

## **Characterisation of the bacteria associated with barnacle, *Balanus amphitrite*, shell and their role in gregarious settlement of cypris larvae**

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### **Abstract**

The acorn barnacle *Balanus amphitrite* (syn. *Amphibalanus amphitrite*) is a model organism to investigate pelago-benthic transitions in marine invertebrates. A driver for larval settlement in this organism is the need to attach close to conspecifics, to allow reproduction to take place. Adult barnacles are covered by microbial biofilms and the contribution of these biofilms to conspecific recognition is not fully understood. Little information is available on microbial communities associated with *B. amphitrite*. We compared biofilm communities from the barnacle shell surface with those from the surrounding rocks using the culture-independent methods of quantitative PCR and denaturing gradient gel electrophoresis. Quantification of the relative abundances of higher bacterial taxa showed that barnacles hosted a greater proportion of  $\alpha$ -Proteobacteria compared to rock-associated biofilms ( $p < 0.01$ ). Differences in relative abundances of other taxa were not observed but DGGE profiling suggested that differences were present at lower taxonomic levels. The capacity of these communities to influence larval settlement was assessed by growing multispecies biofilms on artificial medium, obtained by extracting nutrients from adult barnacles. Biofilms composed of shell-associated bacteria were capable of promoting conspecific settlement by 67% compared to control surfaces ( $p < 0.05$ ), while rock-associated communities showed contrasting effects. A taxonomic comparison of settlement-stimulating and -inhibiting bacteria was performed by DGGE and band sequencing. All partial 16S rRNA genes sequenced were similar to members of the *Vibrio* and *Pseudoalteromonas* genera, suggesting that larvae can detect and respond to variations in the composition of microbial biofilms at low taxonomic levels. Our results indicate that barnacle larvae may be able to detect parentally-associated biofilms and use this information to settle close to members of its own species.

## Introduction

Marine bacteria have been shown to play important roles in the life cycles of different invertebrates (McFall-Ngai and Ruby, 2000). In biofouling research, attention has focused on the effect of microbially-produced compounds on invertebrate settlement (Dobretsov *et al.*, 2006; Hadfield, 2011). Bacteria can induce permanent attachment in a variety of taxa, including the alga *Ulva* sp. (Joint *et al.*, 2000), the coral *Acropora microphthalma* (Webster *et al.*, 2004) and the polychaete *Hydroides elegans* (Lau *et al.*, 2003). However, the literature regarding barnacles is equivocal. For the cirripede *Balanus amphitrite* (syn. *Amphibalanus amphitrite*; Clare and Høeg, 2008), two main types of experiments have been conducted to assess the effect of microbes on larval settlement: those testing natural, multispecies biofilm developed on submerged panels, and those using single bacterial strains grown on artificial media. Initial tests using multispecies biofilms indicated that older ones promoted while younger biofilms inhibited settlement (Wieczorek *et al.*, 1995) but the opposite effect was also reported (Oliver *et al.*, 2000; Faimali *et al.*, 2004), often depending on the site from which the biofilm originated (Qian *et al.*, 2003; Hung *et al.*, 2007). In contrast, monospecific biofilms generally elicited more reproducible effects, with some strains that are capable of promoting (Khandeparker *et al.*, 2002; Khandeparker *et al.*, 2003) and some inhibiting (Maki *et al.*, 1992; Avelin Mary *et al.*, 1993; O'Connor and Richardson, 1998), even though the majority of tested isolates appear to have no effects on settlement. However, despite these efforts, a clear mechanism underlying the influence of bacteria on *B. amphitrite* settlement has yet to emerge.

In barnacles, the cyprid has evolved as a transitional phase between the pelagic larva and the benthic adult (Walker *et al.*, 1987; Glenner, 1999). Cyprids that are developmentally competent to settle display an adaptive ability to delay permanent attachment and discriminate between potential settlement sites. Although the barnacle pelago-benthic transition is influenced by multiple factors (Rittschof *et al.*, 1984; Maki *et al.*, 1988; Mullineaux and Butman, 1991), cross-fertilisation, which is important for many species, including *B. amphitrite*, is the driver of gregarious settlement ensuring adult barnacles are sufficiently close to mate, i.e. within the range of the extensible penis (Clare, 2011).

Recognition of conspecifics by cyprids is mediated by an adult glycoprotein (Knight-Jones, 1953) – the settlement-inducing protein complex (SIPC) (Matsumura *et al.* 1998b) – recently identified in *B. amphitrite* as an  $\alpha$ 2-macroglobulin-like molecule (Dreanno *et al.*, 2006b). The SIPC is a cuticular protein (Dreanno *et al.*, 2006a) and once isolated is maximally effective when bound to a surface (Crisp and Meadows, 1963). Accordingly, the SIPC is posited to act as a

contact pheromone (Clare, 2011). However, barnacle shells in their natural environment are covered by biofilm; a thick layer composed of microbial cells embedded in a layer of polymeric substances (Branda *et al.*, 2005). For this reason, a putative role for bacteria and their exudates as intermediaries in barnacle gregariousness deserves further attention. Furthermore, it can be expected that the existence of bacteria that promote barnacle settlement would favour reproductive success, while the growth of cyprid-repelling bacteria around adult shells would not be favoured in an evolutionary sense. Few attempts have been made to isolate bacteria associated with barnacles and to test their effect on larval behaviour (Avelin Mary *et al.*, 1993; Khandeparker *et al.*, 2002; 2006) but a systematic survey of bacterial communities associated with *B. amphitrite* has yet to be undertaken.

Our aim was to investigate whether some bacterial taxa are specifically associated with *B. amphitrite* and influence settlement of conspecific cyprids. Initially, bacterial communities from the external shell of *B. amphitrite* were compared to those colonising the surrounding rocks by determining the abundance and diversity of 16S rRNA genes belonging to the broad taxonomic units Firmicutes, Actinobacteria, Bacteroidetes and  $\alpha$ -Proteobacteria. Subsequently, a microbiological medium composed of barnacle extract as the only source of nutrients was used to favour the isolation of barnacle-associated bacteria. The ability of the resulting multispecies biofilms to affect the behaviour of cypris larvae was assessed by bioassay. Finally, the bacteria present in settlement-inhibiting and -promoting biofilms were identified by DGGE band sequencing. Our results suggest that bacteria may have an overlooked role in the establishment of gregarious colonies of *B. amphitrite*.

## **Materials and methods**

### **Natural biofilm sampling and DNA extraction**

Samples were collected between January and February 2010 in the intertidal area of Dona Paula Bay, Goa, India (15°27.5'N, 73°48'E). Bacteria attached to 4 different *B. amphitrite* shells were sampled as well as those adherent to the rock surface, both 2 and 4cm away from the calcareous basis of each barnacle shell sampled. Prior to collecting the bacterial communities, sample areas were washed with a copious amount of sterile seawater to remove loosely attached organisms and detritus. Biofilms were collected from all surfaces using a sterile cotton swab. Biofilm material was removed with a sterile scalpel and total DNA extracted (GenElute, Sigma-Aldrich) directly from the cotton. DNA was precipitated and kept in ethanol until required.

### **Taxon-specific quantitative PCR**

Microbial assemblages were characterised by quantitative PCR (qPCR) using 16S rRNA gene-targeting primers specific to Firmicutes, Actinobacteria, Bacteroidetes and  $\alpha$ -Proteobacteria. Primers and reaction conditions were as described (Bacchetti De Gregoris *et al.*, 2011). Quantitative sample similarities were investigated with the Kulczynski index (Clarke and Warwick, 2001) using the relative proportion of higher bacterial taxa as variables. A non-metric multi-dimensional scaling (MDS) analysis was performed on the resemblance matrix obtained and 1000 restarts were carried out to draw a 2-D scatter plot with Primer 6 (Clarke, 1993). For each bacterial group investigated, a one-way ANOVA was performed to assess the significance of the difference found between shell- and rock-associated communities.

### **Denaturing gradient gel electrophoresis (DGGE) of qPCR products**

A nested-PCR approach was employed to profile taxon-specific qPCR products by DGGE. This method was presented by Bacchetti De Gregoris *et al.* (2011) and used here with optimised primers: for the Bacteroidetes, (f) AACAGGATTAGATACCCTG and (r) \*TTCCTCGCGTATCATCGAAT; for the Firmicutes, (f) TGAAACTYAAAGGAATTGACG and (r) \*ACCATGCACACCTGTC; for the Actinobacteria (f) TTAATTCGATGCAACGCG and (r) \*AACATCTCACGACACGAGCT; and for the  $\alpha$ -Proteobacteria (f) TTAGATACCCTGGTAGTCCA and (r) \*CCCCGTCAATTCCTTTGAGTT. Primers marked with \* contained the GC-clamp required for DGGE analysis (Muyzer *et al.*, 1993). One  $\mu$ l of qPCR products was re-amplified with taq polymerase in a 60 $\mu$ l reaction containing 0.3 $\mu$ M each of the abovementioned primers for the corresponding taxon.

GC-clamp-containing PCR products were analysed using polyacrylamide gel electrophoresis (Muyzer *et al.*, 1993) (10% w/v of acrylamide/bisacrylamide 37.5:1 in 1X TAE buffer) with various denaturing gradients: 35-55% for the Firmicutes, 25-45% for the Bacteroidetes, 25-50% for the Actinobacteria and 25-55% for the  $\alpha$ -Proteobacteria. At the end of the run, bands were stained for 30 min with 1X SYBR gold, washed for 5 min in water and visualised under UV light (Bio-Rad Fluor-S imager). A binary matrix (1=present and 0=absent) was determined from DGGE profiles by visual inspection of digitised gel images. The similarity matrix for the profiles was evaluated by determining Jaccard distances and clustering samples using the unweighted pair group method with arithmetic averages (UPGMA).

### ***Balanus amphitrite* culture**

The barnacle breeding stock used in this study was obtained by collecting adults from the Duke Marine Laboratory, Beaufort, North Carolina, USA (34°40'N, 76°40'W). Adults were maintained in the aquarium in Newcastle as described elsewhere (Bacchetti De Gregoris *et al.*, 2009). Hatched larvae were released by the adults and attracted to a point light source for collection by pipette. Nauplii were cultured at a density of  $\sim 1$  larva ml<sup>-1</sup> at 28°C on a 12:12 light:dark cycle. The nauplii were fed with 1000ml a day of *Skeletonema costatum* ( $\sim 2 \times 10^5$  cells/ml) until they reached the cyprid stage (approx. for 4–5 days). Cyprids were collected by filtering through a tier of filters (pore sizes of 350 and 250  $\mu$ m) in order to discard nauplii and microalgae, and stored in filtered seawater at 6°C for 3 days before use. At this point, cyprids were ready to be used for settlement assays.

### **Media preparation, bacterial isolation and settlement assay**

A growth medium containing barnacle adult extract (AE medium) was prepared to increase the likelihood of culturing barnacle-associated bacteria. Adult barnacles were ground using a mortar and pestle and boiled in seawater for 30 min ( $\sim 100$  individuals per 1 litre). The solution was centrifuged at 1,200g for 10 min to remove particulates before autoclaving. The final protein content of the AE medium was  $\sim 170\mu$ g/ml (Lowry, *et al.*, 1951).

To isolate barnacle-associated bacteria (BAB), a living *B. amphitrite* adult from our breeding stock was rinsed with sterile seawater and added to 20ml of AE medium. For rock-associated bacteria (RB), a stone of roughly the same size as a barnacle was taken from the aquarium and used as inoculum. Samples were shaken at 90rpm for 24h at 20°C to allow enrichment. Bacterial cells were then collected by centrifugation at 12,000g for 5 min and washed twice with sterile seawater in order to remove AE medium. The microbial mixtures were re-suspended in seawater at the same concentration (cell/ml) by checking their turbidity at an optical density of 600nm. Aliquots of 2ml were used to inoculate each well of a 24-well microtiter plate. A biofilm was allowed to develop for 20h before discarding the culture medium and rinsing the wells 3X with 2ml of sterile seawater. Cyprids ( $\sim 15$  in 2ml) were added to each well and the plates incubated at 28°C. Settlement was checked after 24h.

Biofilm growth was estimated visually by growing them on a parallel set of plates treated as described above. Quantification of attached bacteria was done by staining cells with 20 $\mu$ l/ml of acridine orange and viewing with a Nikon eclipse 80i microscope equipped with an 100x oil objective and a fluorescent illumination system (Exfo, X-Cite 120). Changes in settlement behaviour of larvae resulting from the treatments were normalised against the control and their

significance was statistically assessed by ANOVA. The experiment was repeated 3X and no difference were detected between repeats. Therefore, results of the settlement assay are presented as pooled data.

### **DGGE of settlement-inducing biofilms**

After rinsing the wells and before adding the cyprids for the settlement assay, two replicates of biofilms formed by BAB and RB were collected. Bacterial cells were removed from the bottom of the wells using sterile cotton swabs and total DNA extracted as described above. 16S ribosomal genes were partially amplified with universal primers 2 and 3 (Muyzer *et al.*, 1993). PCR products were run in a 30-55% denaturing gradient acrylamide gel and bands were excised aseptically from the gel and kept in 50µl of TE buffer at -20°C. For sequencing, single PCR products were reamplified with primers 1 and 2 (Muyzer *et al.*, 1993). Successful PCR products were purified and sequenced with a BigDye™ sequencer (Applied Biosystems). Both the quality-clipping and the subsequent base calling steps on the sequences were performed using the Phred13 software (Ewing *et al.*, 1998), resulting in a good quality, average read length of 161 nucleotides (trimmed of primers). A taxonomical analysis of the BAB and RB sequences was obtained using BLAST.

## **Results and Discussion**

Stable associations between bacteria and animals are widely distributed in both marine and terrestrial ecosystems. Many of these partnerships involve mutualistic symbiosis between a single bacterial strain and its animal host (McFall-Ngai and Ruby, 1991; Polz *et al.*, 1994). However, conserved associations with a more diverse microbial assemblage have also been reported, and bacteria living on sponges and corals are typical examples (Hentschel *et al.*, 2002; Bourne *et al.*, 2008). Furthermore, by studying coral microbiology, Rosenberg and colleagues presented evidence that a dynamic relationship is established between microorganisms and corals, and that the best-suited holobiont (the host species and its associated symbiotic microbiota) is selected according to the prevailing environmental conditions (Rosenberg *et al.*, 2007). This led them to propose the “hologenome theory of evolution” (Rosenberg *et al.*, 2009), which posits that the holobiont is a unit of selection in evolution, and they elegantly demonstrated this theory by showing that gut microbiota were capable of influencing mating preference in *Drosophila melanogaster* (Sharon *et al.*, 2010). As barnacles essentially decide their mating preference at

settlement in relation to a small number of neighbours, the discovery of settlement-inducing bacteria associated with barnacle species could also support this theory. The experiments presented here were designed to begin exploring this possibility.

### **Quantitative fingerprinting of rock- vs. shell-associated biofilms**

Several studies have investigated the effect of bacteria on *B. amphitrite* cyprid settlement, testing either natural biofilms (Wieczorek *et al.*, 1995; Qian *et al.*, 2003; Hung *et al.*, 2007) or isolated strains (Maki *et al.*, 1988; 1992; Wieczorek *et al.*, 1995; Khandeparker *et al.*, 2006; Zardus *et al.*, 2008). However, this is the first attempt to characterise bacterial communities associated with this barnacle. The non-metric multi-dimensional scaling of quantitative data showed a stronger clustering effect between biofilms collected from barnacles than from the surrounding rocks, suggesting that bacterial communities attached to *B. amphitrite* shells had similar relative abundances of higher taxa (Figure 1A). Some rock biofilms clustered together with those from barnacles, particularly the sample collected around shell number 1, but they generally tended to be more diverse between themselves. Of the 4 taxa investigated, only the proportion of  $\alpha$ -Proteobacteria was statistically influenced by the sampling location ( $p < 0.01$ ; Figure 1B). *B. amphitrite*-associated communities were dominated by  $\alpha$ -Proteobacteria and Bacteroidetes, with the first group being particularly abundant. The proportions of these two taxa in rock-attached biofilms were more variable, with the  $\alpha$ -Proteobacteria ranging between 1 and 22% and the Bacteroidetes between 3 and 33%. The proportion of both Actinobacteria and Firmicutes was similar in all biofilms sampled, with the first group always less than 4%.

The prevalence of  $\alpha$ -Proteobacteria in marine biofilms is known, particularly in well-established communities (Dang and Lovell, 2000; Jin-Woo *et al.*, 2008). However, our results showed a significant difference in the proportion of this taxon in „shell“ versus „rock“ biofilms but the mechanism by which the barnacles foster this group remain unclear. As the interplay between surface physicochemical properties and cell-wall characteristics is known to affect bacterial adhesion (Neu, 1996), the specific shell chemistry may favour the attachment of specific groups of bacteria. Although shell chemistry may influence biofilm composition, barnacle-secreted compounds may also have an effect.

Quantitative analysis of higher taxa suggested that *B. amphitrite* statistically influenced the abundance of  $\alpha$ -Proteobacteria alone, but community fingerprinting also revealed an effect on other bacterial taxa. Phylotype diversity within each higher taxonomic group was investigated by comparing DGGE profiles of two barnacle-associated communities and two rock-attached

biofilms (Figure 2). For each sample, the number of bands found in the four bacterial groups (within taxa diversity) was similar, with only the Actinobacteria showing a proportionally lower diversity in certain profiles. This suggests that the dominance of  $\alpha$ -Proteobacteria found by qPCR resulted from the higher abundance of certain bacterial species rather than from increased within-taxa diversity. However, the microbial diversity observed is likely to underestimate the real diversity, as it is known that DGGE profiles tend to miss less abundant taxa (Muyzer *et al.*, 1998).

Resemblance analysis of the binary matrixes obtained (band presence/absence) indicated that samples of the same type (shell or rock) always clustered together, with their branches on the UPGMA tree that departed between 6% and 33% similarity. Of the 121 unique bands generated, 12% were found in all samples, 35% appeared in only 1 sample, and 22% and 23% were found in either both shell or both rock profiles respectively. The shell- and rock-specific bands appeared to be evenly distributed across the four taxa investigated, suggesting that the capacity of bacteria to show a preference for the surface they grow attached to is not a feature specific to certain higher taxa. These results suggest that the outer shell of *B. amphitrite* is host to bacteria that are not present on nearby rocks, and that the shell surface favours the growth of  $\alpha$ -Proteobacteria providing the first indication there is a specific association between barnacles and their microflora.

### **Effect of bacteria on larval settlement**

Ideally, the settlement assays would have directly complemented the biofilm characterisation but due to time constraints and availability of cyprids, the experiments were performed with barnacles from the US, which are used routinely at Newcastle. The effect on settlement of bacteria growing on barnacle shells (BAB) was compared with those growing on rocks from the aquarium (RB). The latter was considered a good control as both microbial inocula were subjected to similar environmental constraints for several weeks, increasing the likelihood of generating uniform bacterial communities in case the barnacle itself did not play an active role in modulating its epibionts. Nevertheless, bacteria originating from bred barnacles were capable of inducing permanent attachment of conspecifics (Figure 3). The cyprid's capacity to settle often varies between batches and results were therefore normalised as a percentage (+ or -) of the settlement that occurred in the control wells. The average settlement in control wells was  $21.5 \pm 11\%$  after 24h. BAB produced an average increase in settlement of 67%, ( $p < 0.05$ ). For RB, the results were more variable; a picture reflected in the literature. Biofilm structure was assessed with fluorescent microscopy and it was noted that 20h-old communities grew as multispecies microcolonies that created a patchiness on the surface (controls were biofilm-free). This biofilm architecture likely reflected the existence of bacterial aggregates growing in the original AE



medium that had subsequently settled on the well bottom.

BAB, which had a promoting effect on settlement (+73%) and RB, which was inhibitory (-37%), were removed from the surfaces by swabbing inoculated wells at the beginning of the cyprid settlement assay (these wells were not used for the settlement assay) and their DNA was extracted. The PCR-DGGE profiles obtained suggested that single biofilms were dominated by few bacterial strains as profiles were characterised by a low number of bands (Figure 4). Replicate biofilms gave rise to similar banding patterns, but band intensity differed markedly. This indicates that even though the wells were inoculated with the same bacterial culture, well-specific microbial interactions led to the establishment of different community structures. However, the settlement assay indicated that BAB communities possessed a conserved capacity to stimulate permanent larval attachment. For this reason, a number of bands were sequenced from both profiles, and the nearest neighbours of these sequences are reported in Table 1. All sequenced bands fell within the  $\gamma$ -Proteobacteria class, and were similar to members of the genera *Pseudoaltermonas* and *Vibrio*. Bands 39 and 42 appeared to be present only in BAB biofilms, and both were tentatively identified as belonging to members of the genus *Vibrio* (Zanetti *et al.*, 1999; Le Roux *et al.*, 2009).

It is known that the influence of bacteria on *B. amphitrite* settlement is subject to the complex interplay between taxonomic composition, nutrients, secreted compounds and biofilm structure (Khandeparker *et al.*, 2002; Khandeparker *et al.*, 2003). Interestingly, Khandeparker *et al.* (2003) found that the inductive effect of certain biofilms could be negated by addition of lectins, perhaps analogous to modulation of the settlement-promoting effect of the SIPC by lectin treatment (Matsumura *et al.*, 1998a). Lectins are a diverse group of proteins with carbohydrate-binding activity that are often involved in non-self recognition and innate immunity (Kilpatrick, 2002; Cambi and Figdor, 2003), and there is some evidence that the molecular signal recognised by exploring *B. amphitrite* cyprids resides in the carbohydrate's configuration of SIPC (Matsumura *et al.*, 1998b). In particular, settlement promotion was blocked by mannose-binding lectins (*Lens culinaris* agglutinin, LCA) in both adult extract and biofilms (Matsumura *et al.*, 1998a; Khandeparker *et al.*, 2003). Consequently, it cannot be excluded that the induction of cyprid settlement by conspecific barnacles and by bacteria is a consequence of similar carbohydrate composition of both the SIPC and the biofilm matrix. Alternatively, it has been suggested that biofilm can absorb invertebrate waterborne cues (Chan and Walker, 1998; Hadfield, 2011), of which some evidence exist for *B. amphitrite* (Elbourne and Clare, 2010). Although our experiments were not designed to explore this hypothesis, the results indicate that, if this was the causative mechanism, the absorption capacity of biofilm changes under the influence of its species composition. For this reason, whichever the underlying biological mechanism, the reproducible

settlement-inducing effect of adult-associated communities suggests that *B. amphitrite* may have evolved mechanisms to harbour bacteria that facilitate cyprid attachment in proximity to conspecifics. This is in line with the hologenome theory introduced by Rosemberg *et al.* (2009), which in our view represents a fertile ground to understand invertebrate-bacteria interactions in the marine environment.

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## Figures

### Figure 1 - **Comparison of biofilms growing on rocks and *Balanus amphitrite* shells.**

A: quantitative sample similarities were investigated with the Kulczynski index using the relative proportion of higher bacterial taxa as variables. A non-metric multi-dimensional scaling (MDS) analysis was performed on the resemblance matrix obtained and 1000 restarts were carried out to draw a 2D scatter plot. Bacterial biofilms were sampled from the external shell of four adult barnacles (triangles facing up) and the rock they lived attached to, respectively 2 cm (triangles facing down) and 4 cm away from the edge of the shell. B: means and standard deviations of the proportion of the four taxa investigated are represented for the „shell“ and „rock“ samples. Differences in the proportion of \* were statistically significant ( $p < 0.01$ ).

### Figure 2 - **Taxon-specific DGGE profiles of rock- and shell-associated bacterial communities**

Two replicates for each sampling site were run for each taxon investigated.

### Figure 3 - **Effect of biofilm on larval settlement**

Comparison of *Balanus amphitrite* settlement between biofilm-free surface (Control), barnacle-associated biofilms (BAB) and rock-associated biofilms (RB). BAB and RB are expressed as +/- percentage of settlement on control surface. Bars represent the average results of 3 replicate experiments measured after 24h. \* was  $p < 0.05$  significant.

### Figure 4 - **DGGE profiles of settlement-inducing or -inhibiting biofilms.**

Microbial biofilms were sampled from *B. amphitrite* shell (BAB) or from the surrounding rock (RB).

Table 1 - **Sequenced bands from biofilms inducing and inhibiting settlement**

Band	Isolated from	Closest relative	Sequence length	Similarity
34	RB	<i>Pseudoalteromonas mariniglutinosa</i>	163	99%
36	RB	<i>Vibrio rotiferianus</i>	162	100%
37	RB	<i>Pseudoalteromonas prydzensis</i>	162	99%
39	BAB	<i>Vibrio ichthyenteri</i>	161	97%
40	BAB	<i>Pseudoalteromonas piscicida</i>	164	100%
41	BAB	<i>Vibrio ponticus</i>	164	97%
42	BAB	<i>Vibrio changasii</i>	158	100%
43	BAB	<i>Vibrio alginolyticus</i>	158	96%



Figure 1

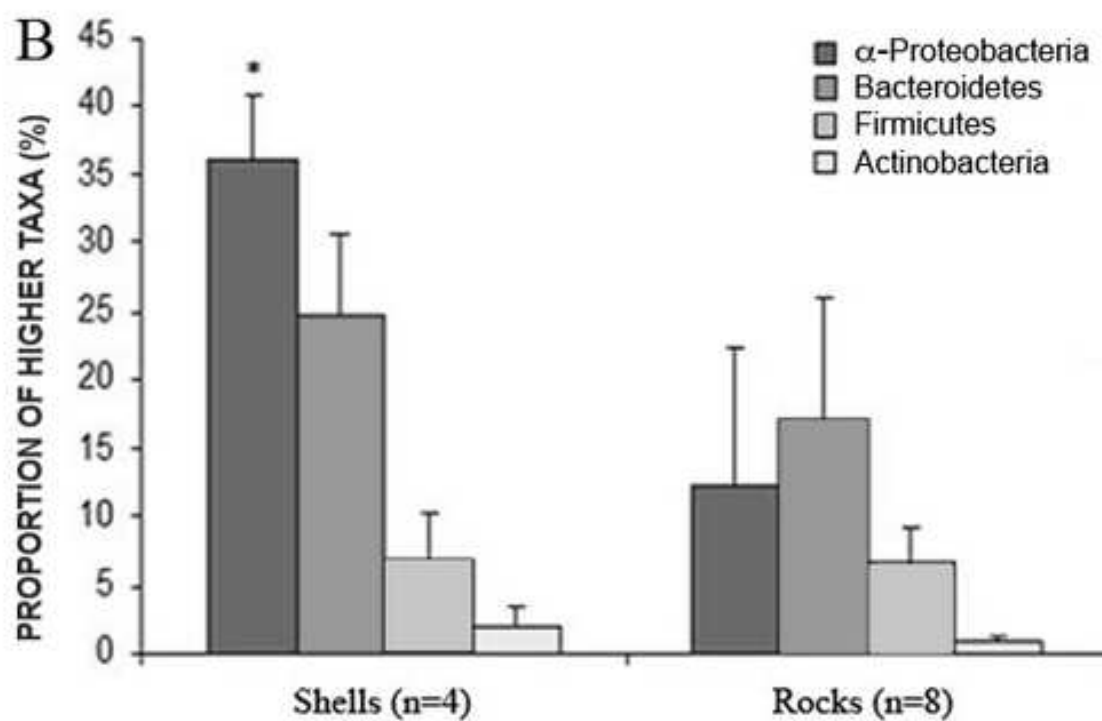
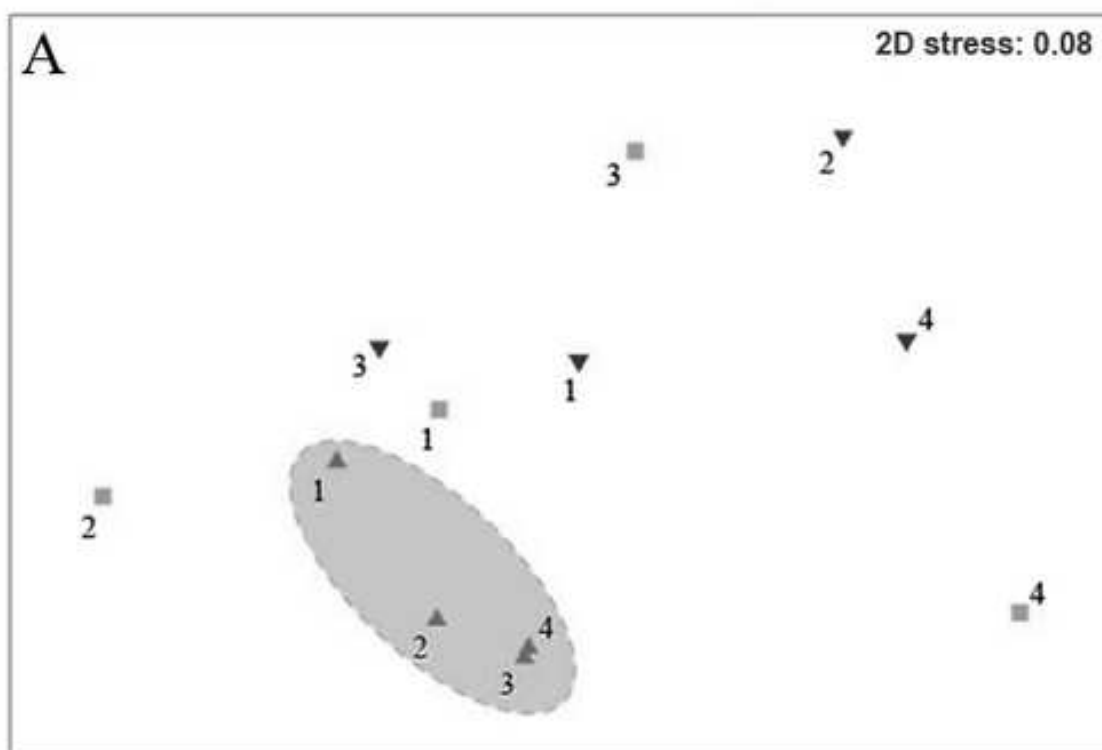


Figure 2

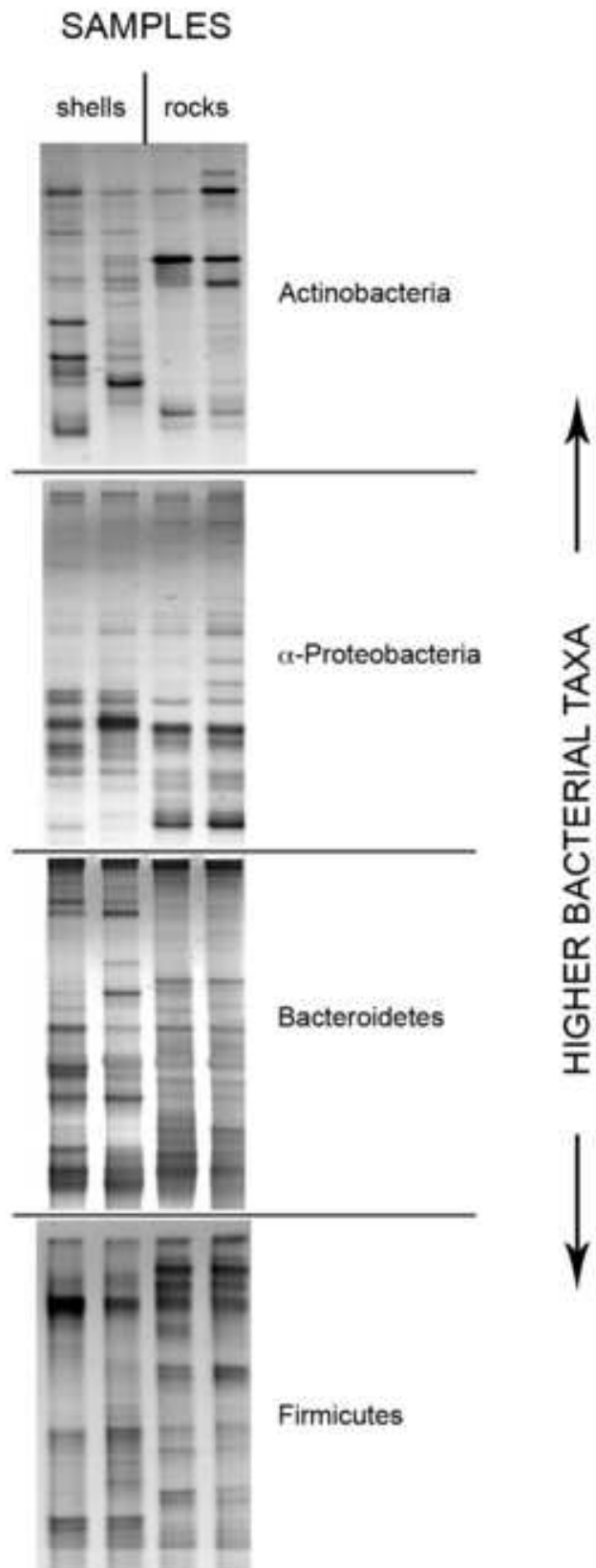


Figure 3

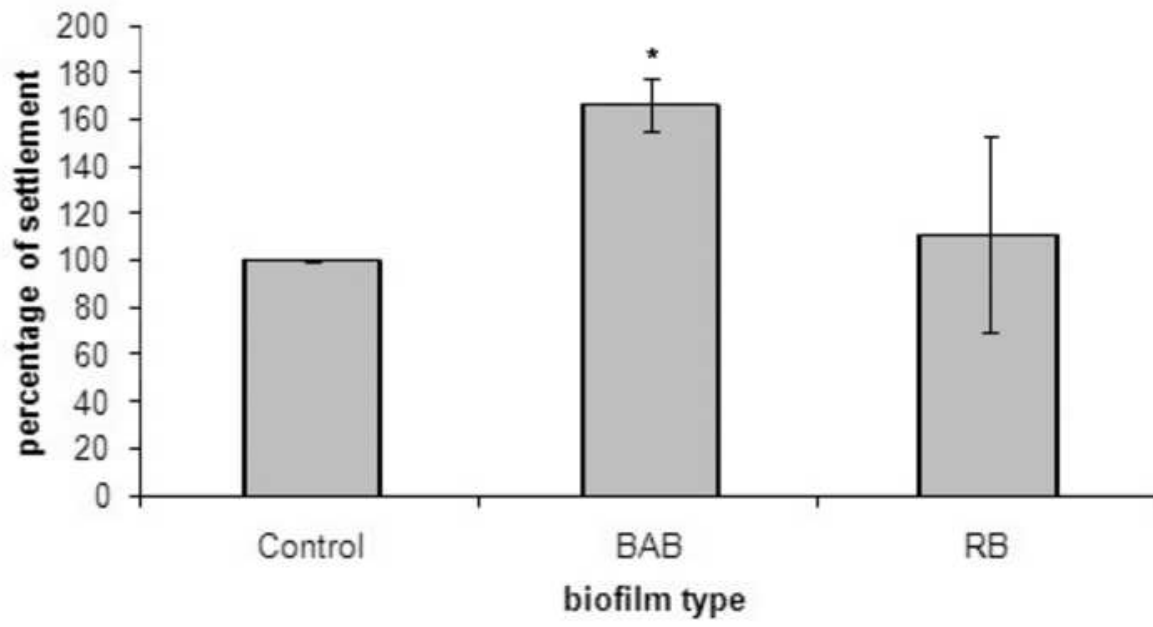


Figure 4

