Microbiome acquisition during larval settlement of the barnacle *Semibalanus balanoides*

Nick Aldred[1] & Andrew Nelson[2]

[1]School of Natural and Environmental Sciences, Newcastle University, NE1 7RU, UK

[2]Faculty of Health and Life Sciences, Northumbria University, NE1 8ST, UK

Abstract

Barnacles are conspicuous members of rocky intertidal communities and settlement of the final larval stage, the cyprid, is influenced by the presence of biofilms. While modulation of cyprid settlement by biofilms has been studied extensively, the acquisition of a specific microbiome by the settling larva has not. This study investigated settlement in the field of *Semibalanus balanoides* in two consecutive years when the composition of the benthic bacterial community differed. In both years, settling cyprids adopted a specific sub-set of benthic bacteria that was distinct from the planktonic cyprid and the benthos. This microbiome was consistent, regardless of annual variability in the benthic community structure, and established within hours of settlement. The results imply that a natural process of selection occurs during the critical final transition of *S. balanoides* to the sessile form. The apparent consistency of this process between years suggests that optimal growth and survival of barnacles could depend upon a complex inter-kingdom relationship, as has been demonstrated in other animal systems.

Introduction

Barnacles are key ecosystem engineers [1] as well as commercially and environmentally important marine biofouling species [2]. As adults they are mostly sessile and gregarious, the latter being critical during larval settlement and for reproduction as mature adults. The cypris larva explores immersed surfaces to determine suitability for permanent attachment. Cyprids are highly discriminatory and surface selection is informed by a range of physical and biological cues [3].

Benthic biofilms are important stimuli for some settling organisms, such as tubeworms [4, 5] and algal spores [6, 7]. In some cases, specific bacterial taxa are required [8]. The barnacle literature is ambiguous, with conflicting observations from different barnacle species, and biofilms of varying composition [9-13]. Experiments using bacterial isolates from laboratory-cultured barnacles have suggested that the bacterial flora of barnacle shells and the adjacent substratum differ, and that shell-associated bacteria have an inductive effect on settlement [14]. The biofilms present in the barnacle's natural environment, however, are more complex and dynamic than those that survive in the laboratory. Suffice it to say that bacterial films are one of several important cues that enable cyprids to locate a suitable habitat [3].

There is growing evidence that during metamorphosis from the cyprid to the juvenile barnacle [15], and subsequent growth to adulthood [16], bacteria between the metamorphosing cyprid and the surface to which it is attached are either removed or killed. Barnacles nevertheless retain a significant population of bacterial cells post-metamorphosis, and their intervention in the natural development of their microbiome suggests a relationship between barnacles and bacteria that is more complex than simple acquisition of the local microbial consortium. The suggestion that benthic bacteria could, in addition to serving as a marker of a suitable habitat, also serve as an inoculum for the barnacle 'holobiont' [e.g. 17] has never been explored.

The annual settlement of *Semibalanus balanoides* in the North Sea provides an opportunity to study this process *in situ*. Adults of the species release nauplius larvae into the water column only once per year, in early spring. The larvae progress through six ecdyses to the cyprid stage, which settles over a short and intense settlement season of around two weeks in late April/early May. This window of settlement activity was exploited to chart the early

development of the barnacle microbiome relative to its surroundings. It was presumed that settling larvae would, over the course of their early development, acquire a bacterial consortium similar to that of the surrounding benthos. It is also intuitive that the most dramatic shift in the barnacle's microbial community might be immediately following metamorphosis from the settled cyprid to the juvenile barnacle, at which point feeding commences. Clarification of these points was considered to be an essential basis for further investigation of the natural relationship between barnacles and bacteria.

Materials and Methods

Sample collection: Experiments were conducted in two settlement seasons, May 2017 and May 2018. The site chosen was the north sea-wall at Cullercoats Bay, UK (Figure 1A; 55°02'07.1"N 1°25'51.7"W). During this period, cyprids of *Semibalanus balanoides* are the dominant zooplankton and easily distinguishable from other species by their large size (approx. 1mm in length). In both seasons, cyprids were collected by trawling the water of the bay immediately below the surface, as well as by collecting settled cyprids and juvenile barnacles in three locations along the wall, each separated by 3 m horizontally. Between trawls, the plankton net was soaked overnight in 5% Decon 90® and rinsed thoroughly before re-use. Trawls of <30 mins were conducted twice in 2016 and three times in 2017 on consecutive high-tides. 30 Larvae were selected at random for sequencing on each occasion from the thousands collected and all samples were processed immediately after collection. Settled individuals were collected 1) after permanent attachment but before metamorphosis to a juvenile, 2) shortly after metamorphosis to a juvenile but before calcification, and 3) post-metamorphosis during initial calcification (Figure 1B). 30 individuals of each life stage were collected in each of the three locations 5 h after each high tide. Care was taken during removal of the individuals to contact only the animal using a sterile mounted needle – one per location & life stage. Laboratory experiments were then designed to replicate the natural settlement process in the absence of the benthic biofilm. Cyprids collected from the final plankton trawl in each year were washed thoroughly using 5x changes of autoclaved seawater. In each year several hundred larvae were then allowed to settle, metamorphose and calcify in the laboratory in a single 2 L beaker of autoclaved seawater, circulated using a magnetic stirrer and containing a sterile slate block as a settlement substrate, from which settled individuals were sampled. Environmental samples included scrapings from barnacle shells adjacent to settled larvae in the field, rock containing no settled individuals and, in the laboratory, scrapings of the slate substrate. Analysis by

PERMANOVA (SUP1) was used to distinguish 'lab vs field' samples and 'planktonic vs pooled-settled stages vs benthos' samples. For the planktonic cyprid sample n = 7 (2016/17 combined), for pooled settled stages (settled, metamorphosed and calcified) n = 27 (2016/17 combined) and for benthos n = 10 (2016/17 combined). Differences between specific benthic life stages (settled, metamorphosed, calcified) and between years were not analysed formally, and discussion of them is therefore based upon visual interpretation of principal coordinate analysis (PCoA) plots and Bray-Curtis distance (SUP1).

16S DNA sequencing and analysis: For details of the sequencing procedure, please refer to supplementary file SUP1. Raw data are available in SUP2.

Results and Discussion

Although adhered to a surface, the settled cyprid prior to metamorphosis is fundamentally unchanged from the planktonic, lecithotrophic stage. During metamorphosis to a juvenile, the cyprid carapace is lost through ecdysis along with any associated epibionts, and feeding commences. A consequential shift in the bacterial community was expected. It was surprising, therefore, when results indicated that the greatest difference in associated bacterial community was between the planktonic and settled cyprids. Shannon diversity was significantly higher ($p = 0.043$) in the combined settled samples (Figure 1C), which had greater relative abundance of e.g. *Flavobacteriaceae* (unclassified), *Lewinella*, *Granulosicoccus* and *Maribius* (Figure 1D). Planktonic cyprids had higher abundance of e.g. *Bizionia*. While it appeared that settled cyprids and metamorphosed individuals differed in terms of their bacterial communities ('Metamorphosis' stage; Figure 1E), the magnitude of this difference was less than that between planktonic and settled cyprids ('Settlement' stage; Figure 1E). The difference in community structure between planktonic cyprids, settled individuals (all stages) and the benthos was significant (PERMANOVA $R^2=0.143$, $F=3.25$, $p=<0.001$). PERMDISP and ANOVA were performed to rule out dispersal as a factor and found to be insignificant ($p=0.29$).

There was considerable overlap between the taxa associated with the barnacle shell and the adjacent rock, so for presentation in Figure 1E these were combined together as 'benthos'. Principle coordinate analysis did not cluster benthos data by location, but there was broad separation by year with samples from 2016 and 2017 being

104 separated across principle component 2, suggesting differing community structures (Figure 1E). The same was
105 not true for settling barnacles, where 2016 and 2017 data visually clustered in terms of their bacterial
106 communities at all life-stages. The microbiome of early barnacle life-stages therefore appeared independent of
107 their immediate environment and maintained despite temporal changes in the community structure of the
108 benthos. Since settled cyprids metamorphose to the juvenile within a day, the adoption of the settled-barnacle
109 microbiome must be rapid. After settlement, changes to the microbiome were less dramatic, although there
110 appeared to be a progression through metamorphosis to calcification (Figure 1E).
111
112 The dramatic shift in the composition of the cyprid microbiome upon settlement in the field did not occur in the
113 laboratory (Figure 1E; PERMANOVA R^2=0.124, F=5.52, p=<0.001), confirming that the inoculum for development
114 of the juvenile barnacle microbiome was not present on the swimming cyprid and must have been acquired from
115 the benthos. The number of recorded OTUs (Figure 2A) was higher in the laboratory, but the abundance of e.g.
116 *Shewanella* sp. and *Colwellia* sp. (Figure 2B) on laboratory-reared individuals agrees with their documented
117 tolerance for a broad range of environmental conditions, perhaps enabling them to better survive transfer out of
118 the natural habitat of the host (See SUP3 for alternative presentation of these data).
119
120 It was evident that the microbiome of cyprids changed fundamentally within hours of settlement, and that the
121 bacterial consortium acquired from the benthos evolved through metamorphosis and calcification of the juvenile.
122 The results suggest that the same consortium of bacteria was associated with settling larvae in two years when
123 the composition of the benthic biofilm differed. However, it is known that bacterial assemblages differ spatially
124 as well as temporally [18]. Pertinent questions for future work would therefore be, how resistant is the barnacle
125 microbiome to environmental change? And how important is microbiome composition to the overall fitness of
126 these important intertidal species [17]?
127
128 **Conclusion**
129 While bacteria were relatively scarce on planktonic cyprids, naturally settled cyprids were colonised rapidly by a
130 precise bacterial consortium that differed relative to planktonic cyprids and the surrounding benthos. Laboratory
131 experiments confirmed that the natural microbiome of juvenile barnacles originated from the benthos. The
132 benthic community differed in two sampling years, but the consortium associated with newly settled barnacles

did not. The composition of the benthos was therefore not the major determinant of the barnacle microbiome and this observation provides the first evidence for selective association of specific bacterial taxa with developing barnacles. At this stage we do not suggest either that the barnacle attracts, passively or actively, specific bacterial strains or that the bacteria most strongly associated with barnacles require the barnacle as a host. Evidently, however, there has evolved a complex inter-kingdom relationship between barnacles and bacteria that may have implications for barnacle growth and survival, and this will be subject of future work.

Ethics

There are no local or national restrictions on sampling marine invertebrate larvae.

Data Accessibility

Raw data are available in supplementary file SUP2 and Dryad DOI: https://doi.org/10.5061/dryad.85g65v8.

Authors' Contributions

NA and AN designed the study. NA collected and prepared samples, while AN conducted the 16S sequencing. NA and AN contributed equally to the preparation of the manuscript. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing Interests

The authors declare no competing interests.

Funding

The work was funded by a Newcastle University SAgE Faculty Research Fellowship to N. Aldred as well as Office of Naval Research Award N00014-16-1-3125 to A. S. Clare and N. Aldred.

References

1. Martins GM, Neto AI, Cacabelos E. 2016 Ecology of a key ecosystem engineer on hard coastal infrastructure and natural rocky shores. *Mar. Environ. Res.* **113**, 88-94.

2. Davidson I, Scianni C, Hewitt CL, Holm E, Tanburri M, Ruiz G. 2016 Mini-review: assessing the drivers of ship biofouling management—aligning industry and biosecurity goals. *Biofouling* **32**, 411–428.

3. Aldred N, Clare AS. 2008 The adhesive strategies of cyprids and development of barnacle-resistant marine coatings. *Biofouling* **24**, 351-363.

4. Shikuma NJ, Pilhofer M, Weiss GL, Hadfield MG, Jensen GJ, Newman DK. 2014 Marine tubeworm metamorphosis induced by arrays of bacterial phage tail–like structures. *Science* **31**, 529-533.

5. Shikuma NJ, Antoshechkin I, Medeiros JM, Pilhofer M, and Newman DK. 2016 Stepwise metamorphosis of the tubeworm *Hydroides elegans* is mediated by a bacterial inducer and MAPK signaling. *Proc. Nat. Acad. Sci. USA* **113**, 10097-10102.

6. Joint I, Tait K, Callow ME, Callow JA, Milton D, Williams P, Camara M. 2002 Cell-to-cell communication across the prokaryote-eukaryote boundary. *Science* **298**, 1207.

7. Joint I, Tait K, Wheeler G. 2007 Cross-kingdom signaling: exploitation of bacterial quorum sensing molecules by the green seaweed *Ulva*. *Phil. Trans. R Soc. B*. **362**, 1223–1233.

8. Freckelton ML, Nedved BT, Hadfield MG. 2017 Induction of Invertebrate Larval Settlement; Different Bacteria, Different Mechanisms? *Scientific Reports* **7**, 42557.

9. Maki JS, Rittschof D, Costlow JD, Mitchell R. 1988 Inhibition of attachment of larval barnacles, *Balanus amphitrite*, by bacterial surface films. *Mar. Biol.* **97**, 199–206.

10. Neal AL, Yule AB. 1994 The interaction between *Elminius modestus* Darwin cyprids and biofilms of *Deleya marina* NCMB 1877. *J. Exp. Mar. Biol. Ecol.* 176, 127–139.

11. Wieczorek SK, Clare AS, Todd CD. 1995 Inhibitory and facilitory effects of microbial films on settlement of *Balanus amphitrite amphitrite* larvae. *Mar. Ecol. Prog. Ser.* **119**, 221–228.

12. Olivier F, Tremblay R, Bourget E, Rittschof D. 2000 Barnacle settlement: field experiments on the influence of larval supply, tidal level, biofilm quality and age on *Balanus amphitrite* cyprids. *Mar. Ecol. Prog. Ser.* **199**, 185–204.

185 13. Khandeparker L, Anil AC, Raghukumar S. 2006 Relevance of biofilm bacteria in modulating the larval
186 metamorphosis of *Balanus amphitrite*. *FEMS Microbiol. Ecol.* **58**, 425-438.

187 14. Bacchetti De Gregoris T, Khandeparker L, Anil AC, Mesbahi E, Burgess JG, Clare AS. 2012 Characterisation of
188 the bacteria associated with barnacle, *Balanus amphitrite*, shell and their role in gregarious settlement of cypris
189 larvae. *J. Exp. Mar. Biol. Ecol*. **413**, 7-12.

190 15. Essock-Burns T, Gohad NV, Orihuela B, Mount AS, Spillmann CM, Wahl KJ, Rittschof D. 2016 Barnacle biology
191 before, during and after settlement and metamorphosis: a study of the interface. *J. Exp. Biol.* **220**, 194-207.

192 16. Fears KP, Orihuela B, Rittschof D, Wahl KJ. 2018 Acorn barnacles secrete phase-separating fluid to clear
193 surfaces ahead of cement deposition. *Advanced Sci*ence **5,** 1700762.

194 17. Marzinelli EM, Qiu Z, Dafforn KA, Johnston EL, Steinberg PD, Mayer-Pinto M. 2018 Coastal urbanisation
195 affects microbial communities on a dominant marine holobiont. Biofilms and Microbiomes **4**, 1.

196 18. Campbell AH, Marzinelli EM, Gelber J, Steinberg PD. 2015 Spatial variability of microbial assemblages
197 associated with a dominant habitat-forming seaweed. *Front. Microbiol*. **6**, 230.

198

199 **Figure 1: (A)** The sampling location at Cullercoats Bay, North-East England. **(B)** Barnacles (*Semibalanus balanoides*)
200 at different life-stages. A = adult, S = settled cyprid, M = metamorphosed juvenile, C = calcified juvenile. **(C)**
201 Shannon diversity index (y-axis), indicating significant differences between planktonic cyprids and pooled settled
202 samples. **(D)** Bacterial taxa that differentiated significantly between planktonic cyprids and pooled settled
203 samples. **(E)** PCoA of all developmental stages and replicates for two sampling years in the laboratory and field
204 (statistics refer to PERMANOVA-based comparisons). There is clear progression of microbiome development in
205 the field (orange ellipse) from planktonic cyprids (black) to settled cyprids (brown), metamorphosed juveniles
206 (orange) and calcified juveniles (light orange), with samples from both years (2016/2017) clustering at each life
207 stage. The same was not true in the laboratory (blue ellipse – settled cyprids dark blue, calcified juveniles lightest
208 blue). Environmental samples from local rocks and barnacle shells were highly variable (green) but appeared to
209 separate by year, in contrast to barnacle samples.

Figure 2: (A) The number of bacterial OTUs recorded in field and laboratory experiments. **(B)** A linear discriminant analysis effect size (LEfSe) plot, presenting bacterial taxa that differentiated between barnacles settled in the laboratory and in field. Only significant taxa are displayed on the plot ($P<0.05$).

SUP3: A linear discriminant analysis effect size (LEfSe) plot, indicating bacteria that differentiated between barnacles settled in the laboratory and the natural environment. Concentric rings indicate progressively lower taxonomic levels outwards from the center. Taxa highlighted in different colours are significantly differentiated.

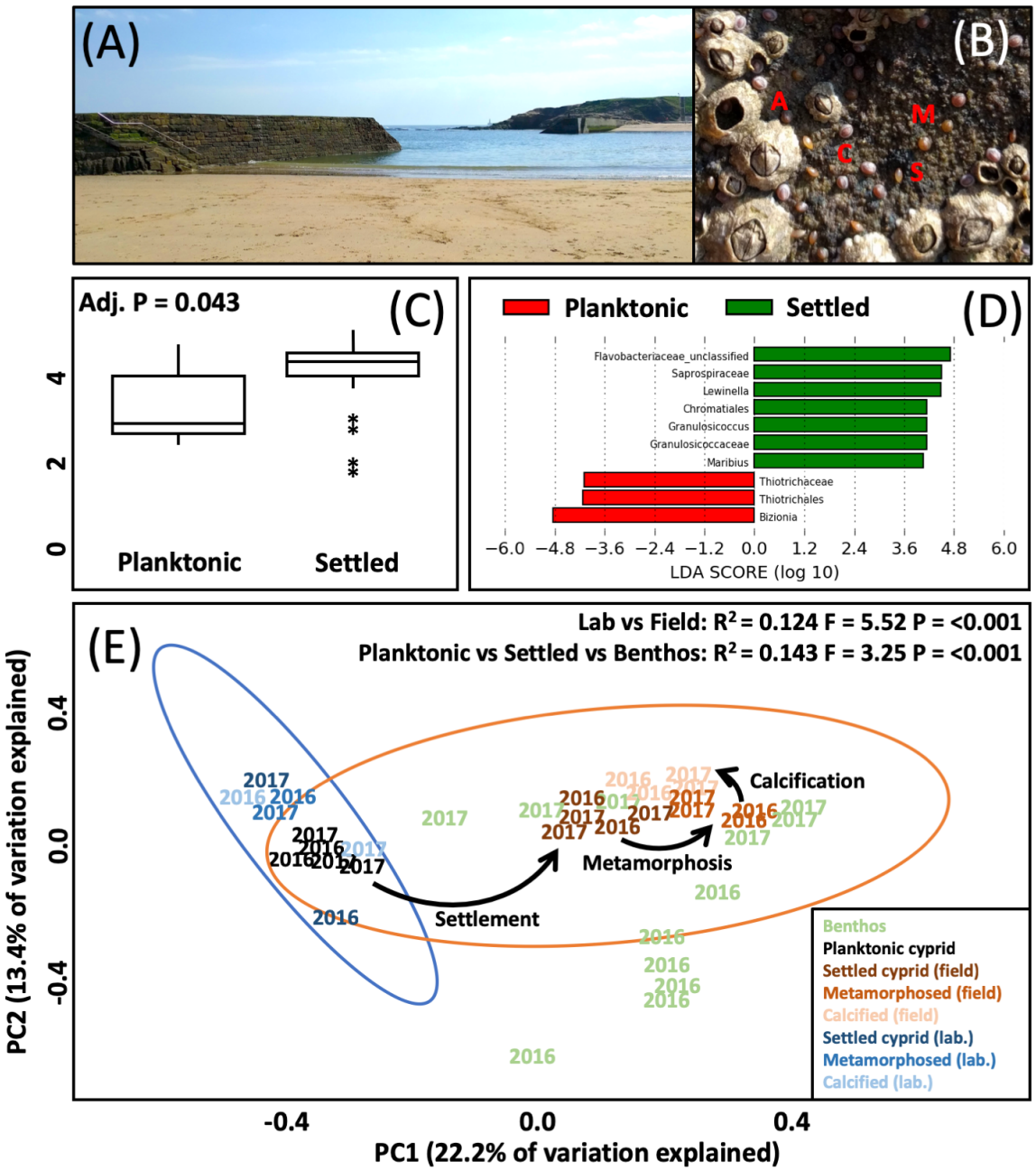
(A)
(B)
A
M
C
S
Adj. P = 0.043
(C)
Planktonic
Settled
(D)
Flavobacteriaceae_unclassified
Saprospiraceae
Lewinella
Chromatiales
Granulosicoccus
Granulosicoccaceae
Maribius
Thiotrichaceae
Thiotrichales
Bizionia
LDA SCORE (log 10)
(E)
Lab vs Field: R² = 0.124 F = 5.52 P = <0.001
Planktonic vs Settled vs Benthos: R² = 0.143 F = 3.25 P = <0.001
Calcification
Metamorphosis
Settlement
Benthos
Planktonic cyprid
Settled cyprid (field)
Metamorphosed (field)
Calcified (field)
Settled cyprid (lab.)
Metamorphosed (lab.)
Calcified (lab.)
PC1 (22.2% of variation explained)
PC2 (13.4% of variation explained)

Figure 1

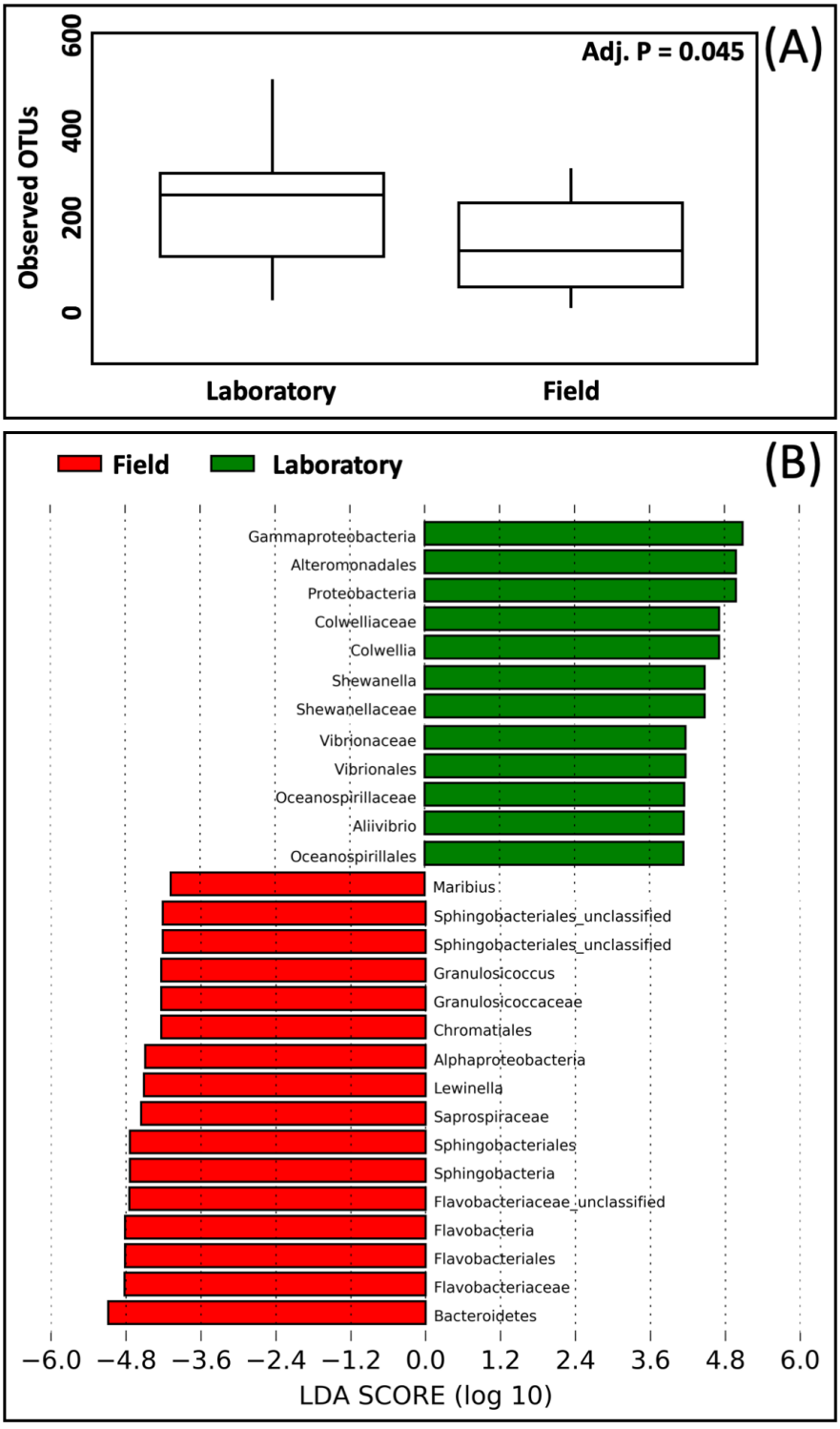
Adj. P = 0.045
(A)
Observed OTUs
0
200
400
600
Laboratory
Field
Field
Laboratory
(B)
Gammaproteobacteria
Alteromonadales
Proteobacteria
Colwelliaceae
Colwellia
Shewanella
Shewanellaceae
Vibrionaceae
Vibrionales
Oceanospirillaceae
Aliivibrio
Oceanospirillales
Maribius
Sphingobacteriales_unclassified
Sphingobacteriales_unclassified
Granulosicoccus
Granulosicoccaceae
Chromatiales
Alphaproteobacteria
Lewinella
Saprospiraceae
Sphingobacteriales
Sphingobacteria
Flavobacteriaceae_unclassified
Flavobacteria
Flavobacteriales
Flavobacteriaceae
Bacteroidetes
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

Figure 2

SUP3: A linear discriminant analysis effect size (LEfSe) plot, indicating bacteria that differentiated between barnacles settled in the laboratory and the natural environment. Concentric rings indicate progressively lower taxonomic levels outwards from the center. Taxa highlighted in different colours are significantly differentiated.