Chemical Mediation of Surface Colonization

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10 Chemical Mediation of Surface Colonization

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CONTENTS

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I. INTRODUCTION

There is strong evidence for the importance of naturally produced compounds as mediators of ecological interactions between marine benthic consumers and their prey, e.g., in plant–herbivore and predator–prey interactions (reviewed by Hay,[1,2] Hay and Steinberg,[3] McClintock and Baker,[4] Paul,[5] and Pawlik[6]). Dozens of characterized, ecologically relevant feeding deterrents are known from marine benthic organisms (reviewed by Hay[1] and Paul[5]). As a consequence, studies of chemically mediated plant–herbivore or predator–prey interactions have increasingly moved beyond a consideration of simple feeding deterrence to address more complex ecological and evolutionary issues such as induction of defenses,[7,8] specialization of consumers,[3] and geographic variation in defenses.[9–11]

Naturally produced compounds from marine organisms also mediate colonization of surfaces, acting as both positive (inducers) and negative (deterrents) cues for settlement and colonization of animate and inanimate surfaces. However, our understanding of the chemical ecology of colonization of surfaces in marine benthic systems is less advanced than for studies of plant–herbivore and predator–prey interactions. For example, although there is extensive literature on the biology of chemical induction of settlement of invertebrate larvae,[12–14] there is not a single known inducer of invertebrate larval settlement that has been chemically characterized, quantified *in situ,* and demonstrated to be ecologically relevant to the target organism. Characterized, quantified, ecologically relevant examples of deterrent cues — natural antifoulants — are also rare,[15,16] and chemical cues which mediate colonization of surfaces by marine bacteria are largely unknown.

This discrepancy in the amount of direct evidence on the effects of naturally produced compounds in different interactions is probably due to a number of factors intrinsic to these systems. As Pawlik[12] and others have pointed out, invertebrate larvae are often very unpredictable in time and space, making *in situ* ecological studies of many species problematic. In contrast, herbivory and predation are often predictable, pervasive, and intense in marine systems,[2,18–20] and the study organisms often common, macroscopic, and amenable to observations of feeding. The impact of herbivores or predators on their prey in marine benthic systems is well documented,[2,18–21] and strong selection for the evolution of prey chemical defenses is likely to have occurred.[2,4,22] In contrast, demonstrations of the direct[23–26] or indirect[27] impact of colonization by epibiota (fouling) of marine organisms are rare,[28] and the general ecological or evolutionary importance of positive signals (inducers) for colonization is debated, particularly in the context of the effectiveness of chemical cues at different scales in natural flow regimes.[29,30]

Methodologically, both laboratory and field methods for studying the effects of prey chemical defenses on consumers in the laboratory and *in situ* are well established.[31] Similarly, characterizing and then quantifying levels of relevant metabolites in whole marine plants or animals is often relatively straightforward.[32] In contrast, the microscale distribution of chemical cues on or near a surface can crucially determine the efficacy of those cues *in situ*, and methods for the collection and analysis of such samples are not well established. Likewise, for studies of colonization, fewer general, realistic bioassay procedures are available, particularly for *in situ* tests. Many cues for settlement are also water soluble[14,33–35] (see Section III below). Such metabolites are less amenable to traditional techniques of separation chemistry and have thus been harder to characterize and quantify than the small lipophilic secondary metabolites which are the best known examples of chemical mediators of consumer–prey interactions. Finally, for plant–herbivore interactions in particular, there is substantial theoretical literature (mostly derived from terrestrial studies) that has guided empirical studies on the ecology and evolution of these systems.[36]

Perhaps because of these intrinsic difficulties with assessing ecological roles for chemical signals at surfaces, generalities regarding the role of naturally produced metabolites as mediators of surface–based interactions are relatively rare.[34] The generalizations that do exist mostly focus on (1) the physiological mechanisms of action of the metabolites, a focus of much of the research on larval inducers for invertebrates,[37] or (2) contrasting the role of hydrodynamics vs. chemical

(and other) settlement cues, and in particular the extent to which hydrodynamic factors modulate the effects of chemical cues.[29] While mechanistic studies of modes of action are valuable in their own right, they do not necessarily speak to the demographic or community effects of natural cues for colonization. Moreover, integrative studies of chemistry and hydrodynamics, while fundamental to our understanding of these systems, are, in all but a few instances, hindered by a lack of sufficiently detailed knowledge of the cues themselves.

This chapter has one main premise and two general goals. The premise is that the chemical ecology of surface-based phenomena such as colonization are different in important ways from the chemical ecology of consumer–prey interactions, requiring different methods, different generalizations, and perhaps different compounds. Our first goal then, is to see whether any generalizations — methodological, chemical, or ecological — might emerge from a broad consideration of colonization phenomena in benthic habitats. This chapter makes no attempt to comprehensively review the field for either positive or negative cues for colonization, as a number of recent reviews are available in each case.[12–14,28,38–42] Rather, it focuses on representative examples from the work of the authors and the broader literature.

Second, in order to try and understand whether there are similarities in these phenomena among macroalgae, invertebrates, and bacteria, this chapter includes examples of chemical mediation of surface colonization for both eukaryotes and prokaryotes. Chemical cues can be fundamental for colonization processes for all three of these groups, but studies of these different taxa often occur largely independently of each other, in disparate literatures. Throughout this chapter, methodological issues relevant to studies of colonization are highlighted, since the field overall is at a stage where progress is particularly reliant on the development of new or improved techniques.

II. DETERRENTS OF COLONIZATION

A. Inhibition of Fouling by Seaweeds

The hypothesis that secondary metabolites produced by macroalgae (seaweeds) deter colonization of algal thalli by epibiota (fouling organisms) is at least 50 years old[43] (the chemical claw). Only recently, however, have more rigorous examinations of this hypothesis been done. For a metabolite to be a natural antifoulant, it must be present at the surface of the producing organism, or released, at a concentration which deters ecologically relevant fouling organisms. Our research in this area has focused on the Australian red alga *Delisea pulchra*, which for much of the year is unfouled in the field. *D. pulchra* produces a range of nonpolar halogenated furanones (Figure 10.1), typically between concentrations of 0.5 and 1.5% of the dry weight of the thallus.[44,45] These compounds occur in vesicles in gland cells, which occur at the surface of the plant (as well as in the interior of the thallus), and release furanones to the surface of the thallus.[15] These compounds can be extracted off the surface without lysing cells, enabling surface concentrations of furanones to be quantified.[46] Total levels of furanones on the surface are typically in the range of 100 to 500 ng/cm^2,[15,46] which on average represents between 0.2 and 0.4% of the total amount of furanones in the alga. Naturally occurring concentrations of furanones applied to test surfaces in laboratory or field assays strongly deter ecologically relevant macro- and microbiota.[16,47,48] Surface concentrations of furanones and fouling also vary seasonally and with depth,[48] such that increased fouling on *D. pulchra* on shallow plants in summer corresponds to a significant drop in levels of surface furanones on these plants[48] (relative to plants occurring at greater depths). The experimental and observational data cited above indicates that furanones in *D. pulchra* act as *in situ* natural antifoulants.

For nonpolar secondary metabolites (e.g., metabolites with no known primary function in the organism's physiology) from macroalgae, only two other studies (besides those on *D. pulchra*) are known in which care has been taken to test metabolite concentrations at ecologically realistic levels. The first is the study of Schmitt et al.[17] for terpenoid metabolites from the brown alga *Dictyota*

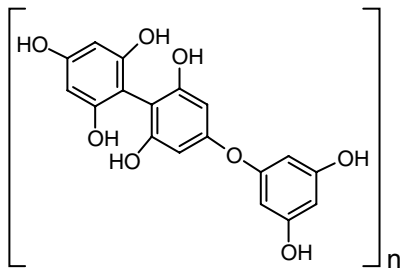
menstrualis. Schmitt et al.[17] found that *D. menstrualis* was typically unfouled in the field and was avoided by larvae of the epiphytic bryozoan *Bugula neretina* in multialgal settlement preference assays in the laboratory. Following these observations, secondary metabolites were "harvested" (as crude extracts) from a known area of the surface of the alga using cotton swabs, and were reapplied to the same areas of bioassay dishes, resulting in significant deterrence of larvae of *B. neretina*. Purified metabolites [pachydictyol A and dictyol E (Figure 10.1)] from the alga also deterred settlement or metamorphosis of larvae. Thus, metabolites appear to be present on the surface of *D. menstrualis* at concentrations high enough to deter an ecologically relevant epiphyte. Caveats to this conclusion are (1) swabbing can damage (lyse) surface cells,[46,48] potentially resulting in the harvesting of nonsurface borne metabolites, and (2) actual surface concentrations of pure metabolites were not determined.

The second example is for terpenoid metabolites from *Laurencia obtusa*.[46] *L. obtusa* in shallow habitats near Sydney is relatively free of fouling when it first appears in the field, becoming increasingly fouled over subsequent weeks. *L. obtusa*, like many species in the genus, produces a variety of sesquiterpenoids which have strong biological activity.[49,50] Two metabolites isolated from *L. obtusa* from the Sydney region, palisadin A and 5 β-hydroxyaplysistatin (Figure 10.1) inhibit settlement of spores of the green alga *Ulva lactuca* and larvae of *B. neretina* (common epiphytes in these habitats) at concentrations of 0.1 and 1 μg/cm^2, respectively.[46,51] However, levels of palisadin A and 5 β-hydroxyaplysistatin on the surface of the plant were less than 5 ng/cm^2 (at most).[46] These levels represent less than 0.1% of the total amount of terpenoids in the alga[46] and were orders of magnitude below levels needed to deter the two species of common epiphytes. The almost complete absence of these metabolites on the surface of the alga is not surprising, in light of the morphology of the plant. Terpenoids in *Laurencia* species are localized in intracellular vesicles known as *corp en cerise*,[52] which — unlike the gland cells in the Bonnemaisoniaceae (including *Delisea pulchra*) — are not known to come to the surface of the thallus. Thus, there is no obvious mechanism for the release of metabolites to the surface of undamaged individuals of *L. obtusa*.

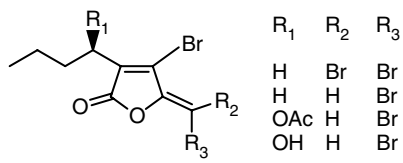
In contrast to the studies described above on nonpolar secondary metabolites, early research on algal secondary metabolites as deterrents of epibiota focused on phlorotannins (Figure 10.1)[53] large water-soluble (polar) metabolites found in brown algae. Phlorotannins would not be efficiently sampled or collected by the dipping or swabbing procedures described above. Phlorotannins in brown algae occur in small vesicles known as physodes, and there are a number of studies demonstrating that phlorotannins are exuded into the water column from brown algae[54,55] (reviewed by Ragan and Glombitza[56]). Several studies have also demonstrated deterrent or toxic effects of phlorotannins against various epibiota or other invertebrates.[56,57]

As for nonpolar metabolites, the effectiveness of phlorotannins as deterrents depends on the relationship between *in situ* concentrations on or near the plant and the concentrations needed to deter fouling organisms. Jennings and Steinberg[54] measured *in situ* exudation of phlorotannins in the sublittoral kelp *Ecklonia radiata*, and found that exudation by undamaged, unstressed plants was much lower than in most previous studies. Based on these exudation rates, they[57] calculated that levels of phlorotannins, either in the boundary layer adjacent to *E. radiata* or in the surrounding water column, were too low to deter settlement of the epiphytic green alga *U. lactuca*. Phlorotannins from a co-occurring alga, *Sargassum vestitum*, also failed to inhibit settlement of *Ulva* at these concentrations. These levels of phlorotannins were much lower than literature values reported to deter other epiphytes (reviewed Jennings and Steinberg[57]), with the exception of peritrichs of the protozoan *Vorticella marine*,[58] and it was concluded that there was no evidence that undamaged *E. radiata* used phlorotannins to inhibit settlement onto their surfaces.

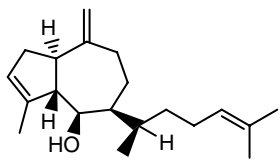
Jennings and Steinberg[57] also pointed out that no other studies of fouling of macroalgae have measured *in situ* rates of exudation of phlorotannins (or any other algal deterrent). Exudation rates of phlorotannins from brown algae in the laboratory, or from stressed thalli, can be substantially elevated,[54,59] and, thus, evidence for the deterrent effects of phlorotannins from other brown algae may be based on unnaturally high concentrations.[60] Possible exceptions to this conclusion are



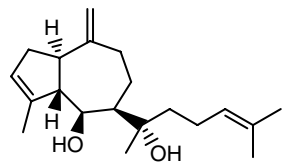
phlorotannins
Phaeophyta



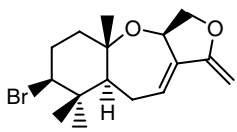
furanones
Delisea pulchra



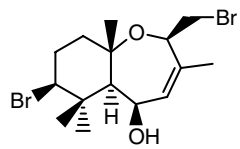
pachydictyol A
Dictyota menstrualis



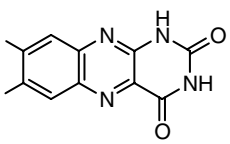
dictyol E
Dictyota menstrualis



palisadin A
Laurencia obtusa

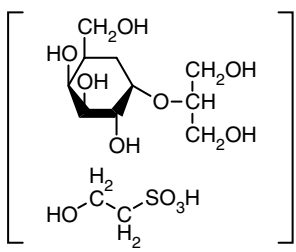


5 β-hydroxyaplysistatin
Laurencia obtusa

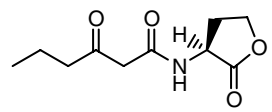


lumichrome
Halocynthia roretzi

floridoside



isethionic acid
Delisea pulchra



N-3-oxo-hexanoyl-L-homoserine
lactone (OHHL)
Gram negative bacteria

FIGURE 10.1 Structures of selected metabolites investigated for their role as inhibitors or inducers of prokaryote and eukaryote colonization.

intertidal algae, which undergo a burst of exudation following tidal immersion.[55] Such bursts may inhibit settling epibiota for short periods, as may accumulation of phlorotannins in tidepools at low tide.[61]

These contrasting examples for the efficacy of algal secondary metabolites as natural antifoulants raise the question: how commonly do such metabolites act as inhibitors of colonization of algal surfaces? Ideally, this question should be answered on a species-by-species basis first by quantifying surface metabolites in *in situ* or near *in situ* conditions, then performing laboratory bioassays of relevant concentrations of metabolites against relevant epibiota, correlating variation in surface metabolites and fouling of the alga in the field,[48] and finally by field tests of fouling in which metabolites are incorporated into artificial media and released at concentrations comparable to those released by the plant. Unfortunately, development of the suite of methods implied by this protocol is decidedly nontrivial, even for a single species. Formal quantification of surface metabolites has only been done for two species (above), and no published methods are known — with the exception of very short term tests of furanones against bacteria[16] — for field tests using artificial release systems in which metabolites are known to be presented or released at realistic concentrations.

Development of such protocols are necessary for a full understanding of antifouling by algae or other benthic organisms, but simpler alternatives exist that can start to give us an understanding of these processes. One such alternative is to study the morphology and ultrastructure of algae with respect to the production of secondary metabolites (Table 10.1). Given that algae must avoid autotoxicity, metabolites will probably be encapsulated in specialized cells, analogous to the cellular and multicellular structures in which terrestrial secondary plant metabolites are usually found.[62] However, we have relatively little general knowledge of the localization of secondary metabolites for most algae or benthic invertebrates (Table 10.1). Given that in most instances it is not known if the metabolites are able to be presented at the surface of the producing organism, we should be cautious in inferring that antifouling by secondary metabolites from benthic organisms is a general phenomenon.

A second approach to the question of the generality of chemical deterrence of epibiota by algae is to use techniques whereby metabolites can be harvested from the surface of the plant and then reapplied to test surfaces in bioassays. This was used by Schmitt et al.[17] in their study of *Dictyota menstrualis*, and has been used by these authors via the surface dip technique of de Nys et al.[46] (we recommend the latter technique since it has a greater consistency of extraction efficiency and is less damaging to surface cells).[46] In such techniques for harvesting metabolites, the metabolites are not quantified, but as long as metabolites are harvested from a known surface area of an alga, they can be reapplied to the same area of a test surface, resulting in ecologically realistic concentrations.

Using this technique, metabolites from measured areas of the surface of 10 species of chemically rich algae from the Sydney region were harvested (Figure 10.2). These extracts were then reapplied to test surfaces and assayed for their effects against settlement of larvae of the epiphytic bryozoan *Bugula neretina* using standard bioassay protocols (de Nys et al.;[63] Figure 10.2). Extracts were applied to test surfaces (petri dishes) at both average natural concentrations (the surface area of the petri dish was the same as that of the area of alga dipped) and twice natural concentrations (area of alga dipped was twice that of the test surface).

Of the ten species of chemically rich algae tested, only surface extracts from one — *Delisea pulchra* — significantly inhibited settlement of the bryozoan larvae at either natural or two times the average natural concentrations (Figure 10.2). These data are from a single experiment, extracts were collected at only one time and place, and only one species of epibiota was tested (although results for other epiphyte species are similar[64]). However, these algae are both taxonomically and chemically diverse, and the data in Figure 10.2 arguably represent the first broad, ecologically realistic screen for the widespread use of algal nonpolar secondary metabolites as antifoulants. The results of the experiment are not consistent with a ubiquitous role for algal nonpolar metabolites as natural antifoulants.

TABLE 10.1
Localization of Secondary Metabolites in Marine Macroalgae and Invertebrates

Taxa	Metabolite	Localization	*Comes to Surface?	Reference
		Algae		
Rhodophyta				
Laurencia spp. (e.g., *L. synderae, L. obtusa*)	Halogenated terpenes	*Corps en cerise*	No	52 46
Bonnemaisoniaceae (e.g., *Bonnemaisonia nootkana*, *Delisea pulchra*)	Furanones, other halogenated metabolites	Vesicles within gland cells	Yes	225 15
Desmarestia firma	H_2SO_4	Cell vacuoles	ND	226
Phaeophyta				
All Phaeophyta	Phlorotannins	Physodes	Yes	56
		Invertebrates		
Porifera				
Dysidea herbacea	2-(2′,4′-dibromophenyl)-4,6-dibromophenol	Cyanobacterial symbiont-*Oscillatoria spongeliae*	ND	88
	Spirodysin, dihydrodysamide C, didechlorodihydrodysamide C	Cyanobacterial symbiont-*Oscillatoria spongeliae* archaeocytes and choanocytes	No No	87
Dysidea avara	Avarol	Choanocytes	No	84
Dysidea fragilis	Ent-furodysinin	Vesicles within unspecifed cells	Yes	85
Crambe crambe	Unspecified (measured as toxicity)	Spherulous cells	Yes	86
	Guanidine alkaloids	Spherulous cells	Yes	83
Theonella swinhoei	Theopalauamide	Eubacteria symbiont	No	90
	Swinoholide A cyclic peptide (antifungal)	Bacterial symbionts (unicellular heterotroph) bacterial symbiont (filamentous heterotroph)	ND ND	89
		Ascidians		
Ascidia nigra	Vanadium	Vanadocytes	Yes	72

Note: ND = not determined.

* of undamaged cells.

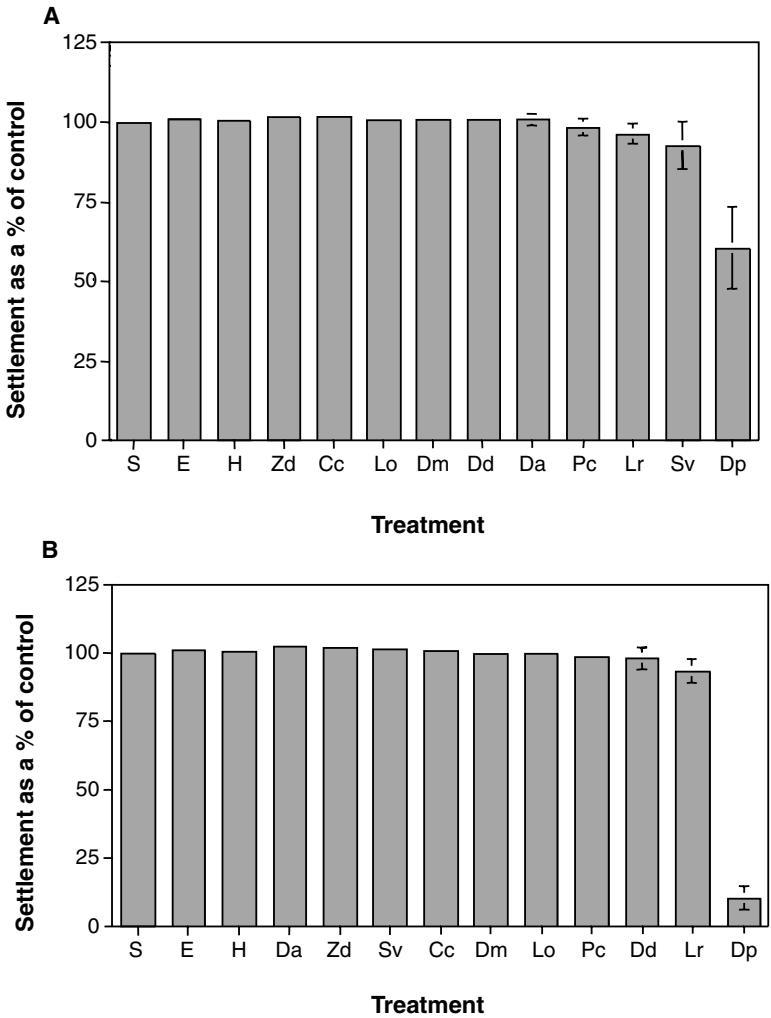


FIGURE 10.2 The effect of surface extracts of ten species of algae from the Sydney area on the settlement and growth of the common fouling bryozoan *Bugula neretina*. The algae used were the red algae *Delisea pulchra* (Greville) Montagne (abbreviated as Dp); *Laurencia obtusa* Lamouroux (Lo); *Laurencia rigidg* Agardh (Lr); *Pterocladia capillacea* (S.G. Gmelin) Bornet (Pc); *Champia compressa* Harvey (Cc); the brown algae *Dictyopteris acrostichoides* (J. Agardh) Boergesen (Da); *Dictyota dichotoma* (Hudson) Lamouroux (Dd); *Dilophus marginatus* J. Agardh (Dm); *Zonaria diesingiana* J. Agardh (Zd); and *Sargassum vestitum* (R. Brown ex Turner) C. Agardh (Sv); All species were collected in the subtidal zone (3 to 5 m depth) at Nielsen Park, Port Jackson (33˚51'04"S, 151˚16'12"E); Bare Island, Botany Bay (33°59'38"S, 151°14'00"E); or Shark Point (33°55'05"S, 151°16'12"E) New South Wales, Australia. Surface-borne compounds from all species were obtained by extraction in hexane as described by de Nys et al.[46] Pieces from freshly collected individuals with a surface area of 9 or 18 cm^2 were cut and extracted for 20 s in double-distilled hexane (AR grade). After extraction, the algal pieces were removed and the hexane taken to dryness at room temperature. The extracts were re-dissolved in 500 µL of ethanol (99.7% or higher purity) and applied to the surface of treatment dishes (9 cm^2). Extracts were therefore tested at mean natural (A) and twice mean natural (B) concentrations. Seawater (S), and two solvent controls — hexane (H) and ethanol (E) — were also prepared. Larvae of *Bugula neretina* were cultured and prepared for settlement assays as described by de Nys et al.[63] Settlement assays were done by adding 15 larvae to either treatment, solvent control, or untreated dishes, each containing 4 ml of sterilized filtered seawater. Test dishes were incubated for 24 h at 28°C in a 15/9 h light–dark cycle. After this time, the percentage of settlement was determined by counting the number of attached (cont.)

Finally, a strategy quite different from those discussed above for algal chemical defense against microorganisms is the recent suggestion by Weinberger et al.[65,66] that seaweeds use oxidative bursts — specifically the production of hydrogen peroxide (H_2O_2) — to inhibit microbial attack. The mechanism is reliant on bacterial degradation of algal cell wall polysaccharides,[65] and it is perhaps more appropriately considered as a defense against pathogenesis. However, depending on the ecological context[66] of the algal–bacterial interaction, it may also serve to generally inhibit bacterial colonization of an alga's surface. Interestingly, the bioluminescent symbiotic bacterium *Vibrio fisheri* in the light organ of the squid (*Euprymna scolopes*) is also controlled, in part, by oxidative bursts from the squid.[200]

B. Inhibition of Fouling by Benthic Invertebrates and Seagrasses

Other benthic organisms such as invertebrates and seagrasses also produce secondary metabolites which deter the settlement of fouling organisms.[28,38,42,68–70] Much of the research in this area has focused on the development of commercial alternatives to current commercial antifouling paints (reviewed by Clare[68]). While some very active metabolites have been discovered, these studies do not generally address the ecological role of these metabolites. For the few studies that have been placed in an ecological context, active deterrents have often been found, but surface concentrations of the metabolites have not been quantified or the compounds have not been tested against ecologically relevant fouling organisms. For example, the ascidian *Eudistoma olivaceum* produces a range of alkaloids including eudistomin G and H, which deter ecologically relevant fouling organisms at low concentrations.[71] However, localization or quantification of these compounds at the surface of the ascidian has yet to be determined. Inorganic vanadium, found in vanadocytes and released to the surface of *Ascidia nigra,* has also been proposed as a mechanism of deterring fouling in ascidians.[69,72] The seagrass *Thalassia testudinum* produces a sulfated flavone glycoside which deters attachment and growth of the marine thraustochytrid protist (fungus) *Schizochytrium aggregatum.*[73] Zosteric acid and other simple phenolic acids from seagrasses deter settlement of barnacles and bacteria.[74] Again, surface concentrations of the seagrass metabolites are not known, although the compound from *T. testudinum* was deterrent at one-fifteenth of whole plant concentrations.[73]

In research that was, in many ways, ahead of its time, Thompson et al.[75] described localization of the brominated secondary metabolites aerothionin and homoaerothionin in the sponge *Aplysina fistularis* in spherulous cells. They further quantified *in situ* release rates of these metabolites,[76,77] although measured release rates may have been at the high range of natural concentrations as they were measured immediately following reimmersion of the sponge.[77] Although the exudates and compounds were not active in antifouling bioassays,[76,78] they did inhibit the feeding response of potential fouling organisms,[76,78] and, thus, may have significant effects on epibiota postcolonization.

A somewhat different example of natural antifouling by invertebrates comes from the work of Woodin, Lincoln, and colleagues[79–81] who have shown that a wide array of infaunal invertebrates in sediment communities produce organohalogens such as bromophenols.[81] In laboratory assays, purified bromophenols at concentrations which occur naturally in the field in beds of the polychaete *Notomastus lobatus*[82] inhibited burrowing activities of recently metamorphosed juveniles of several infaunal species.[80] The taxonomically widespread occurrence of such metabolites and the nature of sediments as a medium capable of accumulating inhibitory metabolites suggest that these compounds may be important in determining spatial distributions of infauna,[80,81] either via inhibition

FIGURE 10.2 (CONTINUED) and unattached larvae. N = six (6) replicates (dishes) were done for all treatments. The results of the assay are expressed as percentage settlement of the seawater (untreated) control. Data are mean ± S.E. Treatments lacking error bars indicate 100% settlement in all replicates. Statistical analysis of the data (separate one-factor analysis of variance ANOVA for each of Figure 10.2A and 10.2B, followed by Tukey's post-hoc comparison among means) showed that only extracts from *D. pulchra* significantly deterred settlement (at both natural and twice natural concentrations).

of settlement or allelopathy (the lines between the two may blur for mobile infauna, particularly juveniles).

As with seaweeds, determining the localization of putative inhibitors and their quantitative distribution is an important step in understanding their ecological roles (Table 10.1). Significant progress has been made in localization of secondary metabolites for sponges in particular (Table 10.1). In some cases, secondary metabolites are in cells that are released to the surface of the producing organism, while in others they remain internal to the organism. In the sponge *Aplysina fistularia*, the secondary metabolites aerothionin and homoaerothionin are contained within spherulous cells adjacent to the exhalent canals.[75] These cells rupture and are the source of the aerothionin and homoaerothionin found in exudates from the sponge.[77] Spherulous cells are also the location of the biologically active secondary metabolites, guanidine alkaloids, in the sponge *Crambe crambe*.[83] At least some of the spherulous cells from *C. crambe* occur outside the sponge exopinacoderm, suggesting release of the compounds and a potential role against fouling organisms.[83,86] In the sponge *Dysidea fragilis*, the furanosesquiterpene, ent-furodysinin, is located in vesicles within cells (cell type not specified). These vesicles appear to open into intercellular spaces, also suggesting release of the compound.[85] No studies of the activity of these furanosesquiterpenes against epibiota have been performed.

In other sponges, secondary metabolites are maintained internally. Avarol, the major active secondary metabolite in *Dysidea avara*, is localized in choanocytes within the sponge.[84] In the related *D. herbacea*, the terpene spirodysin is present within archaeocytes and choanocytes, while another class of secondary metabolite, chlorinated diketopiperazines, occurs within the symbiotic filamentous unicellular cyanobacterium *Oscillatoria spongeliae*.[87] Flowers et al.[87] have also isolated a sample of *O. spongeliae* from *D. herbacea* which do not contain the diketopiperazines. These later examples illustrate the importance of determining the localization of bioactive metabolites for understanding host–symbiont interactions as well as colonization phenomena, and there are now several studies demonstrating the production of secondary metabolites by symbiotic bacteria in host sponges.[88–90] Bacteria may also be associated with the production of secondary metabolites in the bryozoans *Amathia wilsoni* and *Bugula neretina.* In *A. wilsoni*, brominated amathamides occur on the surface of the zooids in association with a rod shaped bacterium. However, the role of these compounds and their origin have yet to be firmly established.[91] Davidson et al.[92] present evidence suggesting that bryostatin from *B. neretina* is produced by a nonculturable bacterial symbiont.

C. Inhibition of Epibiota by Biofilm Bacteria

Bacterial biofilms are ubiquitous in the marine environment and play an important role in mediating interactions at surfaces.[93,94] Some invertebrates (e.g., *Hydroides elegans*[95]) require a biofilm for successful settlement and metamorphosis. Settlement in other species can be either facilitated or inhibited by natural biofilms, with the response often specific to the biofilm and the invertebrate investigated. For example, biofilms deter settlement of the bryozoan *Bugula flabellata* but facilitate settlement of the ascidian *Ciona intestinalis*.[96] Similarly, biofilms of differing ages have significantly different effects on settlement, with the effect again dependent on the biofilm and the invertebrate larvae being tested.[97–99] Larvae also respond differentially to biofilms as a function of their metabolic activity, density, and composition.[93]

As with inhibitors produced by eukaryotes, few studies have characterized and quantified inhibitors produced by natural biofilms. Rather, most studies have focused on isolated bacterial strains, or biofilms, cultured in laboratories.[98,100–103] Only one inhibitory metabolite from biofilms has been fully characterized, the natural product ubiquinone-8 from the culture supernatant of the bacteria *Alteromonas* sp.,[103] a bacterium isolated from the sponge *Halichondria okadai*.

Several partially characterized inhibitors have been described from the marine bacterium *Pseudoalteromonas tunicata*, isolated from the tunicate *Ciona intestinalis*. This bacterium produces a diversity of metabolites, each of which specifically inhibits the settlement of invertebrate larvae

and algal spores or growth of bacteria, fungi, or diatoms (see Holmström and Kjelleberg[104] for review). Production of the inhibitors is associated with production of a pigment, as appears to be the case for a number of inhibitory strains of bacteria isolated from the surface of marine eukaryotes.[102] Several of the compounds are partially characterized and water-soluble,[101,104,105] in contrast to the hydrophobic ubiquinone-8 and the nonpolar deterrents discussed above. However, treatment of *P. tunicata* cells with periodate to oxidize cell surface polysaccharides enhances the inhibitory effect of the antilarval compound.[101] This suggests that this metabolite is immobilized or associated with the exopolysaccharide coat.

Both *Alteromonas* sp. and *Pseudoalteromonas tunicata* were isolated from eukaryote hosts. This raises the possibility, suggested by several authors,[91,93] that inhibition of fouling by some eukaryotes may be accomplished by specific bacterial biofilms living on their surfaces. In support of this, the known hosts for *P. tunicata* are generally unfouled in the field, but are not known themselves to produce bioactive secondary metabolites. However, the hypothesis that bacteria colonize a host and then provide inhibitory cues to prevent prokaryote or eukaryote fouling is untested in an ecologically realistic context. Experiments using biofilms in the laboratory may be difficult to relate to field conditions[99] given the difficulties both in identifying and measuring cues *in situ* and in identifying and characterizing the biofilm itself.

The fundamental need to characterise and quantify bacterial strains in natural biofilms in order to understand their potential role as producers of settlement signals highlights the need for molecular techniques which can be used to identify and quantify bacteria *in situ*. For example, low abundance of putatively deterrent bacterium on surfaces *in situ* may preclude the production of sufficient quantities of deterrent metabolites. Appropriate techniques for such characterization include denaturing gradient gel electrophoresis (DGGE)[107] and fluorescence *in situ* hybridization (FISH)[108] and would in principle allow for the detection and quantification of all species in the biofilm.

D. Summary of Deterrence of Epibiota

Davis et al.[69] summarized the state of play of natural antifoulants over a decade ago, and some progress has been made since then. We now have a few examples where metabolites at or near the surface of the producing organism have been quantified or extracted and realistic concentrations tested against ecologically meaningful epibiota in the lab or field.[16] Significant methodological challenges remain, but progress has been made in localizing metabolites within or on producing organisms (Table 10.1), quantifying metabolites in a realistic way,[46,57,77] harvesting surface metabolites for bioassays[17] (Figure 10.2), and developing appropriate field tests.[16]

With regard to field tests, one positive development in the search for methods for realistic assessment of natural antifoulants is the use of durable, readily accessible materials for testing metabolites in the field. Examples of such materials are Phytagel[109] and copolymer resins.[110] Hendrikson and Pawlik[109] showed that an extract from the sponge *Aplysilla longispina* incorporated into Phytagel deterred settlement of fouling organisms for up to a month in the field, and Vasishtha et al.[110] used a commercially available resin (VYHH) to measure antifouling activity and release rates of an organic biocide (an isothiazolone). While those are important steps in the right direction, the challenge with such artificial matrices is to achieve a release rate/presentation of metabolites that mimics that of the producing organism. As any manufacturer of antifouling paints can attest, it is quite easy to develop materials that either rapidly release all the incorporated active ingredient or barely release any at all. Different active ingredients (e.g., secondary metabolites) also differ dramatically in their behavior in different media, and, thus, for any long-term field test of a natural antifoulant, it is necessary to calibrate the release rate of a specific metabolite from a specific substance (matrix). This requires the same sort of analytical methods necessary for the quantification of metabolites *in situ* on or around the producing organism.

Are there general patterns in the production of natural antifoulants by marine organisms? Research on seaweeds (reviewed above and by de Nys and Steinberg[28]) and evidence from marine

invertebrates[78] suggest that natural antifoulants will primarily be nonpolar secondary metabolites localized within an organism in a fashion that will enable the metabolites to be released to the surface. Polar metabolites such as phlorotannins are likely to be less effective because of the rapid dissolution of such metabolites away from the surface of the producing organism. In contrast, hydrophobic metabolites such as the furanones from *Delisea pulchra* can remain adsorbed to or associated with the organic surfaces of the producing organism, providing a more persistent concentration of deterrent metabolites at the surface. Possible exceptions to this prediction are (1) sponges, which by virtue of their complex system of channels and cavities may be able to concentrate polar metabolites within internal spaces, which may also result in high concentrations of metabolites near their surface, (2) the research of Weinberger et al. [65] on the possible role of hydrogen peroxide as a deterrent of bacterial pathogens, and (3) evidence that at least some bacterially derived deterrents are small, water-soluble metabolites (e.g., *Pseudoalteromonas tunicata*[111]).

III. INDUCTION OF SETTLEMENT OF EUKARYOTE PROPAGULES

Chemical inducers for the settlement or metamorphosis of larvae of marine invertebrates have been comprehensively reviewed during the last decade,[12,13,39] as well as in this volume.[14] Considerably less information is available for algal propagules.[112] Rather than reiterating these reviews, this section focuses its consideration of induction of colonization of surfaces by eukayotes on two questions: (1) what kinds of chemicals are used and why, and, (2) are there habitat or life historical correlates of particular kinds of cues? For the purposes of this chapter, settlement is defined as the sum total of processes that result in the transition from a planktonic existence to a benthic one, which generally incorporates settlement to the bottom per se (a behavioral phenomenon) followed by various morphological/developmental changes such as metamorphosis (animals) or germination (algae). We note, however, that the behavioral components of settlement, vs. metamorphosis or germination, may be affected by different cues.[14]

As with natural antifoulants, there are few (if any) fully documented examples of natural, ecologically relevant inducers of settlement; that is, characterized cues which induce settlement, have been quantified *in situ,* are active at *in situ* concentrations against the relevant target organism, and can be related to patterns in the demography of that organism. However, there are a wealth of examples, particularly for invertebrates, in which various pieces of this puzzle are known, and that information is summarized here. The information that is available points to the widespread use of primary metabolites, particularly water-soluble (polar) peptides and carbohydrates, as natural cues.

A. What Chemicals Are Used for Induction of Invertebrate Settlement?

1. Peptides

Probably the most widespread evidence for the importance of soluble primary metabolites as inducers of invertebrate settlement comes from studies of peptides, amino acids, or other proteinaceous cues. Their importance has been suggested by a number of authors over the years,[33,113–115] and it is now clear that such metabolites affect settlement in a wide taxonomic array of invertebrate larvae. Research on oysters and barnacles are among the more prominent examples of peptides as inducers. Zimmer-Faust and colleagues[115–117] have shown that larvae of the oyster *Crassostrea virginica* respond to a small (approximately 500 to 1000 Da) peptide from oyster shells by dropping rapidly from the water column and starting settlement behavior.[115] The natural peptide is uncharacterized but is mimicked by the tripeptide glycince–glycine–arginine (GGR). The inducer is active in both still water and flow (in a flume).[117,118] The source of the cue — adult oysters or their shells vs. shell-associated biofilms — has not yet been determined.[116,119]

Settlement and metamorphosis of barnacles in response to chemical cues appears more complex than for oysters, with several materials functioning as inducers of settlement (reviewed by Clare

and Matsumura[40]). First, barnacles settle and metamorphose in response to a partially characterized proteinaceous cue termed "settlement-inducing protein complex" (SIPC) which is associated with the adult shell. The SIPC is a glycoprotein complex with four subunits, each of which has a similar effect to the intact glycoprotein complex.[120,121] Second, proteinaceous adhesives left by settling cyprids induce settlement of conspecifics in *Semibalanus balanoides* and *Balanus amphitrite.*[122,123] Finally, a smaller, water-soluble peptide settlement cue has also been partially purified from water conditioned by adult barnacles.[124,125] The three cues may be related; Clare and Matsumara[40] have suggested similarities between the adhesive-derived cue and SIPC, and Harrison[126] suggested that the waterborne cue is derived from the SIPC. Interestingly, the same tripeptide — GGR — that induces settlement of oyster larvae also induces metamorphosis in barnacles.[125]

Amino acids and peptides are also implicated in settlement and metamorphosis of abalone (*Haliotis rufescens*). Uncharacterized small peptides (approximately 1000 Da), which are subunits of larger protein moieties associated with the surface of coralline algae, induce settlement of *H. rufescens.*[127,128] The activity of these peptides is mimicked by GABA — γ-amino-butyric acid — in inducing the settlement of abalone larvae.[129] While the signal transduction pathway for GABA and GABA analogues has been a focus of investigation (see Slattery[13] for review), the nature, specific activity, and source of the natural inducer are still not fully understood. In particular, the role of bacteria on the surface of crustose coralline algae in the production of GABA and/or the settlement inducer remains to be resolved. Marine bacteria are able to produce and metabolize GABA,[130,131] and Johnson et al.[106] argue that bacteria associated with the surface of coralline algae may be responsible for the induction of abalone larval settlement (either through production of GABA or other inducers). There also appear to be species-specific responses by abalone to settlement cues, with some abalone species induced to settle by diatom films alone.[132]

One of the most intriguing examples of peptides as cues for settlement and metamorphosis comes from the rotting leaves of the mangrove *Rhizophora mangle.*[133] An inducer of the jellyfish *Cassiopea xamachana* is produced by bacterial decomposition of the mangrove leaves[133] and has been identified as a water-soluble proline- and glycine-rich protein.[134] The inductive compound appears to be a nonspecific by-product of bacterial degradation of proteinaceous plant matter, although the possibility that the inducer is produced by the degradative bacteria themselves cannot be ruled out.

A variety of other amino acid- or peptide-based cues also induce larval settlement and metamorphosis. For example, larvae of the sand dollar *Dendraster excentricus* settle and metamorphose in response to (1) a peptide-based cue from sand associated with adults, and (2) to water conditioned by adults.[135–137] The nudibranch *Adalaria proxima* is induced to settle and metamorphose by a water-soluble-peptide-based cue from its bryozoan host *Electra pilosa.*[138,139] A surface-associated peptide inducer has been proposed for the tube worm *Phragmatopoma lapidosa californica*,[140] but its role as a natural inducer remains to be defined.

2. Carbohydrates

Sugars and their derivatives are increasingly implicated as inducers of settlement. Williamson et al.[35] studied induction of metamorphosis of larvae of the Australian urchin *Holopneustes purpurascens*. This echinoid occurred primarily (in more than 95% of individuals) on two host plants at their study site, the red alga *Delisea pulchra* and the kelp *Ecklonia radiata*. While densities of the urchin on its two host plants did not significantly differ, urchins on *D. pulchra* were significantly smaller than those on *Ecklonia radiata*, and urchins in the smallest size class (e.g., new recruits) were only found on *D. pulchra.*[35] In laboratory bioassays, competent larvae of *H. purpurascens* rapidly metamorphosed (3 to 7 h) when in the presence of pieces of *D. pulchra* or a polar (water-soluble) extract of this alga. No metamorphosis was observed in response to *E. radiata* or its extracts. Bioassay guided fractionation resulted in the characterization of a specific chemical cue from *D. pulchra*, a noncovalently bound complex between the red algal sugar floridoside (Figure 10.1)

and the small organic acid, isethionic acid (Figure 10.1), which induced metamorphosis within 2 h at a concentration of 25 μM. Seawater collected *in situ* within centimeters of *D. pulchra* also induced metamorphosis, while seawater collected near *E. radiata* or distant (meters) from other algae did not. Finally, in laboratory assays, other red algae from *H. purpurascen*'s habitat also induced metamorphosis of larvae, although at lower levels than for *D. pulchra*. The larvae did not metamorphose in the presence of brown or green algae, consistent with the fact that floridoside is widespread within the red algae but not found in browns or greens.[141]

Larvae of many species of corals are induced to metamorphose by a carbohydrate-based cue from algal cell walls, in particular those of crustose coralline algae.[142,143] The inducer is apparently conserved across many algal species in the Pacific and Caribbean,[144] and it has been identified as a high molecular weight polysaccharide with an active, water-soluble subunit.[145] The isolated subunit has been adsorbed onto a resin base, which had activity similar to the natural inducer in laboratory and field studies.[145]

Host-plant-derived carbohydrates induce settlement and metamorphosis in Opisthobranch molluscs. The ascoglossan *Alderia modesta* is induced to settle by three carbohydrate cues isolated from the yellow-green alga *Vaucheria longicaulis,* the host of *A. modesta.*[146,147] These include a soluble low molecular weight cue, a soluble high molecular weight cue, and an insoluble surface-associated high molecular weight cue.[146] The nudibranch *Eubranchus doriae* is induced to settle and metamorphose by a soluble carbohydrate isolated from its primary prey, the hydroid *Kirchenpaueria pinnata.*[148] An isolated polysaccharide containing galactosidic residues, as well as purified hexoses and galactosamine, all induced metamorphosis. Only sugars with the hydroxyl groups at carbons 3 and 4 with a *cis*-conformation induced metamorphosis, suggesting a stereospecific response from the larvae.[148] This conformation is the same as that for the sugar component of the floridoside–isethionic acid inducer of *Holopneustes purpurascens.*[35]

Finally, Forward et al.[149] provide further evidence for a role for plant carbohydrates as larval inducers. They showed that humic acids either from a commercial source or extracted from estuarine water (the latter presumably complexes derived from the degradation of plant carbohydrates) enhanced the rate of metamorphosis of blue crab (*Callinectes sapidus*) larvae.[149]

3. Fatty Acids

Arguably, the best known example for induction of invertebrate larvae by fatty acids is the work of Pawlik and others on settlement by *Phragmatopoma lapidus californica* in response to cues associated with sand from adult worm tubes.[12,150,151] Free fatty acids were isolated from the sand surrounding tubes and induced settlement and metamorphosis of the worms.[151–153] However, these findings have been challenged by Jensen and Morse[140,154] and Jensen et al.,[155] who argued that the fatty acids were contaminants, with settlement and metamorphosis actually induced by a polymeric protein containing L-DOPA subunits. The relative role of free fatty acids or L-DOPA in induction of settlement and metamorphosis of *Phragmatopoma* is unresolved to date and warrants further investigation.

Free fatty acids as inducers of settlement and metamorphosis have also been suggested for echinoderms.[156] However, there are, at present, no studies which unequivocally support the role of free fatty acids as settlement inducers for marine invertebrates.

4. Other Metabolites

Lumichrome (Figure 10.1), a water-soluble degradation product of riboflavin,[157] appears to be the first characterized gregarious (produced by conspecifics) cue for induction of metamorphosis of a marine invertebrate. Lumichrome is exuded into aqueous media from cultures of larvae of the ascidian *Halocynthia roretzi*, and was also found in the eggs, gonads, and tunic of the adults following extraction in methanol.[157] Metamorphosis of larvae was induced by purified lumichrome

with an EC_{99} value of 100 nM. The effect is species-specific to at least some extent, as lumichrome had no effect on larvae of another ascidian, *Ciona sauvignyi.* As with other chemical inducers, several questions about the activity of lumichrome *in situ* remain. For example, if larvae respond to adult *H. roretzi* in the field, how is lumichrome released from the adults? Moreover, only media from high density larval cultures induced metamorphosis.[157] Thus, although *H. roretzi* produces synchronously developing larvae, unless these larvae remain together, and settle together, it is unclear how larvae alone could be the source of the cue *in situ*.

Several low molecular weight, nonpolar secondary metabolites also induce settlement of invertebrate larvae. These include jacaronone from the red alga *Delesseria sanguinea*, which induces settlement of the scallop *Pecten maximus*,[158] and α-tocopherol epoxide from *Sargassum tortile,* which induces settlement of the hydroid *Coryne uchidai.*[159] However, both jacaronone and the tocopherol epoxide have questionable ecological relevance. Scallop settlement is unrelated to the occurrence or distribution of *D. sanguinea*,[160] and a number of unresolved chemical and biological questions remain regarding the putative inducers of *C. uchidai* (for details, see Pawlik[12]).

There are also a number of examples of chemical cues inducing settlement and metamorphosis, the nature of which have not been determined. An uncharacterized waterborne cue of approximately 500 Da in size from the massive coral *Porites compressa* induces metamorphosis of the nudibranch *Phestilla sibogae* which feeds on these corals.[161,162] The queen conch *Strombus gigas* is induced to settle and metamorphose by red algae, in particular *Laurencia poitei,* the dominant red alga in conch shell nurseries.[163] The cue is a low molecular weight, water-soluble compound (less than 1000 Da) which induces settlement at levels comparable to *L. poitei*. The structure of the cue remains unresolved, although metamorphosis of *S. gigas* is induced by amino acids and peptides.[164] A cue for the metamorphosis of larvae of the mollusc *Haminaea callidegenita* has been partially purified from adult tissue and from the egg masses of this and four other species of opisthobranch molluscs. The cue is a polar nonproteinaceous compound with a molecular weight of less than 1000 Da.[165]

5. Biofilms

Biofilms fundamentally affect settlement for many invertebrate larvae (reviewed by Wieczorek and Todd[94] and Holmström and Kjelleberg[111]), and the prevalence of biofilms on marine benthic surfaces may provide a general habitat signal for settling marine organisms.[106] Settlement and metamorphosis of the tube worm *Hydroides elegans* is (obligately) triggered by bacterial biofilms[95,166] (see Chapter 13 in this volume). Unabia and Hadfield[95] isolated a number of bacterial strains which induced settlement and metamorphosis of the worms. However, this occurred at a lower frequency than for mixed natural biofilms, suggesting the possibility of multiple cues. The related *Hydroides dianthus* also settles in response to biofilms, but this species responds to live *H. dianthus* as well.[167] This polytypic response may provide initial founder and subsequent aggregator communities of *H. dianthus*, thereby supporting the establishment of dense colonies.[167]

Polysaccharides are perhaps the most studied of the partially characterized larval cues from biofilms. Exopolysaccharides from the bacterium *Halomonas* (*Deleya*) *marina* stimulated settlement in the spirobid polychaete *Janua brasiliensis* through surface recognition of a galactose–galactose subunit within the polymer.[168,169] Settlement of *J. brasiliensis* could be blocked by surface coating the biofilm with specific lectins, strongly suggesting that the cue is surface-bound, as opposed to a water-soluble subunit broken off from the polymer. Exopolysaccharides from *Pseudoalteromonas* (*Pseudomonas*) sp. S9 also induce larval settlement, in this case of the ascidian *Ciona intestinalis*. Both recognition of the surface-bound polymer and entrapment of larvae in the exopolymer-enhanced larval settlement and recruitment of *C. intestinalis.*[170] Both of these examples in which larvae respond to surface-bound cues appear to represent exceptions to the more frequent pattern of water-soluble inducers described above.

As discussed earlier for inhibitory biofilms (Section II.C), in general, neither the strains of biofilm bacteria responsible for inducing settlement of invertebrate larvae nor the relevant signals have been characterized. Thus, it is not clear whether biofilm-derived signals differ in general from those produced by eukaryotes. Moreover, responses to monospecific biofilms in the laboratory may not reflect responses of propagules to biofilms in the field. In addition to field biofilms containing a diversity of bacteria, biofilms also contain other microorganisms besides bacteria which may be responsible for induction of settlement. For example, diatom films are commonly used in abalone aquaculture to induce settlement,[132] and fungi appear to be a common component of marine biofilms.[171]

B. Induction of Macroalgal Settlement

While it is generally considered that, "direct evidence for chemical signals associated with settlement is lacking in algae…,"[112] a few examples of such settlement are known. Amsler and Neushul[172] showed that settlement of motile spores of the kelps *Macrocystis pyrifera* and *Pterygophora californica* was enhanced in the presence of inorganic nutrients and the amino acids glycine and aspartate (*M. pyrifera* only). The kelp spores also exhibited positive chemotaxis in response to several simple inorganic and organic nutrients, with iron and high concentrations (1 mM) of ammonium eliciting negative chemotaxis. Nutrients do not elicit chemotaxis or enhanced settlement in spores of *Ectocarpus siliculosus*,[173] however, indicating that such responses are not universal for brown algal spores. Relatively little is known in this regard for other taxa of macroalgae (reviewed by Amsler et al.[173]). Similarly, little is known regarding the role of biofilms in macroalgal settlement. Dillon et al.[174] demonstrated enhanced adhesion of swarmers of the green alga *Enteromorpha* to biofilms, and Thomas and Allsopp[175] showed enhanced attachment and germination of zoospores of *Enteromorpha* in response to several bacterial biofilms.

C. Why Soluble Primary Metabolites as Inducers?

The examples above suggest that inducers of settlement differ in some important ways from deterrents. The few ecologically relevant deterrents of settlement that are known are nonpolar secondary metabolites. In contrast to deterrents and some earlier suggestions,[12] that inducers are primarily surface associated, inducers of invertebrate (at least) settlement are generally primary metabolites such as carbohydrates or peptides and are commonly water-soluble.[146] Exceptions to this pattern in regard to the latter criterion (water solubility) appear to be twofold. First, for barnacles[40] and the sea slug *Aldaria*,[146] large, insoluble, surface-associated cues are present in addition to smaller water-soluble ones. Second, cues for coral planula[142] and exopolymers of biofilm bacteria such as *Pseudomonas sp. S9*[170] and *Deleya marina*[168,169] are surface associated. However, some of these cues,[143] when hydrolyzed or otherwise degraded, often reveal active, water-soluble subcomponents, suggesting that there may also be smaller soluble cues present in these examples as well (although this is not supported for the *Deleya marina/Janua brasiliensis* interaction[168,169]). A number of hypotheses may explain the prevalence of water-soluble primary metabolites as inducers.

1. The Signal Should Extend beyond Its Source

Many invertebrate larvae, when competent to settle, and many algal propagules in general, exhibit tropisms (e.g., photonegativity) or other behaviors which cause them to associate with the benthos rather than move more broadly through the water column.[41,176,177] This facilitates the ability of a propagule to detect a surface-associated cue. However, the optimal inducer should be able to be detected as far from the source surface as possible, maximizing the target area for a settling propagule. Water solubility of a cue ensures that it will diffuse readily into the water column,

increasing the area in which a settling propagule can encounter the cue. (Note that the distances that a cue diffuses does not need to be far to significantly enhance the area of detection. Diffusion of a cue by 2 cm from a hemispherical source with a radius of 5 cm doubles the surface area of the volume containing the cue.) Nonpolar metabolites are likely to be less effective, because they tend to remain adsorbed to organic surfaces of the producing organisms, and are also likely to rapidly partition to organic aggregates once in the water column. Both phenomena will decrease the volume in which they are available as cues.

In contrast, the optimal strategy for hosts producing deterrents is to maximize the concentration of metabolites at, or as close as possible to, their surface.[57] This is best achieved by producing nonpolar metabolites such as furanones or terpenoids which will adsorb to their surface, or metabolites which are complexed or otherwise immobilized at the surface, as suggested for inhibitory compounds from the bacterium *Pseudoalteromonas tunicata* (reviewed by Holmström and Kjelleberg[111]). The tendency for many nonpolar metabolites to be unique or idiosyncratic to particular taxa of benthic organisms[178] also means that these cues are unlikely to be very widespread in the habitat, lessening the probability of generalist epibiota[179] evolving resistance to common deterrent cues.

These differences between deterrents and inducers highlight differences in selective pressures relevant to the two interactions. For deterrence, selection should be primarily on the host. While consequences of fouling to the host can, at least putatively, be severe, from the colonizer's perspective, there are usually many other sites on which to settle, especially given the generalist nature of many epibiota.[179] In contrast, for induction of settlement, there should be strong selection on colonizers to be able to detect signals and follow concentration gradients as efficiently as possible. In such cases, there may be no selection on the producer of the cue at all; production of the cue may simply be the consequence of natural leakage of metabolites from a producing organism (see below).

A main constraint on the detection of a water-soluble cue *in situ* is the extent to which it is diluted or dispersed by hydrodynamic factors. This topic has been discussed extensively in the literature, and will not be reviewed in depth here (see Chapter 12 in this volume). It has been argued that such cues may not be generally important in the real world because of rapid dilution or dispersion in the field (as Jennings and Steinberg[57] have argued for the deterrent effects of phlorotannins from *Ecklonia radiata*) or because of an inability of weakly swimming larvae to follow a concentration gradient in flow.[180–182] However, such conclusions are generally drawn without much specific knowledge of the cues, although some experiments with isolated cues in flow have been performed.[118,183] With regard to the weakly swimming hypothesis, we note that organisms both larger (larval fish[184]) and smaller (bacteria[185,186]) than invertebrate larvae are capable of strong directional movement in response to cues. Moreover, the observation that settlement patterns of some larvae at a variety of spatial scales do not differ from passive settlement patterns[30,187] is perhaps not surprising, since presumably not all larvae or spores respond strongly (or at all) to chemical signals. It is also not known whether settlement in response to chemical cues is generally concentration dependent, requiring a propagule to follow a diffusion gradient, or a threshold phenomena, in which detection of a cue above a threshold concentration induces settlement. The latter appears to be the case for oyster larvae, which drop out of the water column upon detecting a water-soluble peptide cue.[117,118] Similarly, metamorphosis of the echinoid *Holopneustes purpurascens* in response to the floridoside–isethionic acid (Figure 10.1) complex is only concentration dependent in a narrow range (2.5 to 12.5 μM). At test concentrations above 12.5 μM (125 μM was the highest concentration tested), all larvae rapidly metamorphosed.[35]

A number of authors[29,30] have addressed the potentially contrasting roles of larval cues vs. hydrodynamics by highlighting the need to understand the different spatial scales at which cues are effective. One simple experiment which would address this issue is to collect water samples at varying distances from the putative source of a cue and test their effectiveness at inducing settlement.

2. The Signal Should Be Present (and Accessible to Detection) in as High a Concentration as Possible

A second argument for the prevalence of primary metabolites as inducers is that they are generally the most common kinds of metabolites produced by organisms, and thus, all else being equal, they should be more abundant and therefore easier to detect than less common compounds. Total nonpolar secondary metabolites in many benthic organisms typically represent 1 to 2% or less of the dry weight of the organism.[188] In contrast, primary metabolites, particularly sugars, can constitute significant fractions of the mass of an organism. The red algal sugar floridoside (Figure 10.1), one component of the characterized inducer of metamorphosis for *Holopneustes purpurascens*,[35] can comprise over 30% of the mass of some red algae,[189] and levels of 8 to 10% are common.[141,189] By comparison, furanones, inhibitory compounds in *Delisea pulchra* (from which the floridoside–isethionic acid inducer was first isolated), typically occur at less than 1% by weight, of which 0.2 to 0.4% of the total is on the surface.

Water-soluble primary metabolites should also be more generally accessible to settling propagules than either secondary metabolites or insoluble primary metabolites. Though sequestration of secondary metabolites is much better known in terrestrial[62] than marine organisms (Table 10.1), benthic organisms must also avoid autotoxicity, and their secondary metabolites are also likely to be encapsulated in various ways. As discussed in Section II, sequestered metabolites may not be presented at the surface of undamaged individuals. Insoluble inducers may be contained within cell walls and only released through hydrolysis or other damage to the cell walls.[127,142] In contrast, primary metabolites are often not encapsulated and readily leak or are exuded out of the organism. This is well known for algal sugars and polysaccharides.[190] Peptides and amino acids also leak from algae, biofilms, and invertebrates.[191]

Finally, the strength of a signal from a biological source should also be enhanced the greater the abundance of the producing organism and the greater its tendency to occur in monotypic stands. Not surprisingly, some of the best-known examples of inducers for invertebrates are produced by organisms which occur in dense, nearly monospecific stands, e.g., oysters,[115,116] barnacles,[40] and several species of algae.[35,146]

3. Organisms May Be Pre-Adapted to Use Primary Metabolites as Positive Signals for Colonization

This pre-adaptation of organisms may occur for at least two reasons. First, bacteria, macroalgal spores or gametes and some invertebrate larvae[192] absorb nutrients directly from the environment, including amino acids, peptides, and sugars, and in many cases respond to concentration gradients in these compounds via chemotaxis. Thus, for some organisms, organic nutrients function directly as signals. This is perhaps most evident for bacteria, such as *Myxococcus xantos*, which uses amino acids both as differentiation signals[193] and nutrients. However, echinoderm larvae also use simple waterborne amino acids and sugars as signals for morphological change.[192] Given that nutrients often accumulate at surfaces,[42,194] initial attraction to particular surfaces may, for many organisms, simply be a function of following concentration gradients to high concentrations of nutrients or dissolved organics at a surface. The evolution of recognition of particular individual signals could have then occurred as responses to particular small organic molecules became more specific, even if organisms no longer relied on the absorption of the signal molecules as nutrients.

Second, a significant proportion of the known organic laboratory inducers of invertebrate settlement or metamorphosis are peptides, amino acids, or their derivatives.[37,152] These neurotransmitters and neurotransmitter mimics (e.g., L-DOPA and GABA) may not be presented exogenously to the larvae in the field at active concentrations and, thus, may not be ecologically relevant inducers. Rather, they may act as internal signals in signal transduction pathways. Nonetheless, they provide further evidence for the general importance of peptides and amino acids in the overall process of

larval settlement. Existing responses and sensory machinery to internal signals may have pre-adapted organisms to respond to similar metabolites as exogenous signals. Rittschof[114] develops this theme more generally, arguing that peptide cues are widespread, conserved signal molecules which induce a variety of behavioral and ecological responses of marine invertebrates.

4. The Signals Should Be Resistant to Degradation

Though resistance to degradation is not restricted to polar primary metabolites, if such metabolites predominate as signals for other reasons, then it would also be advantageous to be resistant to degradation. Decho et al.[34] have shown that particular peptides — those with a basic amino acid terminus — are more resistant to degradation by marine bacteria than those with acidic termini. As a consequence, particular peptides may be more common as signals than others.[34] Resistance to degradation or uptake by marine bacteria may be a particularly important consideration because marine bacteria typically have a greater uptake affinity for dissolved organics than do marine eukaryotes.[195]

In sum, inducers will be easiest to detect if they are produced in high concentrations by abundant organisms which are persistent features of habitats, leak readily out of organisms, and resist bacterial degradation.

D. Ecological Correlates of Inducers

1. Habitat-Specific vs. Habitat-General Cues

To date, general classifications of inducers have been primarily based on the sources of the cues, with a variety of authors[12,13] distinguishing between gregarious (conspecifically derived) cues, associative cues derived from species other than the colonizing organism, and cues derived from biofilms. However, the importance of distinguishing between these categories may not always be clear. For example, unless adult organisms or newly settled juveniles have the capability of preferentially attracting related conspecific propagules (thereby enhancing their own extended fitness), there would seem to be few important ecological or evolutionary distinctions between many gregarious and associative cues. That is, a larva attracted to an assemblage of conspecifics or a dense stand of a preferred prey or host plant[35,146] is, in both instances, responding to a signal representing a suitable habitat. The distinction is further blurred by the realization that in many instances it is not known if gregarious cues are, in fact, produced by conspecifics or by a biofilm associated with the organism, in which case it becomes an associative cue (for eukaryote propagules, at any rate).

An alternative approach is to consider whether the cues are produced broadly across the habitat or are specific to particular source organisms. Similarly, one could ask whether the colonizing organisms are habitat generalists or specialists. This approach develops the theme of Johnson et al.,[106] who argue that specificity of cues may be due to the presence of host-specific vs. more generalized biofilms, which are, respectively, the source of habitat-specific or more generalized habitat cues. However, the only two characterized inducers of larval metamorphosis are derived from eukaryotes.[35,157] Thus, the argument of Johnson et al.[106] can be considered more generally, by asking whether cues are broadly distributed across a habitat and whether organisms exhibiting different breadth of settlement preferences use different cues. One possibility is that deterrents will tend to be very specific metabolites associated with specific sources, whereas inducers will be more broadly based (e.g., as suggested for peptides[34,114]). The work of the authors supports this distinction. Negative cues (furanones) from *Delisea pulchra* are unique to the genus, while the components of the inducer from this species — floridoside and isethionic (Figure 10.1) — occur broadly across the red algae. The polysaccharide cues from corallines which induce settlement of planula larvae are also thought to be broadly distributed across reef habitats as well as active against a broad range of species of corals.[144]

2. Life History of the Propagules

The range of life history characteristics among eukaryote propagules is substantial. Algal propagules and invertebrate larvae vary in their sensory capacity, length of life (from hours to months), swimming ability (from nonmotile to active swimmers), ability to absorb nutrients, and many other factors. Algal propagules tend to be short lived relative to larvae and, due to their small size and lack of swimming appendages other than flagella, may be weaker swimmers. Such properties would be expected to affect the ability of propagules to respond to chemical cues. For example, given their inability to respond actively to chemical cues, nonmotile spores (such as those from the red algae) should be more resistant to natural antifoulants than longer-lived propagules which are active swimmers. Given their constraints on active habitat selection, nonmotile propagules are also not expected to rely heavily on positive chemical cues. In a similar vein, Wahl and Mark[179] suggest that sessile epibiota are mostly habitat generalists and facultative with regards to settlement on living vs. nonliving hosts. In one of the few direct comparisons of the response of propagules with different life histories to chemical cues, Krug and Zimmer[147] found minimal differences in the responses of lecithotrophic vs. planktotrophic larvae of the sea slug *Alderia modesta* to chemical cues from their host alga. Given the role that comparisons among propagules with different life histories has played in larval biology, further comparisons of responses to signals by propagules with different life histories would be a fruitful area for further study.

IV. SIGNAL-MEDIATED BACTERIAL COLONIZATION

Effective colonization of a surface by bacteria can occur in a number of different stages and occurs at both the level of the individual cell and in multicellular populations and communities. Pragmatically, the time course of bacterial colonization from initial attachment to complex biofilm formation is also often short in comparison to the time course of settlement and metamorphosis for larvae of many invertebrates. Thus, bacterial colonization is broadly considered here to include directional swimming (e.g., the ability to approach a surface) (see also Chapter 12 in this volume), attachment, various kinds of surface motility, and biofilm formation. Chemical signals can affect all stages of this process for bacteria, although direct evidence is sparse for marine bacteria.

A. Bacterial Chemotaxis and Swimming

Standard models for bacterial chemotaxis are based on the behavior of nonmarine enteric bacteria.[196] Chemotactic behavior of nonmarine bacteria consists of discrete steps of short runs interspersed with tumbling, resulting in the random repositioning of the cells, i.e., the classical random walk. As a consequence, the net speed up a chemical gradient via the random-walk response is only a few percent of the swimming speed. The relatively slow speed and mode of chemotaxis displayed by nonmarine enteric bacteria would restrict the ability of marine bacteria to respond to chemical gradients in the sea and hence cast doubt on the importance of chemotaxis for bacteria in turbulent marine environments.

However, Mitchell et al.[185] have recently shown that both heterotrophic marine bacteria from cultures and enriched natural samples of marine bacteria can swim at speeds up to 400 μm s^{-1}. This is at least an order of magnitude faster than enteric bacteria and forces a re-evaluation of the potential role of chemotaxis as a means of approaching nutrient-rich surfaces or patches in the sea. The speed at which marine bacteria move, the pattern at which they travel, and their rapid changes in speed all imply fast reaction times for sensing and responding to nutrients in the environment and the ability to respond to nutrient gradients around micropatches even in turbulent waters.[185,186] Through rapid speed changes and reversals, marine bacteria can detect the gradient edge and maintain themselves in a micropatch, away from turbulent shear that would otherwise remove them from the nutrient patch.

These results have implications for propagules of marine eukaryotes as well as for marine bacteria. The ability of bacteria to rapidly respond to chemical signals as they approach a surface indicates that small organisms can detect and respond in an active way to chemical signals. The chemotactic response of bacteria to nutrients is also consistent with the suggestion above (Section III.C.3) that signal-mediated settlement by propagules of higher organisms could have evolved from chemotactic responses to high localized concentrations of nutrients, such as at surfaces.

B. Colonization: Attachment, Surface Motility, and Biofilm Formation

Bacteria respond to chemical signals when attaching to and moving about on surfaces and developing biofilms. For example, signal-based regulatory systems such as the AHL (acylated homoserine lactone, Figure 10.1) system are important for colonization of surfaces (including those of higher organisms) for a number of bacteria common in aqueous (though mostly nonmarine) environments.[197] One of the most compelling examples of the role of AHL regulatory systems in bacterial colonization is the formation of multicellular clusters in biofilms of *Pseudomonas aeruginosa*[198] and *Aeromonas hydrophila*.[199] Davies et al.[198] have shown that mutant strains (*lasI*-) of *P. aeruginosa* that lack the ability to produce relevant AHL form flat biofilms in which the bacteria are tightly packed. The addition of exogenous AHL restores the more complex, three-dimensional structure of wild-type biofilms. Similarly, in the best known example of AHL regulation from marine systems, the marine bacterium *Vibrio fischeri* relies on its AHL system for successful colonization of the light organ in squid (reviewed by Visick and Ruby[200]). As with *P. aeruginosa*, mutant strains which lack AHL (*luxI-)* fail to colonize the light organ.[200]

Other specific colonization traits mediated by the AHL signaling system include attachment[201] and surface motility or swarming[202] of *Serratia liquefaciens*. Swarming is apparently common in marine bacteria,[16] although the underlying regulatory control is not known. Bacteria also express extracellular products, via the AHL regulatory system, which facilitate colonization of eukaryotes. For example, exoenzymes accounting for virulence (and thus effective colonization of hosts) by the plant pathogens *Erwinia carotorora* and *E. stewartii* are not produced by mutant strains lacking the AHL system. Similarly, regulation of Ti plasmid conjugation in *Agrobacterium tumefaciens*, and, hence, virulence on host plants, depends on the presence of AHL.[203,204] Colonization by cells in beneficial bacterial–host associations can also rely on AHL-mediated gene regulation. Examples include the nitrogen-fixing symbiotic bacteria *Rhizobium leguminosarum*[203] and *Vibrio fischeri* in the light organ of the squid (above).

Indirect evidence for the importance of AHL regulation for colonization by marine bacteria comes from the observations that furanones from the red alga *Delisea pulchra* specifically interfere with AHL systems[205,206] and inhibit colonization of surfaces (attachment, swarming) by marine bacteria.[16] Involvement of AHL regulation in marine eukaryote–prokaryote interactions is further suggested by recent identification of an AHL-producing bacterium, *Vibrio campbelli*, as a common component of the culturable bacterial fraction from several species of sponges near Sydney.[207]

More generally, there exists a wide range of bacterially produced extracellular signaling molecules.[197] The role of these signals in colonization of surfaces by marine bacteria is largely unknown, although some signals are widespread across many bacteria. These include cyclic dipeptides[208] and the apparently ubiquitous, water-soluble *luxS* encoded, AI2 (AutoInducer 2) signal, first discovered in the marine bacterium *Vibrio harveyi.*[209] One function of the AI2 system may be to facilitate surface colonization by bacteria.[209]

As with eukaryote signals, the presentation and microscale distribution of bacterial signals *in situ* is largely unknown. The data that is available suggest that understanding the distribution of the compounds will be crucial for understanding colonization processes. Charlton et al.[210] measured concentrations of 3-oxo-acylated homoserine lactones (Figure 10.1) in flow cells in which they grew biofilms of *Pseudomonas aeruginosa*. Concentrations of AHL differed strikingly between the

biofilm itself and the culture medium exudate, and between bacteria grown separately in bulk liquid cultures (up to one thousand-fold greater concentrations in the biofilm).

V. OTHER CHEMICALLY MEDIATED SURFACE-BASED INTERACTIONS

Many other ecological phenomena besides colonization rely on interactions at surfaces, and many of the issues raised above are also relevant to these phenomena. Allelopathy, the chemical mediation of competition, relies on the production of surface or near-surface cues to deter competitors. Although there are a number of intriguing studies of allelopathy in the literature,[211,212] presentation and surface quantification of the putative allelopathic chemical(s) are largely unknown. Determination of suitable prey by a predator may often occur as a response to an olfactory or gustatory cue present at the surface of the prey. Failure to localize and present a predator deterrent in an appropriate way may, in fact, result in accidental consumption of the prey by a predator, with consequences just as severe as purposeful consumption. The failure of many aplysiid sea hares to localize sequestered algal secondary metabolites at their surface has, in fact, resulted in Pennings and Paul[213] calling into question the utility of sea hare acquired algal defenses. The use of sex pheromones by eggs to attract sperm, well known from both macroalgae and invertebrates,[214,215] is an additional surface-based interaction that is chemically mediated, although, in this instance, the surface in question is the exterior of an egg. Although the inducers in this case are generally nonpolar hydrocarbons, they are effective at concentrations in which they are soluble in water.[214,215]

Finally, an aspect of chemical mediation of surface interactions not covered here in depth is the role that naturally produced chemicals may play as modifiers of the physical characteristics of a surface (e.g., physicochemical effects such as hydrophobicity or surface energy). For example, a number of studies have shown that colonization by some fouling organisms is reduced on artificial surfaces that have surface free energies in a particular range (typically 20 to 30 mN/m[216,217]). Vrolijk et al.,[218] in one of the few instances of an analogous study with marine organisms, showed that the surface free energy of two species of typically unfouled gorgonians corresponded to the energy minima associated with low fouling artificial surfaces. Thus, one chemical strategy for minimizing surface colonization may be to deposit or present at the surface molecules that affect the physical properties of the surface in particular ways. Difficulties in assessing the generality of this proposal in a realistic ecological context for colonization of marine organisms include (1) physical properties of surfaces tend to be rapidly modified by molecular conditioning films[219] or biofilms[220] once immersed, (2) most studies to date have been done on artificial surfaces, often in the context of the development of new antifouling technologies,[221] (3) colonization by propagules of a variety of organisms does not vary predictably with variation in surface free energy,[222,223] (4) measurements of physical characteristics are generally not done when the surface is immersed,[224] which may potentially change hydration and other parameters of the surface relative to the *in situ* condition, and (5) processing of samples (e.g., fixation) prior to analysis may also alter surface characteristics from the *in situ* condition. Vrolijk et al.[218] discuss some of the difficulties associated with measuring and interpreting physicochemical data for living marine surfaces.

VI. OVERVIEW AND CONCLUSIONS

This chapter attempted to understand whether there are common themes in the use of chemical cues to mediate colonization of surfaces by propagules. Few studies have definitively demonstrated that a characterized chemical, at a known concentration, either positively or negatively mediates colonization in the field. Nonetheless, some general patterns appear to emerge (Table 10.2).

Of obvious importance for the success of any cue is that it persists at effective concentrations long enough for the receiving organisms to respond. Where the production of the cue represents a potential cost to the producing organism, there should be selection to achieve this as efficiently as

TABLE 10.2
Comparison between Chemical Deterrents of Colonization vs. Inducers as Proposed in This Chapter

	Deterrents	Inducers
Chemistry	Nonpolar secondary metabolites	Primary metabolites (carbohydrates, peptides) Most water soluble
Taxonomic breadth of production	Narrow	Broad
Quantities produced	Small	Large
Natural selection strongest on	Signal producer	Signal receiver
Importance of hydrodynamic factors for the interaction	Low	High

possible. For deterrents, this argues for deployment of nonpolar metabolites which will adsorb to hydropobic (cell) surfaces of producing organisms with a minimal rate of dissolution into the water column. This may be particularly important for highly pliable organisms such as seaweeds or many benthic invertebrates, for which the establishment of persistent surface boundary layers may be rare.

The ecological context for inducers is different from that of deterrents. First, selection on the receiving organism should be more stringent for positive cues, as the inability to detect a signal may result in a failure to successfully recruit. To maximize the probability of reception by a settling propagule, an inducer should be present in high concentrations and persist in the habitat. These authors suggest that water-soluble primary metabolites, from either benthic eukaryotes or biofilms, best fit these criteria.

The importance of hydrodynamic factors should also differ for inducers vs. deterrents. Hydrodynamic events may greatly reduce the efficacy of inducers,[29] rendering them undetectable by propagules. In contrast, hydrophobic deterrents only need to act right at the point of contact between the settling propagule and the surface of the host. Any propagules that miss the target due to flow or turbulence are irrelevant to the host and its defenses.

Too little is known in a realistic ecological context about the role of chemical signals as mediators of colonization by marine bacteria to generally compare these processes between bacteria and eukaryotes. For at least some (not necessarily marine) bacteria, signals mediate several stages of colonization, including attachment, surface motility, and biofilm formation. In many instances (e.g., between bacterial cells in a biofilm), these signals may be operating over the scale of micrometers, in contrast to the hundreds of micrometers to centimeters that may be more typical of invertebrate larvae.

Finally, new methodologies are likely to lead the way in our exploration of chemically mediated interactions at surfaces. New and improved methods for detection and quantification of signals *in situ* are needed, as are development of *in situ* bioassays and enhanced molecular methods for characterizing bacterial communities.

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