Review of factors contributing to the settlement and recruitment of barnacles

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Introduction

Barnacles are popular study organisms for a variety of reasons. As adults, they are sessile, and they are often small and numerous, making them easy to manipulate in ecological experiments. A researcher can be sure that an adult barnacle that disappears between observations has died, and has not moved away. As larvae, they develop through a series of six naupliar instars in the plankton. Nauplii are easy to identify in plankton samples, and are easy to collect from the egg lamellae of adult barnacles and culture in the laboratory. Nauplii metamorphose into the non-feeding cyprid stage, at which point they attempt to contact and stick to hard substrata. Depending on condition of the substrate, cyprids metamorphose into juvenile barnacles or return to the water column. A juvenile barnacle feeds and grows at its site of metamorphosis until it dies.

Barnacles can be a costly nuisance to human society. Along with other sessile marine invertebrates and algae, barnacles foul boat hulls and other man-made structures, reducing fuel efficiency and costing time and money in removal efforts. Much research into barnacles has been motivated by preventing the fouling of man-made substrata. Because of their affinity for man-made structures, some species of barnacle have successfully invaded new habitats, and have, in some cases, been spread worldwide by human shipping and mariculture practices.

Much effort has gone into research on settlement and recruitment of barnacles. In the marine biological literature, settlement refers to the process by which a planktonic larva leaves the plankton for the benthos. A cyprid is transported shoreward and then contacts and explores a substrate, then undergoes metamorphosis into a juvenile barnacle. The moment that a cyprid contacts its site of metamorphosis can be called the moment of settlement (Keough and Downes 1982, Connell 1985). Metamorphosis is irreversible and has important consequences for the barnacle, because it must remain on the exact point of metamorphosis for the remainder of its life. Failure by the cyprid to choose a suitable settlement site results in death before reproduction. There are many factors affecting settlement and those factors apply to the different aspects of the settlement process, and will be discussed in the context of historical and recent research. Recruitment is the process by which larval or juvenile organisms join the adult population. Barnacle recruitment encompasses settlement and post-settlement processes, from larval stages to early juvenile, after which a barnacle has "recruited" and is considered to have joined the adult population. These terms were once used interchangeably (Keough and Downes 1982), but the distinction has been clear in recent literature. Many factors can affect both settlement and recruitment, and are the focus of much research. Table 1 summarizes these and their importance for the different life stages of the barnacle.

Research on barnacles is a common pursuit among ecologists and larval biologists, because of their ease of study and because results may generalize to other species with planktonic larvae. The abundant literature on barnacles necessitates occasional review. The intent of this essay is to review historical and recent research on settlement and recruitment of barnacles and make suggestions for future research that will lead to a comprehensive understanding of the processes which influence barnacle settlement and recruitment.

Larval Condition

Larval condition has an impact on settlement ability and post-settlement mortality. Cyprids are non-feeding, and they must rely on stored energy in the form of lipid and protein reserves while they swim in the plankton, contact and explore a surface, and metamorphose into a juvenile barnacle. Lucas et al. (1979) showed that lipid and protein content of *Balanus balanoides* cyprids decreases with the number of weeks spent in laboratory. A cyprid's ability to successfully undergo metamorphosis decreases after 3 or 4 weeks containment in the laboratory. This shows that cyprids have a limited amount of time to find a suitable settlement substrate before they no longer have enough energy to successfully metamorphose. This has implications for settlement tendencies of different ages of cyprid. Young cyprids are more "choosy" than older cyprids in their settlement site-specificity (Rittschof et al. 1984). The tendency of older, non-feeding planktonic larvae to settle more readily than young larvae is referred to in the literature as the desperate larva hypothesis (Knight-Jones 1953). This has been demonstrated behaviorally in barnacles by Miron et al. (2000), who examined the effect of larval condition, as measured by the ratio between triacylglycerol (TAG) and DNA, on substrate exploration in cyprids of *B. amphitrite*. TAG/DNA ratio decreased with larval age, and older cyprids explored "unsuitable" substrata more often than younger cyprids. Barnacle cyprids have a limited amount of time to settle since they can not acquire new energy, though they may be able to extend this time period. Pineda et al. (2005) reported the ability of cyprids of the barnacle *Semibalanus balanoides* to survive while embedded in sea ice, and retain the ability to metamorphose after thawing. Cyprids that were frozen were able to maintain this ability for a maximum of 38 days, compared to non-frozen cyprids, which would lose the ability after a maximum of 26 days. The ability to survive freezing may allow cyprids to disperse further and may extend the recruitment season of *S. balanoides*.

Jarrett and Pechenik (1997) measured organic content of cyprids of *Semibalanus balanoides* on five different days to determine temporal variation in larval quality. Juveniles that settled on these days were raised in the laboratory to determine growth capacity. Organic content of cyprids significantly varied by day collected, as did juvenile growth rate. This study demonstrated that larval condition can vary by day and impacts post-settlement processes. Jarrett (2003) went on to demonstrate that quality of cyprids (in terms of organic content) of *S. balanoides* impacts juvenile growth in the field, confirming that the results of the previous studies are applicable to natural populations of the barnacle.

Larval condition varies with cyprid age because energy reserves are used by cyprids to swim and explore surfaces. It also varies by the initial energy investment of the parent barnacle into the egg and by the success of naupliar feeding in the plankton. These factors vary with parental condition and environmental variables. Barnes and Barnes (1965) described egg and nauplius sizes of some barnacle species, and the variation of these factors with environmental conditions. They found that egg size in *Balanus balanoides* was larger in regions with severe winters and cool summers, and they suggest that this trait is adaptive for the climate. Warm-water barnacle species have smaller egg sizes than *B. balanoides*, which may be due to the relatively frequent reproduction rate in warm-water barnacles.

Further, larval production rate is tied to environmental conditions. Leslie et al. (2005) found that barnacle larval production in *B. glandula* along the Oregon coast was 5 times higher in a region of high primary productivity compared to a region of lower primary productivity, approximately 80 km away.

In addition to the condition of individual larvae, the genetics of the larval pool is also important, and this factor might be viewed as larval condition in that an individual cyprid can show heritable traits of settlement preferences (Toonen and Pawlik 2001), and the genetic diversity of a pool of larvae has an impact on their settlement range and intensity. Gamfeldt et al. (2005) recently presented a study on the impact of intraspecific diversity on settlement in *Balanus improvisus*. Increased number of parental broods (1, 2, or 3) resulted in increased settlement. The authors suggest several mechanisms by which genetic diversity may influence settlement rate: by diversifying settlement preferences, by increasing frequency of founder larvae in the settlement assays, by stimulating cyprid response to individuals with complementary genotypes, or by increasing the ability of the brood to deal with stress. Research that includes the use of settlement assays should take these results into account: the use of larvae from multiple parents may yield higher settlement rates than what occurs for larvae cultured from a single parent.

Larval condition is an important consideration in settlement research. Condition impacts settlement ability, preference, and post-settlement performance. Researchers should be aware that field-collected barnacle larvae will vary in physiological quality, genetic diversity, and age, and these factors will influence their settlement. Because environmental conditions impact quality, the condition of larvae in the field varies spatially and temporally. The condition of larvae cultured in the laboratory may therefore differ greatly from those in nature, and this should be considered in settlement studies.

Larval Supply

Settlement rate depends on the passive process of contact and the active process of metamorphosis by cyprids. Contact rate is regulated by flow, surface contour, and larval supply. Larval supply is largely a passive process, controlled by currents, winds, and other large-scale oceanographic processes. Bousfield (1955) demonstrated an exception in *Balanus improvisus*, which is retained in its home estuary via vertical migration during the naupliar stages. Nauplii sink to the bottom of the water column during the ebb of the tide to avoid being flushed into the ocean and dispersed. In this case, larval behavior regulates larval supply in the estuary. Passive retention due to the enclosed geography of the Lough Hyne Marine Reserve in Ireland facilitated the invasion of the non-native barnacle *Elminius modestus* (Watson 2005). This study is another example of the effect of retention on larval supply.

Berntsson et al. (2004) found that larval rejection of substrata can lead to aggregated settlement on nearby settlement-worthy substrata for *Balanus improvisus*. They placed microtextured panels in the field and only 1-5% of cyprids settled on the panels after initial contact, compared with 28% settlement after contact on smooth panels. Exploration behavior of individual cyprids was observed on each type of substrate. Cyprids demonstrated higher crawling speed and dispersal rate on textured substrata than on smooth substrata, indicating less exploration on the unsuitable substrate. Rejection of substrata led to increased contact on adjacent substrata, suggesting that rejection behavior may be a factor in aggregated settlement. This is similar to the findings of Pineda and Caswell (1997), who found disproportionately high settlement on surfaces in areas of low substrate availability. In this case, it is not rejection of unsuitable substrata, but unavailability of substrata that leads to aggregated settlement. The authors called this phenomenon the intensification effect.

Mace and Morgan (2006) examined settlement of invertebrates, including the barnacles *Balanus* spp. and *Lepas* spp., on either side of a headland in California. They found that settlement was higher in the lee of the headland due to accumulation of larvae in that area. Jenkins and Hawkins (2003) reported an example of the impact of small-scale larval supply on settlement. They examined larval supply of *Semibalanus balanoides* to sheltered rocky shores in northwest Europe. There is a limited abundance of adult *S. balanoides* among fucoid-dominated sheltered rocky shores. The authors found that differences in flow rates between sheltered and rocky shores did not account for the difference in adult abundance. Larval supply was 14 times greater in the sheltered area with low flow compared to the exposed area. However, fucoid algae was more abundant at the sheltered site and acted as a physical barrier, limiting larval supply to the substratum below.

Larval supply is influenced by both passive and active processes operating on both large-scales and small-scales. When studying the factors affecting barnacle settlement in the field, supply is an important consideration, and researchers should be aware of the factors that influence supply of their study species. In the absence of this information, researchers should be aware of the factors that operate on larval supply of barnacles that share a similar habitat with their study species.

Chemical sense

Cyprids determine the suitability of a substrate for settlement by interpreting chemical cues present on the substratum. These cues include salinity of surrounding water (Dineen and Hines 1992), biofilm condition, presence of toxic or otherwise inhibitive antifouling compounds, and presence or absence of congeners, spatial competitors, and predators. While cyprid chemosensory capabilities are not fully understood, they are known to detect the aforementioned factors during exploration of a potential settlement site. The relative role of each of the cues is not known (Rittschof et al. 1998). Rittschof et al. 1984 described the protocol for preparation of settlement factor to induce settlement of cyprids onto a surface. This recognition of conspecific extract by cyprids is an example of their chemosensory ability. Cyprids temporarily adhere to the substratum using attachment discs on the 3^{rd} segment of the antennules, and a sensory seta, protruding from the center of the disc, facilitates the chemosensory ability (Anderson 1994, Blomsterberg et al. 2004).

Recent evidence supporting the chemosensory ability of barnacle cyprids comes from studies of commensal and parasitic barnacles. Their need to identify substrata as their specific host species necessitates well-developed chemosensory abilities. The parasitic rhizocephalan barnacle, *Sacculina carcini*, has accessory chemosensory setae (aesthetascs) with many ciliary branches originating from the 3^{rd} and 4^{th} antennule segments, which are though to be used for long-range detection of dilute chemical cues (Pasternak et al. 2005). Nogata and Matsumura (2006) recently described larval development of *Coronula diadema*, the whale barnacle. They found that it did not settle in seawater unless there was a piece of skin from the host whale present in the settlement culture, indicating the necessity of a host-specific chemical cue for settlement to occur. The rhizocephalan barnacle, *Loxothylacus texanus*, must settle on its host species, *Callinectes sapidus*, within 3 days after metamorphosis from the last naupliar stage. Boone et al. (2003) determined that chemical cues in the crab exoskeleton epicuticle layer were responsible for inducing settlement. Bacterial biofilms associated with the host exoskeleton were isolated and did not induce settlement alone, indicating that they had no role in the settlement of cyprids of *L. texanus*. The authors suggest that biofilms may actually protect host species from parasitic barnacles by shielding cyprids from the cues located on the epicuticle.

Cyprids tend to settle gregariously in the presence of adult barnacles and/or on areas previously explored by other cyprids (Head et al. 2004). Presence of conspecific adults and cyprids on a substratum indicates suitability of the substratum for post-settlement survival. Rittschof et al. (1984) described the protocol for preparation of adult barnacle extract and its application to substrata to invoke gregarious response of cyprids in settlement studies. The specific compound responsible for gregarious settlement was recently determined by Dreanno et al. (2006 a). They identified and described the settlement-inducing protein complex (SIPC), isolated from adult *Balanus amphitrite*. A settlement assay with a treatment of purified SIPC induced settlement at a concentration 100 times lower than that of crude adult extract. Dreanno et al. (2006 b) determined similarity between the cyprid temporary adhesive and the SIPC using antibodies of two different peptides of the SIPC in *Balanus amphitrite*. The antibodies bound to the cyprid footprint protein left behind on nitrocellulose paper, as well as the antennular discs of cyprids. The authors suggest that SIPC is either a component of, or identical to, the cyprid footprint protein.

Surface wettability is the degree to which a surface is hydrophobic or hydrophilic, as measured by the contact angle of a drop of water, and it impacts settlement of the barnacle *Balanus improvisus* (Dahlstrom et al. 2004). Polystyrene petri dishes were made hydrophilic and hydrophobic, and hydrophilic surfaces inhibited settlement compared to hydrophobic surfaces. The authors suggest that hydrophilic surfaces impede the chemosensory ability of barnacle cyprids, leading to rejection of the surface. Qian et al. (2000) obtained the opposite result using cyprids of *B. amphitrite.* They examined settlement of *B. amphitrite* and the bryozoan *Bugula neritina* in flow-through tubes of 7 different materials of varying wettability. *B. amphitrite* preferred hydrophilic surfaces and *B. neritina* preferred hydrophobic surfaces. They also found that biofilm formation affected surface wettability differently for each type of material. Researchers should be careful not to assume surface wettability remains the same on a surface after it has been conditioned in seawater.

Cyprids have chemosensory abilities that allow them to identify suitability of substrata for settlement. There are many chemical cues that barnacles respond to, and they can be inhibitive or inductive to settlement. These cues are not always shared by barnacles of different species, or even of the same species in different circumstances (e.g. response of *B. amphitrite* to surface wettability). Researchers should be aware of the cues that have been shown to be important factors to the settlement of their study organism.

Antifouling Research

Much of the barnacle settlement research is aimed at preventing it. Antifouling products are typically chemically inhibitive to settlement, though some physical methods have been described, such as use of sound waves to inhibit settlement (Branscomb and Rittschof 1984), fouling release coatings (Berglin et al. 2001), and microtextured surfaces (Berntsson et al. 2004) unsuitable for settlement. I have summarized recent findings in antifouling research in Table 2. Most researchers follow the protocol described by Rittschof et al. (1992). *Balanus amphitrite amphitrite* is used because: 1) it reproduces frequently throughout the year, 2) it can be found in temperate and tropical habitats throughout the world, and 3) it is easily kept in culture. Twenty-five to 30 cyprids are placed in polystyrene petri dishes containing 5 ml of experimental or control solution and incubated for 22 hours at 28º C on a 15h:9h L:D cycle. Cyprids and settlers are counted afterwards to determine the effictiveness of the treatment vs. the control.

The weakness of this method is that it does not conform easily to studies of antifouling compounds bound in surface coatings, only compounds in solution. Head et al (2003) point out that the protocol of Rittschof et al. (1992) may be biased by the effects of gregariousness. Head et al. (2003) reported significance of surface area to volume ratio of the container used in bioassay, significance of number of cyprids in the assay, and a significant interaction between the two. These effects can be seen in the contrast between effectiveness values of the settlement inhibitor phloroglucinol obtained from two different studies (Table 2). Head et al. (2004) discovered further complications in the seemingly simple settlement bioassay protocol. They found that gregariousness is reduced with cyprid age and experiment duration increases gregarious effects. They suggested that *B. improvisus* exhibits a greater gregariousness than *B. amphitrite* because *B. improvisus* deposits much more temporary adhesive during exploration.

A recent trend in antifouling research is the use of extracts of marine organisms and their synthetic analogs, which tend to be much less toxic than traditional antifouling compounds, such as copper-based paints. Toxicity is expressed as the LD_{50}, the concentration of compound which kills 50% of larvae in a treatment. Effectiveness is expressed as EC_{50}, the concentration of compound which prevents 50% of larvae from settling in a treatment. When toxicity and effectiveness values are similar, it indicates that the compound prevents settlement by killing the larvae, rather than by directly inhibiting settlement. An ideal antifouling compound has an extremely low effectiveness value and a very high toxicity concentration. Toxicity in antifouling compounds is undesirable because of their tendency to pollute estuaries and negatively impact non-fouling invertebrates. Researchers tend to use extracts from marine organisms that are unfouled in the field. Two groups of organisms that tend to fit this designation are red algae (Hellio et al. 2004, Nylund and Pavia 2003) and sponges (Hellio et al.

2005, Sjogren et al. 2006). The use of Cycloviolacin O2 as an anti-fouling compound (Goransson et al. 2004) is surprising because it is isolated from a terrestrial plant, *Viola odorata.* Bioprospecting for organic extracts to prevent fouling seems to be a field of research that yields, and will continue to yield, valuable information about fouling and larval chemosensory abilities in general.

Despite the acceptance of the protocols of Rittschof et al. (1992) for toxicity and settlement assays, results are often reported that do not follow the protocols and are thus difficult to understand when compared to other results. The results compiled in Table 2 show the use of various units of concentration, the use of varying species of barnacle, and varying methods of calculating effectiveness and toxicity. Authors should strive to follow standards when reporting this type of research for the sake of ease of comprehension, but this is not always possible. Non-toxic compounds, for example, may have toxicity values that are too high to be ecologically significant. Availability of study species will take precedence over the use of the preferred standard, *B. amphitrite.* Researchers should be aware that results may be species-specific, so potential antifouling compounds should ideally be tested on several fouling species to determine the potential for general use of the compound. Time, money, and availability of the compounds may constrain the ability of researchers to determine proper resolution of effectiveness values.

Biofilms

Barnacle researchers have long been aware of the necessity to "condition" a substrate before it can be used in a settlement assay. The conditioning usually involves placing the substrate into seawater for several days so that it develops a biofilm, a layer of marine microorganisms, such as bacteria, protozoans, fungi, and diatoms (Faimali et al. 2004). The biofilm is the first stage in the succession of a fouling community, and provides a more important cue for settlement than do early invertebrate settlers (Keough 1998). Biofilm condition and composition influences settlement in many invertebrate taxa, such as sea urchins (Swanson et al. 2006), polychaetes (Lau et al. 2002, Shikuma and Hadfield 2006), corals (Webster et al. 2004), oysters (Anderson 1996), crabs (Rodriguez and Epifanio

2000), and barnacles (Crisp 1974, Qian et al. 2003).

Recent research has examined the role of biofilm composition and condition on barnacle settlement and post-settlement processes. Faimali et al. (2004) examined the interaction of substrate type and biofilm structure, as well as the effects on settlement of *Balanus amphitrite*. They found that biofilm age and different substrata (glass, marble, quartz, and cembonit) had significant effects on biofilm structure. Settlement varied significantly with substrate type, biofilm structure, and the interaction of the two. Anderson (1996) obtained a similar result in examining settlement of an oyster, *Saccostrea commercialis*, on cement, sandstone, sand, or various mixtures of calcium hydroxide and sand. In this study, there was a significant interaction of biofilm and substrata. Qian et al. (2000) studied settlement of *Balanus amphitrite* in tubes of different type, surface wettability, biofilm condition, and flow rates. They also found a significant effect of interaction between substrate type and biofilm on settlement. The results of these studies have implications in barnacle settlement research, since substrate preference is likely to be confounded by substrate-dependent biofilm composition and condition.

Diatoms within the biofilm can also have an inductive effect on larval settlement. Harder et al. (2002) showed this by settling the polychaete *Hydroides elegans* on monocultures of various diatom species. Settlement rates on each diatom film was categorized as being inductive, weakly inductive, or having no effect in comparison to a natural biofilm (positive control) and a clean glass slide (negative control). They found that settlement induction varied by species of diatom, but was not general within genera. Patil and Anil (2005) examined the influence of several species of diatom and diatom extracellular polymeric substances (EPS) on settlement of the barnacle *B. amphitrite*. They collected free and biofilm EPS from axenic monocultures of five diatom species and non-axenic monocultures of two of these species to isolate effects of bacterial EPS for use in settlement assays. Settlement induction was categorized according to Harder et al. (2002). Free EPS, which are exuded by the diatoms into the environment, did not induce settlement. Biofilm EPS, which remains in the biofilm matrix, did enhance settlement. Non-axenic diatom EPS were more settlement-inductive than axenic diatom EPS, indicating that bacteria have an important role as a settlement cue within the biofilm. The EPS of different diatom species exhibited varying inductive activity. This study highlights the importance of biofilm composition to barnacle settlement.

Biofilm research can be an interdisciplinary endeavor, requiring knowledge of marine ecology and microbiology. Many marine bacterial species are unculturable, and thus are difficult to identify (Lau et al. 2002). These factors hinder understanding of the bacterial composition of biofilms and the role of this composition in settlement. Lau et al. (2002, 2005) incorporated molecular techniques to the study of biofilm bacterial composition. These techniques were used to determine strains of bacteria. Settlement of the polychaete *Hydroides elegans* was induced by 38 bacterial strains across 3 phylogenetic branches, as determined by comparison of 16S rRNA gene sequences. Bacteria of the same genus exhibited different inductive capability, demonstrating that each bacterial strain plays a different role as settlement cue and/or inducer within the biofilm (Lau et al. 2002) for polychaetes. Lau et al. (2005) examined the settlement inducing activity of nine different biofilm growth treatments (3 temperatures: 16, 23, and 30ºC by 3 salinities: 20, 27, 34 ppt). The results indicated different bacterial community composition in the high temperature (23 and 30ºC) treatments and low temperature (16ºC) treatments, while salinity did not have an effect. Settlement of *Balanus amphitrite* and *B. trigonus* was induced by biofilms grown at the high temperatures, and the biofilm grown at a low temperature inhibited settlement of *B. trigonus*, though the researchers point out that the bacteria may have been stressed when moved from 16 to 25ºC for the settlement assay. Settlement of the 2 barnacle species was not correlated with biomass or bacterial density within the biofilm, but it was correlated with bacterial composition, as determined by terminal restriction fragment length polymorphism analysis. Settlement of *H. elegans* was not correlated with bacterial composition or biofilm culture temperature, but with bacterial density. These results suggest a difference in settlement response to biofilm attributes across phyla. The authors suggest that invertebrate larvae may use environmentallydetermined biofilm attributes to identify appropriate settlement habitats. This conclusion is supported by the results of Qian et al. (2003), who conditioned settlement substrata at 3 intertidal heights (subtidal, mid-, and high intertidal). Cyprids of *B. amphitrite* preferentially settled on plates conditioned at the mid-intertidal height, regardless of height of the plate during settlement, indicating that biofilm community composition was responsible for settlement induction.

The age of the biofilm is also an important factor. To determine the effect of biofilm age on settlement, a clean surface is placed in running seawater for various amounts of time before being placed into a settlement container. Biofilm biomass, bacterial density, and community composition changes with time, and these biofilm conditions influence settlement. Qian et al. (2003) found that cyprids of *Balanus amphitrite* settled preferentially on 6-day-old intertidal biofilm compared to biofilms aged 3, 9, and 12 days. Neal and Yule (1994) found that *Elminius modestus* settled more frequently on a 4-day-old biofilm than a 1-month-old biofilm. After settlement, juvenile barnacles adhered more strongly onto the 4-day-old biofilm than the 1-month-old biofilm. This result suggests that the avoidance of the older biofilm may be adaptive since cyprids tended to reject the surface that is less suitable for post-settlement survival. In an interesting approach to the study of biofilm age, Khandeparker et al. (2005) examined the effect of biofilm age on settlement of *B. albicostratus,* as well as the effect of Irgarol 1051, a biocidal antifouling compound, on biofilm settlement inductivity. Aged biofilms that significantly promoted barnacle settlement inhibited settlement when they were treated with high concentrations of the biocide. The compound stressed the biofilm bacteria, causing the biofilm to become toxic and unsuitable for settlement. This effect can confound toxicity and antifouling research in the field, in that it may be impossible to determine whether a compound is directly inhibiting larval settlement or is affecting biofilm condition.

Biofilms have sometimes been shown to inhibit settlement of barnacles. Olivier et al. (2000) found that cyprids of *Balanus amphitrite* settled more often on clean surfaces than those with a biofilm. This result is anomalous with respect of most biofilm research. Theirs was a multifactorial study of barnacle settlement, examining effects of larval supply, tidal level, and biofilm condition on settlement. They conditioned Plexiglas disks at 3 heights spanning the vertical range of *B. amphitrite,* for 21, 14, or 7 days. The disks, along with clean disks with no biofilm, were placed at three intertidal heights in the field and were then examined for barnacle settlement. Biofilm age was the most important factor affecting settlement, with the significantly greater settlement on clean plates compared to those with a biofilm. Bacterial density was correlated to biofilm age. Lau et al. (2003) also demonstrated an inhibitory effect of biofilm with settlement preference experiments using three isolated strains of biofilm bacteria. *B. amphitrite* cyprids chose to settle more often on clean surfaces rather than those with a monoculture of each of the three strains. They demonstrated that the bacteria retain settlement-inhibitory activity even after they were killed by UV radiation or formaldehyde. Hung et al. (2005) studied the effect of UV-A and UV-B radiation on the ability of biofilms to influence settlement of *B. amphitrite.* In accordance with the results of Lau et al. (2003), they found that treating the biofilms with UV radiation did not affect subsequent barnacle settlement, though it did kill the bacteria. In this case, barnacles settled more frequently on unfilmed surfaces.

Biofilms influence settlement of barnacles in a variety of ways. Biofilms vary greatly in their condition by location and age, and barnacles respond to the condition when settling. Cyprids exhibit a settlement response to biofilms after the bacteria have been killed by radiation, indicating that they respond to chemical cues generated by the organisms within the biofilm. Molecular techniques are used to identify species of bacteria that are unculturable, and can be used to assess the diversity of the biofilm community. Researchers should be aware of how their study species responds to biofilms and should condition experimental substrata according to their responses.

Substratum Characteristics

Barnacles researchers have long been aware that physical conditions of a potential settlement site can impact settlement. Wethey (1984) examined daily changes in settlement of *Balanus balanoides* by taking daily photographs of substrata in the intertidal zone. He found that barnacles preferred to settle in pits and cracks over flat surfaces on the substrate. Wethey (1986) also investigated the role of surface contour, independent of chemical cues, by making plastic casts of natural rock surfaces and placing them into the field. About 30% of cyprids of *Chthamalus fragilis* and *Semibalanus balanoides* settled in the same microsites on replicate casts. These microsites composed 7% of the total available settlement area, indicating that contour alone influenced settlement. Larvae tended to settle in areas where sand settled in flume trials, indicating the potential importance of passive processes (flow and substrate contour) on settlement. Wethey's results demonstrate the importance of the topography of the the substratum surface.

Herbert and Hawkins (2006) studied the effect of rock type on settlement and post-settlement mortality of *Chthamalus montagui*. They measured the hardness and roughness of the four rock types: Kimmeridge Cementstone, Blue Lias Limestone, Chalk, and Bembridge Limestone. The hardest and least rough was Kemmeridge Cementstone, which exhibited significantly lower recruitment than the other 3 types. Rock type had a significant effect on mortality, which was highest on chalk because it tends to chip and flake easier than the harder rock types. The authors suggest that *C. montagui* will have difficulty in colonizing the English Channel because of its abundance of chalk platforms.

Barnacle cyprids can assess and respond to physical substratum characteristics during exploration. These characteristics include rugosity (roughness), hardness, contour, and topography. Researchers should be aware of the specific responses to these characteristics in their study species. If this is not known for the species, then researchers should make themselves aware of the potential importance of these factors in their studies. Knowledge of the geology of the benthos in an environment, along with knowledge of barnacle response to certain rock types, allows researchers to make generalized settlement predictions within areas with similar geography (e.g. - Herbert and Hawkins 2006).

Larval Exploration

Initial contact of a surface by the cyprid is considered to be a passive process, dependent on flow and surface contour (Wethey 1986). After contact, cyprids adhere to the surface using ciliated attachment discs on the 3rd segment of the antennules (Anderson 1994). They release a proteinaceous secretion to temporarily adhere to the surface (Walker and Yule 1984), which can be detected after exploration of the surface (cyprid footprints). Cyprids use the footprint protein to explore the surface beyond the point of contact to find a suitable microsite on which to settle.

Studies of surface exploration prior to settlement in cyprids are few because of the difficulty of observing the movement of a single cyprid under a microscope in still water or in a flume using an endoscopic apparatus to record video of a surface in flow (Walters et al. 1999). While in contact with a surface, cyprids can exhibit different exploratory behaviors, such as: sit still, crawl, "hop" by swimming briefly, swim, side-crawl, or rotate (Walters et al. 1999). The amount of time devoted to each behavior depends on flow speed in *Balanus amphitrite* cyprids. Lagersson and Høeg (2002) recently described additional behaviors in *B. amphitrite* cyprids during surface exploration. Cyprids "walk" in a straight line, taking steps approximately equal to half of their carapace length, during initial exploration, and then they may utilize directional changes to explore a small surface area with greater intensity than they do while walking straight. The antennules are able to bend and articulate to a greater degree than that of the rhizocephalan barnacle *Lernaeodiscus porcellanae*. The authors suggest that the difference is due to the need for maneuverability during intertidal exploration in *B. amphitrite*, compared to *L. porcellanae*.

The behavior of cyprids during settlement is difficult to study, but Marechal et al. (2004) have recently described the use of EthoVision 3.0, a video tracking software, for the purpose of tracking individual cyprids on video. The software can measure parameters that describe cyprid exploration behavior, such as distance, velocity, turn angle, and meander, where these measurements previously needed to be determined by first watching a video in real-time and marking the path of an individual cyprid, and then making calculations from the drawing. This represents an improvement in the ability to quantify cyprid exploration behavior.

Because of the difficulty of observation of cyprid exploration of substrata, both in the laboratory and in the field, there are few studies. Researchers should make themselves aware of cyprid behaviors, though they may not be applicable for different species. Future research is needed to determine if previously observed behaviors are universal for certain taxa of barnacles, or if they are species-specific. The use of new tools, such as the EthoVision software (Marechal et al. 2004), might make it easier to conduct studies of this kind.

Local Flow Speeds

Flow is an important factor that can influence barnacle settlement, juvenile growth, and mortality. After initial contact, a cyprid must select a settlement site with an appropriate flow regime such that it will be able to feed effectively and obtain adequate food throughout its lifetime, thus selection of sites with appropriate flow is argued to be an adaptive trait (Crisp 1955). Where flow is too slow, cyprids will not contact the substrate often, and will reject the substrate. Rejection of sites with low flow is adaptive since low flow speeds will result in low amounts of food available to the growing juvenile after metamorphosis. Where flow is too fast, cyprids often reject the surface (Mullineaux and Butman 1991), and in very high flow, they may be unable to adhere to surfaces and will fall off. Rejection rates are higher on the substrata in high flow than those in low flow, though the contact rate is higher on substrata in high flow, resulting in net higher settlement in high flow than in low flow (Mullineaux and Butman 1991).

Larsson and Jonsson (2006) studied juvenile and cyprid *B. improvisus* in the laboratory and in the field in Europe. Juveniles in a flume exhibited reduced feeding rates and efficiency at flow speeds above 10 cm/s. In the field, juveniles exhibited reduced growth and survival in flows beyond 15 cm/s. However, these flow rates are an order of magnitude lower than those found in a small estuary in Oregon, USA (S. Rumrill, pers. comm.) where *B. improvisus* is known to exist, suggesting that results for one species may differ by location. Time spent exploring substrata in a flume was dependent on flow speed, with a peak exploration time at 3.0 cm/s. The authors argue that cyprids reject substrata that are situated in flow speeds that would have otherwise resulted in an increased post-settlement mortality, and that the behavior is therefore adaptive. Jonsson et al. (2004) obtained similar results in a study of cyprid exploration time vs. flow speed. They determined contact rate by capturing cyprids on grease-coated panels and observed cyprid exploration of substrata in a flume. They determined a positive correlation between contact rate and settlement. They found that 75% of the variability in settlement was due to passive contact rate, in accordance with Wethey (1986). Counter to models predicting increased contact rate with increased flow, they determined that contact rate was negatively correlated to flow speed, though this may have been due to failure of silicone grease coatings on test panels to capture and retain cyprids at high flow speeds.

Flow can be the dominant force in determining settlement patterns on substrata (Jonsson et al. 2004, Wethey 1986). Flow and surface contour determine the contact rate of cyprids on substrata, and contact rate and locations approximate settlement rates and locations on substrata (Wethey 1986), even though larvae move across and explore a surface after contact and before metamorphosis. Despite the active role of cyprids in exploring, and then rejecting or metamorphosing on, substrata, the apparent importance of the effect of flow on settlement demonstrates that the process of settlement can be largely passive. The discrepancy in maximum flows tolerable by *Balanus improvisus* in the laboratory (Larsson and Jonsson 2006) and the flows at which adults exist in nature (Rumrill, pers. comm.) suggests that results of studies on the effect of flow on settlement and post-settlement mortality may be different in different locations.

Post-settlement mortality

Cyprids and juvenile barnacles often die days to weeks after settlement, due to biotic (e.g. predation, food availability, spatial competition) and abiotic factors (e.g. dessication, salinity, temperature). The rates of settlement and post-settlement mortality of recruits shapes the ensuing adult community. Classic studies highlight the importance of settlement and post-settlement processes (Connell 1985, Raimondi 1990). Connell (1985) determined that adult density was correlated with recruitment when it was low, and it was not correlated when recruitment was high. Raimondi (1990) made a similar determination. Essentially, post-settlement mortality is density-dependent, and is an important factor in determining adult population when there is high recruitment.

Some recent studies highlight the importance of post-settlement mortality. Pineda et al. (2006) found that the only barnacles that survived to reproductive age after settlement were those that settled within a 21-day "recruitment window". The existence of recruitment windows elsewhere could potentially simplify the study of recruitment, and similar studies should focus on post-settlement mortality from settlement to reproductive age to determine the effective recruitment season. Power et al. (2006) also followed post-settlement mortality until age of reproduction, and they determined that post-settlement mortality determined patterns of distribution of *Chthamalus stellatus* and *C. montagui* in SW Ireland.

Wikstrom and Pavia (2004) examined the antifouling activity of two fucoids, *Fucus evanescens*, and *F. vesiculosus* on the barnacle *Balanus improvisus*. They found that *B. improvisus* occurs less frequently on *F. evanescens* than on *F. vesiculosus*, despite greater inhibitory effect of metabolites extracted from *F. vesiculosus* than those from *F. evanescens*. Post-settlement mortality was greater on *F. evanescens* than on *F. vesiculosus*, demonstrating an antifouling mechanism whose activity is post-settlement rather than settlement-inhibitory. A traditional approach to study of antifouling activity of these two species would have yielded the opposite result.

Long-term post-settlement mortality studies are few because they require a large dedication of time and effort on the part of the researcher(s). The cost of these studies can be tremendous, and the cost and effort represents a large constraint on studies of this kind. Recruitment windows, if they are shown to be predictable, may allow for more long-term studies of post-settlement mortality because a smaller group of organisms can be observed when the recruitment window is known than when it is unknown or nonexistent. The example of Wikstrom and Pavia (2004) shows that post-settlement mortality can be more important than settlement in a fouling study. Without consideration of the post-settlement mortality, the interpretation of results would have been different. Researchers should be aware that post-settlement mortality may be more important than settlement when studying factors that influence recruitment.

Conclusion

The focus of this essay is to review recent and historic research on the factors contributing to settlement and recruitment of barnacles, outlined in Table 1. Recent research has focused on many of these factors. Larval condition has an impact on settlement ability and post-settlement survival in cyprids. Cyprids depend on energy reserves while they undergo settlement, and environmental conditions prior to the cyprid stage have an impact on cyprid physiological quality (Leslie et al. 2005). Cyprid quality has been shown to effect juvenile growth in the field (Jarrett 2003). In addition to the quality of individual larvae, the quality of the larval pool has been shown to impact settlement as well (Gamfeldt et al. 2005). The effects of larval supply on settlement have been examined in recent literature. Physical retention and exclusion of cyprids can have a profound effect on settlement and recruitment (Jenkins and Hawkins 2003, Watson 2005). Rejection of substrata can increase local larval supply in surrounding areas (Berntsson et al. 2004, Pineda and Caswell 1997).

Research on the role of chemical cues in settlement has focused on potential antifouling compounds, though some morphological studies have expanded our understanding of the mechanisms of chemical sense in cyprids (Blomsterberg et al. 2004, Pasternak et al. 2005). Antifouling research has focused on finding natural non-toxic metabolites and extracts that inhibit settlement without toxicity that may pollute the marine environment. Compounds from sponges and red algae have shown antifouling activity and have potential for development into commercial antifoulants. Much research has been done on the role of biofilm composition and condition on barnacle settlement, though some results have been contradictory. Dreanno et al. (2006 a,b) have shown that the protein complex that causes gregarious settlement, and their results suggest that it might be the same compound as the temporary adhesive secretion or footprint protein. The study of larval exploration remains difficult, though the use of programmable software to analyze video for cyprid movement may allow for better studies on cyprid exploration behavior (Marechal et al. 2004).

Future research in barnacle settlement should focus on interdisciplinary approaches to old problems. Bioprospecting for non-toxic antifoulants may yield an effective, yet non-toxic antifouling material for use on man-made marine structures. Molecular techniques should continue to be used to elucidate biofilm composition because unculturable bacterial species can be otherwise indistinguishable. Histological techniques have shown that SIPC and cyprid temporary adhesive may be the same compound, and further work may yield a greater understanding of the foundation of gregariousness in settlement. Use of computer software in analysis of cyprid exploration behavior might make such studies more accessible to researchers.

Summary

Barnacles are important study organisms in marine biology because their sessile adult stage and intertidal presence allow for easy experimental manipulation. Barnacle are models for marine invertebrates with planktonic larva stages for the study of settlement and recruitment. Recruitment is the process of larvae joining the adult population, while settlement is the process of planktonic larvae joining the benthic community by contact with and establishing residence on the substratum. Many factors influence settlement, such as larval condition, larval supply, substrate type, substrate condition, chemical cues, biofilm condition, flow conditions, and the existing benthic community. Recruitment is affected by factors that influence settlement, and by post-settlement processes. Historical and recent literature concerning these factors are reviewed and discussed. Research on barnacle settlement is common in recent literature because of the negative economic impact of barnacle fouling. This critical essay reviews and discusses this research. Suggestions for future research include focus on the use of new methods, new technologies, and interdisciplinary approaches to further understanding of barnacle settlement and recruitment. Interdisciplinary approaches are necessary to understand factors that affect settlement on a small scale (such as biofilm composition and SIPC). Examples of species-specific settlement responses to some factors indicate that researchers should exercise caution when generalizing results. Researchers should be aware of the factors that are known to be and may be important to their specific study species.

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Table 1 – Factors with direct and indirect affects on settlement and recruitment of barnacles.

Stage: Factor	Pre-release stage, reproduction and development of lamellae	Planktonic stage, development through naupliar stages	Settlement stage, cyprid metamorphosis	Recruitment stage, development of post-settler
Intraspecific diversity	X		X	
Physical conditions	X	X	X	X
Food abundance and quality	X	X		X
Environmental Toxicity	X	X	X	X
Predation	X	X	X	X
Oceanographical conditions affecting transport	X	X	X	
Larval swimming behavior		X	X	
Larval age			X	
Larval supply			X	
Local flow rates			X	X
Larval choice			X	
Geography	X	X	X	
Substrate availability			X	
Biofilm composition and quality			X	X
Presence of spatial competitors			X	
Crowding by spatial competitors	X			X

List of factors affecting settlement and recruitment of barnacles at different life stages. Some factors may interact with others.

Table 2 – Effects of various compounds on barnacle cyprid toxicity and settlement.

<table>
<tr><th>Compound</th><th>Source and type of compound</th><th>effectiveness of settlement inhibition</th><th>Toxicity</th><th>Authors</th><th>Species & Comment</th></tr>
<tr><td>Barettin</td><td>Extract of the sponge Geodia barretti</td><td>$EC_{50} = 0.9\ \mu M$</td><td>Non-toxic</td><td rowspan="2">Sjogren et al 2006</td><td rowspan="2">Balanus improvisus settlement assay.</td></tr>
<tr><td>Benzo(g)dipodazine</td><td>Synthetic structural analog of Barettin</td><td>$EC_{50} = 0.034\ \mu M$</td><td>Non-toxic, shown to be reversible</td></tr>
<tr><td>Ircinin I and II</td><td rowspan="11">Extracts and metabolites of Mediterranean sponges Ircinia oros, I. Spinosula, Cacospongia scalaris, Dysidea sp., and Hippospongia communis.</td><td>$EC_{100} = 5.0$ ppm</td><td>$LC_{50} = 4.7$ ppm</td><td rowspan="11">Hellio et al 2005</td><td rowspan="11">B. amphitrite settlement assay.</td></tr>
<tr><td>Ircinin I and II acetates</td><td>$EC_{100} = 5.0$ ppm</td><td>$LC_{50} = 4.9$ ppm</td></tr>
<tr><td>Furodysinin</td><td>$EC_{100} = 5.0$ ppm</td><td>$LC_{50} = 18.1$ ppm</td></tr>
<tr><td>I. oros CH_2Cl_2 extract</td><td>$EC_{100} = 50.0$ ppm</td><td>$LC_{50} = 21.7$ ppm</td></tr>
<tr><td>7-Deacetoxyolepupuane</td><td>$EC_{100} = 100.0$ ppm</td><td>$LC_{50} = 106.2$ ppm</td></tr>
<tr><td>Dysidea sp. CH_2Cl_2 extract</td><td>$EC_{100} = 5.0$ ppm</td><td>$LC_{50} = 4.7$ ppm</td></tr>
<tr><td>Euryfuran</td><td>$EC_{50} < 100\ \mu g/ml$</td><td>Non-toxic</td></tr>
<tr><td>Hydroquinone-A acetate</td><td>$EC_{50} < 100\ \mu g/ml$</td><td>Non-toxic</td></tr>
<tr><td>Dihydrofurospongin II</td><td>$EC_{50} < 100\ \mu g/ml$</td><td>Non-toxic</td></tr>
<tr><td>Hydroquinone-C acetate</td><td>$EC_{100} < 10\ \mu g/ml$</td><td>Non-toxic</td></tr>
<tr><td>Dysidea sp. alcohol extract</td><td>$EC_{100} < 25\ \mu g/ml$</td><td>Non-toxic</td></tr>
</table>

Compound	Source and type of compound	effectiveness of settlement inhibition	Toxicity	Authors	Species & Comment
Isocyanides	12 synthesized analogs of the functional group of Isocyanoterpenoids, extracted from marine organisms.	$EC_{50} = 0.046 - 1.90$ μg/ml	$LC_{50} > 21.28$ μg/ml	Nogata el al 2004	*B. amphitrite* settlement assay.
CuSO4	Known antifouling compound	$EC_{50} = 0.30$ μg/ml	$LC_{50} = 2.95$ μg/ml		
2,3'-Bipyridyl	Synthetic alkaloid from nemertine *Amphiporus angulatus*	$EC_{50} = 4.1$ μM	$LC_{50} = 1.9$ μM	Kem et al 2003	*B. amphitrite* settlement assay.
Anabaseine		$EC_{50} = 1.2$ μM	$LC_{50} = 2.0$ μM		
Anabasine		$EC_{50} = 3.2$ μM	Non-toxic		
Nemertelline	Alkaloid extract from *A. angulatus*	$EC_{50} = 3.0$ μM	Non-toxic		
Floridoside	Extracts of red alga *Grateloupia turuturu*	$EC_{50} < 0.001$ mg/ml	$LC_{50} > 0.2$ mg/ml	Hellio et al 2004	*B. amphitrite* settlement assay.
Isethionic Acid		$EC_{50} < 0.001$ mg/ml	$LC_{50} = 0.017$ mg/ml		
Chondrus crispus extract	Extracts of unfouled species of red algae	$EC_{50} < 25$ μg/cm^2	Non-toxic	Nylund and Pavia 2003	*B. improvisus* settlement assay.
Delesseria sanguina extract		$EC_{50} < 25$ μg/cm^2	Non-toxic		
Osmundea ramosissima extract		$EC_{50} < 2.5$ μg/cm^2	Non-toxic		
Polyides rotundus extract		$EC_{50} < 25$ μg/cm^2	Non-toxic		

Compound	Source and type of compound	effectiveness of settlement inhibition	Toxicity	Authors	Species & Comment
Cycloviolacin O2	Peptide extract from terrestrial plant *Viola odorata*	$EC_{50} = 0.15\ \mu M$	Non-toxic, shown to be reversible	Goransson et al 2004	*B. improvisus* settlement assay.
Irgarol 1051	An algicide with antifouling activity	$EC_{50} < 2$ mg/L	$LC_{50} = 0.556$ mg/L	Khandeparker et al 2005	Work focuses on toxicity of the compound, which is a pollutant of estuaries. *B. albicostratus* settlement assay.
Phloroglucinol	Known settlement inhibitor	$EC_{50} = 1.6 \times 10^{-7}$ M	$LC_{50} = 1.86 \times 10^{-3}$ M	Lau and Qian 2000	*B. amphitrite* settlement assay.
Phloroglucinol	Known settlement inhibitor	$EC_{50} = 3.06 \times 10^{-13}$ M	(not reported)	Head et al 2003	Work focused on effects of gregariousness in *B. amphitrite* on settlement bioassays. 5 cyprids were used in one well to obtain this effectiveness value.

Barnacle settlement inhibition effectiveness and toxicity of various compounds. Entries are arranged primarily by source of the compound. EC_{50} indicates 50% settlement inhibition, and EC_{100} indicates complete inhibition. LC_{50} refers to concentration at which 50% of cyprids died after 48 hours of culture. Compounds listed as "non-toxic" generally resulted in cyprid mortality too low for calculation of LC_{50} value. Studies were generally focused on antifouling potential of compounds, and exceptions are noted.