the ANOVA) and to separate the effects of biofilming from those of the presence of early incumbent settlers (see Chapter 3). In cases where no interaction was detected, but significant Treatment and Experiment "main" effects were indicated, the contrasts were carried out for the pooled data of all eight experiments.

An analysis was considered only for those taxonomic groups and species which settled in more than one season. In the cases of groupings of settlers for which overall counts were low or highly variable within experiments no formal analysis was carried out, but rather a descriptive account of the settlement responses across time is given.

Another way of analysing the data from this experimental series is presented in the original version of this chapter which is included in Appendix 3.

4.3 RESULTS

Total Settlement

Average densities of settlers per panel of treatment C (panel netted for the first half of the experiment prior to exposure to larval settlement for the second half of the experiment) ranged between 0.24·cm^{-2} in the winter experiments and 0.76·cm^{-2} in autumn 1992 (Fig. 4.2). For treatment A (un-netted panels exposed to larval settlement throughout the experiment for 28 d) mean counts per panel of all categories of settlers pooled (Total Settlement) ranged between 0.21·cm^{-2} in winter 1994 and 1.08·cm^{-2} in summer 1994 (Fig. 4.4) (mean counts and standard deviation for the taxonomic groups and species enumerated are listed in Appendix 2 for all treatments and experiments). The ANOVA for Total Settlement (Table 4.1) revealed the factor Block to be only marginally non-significant ($P = 0.002$). There were, however, highly significant effects for Experiment ($P < 0.001$) and Treatment ($P < 0.001$) and the interaction of the latter

FIGURE 4.2

Contrast C *versus* [E_a + D_b]. Seasonal larval settlement of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/*Pomatoceros* spp., Total Ascidians, *Botryllus schlosseri*/*Botrylloides leachi*, Total Bryozoans, Erect Bryozoans and *Crisia* sp.. Plotted values are the mean numbers of settlers per panel and standard errors. Note the differences in the abundance scales applied. The connecting lines between points are for visual aid only and no predictions between points are implied. —□— treatment C, —●— treatment [E_a + D_b].

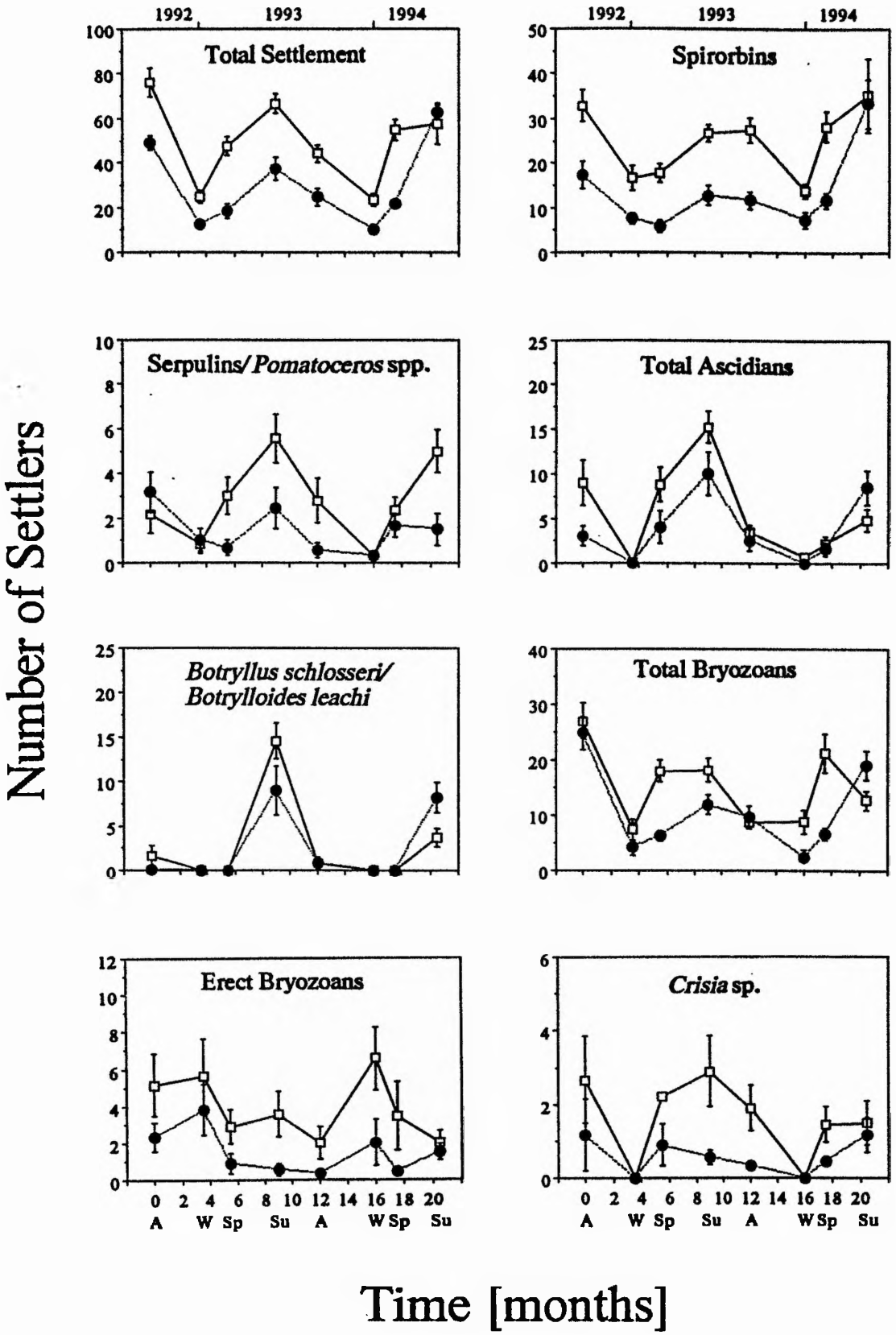

1992
1993
1994
Total Settlement
Spirorbins
Serpulins/ Pomatoceros spp.
Total Ascidians
Botryllus schlosseri/
Botrylloides leachi
Total Bryozoans
Erect Bryozoans
Crisia sp.
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.3

Contrast C *versus* [E_a + D_b]. Seasonal larval settlement of *Alcyonidium* spp., *Tubulipora* sp./*Plagioecia* sp. (= Mound Bryozoans), Sheet Bryozoans, *Celleporella hyalina* and *Electra pilosa*. Plotted values are the mean numbers of settlers per panel and standard errors. Details as for Fig. 4.2.

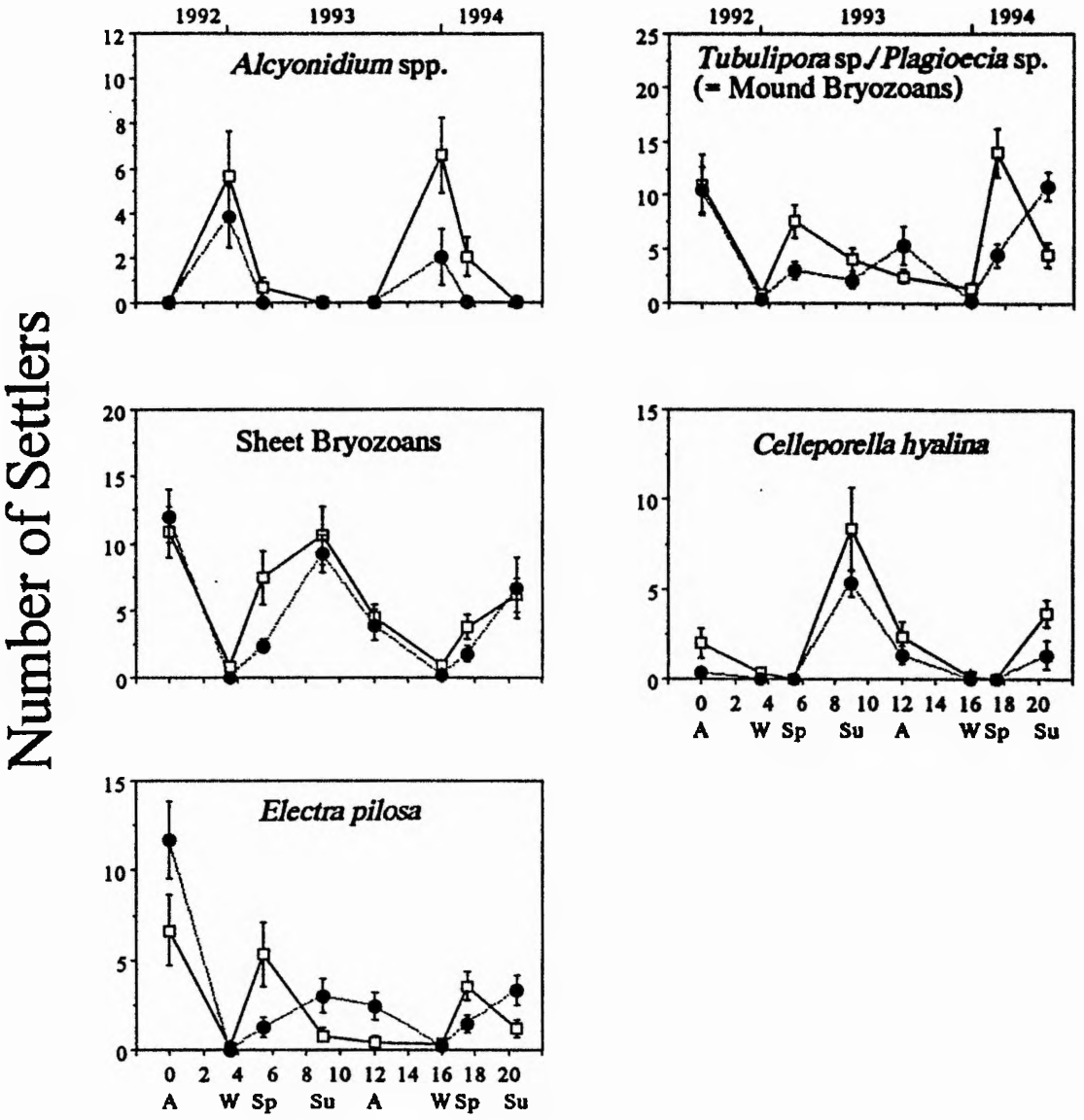

1992
1993
1994
Alcyonidium spp.
Tubulipora sp./Plagioecia sp.
(= Mound Bryozoans)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.4

Contrast A *versus* [C - E_a + D_a]. Seasonal larval settlement of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/*Pomatoceros* spp., Total Ascidians, *Botryllus schlosseri*/*Botrylloides leachi*, Total Bryozoans, Erect Bryozoans and *Crisia* sp.. Plotted are the mean numbers of settlers per panel and standard errors. Note the differences in the abundance scales applied. The connecting lines between points are for visual aid only and no predictions between points are implied. —○— treatment A, ---▲— treatment [C - E_a + D_a].

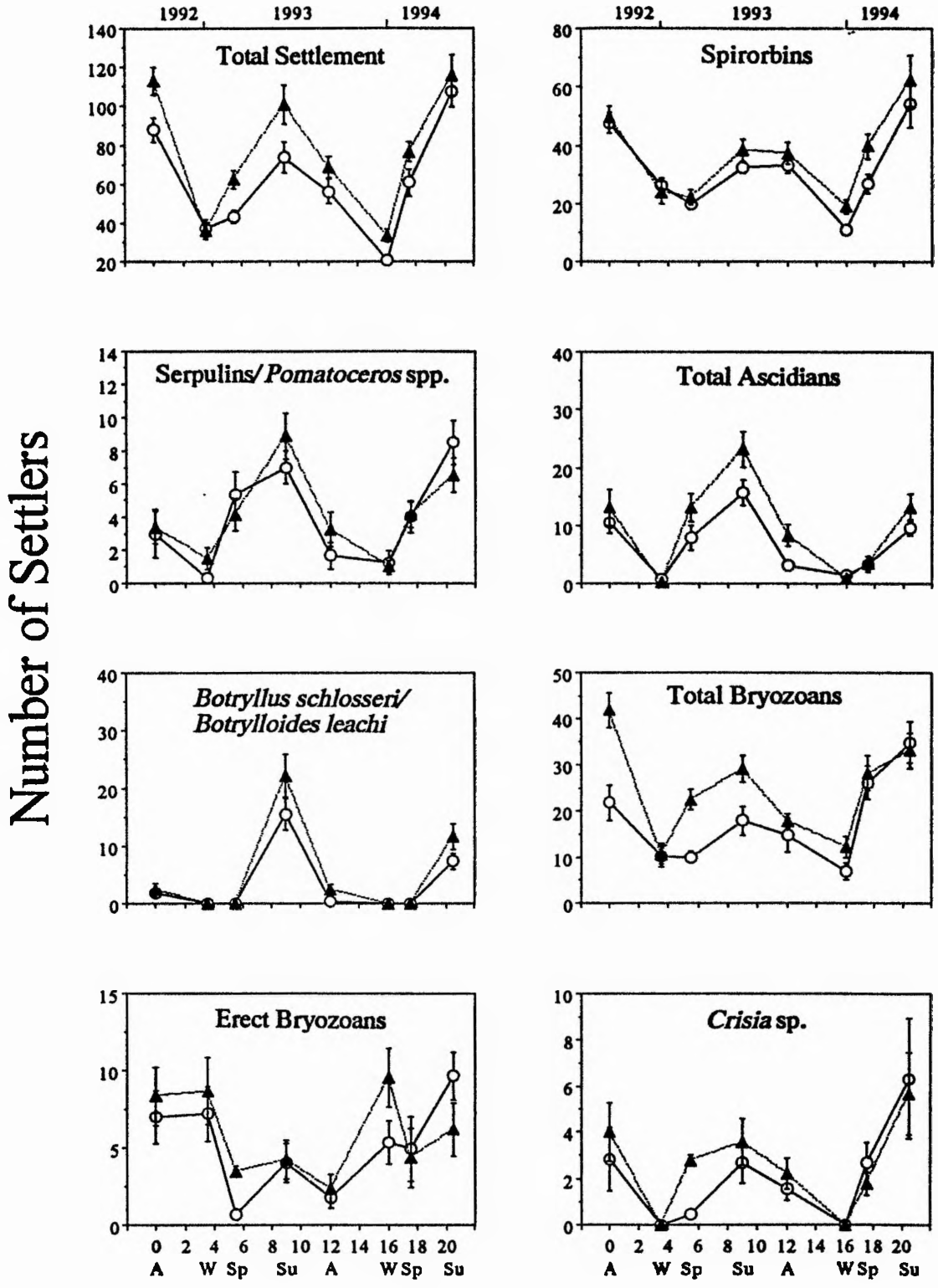

1992
1993
1994
Total Settlement
Spirorbins
Serpulins/ Pomatoceros spp.
Total Ascidians
Botryllus schlosseri/
Botrylloides leachi
Total Bryozoans
Erect Bryozoans
Crisia sp.
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.5

Contrast A *versus* [C - E_a + D_a]. Seasonal larval settlement of *Alcyonidium* spp., *Tubulipora* sp./*Plagioecia* sp. (= Mound Bryozoans), Sheet Bryozoans, *Celleporella hyalina* and *Electra pilosa*. Plotted are the mean numbers of settlers per panel and standard errors. Details as for Fig. 4.5.

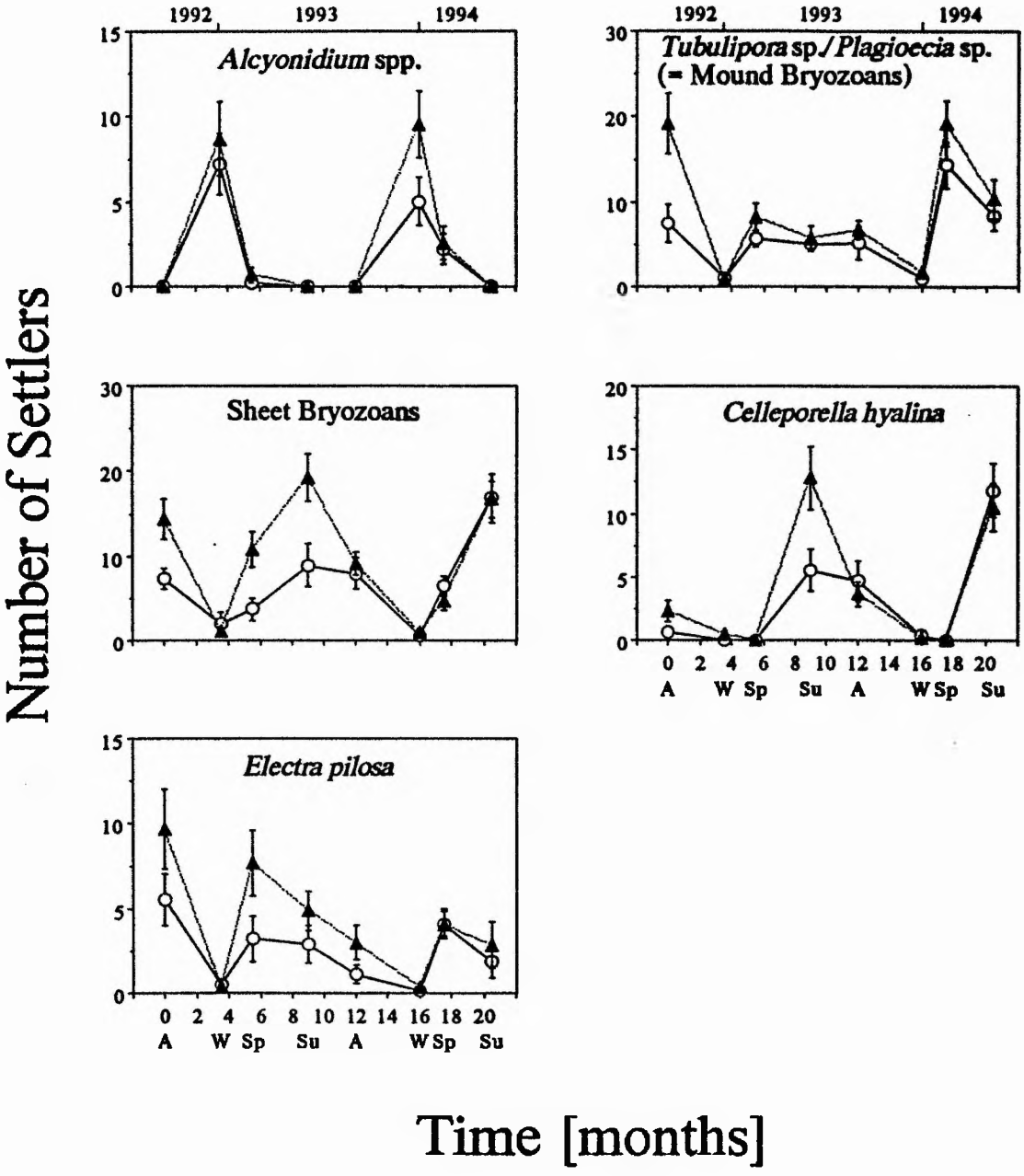

1992
1993
1994
Alcyonidium spp.
Tubulipora sp./Plagioecia sp.
(= Mound Bryozoans)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

Table 4.1. Analysis of variance for all categories of settlers pooled (= Total Settlement).

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Block	2	4808.1	1380.2	690.1	6.38	0.002
Experiment	7	48493.4	48493.4	6927.6	64.07	<0.001
Treatment	5	173197.7	176003.7	35200.7	325.54	<0.001
Experiment*Treatment	35	33765.9	33765.9	964.7	8.92	<0.001
Error	328	35467.3	35467.3	108.1		
Total	377	295732.4				

Table 4.2. Comparisons between treatment means of all categories of settlers pooled (= Total Settlement) for the contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a]. Significant *t*-values ($\alpha_{[2]} \leq 0.003$) are shown in bold face. Significant outcomes with *t*-values preceded by a '-' symbol are experiments for which the left hand term (C or A) was the smaller, '+' symbols identify experiments for which the right hand term was smaller (n = number of observations; A = autumn; W = winter; Sp = spring; Su = summer).

Experiment	n	C *vs.* [E_a + D_b] *t*	A *vs.* [C - E_a + D_a] *t*
1 A 1992	6	**+ 3.600**	2.816
2 W 1993	6	**+ 4.133**	0.160
3 Sp 1993	9	**+ 5.419**	**- 3.301**
4 Su 1993	9	**+ 4.404**	2.498
5 A 1993	9	**+ 3.418**	1.478
6 W 1994	9	**+ 3.801**	3.015
7 Sp 1994	9	**+ 6.851**	1.831
8 Su 1994	6	0.515	0.639

critical value for $t_{0.003(2),\,350}$ = 3.251

critical value for $t_{0.001(2),\,350}$ = 3.319

two factors ($P < 0.001$). The significant effect of the factor Experiment is most likely due to the highly seasonal changes in abundances of most of the groups and species observed in the present study, but between-year variation in both, onset of settlement period and overall abundances might contribute to some extend. Further analysis of the "simple" effects for the contrast C *versus* [E_a + D_b] indicated significantly facilitatory effects of biofilming in the case of all experiments except for summer 1994 (Table 4.2, see also Fig. 4.2). Only for the spring 1993 experiment was an effect of incumbent settlers shown to be significant (and inhibitory) on the larval settlement response (contrast A *versus* [C - E_a + D_a]) (Table 4.2), although Figure 4.4 shows that mean settlement was overall lower on panels bearing early settlers from the first half of the experiments (treatment A).

Spirorbins

This grouping clearly accounted for the largest proportion of settlers across all experiments, and thus overall settlement patterns are largely reflected by spirorbin responses to substrata. It is possible that this group comprised more than one *Spirorbis* species.

Average densities for this group on treatment C panels ranged between 0.1· cm^{-2} in winter 1994 and 0.4·cm^{-2} in summer 1994 (Fig. 4.2) and between 0.1·cm^{-2} in winter 1994 and 0.5·cm^{-2} in summer 1994 on treatment A panels (Fig. 4.4). As for Total Settlement highly significant Experiment, Treatment and interaction effects ($P < 0.001$) were shown for Spirorbins (Table 4.3), and Block was marginally non-significant ($P = 0.004$).

In all experiments except in summer 1994 settlement on biofilmed panels was higher than for the composite treatment [E_a + D_b], although the analysis of the "simple" effects showed significant facilitation of Spirorbin settlement on biofilmed substrata (treatment C) for only five of the eight experiments (autumn 1992, spring 1993, summer 1993, autumn 1993 and spring 1994) (Table 4.4, Fig. 4.2). No biofilming

Table 4.3. Analysis of variance for Spirorbins.

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Block	2	1769.7	490.9	245.4	5.51	0.004
Experiment	7	10508.7	10508.7	1501.2	33.68	<0.001
Treatment	5	43054.9	44663.6	8932.7	200.40	<0.001
Experiment*Treatment	35	7642.7	7642.7	218.4	4.90	<0.001
Error	328	14620.3	14620.3	44.6		
Total	377	77596.3				

Table 4.4. Comparisons between treatment means of Spirorbins for the contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a]. Details as for Table 4.2.

		C *vs.* [E_a + D_b]	A *vs.* [C - E_a + D_a]
Experiment	n	*t*	*t*
1 A 1992	6	+ 3.708	0.388
2 W 1993	6	2.931	0.350
3 Sp 1993	9	+ 4.975	0.177
4 Su 1993	9	+ 4.928	1.065
5 A 1993	9	+ 4.432	0.660
6 W 1994	9	2.763	2.776
7 Sp 1994	9	+ 4.520	2.572
8 Su 1994	6	0.188	0.769

critical value for $t_{0.003(2),\,350}$ = 3.251

critical value for $t_{0.001(2),\,350}$ = 3.319

effects were indicated for the winter experiments, although it should be noted that here abundances of the group were lowest (Fig. 4.2). For none of the experiments did the contrast A *versus* [C - E_a + D_a] reveal an effect of incumbents (see also Fig. 4.4).

Serpulins/*Pomatoceros* spp.

Settlement of this group was overall low, with largest mean numbers around 0.09·cm^{-2} in summer 1994 on panel treatment A, and around 0.06·cm^{-2} in summer 1993 on panel treatment C (Figs. 4.2 and 4.4); therefore no formal analysis was carried out. Figure 4.2 shows that except for the autumn 1992 experiment and the winter 1993 experiment settlement on biofilmed substrata was higher than on unfilmed substrata. The numbers of settlers of the Serpulins/*Pomatoceros* spp. grouping on substrata bearing incumbents appears to be similar to those on substrata without incumbents (Fig. 4.4).

Total Ascidians

This grouping included various solitary ascidians of rare occurrence (e.g. *Dendrodoa grossularia* and *Ascidiella scabra*), but primarily consisted of various colonial didemnid and tridemnid species and *Botryllus schlosseri* and *Botrylloides leachi* (see below), depending on seasonal availability. Average densities of this grouping ranged between 0.01·cm^{-2} in winter 1993 and 0.16·cm^{-2} in summer 1994 on panels of treatment A, and between < 0.01·cm^{-2} in winter 1993 and 0.15·cm^{-2} in summer 1993 on panels of treatment C. The ANOVA revealed significant effects of the factors Experiment and Treatment (P< 0.001), but no Experiment*Treatment interaction was indicated (Table 4.5). The "main" effect analysis, however, showed the differences between treatment means to be non-significant in both contrasts (t= 1.997 for the contrast C *versus* [E_a + D_b], and t= 2.455 for the contrast A *versus* [C - E_a + D_a];

Table 4.7. Analysis of variance for Total Ascidians.

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Block	2	134.03	93.82	46.91	2.45	0.089
Experiment	6	3186.77	3186.77	531.13	27.73	<0.001
Treatment	3	385.73	385.34	128.45	6.71	<0.001
Experiment*Treatment	18	543.02	543.02	30.17	1.58	0.069
Error	198	3792.18	3792.18	19.15		
Total	227	8041.73				

critical $t_{0.001(2),\,200} = 3.340$), thus, no significant biofilming or incumbent effects were revealed (see also Figs. 4.2 and 4.4).

Botryllus schlosseri/Botrylloides leachi

Settlement of this group was highly seasonal, with low overall numbers peaking in summer (highest average density on panel treatments A and C of 0.15·cm^{-2}) (Figs. 4.2 and 4.4). No formal analysis was carried out for this grouping.

Total Bryozoans

Average densities for this composite category, which included the sub-categories Erect Bryozoans, Mound Bryozoans and Sheet Bryozoans (see below), on treatment A were lowest in winter 1994 (0.07·cm^{-2}), and highest in summer 1994 (0.35·cm^{-2}). Mean Settlement on treatment C ranged between 0.07·cm^{-2} in winter 1993 and 0.27·cm^{-2} in autumn 1992. The ANOVA (Table 4.6) indicated highly significant Experiment, Treatment and interaction effects for Total Bryozoans ($P < 0.001$). However, only in spring 1993 was there a significant facilitation of settlement on biofilmed substrata (Table 4.7, see also Fig. 4.2). Inhibitory effects of incumbent settlers were shown for autumn 1992 and spring 1993 (Table 4.7, see also Fig. 4.4).

Erect Bryozoans

This category mainly comprised an unidentified *Crisia* species (see below), and several *Scrupocellaria* and *Alcyonidium* (see below) species, which. Within treatment and experiment variances for this category were comparatively high (Figs. 4.2 and 4.4; Appendix 2), thus no formal analysis was carried out. Figure 4.2 shows that settlement

Table 4.10. Analysis of variance for Total Bryozoans.

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Block	2	887.11	298.36	149.18	3.71	0.026
Experiment	7	7313.98	7313.98	1044.85	26.01	<0.001
Treatment	3	3149.00	2880.15	960.05	23.90	<0.001
Experiment*Treatment	21	3548.96	3548.96	169.00	4.21	<0.001
Error	218	8756.81	8756.81	40.17		
Total	251	23655.85				

Table 4.11. Comparisons between treatment means of Total Bryozoans for the contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a]. Details as for Table 4.2.

Experiment	n	C *vs.* [E_a + D_b] *t*	A *vs.* [C - E_a + D_a] *t*
1 A 1992	6	0.483	- 3.650
2 W 1993	6	1.337	0.157
3 Sp 1993	9	+ 4.927	- 4.982
4 Su 1993	9	2.303	2.627
5 A 1993	9	0.399	0.742
6 W 1994	9	1.113	1.813
7 Sp 1994	9	1.104	0.406
8 Su 1994	6	2.037	0.278

critical value for $t_{0.003(2),\,250}$ = 3.261

critical value for $t_{0.001(2),\,250}$ = 3.330

of Erect Bryozoans in general was higher on filmed substrata than on the composite treatment [E_a + D_b]. With the exception of the spring 1994 and the summer 1994 experiments, settlement was lower on panels bearing early settlers from the first halves of the experiments (treatment A) (Fig. 4.4).

Crisia sp.

Overall numbers of this group were considered too low to justify formal analysis (highest mean density on treatment A = 0.06·cm^{-2} for summer 1994). The settlement patterns observed on the various panel treatments were similar to those of the pooled Erect Bryozoans (Figs. 4.2), in that *Crisia* sp. counts, too, were generally higher on panels with biofilms than on unfilmed substrata. The patterns for substrata with incumbents were, however, less clear, and substrata without incumbents did not appear to receive less settlement than treatment A panels (Fig. 4.4).

Alcyonidium spp.

Figures 4.3 and 4.5 show the strong seasonality of this group, with settlement peaking in winter. However, densities of this groups even in winter did not exceed a mean of 0.07·cm^{-2} in 1993 on treatment A. Thus, no formal analysis was carried out. The group was represented in higher numbers on filmed panels than on unfilmed panels in both winter experiments, and for both winter experiments settlement was lower on incumbent bearing panels as compared to substrata without settlers from the first half of the experiment.

Tubulipora sp./*Plagioecia* sp.(= Mound Bryozoans)

This composite category comprised the only two mound forming bryozoan groups found on the substrata in the present study, both of which appeared to be present in more or less equal abundance in those experiments in which they occurred (i.e. neither of the species seemed to dominate the grouping in any of the experiments, although it should be noted, that it is impossible to confidently distinguish between the very early stages of these species). Average numbers of settlers on treatments A and C ranged between 0.01·cm^{-2} in the winter experiments and 0.14·cm^{-2} in spring 1994.

Table 4.8 shows the results of the ANOVA for the *Tubulipora* sp./*Plagioecia* sp. grouping: no differences between frames (blocks) were indicated by the analysis, but there were significant Experiment ($P < 0.001$), Treatment ($P = 0.001$) and interaction effects ($P < 0.001$). Figure 4.3 shows that mean numbers of settlers of this category were higher on biofilmed surfaces than on unfilmed surfaces in both spring experiments. This numerical relation reverses in autumn 1993 and summer 1994, with higher mean densities on unfilmed substrata. The comparisons between treatment means for the contrast C *versus* [E_a + D_b] (Table 4.9), however, showed differences to be significant for the winter 1994 experiment, the spring 1994 experiment (C > [E_a + D_b]) and the summer 1994 experiment ([E_a + D_b] > C) only. No significant effects of early incumbent settlers was revealed in the contrast A *versus* [C - E_a + D_a] (Table 4.9, see also Fig. 4.5).

Sheet Bryozoans

This group included the sheet forming bryozoan species *Celleporella hyalina* (see below), *Electra pilosa* (see below), *Escharoides coccinea* and several less frequently found species (e.g. *Schizoporella unicornis*, *Microporella* sp., *Cribrilina* sp.). Mean densities of Sheet Bryozoans on treatment A ranged between 0.01·cm^{-2} in winter 1994 and 0.17·cm^{-2} in summer 1994. Average settlement densities on treatment

Table 4.15. Analysis of variance for *Tubulipora* sp./*Plagioecia* sp. (= Mound Bryozoans).

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Block	2	86.69	40.11	20.05	1.38	0.253
Experiment	7	2540.22	2540.22	362.89	25.04	<0.001
Treatment	3	352.46	263.43	87.81	6.06	0.001
Experiment*Treatment	21	972.44	972.44	46.31	3.20	<0.001
Error	218	3158.84	3158.84	14.49		
Total	251	7110.65				

Table 4.16. Comparisons between treatment means of *Tubulipora* sp./*Plagioecia* sp. (= Mound Bryozoans) for the contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a]. Details as for Table 4.2.

Experiment	n	C *vs.* [E_a + D_b] *t*	A *vs.* [C - E_a + D_a] *t*
1 A 1992	6	0.140	2.814
2 W 1993	6	1.101	0.278
3 Sp 1993	9	2.560	1.366
4 Su 1993	9	1.509	0.426
5 A 1993	9	1.531	0.700
6 W 1994	9	+ 5.500	1.283
7 Sp 1994	9	+ 3.792	1.288
8 Su 1994	6	- 3.572	0.708

critical value for $t_{0.003(2),\,250}$ = 3.261

critical value for $t_{0.001(2),\,250}$ = 3.330

C ranged between 0.01·cm^{-2} in the winter experiments and 0.11·cm^{-2} in autumn 1992 and summer 1993.

The ANOVA did not show any Block effect on larval settlement, but significant Experiment ($P < 0.001$), Treatment ($P = 0.001$) and interaction effects ($P < 0.001$) were indicated (Table 4.10). However, the comparisons between treatment means failed to reveal any significant effects of either biofilming or incumbents (Table 4.11, see also Figs. 4.3 and 4.5).

Celleporella hyalina

Settlement of this species was comparatively low and seasonal, with highest mean densities found in the summer experiments (mean settlement on treatment A = 0.06·cm^{-2} in summer 1993 and 0.12·cm^{-2} in summer 1994). No formal analysis was carried out for this species. Figure 4.3 shows that in those experiments in which *Celleporella hyalina* was present, settlement was higher on surfaces bearing biofilms than on unfilmed substrata. Only in the summer 1993 experiment does mean settlement appear to be lower on panels with incumbents than on surfaces bare of early settlers (Fig. 4.5).

Electra pilosa

Average counts of *Electra pilosa* were comparatively low and ranged for treatments A and C between < 0.01·cm^{-2} in the winter experiments and 0.05·cm^{-2} and 0.06·cm^{-2} respectively in the autumn 1992 experiment. Some patterns emerge from the plots of the mean numbers of settlers for the treatment contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a] (Figs. 4.3 and 4.5): settlement was higher on biofilmed substrata (treatment C) than on unfilmed surfaces ([E_a + D_b]) in spring, but lower than on the composite treatment [E_a + D_b] in autumn and summer, which might indicate a

Table 4.17. Analysis of variance for Sheet Bryozoans.

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Block	2	48.17	40.25	20.12	1.18	0.308
Experiment	6	1983.68	1983.68	330.61	19.45	<0.001
Treatment	3	283.05	279.75	93.25	5.49	0.001
Experiment*Treatment	18	819.89	819.89	45.55	2.68	<0.001
Error	186	3161.64	3161.64	17.00		
Total	215	6296.44				

Table 4.18. Comparisons between treatment means of Sheet Bryozoans for the contrasts C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a]. Excluded is Experiment 6 for which mean counts of settlers are < 1 per panel for all treatments. Details as for Table 4.2.

Experiment	n	C *vs.* [E_a + D_b] *t*	A *vs.* [C - E_a + D_a] *t*
1 A 1992	6	0.429	2.657
2 W 1993	6	2.713	0.632
3 Sp 1993	9	2.503	2.882
4 Su 1993	9	0.520	2.749
5 A 1993	9	0.457	0.530
7 Sp 1994	9	1.786	1.125
8 Su 1994	6	0.195	0.047

critical value for $t_{0.003(2),\,190} = 3.273$

critical value for $t_{0.001(2),\,190} = 3.342$

seasonal reversal of the effect of biofilming on the settlement response of *Electra pilosa*. However, it remains speculative whether this apparent reversal in the biofilming effect issignificant, as the numbers of this species were considered too low and variable for formal analysis.

4.4 DISCUSSION

The use of netted panel substrata in the present study allowed the separation of the effect of biofilms on larval settlement from any potential confounding effects of early incumbent post-larvae. Such species-specific effects of incumbents were observed in the comparison A *versus* [C - E_a + D_a] for Total Settlement and Total Bryozoans in the present experiments, and in Chapter 3, and their occurrence was found to vary between experiments. These variations could reflect the seasonality of certain macroinvertebrate species – either in their reproductive cycles, and thus larval supply – or perhaps in their production of allelochemical cues (Dyrynda, 1985; Bakus *et al.*, 1986; Davis *et al.*, 1991; Martin & Uriz, 1993; Maida *et al.*, 1995). The avoidance of future dominant competitors for primary space or food at the stage of larval settlement and the use of co-occurring species as indicators for favourable settlement sites are likely to be of high adaptive importance, and there are a number of examples of intra-specific inhibition (Goodbody, 1961; Grosberg, 1981; Young & Chia, 1981; Young, 1989; Martin & Uriz, 1993; Todd & Keough, 1994) and facilitation (Standing *et al.*, 1984; Raimondi, 1988; LeTourneux & Bourget, 1988; Todd & Keough, 1994) among sessile marine invertebrate species. The inhibitory effects on newly arriving recruits related to production of allelochemicals, feeding activity or pre-emption of space by established filter-feeding hard substratum organisms have been documented in the past (Goodbody, 1961; Connell & Slayter, 1977; Grosberg, 1981; Sousa, 1984a; Todd & Turner, 1986; Wahl, 1989; Martin & Uriz, 1993; Todd & Keough, 1994; Osman & Whitlatch, 1995a, 1995b). It should be noted, however, that the present experiments did not reveal many significant effects of the presence of early incumbent settlers (but see Chapter 3), and

although the presence of early incumbent post-larvae had an inhibitory effect on some of the present taxa this inhibition does not appear to be bound to the seasonally timed occurrence of a particular competitor species. Nevertheless, it must be borne in mind that the application of a very conservative significance criterion of $\alpha_{[2]} = 0.001$ might have resulted in making type II errors in some cases. The consistently higher settlement on the composite treatment [C - E_a + D_a] with respect to treatment A (although this difference is non-significant for the majority of groups) is somewhat surprising as the inhibitory incumbent effects are superimposed on the generally facilitatory biofilming effect and certain settlement enhancing effects related to gregarious behaviour in particular groups of settlers, such as Spirorbins.

The significant Block effects, found for some taxonomic groups and species, were most likely due to more or less systematic small-scale differences in larval supply, gregariousness of certain species (e.g. Jackson, 1985; Pawlik *et al.*, 1991; Toonen & Pawlik, 1994) and/or small-scale habitat heterogeneities (see also Roberts *et al.*, 1991; Todd & Keough, 1994). Block effects were expected because they are more or less inevitable in this type of field experimental design.

The between-experiment differences of the species-specific effects of biofilming on some of the year-round settling invertebrate groups and taxa observed in the present study could partly be ascribed to changes in the numbers of available settlers at particular times of the year or between years (e.g. timing of the onset of the settlement season: see also Sutherland & Karlson, 1977; Harms & Anger, 1983; Todd & Turner, 1986; Turner, 1988), especially in the case of composite groupings containing more than one species (e.g. Spirorbins, Serpulins/*Pomatoceros* sp.). However, differential larval availability and patchy settlement alone are unlikely to have caused the changes from inhibition/facilitation to facilitation/inhibition shown for the response of some taxonomic groups and species (*Electra pilosa*, *Tubulipora* sp./*Plagioecia* sp.). Neither can it be ruled out that the temporal variations in the settlement patterns observed were caused by differential predation/mortality of newly metamorphosed larvae, but this seems unlikely given the relatively short immersion periods (14d), which have allowed the assessment

of settlement rather than recruitment (Keough & Downes, 1986; Todd & Keough, 1994).

General non-uniformity of the effect of a settlement cue can result from either of two causes: (1) intrinsic variability in larval behaviour, or (2) differential responses to variable, uncontrolled external stimuli (Raimondi & Keough, 1990). Thus, one possible explanation of the observed temporal variations in settlement responses to biofilming could be seasonal variations in the "sensitivity" of the larvae to biofilm cues (intrinsic variability). There is substantial evidence in the literature of the occurrence of behavioural variations between different larval populations, "batches" or generations (see Raimondi & Keough, 1990, for review of variation in larval behaviour). Toonen & Pawlik (1994) proposed the existence of two behaviourally distinct subpopulations of larvae of the serpulid *Hydroides dianthus*: one group colonized uninhabited substrata ("founders") and responded to biofilm cues, and the other group ("aggregators") only settled in response to cues associated with conspecifics. Seasonally distinct behavioural responses at settlement would appear to be adaptive where these are related to variations in selective pressure, such as the seasonality of dominant space competitors or predator species, but evidence for such intrinsic seasonal changes in behavioural patterns of marine invertebrate larvae in the literature is extremely rare (Keough, 1986; Raimondi & Keough, 1990). There is, however, strong evidence that the presence of recruits of competing species can exert inhibitory effects on other species (above, see also Todd & Keough 1994; Keough & Raimondi, 1995). It is possible that biofilming as a settlement cue simply decreases in importance for the larvae of certain taxa which encounter seasonal increases in the effects of other competitor species and/or their production of allelochemical metabolites.

Nevertheless, the results of Chapter 3 support the idea that the observed between-season differences in the effects of biofilm cues are most likely a reflection of seasonal changes of these cues themselves (external variability), which may result in alterations of larval settlement responses. It was shown in Chapter 3 that there are marked quantitative and qualitative differences in biofilm composition between two local sites of contrasting flow regime within Clachan Seil, and that larvae respond

differentially to these during settlement. Composition, metabolic activity and quantitative characteristics of biofilm communities are known to change as a consequence of seasonal succession in the field (Hudon & Bourget, 1981; Munteanu & Maly, 1981; Underwood, 1984; MacLulich, 1987; Anderson, 1995), and it appears very likely that larvae are able to detect and respond to these changes.

The present study showed marked seasonal variations in the effects of biofilming cues on the larval settlement of certain marine invertebrate groups and taxa under natural conditions, which cannot be explained by changes in larval supply alone. Moreover, a reversal of the effect of filming on larval settlement response - from inhibitory/facilitatory to facilitatory/inhibitory - with season was noted in the case of certain species. Although there were only few examples of statistically significant biofilming effects on larval settlement (the highly conservative significance criterion of $\alpha_{[2]} = 0.001$, which was necessitated by the analysis of the data in their natural scale, could have led to type II errors in some cases), an overall facilitatory effect of biofilming was shown. The study also indicated that superimposed on the biofilming effects on larval settlement is the overall inhibitory impact of the presence of incumbent settlers. These observations are of particular interest with regard to the interpretation of events following disturbances that create open spaces within natural fouling communities, in which space commonly is a limiting resource (Sousa, 1979; Sutherland, 1984; Butler & Chesson, 1990). Such disturbances can be highly seasonal physical or biological events, and their timing can be of major importance to patterns of recolonization and succession (Sousa, 1984). The results of the present study imply that seasonal patterns of recolonization of newly disturbed areas can be only partly explained by changes in species availability and temporal variations in larval supply (Osman 1977; Smedes, 1984; Turner & Todd, 1993), and that seasonal variations in the effects of substratum associated biofilm cues (and/or in the effect of other incumbent recruits) on year-round settling species may also be important. These results emphasize the need for long term assessments of the effects of biofilming cues, under field conditions, if any conclusions about species-specific larval responses and their ecological significance in relation to fouling community structure are to be drawn.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Settlement site is of particular importance to the many sessile suspension feeding invertebrate species characteristic of fouling assemblages, which after attachment and metamorphosis are essentially immobile and can invade new spaces during postlarval life only by horizontal modular growth. Groups capable of horizontal space invasion include many colonial ascidians, and in particular species of indeterminate growth, such as sponges, and many sheet forming bryozoans. Nonetheless, tropical (Ryland *et al.*, 1984) and temperate (C.D. Todd, pers. comm.) didemnid ascidians are unusual in being able to move across substrata in an amoeboid fashion and may relocate on a substratum as a fully formed colony (see also Birkeland *et al.*, 1981). The aforementioned constraints characteristic of sessile life are known to lead to competition for primary substratum (see below) and food (Buss, 1979a; Mook, 1981; Best & Thorpe, 1986; Okamura, 1988; 1992; Nandakumar & Tanaka, 1994). Large numbers of long-lived larvae are produced by many solitary organisms (e.g. barnacles), apparently facilitating long distance dispersal (Thorson, 1950; Scheltema, 1974). Other species produce larvae of short planktonic life (Thorson, 1950; Ryland, 1974, 1976), with time from liberation to attachment to the substratum ranging from perhaps a few hours (e.g. *Spirorbis spirorbis*) to up to 1d (*Bugula flabellata*); this presumed reduced dispersal capacity results in certain species, such as *Ciona intestinalis*, settling in close proximity to the parent organisms (see also Keough & Chernoff, 1987). Larval dispersal is further counteracted by gregarious settlement behaviour in some species (e.g. spirorbid worms, barnacles) (Knight-Jones, 1951; Jackson, 1985; Gotelli, 1990; Pawlik *et al.*, 1991; Toonen & Pawlik, 1994).

Benthic sublittoral hard substratum communities are generally considered to be space limited (e.g. Jackson, 1977; Sebens, 1982; Paine, 1984). Many studies in the past investigated how fouling assemblages are influenced by space competition (e.g. Stebbing, 1973a, 1973b; Buss & Jackson, 1979; Keen & Neill, 1980; Grosberg, 1981;

Sebens, 1986; Todd & Turner, 1988; Lopez Gappa, 1989; Meyers, 1990; Steneck *et al.*, 1991; Nandakumar & Tanaka, 1994; Turner & Todd, 1994). New space for settlement can be generated in two ways: by clearance of formerly unavailable areas through disturbances (primary space) and by introduction of new substrata, for example in the form of piers and pilings (Karlson, 1978; Keough & Butler, 1979) or through invasion of species with hard skeletons (such as molluscs or tubiculous worms), which themselves can be overgrown and encrusted by other fouling organisms (secondary space) (Keough, 1984b; Lohse, 1993). Disturbances leading to the development of new space in epibenthic communities are thought to be generated either by physical activities, such as wave or current action, abrasion (by sediments, ice scour, or large water borne objects), desiccation and abrupt changes in temperature or salinity (e.g. Dayton, 1971; Osman, 1977; Sousa, 1979, 1980; Underwood & Jernakoff, 1984, and references in Connell & Keough, 1985, and Sousa, 1985), or biological activities, such as predation, grazing, interference competition and adult mortality through senescence (e.g. Sutherland & Karlson, 1977; Keen & Niell, 1980, Ayling, 1981; Smedes & Hurd, 1981, and references in Connell & Keough, 1985, and Sousa, 1985). Local pollution can also act as a space-creating source (Chapman *et al.*, 1995). Theory implies that disturbances "reset the successional clock" (Begon *et al.*, 1990) by creating heterogeneity and renewing a limiting resource (e.g. space) and thereby permitting its utilization by competitively inferior species, thus preventing their extinction (Levin & Paine, 1974). Conversely, this is thought to prevent dominant competitors from total resource monopolization, and intermediate levels of disturbance (in intensity and frequency) will create the highest levels of species richness and thus diversity (Connell, 1978; Lubchenco, 1978; Pickett & White, 1985). Some disturbances can be highly phased or seasonal events (Begon *et al.*, 1990).

The fouling assemblages at Clachan Seil are unlikely to be exposed to a high degree of physical disturbances. This study site is at a very sheltered location (see Chapter 3, Material and Methods), and therefore strong wave action is unlikely to be of major importance in creating new patches of open space. However, strong storms in autumn and early winter may be the source of some disturbance. There is some

freshwater run-off from a small stream close to the experimental site which might create some temporal variations in salinity (Todd & Turner, 1986). There are no major human inferences at the site, although planned building activities may create some local disturbances in the future. Because of the Seil's topography (see Chapter 3, Materials and Methods) the study site is flushed regularly, and the impact of domestic sewage or road washings diverted into the Seil can be regarded as negligible. Although surface temperatures and salinities rise at spring ebb tides during summer, temperature stress can be ruled out as a major factor of disturbance at this site (Todd & Turner, 1986). As is characteristic for subtidal epibenthic communities, patch formation in the fouling assemblages at Clachan Seil is much more likely to be determined by senescence, natural sloughing or by predation rather than physical disturbances. Several mollusc species (*Nucella lapillus* (L.), *Gibbula cineraria* (L.), *Littorina* spp., *Calliostoma zizyphinum* (L.), *Lacuna* spp.) have been commonly found preying or grazing on the experimental panels in the present study (see also Todd & Turner, 1986; Turner & Todd, 1991; Turner, 1988), and could have marked effects on recruitment to the assemblage.

Open patches in substratum limited communities are created at different spatial and temporal scales (Connell & Keough, 1985), and the availability of cleared patches may often exhibit a characteristic seasonal pattern, which could have a particularly strong impact on the recruitment of those species with seasonally regular times of settlement (Keough, 1983); thus, the patterns of recolonization can depend to a large extent on the season in which a patch was created and the site of substratum clearance (Dayton, 1971; Lubchenco & Menge, 1978; Paine & Levin, 1981). The timing of larval release and settlement can be as crucial to the survival of some animals as is choice of settlement site. This is of especial importance if, for example, the annual growth of a host plant (and thus renewal of secondary space) is strongly seasonal (Seed & Hughes, 1992), or if food for the settled postlarvae is limited to a certain short period of the year (Todd & Doyle, 1981). Thus, settlement of many marine invertebrates and recruitment patterns found under natural conditions are highly seasonal. While species that brood their larvae, such as *Spirorbis spirorbis*, *Bugula flabellata* and *Celleporella hyalina*, release these over longer periods, or have several settlement peaks during the year, sexual reproduction can

be more or less strictly seasonal for some fouling species (e.g. *Alcyonidium* spp., *Flustrellidra hispida*, certain didemnid species and *Semibalanus balanoides*, *Semibalanus (= Balanus) crenatus*). In habitats where space is the limiting factor for larval settlement, single annual mass spawnings appear to be a poor strategy, and prolonged larval release over the year appears to be advantageous (Connell & Keough, 1985; Seed & Hughes, 1992).

Patch dynamics theory distinguishes between two kinds of open space generated by disturbances: (i) spaces within existing assemblages (patch type 1), and (ii) spaces isolated from occupied sites (type 2 patches) (Connell & Keough, 1985; Sousa, 1985). The main difference between these two types of patches in encrusting benthic subtidal communities is that in the first case newly available space may be invaded and gained by means both of spreading and expansion of neighbouring organisms (modular growth) as well as by propagules arriving from the water column; in the second case, however, occupation of newly available space by macrofouling organisms is solely determined by larval recruitment (Connell & Keough, 1985). The artificial substrata used in the present study (as well as the settlement panels used in many other fouling studies) essentially represent type 2 patches because, through their (often) being mounted on a frame, they are not in direct contact with the surrounding assemblage. One could therefore argue that this type of patch is rather uncharacteristic of the situation of hard substratum subtidal assemblages, in which open spaces created by disturbances are much more likely to be non-isolated and invaded by surrounding organisms as well as by settling larvae from outside (Kay & Keough, 1981; Palumbi & Jackson, 1982); this experimental artefact is quite likely to influence the patterns of recruitment (see Discussions of the experimental chapters for details on incumbent effects). While this might pose a problem in many other studies utilizing fouling panels, an isolation of the effects of biofilming and early incumbent postlarvae was intentional and important to the underlying rationale in the present field experiments. Additional variation from naturally occurring free substrata might be introduced by the experimentally standardized size of the fouling panels. Naturally generated patches occur in various shapes and sizes. Patch size as well as patch location, however, are known to have a marked impact on recruitment composition,

species richness and distribution (Keough, 1984c; Sousa, 1985; Butler, 1991) (for a discussion of artificial substrata and panel effects see Jackson, 1977b; Osman, 1977, 1982; Schoener, 1982, 1984, and Bakus, 1988).

It has been proposed that species succession in natural communities is based on three possible mechanisms (Connell & Slayter, 1977): the resident species (i) enhance the recruitment, establishment or growth of the settling/invading species (facilitation), (ii) depress the recruitment, establishment or growth of the settling/invading species (inhibition), or (iii) have no effect on the successionally settling/invading species (tolerance). Van Tamelen (1987), however, points out that dynamic factors in a natural community, such as seasonality of recruitment, growth and mortality, can lead to temporal and spatial changes, and even reversal, of those intra-specific interactions during succession. While species distribution and succession in fouling communities is usually discussed with emphasis on the macrofouling species, spatial heterogeneities exist, and successional changes take place also, in the microfouling assemblages of biofilms (see Chapters 1 and 2). The present study shows that the above concepts extend also to microbial-macroinvertebrate interactions. Turner (1983) argued that inhibition occurs more frequently than does facilitation in many communities, and among marine environments in particular (see for example Standing, 1976; Connell & Slayter, 1977, and Sutherland & Karlson, 1977). Connell & Slayter (1977) suggest that the mechanism of facilitation is more important to assemblages on substrata in harsh physical environments. This is in contrast to the present results indicating the majority of the effects of biofilming of substrata on larval settlement to be facilitatory rather than inhibitory for the sheltered field site at Clachan Seil (Chapters 3 and 4).

Many of the species found on the settlement panels in the present field experiments were colonial, such as the ascidians *Botryllus schlosseri* and *Botrylloides leachi*, the slime sponges and the bryozoan taxa. These findings are in agreement with the general opinion that encrusting epizooic subtidal assemblages are dominated by modular organisms (Jackson, 1977a; Buss, 1979b; Buss & Jackson, 1979; Woodin & Jackson, 1979; Jackson *et al.*, 1985; Todd & Turner, 1989), at least during the early stages of succession, whereas solitary forms dominate intertidal communities. With

respect to this proposed intertidal/sublittoral dichotomy (see above), it would be interesting to test the hypothesis that intertidal organisms are adapted to recognize and settle preferentially onto biofilms typical for that zone and *vice versa* for subtidal organisms (see also Strathmann *et al.*, 1981).

The majority of colonial organisms are thought to be successful space invaders, due to their capacity to grow indeterminately and fast, and to retain space successfully (but see also Ayling, 1981, and Greene *et al.*, 1983). Colonial marine species also commonly brood embryos and their planktonic larval life is shortened (Strathmann, 1990). With respect to their utilization of settlement space, Jackson (1979) categorized sedentary modular organisms by their basic shapes (see also Greene *et al.*, 1983; Barnes and Hughes, 1988; Bishop, 1989). It is difficult to generalize about the advantages of certain growth forms, but there is some evidence that "runner-like" encrusting bryozoans, such as *Electra pilosa*, maintain themselves as important members of fouling communities, despite their competitive inferiority, by opportunistically being among the first in the succession of the invading settlers arriving on a newly created free patch of space, and by being ephemeral and fast growing (Stebbing, 1973a; Seed & Hughes, 1992). Stebbing (1973a) observed that the larvae of *Electra pilosa* preferentially settled onto the distal ends of *Fucus serratus* fronds. These younger parts are less densely encrusted by epiphytes and presumably are characterized by less developed microbial surface films. Conversely, settlement of *Alcyonidium hirsutum* was found to be highest on the middle regions of *Fucus serratus* fronds (Hayward & Ryland, 1975), bearing biofilms of intermediate age. Colonial organisms of varying growth forms may differ in their vulnerability to certain disturbances (Connell & Keough, 1985). Sheet bryozoans are less likely to be affected by strong wave action, and more likely to be affected by overgrowth than are erect bryozoans (Walters & Wethey, 1991). It is therefore not surprising that in the flow regime experiments (Chapter 3), there were generally higher abundances of the settler category Erect Bryozoans on panels at the low flow rate site (LowFRS) than on panels at the high flow rate site (HighFRS). Todd & Turner (1989) describe the opportunistic settlement behaviour of the colonial ascidians *Botryllus schlosseri* and *Botrylloides leachi*, and suggest that these species might not be able to

settle into already colonized patches of substrata, thus supporting the idea of colonial organisms as early settlers with fast growth abilities. It is possible that species with erect growth forms, such as the bryozoans *Scrupocellaria scruposa* and *Crisia* sp. are less likely to be overgrown by space competitors once they have reached a certain size (Barnes & Hughes, 1988). These species, too, would therefore seem to benefit from rapidly colonizing newly available substrata, in order to "escape their competitors in size" (see also Sebens, 1982). Both of the above bryozoan species were found in relatively high abundances on panels in the present field experiments (see Chapters 3 and 4).

Seed & Hughes (1992) discuss the reproductive strategies of epialgal bryozoans with respect to their abilities to successfully colonize space. They describe how the coexistence of the competitively inferior species *Electra pilosa*, *Celleporella hyalina* and *Membranipora membranacea* with the spatially dominant species *Flustrellidra hispida* and *Alcyonidium* spp., is possible due to their temporally segregated patterns of reproduction and settlement. While *Flustrellidra* releases its lecithotrophic larvae between May and November, with settlement peaking in spring and early summer, larval release in *Alcyonidium* is temporally separated in occurring between October and January, with peak settlement during October and November (see also Wood & Seed, 1992); thus, these species avoid competition for primary substratum in the early stages of their colony development. The recruitment of the ephemeral encrusting species, however, is relatively time-independent, which means that small numbers of larvae are more or less always available in the plankton. This allows them to opportunistically settle onto newly available patches of substratum and to grow rapidly to reproductive maturity before being overgrown by the dominant species.

From an adaptational point of view it is possible that larvae of sessile benthic hard substratum species settling at an early successional stage ("pioneer species", or according to MacArthur and Wilson's concept, r-selected species (Pianka, 1970); but see also Seed & Hughes, 1992; Stearns, 1992) have evolved to recognize and respond positively to such "young" microbial films, which are associated with newly created patches of free substratum. Being able to distinguish between new free space and patches already partly occupied by recruits of potential future dominant competitors would be especially

important for the survival of short lived species, whose strategy is to produce large numbers of larvae with short planktonic phases, and to rapidly settle onto and utilize newly available substrata created by disturbances or algal growth. Conversely, later arriving settlers possibly benefit from recognizing and settling into already established fouling assemblages, characterized by a more "mature" biofilm and the presence of incumbents. It is possible also that biofilms in the latter case are of less importance as settlement cues than is the presence or absence of early recruiting taxa in affecting the settlement behaviour of such successionally late species, which are thought to be of greater longevity and higher competitive ability (K-selected species, Pianka, 1970; see also Barnes & Hughes, 1988).

In the present study the use of microbial surface films as settlement cues by marine invertebrate larvae was shown to play an important rôle in the first stages of substratum colonization. It was further revealed that the larvae of certain macrofouling species are able to differentiate between natural multi-species biofilms of varying compositions, densities and ages, and to respond to these differences during settlement under both laboratory and field conditions. The larvae of a number of taxa were able also to distinguish between substrata preconditioned with biofilms at sites of contrasting flow regimes in the field. The larvae of certain year-round settling species were found to vary in their response to biofilmed substrata depending on the season. These subtle larval responses to small changes in microbial surface characteristics of a substratum are likely to be of great importance in the determination of local and temporal patterns of recruitment and in pre-defining part of the successional development of an assemblage. Indeed, some of the spatial and temporal variations in macroinvertebrate community patterns may be a direct function of variability in the development of the substratum-associated biofilms.

It must, however, be borne in mind that the settlement responses of species which do not use biofilms as cues, and the effect of other settlement cues (or the confounding responses to incumbent settlers), might be superimposed onto the patterns of substratum selection displayed by a certain species with respect to biofilm type (Keough & Raimondi, 1995). A whole hierarchy of settlement cues might be operative at different

spatial and temporal scales, as has been shown for the barnacle species *Chthamalus fragilis* and *Semibalanus balanoides* (Wethey, 1986) and *Semibalanus* (= *Balanus*) *crenatus* (Hudon *et al.*, 1983). Knowledge of the finely tuned response repertoire of a broad spectrum of macroinvertebrate settlers to microbial surface film cues is thus of obvious value in understanding re-colonization processes of newly available substratum space, following small disturbances.

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APPENDIX 1

Expeiment 1. ANOVA of larval settlement including treatments A, C, D_a, D_b, E_a, and E_b.

Total Settlement

Source	DF	SS	MS	F	P
Site	1	19045	19045	13.92	<0.001
Block (Site)	2	3200	1600	1.17	0.318
Treatment	5	455378	91076	66.54	<0.001
Site*Treatment	5	23982	4796	3.50	0.008
Error	58	79382	1369		
Total	71	580987			

Spirorbins

Source	DF	SS	MS	F	P
Site	1	854	854	1.09	0.300
Block (Site)	2	2362	1181	1.51	0.229
Treatment	5	176469	35294	45.14	<0.001
Site*Treatment	5	9069	1814	2.32	0.055
Error	58	45353	782		
Total	71	234108			

Serpulins/*Pomatoceros* spp.

Source	DF	SS	MS	F	P
Site	1	120.125	120.125	33.16	<0.001
Block (Site)	2	47.028	23.514	6.49	0.003
Treatment	5	407.903	81.581	22.52	<0.001
Site*Treatment	5	73.792	14.758	4.07	0.003
Error	58	210.139	3.623		
Total	71	858.986			

Total Ascidians

Source	DF	SS	MS	F	P
Site	1	316.68	316.68	12.38	0.001
Block (Site)	2	7.36	3.68	0.14	0.866
Treatment	5	1681.90	336.38	13.15	<0.001
Site*Treatment	5	210.57	42.11	1.65	0.162
Error	58	1483.14	25.57		
Total	71	3699.65			

Botryllus schlosseri/Botrylloides leachi

Source	DF	SS	MS	F	P
Site	1	0.889	0.889	0.11	0.738
Block (Site)	2	30.278	15.139	1.92	0.156
Treatment	5	172.444	34.489	4.37	0.002
Site*Treatment	5	46.944	9.389	1.19	0.325
Error	58	457.389	7.886		
Total	71	707.944			

Didemnid Ascidians

Source	DF	SS	MS	F	P
Site	1	21.125	21.125	2.98	0.089
Block (Site)	2	12.472	6.236	0.88	0.420
Treatment	5	346.958	69.392	9.80	<0.001
Site*Treatment	5	26.625	5.325	0.75	0.588
Error	58	410.694	7.081		
Total	71	817.875			

Total Bryozoans

Source	DF	SS	MS	F	P
Site	1	3348.3	3348.3	36.22	<0.001
Block (Site)	2	663.0	331.5	3.59	0.034
Treatment	5	17147.2	3429.4	37.10	<0.001
Site*Treatment	5	2471.6	494.3	5.35	<0.001
Error	58	5361.1	92.4		
Total	71	28991.3			

Erect Bryozoans

Source	DF	SS	MS	F	P
Site	1	1233.39	1233.39	66.53	<0.001
Block (Site)	2	32.11	16.06	0.87	0.426
Treatment	5	1549.44	309.89	16.72	<0.001
Site*Treatment	5	1295.11	259.02	13.97	<0.001
Error	58	1075.22	18.54		
Total	71	5185.28			

Scrupocellaria scruposa

Source	DF	SS	MS	F	P
Site	1	734.72	734.72	44.54	<0.001
Block (Site)	2	10.00	5.00	0.30	0.740
Treatment	5	968.94	193.79	11.75	<0.001
Site*Treatment	5	891.61	178.32	10.81	<0.001
Error	58	956.67	16.49		
Total	71	3561.94			

Crisia sp.

Source	DF	SS	MS	F	P
Site	1	64.222	64.222	47.62	<0.001
Block (Site)	2	8.111	4.056	3.01	0.057
Treatment	5	89.944	17.989	13.34	<0.001
Site*Treatment	5	65.944	13.189	9.78	<0.001
Error	58	78.222	1.349		
Total	71	306.444			

Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)

Source	DF	SS	MS	F	P
Site	1	56.889	56.889	11.30	0.001
Block (Site)	2	1.389	0.694	0.14	0.871
Treatment	5	536.778	107.356	21.33	<0.001
Site*Treatment	5	81.611	16.322	3.24	0.012
Error	58	291.944	5.034		
Total	71	968.611			

Sheet Bryozoans

Source	DF	SS	MS	F	P
Site	1	401.39	401.39	7.67	0.008
Block (Site)	2	198.72	99.36	1.90	0.159
Treatment	5	4630.11	1526.02	29.17	<0.001
Site*Treatment	5	452.28	90.46	1.73	0.142
Error	58	3034.61	52.32		
Total	71	11717.11			

Celleporella hyalina

Source	DF	SS	MS	F	P
Site	1	43.556	43.556	6.71	0.012
Block (Site)	2	14.444	7.222	1.11	0.335
Treatment	5	259.778	51.956	8.01	<0.001
Site*Treatment	5	70.444	14.089	2.17	0.070
Error	58	376.222	6.487		
Total	71	764.444			

Electra pilosa

Source	DF	SS	MS	F	P
Site	1	91.13	91.13	10.36	0.002
Block (Site)	2	15.25	7.62	0.87	0.425
Treatment	5	1347.90	269.58	30.66	<0.001
Site*Treatment	5	147.13	29.42	3.35	0.010
Error	58	509.92	8.79		
Total	71	2111.32			

Escharoides coccinea

Source	DF	SS	MS	F	P
Site	1	0.125	0.125	0.01	0.905
Block (Site)	2	39.806	19.903	2.30	0.110
Treatment	5	211.403	42.281	4.88	0.001
Site*Treatment	5	36.292	7.258	0.84	0.529
Error	58	502.694	8.667		
Total	71	790.319			

Slime Sponges

Source	DF	SS	MS	F	P
Site	1	3133.7	3133.7	31.12	<0.001
Block (Site)	2	297.0	148.5	1.47	0.237
Treatment	5	6674.1	1334.8	13.25	<0.001
Site*Treatment	5	6664.9	1333.0	13.24	<0.001
Error	58	5841.1	100.7		
Total	71	22610.9			

Leucosolenia sp.

Source	DF	SS	MS	F	P
Site	1	1404.50	1404.50	17.59	<0.001
Block (Site)	2	442.00	221.00	2.77	0.071
Treatment	5	687.28	137.46	1.72	0.144
Site*Treatment	5	727.17	145.43	1.82	0.123
Error	58	4632.33	79.87		
Total	71	7893.28			

Expeiment 2. ANOVA of larval settlement including treatments A, C, D_a, D_b, E_a, and E_b.

Total Settlement

Source	DF	SS	MS	F	P
Site	1	18883	18883	34.27	<0.001
Block (Site)	2	44	22	0.04	0.961
Treatment	5	116536	23307	42.30	<0.001
Site*Treatment	5	49110	9822	17.83	<0.001
Error	58	31956	551		
Total	71	216529			

Spirorbins

Source	DF	SS	MS	F	P
Site	1	10755.6	10755.6	32.12	<0.001
Block (Site)	2	16.1	8.0	0.02	0.976
Treatment	5	66788.4	13357.7	39.89	<0.001
Site*Treatment	5	22133.6	4426.7	13.22	<0.001
Error	58	19420.3	334.8		
Total	71	119113.9			

Serpulins/*Pomatoceros* spp.

Source	DF	SS	MS	F	P
Site	1	13.347	13.347	1.61	0.209
Block (Site)	2	27.694	13.847	1.67	0.197
Treatment	5	104.569	20.914	2.52	0.039
Site*Treatment	5	51.236	10.247	1.24	0.304
Error	58	480.472	8.284		
Total	71	677.319			

Total Ascidians

Source	DF	SS	MS	F	P
Site	1	32.00	32.00	1.59	0.213
Block (Site)	2	26.72	13.36	0.66	0.520
Treatment	5	165.28	33.06	1.64	0.164
Site*Treatment	5	79.00	15.80	0.78	0.566
Error	58	1170.28	20.18		
Total	71	1473.28			

Didemnid Ascidians

Source	DF	SS	MS	F	P
Site	1	11.68	11.68	1.08	0.304
Block (Site)	2	5.69	2.85	0.26	0.770
Treatment	5	120.46	24.09	2.22	0.064
Site*Treatment	5	45.90	9.18	0.85	0.523
Error	58	629.14	10.85		
Total	71	812.88			

Total Bryozoans

Source	DF	SS	MS	F	P
Site	1	210.13	210.13	4.99	0.029
Block (Site)	2	14.81	7.40	0.18	0.839
Treatment	5	519.24	103.85	2.47	0.043
Site*Treatment	5	463.13	92.62	2.20	0.066
Error	58	2441.36	42.09		
Total	71	3648.65			

Erect Bryozoans

Source	DF	SS	MS	F	P
Site	1	6.1250	6.1250	13.31	0.001
Block (Site)	2	2.8056	1.4028	3.05	0.055
Treatment	5	2.6250	0.5250	1.14	0.349
Site*Treatment	5	2.6250	0.5250	1.14	0.349
Error	58	26.6944	0.4602		
Total	71	40.8750			

Mound Bryozoans (*Tubulipora* sp./*Plagioecia* sp.)

Source	DF	SS	MS	F	P
Site	1	8.681	8.681	6.73	0.012
Block (Site)	2	0.361	0.181	0.14	0.870
Treatment	5	6.403	1.281	0.99	0.430
Site*Treatment	5	9.403	1.881	1.46	0.218
Error	58	74.806	1.290		
Total	71	99.653			

Sheet Bryozoans

Source	DF	SS	MS	F	P
Site	1	82.35	82.35	2.81	0.099
Block (Site)	2	3.47	1.74	0.06	0.942
Treatment	5	353.90	70.78	2.42	0.046
Site*Treatment	5	300.57	60.11	2.05	0.084
Error	58	1696.69	29.25		
Total	71	2436.99			

Celleporella hyalina

Source	DF	SS	MS	F	P
Site	1	8.000	8.000	3.15	0.081
Block (Site)	2	0.556	0.278	0.11	0.896
Treatment	5	29.278	5.856	2.31	0.056
Site*Treatment	5	12.833	2.567	1.01	0.419
Error	58	147.111	2.536		
Total	71	197.778			

Electa pilosa

Source	DF	SS	MS	F	P
Site	1	8.681	8.681	1.52	0.223
Block (Site)	2	2.694	1.347	0.24	0.791
Treatment	5	51.236	10.247	1.79	0.129
Site*Treatment	5	70.903	14.181	2.48	0.042
Error	58	332.139	5.727		
Total	71	465.653			

Escharoides coccinea

Source	DF	SS	MS	F	P
Site	1	2.722	2.722	1.93	0.170
Block (Site)	2	1.389	0.694	0.49	0.613
Treatment	5	7.000	1.400	0.99	0.429
Site*Treatment	5	7.278	1.456	1.03	0.406
Error	58	81.611	1.407		
Total	71	100.000			

Slime Sponges

Source	DF	SS	MS	F	P
Site	1	112.50	112.50	10.34	0.002
Block (Site)	2	0.72	0.36	0.03	0.967
Treatment	5	46.28	9.26	0.85	0.520
Site*Treatment	5	57.33	11.47	1.05	0.395
Error	58	630.94	10.88		
Total	71	847.78			

Leucosolenia sp.

Source	DF	SS	MS	F	P
Site	1	0.500	0.500	0.14	0.710
Block (Site)	2	12.556	6.278	1.75	0.182
Treatment	5	18.444	3.689	1.03	0.409
Site*Treatment	5	25.333	5.067	1.41	0.233
Error	58	207.778	3.582		
Total	71	264.611			

Expeiment 3. ANOVA of larval settlement including treatments A, C, D_a, D_b, E_a, and E_b.

Total Settlement

Source	DF	SS	MS	F	P
Site	1	25947	25947	30.46	<0.001
Block (Site)	2	10605	5303	6.22	0.005
Treatment	5	235977	47195	55.40	<0.001
Site*Treatment	5	60892	12179	14.30	<0.001
Error	34	28963	852		
Total	47	362385			

Spirorbins

Source	DF	SS	MS	F	P
Site	1	16838	16838	22.46	<0.001
Block (Site)	2	7199	3599	4.80	0.015
Treatment	5	139023	27805	37.10	<0.001
Site*Treatment	5	39401	7880	10.51	<0.001
Error	34	25483	750		
Total	47	227943			

Serpulins/*Pomatoceros* spp.

Source	DF	SS	MS	F	P
Site	1	27.000	27.000	4.54	0.040
Block (Site)	2	9.417	4.708	0.79	0.461
Treatment	5	270.667	54.133	9.11	<0.001
Site*Treatment	5	68.750	13.750	2.31	0.065
Error	34	202.083	5.944		
Total	47	577.917			

Total Ascidians

Source	DF	SS	MS	F	P
Site	1	70.08	70.08	3.93	0.055
Block (Site)	2	12.42	6.21	0.35	0.708
Treatment	5	455.42	91.08	5.11	<0.001
Site*Treatment	5	48.17	9.63	0.54	0.744
Error	34	605.58	17.81		
Total	47	1191.67			

Botryllus schlosseri/Botrylloides leachi

Source	DF	SS	MS	F	P
Site	1	96.33	96.33	6.80	0.013
Block (Site)	2	4.08	2.04	0.14	0.866
Treatment	5	253.42	50.68	3.58	0.010
Site*Treatment	5	80.67	16.13	1.14	0.359
Error	34	481.42	14.16		
Total	47	915.92			

Total Bryozoans

Source	DF	SS	MS	F	P
Site	1	963.02	963.02	33.23	<0.001
Block (Site)	2	168.54	84.27	2.91	0.068
Treatment	5	963.02	1190.47	41.08	<0.001
Site*Treatment	5	1352.85	270.57	9.34	<0.001
Error	34	985.21	28.98		
Total	47	9421.98			

Erect Bryozoans

Source	DF	SS	MS	F	P
Site	1	426.02	426.02	39.90	<0.001
Block (Site)	2	21.21	10.60	0.99	0.381
Treatment	5	782.10	156.42	14.65	<0.001
Site*Treatment	5	529.10	105.82	9.91	<0.001
Error	34	363.04	10.68		
Total	47	2121.48			

Crisia sp.

Source	DF	SS	MS	F	P
Site	1	426.02	426.02	41.03	<0.001
Block (Site)	2	22.71	11.35	1.09	0.347
Treatment	5	777.94	155.59	14.98	<0.001
Site*Treatment	5	536.10	107.22	10.33	<0.001
Error	34	353.04	10.38		
Total	47	2115.81			

Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)

Source	DF	SS	MS	F	P
Site	1	192.00	192.00	21.43	<0.001
Block (Site)	2	75.33	37.67	4.20	0.023
Treatment	5	1281.92	256.38	28.61	<0.001
Site*Treatment	5	467.75	93.55	10.44	<0.001
Error	34	304.67	8.96		
Total	47	2321.67			

Sheet Bryozoans

Source	DF	SS	MS	F	P
Site	1	12.000	12.000	2.85	0.101
Block (Site)	2	9.750	4.875	1.16	0.326
Treatment	5	192.167	38.433	9.12	<0.001
Site*Treatment	5	50.750	10.150	2.41	0.057
Error	34	143.250	4.213		
Total	47	407.917			

Celleporella hyalina

Source	DF	SS	MS	F	P
Site	1	2.083	2.083	1.15	0.292
Block (Site)	2	0.750	0.375	0.21	0.814
Treatment	5	17.417	3.483	1.92	0.117
Site*Treatment	5	7.667	1.533	0.84	0.528
Error	34	61.750	1.816		
Total	47	89.667			

Electra pilosa

Source	DF	SS	MS	F	P
Site	1	0.021	0.021	0.02	0.901
Block (Site)	2	5.708	2.854	2.13	0.134
Treatment	5	57.354	11.471	8.56	<0.001
Site*Treatment	5	13.854	2.771	2.07	0.094
Error	34	45.542	1.339		
Total	47	122.479			

Expeiment 3. ANOVA of total bacterial cell counts and percentage dividing cells (arcsine-transformed data) obtained by AODC, including treatments A, C, D_a, D_b, E_a, and E_b.

Total Bacteria

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	3.767×10^{13}	2.878×10^{13}	2.878×10^{13}	22.65	<0.001
Block (Site)	2	1.236×10^{12}	1.328×10^{12}	6.639×10^{11}	0.52	0.593
Treatment	5	2.612×10^{14}	2.254×10^{14}	4.507×10^{13}	35.47	<0.001
Site*Treatment	5	4.498×10^{12}	6.029×10^{12}	1.206×10^{12}	0.95	0.448
Block (Site)*Treatment	10	1.442×10^{13}	1.442×10^{13}	1.442×10^{13}	1.13	0.333
Error	1056	1.342×10^{15}	1.342×10^{15}	1.271×10^{12}		
Total	1079	1.661×10^{15}				

Dividing Cells

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	205.7	116.0	116.0	0.70	0.403
Block (Site)	2	134.2	71.4	35.7	0.21	0.807
Treatment	5	553.9	458.5	91.7	0.55	0.737
Site*Treatment	5	416.0	612.2	122.4	0.74	0.596
Block (Site)*Treatment	10	1962.6	1962.6	196.3	1.18	0.299
Error	1056	175386.7	1.75396.7	166.1		
Total	1079	178659.1				

Expeiment 3. ANOVA of biofilm components (arcsine-transformed data) obtained by SEM, including treatments A, C, D_a, D_b, E_a, and E_b.

Bacteria

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	3493.6	3419.4	3419.4	59.63	<0.001
Block (Site)	2	199.9	181.1	90.6	1.58	0.207
Treatment	5	20117.4	19333.5	3866.7	67.42	<0.001
Site*Treatment	5	820.3	1027.0	205.4	3.58	0.003
Block (Site)*Treatment	10	844.8	844.8	84.5	1.42	0.144
Error	840	48172.4	48172.4	57.3		
Total	863	73648.3				

EPS

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	15908.6	14844.8	148448	257.61	<0.001
Block (Site)	2	73.2	99.9	49.9	0.87	0.421
Treatment	5	14657.9	12647.7	2529.5	43.90	<0.001
Site*Treatment	5	5314.1	4680.1	936.0	16.24	<0.001
Block (Site)*Treatment	10	779.7	779.7	78.0	1.35	0.198
Error	840	48405.5	48405.5	57.6		
Total	863	85139.0				

Diatoms

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	4.5	6.9	6.8	0.43	0.514
Block (Site)	2	22.0	23.2	11.6	0.72	0.488
Treatment	5	374.0	277.5	55.5	3.44	0.004
Site*Treatment	5	24.7	45.3	9.1	0.56	0.729
Block (Site)*Treatment	10	162.6	162.6	16.3	1.01	0.436
Error	840	13563.0	13563.0	16.2		
Total	863	14150.8				

Protozoans

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	56.0	75.0	75.0	3.02	0.083
Block (Site)	2	36.5	29.2	14.6	0.59	0.556
Treatment	5	300.9	276.0	55.2	2.22	0.050
Site*Treatment	5	70.7	73.1	14.6	0.59	0.709
Block (Site)*Treatment	10	187.9	187.9	18.8	0.76	0.672
Error	840	20877.8	20877.8	24.9		
Total	863	21529.8				

Fungi

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	8.6	1.6	1.58	0.11	0.746
Block (Site)	2	112.8	139.3	69.6	4.63	0.010
Treatment	5	316.6	290.4	58.1	3.86	0.002
Site*Treatment	5	68.7	63.8	12.8	0.85	0.516
Block (Site)*Treatment	10	95.3	95.3	9.5	0.63	0.786
Error	840	12637.1	12637.1	15.0		
Total	863	13239.2				

Organic Debris

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	27289.1	24222.3	24222.3	523.32	<0.001
Block (Site)	2	232.2	649.5	324.8	7.02	0.001
Treatment	5	7924.7	7537.0	1507.4	32.57	<0.001
Site*Treatment	5	2320.8	2034.4	406.9	8.79	<0.001
Block (Site)*Treatment	10	438.0	438.0	43.8	0.95	0.490
Error	840	38880.4	38880.4	46.3		
Total	863	77085.2				

Sediment Particles

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	363.4	225.9	225.9	6.18	0.013
Block (Site)	2	331.7	416.1	208.1	5.69	0.004
Treatment	5	1290.7	839.6	167.9	4.59	<0.001
Site*Treatment	5	691.2	507.6	101.9	2.78	0.017
Block (Site)*Treatment	10	691.1	691.1	69.1	1.89	0.043
Error	840	30719.1	30719.1	36.6		
Total	863	34087.2				

Panel Surface

Source	DF	SeqSS	Adj SS	AdjMS	F	P
Site	1	1720.0	1537.1	1537.1	18.00	<0.001
Block (Site)	2	169.4	377.6	188.8	2.21	0.110
Treatment	5	91373.5	82921.9	16584.4	194.19	<0.001
Site*Treatment	5	414.4	668.7	133.7	1.57	0.167
Block (Site)*Treatment	10	1551.0	1551.0	155.1	1.82	0.054
Error	840	71737.4	71737.4	85.4		
Total	863	166965.7				

Experiment 1. Mean counts (and standard deviations) of larval settlement for treatments A, C, D_a, D_b, E_a, and E_b (HighFRS = high flow rate site; LowFRS = low flow rate site).

Total Settlement

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	192.50	176.67	41.33	80.50	17.50	12.83
	69.93	15.297	17.84	25.19	10.60	3.54
LowFRS	265.83	174.33	116.67	137.50	12.50	9.67
	75.45	45.71	31.78	16.81	7.31	3.98

Spirorbins

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	152.67	134.50	26.00	48.17	17.17	12.17
	55.67	39.10	13.33	19.89	10.15	2.99
LowFRS	126.67	93.83	45.17	69.83	6.17	7.67
	55.44	26.61	14.89	12.89	6.31	4.55

Serpulins/*Pomatoceros* spp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	4.333	3.667	1.000	1.333	0.000	0.000
	2.658	2.582	0.633	1.033	0.000	0.000
LowFRS	9.333	5.167	4.500	6.333	0.333	0.167
	3.502	3.312	2.588	2.422	0.516	0.408

Total Ascidians

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	8.667	7.667	3.333	5.667	0.000	0.000
	5.086	7.633	1.033	7.118	0.000	0.000
LowFRS	17.167	12.500	11.500	9.333	0.000	0.000
	6.432	7.556	5.718	5.574	0.000	0.000

Botryllus schlosseri/Botrylloides leachi

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	4.667	1.500	1.333	4.000	0.000	0.000
	4.033	1.378	1.033	6.899	0.000	0.000
LowFRS	3.000	2.333	4.667	2.833	0.000	0.000
	1.673	1.751	4.719	1.602	0.000	0.000

Didemnid Ascidians

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	4.167	4.500	1.667	1.667	0.000	0.000
	2.563	4.416	0.516	0.516	0.000	0.000
LowFRS	7.667	4.833	3.000	3.000	0.000	0.000
	4.179	5.076	2.828	2.608	0.000	0.000

Total Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	22.833	21.167	10.333	23.333	0.167	0.333
	14.372	7.441	5.354	9.933	0.408	0.516
LowFRS	56.667	39.500	24.000	39.833	0.000	0.000
	15.667	19.388	10.826	8.750	0.000	0.000

Erect Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	1.167	2.333	0.333	0.167	0.000	0.000
	1.941	3.327	0.816	0.408	0.000	0.000
LowFRS	26.333	9.167	11.000	7.167	0.000	0.000
	8.042	8.424	2.367	6.047	0.000	0.000

Scrupocellaria scruposa

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.333	1.500	0.167	0.000	0.000	0.000
	0.516	2.739	0.408	0.000	0.000	0.000
LowFRS	21.333	7.833	6.167	5.000	0.000	0.000
	8.140	8.727	3.869	5.292	0.000	0.000

Crisia sp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.833	0.833	0.167	0.167	0.000	0.000
	1.602	0.753	0.408	0.408	0.000	0.000
LowFRS	5.000	1.333	4.833	2.167	0.000	0.000
	1.549	1.211	2.714	1.602	0.000	0.000

Mound Bryozoans (*Tubulipora* sp./*Plagioecia* sp.)

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	5.833	4.000	2.500	0.833	0.167	0.167
	4.070	2.530	1.643	0.753	0.408	0.408
LowFRS	10.500	3.667	5.000	5.167	0.000	0.000
	1.893	0.843	0.447	0.910	0.000	0.000

Sheet Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	15.833	14.833	7.000	22.333	0.000	0.167
	12.449	5.707	4.604	10.132	0.000	0.408
LowFRS	26.667	26.667	7.667	27.500	0.000	0.000
	10.820	12.388	6.470	4.764	0.000	0.000

Celleporella hyalina

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	2.000	2.833	2.500	1.667	0.000	0.000
	2.280	2.229	2.074	2.251	0.000	0.000
LowFRS	5.333	7.833	2.167	3.000	0.000	0.000
	3.141	6.306	2.317	1.897	0.000	0.000

Electra pilosa

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	2.167	5.333	1.000	7.833	0.000	0.000
	1.941	4.457	0.633	3.971	0.000	0.000
LowFRS	4.000	9.167	1.000	15.667	0.000	0.000
	4.243	5.382	0.894	4.179	0.000	0.000

Escharoides coccinea

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	4.833	1.000	1.667	3.667	0.000	0.000
	8.377	0.894	1.211	4.321	0.000	0.000
LowFRS	2.167	1.000	3.167	5.333	0.000	0.000
	1.602	1.095	2.927	2.251	0.000	0.000

Slime Sponges

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.333	5.333	0.000	0.167	0.000	0.333
	0.516	12.094	0.000	0.408	0.000	0.816
LowFRS	55.333	13.00	13.167	3.167	0.167	0.500
	29.487	11.747	8.110	2.563	0.408	0.548

Leucosolenia sp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.167	0.833	0.000	0.167	0.167	0.333
	0.408	1.169	0.000	0.408	0.408	0.816
LowFRS	19.333	7.667	15.500	5.167	5.667	1.333
	26.741	7.230	13.546	5.565	5.317	1.506

Experiment 2. Mean counts (and standard deviations) of larval settlement for treatments A, C, D_a, D_b, E_a, and E_b (HighFRS = high flow rate site; LowFRS = low flow rate site).

Total Settlement

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	55.67	20.00	58.83	21.00	4.83	5.83
	16.44	5.97	29.12	5.93	3.87	3.97
LowFRS	195.00	67.50	39.50	54.83	6.50	3.17
	61.17	10.93	20.16	30.06	2.66	1.47

Spirorbins

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	46.833	20.833	33.667	17.667	2.833	5.667
	16.412	5.811	18.446	5.428	2.858	3.830
LowFRS	144.167	49.833	29.000	42.333	6.000	2.833
	47.191	10.647	17.877	22.871	2.683	1.169

Serpulins/*Pomatoceros* sp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	1.000	0.667	4.833	0.167	0.333	0.000
	1.265	1.211	1.352	0.108	0.516	0.000
LowFRS	1.500	1.667	3.167	4.000	0.167	1.667
	2.258	1.211	6.338	5.933	0.408	2.251

Total Ascidians

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	5.000	1.833	4.667	1.500	2.167	0.000
	1.789	1.722	4.179	1.378	5.307	0.000
LowFRS	6.500	2.000	4.667	4.000	0.833	5.167
	7.817	2.000	3.777	4.147	1.602	9.304

Didemnid Ascidians

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	4.500	1.667	3.000	1.000	1.667	0.000
	1.378	1.633	2.608	0.894	4.083	0.000
LowFRS	0.5619	0.1799	0.6267	0.5102	0.1667	0.3389
	6.178	0.817	1.871	2.563	1.602	6.831

Total Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	2.000	2.500	11.833	1.667	1.167	0.167
	1.414	1.378	8.519	1.862	2.858	0.408
LowFRS	12.333	6.000	6.667	7.667	0.500	6.667
	11.413	4.517	4.590	7.501	0.837	13.201

Erect Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.000	0.000	0.500	0.000	0.000	0.000
	0.000	0.000	0.548	0.000	0.000	0.000
LowFRS	1.000	1.000	0.667	0.667	0.000	0.667
	1.095	0.894	1.033	0.422	0.000	1.211

Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.000	0.167	1.167	0.000	0.000	0.000
	0.000	0.408	1.602	0.000	0.000	0.000
LowFRS	1.000	1.167	0.667	1.000	0.000	1.667
	1.265	0.983	0.817	0.894	0.000	2.875

Sheet Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	1.833	2.333	9.667	2.000	1.500	0.167
	1.472	1.366	8.847	2.450	2.811	0.408
LowFRS	10.333	3.833	5.333	6.000	0.500	4.333
	9.812	3.251	3.445	6.261	0.837	9.223

Celleporella hyalina

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.833	0.333	2.167	0.167	0.167	0.000
	0.983	0.817	3.061	0.408	0.408	0.000
LowFRS	2.500	1.333	1.500	1.667	0.000	0.667
	2.168	2.160	1.517	2.251	0.000	1.211

Electra pilosa

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.167	1.000	4.333	1.000	0.667	0.167
	0.408	1.673	4.502	1.265	1.633	0.408
LowFRS	3.833	1.333	1.667	2.833	0.167	1.667
	3.817	1.751	1.862	2.787	0.408	3.204

Escharoides coccinea

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.167	0.333	1.167	0.000	0.167	0.000
	0.408	0.817	1.169	0.000	0.408	0.000
LowFRS	1.333	0.167	0.833	0.667	0.000	1.167
	2.066	0.408	0.753	1.033	0.000	2.858

Slime Sponges

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.167	0.167	1.000	0.333	0.167	0.000
	0.408	0.408	1.265	0.817	0.408	0.000
LowFRS	5.167	4.167	1.667	3.000	0.000	2.833
	8.183	4.070	2.733	2.280	0.000	5.231

Leucosolenia sp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.000	0.000	2.833	0.000	0.500	0.000
	0.000	0.000	5.601	0.000	0.838	0.000
LowFRS	0.500	1.333	0.500	0.167	0.833	1.000
	0.548	1.633	0.837	0.408	2.041	2.000

Experiment 3. Mean counts (and standard deviations) of larval settlement for treatments A, C, D_a, D_b, E_a, and E_b (HighFRS = high flow rate site; LowFRS = low flow rate site).

Total Settlement

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	98.50	123.00	45.25	57.25	2.25	4.25
	22.25	31.44	18.91	20.56	1.71	3.77
LowFRS	302.25	142.25	65.50	90.75	3.00	5.75
	94.77	30.36	16.54	26.85	1.41	2.99

Spirorbins

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	75.00	88.75	31.50	37.75	2.00	4.25
	17.61	31.07	19.94	12.45	1.63	3.77
LowFRS	238.50	100.75	46.75	69.50	3.00	5.50
	87.80	27.20	15.69	18.48	1.41	3.11

Serpulins/*Pomatoceros* sp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	0.250	6.000	1.750	2.000	0.250	0.000
	0.500	2.160	0.500	1.414	0.500	0.000
LowFRS	5.750	7.750	5.250	0.500	0.000	0.000
	5.560	4.500	3.304	1.000	0.000	0.000

Total Ascidians

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	8.250	8.750	5.750	8.000	0.000	0.000
	3.202	5.909	6.397	9.626	0.000	0.000
LowFRS	5.500	6.750	1.000	3.000	0.000	0.000
	4.123	2.217	0.817	2.160	0.000	0.000

Botryllus schlosseri/Botrylloides leachi

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	6.500	6.750	5.500	7.500	0.000	0.000
	1.915	4.787	6.608	9.256	0.000	0.000
LowFRS	2.000	5.750	0.500	1.000	0.000	0.000
	1.826	1.258	0.577	0.817	0.000	0.000

Total Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	15.000	18.500	5.750	8.750	0.000	0.000
	4.243	4.509	3.594	5.560	0.000	0.000
LowFRS	46.500	25.250	12.000	17.750	0.000	0.250
	6.137	8.921	5.033	12.633	0.000	0.500

Erect Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	1.250	4.000	2.000	2.500	0.000	0.000
	1.258	3.266	2.160	3.786	0.000	0.000
LowFRS	20.250	12.750	4.000	8.500	0.000	0.000
	3.775	6.185	0.816	6.608	0.000	0.000

Crisia sp.

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	1.250	3.750	2.000	2.500	0.000	0.000
	1.258	2.872	2.160	3.786	0.000	0.000
LowFRS	20.250	12.750	3.750	8.500	0.000	0.000
	3.775	6.185	0.500	6.608	0.000	0.000

Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.)

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	5.500	10.000	2.250	2.750	0.000	0.000
	3.873	3.742	1.258	3.403	0.000	0.000
LowFRS	23.000	9.750	5.000	6.750	0.000	0.000
	2.160	5.679	2.449	6.449	0.000	0.000

Sheet Bryozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	8.250	4.500	1.500	3.500	0.000	0.000
	3.594	3.317	1.291	1.915	0.000	0.000
LowFRS	3.250	2.750	3.000	2.500	0.000	0.250
	0.957	2.630	2.160	3.000	0.000	0.500

Celleporella hyalina

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	1.500	2.250	0.000	1.000	0.000	0.000
	1.291	2.630	0.000	1.414	0.000	0.000
LowFRS	0.250	0.500	0.000	1.500	0.000	0.000
	0.500	1.000	0.000	3.000	0.000	0.000

Electra pilosa

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	3.000	2.000	1.000	1.250	0.000	0.000
	1.633	2.160	0.817	1.258	0.000	0.000
LowFRS	1.750	3.500	2.250	0.000	0.000	0.000
	0.957	1.732	1.893	0.000	0.000	0.000

Experiment 3, AODC. Mean counts·cm^{-2} (and standard deviations) of total biofilm bacteria and dividing cells for treatments A, C, D_a, D_b, E_a, and E_b (HighFRS = high flow rate site; LowFRS = low flow rate site).

Total Bacteria

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	2655562	2788978	1514423	1281653	1426424	1571196
	110898	112096	87250	86873	85949	82939
LowFRS	2577499	2563306	1562680	1610937	1358296	1457649
	101477	111047	91923	93736	101293	94304

Dividing Cells

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	8.384	11.175	10.584	8.775	10.282	8.893
	0.867	1.295	1.584	1.503	1.939	1.149
LowFRS	9.216	10.054	7.313	8.955	9.337	7.980
	0.877	1.627	1.025	1.476	1.463	1.052

Experiment 3, SEM. Mean percentage surface cover (and standard deviations) of the biofilm surface categories for treatments A, C, D_a, D_b, E_a, and E_b. All data are given as arcsine transformed percentages (HighFRS = high flow rate site; LowFRS = low flow rate site).

Bacteria

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	31.238	33.704	24.591	22.016	22.585	26.551
	0.885	0.920	0.868	0.728	0.494	0.956
LowFRS	38.691	37.690	27.519	26.393	26.059	27.086
	0.912	1.040	0.905	1.050	0.866	1.058

EPS

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	36.366	34.049	20.537	23.358	21.301	20.637
	0.944	1.007	0.876	0.747	0.750	0.6542
LowFRS	20.183	19.390	16.465	16.098	16.805	15.816
	0.942	1.117	0.941	0.985	0.911	0.774

Diatoms

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	2.702	2.807	1.433	0.932	1.431	1.355
	0.595	0.576	0.439	0.343	0.407	0.411
LowFRS	3.004	2.516	1.628	1.403	1.158	1.818
	0.596	0.537	0.444	0.425	0.370	0.447

Protozoans

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	3.395	2.714	1.809	1.760	2.168	1.358
	0.684	0.606	0.517	0.546	0.572	0.443
LowFRS	2.844	3.527	3.382	2.480	2.432	1.594
	0.585	0.742	0.665	0.566	0.580	0.460

Fungi

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	2.033	1.791	1.245	1.177	1.988	0.791
	0.504	0.492	0.433	0.404	0.520	0.349
LowFRS	3.126	2.191	0.998	1.712	1.421	0.772
	0.575	0.559	0.340	0.468	0.454	0.309

Organic Debris

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	8.545	6.724	4.232	4.661	3.402	4.316
	0.775	0.790	0.694	0.794	0.621	0.695
LowFRS	23.700	21.010	12.644	11.968	16.697	13.302
	0.771	0.971	0.862	0.927	0.854	0.864

Sediment Particles

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	6.201	4.408	5.089	4.238	4.860	4.772
	0.784	0.716	0.734	0.723	0.758	0.700
LowFRS	9.681	8.213	4.484	4.746	4.698	5.528
	0.830	0.700	0.649	0.715	0.637	0.676

Panel Surface

	Treatment					
	A	C	D_a	D_b	E_a	E_b
HighFRS	30.654	31.494	52.778	53.220	52.034	55.325
	1.408	1.353	0.904	0.930	0.918	0.699
LowFRS	27.142	30.868	49.885	50.946	49.087	50.646
	1.288	1.409	0.966	1.193	0.810	0.964

APPENDIX 2

Experiments 1-8. Mean counts and (standard deviations) of larval settlers. Columns = Treatments (1 = A, 3 = C, 5 = D_a, 6 = D_b, 8 = E_a and 9 = E_b), rows = Experiments 1-8, All = column- and row-totals.

Total Settlement

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	87.833	75.833	41.000	45.167	3.667	2.500	42.667
	15.955	15.867	7.403	9.109	1.633	2.588	34.213
2	6	6	6	6	6	6	36
	37.167	24.833	12.333	11.667	1.000	2.500	14.917
	11.125	6.735	7.992	2.422	0.894	2.739	14.129
3	9	9	9	9	9	9	54
	43.333	47.444	15.667	17.556	0.778	2.667	21.241
	10.000	12.758	5.852	9.787	1.093	2.121	19.922
4	9	9	9	9	9	9	54
	73.556	66.556	35.111	36.333	1.000	3.222	35.963
	22.705	13.087	19.297	14.958	1.118	2.635	31.362
5	9	9	9	9	9	9	54
	56.111	44.111	25.333	24.000	0.778	0.556	25.148
	18.731	12.067	12.298	11.874	1.202	0.527	23.387
6	9	9	9	9	9	9	54
	20.778	23.556	9.778	9.889	0.222	0.444	10.778
	7.242	8.323	5.357	6.566	0.441	0.726	10.554
7	9	9	9	9	9	9	54
	60.667	54.778	23.000	20.333	1.333	1.333	26.907
	20.946	13.470	6.745	5.099	1.658	1.658	25.743
8	6	6	6	6	6	6	36
	107.500	57.667	64.500	56.333	6.333	1.333	48.944
	18.674	22.367	12.582	7.257	3.502	1.506	38.795
ALL	63	63	63	63	63	63	378
	58.492	48.857	26.778	26.222	1.635	1.778	27.294
	29.677	20.961	18.807	17.116	2.302	2.075	28.008

Spirorbins

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	47.667	32.833	20.167	14.000	3.333	1.833	19.972
	8.165	8.773	3.601	5.020	1.633	1.941	17.225
2	6	6	6	6	6	6	36
	26.000	16.500	8.333	6.500	1.000	2.333	10.111
	11.883	6.804	6.501	3.082	0.894	2.733	10.631
3	9	9	9	9	9	9	54
	20.000	17.778	5.000	5.000	0.778	2.556	8.519
	6.519	6.078	4.583	4.031	1.093	2.128	8.709
4	9	9	9	9	9	9	54
	32.444	26.778	12.444	11.778	1.000	2.667	14.519
	11.749	5.674	9.658	6.261	1.118	2.000	13.545
5	9	9	9	9	9	9	54
	33.333	27.444	10.556	10.889	0.778	0.222	13.870
	13.739	8.531	7.055	5.988	1.202	0.441	14.573
6	9	9	9	9	9	9	54
	11.111	13.778	5.333	7.222	0.111	0.333	6.315
	5.207	4.764	4.770	5.118	0.333	0.707	6.425
7	9	9	9	9	9	9	54
	26.889	28.222	12.333	10.778	0.889	0.778	13.315
	8.403	9.718	7.365	4.842	1.691	1.394	12.671
8	6	6	6	6	6	6	36
	54.333	35.167	32.333	28.000	5.333	1.167	26.056
	13.018	20.024	6.314	12.649	3.445	1.602	21.228
ALL	63	63	63	63	63	63	378
	29.873	24.333	12.317	11.143	1.429	1.444	13.423
	15.809	11.264	10.017	8.489	2.100	1.873	14.347

Serpulins/*Pomatoceros* spp.

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	3.0000	2.1667	1.1667	3.1667	0.0000	0.1667	1.6111
	3.5214	2.0412	1.1690	2.2286	0.0000	0.4082	2.2204
2	6	6	6	6	6	6	36
	0.3333	0.8333	0.6667	1.0000	0.0000	0.1667	0.5000
	0.5164	0.9832	1.2111	1.2649	0.0000	0.4082	0.8783
3	9	9	9	9	9	9	54
	5.3333	3.0000	1.1111	0.6667	0.0000	0.0000	1.6852
	4.1833	2.5000	1.5366	1.0000	0.0000	0.0000	2.8004
4	9	9	9	9	9	9	54
	7.0000	5.5556	3.3333	2.4444	0.0000	0.0000	3.0556
	2.9580	3.2447	2.5981	2.7889	0.0000	0.0000	3.4718
5	9	9	9	9	9	9	54
	1.6667	2.7778	0.4444	0.5556	0.0000	0.0000	0.9074
	2.3979	2.9907	0.7265	1.0138	0.0000	0.0000	1.8660
6	9	9	9	9	9	9	54
	1.2222	0.3333	0.7778	0.2222	0.1111	0.1111	0.4630
	2.1082	0.7071	0.6667	0.4410	0.3333	0.3333	1.0226
7	9	9	9	9	9	9	54
	4.0000	2.3333	1.7778	1.6667	0.0000	0.1111	1.6481
	2.8284	1.8028	1.3944	1.5811	0.0000	0.3333	2.0664
8	6	6	6	6	6	6	36
	8.5000	5.0000	1.5000	1.5000	0.0000	0.0000	2.7500
	3.2711	2.2804	1.0488	1.7607	0.0000	0.0000	3.5406
ALL	63	63	63	63	63	63	378
	3.8730	2.7619	1.3810	1.3333	0.0159	0.0635	1.5714
	3.7994	2.7458	1.6405	1.8050	0.1260	0.2458	2.5547

Total Ascidians

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	10.5000	9.0000	1.1667	3.0000	0.0000	0.0000	1.1111
	1.5056	6.2610	1.1460	2.6833	0.0000	0.0000	5.1166
2	6	6	6	6	6	6	36
	0.6667	0.1667	0.0000	0.0000	0.0000	0.0000	0.1389
	0.8165	0.4082	0.0000	0.0000	0.0000	0.0000	0.4245
3	9	9	9	9	9	9	54
	7.8889	8.7778	1.3333	1.0000	0.0000	0.0000	1.1667
	6.1507	5.7179	1.2131	5.5000	0.0000	0.0000	5.5073
4	9	9	9	9	9	9	54
	15.6667	15.2222	8.0000	10.0000	0.0000	0.0000	8.1481
	6.6521	5.3567	7.3993	7.2111	0.0000	0.0000	8.2677
5	9	9	9	9	9	9	54
	3.2222	3.1111	1.7778	2.5556	0.0000	0.0000	2.3333
	2.7739	2.5055	1.9911	3.6780	0.0000	0.0000	3.3365
6	9	9	9	9	9	9	54
	1.5556	0.6667	0.2222	0.0000	0.0000	0.0000	0.1071
	2.1858	0.8660	0.1110	0.0000	0.0000	0.0000	1.0906
7	9	9	9	9	9	9	54
	3.3333	2.2222	1.1111	1.1111	0.1111	0.0000	1.1259
	3.8079	2.1381	2.1552	2.1858	0.3333	0.0000	2.1691
8	6	6	6	6	6	6	36
	9.6667	1.8333	8.6667	8.1667	0.3333	0.0000	5.2778
	3.5021	3.1252	1.6762	1.5350	0.8165	0.0000	5.0006
ALL	63	63	63	63	63	63	378
	6.5079	5.6667	3.9018	3.6319	0.0176	0.0000	3.2937
	6.1879	6.1931	5.0823	5.2157	0.2799	0.0000	5.3186

Botryllus schlosseri/Botrylloides leachi

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	1.8333	1.6667	0.6667	0.1667	0.0000	0.0000	0.7222
	1.4720	2.6583	1.2111	0.4082	0.0000	0.0000	1.4660
2	6	6	6	6	6	6	36
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
4	9	9	9	9	9	9	54
	15.4444	14.5556	7.5556	9.0000	0.0000	0.0000	7.7593
	6.6729	4.9777	7.3333	6.7454	0.0000	0.0000	8.0022
5	9	9	9	9	9	9	54
	0.4444	0.7778	1.5556	0.7778	0.0000	0.0000	0.5926
	1.0138	1.6415	2.4552	0.9718	0.0000	0.0000	1.3807
6	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
7	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
8	6	6	6	6	6	6	36
	7.3333	3.6667	8.3333	7.8333	0.3333	0.0000	4.5833
	3.3862	2.5820	4.6762	4.0702	0.8165	0.0000	4.5316
ALL	63	63	63	63	63	63	378
	3.1429	2.6984	2.1587	2.1587	0.0317	0.0000	1.6984
	6.0930	5.4466	4.5016	4.5159	0.2520	0.0000	4.3834

Total Bryozoans

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	21.833	27.000	15.167	24.500	0.333	0.500	14.889
	9.432	8.050	5.115	7.450	0.516	0.837	12.444
2	6	6	6	6	6	6	36
	10.167	7.333	3.333	4.167	0.000	0.000	4.167
	5.636	4.803	2.422	3.251	0.000	0.000	4.914
3	9	9	9	9	9	9	54
	10.000	17.889	4.556	6.222	0.000	0.111	6.463
	4.000	5.819	2.506	2.682	0.000	0.333	6.960
4	9	9	9	9	9	9	54
	17.889	18.111	11.000	11.889	0.000	0.222	9.852
	9.493	6.373	5.788	5.011	0.000	0.441	9.182
5	9	9	9	9	9	9	54
	14.778	8.667	9.111	9.556	0.000	0.333	7.074
	11.200	2.739	3.756	6.106	0.000	0.500	7.506
6	9	9	9	9	9	9	54
	6.889	8.778	3.444	2.444	0.000	0.000	3.593
	5.645	6.200	2.744	3.575	0.000	0.000	4.970
7	9	9	9	9	9	9	54
	26.111	21.222	7.333	6.333	0.333	0.333	10.278
	10.902	10.366	4.153	3.317	0.500	0.707	11.796
8	6	6	6	6	6	6	36
	34.833	12.667	21.167	18.333	0.667	0.167	14.639
	10.926	4.320	8.727	6.218	0.816	0.408	13.632
ALL	63	63	63	63	63	63	378
	17.175	15.143	8.841	9.683	0.143	0.206	8.532
	11.876	8.821	6.950	7.975	0.396	0.481	9.891

Erect Bryozoans

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	7.0000	5.1667	3.1667	2.3333	0.0000	0.0000	2.9444
	4.1952	4.1673	1.9408	1.8619	0.0000	0.0000	3.5693
2	6	6	6	6	6	6	36
	7.1667	5.6667	3.0000	3.8333	0.0000	0.0000	3.2778
	4.4008	4.8028	2.3664	3.3714	0.0000	0.0000	3.9757
3	9	9	9	9	9	9	54
	0.6667	2.8889	0.5556	0.8889	0.0000	0.1111	0.8519
	0.8660	2.7588	0.7265	1.6915	0.0000	0.3333	1.6530
4	9	9	9	9	9	9	54
	4.0000	3.5556	0.6667	0.5556	0.0000	0.0000	1.4630
	3.6742	3.7786	1.3229	1.0138	0.0000	0.0000	2.7245
5	9	9	9	9	9	9	54
	1.7778	2.0000	0.3333	0.3333	0.0000	0.1111	0.7593
	1.9221	2.6458	0.7071	0.7071	0.0000	0.3333	1.5654
6	9	9	9	9	9	9	54
	5.3333	6.5556	3.0000	2.0000	0.0000	0.0000	2.8148
	4.1533	5.0525	2.8723	3.7081	0.0000	0.0000	4.0051
7	9	9	9	9	9	9	54
	4.8889	3.4444	0.8889	0.4444	0.0000	0.0000	1.6111
	6.2138	5.5478	1.3642	0.7265	0.0000	0.0000	3.7988
8	6	6	6	6	6	6	36
	9.6667	2.0000	4.3333	1.3333	0.1667	0.0000	2.9167
	3.8297	1.5492	3.8816	1.0328	0.4082	0.0000	4.0311
ALL	63	63	63	63	63	63	378
	4.6508	3.8571	1.7778	1.3175	0.0159	0.0317	1.9418
	4.5726	4.1225	2.3722	2.1984	0.1260	0.1767	3.3317

Crisia sp.

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	2.8333	2.6667	1.3333	1.1667	0.0000	0.0000	1.3333
	3.3116	2.8752	1.2111	2.4014	0.0000	0.0000	2.2552
2	6	6	6	6	6	6	36
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
3	9	9	9	9	9	9	54
	0.4444	2.2222	0.5556	0.8889	0.0000	0.1111	0.7037
	0.7265	2.3333	0.7265	1.6915	0.0000	0.3333	1.4093
4	9	9	9	9	9	9	54
	2.6667	2.8889	0.6667	0.5556	0.0000	0.0000	1.1296
	3.3166	3.5512	1.3229	1.0138	0.0000	0.0000	2.3315
5	9	9	9	9	9	9	54
	1.5556	1.8889	0.3333	0.3333	0.0000	0.0000	0.6852
	2.0069	2.5221	0.7071	0.7071	0.0000	0.0000	1.5150
6	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
7	9	9	9	9	9	9	54
	2.6667	1.4444	0.3333	0.4444	0.0000	0.0000	0.8148
	4.7170	3.9721	0.7071	0.7265	0.0000	0.0000	2.6140
8	6	6	6	6	6	6	36
	6.3333	1.5000	4.1667	1.1667	0.0000	0.0000	2.1944
	3.2660	1.2247	3.9200	0.9832	0.0000	0.0000	3.0967
ALL	63	63	63	63	63	63	378
	1.9206	1.6032	0.7937	0.5397	0.0000	0.0159	0.8122
	3.1587	2.6490	1.7704	1.1616	0.0000	0.1260	2.0183

Alcyonidium spp.

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
2	6	6	6	6	6	6	36
	7.1667	5.6667	3.0000	3.8333	0.0000	0.0000	3.2778
	4.4008	4.8028	2.3664	3.3714	0.0000	0.0000	3.9757
3	9	9	9	9	9	9	54
	0.2222	0.6667	0.0000	0.0000	0.0000	0.0000	0.1481
	0.4410	1.3229	0.0000	0.0000	0.0000	0.0000	0.5958
4	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
5	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
6	9	9	9	9	9	9	54
	5.0000	6.5556	3.0000	2.0000	0.0000	0.0000	2.7593
	4.2131	5.0525	2.8723	3.7081	0.0000	0.0000	3.9808
7	9	9	9	9	9	9	54
	2.2222	2.0000	0.5556	0.0000	0.0000	0.0000	0.7963
	2.7285	2.5495	1.3333	0.0000	0.0000	0.0000	1.8159
8	6	6	6	6	6	6	36
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ALL	63	63	63	63	63	63	378
	1.7460	1.8571	0.7937	0.6508	0.0000	0.0000	0.8413
	3.3262	3.5644	1.8242	2.0647	0.0000	0.0000	2.3895

Tubulipora sp./*Plagioecia* sp. (= Mound Bryozoans)

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	7.5000	11.0000	8.3333	10.3333	0.1667	0.0000	6.2222
	5.4681	6.9570	4.9666	5.2409	0.4082	0.0000	6.2797
2	6	6	6	6	6	6	36
	1.0000	0.8333	0.0000	0.3333	0.0000	0.0000	0.3611
	1.0954	0.9832	0.0000	0.5164	0.0000	0.0000	0.7232
3	9	9	9	9	9	9	54
	5.6667	7.5556	0.6667	3.0000	0.0000	0.0000	2.8148
	2.9580	4.7199	0.7071	2.5000	0.0000	0.0000	3.7921
4	9	9	9	9	9	9	54
	5.0000	4.0000	1.6667	2.1111	0.0000	0.0000	2.1296
	2.3979	3.1225	2.5495	2.0883	0.0000	0.0000	2.7475
5	9	9	9	9	9	9	54
	5.1111	2.4444	4.2222	5.3333	0.0000	0.1111	2.8704
	5.7542	1.8782	2.7739	5.3385	0.0000	0.3333	3.9908
6	9	9	9	9	9	9	54
	1.0000	1.3333	0.3333	0.2222	0.0000	0.0000	0.4815
	1.3229	1.4142	0.7071	0.4410	0.0000	0.0000	0.9662
7	9	9	9	9	9	9	54
	14.3333	14.0000	5.4444	4.2222	0.2222	0.3333	6.4259
	8.0623	6.8007	4.2164	3.2702	0.4410	0.7071	7.4419
8	6	6	6	6	6	6	36
	8.3333	4.5000	6.3333	10.3333	0.5000	0.1667	5.0278
	4.1312	2.8810	4.6762	3.1411	0.8367	0.4082	4.7838
ALL	63	63	63	63	63	63	378
	6.0476	5.7460	3.1587	4.1270	0.0952	0.0794	3.2090
	5.9823	5.9865	3.9806	4.6403	0.3461	0.3263	4.8769

Sheet Bryozoans

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	7.3333	10.8333	3.6667	11.8333	0.1667	0.5000	5.7222
	2.9439	4.5350	3.5024	4.8751	0.4082	0.8367	5.6092
2	6	6	6	6	6	6	36
	2.0000	0.8333	0.3333	0.0000	0.0000	0.0000	0.5278
	3.0984	0.7528	0.5164	0.0000	0.0000	0.0000	1.4240
3	9	9	9	9	9	9	54
	3.6667	7.4444	3.3333	2.3333	0.0000	0.0000	2.7963
	3.7749	5.9184	2.3452	1.5811	0.0000	0.0000	3.8967
4	9	9	9	9	9	9	54
	8.8889	10.5556	8.6667	9.2222	0.0000	0.2222	6.2593
	7.6066	6.5213	5.1720	4.0859	0.0000	0.4410	6.4316
5	9	9	9	9	9	9	54
	7.8889	4.5556	4.5556	3.8889	0.0000	0.1111	3.5000
	5.6224	2.6510	3.0459	3.4801	0.0000	0.3333	4.0967
6	9	9	9	9	9	9	54
	0.5556	0.8889	0.1111	0.2222	0.0000	0.0000	0.2963
	0.7265	1.1667	0.3333	0.6667	0.0000	0.0000	0.6904
7	9	9	9	9	9	9	54
	6.4444	3.7778	1.0000	1.6667	0.1111	0.0000	2.1667
	3.5746	2.8626	1.2247	1.7321	0.3333	0.0000	3.0328
8	6	6	6	6	6	6	36
	16.8333	6.1667	10.5000	6.6667	0.0000	0.0000	6.6944
	6.9690	3.0605	4.2308	5.5015	0.0000	0.0000	7.1187
ALL	63	63	63	63	63	63	378
	6.4127	5.5873	3.9048	4.2381	0.0317	0.0952	3.3783
	6.3440	5.2352	4.4927	4.9178	0.1767	0.3461	4.9643

Celleporella hyalina

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	0.6667	2.0000	0.3333	0.3333	0.0000	0.0000	0.5556
	0.5164	2.0000	0.5164	0.5164	0.0000	0.0000	1.0809
2	6	6	6	6	6	6	36
	0.0000	0.3333	0.1667	0.0000	0.0000	0.0000	0.0833
	0.0000	0.5164	0.4082	0.0000	0.0000	0.0000	0.2803
3	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
4	9	9	9	9	9	9	54
	5.5556	8.3333	4.4444	5.3333	0.0000	0.2222	3.9815
	5.0277	6.8920	2.2973	2.3452	0.0000	0.4410	4.6601
5	9	9	9	9	9	9	54
	4.6667	2.3333	1.3333	1.3333	0.0000	0.0000	1.6111
	4.8477	2.4495	1.5811	1.5000	0.0000	0.0000	2.7842
6	9	9	9	9	9	9	54
	0.3333	0.1111	0.1111	0.0000	0.0000	0.0000	0.0926
	0.7071	0.3333	0.3333	0.0000	0.0000	0.0000	0.3512
7	9	9	9	9	9	9	54
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
8	6	6	6	6	6	6	36
	11.8333	3.6667	6.8333	1.3333	0.0000	0.0000	3.9444
	5.1153	1.8619	4.2151	1.9664	0.0000	0.0000	5.0931
ALL	63	63	63	63	63	63	378
	2.6984	2.1111	1.5397	1.1111	0.0000	0.0317	1.2487
	4.7268	3.9477	2.7814	2.1561	0.0000	0.1767	3.0463

Electra pilosa

	1	3	5	6	8	9	ALL
1	6	6	6	6	6	6	36
	5.5000	6.6667	3.1667	11.5000	0.1667	0.5000	4.5833
	3.7283	4.7610	3.3116	5.2058	0.4082	0.8367	5.1402
2	6	6	6	6	6	6	36
	0.5000	0.1667	0.1667	0.0000	0.0000	0.0000	0.1389
	0.8367	0.4082	0.4082	0.0000	0.0000	0.0000	0.4245
3	9	9	9	9	9	9	54
	3.2222	5.3333	2.3333	1.2222	0.0000	0.0000	2.0185
	3.9930	5.3852	2.1794	1.6415	0.0000	0.0000	3.3953
4	9	9	9	9	9	9	54
	2.8889	0.7778	4.1111	3.0000	0.0000	0.0000	1.7963
	3.2575	1.3944	3.1798	2.7839	0.0000	0.0000	2.6874
5	9	9	9	9	9	9	54
	1.1111	0.4444	2.5556	2.4444	0.0000	0.1111	1.1111
	1.6915	1.0138	2.8333	2.2973	0.0000	0.3333	1.9296
6	9	9	9	9	9	9	54
	0.1111	0.3333	0.0000	0.2222	0.0000	0.0000	0.1111
	0.3333	0.7071	0.0000	0.6667	0.0000	0.0000	0.4196
7	9	9	9	9	9	9	54
	4.1111	3.5556	0.6667	1.3333	0.1111	0.0000	1.6296
	2.2608	2.3511	1.0000	1.4142	0.3333	0.0000	2.1831
8	6	6	6	6	6	6	36
	1.8333	1.1667	1.6667	3.3333	0.0000	0.0000	1.3333
	2.2286	1.1690	3.2042	2.0656	0.0000	0.0000	2.0840
ALL	63	63	63	63	63	63	378
	2.3810	2.2540	1.8571	2.5873	0.0317	0.0635	1.5291
	2.9861	3.4964	2.6080	3.8211	0.1767	0.3044	2.8574

APPENDIX 3

ORIGINAL VERSION OF CHAPTER 4

SEASONAL VARIATION IN THE EFFECT OF HARD SUBSTRATUM BIOFILMING ON SETTLEMENT OF MARINE INVERTEBRATE LARVAE IN THE FIELD

4.1 INTRODUCTION

In a novel field experimental approach, in which biofilming of panel substrata under exclusion of larval settlers was achieved by enclosure of panels within tight-fitting, but removable, mesh screens (Todd & Keough, 1994; Keough & Raimondi, 1995, Chapter 3), it was revealed that *in situ* larval responses to microbial cues are highly species-specific, and can be either facilitatory or inhibitory. In Chapter 3 it was shown that larvae of certain hard-substratum species show flow regime related differences in their settlement response to biofilmed surfaces within a local habitat. It was concluded that such differential larval settlement is likely to be due to flow-regime associated microcolonization patterns. Many species also are able to distinguish between microfilms of varying ages developed under similar conditions (Maki *et al.* 1988, 1990, 1992; Pearce & Scheibling, 1991; Holmström *et al.*, 1992; see also Chapter 2). Thus, there is growing evidence that larval responses to microbial cues are indeed very subtle and finely tuned, and that small-scale differences in successional composition, physiological condition and growth phase of the biofilm community may well alter the effect on larval settlement (Neumann, 1979; Szewzyk *et al.*, 1991; Anderson, 1995; Chapter 2, Section 2.2). In this context it has been proposed by several authors that microbial cues can help larvae to recognize settlement sites of high survival value (Strathmann & Branscomb, 1979; Strathmann *et al.*, 1981; Raimondi, 1988; see Chapter 3).

In view of the evidently highly complex interactions between microfilm dynamics, larval responses and environmental fluctuations, it seems futile to study microbial settlement cues as isolated parameters. Most research in the past has been

laboratory based, and often concerned only mono-specific films and a small selection of invertebrate taxa (Neumann, 1979; Weiner *et al.*, 1985; Fitt *et al.*, 1990; Szewzyk *et al.*, 1991; Holmström *et al.*, 1992, Leitz & Wagner, 1993; Neal & Yule, 1994a). Whilst such controlled laboratory assays increase experimental tractability, they fail to consider the complexity of natural larva-biofilm interactions. Recent field studies examining larval settlement patterns in response to biofilming were short-term experiments and which did not allow the assessment of seasonal and annual changes in the effect of filming (Todd & Keough, 1994; Keough & Raimondi, 1995). Seasonal changes of the composition, density and/or physiological activity of epibenthic microfilms have been reported (e.g. Hudon & Bourget, 1981; Munteanu & Maly, 1981; Underwood, 1984; MacLulich, 1987; Anderson, 1995), and intuitively these will be important in explaining variations of settlement patterns for those species that reproduce throughout most parts of the year. Raimondi and Keough (1990), who discussed the marked plasticity in behavioural responses of larvae to settlement cues, also pointed to the possible impact of temporal variations in the environment. It is possible, for example, that the importance of biofilming as a positive or facilitatory settlement cue for the larvae of certain taxa might vary inversely with a seasonal increase in the effects of the presence of dominant competitor species.

The present series of experiments is the first to investigate the seasonality of the effects of biofilms on larval settlement in the field. The objective was to assess any temporal variation in the species-specific responses for a range of taxa, and to separate the effects of biofilming from those of the presence of other early incumbent post larvae (Chapter 3; see also Todd & Keough, 1994; Keough & Raimondi, 1995).

4.2 MATERIALS AND METHODS

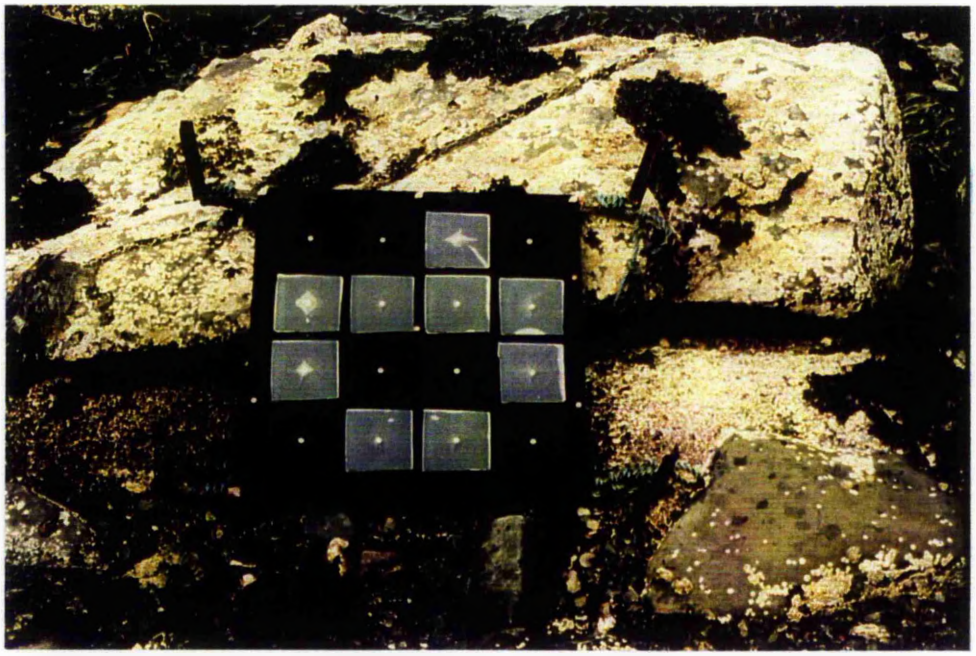
Field Experiments

The field experimental location was at Clachan Seil, western Scotland (see Chapter 3, and Todd & Turner, 1986; Turner, 1988). The general experimental protocols followed those of Chapter 3 (see also Todd & Keough, 1994, and Keough & Raimondi, 1995): black plastic (perspex) panels (0.5 mm thickness, 11 x 11 cm square) were used as settlement substrata. Preliminary field experiments that compared settlement rates onto different types of artificial substrata (perspex, natural slate, darkened slate) revealed black Perspex to be a most suitable material, which has the advantage also of being easily cut to size and of light weight. Panels were bolted to square mounting plates (also made of black perspex) by means of plastic screws running through counter-sunk holes in the centres of the panels and retained by plastic wing nuts. Each frame could hold 16 panels in a 4 x 4 array on a mounting plate (Plate 4.1), leaving spaces of 3 cm between adjacent panels. The mounting plates were attached horizontally in an inverted position to four-legged steel frames which were coated in epoxy resin to prevent corrosion. The downward facing sides of the panels comprised the experimental substrata. This orientation of the panels was chosen to prevent any confounding effects of sediment deposition. Prior to their use in experiments, all panels were sanded with medium coarse sandpaper to remove the original surface-sheen of the material, and then soaked in freshwater for 2d to leach any contaminants. The surfaces of the panels then were lightly marked in a 1 x 1 cm grid to facilitate the scoring of established organisms at the end of the experiments.

The experiments included 5 panel treatments (Fig. 4.1) and these were letter coded as by Todd & Keough (1994). In treatment C initial biofilming of the panel substrata, whilst excluding larval settlement, was achieved by the enclosure of experimental panels within tight-fitting (but removable) polyester mesh screens of 100μm pore size (see Todd & Keough, 1994 for the effect of mesh sizes). These screens were folded tightly beneath the edges of the panels where they were securely held between the panel and the

PLATE 4.1

The frames used in the time series experiments holding the experimental panel substrata (viewed from below).



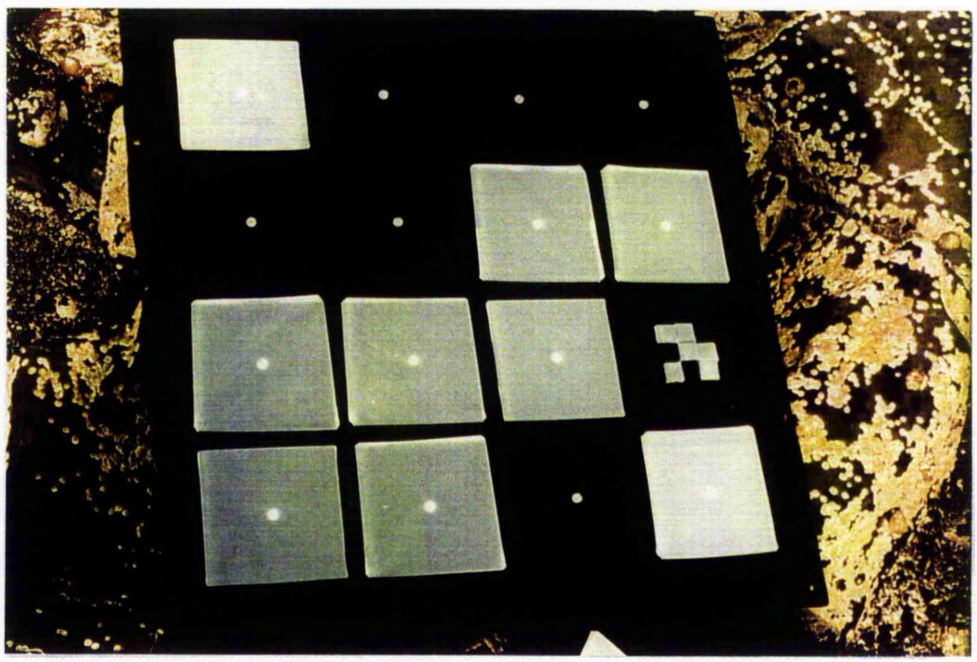
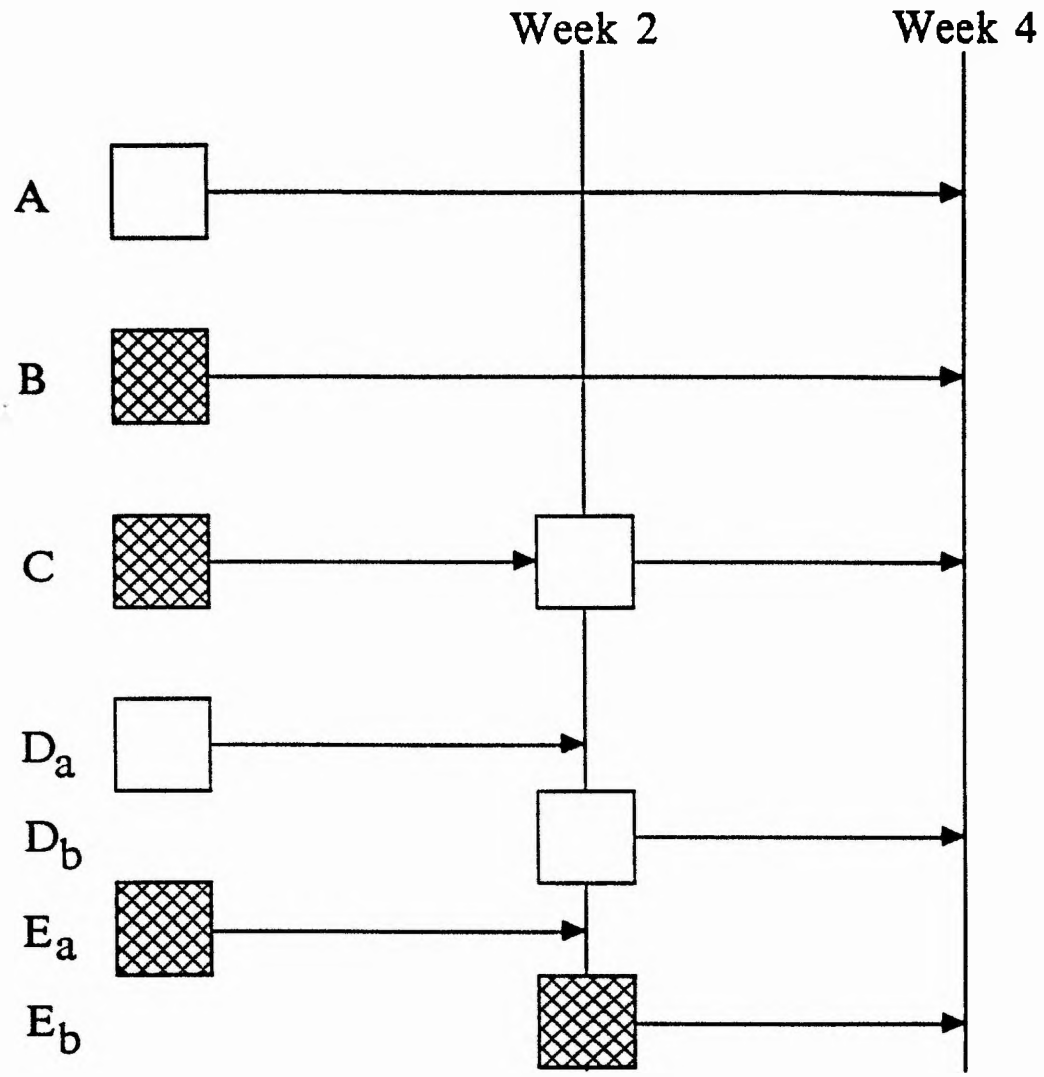


FIGURE 4.1

Schematic diagram of the experimental treatments and their codes for the first and second halves of the experiments. Netted and un-netted panels are represented by hatched and unhatched squares respectively.


Week 2
Week 4
A
B
C
D_a
D_b
E_a
E_b

mounting plate (Plate 4.1). After an initial 14d period of immersion, allowing the development of a biofilm but an essential exclusion of larval settlers, the meshes were removed and the experimental surfaces exposed to larval settlement for a further 14d. In Chapter 3 it was shown that netting did not have any major effects on the establishment of microbial films onto panel surfaces (Chapter 3, but see also Keough & Raimondi, 1995). Treatment A panel substrata were un-netted and exposed to larval settlement throughout the experimental time of one month. Treatments D (D_a and D_b) and E (E_a and E_b) were the respective controls for the un-netted and netted treatments and were included to provide separate data for the two halves of the experimental period: D_a/E_a was an un-netted/netted panel, which was replaced after 14d by another un-netted/netted panel, D_b/E_b. In a fifth treatment, B, netted panels remained deployed and unmanipulated throughout the experimental period of 28d. Although initially included in the experiments as additional controls treatments B and E_b subsequently were not used in the statistical analysis.

Following each experiment the panels were transferred into holding containers filled with seawater and transported to St. Andrews, where the settlers were counted under a dissecting microscope and identified to the lowest possible taxon (grouping of taxa into categories as described in Chapter 3). Except for the 1994 spring experiment, in which panels from the first half of the experiment were preserved for 1wk in a 4% formaldehyde seawater solution, all panels were scored within two days of return to the laboratory. During removal and transport of the panels care was taken to prevent them from contacting the air water interface. Only the central areas (10 x 10 cm) of the panels were counted and considered in the analysis, leaving a 1 cm wide margin to allow for potential edge effects and inadvertent handling during retrieval (see for example Keough & Raimondi, 1995).

The frames (= blocks in the statistical analysis) were anchored by four 12 kg concrete cylinders attached to the frame legs. There were three frames per experiment, with the exception of the 1992 autumn experiment, the 1993 winter experiment and the 1994 summer experiment, in which there were two frames only. Each frame included three replicate panels of experimental treatments A, B, C, D and E (= 15 panels) in a

randomized array; the 16th slot of each frame was filled with a combination of nine small Perspex panels (1.5 x 1.5 cm), which were attached to the mounting panel by means of silicon glue. The small panels were assembled in randomized positions in a 3 x 3 array, and three replicates each of treatments A, B and C were used. At the end of the experiments these panels were preserved in a 4% glutaraldehyde solution, because they were initially included for SEM comparison of biofilms. No quantitative examinations were carried out for these film samples, and only a number of them were used for visual comparisons between treatments (for a more detailed quantitative comparison of biofilms from netted and un-netted panel treatments see Chapter 3). No obvious treatment effects were noted between netted and un-netted substrata.

Experiments were repeated twice for each season: autumn (September/October 1992 and 1993), winter (January/February 1993 and 1994), spring (March/April 1993 and 1994) and summer (June/July 1993 and 1994).

Statistical Analysis

The seasonal variations of the effect of biofilming were assessed for all species and taxonomic groups by comparing filmed and unfilmed substrata across all eight experiments using the contrast C *versus* $[E_a + D_b]$. Treatment C and the composite treatment $[E_a + D_b]$ were chosen for the between-season comparison, because the use of panel treatments which were netted during the first half of the experiment allowed the isolation of filming effects from any potentially confounding effects of already established early incumbent settlers for the second half of the experiment. The combination $[E_a + D_b]$ provided a comparable netted unfilmed control to C. Whereas the surface of a treatment C panel was filmed at the onset of the second half of the experiment (after removal of the mesh screen), the derived variable $[E_a + D_b]$ was unfilmed at the commencement of the second half of the experiment.

The comparison A *versus* $[C - E_a + D_a]$, including the pooled data of all eight experiments, was carried out for all species and taxonomic groups to assess seasonal

variations in the effects of early incumbents on subsequent larval settlement (in the presence of biofilming). This contrast was preferred over the comparison [A - D_a] *versus* [C - E_a] because the latter more often resulted in negative terms, which could not be log-transformed (see also Chapter 3).

The eight experiments were treated as a time series. The comparisons were made by Linear Contrasts of the treatment combinations C *versus* [E_a + D_b] and A *versus* [C - E_a + D_a], and were carried out for the total counts of settlers on all three replicate panels per frame (block) within each experiment using Genstat (version 5.3, 1994). The use of total counts per treatment across frames rather than counts per panel was chosen because a random combination of individual panels (within a frame) into composite variables (i.e. [E_a + D_b] and [C - E_a + D_a]) could potentially have confounding effects on the analysis. Comparisons were made for those taxonomic groups and species which settled in more than one season, and those which were too heterogeneous in their variances and numerical abundances were excluded. Although some intra-specific interactions were anticipated (due to possible allelochemical inhibition or gregarious behaviour at settlement), and tested for in the contrast A *versus* [C - E_a + D_a], no major clustering of settlers was expected and a Poisson distribution was assumed for the counted larval settlers. All data were log-transformed prior to analysis.

The overall "sources" of variation considered in the experimental rationale were temporal (between-year differences and seasonal effects), spatial (positional effects of the frames holding the panels) and experimental (treatment effects). Intra-annual variations were thought to have two potential sources, between-year differences in the onset of the settlement periods of particular species and between-year differences in the overall levels of settlement for some taxa. The onset of settlement is well known and predictable for most of the species enumerated here from previous studies at Clachan Seil (e.g. Todd & Turner, 1986; Turner, 1988), and the times at which the experiments were carried out had been chosen accordingly. The main source of the expected between-year variations should therefore be in the average numbers of settlers per year. Strong seasonal variations were expected and it was of particular interest to assess any

overall seasonal patterns of the biofilming effects, and hence Season*Treatment interactions were of especial importance. The frames were placed in the same locations (marked by the concrete anchors) for all of the eight experiments, and therefore the factor Block did not require to be nested within the factors Experiment or Season. Block effects were assumed to be mostly due to positional variations in settlement between frames, thus to be of a fixed rather than a random nature, and had to be accounted for in the analysis. Block interaction terms with other factors were omitted from the model, because their contribution to the variance was considered to be largely random and of little interpretational value.

The experimental design was non-orthogonal because Block degrees of freedom varied between experiments, hence the sequence in which the factors were fitted had to be considered. A hierarchical order of fitting was chosen (according to "source" of variation and scale) for the model, adding Block (with either two or three levels) first, followed by Experiment (eight levels), Season (four levels), Treatment (two levels) and the interaction terms of the latter three factors. It should, however, be noted that fitting the terms in different orders did not markedly change the analytical results and mostly led to similar conclusions. The significance criterion for all tests was $\alpha_{[2]} \leq 0.05$.

4.3 RESULTS

Contrast C *versus* [Ea + Db] and biofilming effects

The summary of results of the Analysis of Deviance contrast C *versus* [Ea + Db] are shown in Table 4.1. An example of a full Analysis of Deviance Table for the pooled counts of all species and taxa (= Total Settlement) is given in Table 4.2 (the complete set of Analysis of Deviance tables for all species and taxonomic groups enumerated is included at the end of the chapter). There were highly significant main effects of the factor Season for all of the species and taxonomic groups enumerated, and 12 out of the

Table 4.1. Contrast C *versus* [E_a + D_b]. Summary of the Analysis of Deviance (full tables for each category of settlers are presented at the end of the chapter). + = significant effect at $\alpha_{[2]} = 0.05$.

	C *vs.* [E_a + D_b]							
	Block	Experiment	Season	Treatment	Season*Experiment	Experiment*Treatment	Season*Treatment	Season*Experiment*Treatment
Total Settlement	+	+	+	+	+		+	+
Spirorbins	+	+	+	+	+		+	
Serpulins/ *Pomatoceros* spp.			+	+				
Total Ascidians		+	+	+				
Botryllus schlosseri/ *Botrylloides leachi*	+	+	+			+		
Didemnid Ascidians			+					
Total Bryozoans	+	+	+	+	+		+	+
Erect Bryozoans			+	+				
Scrupocellaria scruposa	+	+	+	+	+			
Crisia sp.			+	+				
Alcyonidium spp.	+	+	+	+		+	+	
Mound Bryozoans (= *Tubulipora* sp./ *Plagioecia* sp.)	+		+	+	+		+	+
Sheet Bryozoans		+	+	+			+	
Celleporella hyalina	+	+	+	+	+			
Electra pilosa		+	+		+		+	
Escharoides coccinea		+	+		+			
Slime Sponges		+	+				+	+

Table 4.2. Contrast C *versus* [E_a + D_b]. Sample Analysis of Deviance table for all categories of settlers pooled (Total Settlement). Significance criterion is $\alpha_{[2]}$ = 0.05.

	d.f.	deviance	mean deviance	deviance ratio	*P*
Block (Frame)	2	54.427	27.213	8.19	0.002
Experiment	1	26.404	26.404	7.95	0.009
Season	3	642.070	214.023	64.43	< 0.001
Treatment	1	366.826	366.826	110.43	< 0.001
Season*Experiment	3	97.886	32.629	9.82	< 0.001
Experiment*Treatment	1	5.124	5.124	1.54	0.226
Season*Treatment	3	80.551	26.850	8.08	< 0.001
Season*Experiment*Treatment	3	35.922	11.874	3.60	0.028
Residual	24	79.721	3.322		
Total	41	1388.932	33.876		

17 groups displayed marked Experiment main effects, which emphasize the overall changes in the abundances of the individual groups and taxa over the course of the year and between years respectively. The analysis further indicated significant Block effects for eight of the groups. Marked differences in the numbers of settlers between biofilmed and unfilmed panels are reflected by significant Treatment effects for the majority of the species and taxa enumerated, exceptions being the composite grouping of the colonial ascidian species *Botryllus schlosseri* (Pallas) and *Botrylloides leachi* (Savigny), Didemnid Ascidians, the sheet forming bryozoans *Electra pilosa* (L.) and *Escharoides coccinea* (Abildgaard), and Slime Sponges. There were significant Season*Experiment interaction effects for all species and taxonomic groups pooled (= Total Settlement), Spirorbins, Total Bryozoans (Mound Bryozoans, Sheet Bryozoans and Erect Bryozoans pooled), the erect bryozoan species *Scrupocellaria scruposa* (L.), Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.), and the sheet bryozoan species *Celleporella hyalina* (L.), *Electra pilosa* and *Escharoides coccinea*, suggesting between-year differences in the onset and overall abundance of these groups. The *Botryllus schlosseri/Botrylloides leachi* grouping and *Alcyonidium* spp. showed marked interactions for the factors Experiment and Treatment. Most important, there were highly significant Season*Treatment interactions for Total Settlement, spirorbin polychaetes, Total Bryozoans, the erect bryozoan species *Alcyonidium* spp., Mound Bryozoans (= Tubulipora sp./*Plagioecia* sp.), Sheet Bryozoans, *Electra pilosa* and Slime Sponges. An interaction of the three terms Season, Experiment and Treatment was indicated for Total Settlement, Total Bryozoans, Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.) and Slime Sponges. The totals (counts for three replicate panels per treatment) as predicted by the analytical model for treatment C and the composite treatment [Ea + Db] are displayed for all species and taxonomic groups enumerated in the Figures 4.2-4.4. Predicted numbers of total settlers across all three replicate panels per frame on treatment C ranged between 71.6 in winter 1994 (approximately $0.24 \cdot cm^{-2}$) and 227.5 in autumn 1992 (approximately $0.76 \cdot cm^{-2}$), and for the composite treatment [Ea + Db] between 188.0 in summer 1994 (approximately $0.63 \cdot cm^{-2}$) and 30.8 in winter 1994 (approximately $0.10 \cdot cm^{-2}$).

FIGURE 4.2

Contrast C *versus* [E_a + D_b]. Seasonal larval settlement of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/*Pomatoceros* spp., *Botryllus schlosseri/Botrylloides leachi*, Didemnid Ascidians, Total Bryozoans and Erect Bryozoans. Plotted values are the total numbers of settlers on three replicate panels per frame and standard errors as predicted by the analytical model. Note the differences in the abundance scales applied. The connecting lines between points are for visual aid only and no predictions between points are implied. —△— treatment C, —●— treatment [E_a + D_b].

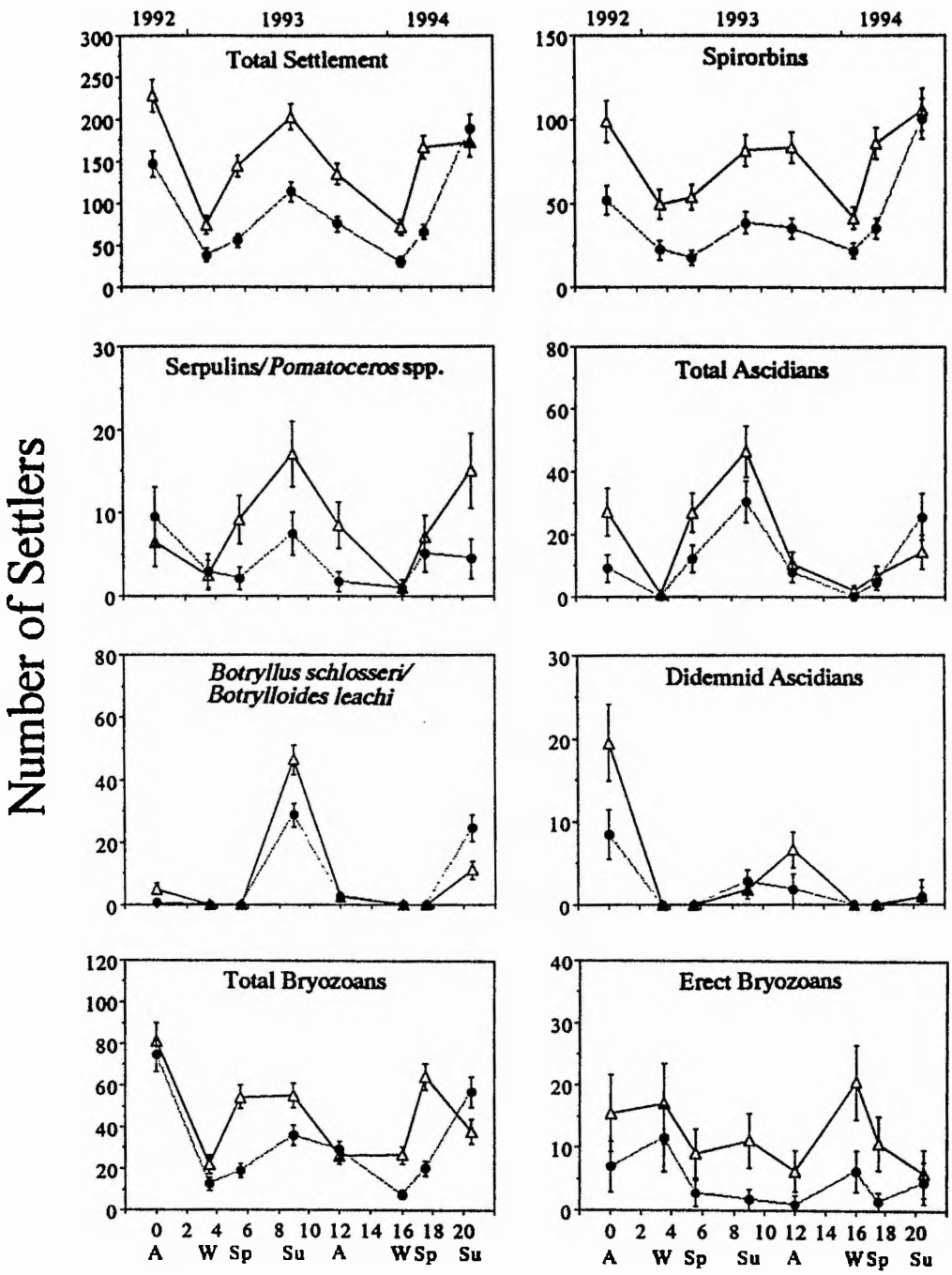

1992
1993
1994
Total Settlement
Spirorbins
Serpulins/*Pomatoceros* spp.
Total Ascidians
Botryllus schlosseri/
Botrylloides leachi
Didemnid Ascidians
Total Bryozoans
Erect Bryozoans
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.3

Contrast C *versus* [E_a + D_b]. Seasonal larval settlement of *Scrupocellaria scruposa*, *Crisia* sp., *Alcyonidium* spp., *Tubulipora* sp./*Plagioecia* sp. (= Mound Bryozoans), Sheet Bryozoans, *Celleporella hyalina*, *Electra pilosa* and *Escharoides coccinea*. Plotted values are the total numbers of settlers on three replicate panels per frame and standard errors as predicted by the analytical model. Details as for Fig. 4.2.

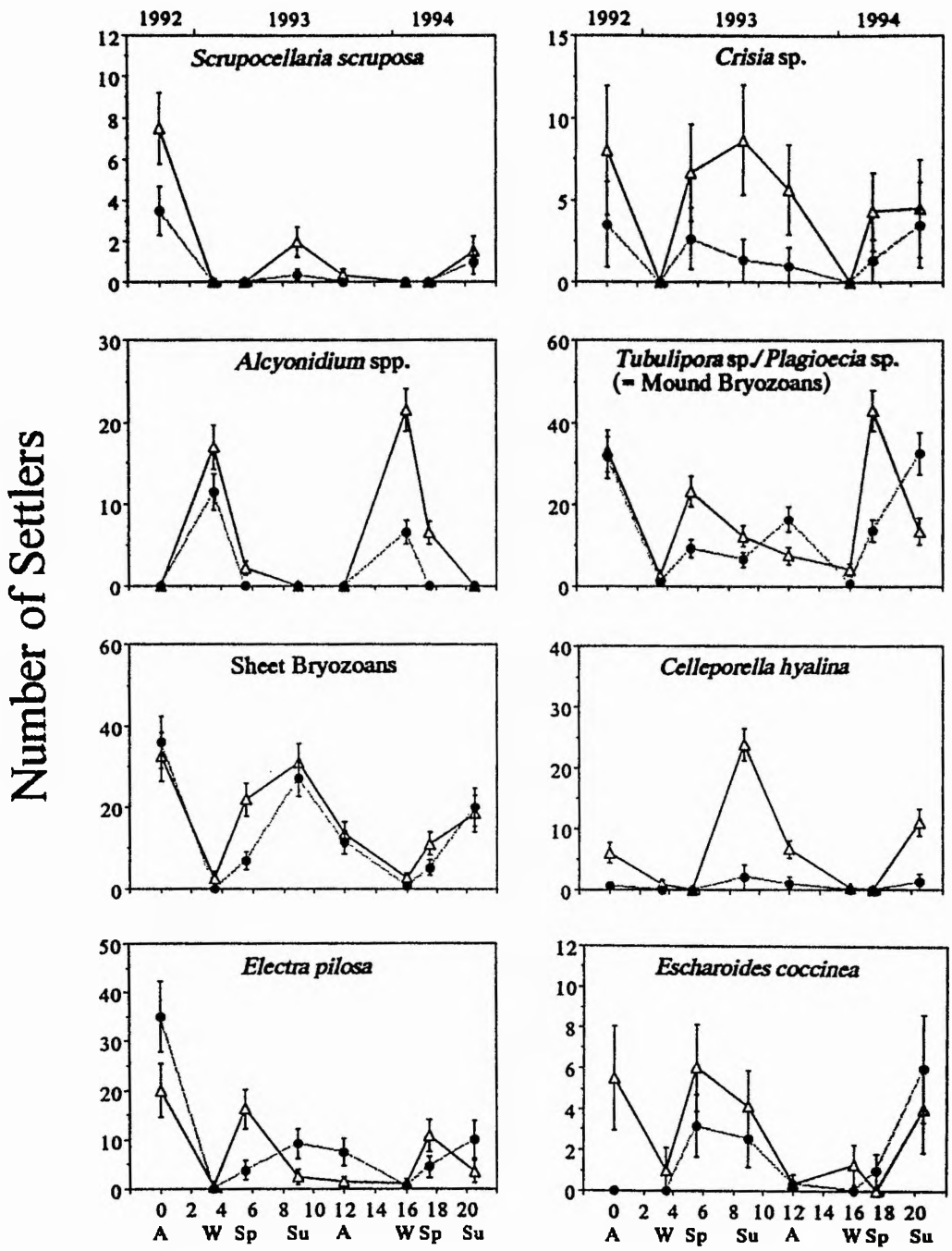
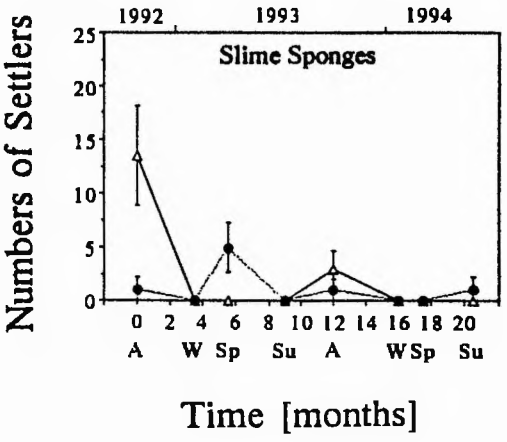

1992
1993
1994
Scrupocellaria scruposa
Crisia sp.
Alcyonidium spp.
Tubulipora sp./Plagioecia sp.
(= Mound Bryozoans)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
Escharoides coccinea
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.4

Contrast C *versus* [E_a + D_b]. Seasonal larval settlement of Slime Sponges.

Details as for Fig. 4.2.


1992
1993
1994
Slime Sponges
Numbers of Settlers
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

The graphs (Figs. 4.2-4.4) show that the majority of the species and taxonomic groups enumerated settled in higher numbers onto biofilmed surfaces (treatment C) rather than unfilmed surfaces (treatment combination [Ea + Db]), thus indicating an overall facilitatory rather than inhibitory effect of biofilming on larval settlement (see also Chapter 3). For most of the species and groupings for which the analysis indicated Treatment*Season interaction effects, these were largely due to between-season variations in the "intensity" of the biofilming effect (e.g. differences in the numbers of the sheet forming bryozoan species *Celleporella hyalina* onto filmed and unfilmed surfaces were highest in summer, and virtually absent in winter and spring, see Fig. 4.3) rather than its "direction". However, the effects of biofilming on larval settlement appear to revert from inhibitory (in summer and autumn) to facilitatory (in spring) for *Electra pilosa* (an unequivocally identifiable sheet forming bryozoan species) (Fig. 4.3). In the case of the mound bryozoan *Tubulipora* sp./*Plagioecia* sp. grouping (Fig. 4.3) settlement appears to be facilitated by the presence of biofilming in the spring experiments, but inhibited on filmed panels (treatment C) in one of the two repeat experiments each in autumn (1993) and summer (1994). There are other groups for which the numerical relation of settlement onto biofilmed and unfilmed panels reverses between seasons and experiments (e.g. Serpulins/*Pomatoceros* sp., Total Ascidians, *Botryllus schlosseri*/*Botrylloides leachi*, Total Bryozoans, *Escharoides coccinea* and Slime Sponges) but here, these reversals are less clear in their magnitude and less systematic, and more likely due to random outliers and chance (especially in the cases of the groupings with lower numerical abundances) (Figs. 4.2-4.4).

Contrast A *versus* [C - Ea + Da] and incumbent effects

The results of the Analysis of Deviance for the contrast A *versus* [C - E_a + D_a] are summarized in Table 4.3. An example of a full Analysis of Deviance Table for the Total Settlement is given in Table 4.4 (the complete set of Analysis of Deviance tables for all species and taxonomic groups enumerated is included at the end of the chapter).

Table 4.3. Contrast A *versus* [C - E_a + D_a]. Summary of the Analysis of Deviance (full tables for each species and taxonomic group are presented in the Appendix 2). + = significant effect at $\alpha_{[2]} = 0.05$.

	A *vs.* [C - E_a + D_a]							
	Block	Experiment	Season	Treatment	Season* Experiment	Experiment* Treatment	Season* Treatment	Season*Experiment* Treatment
Total Settlement	+		+	+	+			
Spirorbins	+		+	+	+			
Serpulins/ *Pomatoceros* spp.			+					
Total Ascidians		+	+	+				
Botryllus schlosseri/ *Botrylloides leachi*	+	+	+	+				
Didemnid Ascidians			+					
Total Bryozoans	+		+	+	+	+		
Erect Bryozoans	+				+			
Scrupocellaria scruposa	+	+	+	+	+	+		
Crisia sp.			+					
Alcyonidium spp.	+	+	+	+	+			
Mound Bryozoans (= *Tubulipora* sp./ *Plagioecia* sp.)			+	+	+			
Sheet Bryozoans			+	+		+		
Celleporella hyalina			+			+		
Electra pilosa		+	+	+				
Escharoides coccinea								
Slime Sponges	+	+	+					+

Table 4.4. Contrast A *versus* [C - E_a + D_a]. Sample Analysis of Deviance table for all categories of settlers pooled (Total Settlement). Significance criterion is $\alpha_{[2]}$ = 0.05.

	d.f.	deviance	mean deviance	deviance ratio	*P*
Block (Frame)	2	127.159	63.579	8.62	0.002
Experiment	1	10.626	10.626	1.44	0.242
Season	3	1132.622	377.541	51.16	< 0.001
Treatment	1	114.618	114.618	15.53	< 0.001
Season*Experiment	3	165.284	55.095	7.47	0.001
Experiment*Treatment	1	2.625	2.625	0.36	0.557
Season*Treatment	3	2.747	0.916	0.12	0.945
Season*Experiment*Treatment	3	23.603	7.868	1.07	0.382
Residual	24	177.115	7.380		
Total	41	1756.400	42.839		

As for the contrast C *versus* [E_a + D_b], here too, highly significant main effects of the factor Season were revealed for all species and groupings, with the exceptions of Erect Bryozoans and *Escharoides coccinea*. Six of the 17 species and taxonomic groups enumerated displayed marked between-year variations in settlement numbers: significant Experiment main effects were recorded for Total Ascidians, the *Botryllus schlosseri/Botrylloides leachi* grouping, the erect bryozoan species *Scrupocellaria scruposa* and *Alcyonidium* spp., the sheet forming bryozoan *Electra pilosa* and Slime Sponges. Again the latter two main effects are most likely a reflection of between-season and between-year differences respectively in the onset of settlement and overall abundances of the particular groups and species. Block main effects were recorded for 8 groups and taxa. For the majority of the groupings (10 out of 17) the numbers of settlers between treatments varied significantly, indicating marked effects of early incumbents, established during the first half of the experiments, on subsequent larval settlement in the second half of the experiments. Significant Season*Experiment interactions shown for Total Settlement, Spirorbins, Total Bryozoans, Erect Bryozoans, *Scrupocellaria scruposa*, *Alcyonidium* spp., and Mound Bryozoans (= *Tubulipora* sp./*Plagioecia* sp.) once again emphasize the between-year variations in onset and average abundance of settlers. Marked Experiment*Treatment interactions were indicated for Total bryozoans, *Scrupocellaria scruposa*, Sheet Bryozoans and *Celleporella hyalina*. There were no significant Season*Treatment interaction effects, and only for Slime Sponges did the analysis show significant interactions between the factors Season, Experiment and Treatment.

Figures 4.5-4.7 show the totals (counts for three replicate panels per treatment) for all species and taxonomic groups as predicted by the analytical model for treatment A and the composite treatment [C - E_a + D_a]. The predicted densities of total larval settlers on the three replicate un-netted panels per frame exposed throughout the experiments (treatment A) ranged between 63.4 in winter 1994 (approximately 0.21·cm^{-2}) and 322.5 in summer 1994 (approximately 1.08·cm^{-2}). For the composite treatment [C - E_a + D_a] total predicted settlement for three replicate panels per frame ranged between 101.1 in winter 1994 (approximately 0.34·cm^{-2}) and 347.5 in summer 1994 (approximately

FIGURE 4.5

Contrast A *versus* [C - E_a + D_a]. Seasonal larval settlement of all categories of settlers pooled (Total Settlement), Spirorbins, Serpulins/*Pomatoceros* spp., *Botryllus schlosseri*/*Botrylloides leachi*, Didemnid Ascidians, Total Bryozoans and Erect Bryozoans. Plotted values are the total numbers of settlers on three replicate panels per frame and standard errors as predicted by the analytical model. Note the differences in the abundance scales applied. The connecting lines between points are for visual aid only and no predictions between points are implied. —□— treatment A, —▲— treatment [C - E_a + D_a].

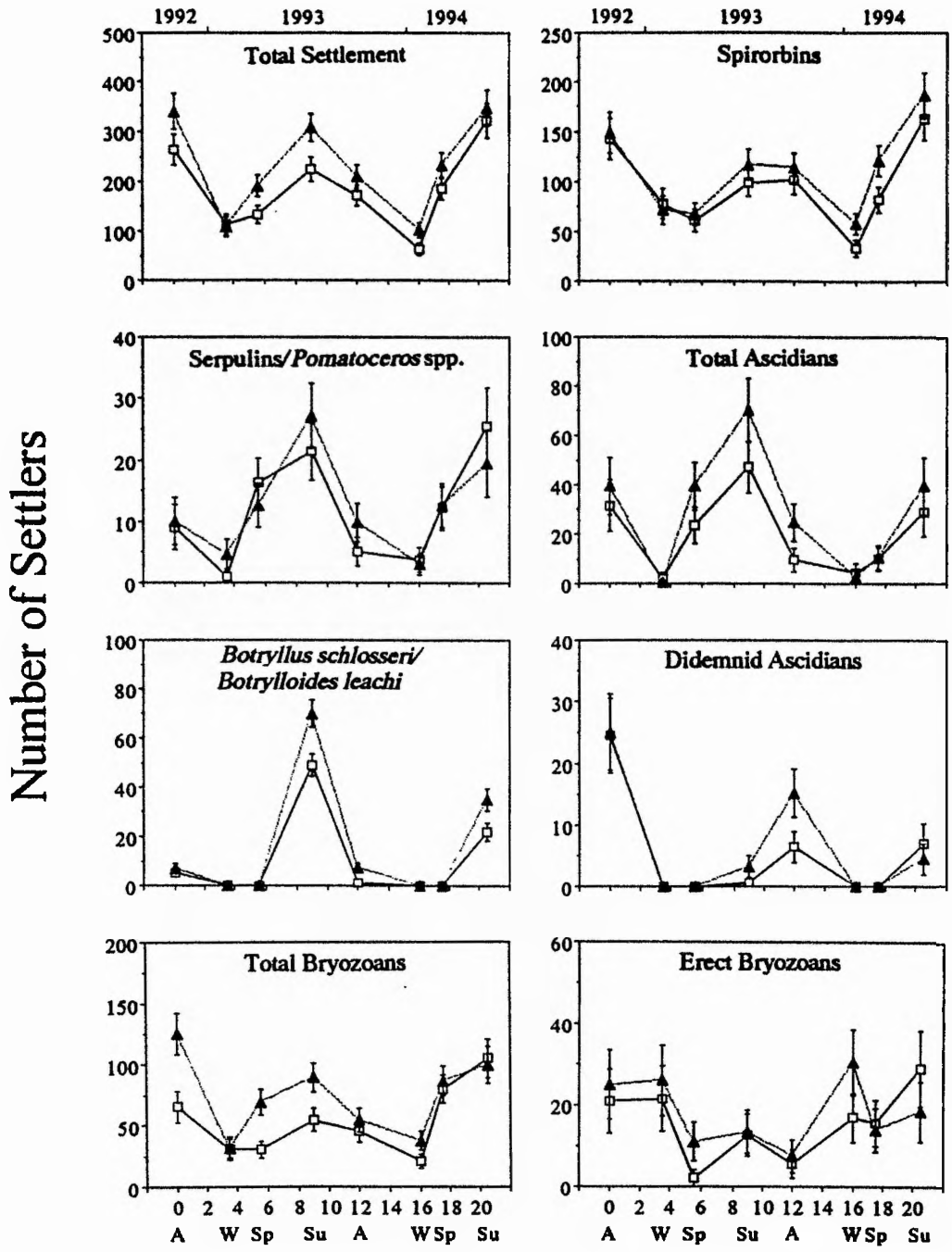

1992
1993
1994
Total Settlement
Spirorbins
Serpulins/*Pomatoceros* spp.
Total Ascidians
Botryllus schlosseri/
Botrylloides leachi
Didemnid Ascidians
Total Bryozoans
Erect Bryozoans
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.6

Contrast A *versus* [C - E_a + D_a]. Seasonal larval settlement of *Scrupocellaria scruposa*, *Crisia* sp., *Alcyonidium* spp., *Tubulipora* sp./*Plagioecia* sp. (= Mound Bryozoans), Sheet Bryozoans, *Celleporella hyalina*, *Electra pilosa* and *Escharoides coccinea*. Plotted values are the total numbers of settlers on three replicate panels per frame and standard errors as predicted by the analytical model. Details as for Fig. 4.5.

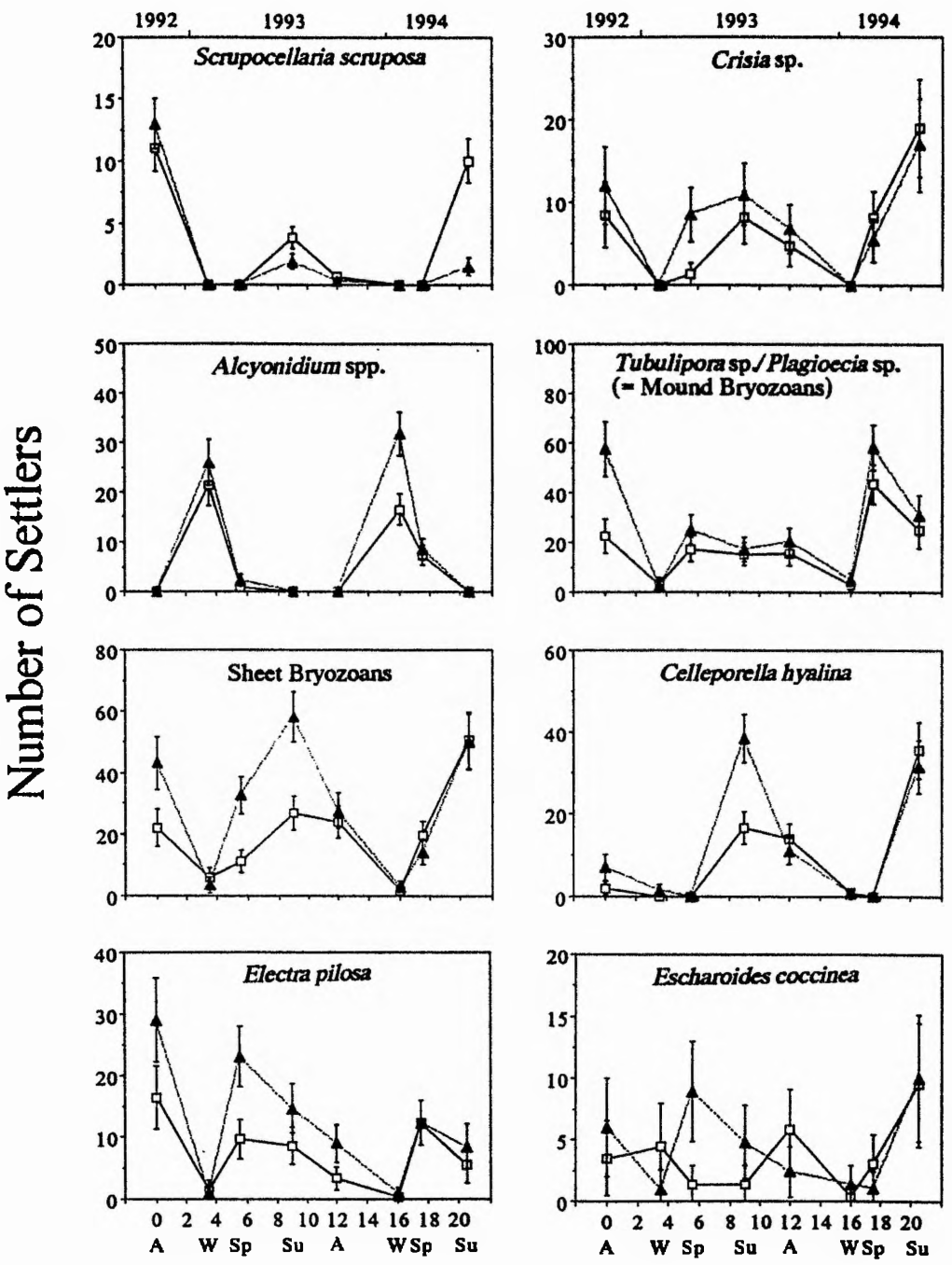
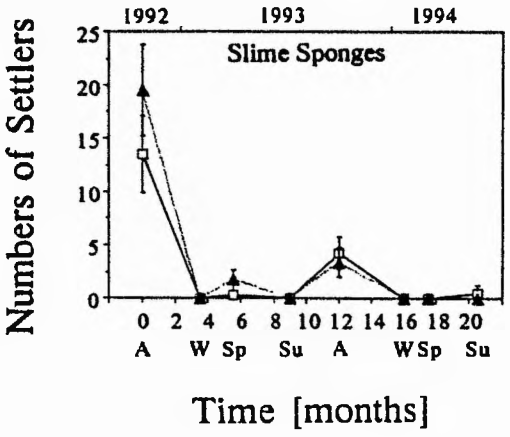

1992
1993
1994
Scrupocellaria scruposa
Crisia sp.
Alcyonidium spp.
Tubulipora sp./Plagioecia sp.
(= Mound Bryozoans)
Sheet Bryozoans
Celleporella hyalina
Electra pilosa
Escharoides coccinea
Number of Settlers
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

FIGURE 4.7

Contrast A *versus* [C - E_a + D_a]. Seasonal larval settlement of Slime Sponges. Details as for Fig. 4.5.


1992
1993
1994
Slime Sponges
Numbers of Settlers
25
20
15
10
5
0
0 2 4 6 8 10 12 14 16 18 20
A W Sp Su A W Sp Su
Time [months]

1.16·cm^{-2}). Figures 4.5-4.7 illustrate that the presence of incumbents overall had an inhibitory rather facilitatory effect on the settlement of the majority of species and groups enumerated (predicted numbers of settlers are consistently higher on the composite panel treatment [C - E_a + D_a] than on treatment A). The only group which displayed significant facilitation of settlement in the presence of incumbents from the first half of the experiment in the present analysis was *Scrupocellaria scruposa*, and the only species for which the highest numbers of settlers varied between treatments more or less from experiment to experiment was *Escharoides coccinea*. However, these results should be interpreted with caution, as the total numbers of settlers predicted for three replicate panels were mostly less than 10 in both cases.

4.4 DISCUSSION

The use of netted panel substrata in the present study allowed the separation of the effect of biofilms on larval settlement from any potential confounding effects of early incumbent post-larvae. Such species-specific effects of incumbents were observed in the comparison A *versus* [C - E_a + D_a] of the present chapter, and in Chapter 3 where they were found to vary between experiments. These variations could reflect the seasonality of certain macroinvertebrate species – either in their reproductive cycles, and thus larval supply – or perhaps in their production of allelochemical cues (Dyrynda, 1985; Bakus *et al.*, 1986; Davis *et al.*, 1991; Martin & Uriz, 1993; Maida *et al.*, 1995). The avoidance of future dominant competitors for primary space or food at the stage of larval settlement and the use of co-occurring species as indicators for favourable settlement sites are likely to be of high adaptive importance, and there are a number of examples of intra-specific inhibition (Goodbody, 1961; Grosberg, 1981; Young & Chia, 1981; Young, 1989; Martin & Uriz, 1993; Todd & Keough, 1994) and facilitation (Standing *et al.*, 1984; Raimondi, 1988; LeTourneux & Bourget, 1988; Todd & Keough, 1994) among sessile marine invertebrate species. The inhibitory effects on newly arriving recruits related to production of allelochemicals, feeding activity or pre-

emption of space by established filter-feeding hard substratum organisms have been documented in the past (Goodbody, 1961; Connell & Slayter, 1977; Grosberg, 1981; Sousa, 1984a; Todd & Turner, 1986; Wahl, 1989; Martin & Uriz, 1993; Todd & Keough, 1994; Osman & Whitlatch, 1995a, 1995b). It should be noted that the present experiments did not reveal any interaction effects between the factors Season and Treatment in the contrast A *versus* [C - E_a + D_a] (and only few significant Experiment*Treatment interactions), and although the presence of early incumbent post-larvae had an inhibitory effect on the majority of the present taxa this inhibition does not appear to be bound to the seasonally timed occurence of a particular competitor species. The consistently higher settlement on the composite treatment [C - E_a + D_a] with respect to treatment A is somewhat surprising as the inhibitory incumbent effects are superimposed on the generally facilitatory biofilming effect and certain settlement enhancing effects related to gregarious behaviour in particular groups of settlers, such as Spirorbins.

The significant Block effects, found to some extent for all experiments and seasons, were most likely due to more or less systematic small-scale differences in larval supply, gregariousness of certain species (e.g. Jackson, 1985; Pawlik *et al.*, 1991; Toonen & Pawlik, 1994) and/or small-scale habitat heterogeneities (see also Roberts *et al.*, 1991; Todd & Keough, 1994). Block effects were expected because they are more or less inevitable in this type of field experimental design.

The between-experiment differences (Experiment*Treatment interactions) and seasonal variations (Season*Treatment interactions) of the species-specific effects of biofilming on many of the year-round settling invertebrate groups and taxa observed in the present study could partly be ascribed to changes in the numbers of available settlers at particular times of the year or between years (e.g. timing of the onset of the settlement season: see also Sutherland & Karlson, 1977; Harms & Anger, 1983; Todd &1 Turner, 1986; Turner, 1988), especially in the case of composite groupings containing more than one species (e.g. Spirorbins, Serpulins/*Pomatoceros* sp.). However, the statistical analysis accounted for such changes in sample size and variance for the individual species enumerated, and differential larval availability and patchy settlement alone are

unlikely to have caused the changes from inhibition/facilitation to facilitation/inhibition shown for the response of some taxonomic groups and species (*Electra pilosa*, *Tubulipora* sp./*Plagioecia* sp.). Neither can it be ruled out that the temporal variations in the settlement patterns observed were caused by differential predation/mortality of newly metamorphosed larvae, but this seems unlikely given the relatively short immersion periods (14d), which have allowed the assessment of settlement rather than recruitment (Keough & Downes, 1986; Todd & Keough, 1994).

General non-uniformity of the effect of a settlement cue can result from either of two causes: (1) intrinsic variability in larval behaviour, or (2) differential responses to variable, uncontrolled external stimuli (Raimondi & Keough, 1990). Thus, one possible explanation of the observed temporal variations in settlement responses to biofilming could be seasonal variations in the "sensitivity" of the larvae to biofilm cues (intrinsic variability). There is substantial evidence in the literature of the occurrence of behavioural variations between different larval populations, "batches" or generations (see Raimondi & Keough, 1990, for review of variation in larval behaviour). Toonen & Pawlik (1994) proposed the existence of two behaviourally distinct subpopulations of larvae of the serpulid *Hydroides dianthus*: one group colonized uninhabited substrata ("founders") and responded to biofilm cues, and the other group ("aggregators") only settled in response to cues associated with conspecifics. Seasonally distinct behavioural responses at settlement would appear to be adaptive where these are related to variations in selective pressure, such as the seasonality of dominant space competitors or predator species, but evidence for such intrinsic seasonal changes in behavioural patterns of marine invertebrate larvae in the literature is extremely rare (Keough, 1986; Raimondi & Keough, 1990). There is, however, strong evidence that the presence of recruits of competing species can exert inhibitory effects on other species (above, see also Todd & Keough 1994; Keough & Raimondi, 1995). It is possible that biofilming as a settlement cue simply decreases in importance for the larvae of certain taxa which encounter seasonal increases in the effects of other competitor species and/or their production of allelochemical metabolites.

Nevertheless, the results of Chapter 3 support the idea that the observed between-season differences in the effects of biofilm cues are most likely a reflection of seasonal changes of these cues themselves (external variability), which may result in alterations of larval settlement responses. It was shown in Chapter 3 that there are marked quantitative and qualitative differences in biofilm composition between two local sites of contrasting flow regime within Clachan Seil, and that larvae respond differentially to these during settlement. Composition, metabolic activity and quantitative characteristics of biofilm communities are known to change as a consequence of seasonal succession in the field (Hudon & Bourget, 1981; Munteanu & Maly, 1981; Underwood, 1984; MacLulich, 1987; Anderson, 1995), and it appears very likely that larvae are able to detect and respond to these changes.

The present study showed marked seasonal variations in the effects of biofilming cues on the larval settlement of certain marine invertebrate groups and taxa under natural conditions, which cannot be explained by changes in larval supply alone. Moreover, a reversal of the effect of filming on larval settlement response - from inhibitory/facilitatory to facilitatory/inhibitory - with season was noted in the case of certain species. The study also showed that superimposed on the biofilming effects on larval settlement is the overall inhibitory impact of the presence of incumbent settlers. These observations are of particular interest with regard to the interpretation of events following disturbances that create open spaces within natural fouling communities, in which space commonly is a limiting resource (Sousa, 1979; Sutherland, 1984; Butler & Chesson, 1990). Such disturbances can be highly seasonal physical or biological events, and their timing can be of major importance to patterns of recolonization and succession (Sousa, 1984). The results of the present study imply that seasonal patterns of recolonization of newly disturbed areas can be only partly explained by changes in species availability and temporal variations in larval supply (Osman 1977; Smedes, 1984; Turner & Todd, 1993), and that seasonal variations in the effects of substratum associated biofilm cues (and/or in the effect of other incumbent recruits) on year-round settling species may also be important. These results emphasize the need for long term assessments of the effects of biofilming cues, under field conditions, if any conclusions

about species-specific larval responses and their ecological significance in relation to fouling community structure are to be drawn.

```
"Program to analyse settlement data of Chapter 4
for the contrast C vs Ea+Db
"
"
lines to change are marked
## here!
"
"
change file names according to species/taxonomic group to be analysed
"
"file for output of results"
open 'newsp1.lis'; 2; out  "## here!"
"file for Genstat postscript graphics"
open 'newsp1.ps'; 4; filetype=graphics "## here!"
device 4
open 'bigall.dat'; 2; in
input 2  "open file with data and factor definitions"
input 2  "run extra section to reduce data"
"
Combine within-frame totals for treatments Ea and Db"
                                  "A,B,C,D,Da,Db,E,Ea,Eb"
calc cvseadb=newlevels(Treat;!(3,3,1,3,3, 2, 3,2, 3))
factor [lev=3; lab=!t(C,'[Ea+Db]',rest)] CvsEaDb; val=cvseadb
"
form totals over reps with frames for each treatment"
table [class=Time,CvsEaDb,Frame] tab[1...23]
restrict c[]; CvsEaDb<>3
tabulate c[]; tot=tab[]
"2 treats, 3 expts with 2 frames, 5 expts with 3 frames"
calc n1=(2*2*2)+(2*3*2)
calc n2=(2*3*3)+(2*2*1)
calc nn=n1+n2
unit [nn]
vari [nn] c[1...23]; extra=sp[]
vari old; !(((2,-1)2,-3)2,((3)2,-3)5,((2,-1)2))
for _tab=tab[]; _c=c[]
   equa [old=old] _tab; _c
endfor
"print[2] tab[5]; dec=0"
delete [y] tab[],treats,Experiment,Season,Treat,CvsEaDb,Frame,Time,time
text treats; !t(C,'[Ea+Db]')
Factor [lev=2;lab=treats] Treat;valu=!(2(1,2)2,3(1,2)5,2(1,2));dec=0
Factor [lev=3] Frame; valu=!(((1,2)2)2,((1,2,3)2)5,(1,2)2); dec=0
Factor [lev=2] Experiment; valu=!(#n1(1),#n2(2)); dec=0
Factor [lev=4; lab=!t(Autumn,Winter,Spring,Summer)] Season;\
  valu=!(4(1,2),6(3,4,1,2,3),4(4))
Factor [lev=8] Time; valu=!(4(1,2),6(3,4,5,6,7),4(8)); dec=0
"return"
"print[2] Time,Experiment,Season,Treat,Frame,c[1];dec=0" "## here!"
"
setup Generalized Linear Model
change c[n] to species to be analysed"
model [dist=poisson;link=log;disp=*] c[1]  "## here!"
terms Frame*Season*Experiment*Treat
fit [pr=*] Frame
add [pr=*;nomess=alias] Experiment
&   Season
```

```
&   Treat
&   Season*Experiment
&   Experiment.Treat
&   Season.Treat
add [pr=a; fprob=y;nomess=alias] Season.Experiment.Treat
print [2; ipr=*] sp[1]  "## here!"
rdisp [pr=a;fpr=y;ch=2]
"
form tables of predictions from fitted model"
predict [alias=ig;com=pres;ch=2] Experiment
predict [pr=p,s;alias=ig;com=pres;ch=2] Season
predict [pr=p,s;alias=ig;com=pres;ch=2] Season,Experiment
predict [pr=p,s;alias=ig;com=pres;ch=2] Treat
page [ch=2]
predict [pr=p,s;alias=ig;com=pres;ch=2] Treat,Experiment
predict [pr=p,s;alias=ig;com=pres;ch=2] Season,Treat
predict [pr=p,s;alias=ig;com=pres;ch=2] Season,Treat,Experiment
terms Frame*Season*Experiment*Treat
fit [pr=*] Frame
add [pr=*;nomess=alias] Season
&   Experiment
&   Treat
&   Season.Experiment
&   Season.Treat
&   Experiment.Treat
& [pr=a; fprob=y;nomess=alias] Season.Experiment.Treat
page [2]
print [2; ipr=*] '***  Note different order of fitting!'
& [sq=y]'***  Change is due to non-orthogonality of Season and Experiment'
rdisp [pr=a;fpr=y;ch=2]
terms Frame*Season*Experiment*Treat
fit [pr=*] Frame
add [pr=*;nomess=alias] Treat
&   Season
&   Experiment
&   Treat.Season
&   Treat.Experiment
&   Season.Experiment
& [pr=a; fprob=y;nomess=alias] Season.Experiment.Treat
print [2; ipr=*] '***  Note yet another order of fitting!'
rdisp [pr=a;fpr=y;ch=2]
"
now save predictions in a form suitable for graphs"
pointer [nv=!t('C','Ea+Db')] bar,fit
pointer [nv=!t('lower','upper')] bar[]
vari [8] fit[],bar[][]
"
setup time variable to be time in months between mid-points of experiments"
vari [8] t; !(0,3.5,2,3.5,3,4,1.5,3); dec=1  "## here!"
calc t=cum(t) "cumulative time from start of experiments in months"
predict [pr=p,s;alias=ig;comb=pres;pred=preds;se=ses]\
        Experiment,Season,Treat
"print preds"
equa [old=!(1,-1)] preds; fit[1]
equa [old=!(-1,1)] preds; fit[2]
equa [old=!(1,-1)] ses;bar[1][1]
equa [old=!(1,-1)] ses;bar[1][2]
calc bar[1][]=fit[1]+(-1,1*bar[1][])
calc bar[1][1]=bar[1][1]*(bar[1][1]>0)  "make lower bounds zero not negative
equa [old=!(-1,1)] ses;bar[2][1]
```

```
equa [old=!(-1,1)] ses;bar[2][2]
calc bar[2][]=fit[2]+(-1,1*bar[2][])
calc bar[2][1]=bar[2][1]*(bar[2][1]>0)  "make lower bounds zero not negati
"
divide values by total area of panels within a frame to get counts/m^2/day
calc fit[],bar[][]=fit[],bar[][]/(3*0.01*28)  "## here!"
"print t,bar[1][1],fit[1],bar[1][2]"
"print t,bar[2][1],fit[2],bar[2][2]"
"return"

frame 1; 0; 1; 0; 1
"
y-axis units are number per sq meter per day - change yt= if divisor
is changed in program above"
axes 1; yt='count / m^2 / day'; xt='time (months)'; yor=0  "## here!"
pen 1,2; meth=line; line=2,4; symb=5,2
dgraph [t=sp[1]; key=0] fit[]; t; 1,2;\  "## here!"
  ylo=bar[]['lower']; yup=bar[]['upper']; desc='Ea Db','C'
"return"
pen 1,2; meth=line; line=2,4; symb=5,2; col=1
dgraph [t=sp[1]; key=0] fit[]; t; 1,2;\  "## here!"
  ylo=bar[]['lower']; yup=bar[]['upper']; desc='Ea Db','C'
device 1
"return"
close 2,4; out,graph

stop
```

```
"Program to analyse isettlement data of Chapter 4
 for the contrast A vs [C-Ea+Da]
"
"
lines to change are marked
## here!
"
"
change file names according to species/taxonomic group to be analysed
"
"file for output of results"
open 'acedsp1.lis'; 2; out  "## here!"
"file for Genstat postscript graphics"
open 'acedsp1.ps'; ps; graph  "## here!"
open 'bigall.dat'; 2; in
input 2  "open file with data and factor definitions"
input 2  "run extra section to reduce data"

print[2] 'Counts totalled over each frame - all (primary) treatments'"## here
print[2] Time,Experiment,Season,Treat,Frame,c[1]; dec=0; f=10  "## here!"

calc c[]=c[]*(1-(2*(Treat.in.'Ea')))  "multiply Ea by -1"
"
Combine within-frame totals for treatments A and C, -Ea, Da"
                                  "A,B,C,D,Da,Db,E,Ea,Eb"
calc a_ceada=newlevels(Treat;!(1,3,2,3,2, 3, 3,2, 3))
factor [lev=3; lab=!t('A','[C-Ea+Da]',rest)] A_CEaDa; val=a_ceada
"
form totals over reps with frames for each treatment"
table [class=Time,A_CEaDa,Frame] tab[1...23]
restrict c[]; A_CEaDa<>3
tabulate c[]; tot=tab[]
"2 treats, 3 expts with 2 frames, 5 expts with 3 frames"
calc n1=(2*2*2)+(2*3*2)
calc n2=(2*3*3)+(2*2*1)
calc nn=n1+n2
unit [nn]
vari [nn] c[1...23]; extra=sp[]
vari old; !(((2,-1)2,-3)2,((3)2,-3)5,((2,-1)2))
for _tab=tab[]; _c=c[]
   equa [old=old] _tab; _c
endfor

print[2] 'Counts totalled over each frame - A and C-Ea+Db and other' "## here
print[2] tab[1]; dec=0  "## here!"

delete [y] tab[],treats,Experiment,Season,Treat,A_CEaDa,Frame,Time,time
text treats; !t('A','[C-Ea+Da]')
Factor [lev=2;lab=treats] Treat;valu=!(2(1,2)2,3(1,2)5,2(1,2));dec=0
Factor [lev=3] Frame; valu=!(((1,2)2)2,((1,2,3)2)5,(1,2)2); dec=0
Factor [lev=2] Experiment; valu=!(#n1(1),#n2(2)); dec=0
Factor [lev=4; lab=!t(Autumn,Winter,Spring,Summer)] Season;\
  valu=!(4(1,2),6(3,4,1,2,3),4(4))
Factor [lev=8] Time; valu=!(4(1,2),6(3,4,5,6,7),4(8)); dec=0
"return"

print[2] 'totals extracted from table (above) for A and C-Ea+Db' "## here!"
print[2] Time,Experiment,Season,Treat,Frame,c[1];dec=0; f=10  "## here!"
```

```
"
setup Generalized Linear Model
change c[n] to species to be analysed"
model [dist=poisson;link=log;disp=*] c[1]  "## here!"
terms Frame*Season*Experiment*Treat
fit [pr=*] Frame
add [pr=*;nomess=alias] Experiment
&   Season
&   Treat
&   Season*Experiment
&   Experiment.Treat
&   Season.Treat
add [pr=a; fprob=y;nomess=alias] Season.Experiment.Treat
print [2; ipr=*] sp[1]  "## here!"
rdisp [pr=a;fpr=y;ch=2]
"
form tables of predictions from fitted model"
predict [alias=ig;com=pres;ch=2] Experiment
predict [pr=p,s;alias=ig;com=pres;ch=2] Season
predict [pr=p,s;alias=ig;com=pres;ch=2] Season,Experiment
predict [pr=p,s;alias=ig;com=pres;ch=2] Treat
page [ch=2]
predict [pr=p,s;alias=ig;com=pres;ch=2] Treat,Experiment
predict [pr=p,s;alias=ig;com=pres;ch=2] Season,Treat
predict [pr=p,s;alias=ig;com=pres;ch=2] Season,Treat,Experiment
terms Frame*Season*Experiment*Treat
fit [pr=*] Frame
add [pr=*;nomess=alias] Season
&   Experiment
&   Treat
&   Season.Experiment
&   Season.Treat
&   Experiment.Treat
& [pr=a; fprob=y;nomess=alias] Season.Experiment.Treat
page [2]
print [2; ipr=*] '***  Note different order of fitting!'
& [sq=y]'***  Change is due to non-orthogonality of Season and Experiment'
rdisp [pr=a;fpr=y;ch=2]
terms Frame*Season*Experiment*Treat
fit [pr=*] Frame
add [pr=*;nomess=alias] Treat
&   Season
&   Experiment
&   Treat.Season
&   Treat.Experiment
&   Season.Experiment
& [pr=a; fprob=y;nomess=alias] Season.Experiment.Treat
print [2; ipr=*] '***  Note yet another order of fitting!'
rdisp [pr=a;fpr=y;ch=2]
"
now save predictions in a form suitable for graphs"
pointer [nv=!t('C','Ea+Db')] bar,fit
pointer [nv=!t('lower','upper')] bar[]
vari [8] fit[],bar[][]
"
setup time variable to be time in months between mid-points of experiments"
vari [8] t; !(0,3.5,2,3.5,3,4,1.5,3); dec=1  "## here!"
calc t=cum(t) "cumulative time from start of experiments in months"
predict [pr=p,s;alias=ig;comb=pres;pred=preds;se=ses]\
```

```
        Experiment,Season,Treat
"print preds"
equa [old=!(1,-1)] preds; fit[1]
equa [old=!(-1,1)] preds; fit[2]
equa [old=!(1,-1)] ses;bar[1][1]
equa [old=!(1,-1)] ses;bar[1][2]
calc bar[1][]=fit[1]+(-1,1*bar[1][])
calc bar[1][1]=bar[1][1]*(bar[1][1]>0)  "make lower bounds zero not negativ
equa [old=!(-1,1)] ses;bar[2][1]
equa [old=!(-1,1)] ses;bar[2][2]
calc bar[2][]=fit[2]+(-1,1*bar[2][])
calc bar[2][1]=bar[2][1]*(bar[2][1]>0)  "make lower bounds zero not negativ
"
divide values by total area of panels within a frame to get counts/m^2/day"
calc fit[],bar[][]=fit[],bar[][]/(3*0.01*28)  "## here!"
"print t,bar[1][1],fit[1],bar[1][2]"
"print t,bar[2][1],fit[2],bar[2][2]"
"return"

frame 1; 0; 1; 0; 1
"
y-axis units are number per sq meter per day - change yt= if divisor
is changed in program above"
axes 1; yt='count / m^2 / day'; xt='time (months)'; yor=0  "## here!"
pen 1,2; meth=line; line=2,4; symb=5,2
dgraph [t=sp[1]; key=0] fit[]; t; 1,2;\  "## here!"
  ylo=bar[]['lower']; yup=bar[]['upper']; desc='Ea Db','C'
"return"
pen 1,2; meth=line; line=2,4; symb=5,2; col=1
device ps
dgrapn [t=sp[1]; key=0] fit[]; t; 1,2;\  "## here!"
  ylo=bar[]['lower']; yup=bar[]['upper']; desc='Ea Db','C'
device 1
"return"
close 2,ps; out.graph

stop
```

Total Settlement

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	54.427	27.213	8.19	0.002
+ Experiment	1	26.404	26.404	7.95	0.009
+ Season	3	642.070	214.023	64.43	<.001
+ Treatment	1	366.826	366.826	110.43	<.001
+ Season.Experiment	3	97.886	32.629	9.82	<.001
+ Experiment.Treatment	1	5.124	5.124	1.54	0.226
+ Season.Treatment	3	80.551	26.850	8.08	<.001
+ Season.Experiment.Treatment	3	35.922	11.974	3.60	0.028
Residual	24	79.721	3.322		
Total	41	1388.932	33.876		

Spirorbins

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	37.139	18.569	6.30	0.006
+ Experiment	1	19.292	19.292	6.54	0.017
+ Season	3	211.123	70.374	23.86	<.001
+ Treatment	1	240.334	240.334	81.48	<.001
+ Season.Experiment	3	58.394	19.465	6.60	0.002
+ Experiment.Treatment	1	6.609	6.609	2.24	0.147
+ Season.Treatment	3	30.893	10.298	3.49	0.031
+ Season.Experiment.Treatment	3	16.377	5.459	1.85	0.165
Residual	24	70.794	2.950		
Total	41	690.955	16.853		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	9.510	4.755	1.80	0.187
+ Experiment	1	8.677	8.677	3.28	0.082
+ Season	3	72.747	24.249	9.18	<.001
+ Treatment	1	31.215	31.215	11.81	0.002
+ Season.Experiment	3	4.270	1.423	0.54	0.660
+ Experiment.Treatment	1	1.412	1.412	0.53	0.472
+ Season.Treatment	3	5.487	1.829	0.69	0.566
+ Season.Experiment.Treatment	3	16.234	5.411	2.05	0.134
Residual	24	63.418	2.642		
Total	41	212.971	5.194		

Total Ascidians

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	15.572	7.786	1.84	0.180
+ Experiment	1	125.062	125.062	29.57	<.001
+ Season	3	329.283	109.761	25.95	<.001
+ Treatment	1	26.731	26.731	6.32	0.019
+ Season.Experiment	3	12.900	4.300	1.02	0.403
+ Experiment.Treatment	1	12.788	12.788	3.02	0.095
+ Season.Treatment	3	22.350	7.450	1.76	0.181
+ Season.Experiment.Treatment	3	1.328	0.443	0.10	0.957
Residual	24	101.510	4.230		
Total	41	647.523	15.793		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	20.653	10.327	7.43	0.003
+ Experiment	1	72.587	72.587	52.24	<.001
+ Season	3	665.788	221.929	159.71	<.001
+ Treatment	1	3.331	3.331	2.40	0.135
+ Season.Experiment	3	4.414	1.471	1.06	0.385
+ Experiment.Treatment	1	21.195	21.195	15.25	<.001
+ Season.Treatment	3	5.642	1.881	1.35	0.281
+ Season.Experiment.Treatment	3	0.816	0.272	0.20	0.898
Residual	24	33.351	1.390		
Total	41	827.777	20.190		

Didemid Ascidians

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	9.053	4.526	2.11	0.143
+ Experiment	1	7.789	7.789	3.63	0.069
+ Season	3	224.266	74.755	34.85	<.001
+ Treatment	1	7.263	7.263	3.39	0.078
+ Season.Experiment	3	4.908	1.636	0.76	0.526
+ Experiment.Treatment	1	0.075	0.075	0.03	0.853
+ Season.Treatment	3	0.867	0.289	0.13	0.938
+ Season.Experiment.Treatment	3	4.899	1.633	0.76	0.527
Residual	24	51.478	2.145		
Total	41	310.597	7.576		

Total Bryozoans

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	21.017	10.508	5.85	0.009
+ Experiment	1	31.511	31.511	17.54	<.001
+ Season	3	177.905	59.302	33.01	<.001
+ Treatment	1	71.894	71.894	40.02	<.001
+ Season.Experiment	3	88.821	29.607	16.48	<.001
+ Experiment.Treament	1	0.188	0.188	0.10	0.749
+ Season.Treatment	3	93.012	31.004	17.26	<.001
+ Season.Experiment.Treatment	3	19.506	6.502	3.62	0.028
Residual	24	43.112	1.796		
Total	41	546.965	13.341		

Erect Bryozoans

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	21.809	10.905	2.25	0.127
+ Experiment	1	2.445	2.445	0.50	0.484
+ Season	3	46.271	15.424	3.18	0.042
+ Treatment	1	80.689	80.689	16.65	<.001
+ Season.Experiment	3	13.298	4.433	0.91	0.449
+ Experiment.Treatment	1	1.999	1.999	0.41	0.527
+ Season.Treatment	3	4.992	1.664	0.34	0.794
+ Season.Experiment.Treatment	3	12.023	4.008	0.83	0.492
Residual	24	116.332	4.847		
Total	41	299.858	7.314		

Scrupocellaria scruposa

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	6.0400	3.0200	3.83	0.036
+ Experiment	1	17.3033	17.3033	21.95	<.001
+ Season	3	54.1083	18.0361	22.88	<.001
+ Treatment	1	6.6414	6.6414	8.42	0.008
+ Season.Experiment	3	11.6875	3.8958	4.94	0.008
+ Experiment.Treatment	1	0.0786	0.0786	0.10	0.755
+ Season.Treatment	3	0.2961	0.0987	0.13	0.944
+ Season.Experiment.Treatment	3	1.5115	0.5038	0.64	0.597
Residual	24	18.9208	0.7884		
Total	41	116.5874	2.8436		

Crisia sp.

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	16.116	8.058	2.10	0.145
+ Experiment	1	8.943	8.943	2.33	0.140
+ Season	3	70.747	23.582	6.13	0.003
+ Treatment	1	36.166	36.166	9.40	0.005
+ Season.Experiment	3	0.609	0.203	0.05	0.984
+ Experiment.Treatment	1	0.150	0.150	0.04	0.845
+ Season.Treatment	3	0.172	0.057	0.01	0.997
+ Season.Experiment.Treatment	3	6.556	2.185	0.57	0.641
Residual	24	92.292	3.846		
Total	41	231.752	5.652		

Alcyonidium spp.

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	32.2326	16.1163	18.64	<.001
+ Experiment	1	5.6798	5.6798	6.57	0.017
+ Season	3	303.3959	101.1320	116.98	<.001
+ Treatment	1	38.1177	38.1177	44.09	<.001
+ Season.Experiment	3	3.6176	1.2059	1.39	0.269
+ Experiment.Treatment	1	5.9893	5.9893	6.93	0.015
+ Season.Treatment	3	14.3039	4.7680	5.52	0.005
+ Season.Experiment.Treatment	3	0.0000	0.0000	0.00	1.000
Residual	24	20.7484	0.8645		
Total	41	424.0853	10.3435		

Tubulipora sp./Plagioecia sp. (= Mound Bryozoans)

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	17.265	8.632	5.32	0.012
+ Experiment	1	1.923	1.923	1.18	0.287
+ Season	3	233.000	77.667	47.86	<.001
+ Treatment	1	14.733	14.733	9.08	0.006
+ Season.Experiment	3	78.182	26.061	16.06	<.001
+ Experiment.Treatment	1	2.709	2.709	1.67	0.209
+ Season.Treatment	3	71.841	23.947	14.76	<.001
+ Season.Experiment.Treatment	3	16.537	5.512	3.40	0.034
Residual	24	38.947	1.623		
Total	41	475.138	11.589		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	0.939	0.469	0.22	0.807
+ Experiment	1	83.356	83.356	38.42	<.001
+ Season	3	273.491	91.164	42.02	<.001
+ Treatment	1	11.127	11.127	5.13	0.033
+ Season.Experiment	3	14.307	4.769	2.20	0.114
+ Experiment.Treatment	1	0.016	0.016	0.01	0.933
+ Season.Treatment	3	29.311	9.770	4.50	0.012
+ Season.Experiment.Treatment	3	3.999	1.333	0.61	0.612
Residual	24	52.073	2.170		
Total	41	468.617	11.430		

Celleporella hyalina

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	16.4341	8.2170	9.32	0.001
+ Experiment	1	40.0720	40.0720	45.45	<.001
+ Season	3	314.1197	104.7066	118.76	<.001
+ Treatment	1	19.8783	19.8783	22.55	<.001
+ Season.Experiment	3	8.1385	2.7128	3.08	0.047
+ Experiment.Treatment	1	0.4362	0.4362	0.49	0.489
+ Season.Treatment	3	2.9586	0.9862	1.12	0.361
+ Season.Experiment.Treatment	3	4.0666	1.3555	1.54	0.230
Residual	24	21.1604	0.8817		
Total	41	427.2643	10.4211		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	9.287	4.644	1.58	0.227
+ Experime	1	41.290	41.290	14.03	0.001
+ Season	3	155.104	51.701	17.56	<.001
+ Treat	1	1.725	1.725	0.59	0.451
+ Season.Experime	3	47.039	15.680	5.33	0.006
+ Experime.Treat	1	0.159	0.159	0.05	0.818
+ Season.Treat	3	70.426	23.475	7.97	<.001
+ Season.Experime.Treat	3	3.679	1.226	0.42	0.743
Residual	24	70.649	2.944		
Total	41	399.357	9.740		

Escharoides coccinea

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	5.938	2.969	1.28	0.296
+ Experiment	1	17.151	17.151	7.40	0.012
+ Season	3	27.153	9.051	3.91	0.021
+ Treatment	1	6.334	6.334	2.73	0.111
+ Season.Experiment	3	29.442	9.814	4.23	0.015
+ Experiment.Treatment	1	5.932	5.932	2.56	0.123
+ Season.Treatment	3	15.327	5.109	2.20	0.114
+ Season.Experiment.Treatment	3	4.979	1.660	0.72	0.552
Residual	24	55.619	2.317		
Total	41	167.874	4.094		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast C vs Ea+Db ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	2.235	1.117	0.39	0.682
+ Experime	1	18.542	18.542	6.45	0.017
+ Season	3	84.342	28.114	9.78	<.001
+ Treat	1	3.413	3.413	1.19	0.285
+ Season.Experime	3	14.602	4.867	1.69	0.192
+ Experime.Treat	1	0.039	0.039	0.01	0.908
+ Season.Treat	3	48.902	16.301	5.67	0.004
+ Season.Experime.Treat	3	-0.001	0.000	0.00	<.001
Residual	27	77.593	2.874		
Total	41	249.668	6.089		

Total Settlement

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	127.159	63.579	8.62	0.002
+ Experiment	1	10.626	10.626	1.44	0.242
+ Season	3	1132.622	377.541	51.16	<.001
+ Treatment	1	114.618	114.618	15.53	<.001
+ Season.Experiment	3	165.284	55.095	7.47	0.001
+ Experiment.Treatment	1	2.625	2.625	0.36	0.557
+ Season.Treatment	3	2.747	0.916	0.12	0.945
+ Season.Experiment.Treatment	3	23.603	7.868	1.07	0.382
Residual	24	177.115	7.380		
Total	41	1756.400	42.839		

Spirorbins

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	108.221	54.111	9.39	<.001
+ Experiment	1	6.548	6.548	1.14	0.297
+ Season	3	399.203	133.068	23.10	<.001
+ Treatment	1	27.724	27.724	4.81	0.038
+ Season.Experiment	3	133.546	44.515	7.73	<.001
+ Experiment.Treatment	1	7.455	7.455	1.29	0.267
+ Season.Treatment	3	4.850	1.617	0.28	0.839
+ Season.Experiment.Treatment	3	12.026	4.009	0.70	0.564
Residual	24	138.262	5.761		
Total	41	837.836	20.435		

Serpulins

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	7.943	3.972	1.33	0.282
+ Experiment	1	10.253	10.253	3.44	0.076
+ Season	3	184.195	61.398	20.61	<.001
+ Treatment	1	0.508	0.508	0.17	0.683
+ Season.Experiment	3	1.173	0.391	0.13	0.941
+ Experiment.Treatment	1	0.309	0.309	0.10	0.750
+ Season.Treatment	3	4.422	1.474	0.49	0.689
+ Season.Experiment.Treatment	3	9.490	3.163	1.06	0.384
Residual	24	71.482	2.978		
Total	41	289.775	7.068		

Total Ascidians

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	8.353	4.177	0.61	0.554
+ Experiment	1	169.481	169.481	24.56	<.001
+ Season	3	445.882	148.627	21.54	<.001
+ Treatment	1	35.956	35.956	5.21	0.032
+ Season.Experiment	3	28.194	9.398	1.36	0.278
+ Experiment.Treatment	1	0.005	0.005	0.00	0.978
+ Season.Treatment	3	8.644	2.881	0.42	0.742
+ Season.Experiment.Treatment	3	9.528	3.176	0.46	0.713
Residual	24	165.605	6.900		
Total	41	871.650	21.260		

Botryllus schlosseri/Botrylloides leachi

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	24.663	12.332	10.50	<.001
+ Experiment	1	118.014	118.014	100.51	<.001
+ Season	3	1043.319	347.773	296.19	<.001
+ Treatment	1	22.554	22.554	19.21	<.001
+ Season.Experiment	3	4.078	1.359	1.16	0.346
+ Experiment.Treatment	1	1.960	1.960	1.67	0.209
+ Season.Treatment	3	1.570	0.523	0.45	0.722
+ Season.Experiment.Treatment	3	3.642	1.214	1.03	0.395
Residual	24	28.180	1.174		
Total	41	1247.981	30.439		

Didemnid Ascidians

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	10.160	5.080	1.70	0.203
+ Experiment	1	3.760	3.760	1.26	0.273
+ Season	3	391.453	130.484	43.77	<.001
+ Treatment	1	4.800	4.800	1.61	0.217
+ Season.Experiment	3	25.408	8.469	2.84	0.059
+ Experiment.Treatment	1	1.363	1.363	0.46	0.505
+ Season.Treatment	3	0.480	0.160	0.05	0.983
+ Season.Experiment.Treatment	3	11.481	3.827	1.28	0.303
Residual	24	71.549	2.981		
Total	41	520.453	12.694		

Total Bryozoans

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	46.518	23.259	4.94	0.016
+ Experiment	1	0.804	0.804	0.17	0.683
+ Season	3	289.292	96.431	20.50	<.001
+ Treatment	1	68.570	68.570	14.58	<.001
+ Season.Experiment	3	117.684	39.228	8.34	<.001
+ Experiment.Treatment	1	30.201	30.201	6.42	0.018
+ Season.Treatment	3	4.757	1.586	0.34	0.799
+ Season.Experiment.Treatment	3	20.366	6.789	1.44	0.255
Residual	24	112.902	4.704		
Total	41	691.093	16.856		

Erect Bryozoans

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	75.098	37.549	6.46	0.006
+ Experiment	1	2.174	2.174	0.37	0.547
+ Season	3	51.602	17.201	2.96	0.053
+ Treatment	1	5.760	5.760	0.99	0.329
+ Season.Experiment	3	61.913	20.638	3.55	0.029
+ Experiment.Treatment	1	1.589	1.589	0.27	0.606
+ Season.Treatment	3	11.251	3.750	0.65	0.594
+ Season.Experiment.Treatment	3	18.083	6.028	1.04	0.394
Residual	24	139.491	5.812		
Total	41	366.961	8.950		

Scrupocellaria scruposa

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	12.6321	6.3160	9.84	<.001
+ Experiment	1	19.6569	19.6569	30.63	<.001
+ Season	3	132.0446	44.0149	68.58	<.001
+ Treatment	1	4.3827	4.3827	6.83	0.015
+ Season.Experiment	3	47.2081	15.7360	24.52	<.001
+ Experiment.Treatment	1	9.3974	9.3974	14.64	<.001
+ Season.Treatment	3	2.9535	0.9845	1.53	0.231
+ Season.Experiment.Treatment	3	0.0524	0.0175	0.03	0.994
Residual	24	15.4040	0.6418		
Total	41	243.7316	5.9447		

Crisia sp.

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	18.868	9.434	2.58	0.097
+ Experiment	1	0.465	0.465	0.13	0.725
+ Season	3	181.514	60.505	16.53	<.001
+ Treatment	1	3.316	3.316	0.91	0.351
+ Season.Experiment	3	12.913	4.304	1.18	0.340
+ Experiment.Treatment	1	7.361	7.361	2.01	0.169
+ Season.Treatment	3	1.373	0.458	0.13	0.944
+ Season.Experiment.Treatment	3	10.129	3.376	0.92	0.445
Residual	24	87.843	3.660		
Total	41	323.782	7.897		

Alcyonidium spp.

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	75.656	37.828	24.05	<.001
+ Experiment	1	16.931	16.931	10.76	0.003
+ Season	3	501.951	167.317	106.38	<.001
+ Treatment	1	11.813	11.813	7.51	0.011
+ Season.Experiment	3	15.640	5.213	3.31	0.037
+ Experiment.Treatment	1	1.080	1.080	0.69	0.415
+ Season.Treatment	3	0.629	0.210	0.13	0.939
+ Season.Experiment.Treatment	3	2.684	0.895	0.57	0.641
Residual	24	37.747	1.573		
Total	41	664.131	16.198		

Tubulipora sp./Plagioecia sp. (= Mound Bryozoans)

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	3.452	1.726	0.41	0.669
+ Experiment	1	13.643	13.643	3.23	0.085
+ Season	3	350.948	116.983	27.67	<.001
+ Treatment	1	32.535	32.535	7.70	0.011
+ Season.Experiment	3	106.620	35.540	8.41	<.001
+ Experiment.Treatment	1	2.879	2.879	0.68	0.417
+ Season.Treatment	3	6.004	2.001	0.47	0.704
+ Season.Experiment.Treatment	3	6.082	2.027	0.48	0.700
Residual	24	101.457	4.227		
Total	41	623.620	15.210		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	7.688	3.844	1.13	0.340
+ Experiment	1	11.148	11.148	3.27	0.083
+ Season	3	441.802	147.267	43.25	<.001
+ Treatment	1	37.094	37.094	10.89	0.003
+ Season.Experiment	3	11.183	3.728	1.09	0.370
+ Experiment.Treatment	1	35.317	35.317	10.37	0.004
+ Season.Treatment	3	2.141	0.714	0.21	0.889
+ Season.Experiment.Treatment	3	13.045	4.348	1.28	0.305
Residual	24	81.713	3.405		
Total	41	641.131	15.637		

Celleporella hyalina

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	9.268	4.634	1.77	0.192
+ Experiment	1	0.351	0.351	0.13	0.718
+ Season	3	649.471	216.490	82.55	<.001
+ Treatment	1	9.034	9.034	3.44	0.076
+ Season.Experiment	3	8.480	2.827	1.08	0.377
+ Experiment.Treatment	1	26.227	26.227	10.00	0.004
+ Season.Treatment	3	0.188	0.063	0.02	0.995
+ Season.Experiment.Treatment	3	2.671	0.890	0.34	0.797
Residual	24	62.943	2.623		
Total	41	768.633	18.747		

Electra pilosa

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	2.316	1.158	0.37	0.694
+ Experiment	1	48.650	48.650	15.55	<.001
+ Season	3	159.871	53.290	17.04	<.001
+ Treatment	1	28.464	28.464	9.10	0.006
+ Season.Experiment	3	17.005	5.668	1.81	0.172
+ Experiment.Treatment	1	1.826	1.826	0.58	0.452
+ Season.Treatment	3	0.788	0.263	0.08	0.968
+ Season.Experiment.Treatment	3	8.030	2.677	0.86	0.477
Residual	24	75.078	3.128		
Total	41	342.027	8.342		

Escharoides coccinea

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	12.251	6.125	1.15	0.333
+ Experiment	1	0.043	0.043	0.01	0.929
+ Season	3	25.145	8.382	1.57	0.221
+ Treatment	1	2.055	2.055	0.39	0.540
+ Season.Experiment	3	26.732	8.911	1.67	0.199
+ Experiment.Treatment	1	11.593	11.593	2.18	0.153
+ Season.Treatment	3	8.817	2.939	0.55	0.652
+ Season.Experiment.Treatment	3	16.986	5.662	1.06	0.383
Residual	24	127.741	5.323		
Total	41	231.363	5.643		

***** Regression Analysis *****

*** Accumulated analysis of deviance for the contrast A vs C-Ea+Da ***

Change	d.f.	deviance	mean deviance	deviance ratio	
+ Frame	2	28.844	14.422	5.71	0.009
+ Experiment	1	16.242	16.242	6.43	0.017
+ Season	3	268.737	89.579	35.44	<.001
+ Treatment	1	0.229	0.229	0.09	0.765
+ Season.Experiment	3	7.644	2.548	1.01	0.404
+ Experiment.Treatment	1	7.844	7.844	3.10	0.089
+ Season.Treatment	3	2.988	0.996	0.39	0.758
+ Season.Experiment.Treatment	3	-0.002	-0.001	0.00	<.001
Residual	27	68.243	2.528		
Total	41	400.772	9.775		