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Differential microbial fouling on the marine bryozoan *Pentapora fascialis*

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Marine fouling is a commercially important problem affecting abiotic and biotic surfaces. In this study we investigated the surface fouling on a colonial reef-building invertebrate, the bryozoan Pentapora fascialis*, from the Welsh coast. We captured 300 scanning electron microscope (SEM) images of 5 colonies of the bryozoan* P. fascialis *in order to quantify the level of fouling on the exterior surfaces. Evidence for differential fouling was found to occur at several spatial scales, including between older and newer zooids, between proximal and distal regions of the same zooids and between colonies. The current year's growing zooids were found to have a higher level of fouling than older zooids. The difference in the mean level of fouling of proximal regions of zooids compared to distal regions was found to be significant in* P. fascialis*. In agreement with the differential fouling previously observed by other authors in the laminar bryozoan* Flustra foliacea *where the proximal region of a zooid was observed to have a higher level of fouling, the fouling coverage in* P. fascialis *was higher in the proximal region of zooids. A reduction of fouling on some bryozoan surfaces may be caused by production of antimicrobial compounds. Further studies of microbial fouling of a similar quantitative scale in other bryozoans could aid in the identification of novel antimicrobial agents useful for preventing microbial fouling on abiotic surfaces in the marine environment.*

Keywords: microbial fouling, Bryozoa, *Pentapora fascialis*, surface fouling

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INTRODUCTION

Marine fouling is a costly problem: $5 billion per year to the US maritime industry alone (Bhadury & Wright, 2004). As a result, there is a large volume of research into commercial fouling covering several aspects of marine fouling including microbial fouling (Dempsey, 1981), macrofouling (Wieczorek *et al.*, 1996; Qian *et al.*, 1999), and inhibition of fouling, both by synthetic means (Wisely, 1962) and using natural products (Bhadury & Wright, 2004; Dobretsov *et al.*, 2006). There is also research on succession through the life-span of fouling assemblages (Miller *et al.*, 1948; Holmstrom & Kjelleberg, 1994; Harder *et al.*, 2002). However, relatively little published research has considered the fouling of biotic surfaces as opposed to abiotic ones, even though recent studies have focused on invertebrate natural products and particularly their antifouling activity (Dobretsov *et al.*, 2006).

Bryozoans are dominant macrofouling species within fouling assemblages (Key *et al.*, 1995, 1996). Bryozoan colonies are modular, consisting of a large number of units, called zooids. These sessile, colonial animals are found in a diverse range of marine environments, attaching on both abiotic and biotic surfaces. Several marine species have developed defence mechanisms against bryozoan fouling, for example the marine macroalga *Dilsea carnosa* (Schmidel) Kuntze, which sheds its epidermis to remove the fouling bryozoans *Electra pilosa* (Linnaeus) and *Membranipora membranacea* (Linnaeus) (Nylund & Pavia, 2005).

Bryozoans foul other marine surfaces, but in turn are also the subject of marine fouling, both at the macro- and micro-level. Macrofouling epizoites have been described to restrict the growth of *Flustra foliacea* (Stebbing, 1971). A number of reports have illustrated the types of marine micro-organisms that foul bryozoan surfaces (Scholz & Krumbein, 1996; Palinska *et al.*, 1999; Gerdes *et al.*, 2005). High levels of microbial diversity have been reported from four main studies on microbial interactions with bryozoans. These include the ctenostome *Amathia wilsoni* Kirkpatrick (Walls *et al.*, 1993) and the cheilostomes *Orthoscuticella ventricosa* (Busk), *Cellaria pilosa* (Kirchenpauer), *Bugularia dissimilis* (Busk) (Walls *et al.*, 1993), *Bugula pacifica* Robertson and *Tricellaria occidentalis* (Trask) (Shellenberger & Ross, 1998), *Aspidelectra melolontha* (Landsborough), *Electra pilosa* Linnaeus, *Electra monstachys* (Busk) and *Conopeum reticulum* (Linnaeus) (Kittelmann & Harder, 2005) and *Flustra foliacea* (Linnaeus) (Pukall *et al.*, 2001).

Studies on Tasmanian bryozoans, *A. wilsoni*, *O. ventricosa*, *C. pilosa* and *B. dissimilis* showed differential levels of bacterial fouling between these species (Walls *et al.*, 1993). This study also noted that the distribution of bacteria throughout *A. wilsoni* colonies was not homogeneous, but did not attempt to measure this aspect of differential fouling.

Temporal studies on *T. occidentalis* and *B. pacifica* showed that quantitative estimates of bacterial growth on bryozoan

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surfaces were not constant over a one month period (Shellenberger & Ross, 1998).

The study by Kittelmann & Harder (2005) on the encrusting bryozoan species *A. melolontha*, *E. monostachys* and *E. pilosa* collected from the Jade Bight, North Sea indicated that bryozoan colonies were largely free of epibiotic bacterial colonizers. However, bacterial counts of 10 pseudoreplicate samples taken from the surface of *C. reticulum* yielded approximately 4.8×10^7 bacterial cells cm^{-2}.

A study of fouling on the erect, laminar species, *F. foliacea* collected from 3 locations approximately 9 nautical miles north-east from the island of Helgoland in the North Sea, showed that microfouling was most prevalent at the proximal end of the zooids and around the operculum area (Pukall *et al.*, 2001). This is of interest because the presence of differential fouling suggests that there may be a control mechanism for anti-fouling allowing the manipulation of microbial films by the bryozoan colonies (Shellenberger & Ross, 1998). *Flustra foliacea* is also known to produce metabolites that can interfere with bacterial communication (quorum sensing) systems and this may be a mechanism by which fouling inhibition occurs (Peters *et al.*, 2003). It is unclear whether the differential fouling observed in *F. foliacea* is species-specific or whether this is a general trend in Bryozoa. In the *F. foliacea* study (Pukall *et al.*, 2001) evidence showed a qualitative difference in fouling within a zooid.

In our study we investigate the possibility of differential fouling on the surface of another cheilostome, *Pentapora fascialis* (Pallas), a sublittoral species with a distinctive, three-dimensional, approximately hemispherical structure in the Atlantic form (Figure 1). The Mediterranean form has a smaller, more reticulate colony type. Individual colonies of *P. fascialis* may grow up to 1 m in diameter in British waters, reaching much larger sizes in the Mediterranean where colonies often fuse together to form extensive reef-like structures 2–3 m across. *Pentapora fascialis* colonies are usually found below 18 m on current-swept rocky ground or boulders around the west and south coasts and into the English Channel. *Pentapora fascialis* may be a dominant part of the sessile benthos on rocky ground with fast currents, with colonies often reaching a frequency of 1 colony per m^2 (Hayward & Ryland, 1999). The geographical range of *P. fascialis* in the British Isles extends from Shetland in the north, around the west coast and south up into the English Channel (Jackson, 2006). This species is of ecological importance in providing shelter for many invertebrate species, including other bryozoans. The foliose shape of *P. fascialis* provides a refuge from strong currents, as well as increasing the spatial complexity of rocky reef environments. When observed *in situ*, colonies of *P. fascialis* appear distinctly more orange in the new growing tips of the colony than in older, more basal parts of the colony, which are often dull grey-orange in colour.

In this study we use a fully quantitative approach, to investigate differential fouling of bryozoan surfaces using *P. fascialis* as a model. Two main questions are addressed: (i) are the proximal regions of individual zooids more heavily fouled than distal areas of zooids, as is the case in *F. foliacea*; and (ii) are newer parts of colonies less fouled than older parts, due to their shorter exposure period?

Fig. 1. Photograph of a single *Pentapora fascialis* colony *in situ* at Skomer Island Marine Nature Reserve. Scale bar = 35 cm.

MATERIALS AND METHODS

Collection and preparation

A fragment from each of five *P. fascialis* colonies (Figure 1) was hand collected from the Waybench location, North Wall of Skomer Island Marine Nature Reserve (MNR), Wales, UK (OS ref: SM 724 094) by the Skomer MNR dive team in June 2005. Fragments of *P. fascialis* were carefully excised from living colonies and packaged *in situ* in local seawater in individual zip-lock bags. Sub-samples of material from fragments collected from 5 colonies were kept in seawater from the collection site for 24 hours before rinsing 3 times with sterile distilled water (SDW) for 10 minutes. Rinsed samples were immersed in 2.5% (v/v) glutaraldehyde in phosphate buffered saline (PBS) for 1 hour to fix. Fixed samples were washed in PBS twice before dehydrating using an alcohol series from 30% to 100% ethanol. The ethanol was then substituted with acetone and the samples were critical point dried using CO_2. Dried samples were attached to 10 mm diameter microscopy stubs using quick-drying silver paint before sputter-coating with 4 nm platinum/palladium. Following analysis specimens were lodged at the BM NH (2007.11.6.1–9).

Image capture

Samples were examined using a Jeol 840 scanning electron microscope (SEM) with a 7 mm working distance, at an accelerating voltage of 5 kV. Digital images were captured at a magnification of ×1000 using the software PrinterFace (Energy Beam Sciences). Within each colony, 2 regions were designated: the 3–4 rows of zooids closest to the growing edge of the colony and zooids at least 10 zooid rows proximal to the growing region. Within each of these areas 5 zooids were randomly selected. Six separate images were taken of the external surface from each of these 5 zooids; 3 images from the proximal end and 3 images from the distal end of each zooid. This gave a total of 300 images for analysis.

Image analysis

Each of the 300 images was analysed manually using Microsoft Office Picture Manager (Microsoft, 2003). Each

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image was analysed qualitatively for overall impression of level of fouling and also the nature of fouling that occurred. Factors considered were whether the fouling surface coverage was continuous or discontinuous (or patchy), whether the fouling was a simple monolayer or a matrix and the fouling particle types and sizes. These observations were made for zooids from different colonies (Colony, C), from zooids in two different positions within colonies (growing edge and ten zooids back from the growing edge) (Age, A) and from regions within zooids (proximal and distal) (Location, L).

For quantitative analysis of fouling coverage each image was resized to 20 × 18 cm and a regular grid of 336 dots (spaced 1 cm apart) was overlaid. Percentage cover was calculated by dividing the number of dots which directly overlaid surface fouling by the total number of dots (336). Some images contained regions that were difficult to analyse in this way, for example when there were large cracks in the bryozoan surface. Where dots overlaid such regions they were excluded from the calculation.

Statistical analyses

Levene's test for equal variance was used to determine the suitability of the data for parametric analysis. A balanced ANOVA of the percentage surface cover was performed using Minitab 14 (Minitab Ltd., 2006) on the raw data without data transformation, using the percentage surface cover as the variable and 'Colony', 'Age', 'Zooid' and 'Location' as the factors. Only the valid interaction 'Colony x Age', and the three main factors 'Colony' (C), 'Age' (A) and 'Location' (L) were tested.

RESULTS

Qualitative analysis

COLONY

Figure 2 illustrates the difference in fouling cover between colonies 2 and 3, particularly in the older zooids (B & D). The type of cover shown in Figure 4A & C is an agglutinated biofilm; according to Gerdes *et al.* (2005) this is the most common type of microbial fouling. Colonies 1 and 2 have a monolayer in comparison with colonies 3, 4 and 5 which have a complex, multilayered biofilm. Fouling coverage is discontinuous in colonies 1, 2 and 5, whereas fouling coverage is continuous in colonies 3 and 4. The particle size and type in colonies 1 and 2 is quite different from colonies 3, 4 and 5; the mixture of particles on the surface of colonies 1 and 2 is less complex and the particles appear to be smaller.

AGE OF ZOOIDS

Figure 2 shows the difference between the fouling observed from images of zooids selected from the growing edge of colonies (A & C) compared to zooids selected from the older part of colonies (B & D). The images of the older parts of the colonies clearly show a decrease in surface coverage in comparison with newer zooids from the colony edge. Whereas the older

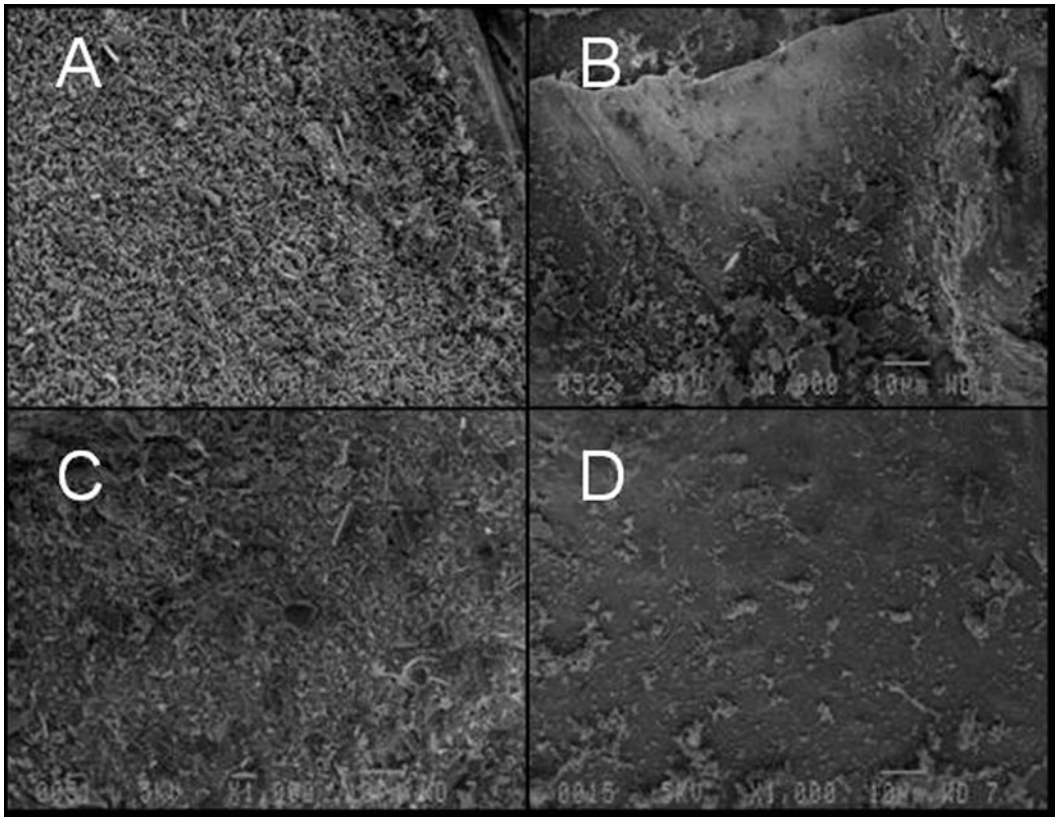

Fig. 2. Typical micrographs taken from colonies 3 (A & B) and 2 (C & D). Images A & C are taken from new zooids at the growing edge of the colony; these surfaces are substantially fouled. B & D are images of zooids from the centre of the colony; these surfaces show partial fouling. Images of colony 3 are representative of colonies 3, 4 and 5. Colony 2 images are representative of both colonies 1 and 2. The scale bar in each image is 10 μm.

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regions consist of a single thin layer of fouling, newer regions have multilayered fouling. The multilayered fouling of newer zooids is a matrix of debris, diatoms, bacterial cells and other fouling particles. In contrast, the fouling on older zooids consists of occasional identifiable micro-organisms such as a diatom.

LOCATION WITHIN ZOOID

There is no appreciable qualitative difference in fouling coverage between distal and proximal regions within the zooids. The surface coverage appears to be the same and the nature of the coverage is also similar.

Quantitative analysis

Levene's test showed that variances between samples were equal (H_o: variances of percentage surface cover between zooids taken from different colonies were equal; $P = 0.1$, $N = 300$, d.f. $= 299$).

INTERACTION COLONY X AGE

ANOVA showed that the interaction 'Colony x Age' is significant (H_o: no interaction between factors 'Colony' and 'Age' in terms of proportion of surface fouled; $P = 0.001$, $N = 300$, d.f. $= 4$). The graph shown in Figure 3 illustrates that the difference in surface coverage between old and new zooids varies between colonies. The smallest difference is observed in colony 4, and the largest difference is observed in colony 2. Although there is some evidence of interaction, fouling cover in new zooids is always greater than fouling cover in older zooids from the same colony. Therefore the factors age and colony are also considered separately, particularly as the F values of these factors are at such a high level of significance ($P = 0.001$).

COLONY

Table 1 shows that this factor is significant at $P = 1.06 \times 10^{-5}$, $N = 300$, d.f. $= 4$ (H_o: there is no difference in the proportion of zooid surface fouled between colonies), therefore at least one colony is significantly more or less fouled than one or more other colonies.

Results of Tukey's test (Table 2) showed that colony 1 is significantly less fouled than colonies 3, 4 and 5. The level of fouling observed on colony 2 is not significantly different to that of colony 1 or colonies 3 and 5, but colony 2 is less fouled than colony 4. Fouling coverage on colonies 3, 4 and 5 are not significantly different.

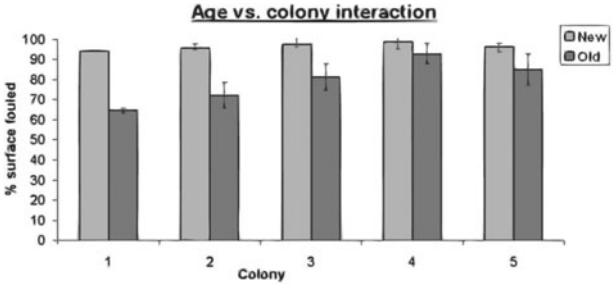

Fig. 3. Bar graph illustrating colony differences split by age of zooids. Dark shading indicates older zooids, light grey shading indicates new zooid growth. Error bars show 1 standard deviation.

Table 1. Estimated surface fouling cover split by colony, age of zooids and position within the zooids. All 3 factors shown here are significant at or below the level of $P = 0.001$ in ANOVA tests.

Factor		% surface cover	*P*
Colony	6	79.33	
	7	84.03	
	8	89.39	1.06×10^{-5}
	9	95.75	
	10	90.59	
Age	Old	79.13	4.46×10^{-12}
	New	96.50	
Location within zooid	Proximal	89.04	0.001
	Distal	86.60	

AGE

ANOVA shows that there is a significant difference in fouling between older zooids and the areas of new growth (H_o: no difference in proportion of surface fouled between old and new regions of bryozoan colonies; $P = 4.46 \times 10^{-12}$, $N = 300$, d.f. $= 1$). Results are given in Table 1, showing that in all colonies, the older parts were less fouled than the newer parts.

LOCATION WITHIN ZOOID

Table 1 illustrates that the average level of surface fouling in the distal areas of the zooids is approximately 87%. This is 2% lower than the average level of fouling found in the proximal areas. Although this difference is small, it is significant at $P = 0.001$, $N = 300$, d.f. $= 50$ (H_o: no difference in proportion of surface fouled between proximal and distal end of individual zooids) in an ANOVA.

COMPARISON OF FOULING STUDIES

Table 3 summarizes the published accounts of the extent of fouling in a range of bryozoan species with various colony morphologies and from different geographical areas. The present study provides the first systematic quantitative analysis of microbial fouling on a bryozoan surface.

DISCUSSION

A previous, largely qualitative, study on fouling of *F. foliacea* by Pukall *et al.* (2001) (Table 3) had indicated that there may be differential fouling of bryozoan zooids. We aimed to undertake a comprehensive analysis of microbial fouling distribution of biotic surfaces to gain systematic quantitative evidence focusing on the model bryozoan *P. fascialis*.

Observations of fouling on a *F. foliacea* zooid (Pukall *et al.*, 2001) suggested that the proximal region was more heavily fouled than the distal region. In our extended analysis of a total of 50 zooids taken from 5 colonies of *P. fascialis*, the increased fouling in proximal regions of zooids compared to distal regions was found to be statistically significant. It has

Table 2. Results of Tukey's test. Underlines indicate series where no significant difference in fouling is evident.

Colony	1	2	3	5	4
Mean % cover	79.33	84.03	89.39	90.59	95.75

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Table 3. Previous studies of bryozoan fouling.

Species	Colony morphology	Location	Fouling observed	Qualitative observation	Quantitative analysis	Reference
Amathia wilsoni	Erect, bushy	Arch Island, Tasmania	Y	Y	Y	Walls *et al.* (1993)
Orthoscuticella ventricosa	Erect, bushy	Arch Island, Tasmania	Y	N	Y	Walls *et al.* (1993)
Cellaria pilosa	Erect, branching	Arch Island, Tasmania	Y	N	Y	Walls *et al.* (1993)
Bugularia dissimilis	Erect, bushy	Arch Island, Tasmania	Y	N	Y	Walls *et al.* (1993)
Electra pilosa	Encrusting	Jade, North Sea	N	Y	N	Kittelmann & Harder (2005)
Electra monostachys	Encrusting	Jade, North Sea	N	Y	N	Kittelmann & Harder (2005)
Aspidelectra melolontha	Encrusting	Jade, North Sea	N	Y	N	Kittelmann & Harder (2005)
Conopeum reticulum	Encrusting	Jade, North Sea	Y	N	Y[1]	Kittelmann & Harder (2005)
Flustra foliacea	Erect laminar	Helgoland, North Sea	Y	Y[2]	N	Pukall *et al.* (2001)
Bugula pacifica	Erect branching	Puget Sound, NW USA	Y	N	Y[3]	Shellenberger & Ross (1998)
Tricellaria occidentalis	Erect branching	Puget Sound, NW USA	Y	N	Y[3]	Shellenberger & Ross (1998)
Pentapora fascialis	Erect foliose	Skomer Island, UK	Y	Y	Y	This study

Y, yes; N, no; [1] 10 pseudoreplicates examined; [2] results recorded from a single zooid; [3]12 replicates from each colony, one colony examined per week for 4 weeks.

been speculated that high levels of fouling encrusting the operculum might interfere with the feeding activity of the zooid (Pukall *et al.*, 2001) and therefore inhibition of surface fouling through production of antimicrobial compounds in this sensitive region may be advantageous to the bryozoan.

Walls *et al.* (1991) demonstrated that metabolites from the bryozoan *Amathia wilsoni* are differentially distributed and Shellenberger & Ross (1998) found higher levels of antibacterial metabolites in bryozoan species which were less fouled than other species. Metabolites produced within Bryozoa may be responsible for anti-predation activity (Montanari *et al.*, 1996); some natural compounds extracted from Bryozoa have been shown to exhibit various activities including antimicrobial, nematocidal and larvicidal activity (Sharp *et al.*, 2007).

In *B. pacifica* and *T. occidentalis* (Table 3), lower levels of fouling had previously been observed at the colony tips (Shellenberger & Ross, 1998). We investigated the distribution of microbial fouling on *P. fascialis* zooids of different ages as we had observed general differences in colour in new growing tips of the colony compared to older, more basal parts of the colony. It is possible that a difference in fouling at this level could arise because new zooids have been subjected to a shorter exposure period than older zooids. In our study, statistical analysis indicated that the greater fouling of younger than older zooids was significant. The complexity and depth of fouling cover was also increased. It has been previously documented that some bryozoan species shed and replenish the exterior cuticle (Winston & Hakansson, 1989). However, if *P. fascialis* zooids were able to shed their outer cuticle, similar levels of fouling might be expected on old and new surfaces. Although we have observed this in SEM photographs of the encrusting bryozoan species, *Umbonula littoralis* Hastings, no evidence for loss of the cuticle was seen in *P. fascialis*.

At the level of the bryozoan colony we found a significant difference in the proportion of zooid surface fouled between colonies. This indicated that at least one colony was significantly more or less fouled than one or more of the other 4 colonies. The colonies from which fragments were collected were of different sizes and therefore of different ages and this may go some way to explaining the differences observed. Some variation may also be expected to occur depending on which side of the bryozoan frond the zooids are sampled (although this factor was not analysed in our study). In this study we have focused specifically on obtaining quantitative information about the distribution of microbial fouling on a biotic surface. However, although outside the scope of the present study, it would also be of interest to characterize the bacterial species present using 16S rDNA analysis.

Studies of similar scope to this one could be performed with a range of other Bryozoa with different colony morphologies. This would help to determine whether the species, colony type, three dimensional structure and habitat contribute to the distribution of microbial fouling on colony surfaces. We can speculate that deterrence of biofilm formation on the surface of bryozoans could be caused by differential spatial production of antibacterial metabolites, or compounds which inhibit bacterial quorum sensing control, produced by the bryozoan or microbial endosymbionts. Differential metabolite production has been shown to occur in *A. wilsoni*, although this has not been conclusively linked to differential fouling within colonies (Walls *et al.*, 1991). Mediterranean populations of *P. fascialis* are known to produce 3 secondary metabolites, pentaporins A, B and C, although no antimicrobial or antifouling activity is recorded from these compounds (Eisenbarth *et al.*, 2002). An interesting question arising from the study is what mechanisms are responsible for the observed localized fouling on *P. fascialis*? In a study of the bryozoan *F. foliacea* by Peters *et al.* (Peters *et al.*, 2003) using quorum sensing based biosensors inducible by *N*-acyl homoserine lactones (Winson *et al.*, 1998; Steidle *et al.*, 2001), extracts of the bryozoan were shown to interfere with the quorum sensing process. These types of interference mechanisms are currently of considerable interest because it is likely that through understanding the processes employed by Bryozoa to reduce fouling on their surfaces we may find a more diverse range of natural compounds for the control of commercial marine fouling on abiotic surfaces.

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