
Defense of Benthic Invertebrates Against Surface Colonization by Larvae: A Chemical Arms Race

P.J. Krug

Abstract. Sessile invertebrates evolved in a competitive milieu where space is a limiting resource, setting off an arms race between adults that must maintain clean surfaces and larvae that must locate and attach to a suitable substratum. I review the evidence that invertebrates chemically deter or kill the propagules of fouling animals and protists under natural conditions, and that chemosensory mechanisms may allow larvae to detect and avoid settling on chemically protected organisms. The fouling process is an ecologically complex web of interactions between basibionts, surface-colonizing microbes, and fouling larvae, all mediated by chemical signaling. Host-specific bacterial communities are maintained by many invertebrates, and may inhibit fouling by chemical deterrence of larvae, or by preventing biofilm formation by inductive strains. Larval settlement naturally occurs in a turbulent environment, yet the effects of waterborne versus surface-adsorbed chemical defenses have not been compared in flow, limiting our understanding of how larvae respond to toxic surfaces in the field. The importance of evaluating alternative hypotheses such as mechanical and physical defense is discussed, as is the need for ecologically relevant bioassays that quantify effects on larval behavior and identify compounds likely to play a defensive role in situ.

1 Introduction

1.1 Multiple Levels of Antifouling Defense

Studies of chemical defense against fouling are generally conducted from the perspective of the chemist or benthic ecologist, striving to uncover the mechanisms that protect biological surfaces from epibiosis. Larvae of fouling organisms are often treated as an enemy to be defeated, rather than a highly adapted marvel to be understood in an ecological context. The

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complexity of the evaluations larvae make before and after contacting a surface is daunting, and given their simple neural wiring, illustrates how natural selection hones the machinery governing settlement behavior. I offer this review from the perspective of a larval biologist interested in chemically mediated behavior, and will examine antifouling defense less as a desired commercial outcome but rather a natural obstacle facing larvae that must recruit to a suitable microhabitat or die. Various mechanistic options will be considered, and the ecological and chemical evidence for each weighed. In particular, four levels of defense will be examined by asking:

- (1) Do invertebrates produce antimicrobial compounds to reduce bacterial abundance on their surfaces, thus eliminating larval settlement cues associated with biofilms?
- (2) Do invertebrates attract specific bacteria to their surfaces, which then (a) chemically inhibit competing, inductive bacteria, or (b) directly deter fouling larvae?
- (3) Do invertebrates rely on lipophilic secondary metabolites to repel exploring larvae, or to kill recently settled larvae and juvenile stages?
- (4) Do invertebrates release waterborne signals that trigger behavioral rejection by larvae prior to contact?

From the marine natural products literature, nearly a hundred compounds demonstrated toxicity towards larvae or antisettlement activity in laboratory bioassays. Antilarval and antisettlement compounds have been comprehensively reviewed at 7–8 year intervals as the field has developed; the reader is referred to the scholarly works of Davis et al. (1989), Clare (1996), and Fusetani (2004). Few compounds have been tested in field assays or in moving water, however, which is needed to evaluate the ecological role of a putative antifouling compound. This review will focus on cases where a chemical defense has been tested against multiple fouling organisms, or ideally against the full guild of potential epibionts in the field.

1.2 Overview of the Fouling Process

Most marine invertebrates and algae have a microscopic, dispersing stage in their life cycle. For sessile, benthic species, commencement of the adult stage requires successful colonization of a suitable surface. This produces intense competition for available space, with prokaryotes, protists, and animal larvae rapidly colonizing inanimate and undefended biological surfaces. Fouling can have severely deleterious effects on organisms, such as inhibition of photosynthesis, blockage of filter feeding, and elevated risk of mechanical dislodgement or predation. In consequence, the

planktonic pool of free bacteria, algal spores and competent larvae imposes a strong selective pressure on benthic invertebrates.

Epibiosis and fouling are common life-history traits in marine organisms, in part because dissolved organic matter and particulate food are effectively distributed throughout the water column. This stands in contrast to terrestrial systems, where few nutrients are suspended in air. Suspension feeding is thus the dominant mode of heterotrophy among the invertebrates that comprise fouling communities. This represents a double-edged sword for sessile organisms; the same currents that supply their food also carry an inexhaustible supply of propagules searching for a surface to which they may attach. Thus, an evolutionary arms race among sessile invertebrates is established: as larvae, they must locate and colonize a surface in order to metamorphose; yet as adults they must keep their own surfaces clean and ward off settlement by larvae. Selective pressures on larvae produce mechanisms for locating, exploring, and attaching to available substrata, while selection on adults drives the evolution of antisettlement strategies.

Competition for space represents an ecological force comparable to predation, yet the field of chemical ecology has disproportionately focused on defenses against predators and herbivores rather than colonization of surfaces (Pawlik 1993; Hay 1996). In part, this stems from the different time scales at which predation and surface colonization occur. Predation is a rapid process, easy to observe and tractable to experimental manipulation; a predator either consumes a prey item, or it does not. This is not to trivialize the ecological complexity of predator-prey or herbivore-algae interactions, but to highlight differences that affect experimental design. Biofouling and epibiosis are multi-step sequences culminating in the establishment of a mature community composed of prokaryotes, fungi, protists and invertebrates. This intrinsically complex process results from the web of interactions in the initial biofilm and subsequent community of colonizers, and is not as easily studied in laboratory or field experiments as predation or competition between adults.

1.3 Biofouling as a Sequential Ecological Process

Biofouling has been described as a four-step sequential process (Wahl 1989). The first two steps, which produce a microbial biofilm, occur in a similar manner whether on a crab carapace in the sea or on a catheter in a hospital room. The next two steps are unique to aquatic habitats, which involve the attachment of unicellular and multicellular eukaryotes to an inorganic or living surface.

The initial step, adsorption of organic macromolecules, occurs almost immediately after submersion of any surface. This “conditioning” process coats surfaces with a film composed chiefly of proteins, glycoproteins and polysaccharides, to which bacteria subsequently attach. The exact nature of the conditioning film depends on surface characteristics of the substrate, and the adsorbed layer may be highly heterogeneous (Taylor et al. 1997; Compere et al. 2001). The second step comprises colonization by prokaryotes and the subsequent development of a bacterial biofilm, within an hour of surface immersion in water. Initial colonizers penetrate the viscous sublayer to contact the surface, either passively carried by eddies or via cellular motility by flagella or pili (O’Toole and Kolter 1998). Cells then make contact with the adsorbed layer of organics through non-covalent interactions with cell-surface carbohydrates or adhesive proteins, and finally through covalent bonds to the outer cell wall. Surfaces of different wettability may require alternative attachment strategies by bacteria, and may also determine the strength of biofilm adhesion (Baier 1981; Fletcher and McEldowney 1984; Paul and Jeffrey 1985).

Once attachment to the surface has occurred, bacterial cells begin producing a matrix of extracellular polymeric substances (EPS) that is critical for maintaining adhesion and subsequent biofilm development; the EPS is composed of polysaccharides, proteins, and even DNA (Sutherland 2001; Whitchurch et al. 2002; Allison 2003). The chemical nature of EPS is now recognized as a critical determinant of biofilm architecture, strength, and material properties (Hall-Stoodley and Stoodley 2002), but our understanding of the diversity and functional consequences of EPS from different biofilms is in its infancy (see also the chapter by Callow and Callow).

Recent advances in genomics, proteomics, and analytical chemistry have revealed the importance of cell–cell signaling and global regulatory networks in biofilm development. Biofilm formation is an interactive process affected by local hydrodynamics of the fluid environment, physicochemical properties of the surface, and behavioral responses of bacterial colonizers, which quickly change gene expression and phenotype upon attachment (Geesey 2001). Genomic studies of the medically relevant bacterium *Pseudomonas aeruginosa* found changes in regulation of genes associated with flagella or pili and polysaccharide biosynthesis during biofilm formation; proteomic comparisons found differential expression of proteins involved in primary metabolism in biofilm versus planktonic cells (Whitely et al. 2001; Sauer et al. 2002). Biofilm phenotypes change with time and composition of the bacterial community, and represent a growing area of investigation both ecologically and biomedically. Quorum-sensing pathways, which allow density-dependent changes in bacterial phenotype, modulate biofilm

formation as well as virulence and symbiosis (Davies et al. 1998, and chapters by Callow and Callow, and de Nys et al.).

After establishment of the primary biofilm, secondary colonization by unicellular eukaryotes occurs; these include photosynthetic taxa such as diatoms, and heterotrophic suspension feeders and predators (Cuba and Blake 1983). This stage begins days after immersion, and continues as the microbial community coating the immersed surface develops. The final step is the attachment of propagules of multicellular organisms, invertebrate larvae and algal spores. This produces a community of macro-organisms that is subsequently shaped by ecological processes such as competition, predation, and succession. Fouling is an on-going process with no true end, as even a mature fouling community will undergo changes in composition due to season, disturbance, predation, and other biological and abiotic influences. Development and final structure of the community may be strongly subject to supply-side trends such as reproductive seasonality and large-scale oceanographic processes controlling the delivery of larvae (Roughgarden et al. 1988; Thomason et al. 2000).

Fouling progresses differently in distinct habitats, and on nearby yet distinct substrates. The predominant organisms differ in temperate zones and the tropics, as is often reflected in the bioassay organisms used in antifouling research. Primary fouling threats in temperate regions are barnacles and bivalves; in tropical areas tube-building polychaetes like *Hydroides* are a major component of early fouling communities, and at the poles, diatoms are key fouling organisms (Hadfield et al. 1994; Slattery et al. 1995). Selection may drive regional specialization of antifouling defenses, depending on the local assemblage of fouling organisms competing for space. However, different ecological processes can shape the community on spatially proximate surfaces; for instance, Keough (1984) found that recruitment explained the difference between fouling around benthic invertebrates versus on nearby docks.

1.4 Ecological Importance of Antifouling Defense Mechanisms

For any long-lived benthic organism, epibiosis must either be tolerated or combated due to the drawbacks associated with a colonized surface. Epibionts increase weight and drag, can reduce buoyancy, constrict range of motion, slow growth (Wahl 1996, 1997), and increase risk of detachment by flow (Dixon et al. 1981; Witman and Suchanek 1984). They can also facilitate manipulation of prey by predators such as crabs (Enderlein et al. 2003; Manning and Lindquist 2003). Shading can

drastically diminish photosynthesis in algae (Bulthuis and Woelkerling 1983; Sand-Jensen and Revsbech 1987); epibiosis may comparably harm invertebrates that harbor photosynthetic symbionts, such as scleractinian corals and many sponges, but this has yet to be tested. Epibionts impede gas exchange and remove dissolved nutrients before they reach the host, and compete for food while interfering with feeding currents of basibionts (Wahl and Lafargue 1990).

Less considered are the potential benefits of a fouled surface. Epibionts can provide camouflage against predators hunting by visual or chemical cues (Fishlyn and Phillips 1980; Wicksten 1983). Shell fouling by sponges reduces predation risk for bivalves (Bloom 1975; Vance 1978; Forester 1979; Feifarek 1987; Laundien and Wahl 1999); these interactions may be mutualistic, with the sponge benefiting from the water currents or mobility of the overgrown bivalve. Spines in the thorny oyster *Spondylus americanus* did not deter predation, but rather increased fouling and directed the growth of sponges beyond the vulnerable shell margin (Feifarek 1987). Epibionts that are themselves chemically defended can confer protection on their host, and may be intentionally exploited for this purpose (Barkai and McQuaid 1988; Stachowicz and Hay 1999). The mutualistic nature of beneficial epibionts suggests that colonization may be preferentially induced by the basibionts; an improved understanding of such systems might be a valuable counterpoint to antifouling investigations.

2 The Role of Biofilms and their Constituent Microbes in Fouling

Biofilms have long been recognized as fundamental settlement cues for many invertebrate larvae (Crisp and Meadows 1963; Crisp 1974). Microbial films are particularly important cues for sessile species that colonize hard substrata, such as sponges (Woolacott and Hadfield 1996; Maldonado and Young 1999), cnidarians (Leitz and Wagner 1993; Negri et al. 2001), mollusks (Tamburri et al. 1992; Zhao and Qian 2002), tube-building polychaetes (Kirchman et al. 1982; Unabia and Hadfield 1999; Harder et al. 2002a), barnacles (Wieczorek et al. 1995), bryozoans (Mihm and Banta 1981; Brancato and Woolacott 1982; Keough and Raimondi 1995) and ascidians (Szewzyk et al. 1991; Wieczorek and Todd 1997). Bacterial films also provide positive and negative cues for the attachment of algal spores (Joint et al. 2000). However, interpreting the effects of films on the fouling process is complicated. The composition of films and the behavior of invertebrate larvae both change with age, meaning that small changes in experimental

design can profoundly alter results. Further, laboratory and field experiments often yield conflicting data.

2.1

Interpreting the Effects of Biofilms on Larval Settlement

Early studies variously reported microbial induction or inhibition of larval settlement for different species. It is now recognized that the nature of biofilms varies widely, and can present a range of positive and negative stimuli to settling larvae (Keough and Raimondi 1996). Larvae can potentially extract much information about a surface from the microbial film coating it, including length of submergence (Wieczorek et al. 1995), tidal height (Strathmann et al. 1981; Thompson et al. 1998; Qian et al. 2003), and local hydrodynamics (Neal and Yule 1994b). The characteristics of a filmed surface can also be modified by other organisms; for instance, cyprid “footprints” left behind by early explorers are settlement cues for subsequent cyprids (Walker and Yule 1984; Clare and Matsumura 2000). Recent occupation by conspecifics (Thompson et al. 1998) can further affect response to a filmed substrate. Sensitivity to such a broad range of cues is clearly adaptive to settling larvae, but will likely confound lab-based assays that manipulate only one or two parameters at a time. Indeed, a pronouncement that films are inductive or inhibitory for a given species is unlikely to be true in all contexts, given the complex interactions between the age of larvae, age and source of the film, nature of the substratum, and other factors that determine a film’s bioactivity.

As a case study, the effects of biofilms on barnacle settlement have a convoluted history. Early claims that films facilitated cyprid settlement (Crisp and Meadows 1963) were followed by studies showing inhibitory effects (Maki et al. 1988); however, the latter results have been questioned due to the large number of cyprids per assay dish (Wieczorek et al. 1995; Head et al. 2003) and statistical methods used in data analysis (Keough and Raimondi 1995). Recent studies have shown that many factors feed into the interpretation of biofilms by cyprids of *Balanus amphitrite*. In laboratory experiments, settlement was inhibited by young biofilms but induced by mature films, and cyprids discriminated among filmed surfaces from different tidal heights (Wieczorek et al. 1995; Thompson et al. 1998). A separate study found no effect of biofilm age, but did find an effect of cyprid age: young cyprids were inhibited by films whereas older cyprids were induced by a filmed surface (Harder et al. 2001). Thus, using either cyprids or biofilms of a fixed age will miss key interactions between these variables.

Field studies further complicate the picture, often conflicting with laboratory results. Thompson et al. (1998) found that cues from prior

occupation by conspecifics increased cyprid settlement in both lab and field studies, while other factors made a surface attractive either in the laboratory (biofilm age) or in the field (proximity to adult barnacles). These apparent contradictions were attributed to a scaling issue, with adult presence influencing settlement at spatial scales greater than 3 cm and microbiota dictating attachment sites at smaller scales (such as come into play in limited-volume laboratory assays). Studies with other barnacle species have revealed different settlement patterns. For *Elminius modestus*, attachment strength of cyprids was inversely proportional to biofilm age (4-day-old vs. 1-month-old film) (Neal and Yule 1994a). Keough and Raimondi (1995) found that *Balanus variegatus* recruitment decreased with film age, while *E. modestus* was negatively affected by films of all ages.

Complex results have also been reported for arborescent bryozoans and solitary ascidians, major contributors to mature fouling communities that are commonly used in laboratory bioassays. In field experiments, recruitment of the bryozoans *Bugula neritina* and *B. stolonifera* was unaffected by the presence of a film in one trial, but increased with film age in a second trial (Keough and Raimondi 1995). Wiczorek and Todd (1997) reported that *Bugula flabellata* larvae were inhibited by biofilms ranging from 1 to 12 days old. Mihm and Banta (1981) found that a biofilm reversed larval preference in *B. neritina* for unfilmed plastic over glass, and this effect was not related to changes in surface wettability. Tadpole larvae of the ascidian *Ciona intestinalis* were induced to settle by biofilms and the effect increased with biofilm age; this was attributed to a combination of larval preference and passive entrapment on the biofilmed surface (Wiczorek and Todd 1997). However, Keough and Raimondi (1995) found no effect of biofilm presence or age on recruitment of four ascidian species, including *C. intestinalis*. These results emphasize the need for a thorough understanding of factors that contribute to habitat selection in the field, if laboratory data are to be meaningfully interpreted.

The cues from biofilms are thought to be primarily prokaryote-derived biochemical signals. However, microbial eukaryotes such as diatoms may also be important. For instance, larvae of the tube-building polychaete *Spirorbis borealis* preferentially settled on films of the diatom *Navicula* sp. or mixed diatom films, but avoided films of the unicellular green alga *Dunaliella galbana* (Meadows and Williams 1963). Diatom films also influence larval settlement for other polychaetes (Harder et al. 2002b; Lam et al. 2003), echinoderms (Ito and Kitamura 1997), and mollusks (Daume et al. 1999).

2.2 Specific Bacterial Strains within Biofilms as Positive Cues for Settlement of Fouling Larvae

Although biofilms are inherently multi-kingdom assemblages, much evidence now exists that individual bacterial strains can produce specific stimulatory or inhibitory chemical signals. Further, settlement-inducing activity does not appear to be phylogenetically constrained, as closely related strains vary widely in their effects. For instance, the tropical fouling polychaete *Hydroides elegans* preferentially colonizes biofilms at least 3 days old (Hadfield et al. 1994). Although settlement correlated with bacterial abundance in a natural microbial assemblage, 13 strains were isolated that were strongly inductive, and a further 11 were moderately active at triggering metamorphosis; larvae were induced to settle by low molecular weight water-soluble products of the active strains (Unabia and Hadfield 1999). Subsequent work revealed that of four biofilm species, two strains that were 30% divergent at the 16 S rRNA gene were the most inductive to larvae, whereas a third bacterium that was only 3% different from an inductive strain had no activity (Huang and Hadfield 2003). In parallel investigations, over half of 38 bacterial strains were inductive to *Hydroides* larvae, and again no phylogenetic pattern to the activity was evident (Lau et al. 2002); however, bacterial cues were most effective when adsorbed onto a solid substratum (Harder et al. 2002a). A marine *Pseudomonas* sp. strain S9 induced settlement of larvae of *Ciona intestinalis*, and the settlement-promoting activity was due to chemical features of the bacterial exopolysaccharide, which in turn was dependent on the metabolic state of the bacteria (Szewzyk et al. 1991).

3 Modulation of Surface Bacteria by Invertebrates: Direct and Indirect Effects on Fouling

Given that microbial films are a prerequisite for fouling by most larvae, one antifouling strategy is for an invertebrate to lower the abundance of settlement-inducing bacteria on its surface. I will consider three chemically mediated ways such a strategy could operate, with examples of each from the literature. The first is to diminish bacterial surface abundance, thereby removing an inductive cue for larvae. Three possible means to this end are (a) to produce antibacterial metabolites, (b) to chemically repel bacteria, or (c) to inhibit biofilm formation by interfering with bacterial signaling pathways. The second strategy is to maintain a host-specific bacterial community comprising non-inductive strains. This could work by selective chemical attraction of non-inductive strains,

and/or repulsion of inductive strains by host or “host-friendly” bacteria. For the third mechanism, the host relies on surface-associated bacteria to chemically repel larvae or kill juveniles of fouling organisms.

3.1 Antibiotic Chemistry: Maintaining a Bacteria-Free Surface as a Defense Against Fouling

Compounds that inhibit development of a surface biofilm could indirectly prevent fouling by removing this essential settlement cue for larvae. The antimicrobial activity of invertebrate secondary metabolites has been appreciated for decades (Burkholder and Rutzler 1969; Faulkner 1984). Sponges produce a wealth of compounds with antibiotic activity against terrestrial and marine bacteria (Bergquist and Bedford 1978; Amade and Chevolut 1982; Thompson et al. 1985; Newbold et al. 1999). In a survey of Caribbean sponges, 48% of species were antibacterial to at least one of eight assay strains, and 23% of all extract-strain interactions were inhibitory (Newbold et al. 1999). Similar patterns of widespread, broad-spectrum antimicrobial activity have been reported for gorgonians (Jensen et al. 1996; Koh et al. 2002). Antibiotics may be produced by bacterial symbionts, suggesting a role in interspecific competition (Unson and Faulkner 1993; Unson et al. 1994; Bewley et al. 1996); if a consequence of this competition is reduced biofouling or disease of the basibiont, then the stage is set for adaptive coevolution.

Although intuitively appealing, antimicrobial chemistry is not clearly correlated with bacteria-free surfaces. Studies with ascidians and sponges suggest that low bacterial surface counts result from repellent compounds that deter bacterial attachment, rather than from antibiotics. Surface abundance of bacteria in 11 temperate ascidians was negatively correlated with chemistry that deterred attachment, but not with antimicrobial activity (Wahl et al. 1994). For instance, bacteria were over two orders of magnitude more abundant on *Polyclinum planum* than on *Cystodytes lobatus*. In an innovative assay, extracts of *C. lobatus* were highly deterrent to bacterial colonization of agar plugs, an activity that correlated with surface counts; *C. lobatus* also contained mild antimicrobial activity, but this did not explain bacterial abundance. Deterrent and antibiotic activities were attributable to different molecules. A survey of 26 Caribbean sponges produced a similar finding: 81% of sponge extracts reduced bacterial attachment to less than 40% of controls (Kelly et al. 2003). Six of 13 sponge species showed potent inhibition of bacterial attachment, but no detectable antibiotic activity (Newbold et al. 1999; Kelly et al. 2003). These studies suggest that chemically mediated repulsion of bacteria may account for unfouled surfaces more than metabolites that inhibit growth or kill bacteria.

The strongest evidence for this strategy comes from algae that interfere with the cell-cell signaling processes that control biofilm formation. The red alga *Delisea pulchra* produces furanones, small molecules that interfere with the acyl homoserine lactone (AHL) signaling pathway used by many bacteria to cue swarming and biofilm formation (Maximilien et al. 1998; Steinberg et al. 1998, 2001, see also de Nys et al. chapter). Eukaryotic interference with AHL systems is known for vascular plants and marine algae, but not among animals; however, host-specific bacteria may be able to use chemical signals to interfere with AHL systems in competing strains (Bauer and Robinson 2002; Taylor et al. 2004a). Such antagonism between bacteria is considered next.

3.2 Maintaining a Community of Host-Specific Bacteria to Block Inductive Biofilm Formation

Both culturing and molecular studies have shown that many marine organisms maintain species-specific microbial communities upon their surfaces, or within their bodies, that are distinct from the bacterial populations in surrounding seawater or on nearby surfaces (Hentschel et al. 2002). A new phylum-level clade of bacteria, *Poribacteria*, may exclusively associate with sponges (Fiesler et al. 2004). A study of bacteria associated with three sponge species found that all species harbored sponge-specific strains, and each species had a subset of host-specific strains that were absent from other sponges and adjacent seawater (Taylor et al. 2004b). In a survey of hard corals, Rohwer et al. (2002) found over half of bacterial isolates from coral surfaces were new genera or species. Different corals had host-specific microbial communities shared by conspecifics separated widely in space, and maintained for over a year, yet that were distinct from bacteria on adjacent corals of other species (Rohwer et al. 2002). Less is known about the microbial associations of ascidians. A dominant photosynthetic symbiont was found on multiple didemnid ascidians, and its presence was negatively correlated with diversity and abundance of other bacteria (Wahl 1995); the host may provide a particularly favorable environment for this symbiont, and/or the symbiont may out-compete or chemically inhibit competitors.

Instead of itself producing antibacterial compounds, an alternative strategy is for the basibiont to attract select bacteria to its surface, which can then competitively exclude other biofilm-forming bacteria. Relying on interspecific aggression between microbes relieves pressure on the host to synthesize costly chemical defenses, while keeping its surface free from fouling by most bacteria and, by extension, macro-organisms. Support for this comes from studies of algal surfaces. Of 280 microbial isolates from

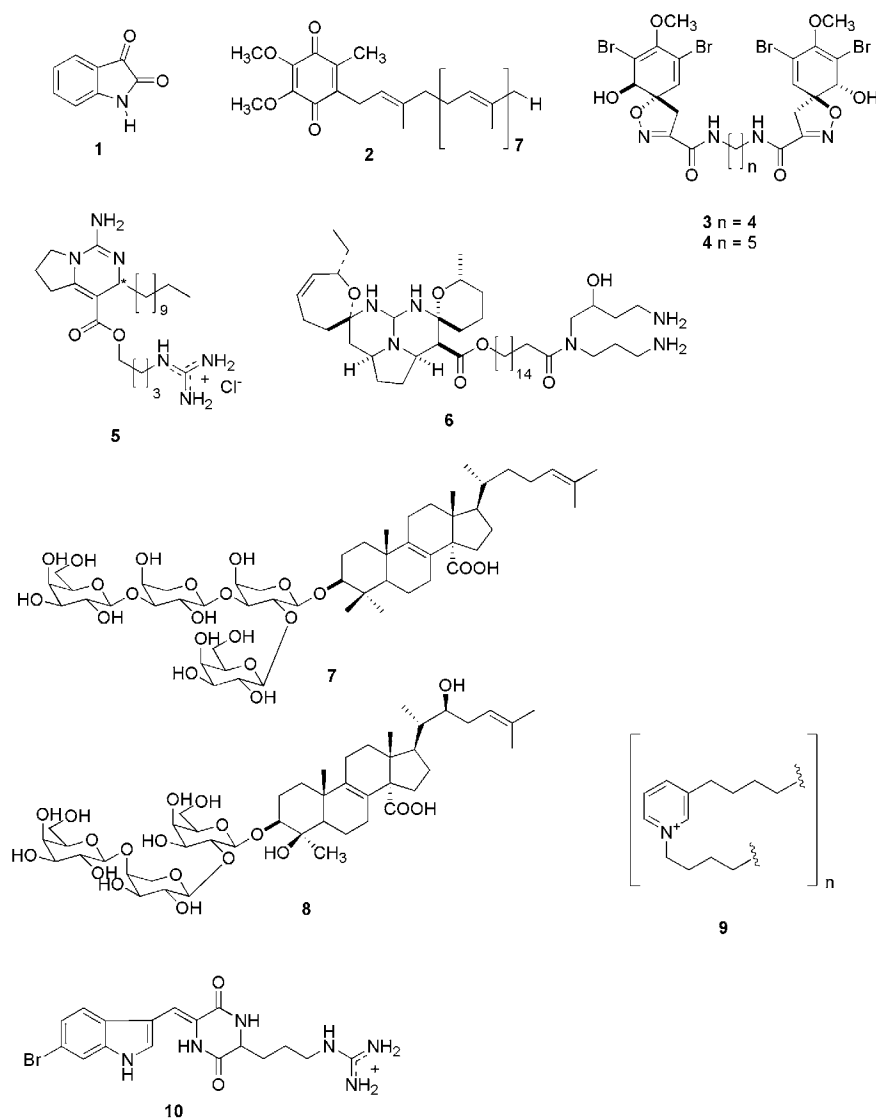
Scottish marine macroalgae, 21% inhibited growth of at least one out of nine marine fouling bacteria isolated from natural biofilms (Boyd et al. 1999). A subset of these strains was then screened for induction of negative chemotaxis in two strains of fouling bacteria, using a novel bioassay. Culture supernatants from algae-associated bacteria were incorporated into agar at the bottom of a spectrophotometer cuvette; a reduction in optical density indicated that bacteria moved away from the agar, and thus that the algae-associated strain released repellent metabolites. Out of 21 strains assayed, 38% triggered avoidance behavior in the fouling bacteria. With only a single exception, none of the strains that induced negative chemotaxis produced antibiotic metabolites in liquid culture (Boyd et al. 1999). However, subsequent studies showed that some algae-associated strains produce antimicrobial compounds when grown as a biofilm (Yan et al. 2002, 2003) or when exposed to potential competitor bacteria or their growth media (Mearns-Spragg et al. 1998).

These studies highlight the dual significance of antagonism between microorganisms and microbial chemotactic behavior in the battle for surface colonization. The data suggest a model in which select, sometimes species-specific, bacterial strains are attracted to a host basibiont's surface. Upon attachment they form a biofilm, and at some critical density begin production of antimicrobial compounds; they also release soluble repellents to trigger avoidance in competing strains of fouling bacteria. In this manner, symbiosis is established between the basibiont and its surface microbiota; the bacteria receive a place to grow in exchange for warding off strains that would otherwise promote fouling, to the detriment both of the host and its associated microbes (Armstrong et al. 2001). Algae that only assume their natural morphology when grown with bacteria may be obligately co-adapted to such mutualistic relationships (Takewati et al. 1983).

Marine bacteria cultured from seawater, sediment, and living or inert surfaces are a diverse source of bioactive chemistry (Fenical 1993; Renner et al. 1999). Bacteria producing antimicrobial and other bioactive substances have been isolated and/or characterized from a range of invertebrate surfaces, including sponges (Unson et al. 1994; Bewley et al. 1996), gorgonians (Tapiolas et al. 1991), other cnidarians (Trischman et al. 1994), molluscs (Armstrong et al. 2000) and echinoderms (Burgess et al. 1999). However, the importance of microbial defense against pathogens, predators and epibionts of host animals remains unclear (Engel et al. 2002). In few cases are both host specificity and the ecological role of a microbe-invertebrate association understood. Embryos of the shrimp *Palaemon macrodactylus* are protected from the pathogenic fungus *Lagenidium callinectes* by the egg-associated bacterium *Alteromonas* sp., which secretes the antifungal compound 2,3-indolinedione (**1**) (Gil-Turnes et al. 1989). Similarly, embryos of the American lobster (*Homarus americanus*) are coated in a monolayer of an unidentified bacterium that

produces 2-(*p*-hydroxyphenyl)ethanol (Gil-Turnes and Fenical 1992). For a marine animal, regulating the bacteria on its surface may be an effective defense (or one component thereof) against fouling and overgrowth.

An intriguing reversal of this paradigm occurs in the ubiquitous fouling bryozoan *Bugula neritina*, which contains low levels of bryostatins, potent cytotoxic macrolides (Pettit et al. 1982). Adult colonies contain a bacterial endosymbiont, *Endobugula sertula*, and vertically transmit it to their lecithotrophic larvae (Davidson and Haygood 1999). Knock-out experiments with antibiotics suggest that the symbiont produces the bryostatins (Davidson et al. 2001). Ecologically, this symbiosis may primarily protect the large, short-lived larvae from predators (Lopanik



et al. 2004). Thus, microbe-larval associations may contribute to aspects of the fouling process beyond settlement induction, such as lowering larval mortality in the plankton.

3.3 Attracting Strains that Chemically Deter Settlement of Fouling Larvae

Just as specific bacterial strains may trigger settlement of certain larvae, others can inhibit surface colonization. Contact with three bacterial isolates inhibited settlement of *Balanus amphitrite* larvae, even in the presence of adult-derived positive cues (Lau et al. 2003). Five of six bacterial strains deterred settlement by *Bugula neritina* larvae, with both surface-bound and soluble compounds showing larval toxicity or settlement inhibition (Bryan et al. 1997). Numerous bacterial strains have been reported to prevent fouling by cyprids (Maki et al. 1988; Avelin et al. 1993), but the complications listed in Sect. 2.1 make the interpretation of cyprid response to single-species films a harrowing task.

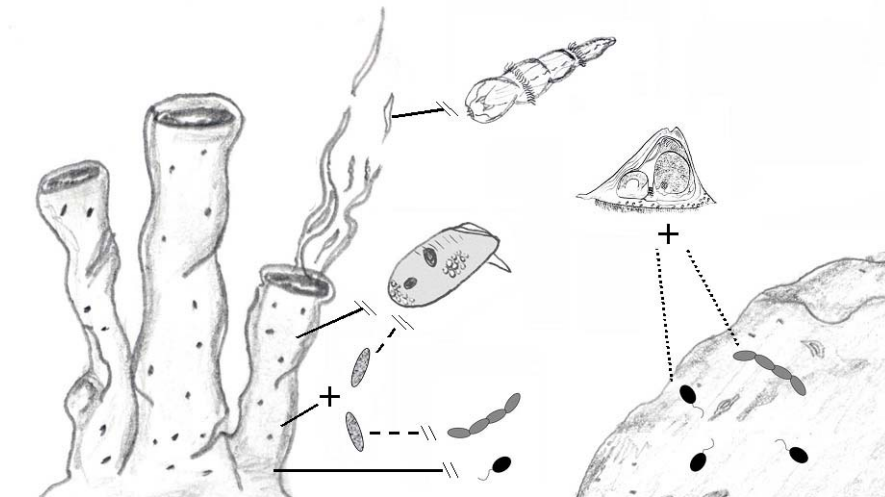


Fig. 1. Possible chemically mediated interactions between a benthic invertebrate, bacteria and other microbiota, and larvae of fouling organisms. Pluses denote positive, or attractive, interactions; double hash marks indicate repellency or toxicity. Solid lines indicate chemicals produced by the invertebrate, a potential basibiont at risk of being fouled. Invertebrates may produce non-polar metabolites concentrated along their surface, or polar compounds released into overlying water, possibly in waste water exiting through excurrent canals. The basibiont may attract species-specific, beneficial bacteria to its surface, while deterring other microbes from attaching. The host-specific bacterial strains may themselves deter or kill microbial competitors through chemistry (dashed lines). Excluded strains are predicted to induce settlement of fouling larvae when coating an undefended organism or inanimate surfaces (dotted lines). Either the host or beneficial bacteria colonizing its surface may produce compounds that deter larval settlement, either by triggering behavioral rejection of the surface, or through sub-lethal toxicity or post-settlement toxic effects on larvae

Given that some bacterial strains inhibit settlement, another possible defense is for an organism to preferentially attract such inhibitory bacteria to its surface (Fig. 1). Bacteria in biofilms may not just repel competing microbes, but could also produce metabolites that deter or kill fouling propagules (Holmström and Kjelleberg 1994). For example, one of five active bacterial strains isolated from the surface of the adult tunic of *Ciona intestinalis* produced cytotoxic, low-molecular weight metabolites that killed both *C. intestinalis* and *Balanus amphitrite* larvae, and a less active, high molecular weight fraction that killed *B. amphitrite* cyprids (Holmström et al. 1992; Holmström and Kjelleberg 1993). A marine *Alteromonas* strain isolated from the sponge *Halichondria okadai* produced ubiquinone-8 (2), which inhibited barnacle cyprid settlement (Kon-Ya et al. 1995). Bacterial isolates from the marine alga *Ulva reticulata* were non-toxic to five *Vibrio* strains and to larvae of *Hydroides elegans*; however, settlement of *H. elegans* larvae was inhibited by a water-soluble compound from *Vibrio* sp. two and by a biofilm of *Pseudoalteromonas* sp. two (Dobretsov and Qian 2002). Microbial metabolites were inhibitory even in the presence of the artificial inducer 3-isobutyl-1-methyl-xanthine (IBMX), suggesting competition for binding sites.

Bacterial strains associated with algal and invertebrate surfaces may be more likely to produce antifouling compounds than free-living strains. Holmström et al. (1996) compared the effects of microbial isolates from rock surfaces with those from algal and animal surfaces against *Balanus amphitrite* larvae, spores of the green alga *Ulva lactuca*, and marine diatoms. Of 93 isolates from rock surfaces, less than 10% blocked larval settlement, fewer than 20% inhibited algal spores from growing, and none of 10 strains affected diatoms; in contrast, 74% of algal isolates and 30% of animal isolates inhibited settlement of barnacle larvae (Holmström et al. 1996). Marine *Pseudoalteromonas* species from invertebrate and algal surfaces have received considerable attention for their potential significance as mediators of antifouling defense (Holmström and Kjelleberg 1999, 2000; Egan et al. 2000; Holmström et al. 2002).

Further support for the hypothesis that bacteria provide antifouling defenses for their invertebrate host comes from the soft coral *Dendronephthya* sp., which is rarely fouled in the field and has a characteristic community of surface bacteria. Chemicals from both the coral and surface-associated bacteria inhibited attachment and growth of other bacteria from nearby abiotic surfaces (Harder et al. 2003). Dobretsov and Qian (2004) isolated 11 bacterial strains that were unaffected by coral antibiotics, of which three inhibited attachment and growth of other marine fouling bacteria. Two strains were subsequently found to inhibit settlement of *Hydroides elegans* larvae, and generated an overall inhibitory effect when mixed with other, inductive bacteria into a biofilm (Dobretsov and Qian 2004). Waterborne, high-molecular weight polysaccharides produced by the two strains inhibited larval settlement of *H. elegans* and *Bugula neritina*. Settlement was blocked in the presence of the inducer IBMX, yet larvae remained viable and settled when transferred

to clean seawater; thus, the water-soluble settlement deterrents were not toxic. Soluble carbohydrates may compete for binding sites on larval lectins, as in other systems (Kirchman et al. 1982; Bahamondes-Rojas and Dherbomez 1990).

Similar results were obtained with the sponge *Mycale adherens*, a member of tropical fouling communities that is not itself fouled. The sponge surface yielded only half the number of bacterial isolates as reference dishes submerged nearby for 5 days, and only 3 of 40 sponge isolates were also found on the reference surfaces; multiple genera of bacteria were found only on the sponge, suggesting a restricted community of microorganisms (Lee and Qian 2003). Whereas 65% of natural biofilm strains induced settlement of *Hydroides elegans* larvae, 75% of sponge-associated strains were non-inductive, and 40% inhibited larval settlement. Dissolved compounds from the sponge were toxic to larvae, but only inhibited the growth of inductive bacteria at 10-fold elevated concentrations.

Taken together, these data suggest a simple hypothesis: bacteria that *repel* fouling larvae are likely to be *attracted* to unfouled invertebrates, whereas bacteria that *induce* settlement in fouling larvae are likely to be *repelled* by the surfaces of unfouled animals. Although chemically mediated repulsion has been documented, attraction of bacteria to the host surface has received little attention for invertebrates. Given the high degree of specialization between marine organisms and their microbial communities, there is reason to expect such chemotactic interactions. Conversely, settlement-inducing strains may be chemically repelled by the basibiont itself (Wahl et al. 1994), or by host-specific bacterial colonists (Armstrong et al. 2001). Thus, there is potential for multi-tiered interactions: animals use chemistry to attract or repel surface-colonizing bacteria; bacteria produce chemicals that induce or deter settlement by other bacteria, algal spores or invertebrates; and the host organism can itself produce chemicals that induce or deter larval settlement (Fig. 1).

Such associations between invertebrates, bacteria, and larvae of epibionts are similar in their ecological complexity to the tri-trophic interactions between plants, herbivorous insects and parasitic wasps (Baldwin and Preston 1999). In these interactions, chemical signals form when caterpillar saliva contacts plant membrane lipids, inducing the attacked plants to produce volatile organic signals; these airborne molecules attract parasitic wasps that paralyze the caterpillars and lay eggs in them. Remarkably, signals between the insect's victim (plant) and parasite (wasp) can be species-specific, with the plant selectively attracting the wasp species that specializes on the type of caterpillar chewing that plant (de Moraes et al. 1998). Similarly complex chemical communication may occur between invertebrates, surface microbes, and fouling organisms, but detailed investigations are needed to define the scope and generality of these interactions.

4 Role of Basibont-Derived Chemistry in Defense Against Eukaryotic Propagules

4.1 Sponges

Many studies have partially or fully characterized sponge metabolites that inhibit settlement, anaesthetize or kill larvae in laboratory still-water assays (Sears et al. 1990). However, with no knowledge of where these compounds are located within the living sponge, or at what concentrations they are present in water overlying a sponge, the ecological significance of antilarval activity is obscured. For instance, a series of non-polar terpenes and steroids from three sponges inhibited cyprid settlement without apparent toxicity; however, as each of these metabolites was cytotoxic or inhibited a key enzyme, their activity in laboratory assays may not reflect their role in nature (Tsukamoto et al. 1997). I will therefore focus on results from studies that tested ecologically relevant aspects of chemical defense against fouling organisms.

In one of the earliest and most cited papers to examine exudation of sponge metabolites, Walker et al. (1985) quantified release rates for the brominated compounds aerothionin (3) and homoaerothionin (4) from the intertidal sponge *Aplysina fistularis*. Rates of exudation were measured (1) in aquaria, using transplanted sponges, and (2) in situ by immersing exposed sponges in containers holding artificial seawater. This study was an admirable first step towards quantifying the release of potential antifouling compounds from a marine invertebrate, and it is striking that few comparable studies have been undertaken in the subsequent quarter-century. The study suffered for lack of replication, as only two sponges were successfully used for in situ work and six for aquaria experiments (Walker et al. 1985). This likely accounted for the high variance between replicates, making the measurements of exudation qualitative rather than quantitative. Release of aerothionin was measured as $\sim 9 \text{ ng min}^{-1} \text{ g}^{-1}$ dry wt of sponge in aquaria, versus $0.8 \text{ ng min}^{-1} \text{ g}^{-1}$ dry wt in situ, further complicating the interpretation of these results. As injured sponges released up to 100 times more aerothionin than uninjured sponges, the higher release rates measured in aquaria may have resulted from stress or damage to sponges during transportation. However, it is clear that these metabolites are released from minimally disturbed sponges into the surrounding seawater at detectable levels.

The importance of aerothionin exudation was supported by ecological evidence and energy dispersive X-ray microanalysis of *Aplysina fistularis* cells (Thompson et al. 1983; Thompson 1985). The brominated metabolites were localized to spherulous cells, which are concentrated just under

the exopinacoderm (the peripheral layer), or beneath the lining of excurrent canals that carry water out of the sponge (Thompson et al. 1983). Electron microscopy showed some spherulous cells degenerating such that their enclosed metabolites would be released into the mesohyl, or liberated into water flowing out of the sponge.

Whether these compounds are responsible for the observed antifouling properties of *Aplysina fistularis* remains unclear. Neither aurothionin nor a mixture of both metabolites prevented germination of brown algal spores of the kelp *Macrocystis pyrifera*, but this is not a fouling organism (Thompson et al. 1985). Neither compound prevented settlement in long-term assays with larvae of the bryozoan *Phidolophora pacifica* and the polychaete *Salmacina tribranchiata*; although these are more ecologically relevant organisms for fouling studies, the laboratory assays were run for exceptionally long periods (10 days and 7 days, respectively). Both aurothionin and the mixture of both metabolites stopped larvae of the abalone *Haliotis rufescens* from completing metamorphosis in the presence of the artificial inducer GABA (Thompson et al. 1985). Given that pesticides also block settlement of GABA-induced *Haliotis* larvae (Morse et al. 1979), the brominated compounds from *Aplysina fistularis* may simply be mildly toxic to abalone larvae, preventing settlement in short-duration (1 h) assays. Indeed, both compounds were toxic to brine shrimp larvae. These metabolites exhibited potent antimicrobial activity, however, which was correlated with reduced surface fouling in a survey of 40 Californian sponges (Thompson et al. 1985). The sponge may thus reduce fouling by slow release of antibiotics from rupturing spherulous cells, preventing growth of a primary biofilm.

Other studies have also indicated that bioactivity or specific metabolites can be concentrated in spherulous cells of sponges. In the Mediterranean sponge *Crambe crambe*, spherulous cells contained most of the toxicity found in whole-sponge extracts, due to alkaloids such as crambescin A (5) and crambescidins (e.g., 6) (Uriz et al. 1996). The defensive chemistry of this sponge inhibited settlement of *Bugula neritina* larvae, and limited the growth of marine bacteria (Beccero et al. 1997). Spherulous cells were concentrated in the exopinacoderm of the sponge, and were microscopically observed exiting the sponge surface. The enclosed compounds are thus likely to be concentrated in, and released onto, the sponge surface, supporting their putative ecological role in antifouling.

The recent study by Kubanek et al. (2002) is an excellent model for how to comprehensively assess the ecological roles of marine natural products. In this investigation, triterpene glycosides from two sponges, *Erylus formosus* and *Ectyoplasia ferox*, were tested for a suite of activities, including predator deterrence, bacterial attachment, fouling, and overgrowth by competitors. Metabolites of *E. formosus* (e.g., formoside, 7) collected from four different sites were effective antifeedants, and

inhibited attachment of a *Vibrio harveyi* strain to agar blocks at concentrations well below those occurring in the sponge. Antifouling activity was assessed in field assays, with compounds incorporated into Phytogel; compounds were initially incorporated into gels at two times whole-sponge concentrations, but as gels lose ~50% of their content over a 3-week deployment, this approximated natural levels in the sponge for the length of the trial. Triterpene glycosides from *E. formosus* strongly inhibited fouling by invertebrates and algae over a 27-day period. Further, at concentrations measured only in the surface layer of the sponge, the compound formoside (8) inhibited fouling for 14 days (Kubaneck et al. 2002). Structurally similar triterpene glycosides from *E. ferox* such as ectyoplaside A (8) were not active in antifouling and bacterial attachment assays, but were antifeedant to fish and allelopathic to another sponge. Given the effects against bacterial attachment, triterpene glycosides might inhibit fouling indirectly by preventing establishment of a biofilm; alternatively, the metabolites could act directly on settling larvae or spores, but this was not tested.

Two methods were used in an attempt to quantify levels of glycosides in seawater around sponges, but neither method was successful, either due to low release rates or to problems with co-eluting contaminants and low recovery efficiency of the sampling apparatus. Concentrations of the active compounds were lowest (2.1 mg ml⁻¹ sponge) in the outermost layer of *Erylus formosus*, but were otherwise uniformly distributed through the remaining sponge (6.3–7.5 mg ml⁻¹ sponge); surface swabs yielded an estimate of 0.04 mg/cm²sponge. Although concentrations were low in the outermost layer, these levels were sufficient to inhibit fouling in a realistic field assay. In contrast, the antifeedant metabolites of *Ectyoplasia ferox* were most concentrated in the outermost 2 mm of the sponge. It is striking that the glycosides of *E. formosus* were antifouling, yet were more concentrated within the sponge, whereas those of *E. ferox* lacked antifouling activity but were present in the outermost layer of the sponge. This illustrates the importance of quantifying where metabolites are present within an invertebrate, as natural distributions may be counter-intuitive to our naïve predictions. Although it is frequently postulated that antifouling metabolites should be concentrated in surface layers of an animal, the study by Kubaneck et al. (2002) demonstrates that natural selection does not necessarily produce such expected patterns.

The effects of antifouling sponge exudates were tested in the field by Dobretsov et al. (2004) by placing settlement dishes at varying distances from the unfouled sponge *Callyspongia pulvinata*, and from physically similar sponge mimics as controls. In laboratory assays, sponge compounds inhibited diatom growth and settlement of the polychaete *Hydroides elegans*, but were not antimicrobial. Analogously, sponges did not affect bacterial density or the settlement-inducing effects of microbial biofilms that grew on nearby plates in the field. Sponge proximity was

negatively correlated with abundance of macroalgae and fouling invertebrates on nearby abiotic surfaces, however, supporting the hypothesis that the sponge's chemistry is responsible for keeping its surface clean. The antifouling strategy of *Callyspongia pulvinata* thus appears to be deterrence of spores and larvae via release of polar metabolites. In contrast, *Aplysina fistularis* may rely on the antimicrobial effects of compounds present at the surface or released into the environment to prevent biofilm development, and thus to inhibit subsequent colonization by larvae.

4.1.1

Potential Non-Toxic Antifoulants, Suggested by Laboratory Bioactivity

Although untested in field assays, some non-toxic sponge compounds inhibit settlement of fouling larvae, suggesting an ecological role. Polymeric 3-alkylpyridinium salts (poly-APS, **9**; see Sepèix and Turk chapter) from the sponge *Reniera sarai* had antisettlement effects on barnacle cyprids that were non-toxic and reversible (Faimali et al. 2003). These compounds have surfactant properties, forming macromolecular assemblages in aqueous solution; they are partly water-soluble, due to their de-localized charge, yet also form a "greasy layer" on the sponge surface, due to alkyl side chains. Poly-APS inhibited settlement of *Balanus amphitrite* cyprids at $1 \mu\text{g ml}^{-1}$, a concentration that did not affect naupliar swimming or survival; further, the effects were fully reversible after 3 days of exposure to 10-fold higher levels (Faimali et al. 2003). In contrast, heavy metal antifoulants were toxic to nauplii, bivalve larvae and microalgae at low concentrations, equal in magnitude to their EC_{50} 's for settlement inhibition. Some marine natural products may thus deter settlement in fundamentally different ways from toxic metals.

The brominated diketopiperazine barretin [(6-bromo-8-tryptophan) arginine, **10**], from the sponge *Geodia barretti*, inhibited barnacle cyprid settlement with an EC_{50} of $0.4 \mu\text{g ml}^{-1}$ (Sjogren et al. 2004); the effects were non-toxic at 10-fold higher doses, and were fully reversed upon transfer to clean seawater. Reduction of the double bond in 8,9-dihydrobarretin caused a 10-fold loss of activity. In still water, *Geodia barretti* released sufficient barretin to inhibit settlement after a 10-fold dilution. This would be an ideal organism for future in situ measurements of exudation rates. Non-toxic brominated settlement inhibitors from other sponges include mauritiamine (**11**, $\text{EC}_{50} = 15 \mu\text{g ml}^{-1}$) and oroidin (**12**, $\text{EC}_{50} = 19 \mu\text{g ml}^{-1}$) from *Agelas mauritiana* (Tsukamoto et al. 1996a), ceratinamide A (**13**, $\text{EC}_{50} = 0.1 \mu\text{g ml}^{-1}$) from *Pseudoceratina purpurea* (Tsukamoto et al. 1996b), and a mildly active bromopyrrole (**14**, $\text{EC}_{50} = 21 \mu\text{g ml}^{-1}$) from *Agelas conifera* (Keifer et al. 1991).

Numerous terpenes with antisettlement activity against cyprids have been isolated from sponges like *Axinyssa* spp. and *Acanthella cavernosa*, and *Phyllidia* nudibranchs that consume them (Okino et al. 1995, 1996a,b;

Hirota et al. 1996, 1998). Potent non-toxic settlement inhibition (EC_{50} 's $<1 \mu\text{g ml}^{-1}$) is associated with isocyano functional groups, which may render molecules more inhibitory than CuSO_4 (EC_{50} 's $<0.15 \mu\text{g ml}^{-1}$) without the associated toxicity. These compounds should be evaluated in the field using slow-release gels, and their exudation from sponges measured in situ.

The role of sterols in antifouling is unclear. Cholesterol endoperoxide (**15**) and the dieterpene manoöl (**16**) from the sponge *Aplysilla glacialis* enhanced the biomass of fouling organisms when painted onto a surface and deployed in the field (Bobzin and Faulkner 1992). Cholesterol inhibited settlement of ascidian larvae in laboratory assays, however (Davis et al. 1991). Neither cholesterol nor ergosterol were effective against cyprids, but halistanol sulfate salts were modestly antisettlement towards cyprids (EC_{50} 's $3\text{--}4 \mu\text{g ml}^{-1}$) (Tsukamoto et al. 1997); further, steroids with a D-seco moiety from the octocoral *Dendronephtya* sp. inhibited cyprid settlement for 7 days, with no toxicity at elevated doses (Tomono et al. 1999).

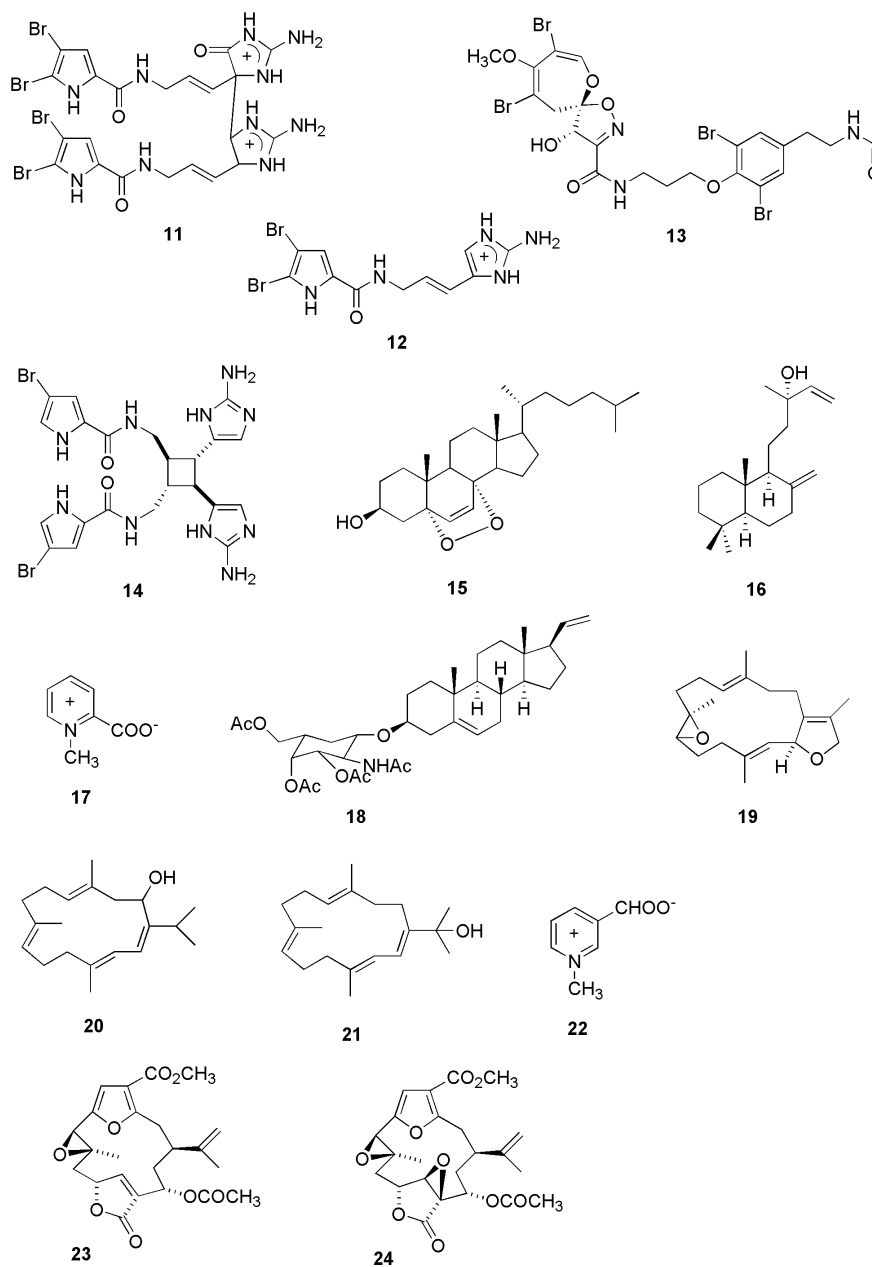
4.2 Cnidarians

4.2.1 *Cnidarian Defense Against Microorganisms and Algae*

Many cnidarians chemically inhibit diatom growth, possibly through non-toxic mechanisms (Wilsanand et al. 2001). Homarine (**17**, *N*-methyl-2-carboxy-pyridine) was isolated from the gorgonians *Leptogorgia virgulata* (0.3% wet weight) and *Leptogorgia setacea* (0.25% wet weight) (Targett et al. 1983). Both water-soluble extracts of the corals and the in situ concentration of homarine (2 mg ml^{-1}) inhibited growth of the co-occurring benthic diatom *Navicula salinicola* by 41%. Although not present in *Leptogorgia* spp., the related compounds nicotinic and picolinic acid were even more inhibitory. Homarine is common in the tissues of many invertebrates (Carr et al. 1996; Polychronopoulos et al. 2001), and may be involved in osmoregulation (Shinagawa et al. 1995), pattern formation during cnidarian development (Berking 1987), and donation of methyl groups in crustacean biosynthesis (Netherton and Gurin 1982). Unlike many nitrogenous polar metabolites, homarine does not stimulate fish feeding (Carr et al. 1996), and deterred predation by an Antarctic asteroid (McClintock et al. 1994).

Bandurraga and Fenical (1985) compared the unfouled octocoral *Muricea fruticosa* with its sympatric congener *M. californica*, which is commonly overgrown by fouling organisms. The unfouled *M. fruticosa* contained the muricins (e.g., **18**), saponins with differently modified aminogalactose residues linked to a degraded sterol derived from pregnane. Although none were active in cytotoxicity or antimicrobial assays, all four compounds inhibited growth of the diatom *Phaeodactylum*

tricornutum by 60–80% at 100 ppm and completely abolished growth at 200 ppm. The basis for inhibition of diatom growth by homarine and the muricins is unknown.



Targett et al. (1983) suggested that soft corals employ a two-level defense against fouling: chemicals slow the growth of photosynthetic protists, and periodic sloughing of the external covering removes the accumulated fouling community. A combination of physical and chemical defenses may operate in many invertebrates, and illustrates the need to consider alternative hypotheses in chemical ecology. For instance, Coll et al. (1987) reported differential patterns of algal fouling among colonies of the soft coral *Lobophylum pauciflorum* that corresponded with distinct chemotypes. Colonies overgrown by the algae *Ceramium flaccidum* and *Enteromorpha* sp. contained diterpene **19** with dihydrofuran and epoxide moieties; structure-activity studies implied that both oxygen functionalities were required for optimal bioactivity. In contrast, co-occurring colonies with clean surfaces contained a pair of cembrane diterpene alcohols (**20–21**). Assays with *Ceramium codii* (a congener of the fouling alga) yielded the counterintuitive result that overgrown colonies contained **19**, which strongly inhibited algal growth, whereas clean-surfaced colonies produced **20–21**, the least inhibitory compounds out of nine tested.

The authors inferred that differences in chemical composition *resulted* from algal overgrowth, postulating that the less-inhibitory compounds were converted to the more active molecule in response to fouling. Clearly, however, if the clean colonies had contained the more-active metabolite, the inference would have been that the diterpenes prevented algal overgrowth. This illustrates the danger in drawing adaptationist conclusions from limited data, since two contradictory conclusions cannot both be right; either the chemical differences play a causative role in observed patterns of fouling, or they don't. There is a tendency in the marine chemical ecology literature to hypothesize causative relationships between an ecological phenomenon and the presence of chemistry, without subsequently testing the hypothesis with appropriate experiments. For instance, in the case of *L. pauciflorum*, the hypothesis that soft coral chemistry changes as an induced response to algal epibiosis could be tested by experimental manipulation of algal cover and subsequent studies of chemical composition. In fact, a survey of 20 soft coral species found no chemical differences between conspecific pairs of overgrown and clean colonies, and colonies were observed to regularly shed their algal epibionts. Chemical variation in soft corals may not explain algal fouling, and physical defenses may be critical to keeping cnidarian surfaces clean.

Slattery et al. (1995) compared two Antarctic soft corals with unfouled surfaces (*Alcyonium paessleri* and *Gersemia antarctica*) to one frequently fouled by diatoms (*Clavularia frankliniana*). Polar extracts of *A. paessleri* and *G. antarctica* had weak antimicrobial activity against sympatric marine bacteria, but chloroform fractions had strong antiattachment activity, showing that different compounds kill bacteria

or inhibit attachment (e.g., Wahl et al. 1994). Both polar and non-polar fractions of *A. paessleri* and *G. antarctica* inhibited growth of the diatom *Navicula* sp. Aqueous homogenates, polar and non-polar fractions all impaired embryonic development of the sea urchin *Sterechinus neumayeri* in 48-h assays, but the relevance of this activity to fouling is unclear. Extracts of *C. frankliniana* had no bioactivity. Field assays were inconclusive; long-term trials with agar gels were not interpretable due to heavy overgrowth on treatment and control gels, and polar extracts quickly diffused from glass surfaces. Chloroform extracts of the unfouled species *A. paessleri* and *G. antarctica* limited diatom growth on treated glass slides over 1 month field deployments (Slattery et al. 1995). Although present in all three soft corals, homarine was not quantified in tested extracts (Slattery 1994). A subsequent study examined the biological activity of water-soluble compounds released by soft coral colonies maintained in aquaria. Water within 1–2 cm of *A. paessleri* contained a mixture of cholesterol and related sterols at a concentration of $1.54 \pm 1.09 \text{ mg l}^{-1}$, while water surrounding *G. antarctica* contained principally homarine and its isomer trigonelline (**22**) at $1.26 \pm 0.98 \text{ mg L}^{-1}$ (Slattery et al. 1997). The polar exudate from *G. antarctica* inhibited growth of 3 bacterial strains, as did pure homarine, but the sterols from *A. paessleri* were not antimicrobial. Trigonelline was also isolated as an antissettlement agent from the octocoral *Dendronephthya* sp. (Kawamata et al. 1994).

4.2.2

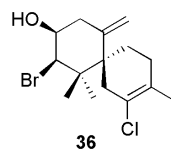
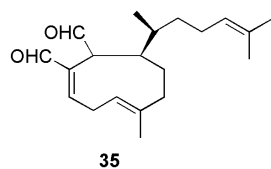
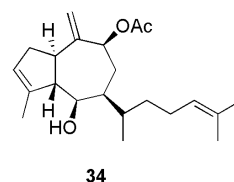
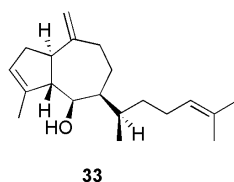
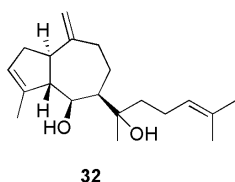
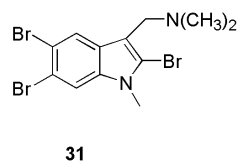
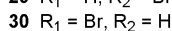
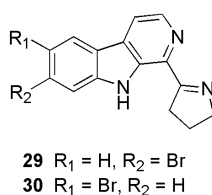
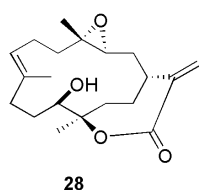
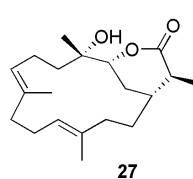
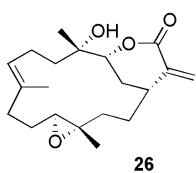
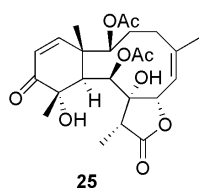
Cnidarian Defense Against Invertebrate Larvae

A low-molecular weight inhibitor of barnacle cyprid settlement was liberated into seawater by *Leptogorgia virgulata* (Standing et al. 1984), the same organism in which homarine slows diatom growth. However, high-molecular weight fractions induced settlement, and the bioassays were not ecologically interpretable. Inhibition was subsequently attributed to the diterpenes pukalide (**23**) and epoxy-pukalide (**24**) (Gerhart et al. 1988). Similar activity against *Balanus amphitrite* cyprids was reported for the renillafoulins (e.g., **25**), diterpenes from the octocoral *Renilla reniformis* (Keifer et al. 1986). Small analogues of these natural products containing furan or lactone rings inhibited cyprid settlement in a reversible manner not due to sub-lethal toxicity (Clare et al. 1999). More studies of how the parent natural products affect larval behavior would clarify the role of diterpenes in cnidarian antifouling defense. For instance, the renillafoulins inhibited settlement of cyprids but not of *Bugula neritina* larvae, and conversely, uncharacterized compounds inhibited *Bugula neritina* larvae but not cyprids, suggesting multiple compounds may be necessary for an effective non-toxic defense against a natural assemblage of fouling taxa (Rittschof et al. 1988).

Soft and hard corals may chemically affect the larvae of other corals, interactions at the interface of antifouling and allelopathy. Scleractinian coral recruitment was depressed in a current-dependent directional manner around the soft corals *Sinularia flexibilis* and *Sarcophyton glaucum*, and settlement did not occur on plates containing an extract of *S. flexibilis* (Maida et al. 1995). The diterpenes flexibilide (26), dihydroflexibilide (27), and sinulariolide (28) from *S. flexibilis* were toxic to fertilized eggs of the hard corals *Montipora digitata* and *Acropora tenuis* during the first 24 hr, but not to sperm, unfertilized eggs, or planulae larvae (Aceret et al. 1995). The ecological significance of toxicity to embryos is unclear, as embryos are neither found near benthic corals nor naturally bathed in coral metabolites for 24 h. Koh and Sweatman (2000) reported that indole alkaloids from the hard coral *Tubastraea faulkneri* were toxic to planulae of 11 other coral species but not to conspecific larvae; however, similar caveats apply, since swimming larvae will not be continuously exposed to solutions of coral metabolites for 4 h prior to settlement. To determine how non-polar metabolites influence settlement by potential competitors, behavior of planulae should be quantified around surfaces exuding diterpenes. For instance, extracts of four hard corals caused reversible changes in larval behavior of the scleractinian *Pocillopora damicornis*, and slowed growth or killed newly settled juveniles (Fearon and Cameron 1997). Such data are necessary to evaluate the role of chemical defense against larvae of fouling organisms and potential competitors for space.

4.3 Ascidians

As a phylogenetic generalization, solitary ascidians are more heavily fouled than colonial ascidians; an exception is the solitary genus *Phallusia*, which was less fouled than co-occurring solitary ascidians in Bermuda, the Mediterranean, and southeastern Australia (Stoecker 1980; Uriz et al. 1991; Davis and White 1994; Bryan et al. 2003). In contrast, most colonial ascidians are extremely well defended against fouling. In Bermuda, 20 of 27 colonial tunicates were free of epibionts (Stoecker 1980); in the Mediterranean 17 of 18 species were unfouled (Uriz et al. 1991); and three of four colonial ascidians from Australia were devoid of fouling (Davis and White 1994). The tunic of solitary ascidians may be a favorable surface for settling larvae of epibionts, but no study has explicitly determined whether solitary and colonial ascidians differ in their degree of chemical protection against fouling larvae.



A combination of mechanisms may generally protect colonial ascidians from fouling. The Mediterranean didemnid *Polysyncraton lacazei* benefits from associational grazing by two arthropods, periodic sloughing of its surface cuticle every 4–15 months, and a diverse chemical defense that variously inhibits growth of bacteria, fungi, diatoms, and sea urchin larvae (Wahl and Banaigs 1991). Surface properties of the tunic were not responsible for antifouling defense. In a study of six colonial ascidians from the Mediterranean, palatability to predatory fish and crustaceans decreased with tissue energy content but was not associated with chemistry or toxicity (Tarjuelo et al. 2002). Physical mechanisms such as

increased proportion of structural materials and lower energy content may thus contribute to antipredator defense in colonial ascidians. The degree to which physical properties of the tunic analogously affect patterns of fouling in solitary versus colonial ascidians remains to be elucidated.

The Atlantic species *Eudistoma olivaceum* produces a suite of over 20 alkaloids termed eudistomins (Rinehart et al. 1987). Early reports attributing the antifouling properties of this species to surface acidity or high levels of vanadium in the blood were subsequently refuted, and antilarval activity was traced to a pair of isomeric β -carboline alkaloids, eudistomins G (29) and H (30) (Stoecker 1978; Davis and Wright 1989; Davis et al. 1989). Both compounds inhibited settlement of *Bugula neritina* larvae at 2 μg per cm^2 , 5% of the concentration of eudistomin H in the ascidian; however, these compounds were toxic to larvae of *Bugula* in bioassays (Davis and Wright 1990). A non-toxic inhibitor was detected but not isolated. In subsequent trials with larvae of two *Bugula* species, an ascidian and a barnacle, the eudistomin fraction inhibited settlement at 5% of the natural concentration. Compared to controls, panels treated with eudistomins were half as fouled by the ascidian *Diplosoma glandulosum* after a 7-h field deployment (Davis 1991). Eudistomins G and H were not concentrated in any part of the colony, did not deter fish feeding, and were only mildly antiviral compared to other eudistomins, implying a specific ecological role as antifoulants (Rinehart et al. 1987; Davis 1991).

The fouled solitary ascidian *Molgula occidentalis* was compared with two unfouled colonial species, *Amaroucium stellatum* and *Botryllus planus* (Bryan et al. 2003). Extracts of the unfouled species actually increased attachment of bacteria, yet the same extracts displayed antibacterial activity against five of six marine bacterial strains in standard disc assays. Extracts of *A. stellatum* were generally inhibitory to barnacle cyprid settlement in lab assays, whereas extracts of *M. occidentalis* were not. However, effects in laboratory and field trials were contradictory for extracts of *B. planus*. A chloroform extract inhibited barnacle settlement in the field, yet increased cyprid settlement in the lab; conversely, a methanol extract was inhibitory to barnacle settlement in the lab, but was non-effective in the field (Bryan et al. 2003). Chloroform extracts of all three species intensified fouling by bryozoans in the field, and no extract prevented fouling by polychaetes. These conflicting data point to the need for field-based evaluations of laboratory findings, as comparable results are not always obtained, particularly against a phylogenetically diverse assemblage of fouling larvae.

4.4 Bryozoans

Despite their calcified exterior, many bryozoan species maintain unfouled surfaces, yet antifouling defenses have rarely been investigated in this phylum. Walls et al. (1993) examined four Australian bryozoans for bacterial abundance, macrofouling, and the presence of antimicrobial chemistry. Bacteria and epibionts were less abundant on *Orthoscuticella ventricosa*, which produced antibiotic extracts, than on *Cellaria pilosa* and *Bugularia dissimilis*, which did not contain antimicrobial compounds. The pattern was obscured by *Amathia wilsoni*, which contained antibacterial secondary metabolites and was relatively unfouled, yet had the highest overall microbial abundance due to high-density patches. The possibility that select bacterial strains were attracted to this species was discussed but not investigated.

The congeners *Zoobotryon verticillatum* and *Z. pellucidum* contained 2,5,6-tribromo-1-methylgramine (**31**), an antibiotic and potent, non-toxic inhibitor of cyprid settlement (Sato and Fenical 1983; Kon-Ya et al. 1994a). One of the most active antisetlement natural products, **31** was six times more inhibitory yet tenfold less toxic to cyprids than tributyltin oxide, and also blocked larval settlement of the mussel *Mytilus edulis* (Kon-Ya et al. 1994a). Simpler indole derivatives also had strong antisetlement activity, repelling but not killing *Balanus* cyprids (Kon-Ya et al. 1994b). Although many bryozoans are protected from fouling larvae by physical defenses such as pincer-like avicularia (Dyrynda 1986), encrusting species in particular deserve more attention for their potential chemical defenses and bacterial symbioses.

5 Investigating Larval Behavior to Understand and Combat Fouling

The structural complexity and biological activity of marine natural products, paired with the observation that few chemically rich species are fouled in the field, has led to the long-standing hypothesis that secondary metabolites defend invertebrates from fouling. Many natural products are toxic to the larvae of common fouling organisms, generally taken as support for the hypothesis that these compounds are antifouling in situ (Fusetani 2004). However, more knowledge is needed about larval behavior during settlement to interpret accurately the role of host chemistry in antifouling defense. Water-soluble, biological macromolecules can also inhibit fouling (Holmström and Kjelleberg 1993; James et al. 1996; Harder and Qian 2000), and despite low solubility in seawater,

some non-polar metabolites diffuse from the surface of producing organisms. This raises fundamental questions about how larvae experience and process chemical deterrents or toxins in nature. Can larvae react to waterborne signals or toxins before contact with a potential basibiont, or are such compounds detected during surface exploration? Do antifouling defenses primarily repel larvae, or kill settled larvae and juveniles? To answer such questions, critical information is needed concerning:

- (1) whether larvae perceive compounds in the water column, prior to surface contact
- (2) the behavior of larvae around live, chemically rich surfaces
- (3) the ecological significance of toxicity versus non-toxic deterrence

5.1 Waterborne Signals and Chemically Mediated Navigation

Historically, waterborne settlement cues were considered unimportant to the recruitment of invertebrate larvae in near-bottom flows, where turbulence would rapidly dilute dissolved compounds to levels below detection thresholds (Crisp 1974; Butman 1987; Pawlik 1992). Further, the slow swimming speeds and small size of larvae were thought to preclude chemotaxis, movement along a chemical gradient, from being effective under field conditions. However, larvae are now known to exhibit behavioral responses to waterborne signals that can enhance settlement success in flow. Dissolved compounds may also function as settlement deterrents, but most studies have hitherto focused on waterborne inducers rather than inhibitors.

The assumption that turbulence would prohibit larval chemotaxis misconstrues how chemically mediated navigation occurs in nature. Both air and water are inherently turbulent milieus, and the level of turbulence dictates how chemical signals propagate and disperse. A source of odorant produces plumes of molecules that are twisted and distorted by the ambient flow, creating filaments containing higher levels of the signal than the surrounding medium (Zimmer and Butman 2000). Many animals use a similar mechanism to navigate in turbulent odor plumes: perception of a chemical signal initiates orientation to a physical parameter, such as wind or current direction (Mafra-Neto and Cardé 1994; Nevitt et al. 1995). For example, upon sensing a pheromone plume, male moths immediately fly upwind; if they lose the plume, they cast back and forth until it is re-encountered (Vickers and Baker 1994; Mafra-Neto and Cardé 1994). Crabs track odor plumes from prey similarly, moving upstream against flow within a plume and tacking laterally to locate lost plumes (Weissburg and Zimmer-Faust 1994). Tracking behaviors evolved in turbulent settings where rheotaxis is a reliable way to trace a scent back to its source; such

behavior is not true “chemotaxis”, as a gradient is not necessary for navigation.

Analogously, dissolved cues can effectively mediate settlement by triggering downward displacement of larvae; when vertical distributions become bottom-skewed, more individuals are swept by turbulent eddies into contact with the bed (Turner et al. 1994; Tamburri et al. 1996). In still water, bivalve and gastropod larvae turned more frequently, decreased speed, and moved downwards when stimulated with waterborne cues (Tamburri et al. 1992; Krug and Zimmer 2000). In flume studies, larvae moved downwards and even swam upstream against flow after passing over a benthic source of settlement cue (Tamburri et al. 1996). Dissolved signals can thus mediate settlement by inducing larvae to orient and move towards the bed, using light or gravity for direction (Forward and Rittschof 1994; Eckman 1996; Tamburri et al. 1996). Indeed, controlled release of an inductive peptide from polyacrylamide gels increased recruitment of barnacle larvae in field assays (Browne and Zimmer 2001).

These studies provide a hypothesis for how waterborne antifouling agents might act. Since dissolved attractants cause larvae to turn more, slow down, and move towards the bed, settlement *inhibitors* should cause larvae to turn less, accelerate, and move upwards or away from a substratum. Such chemically mediated rejection will cause larvae to be swept by eddies or advected away from a repellent surface in the field. Larval behavior should thus be tested in simulated flows, yet surprisingly few studies of fouling behavior have been performed in flume tanks, where flow speed and turbulence can be manipulated as experimental variables. Cyprid settlement was studied in different flows (Mullineaux and Butman 1991), but larval behavior around chemically protected invertebrates has yet to be examined in a flume. The behavior of *Balanus improvisus* cyprids over smooth and textured surfaces was studied in still water and flume assays, testing behavioral rejection of substrata (Berntsson et al. 2004). In still water, cyprids moved faster, continuously and randomly over textured surfaces; however, conspecific extract resulted in slower, more twisted paths, indicating greater surface exploration. In a flume, the proportion of larvae remaining on textured surfaces was lower than on smooth surfaces after 5 min, as larvae that rejected a surface were swept away by the flow.

5.2 Measuring Production and Release of Chemical Deterrents in Situ

Field distributions of water-soluble molecules reflect rates of production and release, followed by transport of chemicals through advection and turbulent mixing (Zimmer and Butman 2000). To assess the ecological

importance of dissolved cues, it is critical to define spatial and temporal scales at which waterborne molecules occur in field habitats (Jennings and Steinberg 1994). This is especially true when a compound is extracted from an intact organism. Molecules localized within the body of an invertebrate may function as antifeedants, acting after a predator punctures the tissue to deter subsequent attacks. However, metabolites present deep inside an organism may not be perceptible to larvae settling on the outer cell layer or cuticle.

Localizing compounds within invertebrate tissues or colonies is critical to the study of fouling defenses. Some sponge compounds are found in spherulous cells, which may be shed into the sponge's environment (Walker et al. 1985; Uriz et al. 1996). In other cases, metabolites are in the outer layers or distributed throughout a sponge (Kubaneck et al. 2002). Metabolites produced by bacterial symbionts may remain inside the microbes (Unson and Faulkner 1993; Unson et al. 1994; Bewley et al. 1996). For instance, the bryostatins are localized inside a bacterial endosymbiont, which is housed inside the larvae, which are brooded within adult colonies (Davidson et al. 2001; Lopanik et al. 2004). Such compounds may not be biologically available for defense at the host's surface.

Despite indirect evidence that waterborne deterrents can inhibit fouling, bioactive compounds have rarely been quantified in seawater overlying invertebrate surfaces. Turbulent eddies dilute and disperse compounds as they are exuded from a point source, so even a continuously released metabolite will not readily accumulate in the water column. Measuring the effective in situ concentrations of putative deterrents is essential to test their ecological role. Few studies have even attempted to measure the concentration of potential antifoulants in seawater. Coll et al. (1982) presented qualitative evidence that non-polar terpenes were exuded by the soft corals *Sarcophyton crassocaule* and *Sinularia flexibilis*. Using a modified apparatus, Schulte et al. (1991) pumped seawater past an *S. flexibilis* colony and over Sep-pak cartridges, trapping sufficient flexibilide (26) to detect by TLC but not by NMR. Two methods failed to recover triterpene glycosides (7–8) from water around two Caribbean sponges, even though glycosides should be more soluble than a terpene like flexibilide (Kubaneck et al. 2002). Clearly, this area is ripe for methodological innovation to quantify in situ rates of antifoulant release.

5.3 Behavior of Larvae around Chemically Defended Surfaces

To assess reliably whether a compound is an antifoulant, it is critical to study how larvae respond to natural concentrations under authentic

conditions. In particular, there is strikingly little information on where rejection of a surface occurs. Larvae may detect a chemically defended organism prior to, or upon, initial contact; alternatively, metamorphosis could be blocked, or juveniles killed, by toxins leaching from a surface. Understanding such processes may determine, for instance, if antifouling paints cause post-settlement mortality whereas invertebrates trigger larval avoidance through waterborne or surface-bound deterrents. Given the importance of reduced exploratory behavior in settlement inhibition (Berntsson et al. 2004), we need to understand the mechanisms that govern site choice by fouling larvae.

Larvae are known to be extraordinarily sensitive to chemical cues during settlement, and as settling on a toxic surface is a fatal mistake, larvae have likely evolved mechanisms for detecting and rejecting poisoned surfaces (either before or upon contact). Evidence of such rejection is indirect, however, due to a lack of research into chemically triggered avoidance behavior. In the field, ascidian tadpole larvae made frequent contacts with the sponge *Mycale* sp. without settling, but almost never contacted the surface of *Crella incrustans* (Davis et al. 1991). As *C. incrustans* was not allelopathic to juvenile ascidians, it likely produces waterborne deterrents that act at a distance to inhibit larval contact. A promising innovation for use with smaller larvae is in situ video monitoring during substratum exploration, and subsequent analysis of behavior (Hills et al. 2000; Thomason et al. 2002). For instance, *Semibalanus balanoides* cyprids were video recorded in the field over three test substrata, and analysis of the video revealed differences in small-scale exploratory behavior corresponding to surface properties of each substratum (Thomason et al. 2002). This technique offers the promise of determining the scale at which larvae recognize and respond to physical and chemical properties of a surface, either attractive or repellent, under natural conditions of flow, conspecific abundance, etc.

The clearest evidence for larval rejection of defended surfaces comes from algae. The thinness of algal blades, compared to invertebrate surfaces, makes algae more amenable to behavioral observations in the laboratory. The brown alga *Dictyota menstrualis* was less fouled than other co-occurring algae, despite having more surface area, and contained diterpenes such as dictyol E (32) and pachydictyol A (33) (Schmitt et al. 1995). In lab assays, larvae of *Bugula neritina* contacted *D. menstrualis* at the same rate as preferred algae, but were 100-fold less likely to settle on *Dictyota*. Thus, larvae displayed no avoidance behavior prior to contacting the alga, where diterpenes are concentrated in a surface layer 1-cell thick. Dictyol E and dictyol B acetate (34) were highly toxic to larvae prior to settlement, when dissolved in seawater at a dose lower than the effective concentration on the algal surface; in contrast, pachydictyol A and dictyodiol (35) were not toxic to larvae, but slowed growth and deformed development of juveniles (Schmitt et al. 1995, 1998). None of the

diterpenes was toxic to larvae of the bryozoan *Amathia convoluta*, but all inhibited metamorphosis and showed post-settlement toxicity at relevant concentrations; further, dictyol E and dictyol B acetate decreased settlement and juvenile development in the hydroid *Eudendrium carneum* (Schmitt et al. 1998). Thus, non-polar metabolites may not affect larvae prior to contact, but can diminish settlement and post-settlement survival of juvenile stages. Whether larvae spend less time exploring dictyol-treated surfaces or change behavior after contact with such a surface is unclear, but evidence suggests no avoidance behavior acts upstream of initial settlement.

In some algae, non-polar metabolites are concentrated at the surface (Dworjanyn et al. 1999). In others, high-molecular weight, biological macromolecules act as waterborne settlement inhibitors (Harder and Qian 2000), but chemical characterization of biomolecules has traditionally lagged far behind that of secondary metabolites. In a comparison of two fucoid algae, cyprid settlement was more inhibited by algal blades and phlorotannins of *Fucus vesiculoides* than *F. evanescens*, although *F. vesiculoides* was more heavily fouled in the field (Wikstrom and Pavia 2004). Post-settlement survival of barnacles was lower on *F. evanescens* due to higher rates of detachment, however; this highlights the need to delineate between pre-settlement deterrents and post-settlement mortality (due to absorption of toxins, inhibitors of metamorphosis, or physical factors).

5.4 Interpreting the Ecological Importance of Larval Toxicity in Laboratory Assays

Laboratory still-water assays have variously demonstrated that extracts of marine invertebrates can (1) inhibit settlement without otherwise impairing larvae, (2) cause developmental abnormalities in embryos, (3) exhibit sub-lethal toxicity to larvae, and (4) kill larvae or juveniles. However, the ecological relevance of such bioactivity is unclear. Marine invertebrates produce a dazzling variety of cytotoxic compounds, active against a wide range of organisms, embryos and cell lines (Faulkner 1984; Pettit et al. 1985). Given the number of metabolites that are cytotoxic or lethal to vertebrates at a sufficient dose, it is to be expected that many natural products are toxic to vulnerable embryonic or larval stages of other species. Killing larvae does not, however, indicate that a compound has an ecological function as an antifoulant, any more than an LD₅₀ for mice indicates that a metabolite naturally functions to protect a sponge from rodents.

Broad-spectrum toxicity is a property of so many marine natural products that care should be exercised in extrapolating ecological function from still-water bioassays. Many natural products bind to

specific intracellular protein targets that are highly conserved among eukaryotes, including kinases (Tosuji et al. 2003), telomerases (Warabi et al. 2003), proteases (Nakao et al. 1999, 2000; Fujita et al. 2001), and ion pumps (Sata et al. 1999). Such mechanisms of action are likely to lead to widespread bioactivity, and indeed, many compounds that inhibit settlement also kill larvae or have other cytotoxic properties. Practical considerations favor the use of standard, still-water bioassays using larvae of easily obtained fouling organisms. However, the widespread reliance on such methods and the lack of field trials has limited our understanding of how chemical defenses protect invertebrates against fouling in nature. Demonstrating that a compound inhibits settlement of fouling larvae in still water is suggestive of a natural role, and reason for further investigation, but does not *a priori* indicate an ecological function. Larvae are inherently mobile, and in the pervasive turbulence of benthic environments, will not remain localized near a surface unless they settle and attach to it. There is no natural situation analogous to larvae that are trapped in a small volume of water containing a chemical solution in the laboratory. Even if a given metabolite is present in the water column at a biologically relevant concentration, swimming larvae will at best be transiently exposed to pulses of a compound while searching for a settlement site.

Fouling is an ecological *process*, involving organisms from different kingdoms and interactions that develop over time. Laboratory assays using larvae of 1–2 test species do not assess whether a metabolite deters fouling in the extracted organism, and cannot be interpreted in an ecological context. I therefore recommend a shift in terminology. The term *antifouling* should be reserved for compounds tested in the field against a natural pool of microbes, algal spores and invertebrate larvae, or for which ecologically relevant data demonstrate a role against fouling in situ. Compounds that inhibit larval settlement in still-water bioassays should be termed *antisettlement*; this reflects their measured biological activity, leaving open the question of whether such activity may be effective against a diverse array of fouling organisms under field conditions. This is particularly relevant to studies showing reversible or non-toxic settlement inhibition by natural products. The term *antilarval* or *larvicidal* should be used for compounds that are toxic to larvae in bioassays, as these terms reflect the observed lethality to larval stages.

Compounds involved in antifouling defense may be broadly toxic, inhibiting larvae from completing metamorphosis via sub-lethal toxicity or killing settled larvae outright. The use of generally toxic compounds in antifouling defense may result from selection against the costs of synthesizing distinct defenses for different threats, leading to multiple ecological roles for a given metabolite (Kubanek et al. 2002). Alternatively, invertebrates may have chemical defenses shaped by natural selection specifically against fouling organisms. Such evolutionarily honed weapons

would deter settlement or be *selectively* toxic to larvae, acting on sensory or internal biochemical pathways unique to larval stages, but would not affect embryos, cell lines, etc. A partnering of traditional, medically driven natural products research and ecologically motivated fouling studies may establish which of these hypothetical mechanisms predominates.

5.4.1

Behavioral Deterrence of Larvae Versus Metabolic Toxicity

Throughout the 1990's, increasing attention was paid to non-toxic inhibition of larval settlement, largely due to the commercial potential of environmentally safe alternatives to current paints (Fusetani et al. 1996; Fusetani 1997). Non-polar metabolites may repel larvae exploring a basibiont's surface; alternatively, polar metabolites liberated into overlying water may be detected by larval receptors and trigger avoidance behavior. Such antifouling chemicals should be classified as *deterrents*, repelling larvae via behavioral mechanisms before or during surface exploration, prior to the irreversible commitment to metamorphosis. Alternatively, waterborne or surface-adsorbed molecules may impair larvae through sub-lethal effects, or kill settled and metamorphosing stages outright. These would be *toxins*, negatively affecting larvae or killing those newly settled or metamorphosing on the organism's surface. Although the effects of sub-lethal toxicity may only be detectable over long periods (Ng and Keough 2003), some testable predictions emerge from this dichotomy that should be amenable to lab experimentation.

Non-toxic antifoulants may act on larval sensory systems, or inhibit early settlement responses without causing damage. Such a molecular defense is akin to bad-tasting but non-toxic antifeedants, like capsaicins from chili peppers or terpenes synthesized *de novo* by some dorid nudibranchs (Okuda et al. 1983; Krug et al. 1995). Non-toxic compounds may repel larvae through behavioral mechanisms, interfere with ligand binding of natural inducers, impair neurotransmission of inductive signals, or cause short-term ciliary arrest. Such metabolites do not likely act post-metamorphosis, and must therefore quickly deter larvae before or upon contact with the host. Marine natural products in this category often contain isocyano, furan, lactone, or bromine functional groups (Hirota et al. 1996; Clare et al. 1999; Sjogren et al. 2004).

One testable prediction is that non-toxic inhibitors may block settlement in the presence of some natural inductive cues, but not others. For example, the inhibitor phloroglucinol diminished only the gregarious component of barnacle settlement, a response to cues from other cyprids, and had no effect on isolated, individual larvae (Head et al. 2003). The selective inhibition of gregariousness among cyprids indicates a non-toxic mechanism of action for phloroglucinol, impairing recognition of

conspecific cues without *a priori* impeding a larva's ability to metamorphose. Another prediction is that non-toxic deterrents should impede settlement in response to natural cues, but *not* in response to pharmacological inducers that act downstream of normal signal transduction pathways. This has not been well studied to date.

In contrast, toxic metabolites could have pronounced post-settlement effects. Although larvae may be adapted to recognize and avoid toxic coatings prior to attachment, surface-bound toxins may require longer contact times than non-toxic deterrents for their effects to manifest, and may act well after settlement (Schmitt et al. 1998). One prediction is that settled stages should be affected by toxins, but not by non-toxic deterrents. A second prediction is that toxins will block settlement in the presence of neuroactive compounds, interfering with the biochemical progression through metamorphosis that such agents artificially trigger. For instance, sub-lethal doses of 3 commercial pesticides (including DDT) blocked 100% of the settlement response in abalone veligers triggered by exogenous addition of the neurotransmitter GABA (Morse et al. 1979). Even at concentrations that do not cause direct mortality, toxins may interfere with the biochemical pathways necessary for normal metamorphosis to occur.

Metals in antifouling paints may not be detectable by larval chemoreceptors, and likely act after larvae have contacted, and potentially attached to, treated surfaces. Indeed, CuSO_4 -treated surfaces did not deter initial exploration or byssal thread attachment by mussel larvae, but eventually affected larval behavior after 2 hr of exposure, and killed 10% of larvae after 24 h (da Gama et al. 2003). In contrast, extracts of the unfouled alga *Laurencia obtusa* containing the terpene elatol (36) were immediately repellent to larvae and deterred attachment, and showed no toxicity for 24 h. Elatol is toxic to barnacle nauplii, bryozoan larvae, fish and insects (Hay et al. 1987; de Nys et al. 1996; König and Wright 1997), yet triggered behavioral rejection of surfaces without observed toxicity in mussel pediveligers. These results suggest larvae have adapted to perceive and avoid algal toxins, which therefore act as settlement deterrents, whereas metals are not detected and therefore act via delayed toxicity.

5.5 An Alternative Bioassay Design to Avoid Artifacts and Concentration Effects

A problem with laboratory bioassays is their reliance on static conditions; virtually all are performed in small volumes of still water. Such designs hinder the development of ecologically meaningful datasets. In still-water assays, larvae experience higher concentrations of toxins than they would encounter in nature, whether the compounds are pre-dissolved into the

assay water or first dried onto a surface and allowed to diffuse into seawater. High levels of antifouling metabolites have never been measured in seawater around a chemically defended invertebrate in the field, where turbulence and advection will dilute and remove compounds exuded from live surfaces. Testing defined concentrations does not even permit comparison of EC_{50} values between studies when different larval densities were used in assays (Head et al. 2003). A more subtle problem may be the lack of hydrodynamics as a stimulus to larvae settling in still water. Flow and chemical cues can interactively determine settlement success (Pawlik and Butman 1993). As most fouling organisms are suspension feeders, their larvae may respond to flow as a cue during habitat choice; assays in static water may fail to trigger natural behaviors. This is an intrinsic limitation on the usefulness of larval bioassays from an ecological perspective, as it is difficult to extrapolate from unnatural assay conditions to the field.

As an alternative to commonly used assay dishes, plastic enclosures could be fashioned with mesh sides of 100- μ m pore size to retain larvae, and a hollow bottom that can be filled with a polymeric gel. Compounds are embedded in a matrix of Phytogel or acrylamide, from which they diffuse at a controlled and calculable rate from the bottom of the container (Henrikson and Pawlik 1995; Browne and Zimmer 2001). The concentration in the gel can be set to match that in an organism, or a rate of release measured in the field. The enclosure is then immersed in seawater, larvae added, and the lid sealed. The whole container is submerged in a large volume of seawater in a flow-through or filtered, recirculating aquarium. Larvae will be exposed to metabolites that are diffusing from the gel, but are free to move away from the gel surface, limiting their exposure to toxins; compounds are continuously diluted and removed by water passing through the mesh sides. This more accurately mimics field conditions, where larvae are not trapped in water with high concentrations of dissolved organic compounds.

To test whether contact with a treated surface is necessary, the gel surface can be covered with crushed shell to which larvae can attach; larvae and settlers will still be exposed to diffusing compounds, but not through direct contact with the surface. This will determine whether a chemical of interest acts as a waterborne or surface-adsorbed agent. Recently settled larvae and juveniles could easily be introduced into the containers, to measure post-settlement effects of metabolites diffusing from the gel. Such assays will be more ecologically meaningful and should provide new insight into how chemical antifouling defenses operate in nature.

Additional factors should be taken into consideration for bioassay design. The importance of density-dependent effects is clear for larvae of species that exhibit gregarious settlement and kin recognition, such as barnacles and bryozoans (Keough 1989). Gregariousness can affect assays

at concentrations as low as 5 barnacle cyprids per dish, and shows unexpected interactions with settlement inducers and inhibitors (Head et al. 2003). Larvae from multiple batches should be used, to examine variation between batches as a factor (Raimondi and Keough 1990; Willemsen et al. 1998). Variation is an important issue in larval biology but has been less considered in fouling research (Holm 1990). Cyprid settlement response can vary over the course of a recruitment season (Jarrett 1997), and other species have inherently dimorphic settlement requirements (Toonen and Pawlik 2001); the impact of such behavioral variance on response to chemically protected surfaces remains unknown. Similarly, whether fouling larvae are locally adapted to the chemistry of common basibiont species remains to be tested; indeed, potential “resistance” to antifouling chemical defense has gone largely unexplored.

Methods using computer-assisted motion analysis to quantify behavior of larvae have been developed, but hitherto have been primarily used to study responses to settlement cues rather than inhibitors (Tamburri et al. 1992, 1996; Krug and Zimmer 2000). However, motion analysis has been used to quantify the behavior of algal spores and diatoms for antifoulant screening programs (Wigglesworth-Cooksey and Cooksey 1996; Iken et al. 2001, 2003); such approaches could be widely applied to study the behavior of fouling larvae during settlement.

In summary, the design of antilarval and antisettlement assays should pay careful attention to the realism of conditions to which larvae are exposed. Larval behavior should be observed and quantified where possible, and the reversibility of inhibition or lethality of treatments assessed. Ideally, assays should expose larvae only to concentrations likely to be encountered in the field, either by reproducing concentrations measured in situ, or by allowing compounds to diffuse into an excess of seawater while larvae are confined near a treated surface. Flume and field experiments may provide missing information on larval behavior around chemically enriched surfaces in realistic flow.

6

The Importance of Alternative Hypothesis Testing: Mechanical and Physical Defense

Science is founded on the testing and rejection of unsupported hypotheses. It is common in the literature for unusual chemistry to be found in an unfouled organism, and if the chemistry is active in antilarval bioassays, a single conclusion is asserted: the chemistry serves an antifouling purpose in nature. This is too often the end of the story, rather than a starting point; thorough testing of many hypotheses is needed to find the best explanation for how an organism maintains a

clean surface. Examples of robust testing of physical, mechanical, behavioral and chemical defense hypotheses can be found in Wahl and Banaigs (1991), Becker and Wahl (1996), and Wahl et al. (1998). In these studies, the authors rejected all but one or two possible antifouling defenses for each species examined, sometimes finding that a combination of different strategies was employed (e.g., sloughing and chemical defense in the ascidian *Polysyncraton lacazei*; Wahl and Banaigs 1991).

A wide range of mechanisms can reduce fouling in invertebrates, yet are frequently overlooked in favor of chemical defenses. Given that mechanical, behavioral and chemical defenses may operate in concert, it is critical to test alternatives to a strictly chemical model of antifouling. Mobile organisms can protect their surfaces through a combination of mechanisms. For example, studies of seven tropical and one temperate crab species found that carapaces were kept unfouled by a combination of behavioral and mechanical strategies, including burrowing to scrape surfaces clean, nocturnal activity patterns that inhibit algal growth, and time spent in air (Becker and Wahl 1996; Wahl et al. 1998). Mutual surface grazing acts as a density-dependent defense in some snail populations (Wahl and Sonnichsen 1992).

Sessile species have fewer non-chemical options, but physical defenses should be considered as alternative hypotheses in any investigation of antifouling (Dyrynda 1986). Microtopography may impede attachment of spores and larvae (Wahl et al. 1998; Scardino et al. 2003; Bers and Wahl 2004). Mechanical defenses include features such as spicules, or miniature pinchers such as echinoderm pedicellaria or bryozoan avicularia (Dyrynda 1986); however, due to size constraints, these structures are only effective against larger organisms such as larvae. Movement of cytoplasm or cells from fouled regions to areas of new growth protects algae (Littler and Littler 1999), and could possibly function in sponges and colonial organisms.

Regular sloughing of outer layers protects certain algae (Filion-Myklebust and Norton 1981; Sieburth and Tootle 1981; Russell and Veltkamp 1984) and sponges (Barthel and Wolfrath 1989) from overgrowth. The study by Barthel and Wolfrath is noteworthy: it is only 3 pages long, yet one of the most cited papers in the fouling literature. More studies on sloughing are clearly needed to determine the general importance of this mechanism. Periodic molting in ecdysozoans or shedding of the tunic in ascidians protects against later stages of fouling such as colonization by invertebrate larvae, but is too infrequent to deter unicellular epibionts (Wahl and Banaigs 1991; Becker and Wahl 1996). Regular shedding of mucus layers may be a significant antifouling mechanism in cnidarians (Johannes 1967; Ducklow and Mitchell 1979). Removal of fouling larvae by suspension feeding has been suggested (Cowden et al. 1984), and bivalve aggregations may reduce fouling

pressure by filtering larvae out of the water column, an effective if indirect defense (Tamburri and Zimmer-Faust 1996; Wahl et al. 1998).

The tight degree of association occasionally observed for specific epibiont-basibiont pairs remains relatively unexplored (Davis et al. 1996). For instance, Davis and White (1994) found distinctive associations between an ascidian and an encrusting sponge, a sponge and a zoanthid, and a bryozoan and an anthozoan. Little has been done to investigate the mechanistic basis for species-specific attraction of epibiont larvae in these presumptive mutualisms, which if co-evolved may not incur the typical costs of fouling to the basibiont. One clear advantage would thus be defense against the broader array of potential fouling organisms, whose larvae may be spatially excluded, chemically repelled, or consumed by the host-associated epibionts.

7 Conclusions

Marine invertebrates utilize an extraordinary range of antifouling strategies, and any given species likely derives some benefit from multiple defense mechanisms. Chemistry can either kill or repel bacteria, or slow the growth of diatoms, thereby removing positive settlement cues that would otherwise encourage attachment of larvae. More coevolved defenses occur where invertebrates selectively attract host-specific microbes to their surfaces, which then displace or inhibit competing bacteria, or chemically deter larval settlement. Basibionts can also directly defend against fouling organisms, either via non-toxic deterrents that induce avoidance behavior in larvae, or through toxins that repel exploring larvae or kill recently attached settlers.

Improved bioassays that allow larvae more realistic freedom of movement and expose them to authentic doses of natural chemicals may shed greater light on how the chemical defenses of marine organisms operate in the field. Quantitative observations of larval behavior should be incorporated into antifouling screening, to provide more sophisticated insight into how larvae act around chemically imbued surfaces, particularly in moving water. Strikingly, it is still unclear whether most natural chemical defenses work by deterring larvae prior to contact, upon initial contact, or through post-settlement toxicity; more comparative studies will be needed to uncover the generality of a given mechanism, and which strategies are effective against particular fouling threats.

Evolutionarily, it would be interesting to learn whether chemical defenses are co-adapted to the regional fouling organisms that threaten a particular organism. However, global fouling communities have become highly homogenized, as human activity moves fouling larvae in ballast

water and adult communities on hulls around the world (Minchin and Gollasch 2003). This may diminish the import of regional specialization in chemical defense against epibiosis before we have a chance to study it, much as invasive species generally threaten biodiversity and overwhelm the fragile adaptations of endemics. The ecological complexity of the interactions and the opportunities for mutualisms and evolutionary arms races between adult invertebrates, microbes, protists and larvae make the study of biofouling an intriguing arena for biologists and chemists alike. Hopefully we may learn from the marvelously adapted invertebrates of the benthos and devise environmentally sound antifouling practices, lest we do further harm to coastal ecosystems in our efforts to ward off the devilishly persistent propagules of fouling organisms.

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References

- Aceret TL, Sammarco PW, Coll JC (1995) Effects of diterpenes derived from the soft coral *Sinularia flexibilis* on the eggs, sperm and embryos of the scleractinian corals *Montipora digitata* and *Acropora tenuis*. *Mar Biol* 122:317–323
- Allison DG (2003) The biofilm matrix. *Biofouling* 19:139–150
- Amade P, Chevolut L (1982) Antimicrobial activities of marine sponges from French Polynesia and Brittany. *Mar Biol* 70:223–228
- Armstrong E, Boyd KG, Pisacane A, Peppiatt CJ, Burgess JG (2000) Marine microbial natural products in antifouling coatings. *Biofouling* 16:215–224
- Armstrong E, Yan LM, Boyd KG, Wright PC, Burgess JG (2001) The symbiotic role of marine microbes on living surfaces. *Hydrobiologia* 461:37–40
- Avelin Mary S, Vitalina Mary S, Rittschof D, Nagabhushanam R (1993) Bacterial/barnacle interaction: potential of using juncellins or antibiotics to alter structure of bacterial communities. *J Chem Ecol* 19:2155–2167
- Bahamondes-Rojas I, Dherbomez M (1990) Purification partielle des substances glycoconjuguees capables d'induire la metamorphose des larves competentes d'*Eubbranchus doriae* (Trinchese, 1879), mollusques nudibranche. *J Exp Mar Biol Ecol* 144:17–27
- Baier R (1981) Early events of micro-biofouling of all heat transfer equipment. In: Somerscales E, Knudsen J (eds) *Fouling of heat transfer equipment*. Hemisphere Publ, Washington, DC, pp 293–304
- Baldwin IT, Preston CA (1999) The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137–145

- Bandurraga MM, Fenical W (1985) Isolation of the muricins: Evidence of a chemical adaptation against fouling in the marine octocoral *Muricea fruticosa* (Gorgonacea). *Tetrahedron* 41:1057–1065
- Barkai A, McQuaid C (1988) Predator–prey role reversal in a marine benthic ecosystem. *Science* 242:62–64
- Barthel D, Wolfrath B (1989) Tissue sloughing in the sponge *Halichondria panicea*: a fouling organism prevents being fouled. *Oecologia* 78:357–360
- Bauer WD, Robinson JB (2002) Disruption of bacterial quorum sensing by other organisms. *Curr Opin Biotechnol* 13:234–237
- Becerro M, Uriz M, Turon X (1997) Chemically mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida). *Hydrobiologia* 356:77–89
- Becker K, Wahl M (1996) Behaviour patterns as natural antifouling mechanisms of tropical marine crabs. *J Exp Mar Biol Ecol* 203:245–258
- Bergquist RP, Bedford JJ (1978) The incidence of antibacterial activity in marine Demospongiae: systematic and geographic considerations. *Mar Biol* 46:215–221
- Berking S (1987) Homarine (*N*-methylpicolinic acid) and trigonelline (*N*-methyl-nicotinic acid) appear to be involved in pattern control in a marine hydroid. *Development* 99:211–220
- Berntsson KM, Jonsson PR, Larsson AI, Holdt S (2004) Rejection of unsuitable substrata as a potential driver of aggregated settlement in the barnacle *Balanus improvisus*. *Mar Ecol Prog Ser* 275:199–210
- Bers AV, Wahl M (2004) The influence of natural surface microtopographies on fouling. *Biofouling* 20:43–51
- Bewley CA, Holland ND, Faulkner DJ (1996) Two classes of metabolites from *Theonella swinhoei* are localized in distinct populations of bacterial symbionts. *Experientia* 52:716–722
- Bloom SA (1975) The motile escape response of a sessile prey: a sponge–scallop mutualism. *J Exp Mar Biol Ecol* 17:311–321
- Bobzin S, Faulkner D (1992) Chemistry and chemical ecology of the Bahamian sponge *Aplysilla glacialis*. *J Chem Ecol* 18:309–332
- Boyd KG, Adams DR, Burgess JG (1999) Antibacterial and repellent activities of marine bacteria associated with algal surfaces. *Biofouling* 14:227–236
- Brancato M, Woollacott R (1982) Effect of microbial films on settlement of bryozoan larvae (*Bugula simplex*, *B. stolonifera* and *B. turrita*). *Mar Biol* 71:51–56
- Browne KA, Zimmer RK (2001) Controlled field release of a waterborne chemical signal stimulates planktonic larvae to settle. *Biol Bull* 200:87–91
- Bryan PJ, Rittschof D, Qian PY (1997) Settlement inhibition of bryozoan larvae by bacterial films and aqueous leachates. *Bull Mar Sci* 61:849–857
- Bryan PJ, McClintock JB, Slattery M, Rittschof DP (2003) A comparative study of the non-acidic chemically mediated antifouling properties of three sympatric species of ascidians associated with seagrass habitats. *Biofouling* 19:235–245
- Bulthuis DA, Woelkerling WJ (1983) Biomass accumulation and shading effects of epiphytes on leaves of seagrass, *Heterozostera tasmanica*, in Victoria, Australia. *Aquat Bot* 16:137–148
- Burgess JG, Jordan EM, Bregu M, Mearns-Spragg, Boyd KG (1999) Microbial antagonism: a neglected avenue of natural products research. *J Biotechnol* 70:27–32
- Burkholder PR, Rutzler K (1969) Antimicrobial activity of some marine sponges. *Nature* 222:983–984
- Butman C (1987) Larval settlement of soft-sediment invertebrates: the spatial scales of pattern explained by active habitat selection and the emerging role of hydrodynamical processes. *Oceanogr Mar Biol Annu Rev* 25:113–165
- Carr WES, Netherton JC, Gleeson RA, Derby CD (1996) Stimulants of feeding behavior in fish: analyses of tissues of diverse marine organisms. *Biol Bull* 190:149–160

- Clare AS (1996) Marine natural product antifoulants: status and potential. *Biofouling* 9: 211–229
- Clare AS, Matsumura K (2000) Nature and perception of barnacle settlement pheromones. *Biofouling* 15:57–71
- Clare AS, Rittschof D, Gerhart DJ, Hooper IR, Bonaventura J (1999) Antisetlement and narcotic action of analogues of diterpene marine natural product antifoulants from octocorals. *Mar Biotechnol* 1:427–436
- Coll JC, Bowden BF, Tapiolas DM, Dunlap WC (1982) In situ isolation of allelochemicals released from soft corals (Coelenterata: Octocorallia): a totally submersible sampling apparatus. *J Exp Mar Biol Ecol* 60:293–299
- Coll J, Price I, Konig G, Bowden B (1987) Algal overgrowth of alcyonacean soft corals. *Mar Biol* 96:129–135
- Compere C, Bellon-Fortaine M-N, Bertrand P, Costa D, Marcus P, Poleunis C, Pradier C-M, Rondot B, Walls MG (2001) Kinetics of conditioning layer formation on stainless steel immersed in seawater. *Biofouling* 17:129–145
- Cowden C, Young CM, Chia F-S (1984) Differential predation on marine invertebrate larvae by two benthic predators. *Mar Ecol Prog Ser* 14:145–149
- Crisp DJ (1974) Factors influencing the settlement of marine invertebrate larvae. In: Grant PT, Machie AM (eds) *Chemoreception in marine organisms*. Academic Press, New York, pp 177–265
- Crisp, Meadows (1963) Adsorbed layers: the stimulus to settlement in barnacles. *Proc R Soc Lond B* 158:364–387
- Cuba TR, Blake NJ (1983) The initial development of a marine fouling assemblage on a natural substrate in a subtropical estuary. *Bot Mar* 26:259–264
- Da Gama BAP, Pereira RC, Soares AR, Teixeira VL, Yoneshigue-Valentin Y (2003) Is the mussel test a good indicator of antifouling activity? A comparison between laboratory and field assays. *Biofouling* 19:161–169
- Daume S, Brand-Gardner S, Woelkerling W (1999) Preferential settlement of abalone larvae: diatom films versus non-geniculate coralline red algae. *Aquaculture* 174: 243–254
- Davidson SK, Haygood MG (1999) Identification of sibling species of the bryozoan *Bugula neritina* that produce different anticancer bryostatins and harbor distinct strains of the bacterial symbiont “*Candidatus* Endobugula sertula”. *Biol Bull* 196:273–280
- Davidson SK, Allen SW, Lim GE, Anderson CM, Haygood MG (2001) Evidence for the biosynthesis of bryostatins by the bacterial symbiont “*Candidatus* Endobugula sertula” of the bryozoan *Bugula neritina*. *Appl Environ Microbiol* 67:4531–4537
- Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP (1998) The involvement of cell-to-cell signalling in the development of a bacterial biofilm. *Science* 280:295–298
- Davis AR (1991) Alkaloids and ascidian chemical defense: evidence for the ecological role of natural products from *Eudistoma olivaceum*. *Mar Biol* 111:375–379
- Davis AR, Wright AE (1989) Interspecific differences in fouling of two congeneric ascidians (*Eudistoma olivaceum* and *Eudistoma capsulatum*): is surface-acidity an effective defense. *Mar Biol* 102:491–497
- Davis AR, Wright AE (1990) Inhibition of larval settlement by natural products from the ascidian, *Eudistoma olivaceum* (Van Name). *J Chem Ecol* 16:1349–1357
- Davis AR, White GA (1994) Epibiosis in a guild of sessile subtidal invertebrates in south-eastern Australia: a quantitative survey. *J Exp Mar Biol Ecol* 177:1–14
- Davis AR, Targett NM, McConnell OJ, Young CM (1989) Epibiosis of marine algae and benthic invertebrates: natural products chemistry and other mechanisms inhibiting settlement and overgrowth. In: Scheuer PJ (ed) *Bioorganic marine chemistry*. Springer, Berlin Heidelberg New York, pp 85–114
- Davis AR, Butler AJ, Vanaltena I (1991) Settlement behavior of ascidian larvae: preliminary evidence for inhibition by sponge allelochemicals. *Mar Ecol Prog Ser* 72:117–123

- Davis AR, Ayre DJ, Billingham MR, Styan CA, White GA (1996) The encrusting sponge *Halisarca laxus*: population genetics and association with the ascidian *Pyura spinifera*. *Mar Biol* 126:27–33
- De Moraes CM, Lewis WJ, Pare PW, Alborn HT, Tumlinson JH (1998) Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573
- De Nys R, Leya T, Maximilien R, Afsar A, Nair PSR, Steinberg PD (1996) The need for standardised broad scale bioassay testing: a case study using the red alga *Laurencia rigida*. *Biofouling* 10:213–224
- Dixon J, Schroeter SC, Kastendiek J (1981) Effects of encrusting bryozoan, *Membranipora membranacea*, on the loss of blades and fronds by the giant kelp, *Macrocystis pyrifera* (Laminariales). *J Phycol* 17:341–345
- Dobretsov SV, Qian PY (2002) Effect of bacteria associated with the green alga *Ulva reticulata* on marine micro- and macrofouling. *Biofouling* 18:217–228
- Dobretsov S, Qian PY (2004) The role of epibiotic bacteria from the surface of the soft coral *Dendronephthya* sp in the inhibition of larval settlement. *J Exp Mar Biol Ecol* 299: 35–50
- Dobretsov S, Dahms HU, Qian PY (2004) Antilarval and antimicrobial activity of waterborne metabolites of the sponge *Callyspongia (Euplacella) pulvinata*: evidence of allelopathy. *Mar Ecol Prog Ser* 271:133–146
- Ducklow HW, Mitchell R (1979) Bacterial populations and adaptations in the mucus layers on living corals. *Limnol Oceanogr* 24:715–725
- Dworjanyn SA, De Nys R, Steinberg PD (1999) Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*. *Mar Biol* 133:727–736
- Dyrynda P (1986) Defensive strategies of modular organisms. *Philos Trans R Soc Lond B* 313:227–243
- Eckman JE (1996) Closing the larval loop: linking larval ecology to the population dynamics of marine benthic invertebrates. *J Exp Mar Biol Ecol* 200:207–237
- Egan S, Thomas T, Holmström C, Kjelleberg S (2000) Phylogenetic relationship and antifouling activity of bacterial epiphytes from the marine alga *Ulva lactuca*. *Environ Microbiol* 2:343–347
- Enderlein P, Moorthi S, Rohrscheidt H, Wahl M (2003) Optimal foraging versus shared doom effects: interactive influence of mussel size and epibiosis on predator preference. *J Exp Mar Biol Ecol* 292:231–242
- Engel S, Jensen PR, Fenical W (2002) Chemical ecology of marine microbial defense. *J Chem Ecol* 28:1971–1985
- Faimali M, Sepcic K, Turk T, Geraci S (2003) Non-toxic antifouling activity of polymeric 3-alkylpyridinium salts from the Mediterranean sponge *Reniera sarai* (Pulitzer- Finali). *Biofouling* 19:47–56
- Faulkner DJ (1984) Marine natural products – metabolites of marine invertebrates. *Nat Prod Rep* 1:551–598
- Fearon RJ, Cameron AM (1997) Preliminary evidence supporting the ability of hermatypic corals to affect adversely larvae and early settlement stages of hard coral competitors. *J Chem Ecol* 23:1769–1780
- Feifarek BP (1987) Spines and epibionts as antipredator defenses in the thorny oyster *Spondylus americanus* Hermann. *J Exp Mar Biol Ecol* 105:39–56
- Fenical W (1993) Chemical studies of marine bacteria – developing a new resource. *Chem Rev* 93:1673–1683
- Fieseler L, Horn M, Wagner M, Hentschel U (2004) Discovery of the novel candidate phylum “*Poribacteria*” in marine sponges. *Appl Environ Microbiol* 70:3724–3732
- Filion-Myklebust C, Norton T (1981) Epidermis shedding in the brown seaweed *Ascophyllum nodosum* (L.) Le Jolis and its ecological significance. *Mar Biol Lett* 2:45–51
- Fishlyn DA, Phillips DW (1980) Chemical camouflaging and behavioral defense against a predatory seastar by three species of gastropods from the surf grass *Phyllospadix* community. *Biol Bull* 158:34–48

- Fletcher M, McEldowney S (1984) Microbial attachment to non-biological surfaces. In: Klug M, Reddy C (eds) Current perspectives in microbial ecology. Proc 3rd Int Symp Microb Ecol, 1983, pp 124–129
- Forester AJ (1979) The association between the sponge *Halichondria panicea* (Pallas) and scallop *Chlamys varia* (L.): a commensal-protective mutualism. J Exp Mar Biol Ecol 36:1–10
- Forward RB, Rittschof D (1994) Photoresponses of crab megalopae in offshore and estuarine waters: implications for transport. J Exp Mar Biol Ecol 182:183–192
- Fujita M, Nakao Y, Matsunaga S, Seiki M, Itoh Y, van Soest RWM, Fusetani N (2001) Ancorinosides B-D, inhibitors of membrane type 1 matrix metalloproteinase (MT1-MMP), from the marine sponge *Penares sollasi* Thiele. Tetrahedron 57:1229–1234
- Fusetani N (1997) Marine natural products influencing larval settlement and metamorphosis of benthic invertebrates. Curr Org Chem 1:127–152
- Fusetani N (2004) Biofouling and antifouling. Nat Prod Rep 21:94–104
- Fusetani N, Hiroto H, Okino T, Tomono Y, Yoshimura E (1996) Antifouling activity of isocyanoterpenoids and related compounds isolated from a marine sponge and nudibranchs. J Nat Toxins 5:249–259
- Geesey GG (2001) Bacterial behavior at surfaces. Curr Opin Microbiol 4:296–300
- Gerhart D, Rittschof D, Mayo S (1988) Chemical ecology and the search for marine antifoulants. Studies of a predator-prey symbiosis. J Chem Ecol 14:1905–1917
- Gil-Turnes MS, Fenical W (1992) Embryos of *Homarus americanus* are protected by epibiotic bacteria. Bioll Bull 182:105–108
- Gil-Turnes MS, Hay ME, Fenical W (1989) Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. Science 246:116–118
- Hadfield MG, Unabia CRC, Smith CM, Michael TM (1994) Settlement preferences of the ubiquitous fouler *Hydroides elegans*. In: Thompson MF, Nagabhushanam R, Sarojini R, Fingerma M (eds) Recent developments in biofouling control. Oxford and IBH, New Delhi, pp 65–74
- Hall-Stoodley L, Stoodley P (2002) Developmental regulation of microbial biofilms. Curr Opin Biotechnol 13:228–233
- Harder T, Qian PY (2000) Waterborne compounds from the green seaweed *Ulva reticulata* as inhibitive cues for larval attachment and metamorphosis in the polychaete *Hydroides elegans*. Biofouling 16:205–214
- Harder TN, Thiyagarajan V, Qian PY (2001) Effect of cyprid age on the settlement of *Balanus amphitrite* Darwin in response to natural biofilms. Biofouling 17:211–219
- Harder T, Lau SCK, Dahms HU, Qian PY (2002a) Isolation of bacterial metabolites as natural inducers for larval settlement in the marine polychaete *Hydroides elegans* (Haswell). J Chem Ecol 28:2029–2043
- Harder T, Lam C, Qian PY (2002b) Induction of larval settlement in the polychaete *Hydroides elegans* by marine biofilms: an investigation of monospecific diatom films as settlement cues. Mar Ecol Prog Ser 229:105–112
- Harder T, Lau SCK, Dobretsov S, Fang TK, Qian PY (2003) A distinctive epibiotic bacterial community on the soft coral *Dendronephthya* sp and antibacterial activity of coral tissue extracts suggest a chemical mechanism against bacterial epibiosis. FEMS Microbiol Ecol 43:337–347
- Hay M (1996) Marine chemical ecology: what's known and what's next? J Exp Mar Biol Ecol 200:103–134
- Hay M, Duffy J, Fenical W (1987) Seaweed chemical defense against diverse coral reef herbivores. Ecology 68:1581–1591
- Head RM, Overbeke K, Klijnstra J, Biersteker R, Thomason JC (2003) The effect of gregariousness in cyprid settlement assays. Biofouling 19:269–278
- Henrikson AA, Pawlik JR (1995) A new antifouling assay method: results from field experiments using extracts of four marine organisms. J Exp Mar Biol Ecol 194:157–165

- Hentschel U, Hopke J, Horn M, Friedrich AB, Wagner M, Hacker J, Moore BS (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol* 68:4431–4440
- Hills JM, Thomason JC, Davis H, Kohler J, Millett E (2000) Exploratory behaviour of barnacle larvae in field conditions. *Biofouling* 16:171–179
- Hirota H, Tomono Y, Fusetani N (1996) Terpenoids with antifouling activity against barnacle larvae from the marine sponge *Acanthella cavernosa*. *Tetrahedron* 52:2359–2368
- Hirota H, Okino T, Yoshimura E, Fusetani N (1998) Five new antifouling sesquiterpenes from two marine sponges of the genus *Axinyssa* and the nudibranch *Phyllidia pustulosa*. *Tetrahedron* 54:13971–13980
- Holm ER (1990) Attachment behavior in the barnacle *Balanus amphitrite amphitrite* (Darwin): genetic and environmental effects. *J Exp Mar Biol Ecol* 135:85–98
- Holmström C, Kjelleberg S (1993) Development of a tissue cell culture bioassay for identifying mechanisms of inhibition of settlement of barnacle and tunicate larvae by surface-colonizing marine bacteria. *Biofouling* 7:329–337
- Holmström C, Kjelleberg S (1994) The effect of external biological factors on settlement of marine invertebrates and new antifouling technology. *Biofouling* 8:147–160
- Holmström C, Kjelleberg S (1999) Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol Ecol* 30:285–293
- Holmström C, Kjelleberg S (2000) Bacterial interaction with marine fouling organisms. In: Evans LV (ed) *Biofilms: recent advances in their study and control*. Harwood Academic Publ, Amsterdam, pp 101–115
- Holmström C, Rittschof D, Kjelleberg S (1992) Inhibition of settlement by larvae of *Balanus amphitrite* and *Ciona intestinalis* by a surface-colonizing marine bacterium. *Appl Environ Microbiol* 58:2111–2115
- Holmström C, James S, Egan S, Kjelleberg S (1996) Inhibition of common fouling organisms by marine bacterial isolates with special reference to the role of pigmented bacteria. *Biofouling* 10:251–259
- Holmström C, Egan S, Franks A, McCloy S, Kjelleberg S (2002) Antifouling activities expressed by marine surface associated *Pseudoalteromonas* species. *FEMS Microbiol Ecol* 41: 47–58
- Huang SY, Hadfield MG (2003) Composition and density of bacterial biofilms determine larval settlement of the polychaete *Hydroides elegans*. *Mar Ecol Prog Ser* 260: 161–172
- Iken K, Amsler CD, Greer SR, McClintock JB (2001) Qualitative and quantitative studies of the swimming behaviour of *Hincksia irregularis* (Phaeophyceae) spores: ecological implications and parameters for quantitative swimming assays. *Phycologia* 40:359–366
- Iken K, Greer SP, Amsler CD, McClintock JB (2003) A new antifouling bioassay monitoring brown algal spore swimming behaviour in the presence of echinoderm extracts. *Biofouling* 19:327–334
- Ito S, Kitamura H (1997) Induction of larval metamorphosis in the sea cucumber *Stichopus japonicus* by periphytic diatoms. *Hydrobiologia* 358:281–284
- James S, Holmström C, Kjelleberg S (1996) Purification and characterization of a novel antibacterial protein from the marine bacterium D2. *Appl Environ Microbiol* 62: 2783–2788
- Jarrett JN (1997) Temporal variation in substratum specificity of *Semibalanus balanoides* (Linnaeus) cyprids. *J Exp Mar Biol Ecol* 211:103–114
- Jennings JG, Steinberg PD (1994) In situ exudation of phlorotannins by the sublittoral kelp *Ecklonia radiata*. *Mar Biol* 121:349–354
- Jensen PR, Harvell CD, Wirtz K, Fenical W (1996) Antimicrobial activity of extracts of Caribbean gorgonian corals. *Mar Biol* 125:411–419
- Johannes RE (1967) Ecology of organic aggregates in the vicinity of a coral reef. *Limnol Oceanogr* 12:189–195
- Joint I, Callow ME, Callow JA, Clarke KR (2000) The attachment of *Enteromorpha* zoospores to a bacterial biofilm assemblage. *Biofouling* 16:151–158

- Kawamata M, Konya K, Miki W (1994) Trigonelline, an antifouling substance isolated from an octocoral *Dendronephthya* Sp. Fish Sci 60:485–486
- Kelly SR, Jensen PR, Henkel TP, Fenical W, Pawlik JR (2003) Effects of Caribbean sponge extracts on bacterial attachment. Aquat Microb Ecol 31:175–182
- Keough MJ (1984) Dynamics of the epifauna of the bivalve *Pinna bicolor*: interactions among recruitment, predation, and competition. Ecology 65:667–688
- Keough MJ (1989) Kin recognition and the spatial distribution of larvae of the bryozoan *Bugula neritina*. Evolution 38:142–147
- Keough MJ, Raimondi PT (1995) Responses of settling invertebrate larvae to bioorganic films: effects of different types of films. J Exp Mar Biol Ecol 185:235–253
- Keifer PA, Rinehart KL, Hooper IR (1986) Renillafoulins, antifouling diterpenes from the sea pansy *Renilla reniformis* (Octocorallia). J Org Chem 51:4450–4454
- Keifer PA, Schwartz RE, Koker MES, Hughes RG, Rittschof D, Rinehart KL (1991) Bioactive bromopyrrole metabolites from the Caribbean sponge *Agelas conifera*. J Org Chem 56:2965–2975
- Keough MJ, Raimondi PT (1996) Responses of settling invertebrate larvae to bioorganic films: Effects of large-scale variation in films. J Exp Mar Biol Ecol 207:59–78
- Kirchman D, Graham S, Reish D, Mitchell R (1982) Lectins may mediate in the settlement and metamorphosis of *Janua* (Dexiospira) *brasiliensis* Grube (Polychaeta: Spirorbidae). Mar Biol Lett 3:131–142
- Koh EGL, Sweatman H (2000) Chemical warfare among scleractinians: bioactive natural products from *Tubastraea falkneri* Wells kill larvae of potential competitors. J Exp Mar Biol Ecol 251:141–160
- Koh EGL, Tan TK, Chou LM, Goh NKC (2002) Antifungal properties of Singapore gorgonians: a preliminary study. J Exp Mar Biol Ecol 273:121–130
- Kon-Ya K, Shimidzu N, Adachi K, Miki W (1994a) 2,5,6-tribromo-1-methylgramine, an antifouling substance from the marine bryozoan *Zoobrottryon pellucidum*. Fish Sci 60:773–775
- Kon-Ya K, Shimidzu N, Miki W, Endo M (1994b) Indole derivatives as potent inhibitors of larval settlement by the barnacle, *Balanus amphitrite*. Biosci Biotechnol Biochem 58:2178–2181
- Kon-Ya K, Shimidzu N, Otaki N, Yokoyama A, Adachi K, Miki W (1995) Inhibitory effect of bacterial ubiquinones on the settling of barnacle, *Balanus amphitrite*. Experientia 51:153–155
- Konig GM, Wright AD (1997) *Laurencia rigida*: chemical investigations of its antifouling dichloromethane extract. J Nat Prod 60:967–970
- Krug PJ, Zimmer RK (2000) Developmental dimorphism and expression of chemosensory-mediated behavior: Habitat selection by a specialist marine herbivore. J Exp Biol 203:1741–1754
- Krug PJ, Boyd KG, Faulkner DJ (1995) Isolation and synthesis of Tanyolides A and B, metabolites of the nudibranch *Sclerodoris tanya*. Tetrahedron 51:11063–11074
- Kubaneck J, Whalen KE, Engel S, Kelly SR, Henkel TP, Fenical W, Pawlik JR (2002) Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 131:125–136
- Lam C, Harder T, Qian PY (2003) Induction of larval settlement in the polychaete *Hydroides elegans* by surface-associated settlement cues of marine benthic diatoms. Mar Ecol Prog Ser 263:83–92
- Lau SCK, Mak KKW, Chen F, Qian PY (2002) Bioactivity of bacterial strains isolated from marine biofilms in Hong Kong waters for the induction of larval settlement in the marine polychaete *Hydroides elegans*. Mar Ecol Prog Ser 226:301–310
- Lau SCK, Thiyagarajan V, Qian PY (2003) The bioactivity of bacterial isolates in Hong Kong waters for the inhibition of barnacle (*Balanus amphitrite* Darwin) settlement. J Exp Mar Biol Ecol 282:43–60
- Laudien J, Wahl M (1999) Indirect effects of epibiosis on host mortality: seastar predation on differently fouled mussels. Mar Ecol 20:35–47

- Lee OO, Qian PY (2003) Chemical control of bacterial epibiosis and larval settlement of *Hydroides elegans* in the red sponge *Mycale adherens*. *Biofouling* 19:171–180
- Leitz T, Wagner T (1993) The marine bacterium *Alteromonas espejiana* induces metamorphosis of the hydroid *Hydractinia echinata*. *Mar Biol* 115:173–178
- Littler MM, Littler DS (1999) Blade abandonment/proliferation: a novel mechanism for rapid epiphyte control in marine macrophytes. *Ecology* 80:1736–1746
- Lopanik N, Lindquist N, Targett N (2004) Potent cytotoxins produced by a microbial symbiont protect host larvae from predation. *Oecologia* 139:131–139
- Mafra-Neto A, Cardé RT (1994) Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144
- Maida M, Sammarco PW, Coll JC (1995) Effects of soft corals on scleractinian coral recruitment. I. Directional allelopathy and inhibition of settlement. *Mar Ecol Prog Ser* 121:191–202
- Maki J, Rittschof D, Costlow J, Mitchell R (1988) Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial biofilms. *Mar Biol* 97:199–206
- Maldonado M, Young CM (1999) Effects of the duration of larval life on postlarval stages of the demosponge *Sigmadocia caerulea*. *J Exp Mar Biol Ecol* 232:9–21
- Manning LM, Lindquist N (2003) Helpful habitant or pernicious passenger: interactions between an infaunal bivalve, an epifaunal hydroid and three potential predators. *Oecologia* 134:415–422
- Maximilien R, de Nys R, Holmstrom C, Gram L, Givskov M, Crass K, Kjelleberg S, Steinberg PD (1998) Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga *Delisea pulchra*. *Aquat Microb Ecol* 15:233–246
- McClintock JB, Baker BJ, Hamann MT, Yoshida W, Slattery M, Heine JN, Bryan PJ, Jayatilake GS, Moon BH (1994) Homarine as a feeding deterrent in common shallow-water Antarctic lamellarian gastropod *Marseniopsis mollis* – a rare example of chemical defense in a marine prosobranch. *J Chem Ecol* 20:2539–2549
- Meadows PS, Williams GB (1963) Settlement of *Spirorbis borealis* Daudin larvae on surfaces bearing films of micro-organisms. *Nature* 198:610–611
- Mearns-Spragg A, Bregu M, Boyd KG, Burgess JG (1998) Cross-species induction and enhancement of anti-microbial activity produced by epiphytic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett Appl Microbiol* 27:142–146
- Mihm JW, Banta WC (1981) Effects of adsorbed organic and primary fouling films on bryozoan settlement. *J Exp Mar Biol Ecol* 54:167–179
- Minchin D, Gollasch S (2003) Fouling and ships' hulls: how changing circumstances and spawning events may result in the spread of exotic species. *Biofouling* 19:111–122
- Morse DE, Hooker N, Duncan H, Jensen L (1979) γ -Aminobutyric acid, a neurotransmitter, induces planktonic abalone larvae to settle and begin metamorphosis. *Science* 204:407–410
- Mullineaux LS, Butman CA (1991) Initial contact, exploration, and attachment of barnacle cyprids settling in flow. *Mar Biol* 110:93–103
- Nakao Y, Masuda A, Matsunaga S, Fusetani N (1999) Pseudotheonamides, serine protease inhibitors from the marine sponge *Theonella swinhoei*. *J Am Chem Soc* 121:2425–2431
- Nakao Y, Fujita M, Warabi K, Matsunaga S, Fusetani N (2000) Bioactive marine metabolites, part 104. Miraziridine A, a novel cysteine protease inhibitor from the marine sponge *Theonella aff. mirabilis*. *J Am Chem Soc* 122:10462–10463
- Neal AL, Yule AB (1994a) The interaction between *Elminius modestus* Darwin cyprids and biofilms of *Deleya marina* NCMB1877. *J Exp Mar Biol Ecol* 176:123–139
- Neal AL, Yule AB (1994b) The tenacity of *Elminius modestus* and *Balanus perforatus* cyprids to bacterial films grown under different shear regimes. *J Mar Biol Assoc UK* 74:251–257

- Negri AP, Webster NS, Hill RT, Heyward AJ (2001) Metamorphosis of broadcast spawning corals in response to bacteria isolated from crustose algae. *Mar Ecol Prog Ser* 223:121–131
- Netherton JC, Gurin S (1982) Biosynthesis and physiological role of homarine in marine shrimp. *J Biol Chem* 257:1971–1975
- Newbold RW, Jensen PR, Fenical W, Pawlik JR (1999) Antimicrobial activity of Caribbean sponge extracts. *Aquat Microb Ecol* 19:279–284
- Nevitt GA, Veit RR, Karieva PM (1995) Dimethyl sulfide as a foraging cue for Antarctic Procellariiform seabirds. *Nature* 376:680–682
- Ng TY-T, Keough MJ (2003) Delayed effects of larval exposure to Cu in the bryozoan *Watersipora subtorquata*. *Mar Ecol Prog Ser* 257:77–85
- Okino T, Yoshimura E, Hirota H, Fusetani N (1995) Antifouling kalihinenes from the marine sponge *Acanthella cavernosa*. *Tetrahedron Lett* 36:8637–8640
- Okino T, Yoshimura E, Hirota H, Fusetani N (1996a) New antifouling kalihipyranes from the marine sponge *Acanthella cavernosa*. *J Nat Prod* 59:1081–1083
- Okino T, Yoshimura E, Hirota H, Fusetani N (1996b) New antifouling sesquiterpenes from four nudibranchs of the family Phyllidiidae. *Tetrahedron* 52:9447–9454
- Okuda RK, Scheuer PJ, Hochlowski JE, Walker RP, Faulkner DJ (1983) Sesquiterpenoid constituents of eight porostome nudibranchs. *J Org Chem* 48:1866–1869
- O'Toole G, Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. *Mol Microbiol* 30:295–304
- Paul J, Jeffrey W (1985) Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio proteolytica*. *Appl Environ Microbiol* 50:431–437
- Pawlik J (1992) Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr Mar Biol Annu Rev* 30:273–335
- Pawlik JR (1993) Marine invertebrate chemical defenses. *Chem Rev* 93:1911–1922
- Pawlik JR, Butman CA (1993) Settlement of a marine tube worm as a function of current velocity – interacting effects of hydrodynamics and behavior. *Limnol Oceanogr* 38:1730–1740
- Pettit GR, Herald CL, Doubek DL, Herald DL (1982) Isolation and structure of bryostatin 1. *J Am Chem Soc* 104:6846–6848
- Pettit GR, Kamano Y, Aoyagi R, Herald CL, Doubek DL, Schmidt JM, Rudloe JJ (1985) Antineoplastic agents 100: the marine bryozoan *Amathia convoluta*. *Tetrahedron* 41:985–994
- Polychronopoulos P, Magiatis P, Skaltsounis AL, Tillequin F, Vardala-Theodorou E, Tsarbopoulos A (2001) Homarine, a common metabolite in edible Mediterranean molluscs: Occurrence, spectral data and revision of a related structure. *Nat Prod Lett* 15:411–418
- Qian PY, Thiyagarajan V, Lau SCK, Cheung SCK (2003) Relationship between bacterial community profile in biofilm and attachment of the acorn barnacle *Balanus amphitrite*. *Aquat Microb Ecol* 33:225–237
- Raimondi PT, Keough MJ (1990) Behavioral variability in marine larvae. *Aust J Ecol* 15:427–437
- Renner MK, Shen YC, Cheng XC, Jensen PR, Frankmoelle W, Kauffman CA, Fenical W, Lobkovsky E, Clardy J (1999) Cyclomarins A-C, new antiinflammatory cyclic peptides produced by a marine bacterium (*Streptomyces* sp.). *J Am Chem Soc* 121:11273–11276
- Rinehart KL, Kobayashi J, Harbour GC, Gilmore J, Mascal M, Holt TG, Shield LS, Lafargue F (1987) Eudistomins A-Q, beta carbolines from the antiviral Caribbean tunicate *Eudistoma olivaceum*. *J Am Chem Soc* 109:3378–3387
- Rittschof D, Hooper IR, Costlow JD (1988) Settlement inhibition of marine invertebrate larvae: comparison of sensitivities of bryozoan and barnacle larvae. In: Thompson MF, Sarojini R, Nagabhushanam R (eds) *Marine biodeterioration*. Oxford and IBJ Publ, New Delhi, pp 599–608
- Rohwer F, Seguritan V, Azam F, Knowlton N (2002) Diversity and distribution of coral-associated bacteria. *Mar Ecol Prog Ser* 243:1–10

- Roughgarden J, Gaines S, Possingham H (1988) Recruitment dynamics in complex life cycles. *Science* 241:1460–1466
- Russell G, Veltkamp CJ (1984) Epiphyte survival on skin-shedding macrophytes. *Mar Ecol Prog Ser* 18:149–153
- Sand-Jensen K, Revsbech E (1987) Photosynthesis and light adaptation in epiphyte-macrophyte associations measured by oxygen microelectrodes. *Limnol Oceanogr* 32:452–457
- Sata NU, Sugano M, Matsunaga S, Fusetani N (1999) Bioactive marine metabolites, part 88. Sinulamide: an H,K-ATPase inhibitor from a soft coral *Sinularia* sp. *Tetrahedron Lett* 40:719–722
- Sato A, Fenical W (1983) Gramine-derived bromo-alkaloids from the marine bryozoan *Zoobotryon verticillatum*. *Tetrahedron Lett* 24:481–484
- Sauer K, Camper A, Erhlich G, Costerton J, Davies D (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184:1140–1154
- Scardino A, de Nys R, Ison O, O'Connor W, Steinberg PD (2003) Microtopography and antifouling properties of the shell surface of the bivalve molluscs *Mytilus galloprovincialis* and *Pinctada imbricata*. *Biofouling* 19:221–230
- Schmitt TM, Hay ME, Lindquist N (1995) Constraints on chemically mediated coevolution – multiple functions for seaweed secondary metabolites. *Ecology* 76:107–123
- Schmitt TM, Lindquist N, Hay ME (1998) Seaweed secondary metabolites as antifoulants: effects of *Dictyota* spp. diterpenes on survivorship, settlement and development of marine invertebrate larvae. *Chemoecology* 8:125–131
- Schulte B, de Nys R, Bakus G, Crews P, Eid C, Naylor S, Manes L (1991) A modified allomone collecting apparatus. *J Chem Ecol* 17:1327–1332
- Sears MA, Gerhart DJ, Rittschof D (1990) Antifouling agents from the marine sponge *Lissodendoryx isodictyalis* Carter. *J Chem Ecol* 16:791–799
- Shinagawa A, Suzuki T, Konosu S (1995) Preliminary studies on the effects of salinity on intracellular nitrogenous osmolytes in various tissues and hemolymph of the Japanese spiny lobster, *Panulirus japonicus* (Vonsiebold, 1824). *Crustaceana* 68:129–137
- Sieburth J, Tootle J (1981) Seasonality of microbial fouling on *Ascophyllum nodosum* (L.) Lejoll, *Fucus vesiculosus* L., *Polysiphonia lanosa* (L.) Tandy and *Chondrus crispus* Stackh. *J Phycol* 17:57–64
- Sjogren M, Goransson U, Johnson AL, Dahlstrom M, Andersson R, Bergman J, Jonsson PR, Bohlin L (2004) Antifouling activity of brominated cyclopeptides from the marine sponge *Geodia barretti*. *J Nat Prod* 67:368–372
- Slattery M (1994) A comparative study of population structure and chemical defenses in the soft corals *Alcyonium paessleri* May, *Clavularia frankliniana* Rouel, and *Gersemia antarctica* Kukenthal in McMurdo Sound, Antarctica. PhD Thesis, University of Alabama at Birmingham, 109 pp
- Slattery M, McClintock JB, Heine JN (1995) Chemical defenses in Antarctic soft corals: evidence for antifouling compounds. *J Exp Mar Biol Ecol* 190:61–77
- Slattery M, Hamann MT, McClintock JB, Perry TL, Puglisi MP, Yoshida WY (1997) Ecological roles for water-borne metabolites from Antarctic soft corals. *Mar Ecol Prog Ser* 161:133–144
- Stachowicz JJ, Hay ME (1999) Reducing predation through chemically mediated camouflage: indirect effects of plant defenses on herbivores. *Ecology* 80:495–509
- Standing J, Hooper IR, Costlow JD (1984) Inhibition and induction of barnacle settlement by natural products present in octocorals. *J Chem Ecol* 10:823–834
- Steinberg PD, de Nys R, Kjelleberg S (1998) Chemical inhibition of epibiota by Australian seaweeds. *Biofouling* 12:227–244
- Steinberg PD, de Nys R, Kjelleberg S (2001) Chemical mediation of surface colonization. In: McClintock J, Baker B (eds) *Marine chemical ecology*. CRC Press, London, pp 355–387
- Stoecker D (1978) Resistance of a tunicate to fouling. *Biol Bull* 155:615–626
- Stoecker D (1980) Relationships between chemical defense and ecology in benthic ascidians. *Mar Ecol Prog Ser* 3:257–265

- Strathmann R, Branscomb E, Vedder K (1981) Fatal errors in set as a cost of dispersal and the influence of intertidal flora on set of barnacles. *Oecologia* 48:13–18
- Sutherland IW (2001) The biofilm matrix – an immobilized but dynamic microbial environment. *Trends Microbiol* 9:222–227
- Szewzyk U, Holmstrom C, Wrangstadh M, Samuelsson MO, Maki JS, Kjelleberg S (1991) Relevance of the exopolysaccharide of marine *Pseudomonas* sp. strain S9 for the attachment of *Ciona intestinalis* Larvae. *Mar Ecol Prog Ser* 75:259–265
- Takewati M, Provasoli L, Pinter IJ (1983) Morphogenesis of *Monostroma oxysperma* (Kütz.) Doty (Chlorophyceae) in axenic culture, especially in bi-algal culture. *J Phycol* 19:404–416
- Tamburri MN, Zimmer-Faust RK (1996) Suspension feeding: basic mechanisms controlling recognition and ingestion of larvae. *Limnol Oceanogr* 41:1188–1197
- Tamburri MN, Zimmer-Faust RK, Tamplin ML (1992) Natural sources and properties of chemical inducers mediating settlement of oyster larvae – a reexamination. *Biol Bull* 183:327–338
- Tamburri MN, Finelli CM, Wethey DS, Zimmer-Faust RK (1996) Chemical induction of larval settlement behavior in flow. *Biol Bull* 191:367–373
- Tapiolas DM, Roman M, Fenical W, Stout TJ, Clardy J (1991) Octalactin A and octalactin B: cytotoxic 8-membered-ring lactones from a marine bacterium, *Streptomyces* sp. *J Am Chem Soc* 113:4682–4683
- Targett NM, Bishop SS, McConnell OJ, Yoder JA (1983) Antifouling agents against the benthic marine diatom, *Navicula salinicola* - homarine from the gorgonians *Leptogorgia virgulata* and *L. setacea* and analogs. *J Chem Ecol* 9:817–829
- Tarjuelo I, Lopez-Legentil S, Codina M, Turon X (2002) Defense mechanisms of adults and larvae of colonial ascidians: patterns of palatability and toxicity. *Mar Ecol Prog Ser* 235:103–115
- Taylor EM, Zheng D, Lee M, Troy PJ, Gyananath G, Sharma SK (1997) Influence of surface properties on accumulation of conditioning films and marine bacteria on substrata exposed to oligotrophic waters. *Biofouling* 11:31–57
- Taylor MW, Schupp PJ, Baillie HJ, Charlton TS, de Nys R, Kjelleberg S, Steinberg PD (2004a) Evidence for acyl homoserine lactone signal production in bacteria associated with marine sponges. *Appl Environ Microbiol* 70:4387–4389
- Taylor MW, Schupp PJ, Dahllöf I, Kjelleberg S, Steinberg PD (2004b) Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity. *Environ Microbiol* 6:121–130
- Thomason JC, Hills JM, Mapson P (2000) The consequences of seasonal reproductive strategies for the interpretation of settlement trials. *Biofouling* 16:323–329
- Thomason JC, Hills JM, Thomason PO (2002) Field-based behavioural bioassays for testing the efficacy of antifouling coatings. *Biofouling* 18:285–292
- Thompson JE (1985) Exudation of biologically active metabolites in the sponge *Aplysina fistularis*. 1. Biological evidence. *Mar Biol* 88:23–26
- Thompson J, Barrow K, Faulkner D (1983) Localization of two brominated metabolites, arothionin and homoarothionin, in spherulous cells of the marine sponge *Aplysina fistularis* (= *Verongia thiona*). *Acta Zool* 64:199–210
- Thompson JE, Walker RP, Faulkner DJ (1985) Screening and bioassays for biologically-active substances from 40 marine sponge species from San Diego, California, USA. *Mar Biol* 88:11–21
- Thompson RC, Norton TA, Hawkins SJ (1998) The influence of epilithic microbial films on the settlement of *Semibalanus balanoides* cyprids – a comparison between laboratory and field experiments. *Hydrobiologia* 376:203–216
- Tomono Y, Hirota H, Fusetani N (1999) Isogosterones A-D, antifouling 13,17-secosteroids from an octocoral *Dendronephthya* sp. *J Org Chem* 64:2272–2275
- Toonen RJ, Pawlik JR (2001) Foundations of gregariousness: a dispersal polymorphism among the planktonic larvae of a marine invertebrate. *Evolution* 55:2439–2454

- Tosuji H, Fusetani N, Seki Y (2003) Calyculin A causes the activation of histone H-1 kinase and condensation of chromosomes in unfertilized sea urchin eggs independently of the maturation-promoting factor. *Comp Biochem Physiol* 135C:415–424
- Trischman JA, Tapiolas DM, Jensen PR, Dwight R, Fenical W, McKee TC, Ireland CM, Stout TJ, Clardy J (1994) Salinamide A and Salinamide B: anti-inflammatory depsipeptides from a marine streptomycete. *J Am Chem Soc* 116:757–758
- Tsakamoto S, Kato H, Hirota H, Fusetani N (1996a) Mauritiamine, a new antifouling oroidin dimer from the marine sponge *Agelas mauritiana*. *J Nat Prod* 59:501–503
- Tsakamoto S, Kato H, Hirota H, Fusetani N (1996b) Ceratinamides A and B: New antifouling dibromotyrosine derivatives from the marine sponge *Pseudoceratina purpurea*. *Tetrahedron* 52:8181–8186
- Tsakamoto S, Kato H, Hirota H, Fusetani N (1997) Antifouling terpenes and steroids against barnacle larvae from marine sponges. *Biofouling* 11:283–291
- Turner EJ, Zimmer-Faust RK, Palmer MA, Luckenback M, Pentcheff ND (1994) Settlement of oyster (*Crassostrea virginica*) larvae: effects of water flow and a water-soluble chemical cue. *Limnol Oceanogr* 39:1579–1593
- Unabia CRC, Hadfield MG (1999) Role of bacteria in larval settlement and metamorphosis of the polychaete *Hydroides elegans*. *Mar Biol* 133:55–64
- Unson MD, Faulkner DJ (1993) Cyanobacterial symbiont biosynthesis of chlorinated metabolites from *Dysidea herbacea* (Porifera). *Experientia* 49:349–353
- Unson MD, Holland ND, Faulkner DJ (1994) A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar Biol* 119:1–11
- Uriz M, Martin D, Turon X, Ballesteros E, Hughes R, Acebal C (1991) An approach to the ecological significance of chemically mediated bioactivity in Mediterranean benthic communities. *Mar Ecol Prog Ser* 70:75–88
- Uriz M, Becerro M, Tur J, Turon X (1996) Location of toxicity within the Mediterranean sponge *Crambe crambe* (Demospongiae: Poecilosclerida). *Mar Biol* 124:583–590
- Vance RR (1978) A mutualistic interaction between a sessile marine clam and its epibionts. *Ecology* 59:679–685
- Vickers NJ, Baker TC (1994) Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc Natl Acad Sci USA* 91:5756–5760
- Wahl M (1989) Marine epibiosis. 1. Fouling and antifouling – some basic aspects. *Mar Ecol Prog Ser* 58:175–189
- Wahl M (1995) Bacterial epibiosis on Bahamian and Pacific ascidians. *J Exp Mar Biol Ecol* 191:239–255
- Wahl M (1996) Fouled snails in flow: potential of epibionts on *Littorina littorea* to increase drag and reduce snail growth rates. *Mar Ecol Prog Ser* 138:157–168
- Wahl M (1997) Increased drag reduces growth of snails: comparison of flume and in situ experiments. *Mar Ecol Prog Ser* 151:291–293
- Wahl M, Lafargue F (1990) Marine epibiosis. 2. Reduced fouling on *Polysyncraton lacazei* (Didemnidae, Tunicata) and proposal of an antifouling potential index. *Oecologia* 82:275–282
- Wahl M, Banaigs B (1991) Marine epibiosis. 3. Possible antifouling defense adaptations in *Polysyncraton lacazei* (Giard) (Didemnidae, Ascidiacea). *J Exp Mar Biol Ecol* 145:49–63
- Wahl M, Sonnichsen H (1992) Marine epibiosis. 4. The periwinkle *Littorina littorea* lacks typical antifouling defenses – why are some populations so little fouled? *Mar Ecol Prog Ser* 88:225–235
- Wahl M, Jensen PR, Fenical W (1994) Chemical control of bacterial epibiosis on ascidians. *Mar Ecol Prog Ser* 110:45–57
- Wahl M, Kroger K, Lenz M (1998) Non-toxic protection against epibiosis. *Biofouling* 12:205–226
- Walker G, Yule AB (1984) Temporary adhesion of the barnacle cyprid: the existence of an antennular adhesive secretion. *J Mar Biol Assoc UK* 64:679–686

- Walker RP, Thompson JE, Faulkner DJ (1985) Exudation of biologically active metabolites in the sponge *Aplysina fistularis*. 2. Chemical evidence. *Mar Biol* 88:27–32
- Walls J, Ritz D, Blackman A (1993) Fouling, surface bacteria, and antibacterial agents of four bryozoan species from in Tasmania, Australia. *J Exp Mar Biol Ecol* 169:1–13
- Warabi K, Matsunaga S, van Soest RWM, Fusetani N (2003) Dictyodendrins A-E, the first telomerase-inhibitory marine natural products from the sponge *Dictyodendrilla verongiformis*. *J Org Chem* 68:2765–2770
- Weissburg MJ, Zimmer-Faust RK (1994) Odor plumes and how blue crabs use them in finding prey. *J Exp Biol* 197:349–375
- Whitely M, Bangera M, Bumgarner R, Parsek M, Teitzel G, Lory S, Greenberg E (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413:860–864
- Wicksten MK (1983) Camouflage in marine invertebrates. *Oceanogr Mar Biol Annu Rev* 21:177–193
- Witman JD, Suchanek TH (1984) Mussels in flow: drag and dislodgement by epizoans. *Mar Ecol Prog Ser* 16:259–268
- Whitchurch CB, Tolker-Nelson T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. *Science* 295:1487
- Wieczorek SK, Clare AS, Todd CD (1995) Inhibitory and facilitatory effects of microbial films on settlement of *Balanus amphitrite amphitrite* larvae. *Mar Ecol Prog Ser* 119:221–228
- Wieczorek SK, Todd CD (1997) Inhibition and facilitation of bryozoan and ascidian settlement by natural multi-species biofilms: effects of film age and the roles of active and passive larval attachment. *Mar Biol* 128:463–473
- Wigglesworth-Cooksey B, Cooksey KE (1996) A computer-based image analysis system for biocide screening. *Biofouling* 10:225–237
- Wikstrom SA, Pavia H (2004) Chemical settlement inhibition versus post-settlement mortality as an explanation for differential fouling of two congeneric seaweeds. *Oecologia* 138:223–230
- Wilsanand V, Wagh AB, Bapuji M (2001) Antifouling activities of octocorals on some microfoulers. *Microbios* 104:131–140
- Willemsen PR, Overbeke K, Suurmond A (1998) Repetitive testing of TBTO, sea-nine 211 and farnesol using *Balanus amphitrite* (Darwin) cypris larvae: Variability in larval sensitivity. *Biofouling* 12:133–147
- Woollacott RM, Hadfield MG (1996) Induction of metamorphosis in larvae of a sponge. *Invert Biol* 115:257–262
- Yan L, Boyd KG, Burgess JG (2002) Surface attachment induced production of antimicrobial compounds by marine epiphytic bacteria using modified roller bottle cultivation. *Mar Biotechnol* 4:356–366
- Yan L, Boyd KG, Adams DR, Burgess JG (2003) Biofilm-specific cross-species induction of antimicrobial compounds in bacilli. *Appl Environ Microbiol* 69:3719–3727
- Zhao B, Qian PY (2002) Larval settlement and metamorphosis in the slipper limpet *Crepidula onyx* (Sowerby) in response to conspecific cues and the cues from biofilm. *J Exp Mar Biol Ecol* 269:39–51
- Zimmer RK, Butman CA (2000) Chemical signaling processes in the marine environment. *Biol Bull* 198:168–187