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HOST MICROBE INTERACTIONS

Dynamics of Bacterial Communities in Cockles (*Cerastoderma edule*) with Respect to Trematode Parasite (*Bucephalus minimus*) Infestation

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Abstract The bacterial communities associated with the cockle (*Cerastoderma edule*) were investigated at the individual level through a 10-month monitoring programme. Temporal changes and those changes associated with a common parasite of the cockle, *Bucephalus minimus*, were investigated by monthly sampling of individuals, selected based on their shell length (cohort monitoring). Cockle bacterial community abundance (CBCA) and diversity (CBCD) were estimated by epifluorescence microscopy counts and automated ribosomal intergenic spacer analysis, respectively. CBCA showed a temporal pattern peaking at 30×10^6 cells per gram of cockle flesh and intervalval liquid in October and a significant 1.8-fold increase linked with *B. minimus* occurrence. CBCD was characterized by 112±26 intergenic transcribed spacer (ITS) per individual and showed a relative homology between individuals (52±6%, Jaccard similarity) in spite of more than 30% of rare ITS. Consistent with an undisturbed evolution of the condition index of the studied cohort individuals as an estimate of their physiological state, neither temporal nor parasite-induced change in CBCA has been related to marked changes in CBCD.

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Introduction

Many types of close associations between marine invertebrates and microorganisms have been described [27]. Some microorganisms are reported to colonize the host tissues as intracellular endosymbionts [8] or endoparasites [2, 4] which can be acquired by vertical transmission from parent to offspring [9] or by bathing in a septic environment including horizontal transmission by contemporary hosts [31, 42]. Other associations between marine invertebrates and microorganisms occur in the digestive tract of the animals [39]. The gut microflora includes mutualistic resident bacteria, commensal or benign parasitic transient bacteria and lysed and absorbed bacteria [27]; the latter represent preys in a trophic interaction sensu stricto. However, literature dealing with the diversity and community dynamics of microorganisms in their host related to the dynamics of host populations remains scarce.

Choosing the cockle (*Cerastoderma edule*, L.), a common European bivalve of many coastal ecosystems, as an example and considering that during their life individuals grow, mature, progressively change their trophic sources [49], harbour different trematode parasites [16]

and face varied climatic situations [19], we can hypothesize that bacterial communities associated with cockles will also undergo variation in structure and abundance.

The cockle represents a good biological model due to its economic value [20] and its ecological relevance for ecosystem functioning (e.g. bioturbation) [26]. Also, previous studies reported the occurrence of food-borne pathogens and faecal indicator bacteria [38] and the infection by mycoplasma-like bacteria [3] or rickettsia-like bacteria [7], or by numerous trematode species suggesting contrasted patterns of interaction among these different organisms [5, 44]. These studies questioned the link between bacteria, macroparasite occurrence and individual cockle fitness.

To address this issue, our study aimed (a) to characterize the structure (density, molecular fingerprints) of cockle bacterial communities (CBC) at the individual scale including methodological settings needed for bacterial genotyping in such a matrix, (b) to assess the CBC dynamics in cockle individuals of a given cohort during 10 months and (c) to estimate whether the occurrence of a trematode parasite in cockles may impair bacterial communities in terms of diversity and abundance. *Bucephalus minimus* was selected because this species uses the cockle as a first intermediate host [13]. It means that the parasite reproduces asexually in the cockle, invades most of its tissues and that strong metabolic disturbances are expected. We monitored on a monthly basis the abundance (epifluorescence microscopy counts) and the genetic diversity (semi-nested automated ribosomal intergenic spacer analysis (ARISA) fingerprinting) of bacterial communities associated with one individual cockle (CBC).

Materials and Methods

Sampling Sites

Cockles were sampled in the National Reserve of Banc d'Arguin situated in Arcachon Bay, a macrotidal lagoon along the French Atlantic coast (44°40′ N, 1°10′ W). At Banc d'Arguin, the habitat consists of moderately sheltered intertidal sand flats (median grain size=350 μm). The water salinity and temperature ranges are 34–35 psu and 9.5–21.0°C, respectively. The benthic community biomass is dominated by cockles with abundance reaching 100 to 200 ind m^{-2} [17]. This population is characterized by high demographic fluctuations, fast individual growth and a relatively short lifespan [24].

Cockle Characterization

Cockles from the May 2006's cohort were sampled monthly at low tide from January to October 2007. This age class was identified by cohort analysis. Individuals were selectively sampled using the shell length. At each occasion, a minimum of 100 buried cockles were collected by hand, measured to 1-mm precision with a calliper and stored at 4°C during transport to the laboratory. Cockles located at the sediment surface were discarded due to poor condition as Blanchet et al. [5] suggested that this abnormal position was a prelude to cockles' death [16]. Prior to analyses, samples were stored at −20°C less than 6 months. After thawing, cockles were washed with tap water and opened under sterile conditions according to the French standards for bacteriological analyses of marine bivalves (AFNOR NF V 08-600 of October 2000). Individual shell mass and flesh and intervalval liquid (FIL) mass were weighed to 0.1 g precision. The wet FIL mass was converted to dry FIL mass using the following equation: dryFILmass = 0.1259 × wetFILmass (de Montaudouin, personal data). Condition index was measured as the ratio of the dry FIL mass (in milligrams) to the shell dry mass (in grams) [51].

B. minimus Occurrence

In order to note the presence/absence of *B. minimus* (Bm + versus Bm−), the foot of cockles was cut, squeezed between two sterile glass slides and observed under a stereomicroscope (NIKON SMZ1500, HR plan Apo 1) [14]. The prevalence was defined as the percentage of parasitized cockles [6]. Then, FIL were shredded (blade OMNI TH) and stored in sterile flasks, at −20°C, for less than 2 years, until bacterial counts and diversity analyses were carried out.

Bacterial Counts

Samples (shredded FIL) were thawed into a preservative buffer (Tris–HCl, 300 mM pH 8; 0.1 M NaCl; 20 mM EDTA). Analyses were performed, for each month, on each FIL of between two and five Bm+ found and from each FIL of three Bm− cockles. An aliquot of 50 μL of each sample was labelled with a mixture containing two volumes of SYBR® Green I (25×, Molecular Probes, Invitrogen Cergy Pontoise, France) for one volume of propidium iodide (60 μM, Molecular Probes, Invitrogen, Cergy Pontoise, France). This mixture is a modification of LIVE/DEAD® BacLight™ Bacterial Viability kit that is usually used to differentiate live from dead bacteria. It was applied to allow a better discrimination between bacteria and tissues of cockles. After fixation for 15 min at room temperature in darkness, samples were filtered onto 0.2-μm GTBP pore-size, 25-mm-diameter Isopore™ membrane filters (Millipore, Molsheim, France) under low vacuum pressure (200 mm Hg). The filters were removed from the filtering

tower and mounted in non-fluorescent immersion oil between a glass slide and cover slip and stored at 4°C until counting with an epifluorescence microscope (Olympus BH2-RFCA, excitation filter BP490, magnification ×1,000) was carried out. Cockle bacterial community abundance (CBCA) was calculated using the following formula: $[CBCA = (FA \times TN)/(SF \times V \times F)]$, with CBCA as the number of cellular units per millilitre of sample, FA as the effective filtration area of the membrane filter (here FA=201 mm^2), TN as the total number of cellular units (here TN ranged between 150 and 250), SF as the surface of microscopic field (0.02 mm^2), V as the filtered volume (millilitres) and F as the number of observation fields investigated (here F=50 minimum). The potential dilution of the FIL was taken into account for calculation of CBCA. The results were expressed as cell units per gram of FIL.

Cockle Bacterial Community Structure Analysis by ARISA

Changes in the cockle bacterial community diversity (CBCD) were assessed using the PCR-based whole-community fingerprinting approach ARISA of bacterial DNA [21]. ARISA amplifies the intergenic transcribed spacers (ITS) between the 16S and 23S rRNA genes using a fluorescent primer, and the ITS size represents the operational taxonomic unit (OTU). Results are displayed as the amount of different PCR products of specific fragment length (ITS size). For each cockle, DNA was extracted from six replicates of 25 mg of FIL, using a bead beating method (FastPrep and lysis matrix A; 6 m s^{-1}; 40 s, MP Biomedicals, Illkirch, France) coupled to QIAamp DNA Mini Kit (QIAgen, Courtaboeuf, France). For each cockle, the pooled amount of DNA extracted from 150 mg (6×25 mg) of cockle FIL was determined by spectrofluorimetry (LS 55, PerkinElmer, Courtaboeuf, France) using SYBR® Green I (Molecular Probes, Invitrogen, Cergy Pontoise, France) and quantified using DNA standard 1 mg mL^{-1} solutions of calf thymus DNA (Sigma Aldrich, St. Quentin Fallavier, France). The same amount of extracted DNA (10 ng) was used for each ARISA amplification assay. For each sample, the first ARISA amplification step was conducted in triplicates. The amplification was run according to Ranjard et al. [47] using universal bacterial primers SDBact (5′-TGC GGC TGG ATC CCC TCC TT-3′ labelled at the 5′ end with the phosphoramidite dye 5-FAM fluorochrome) [41] and LDBact (5′-CCG GGT TTC CCC ATT CGG-3′) [41] (Molecular Probes Invitrogen Cergy Pontoise, France) with the following conditions: (a) 94°C for 5 min; (b) 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min and then (c) 72°C for 5 min. The 25-μL reaction mixtures contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.3 mg mL^{-1} of bovine serum albumin (MP Biomedicals, Illkirch, France), 5% of DMSO, 0.2 mM of each dNTP, 0.1 μM of each primer, 1 U of *Taq* polymerase (Promega, Charbonnières-les-Bains, France) and 10 ng of template DNA. A second PCR amplification was performed on 1 μL of the pooled first step PCR amplicons using the same forward primer (SDBact) and the reverse primer ITSReub (5′-GCC AAG GCA TCC ACC-3′) [34] (Invitrogen, Cergy Pontoise, France) recommended in [35]. Nested (or semi-nested) ARISA, a two successive PCR amplification of the ITS, was shown to enhance the detection threshold of natural microbial communities originating from complex matrices that possibly contain PCR inhibitors such as humic acids [35]. The amplification conditions were held at 95°C for 5 min, 35 cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 90 s and a final extension of 72°C for 10 min. Single-stage (i.e. first ARISA amplification step) and semi-nested PCR products were purified using a QIAquick PCR Purification Kit (QIAgen, Courtaboeuf, France) and quantified by spectrofluorimetry as for extracted DNA. Two nanograms of purified products from each sample were combined with 10 μL Hi Di formamide and an internal LIZ1200 standard (Applied Biosystems Ltd, Courtaboeuf, France) before being heat-treated (95°C, 5 min) and then cooled on ice. ARISA was performed on a 3730XL Capillary Genetic Analyser (Applied Biosystems Ltd, Courtaboeuf, France), using a 50-cm capillary and standard Genemapper protocol, which detects the relative abundance of different sized PCR products labelled with the fluorescent primers (Plateforme Genome-Transcriptome Pierroton INRA, Bordeaux-Aquitaine, www.pierroton.inra.fr/biogeco/site_pole_agro/genoseq.html). Results were read using the ABI Peak scanner software provided by Applied Biosystems (Courtaboeuf, France) where each profile of peaks in an electrophoregram defines bacterial diversity fingerprint. Fluorescence data (peak areas and peak sizes) were exported to Microsoft Excel to allow the data analysis. Peak size values were rounded to the nearest whole number, and peaks with a size inferior to 200 bp were considered as noise and excluded for analysis [35]. According to Osborne et al. [43], an optimal divisor (2,500) was determined to eliminate the background fluorescence (data not shown). Moreover, according to Ramette [46], data were binned (i.e. electrophoretic profiles alignment) using the interactive and automatic binning algorithms (their respective manuals and examples are available online, http://www.ecology-research.com) implemented in the free, R programming language (The R Foundation for Statistical Computing, http://cran.r-project.org/). From Peakscanner output table, custom R binning scripts [46] were applied (WS 2; Sh 0.1). All peaks with relative fluorescence intensity value inferior to 0.004 (1/optimal divisor) were not included in further analyses since

they consisted of background peaks. Therefore, to consider a maximum of peaks while excluding background noise, only fragments with relative fluorescence intensity above a threshold of 0.4% and ranging between 200 and 1,200 bp were considered as a true peak. Each true peak displayed represented one ITS, and the peak area represented the relative abundance of the ITS present. Data were also transformed to abundance matrix. Similarity between profiles (presence/absence) was computed using Primer (PRIMER-E Ltd, Lutton, UK), from the Jaccard similarity index $[J = 100 \times c/(a + b - c)]$ where a is the number of ITS found only in sample A, b the number of ITS found only in sample B and c is the number of ITS shared between samples A and B. Similarity between profiles (relative fluorescence) was computed from the Bray–Curtis similarity index $[\mathrm{BC} = 100 \times (1 - \mathrm{sum}(d - \mathrm{e}))]$ where d is the ITS relative abundance in sample D and e is the ITS relative abundance in sample E. A rarefaction curve based on Michaelis–Menten equation was designed a posteriori from the presence/absence matrix of all samples using Primer (PRIMER-E Ltd, Lutton, UK).

Statistical Analyses

Mann–Whitney and Kruskal–Wallis tests were used to compare CBCA and CBCD similarity indexes (Jaccard and Bray–Curtis) between different sampling times or parasitic states. Statistical differences between groups were tested using analysis of similarity (ANOSIM), a random permutation tests (Monte Carlo test, 100,000 permutations) performed from similarity matrix [32]. Comparison of the ARISA profiles (presence/absence matrix) obtained after single-stage and semi-nested PCR was analysed by non-multidimensional scaling (MDS) [10]. On each MDS chart, every bacterial community was represented as one plot and the relative changes in community structure as the distances between the points. The two MDS were superimposed, and symmetric Procrustes rotation rotates one MDS to maximize similarity, with the other MDS minimizing the sum of squared differences between the plots. Statistical differences between presence/absence matrixes obtained by each method were tested by 1,001 permutation tests. All tests and correlations were considered significant statistically at p value ≤ 0.05.

Results

Cockle Sampling Set

A total of 46 cockles was analysed between January and October 2007, including 16 cockles whose tissues where found to be parasitized by *B. minimus* (Bm+ cockle; Table 1). Cockles were labelled using the sampling month number and a distinctive letter for each individual. Shell lengths ranged from 23±1 mm in January 2007 for 8-month-old cockles to 34±1 mm in October 2007 for 17-month-old cockles. As a consequence of the sampling strategy (2006's cohort cockles were selected), no variation of the shell length was expected within the set of cockles analysed monthly. Individual FIL mass ranged from 1.12 to 5.79 g and varied monthly (p=0.002, Kruskal–Wallis based on Bm− individuals). On June, July, August and September 2007, when the number of collected Bm+ individuals was sufficient, no differences in the cockles FIL mass were found between Bm+ and Bm− individuals (p>0.05, Mann–Whitney). The condition index increased from 32±4‰ in January 2007 to 82±14‰ in June (p=0.006, Kruskal–Wallis) with no significant difference found between May and June. From July to October, the monthly mean condition index reached a plateau around 87±5‰. From June to October, no significant difference in condition index was found between Bm− and Bm+ individuals (p= 1.00, Mann–Whitney; Fig. 1a).

ARISA Settings

DNA extraction yields exhibited a high heterogeneity varying between 0.32 and 16.1 μg g^{-1} [FIL]. One sampled cockle, individual "1c", displaying a totally out of range value (76.9 μg g^{-1} [FIL]), was excluded for further analyses. To analyse the CBCD, the ARISA were based on semi-nested PCR amplification of a fraction of the former amplicon. Indeed, after a single-stage PCR, 21 samples out of 38 did not display an interpretable ARISA profile (no peak detected). For the remaining 17 individuals, the extent of amplification yields obtained by single-stage PCR varied from 0.73 to 237 ng ng^{-1} [DNA template], mean±SE=27± 8 ng ng^{-1} [DNA template] (Fig. 2). Semi-nested PCR amplification yields varied from 349 to 1,683 ng ng^{-1} [DNA template], mean=817±45 ng ng^{-1} [DNA template]. One individual, cockle 9c, displaying a very low semi-nested PCR amplification yield (43 ng ng^{-1} [DNA template]) was excluded for further analyses. No correlation was found between single-stage PCR and semi-nested PCR amplification yields (p=0.56, Spearman correlation). The CBCD profiles recovered from the two amplification strategies were compared using a procrustean rotation of the two non-metric multidimensional scaling corresponding to either the samples amplified by single-stage or those amplified by semi-nested PCR (Fig. 3). Similar within-cluster plotting patterns were found between the two nMDS (p<0.22, Procrustes correlation) indicating that semi-nested PCR amplification did not change the respective CBCD profiles obtained by single-stage PCR amplification. CBCD was consequently analysed by semi-nested PCR ARISA on 37 cockles.

Table 1 Description of the individual 2006's cockle cohort sampled at Banc d'Arguin (Arcachon Bay) in 2007

Individual	Date	Shell length (mm)	FIL mass (g)	*Bucephalus minimus*	DNA extraction yield (μg DNA g^{-1} [FIL])
1a	4 January 2007	26	1.58	−	2.98
1b	4 January 2007	23	1.12	−	0.88
1c	4 January 2007	23	1.23	−	76.9
2a	1 February 2007	26	1.80	−	2.73
2b	1 February 2007	24	1.66	−	2.07
2c	4 February 2007	26	1.75	−	0.32
3a	8 March 2007	26	1.73	−	na
3b	8 March 2007	25	1.55	−	na
3c	8 March 2007	24	1.32	−	na
4a	5 April 2007	26	2.12	−	16.1
4b	5 April 2007	27	2.28		na
4c	5 April 2007	26	2.04	−	1.06
5a	3 May 2007	27	2.18	−	na
5b	3 May 2007	28	2.84	−	na
5c	3 May 2007	28	2.54	−	na
6a	5 June 2007	29	1.88	−	6.93
6b	5 June 2007	29	3.36	−	0.84
6c	5 June 2007	29	2.54	−	0.56
6d	5 June 2007	29	3.39	+	0.58
6e	5 June 2007	29	5.11	+	1.21
7a	4 July 2007	32	4.96	−	0.78
7b	4 July 2007	32	3.94	−	0.96
7c	4 July 2007	32	5.42	−	0.45
7d	4 July 2007	32	3.25	+	0.40
7e	4 July 2007	32	4.41	+	0.50
7f	4 July 2007	32	4.00	+	0.36
7g	4 July 2007	32	5.03	+	0.56
8a	3 August 2007	33	3.08	−	2.92
8b	3 August 2007	33	4.63	−	0.88
8c	3 August 2007	33	5.38	−	0.84
8d	3 August 2007	33	3.39	+	0.46
8e	3 August 2007	33	3.02	+	0.42
8f	3 August 2007	33	3.97	+	1.11
9a	10 September 2007	33	3.53	−	1.09
9b	10 September 2007	33	4.27	−	1.88
9c	10 September 2007	33	5.79	−	0.82
9d	10 September 2007	33	4.54	+	1.61
9e	10 September 2007	33	4.44	+	0.83
9f	10 September 2007	33	2.60	+	2.00
9g	10 September 2007	33	4.85	+	0.36
9h	10 September 2007	33	4.66	+	1.32
10a	9 October 2007	34	4.46	−	0.58
10b	9 October 2007	34	4.34	−	0.65
10c	9 October 2007	34	4.08	−	0.63
10d	9 October 2007	34	2.96	+	0.57
10e	9 October 2007	34	5.39	+	0.59

+ detection in cockle tissues of *B. minimus*, − no detection, *na* not available, *FIL* flesh and intervalval liquid

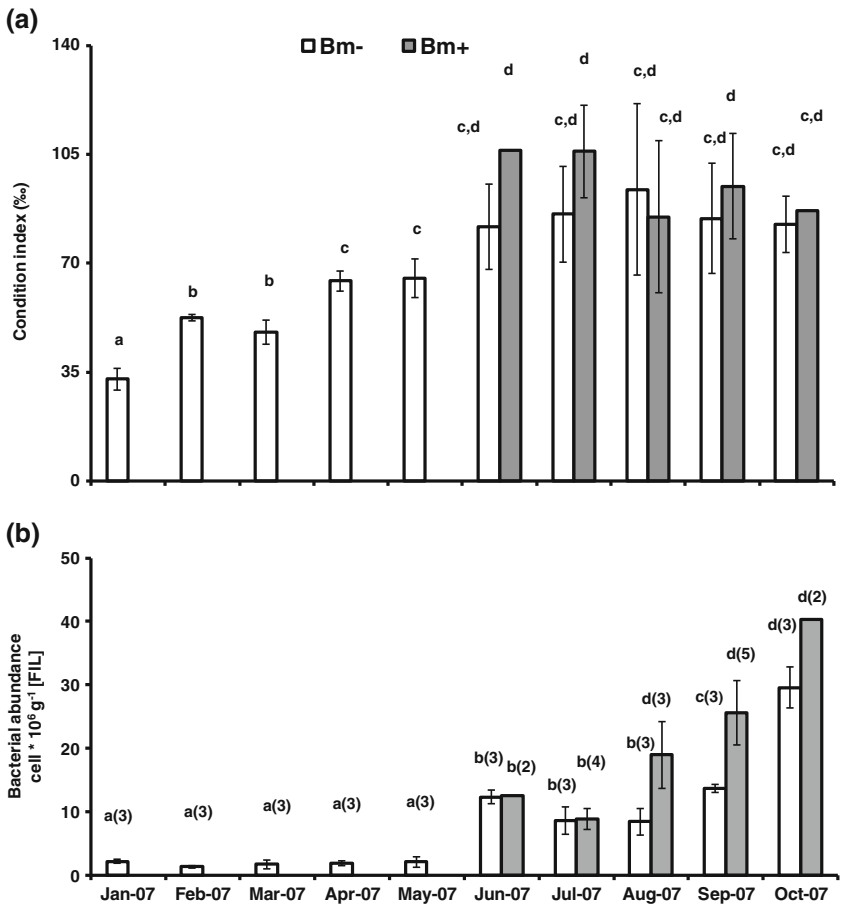

Figure 1 Monthly mean condition index (**a**) and bacterial community abundances (**b**) of non-parasitized (Bm−) or parasitized (Bm+) cockles sampled from January 2007 to October 2007 in Banc d'Arguin (Arcachon Bay). Number of replicates is between *brackets*. *Error bars* represent standard deviations (absence of error bars is due to a lack of replicates). *Similar letters in superscript* indicate no statistical differences among months at $p=0.05$ level (Mann–Whitney)

Cockle Bacterial Community Abundance

The CBCA was measured on sampled Bm− cockle individuals from January to October 2007 (Fig. 1b). During this period, values of individual CBCA ranged from 1×10^6 to 30×10^6 cells g^{-1} [FIL] corresponding to 2×10^6 and 162×10^6 bacterial cells per individual, respectively.

During the first 5 months of the monitoring (January to May 2007), monthly Bm− CBCA were not significantly different ($p=0.34$, Kruskal–Wallis), with an average of $1.9\pm0.3\times10^6$ cells g^{-1} [FIL]. A marked and significant increase in the CBCA was observed between May and June, with a mean value of $12\pm1\times10^6$ cells g^{-1} [FIL] in June ($p=0.02$, Mann–Whitney). From August to October 2007, the monthly mean CBCA significantly increased ($p=0.03$, Kruskal–Wallis), peaking at 30×10^6 cells g^{-1} [FIL] in October.

Bm+ cockles were detected from June 2007 when the mean shell length was 29 mm (Table 1). The temporal pattern of Bm+ CBCA was comparable to the pattern described for Bm− individuals; maximal values occurred in October, mean$=40\times10^6$ cells g^{-1} [FIL].

No significant difference in CBCA was found between Bm+ and Bm− individuals in June and July ($p>0.05$, Mann–Whitney). Conversely, the cockle bacterial community abundance was 1.8 higher in Bm+ individuals than in Bm− individuals in August, September and October ($p<0.05$, Mann–Whitney).

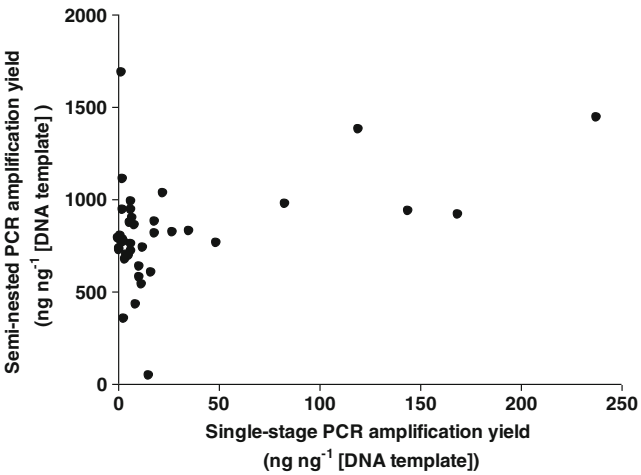

Figure 2 Comparison of DNA amplification yields between semi-nested PCR and single-stage PCR amplification of cockle bacterial communities using Ranjard et al. [47] and Lear and Lewis [35] amplification protocol for ARISA

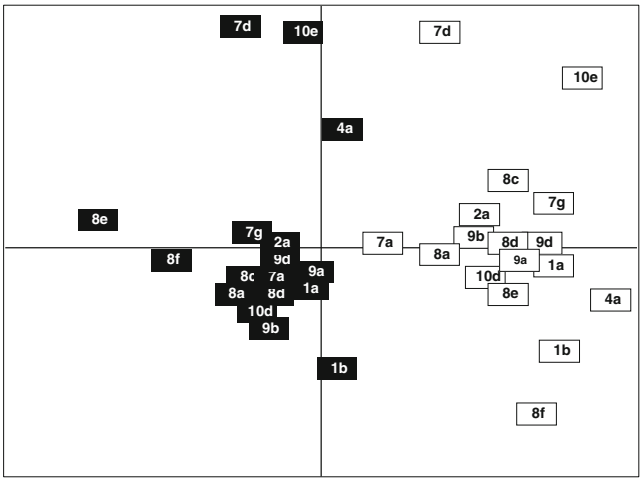

Figure 3 Comparison of bacterial community profiles obtained by two different approaches (single-stage PCR and semi-nested PCR) on cockles sampled from January 2007 to October 2007 in Banc d'Arguin (Arcachon Bay). Plot is derived from non-metric multidimensional scaling of ARISA using a Jaccard distance matrix similarity (ITS presence/absence matrix; *black box* single-stage PCR method, *white box* semi-nested PCR method). Comparison was only realized on the bacterial communities of 17 cockle individuals which display an interpretable ARISA profile with single-stage PCR

Cockle Bacterial Community Diversity

A total of 284 ITS, ranging from 200 to 909 bp in size, was detected by semi-nested PCR ARISA analysis of the bacterial community of 37 cockles. Among the detected ITS, 41 (14% of all ITS) were detected only in Bm− cockle set, 15 (5% of all ITS) were detected only in Bm+ cockle set whereas 228 ITS were shared by the two sets.

Regarding temporal patterns in Bm− and Bm+, respectively, (a) three ITS (ITS# 227, 251, 273) have been detected every month in every tested cockle, (b) 77 ITS out of 269 (Bm−) and 91 ITS out of 243 (Bm+) have been detected every month and (c) 66 of these ITS were found in both Bm− and Bm+ cockles. Third of the detected ITS (93 out of 284) occurred in less than 10% of individuals sampled during the monitoring. A focus on the 108 ITS contributing to more than 1% of total abundance was realized (Fig. 4). Regarding their temporal pattern in Bm− and Bm+, respectively, nine ITS and eight ITS have been detected every time; among these ITS, five were indistinctly found in Bm− and Bm+ cockles. Half of the detected ITS (51 out of 108) were only detected once or twice during the 10-month monthly monitoring. These ITS were moreover characterized by a rare occurrence being generally found in only one of the three replicate individuals.

The high proportion of rare ITS (detected by the two discrimination methods) resulted in a low mean level (mostly <50%) of Jaccard and Bray–Curtis similarities of CBC between individuals. CBC similarities of Bm− individuals collected at different times (e.g. two consecutive months Jaccard similarity=49±10%, Fig. 5a; Bray–Curtis similarity=46±14%, Fig. 5b) were not significantly lower than similarities recorded between individuals collected on the same date (Jaccard similarity=52±8%, Fig. 5a; Bray–Curtis similarity=53±14%, Fig. 5b; $p>0.1$, Mann–Whitney). As a consequence, no temporal pattern could be evidenced using the presence/absence data and Jaccard index or peak relative intensity data and Bray–Curtis index. Moreover, comparison of CBCD among months when CBCA was low (January, February, April) to the ones when CBCA was significantly higher (June, July, August, September, October), using ANOSIM, showed no significant difference ($p>0.05$). Consistently unlinked with the CBCA, no significant difference in the CBCD was found among months from May to October.

Regarding the CBCD of Bm− and Bm+ individuals, no significant difference was found in the number of ITS per individual between Bm+ and Bm− cockles ($p=0.8$, Mann–Whitney), mean=112±26 ITS per individual. Amongst the 15 ITS out of 243 that were detected only in the Bm+ individuals, most of them had an occurrence lower than 25%. Globally rare ITS primarily accounted for differences in CBCD between Bm− and Bm+ individuals. Based on the presence/absence of the ITS, Jaccard similarities between CBCD of Bm− (Jaccard=50±10%) or Bm+ individuals (Jaccard=50±8%) were not significantly different from similarities calculated between individuals belonging to the same set ($p>0.05$, Mann–Whitney; Fig. 6a). Bray–Curtis similarities based on peak relative intensity showed comparable results (Fig. 6b). Similarities between CBCD of Bm− on one side (Bray–Curtis=48±14%) and Bm+ on the other side (Bray–Curtis=33±15%) were not significantly different of similarities between Bm− and Bm+ cockles (Bray–Curtis=38±15%; $p>0.05$, Mann–Whitney; Fig. 6b). Comparison of CBCD among Bm− and Bm+ groups ANOSIM showed no significant difference between Bm− and Bm+ clusters ($p=0.46$).

Discussion

In this study, we applied structural descriptors of bacterial communities to characterize the dynamics of the communities associated with cockles at the individual scale. This sought to document the link between bacteria, parasite occurrence and cockle age.

To characterize cockle bacterial community diversity, a fingerprinting method (ARISA) was used. This culture-independent approach assesses the genetic structure of a bacterial community based on 16S–23S ITS size [21]. Since bacterial species have various numbers and types of ribosomic operon, there is no simple relationship between

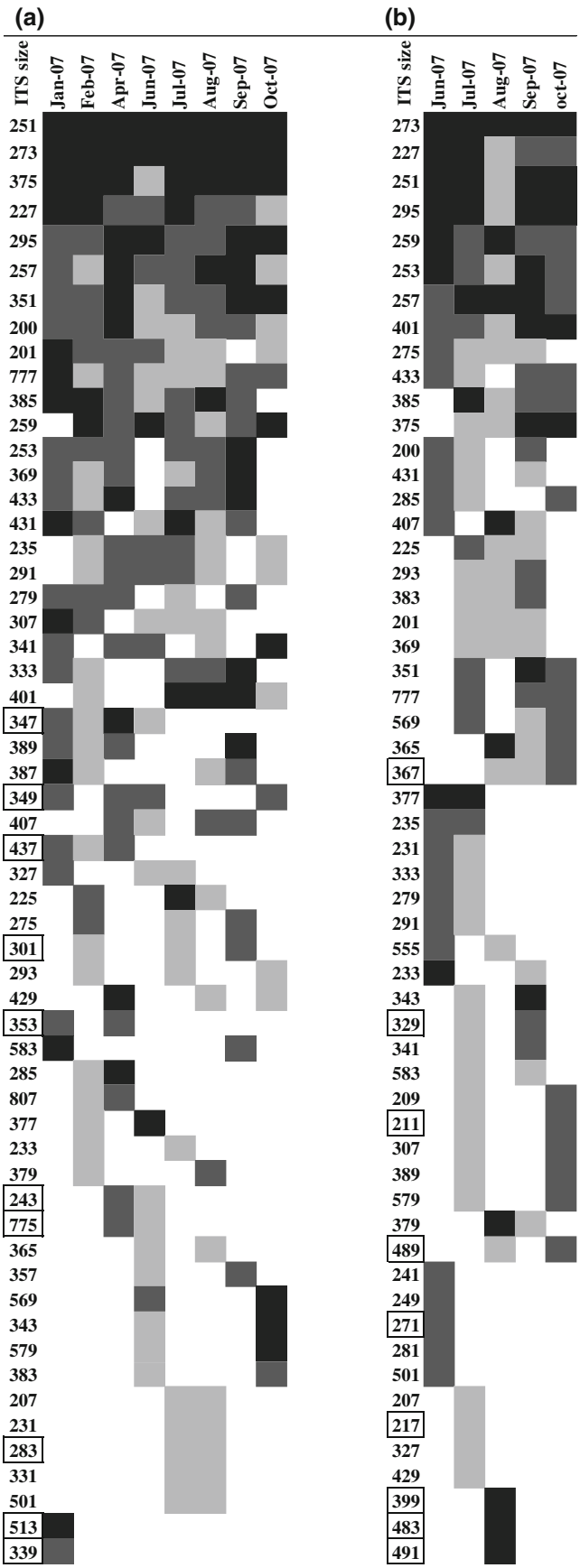

Figure 4 Frequency of bacterial ITS detected from individual cockles sampled from January 2007 to October 2007 in the Arcachon Bay, with a relative abundance higher than 1%. **a** Bm− individuals. **b** Bm+ individuals. ITS monthly frequency is represented by a *shade of grey* where the *colour* symbolizes the ITS frequency: *white* not detected, *light grey* 1% to 33%, *dark grey* 34% to 66%, *black* 67% to 100% of analysed individuals. *Open black boxes* underline the presence of ITS only found in Bm− or Bm+ individuals

the occurrence of a bacterial species and the number and types of retrieved ITS [29]. It is nevertheless assumed that the ITS richness and composition realistically reflect the bacterial taxonomic diversity [23]. An attempt to assess the cultivable diversity of the same samples provided only 17 colonial morphotypes on marine agar (data not shown). Eight out of 17 could be closely related to members of the genus *Bacillus*, i.e. an endospore former, suggesting that sample storage (no preservative such as glycerol added) affected the cultivable fraction. On the other hand, the 2-year long storage of cockle FIL samples at −20°C was unlikely to have affected the sample DNA quality [48]. Neither comparison between molecular and cultivable diversities nor phylogenetic assignment of the recovered ITS was therefore possible. Apart from ITS ascribing, ARISA fingerprinting is intrinsically adapted to assess β diversity (*sensu* Forney et al. [22]), i.e. pairwise comparisons of the composition (Jaccard similarity based on the presence/absence of the ITS) or of the relative abundance (Bray–Curtis similarity based on the relative fluorescence of the peaks) of bacterial communities. From the measured bacterial abundances (maximum 40×10^{6} cells g^{-1} [FIL]) and taking an average individual bacterial mass of 9.5×10^{-13} g $cell^{-1}$ [40], it could be estimated that bacterial biomasses have represented less than 0.1% of the biological material from which DNA was extracted, the remaining 99.9% being cockle tissues. Hence, low quantities of bacterial DNA and thus a low proportion of target DNA were to be recovered in our samples. This could explain why single-stage PCR did not amplify enough DNA in numerous samples. Conversely, semi-nested PCR, known to enhance the sensitivity [35], proved not to distort the CBC diversity pattern and was therefore successfully adopted.

The cockle population of Banc d'Arguin has been monitored monthly since 1999 and is representative of the species on its geographic distribution area [19]. More precisely, the studied 2006 cohort exhibited common temporal patterns of growth as attested by (a) Von Bertalanffy's growth model parameters: $L_{\infty} = 36.75$ mm, $k=1.33$ $year^{-1}$ [25] and (b) comparison of measured condition index (mean=78±24‰, min=30‰, max=122‰) to the values mentioned in de Montaudouin et al. [15] (mean=58‰, min=49‰, max=73‰). The patterns of CBC structure reported in the present study should thus be considered to represent typical features even for parasitized

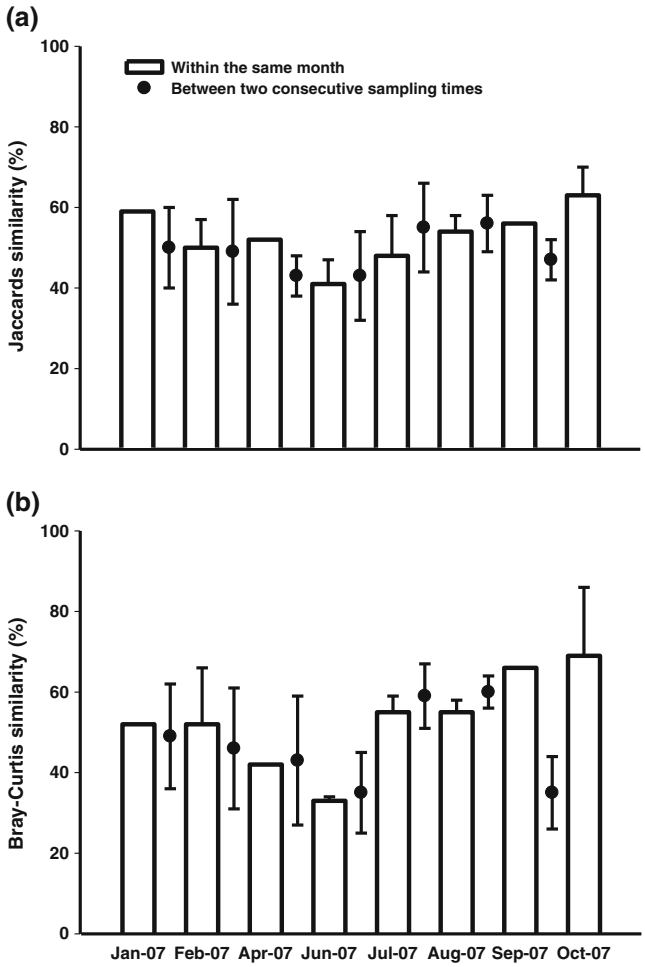

Figure 5 Temporal dynamics of bacterial communities in individual cockles, sampled in Banc d'Arguin (Arcachon Bay) from January 2007 to October 2007, where no *B. minimus* was detected. *Histogram bars with error bars* corresponded to similarities of cockle bacterial communities within a month; *black points with error bars* correspond to similarities of cockle bacterial communities between 2 months. *Error bars* represent standard deviation (absence of error bars is due to a lack of replicates). **a** Similarity based on the presence/absence of the ITS; **b** similarity based on the relative abundance of ITS (peak relative fluorescence)

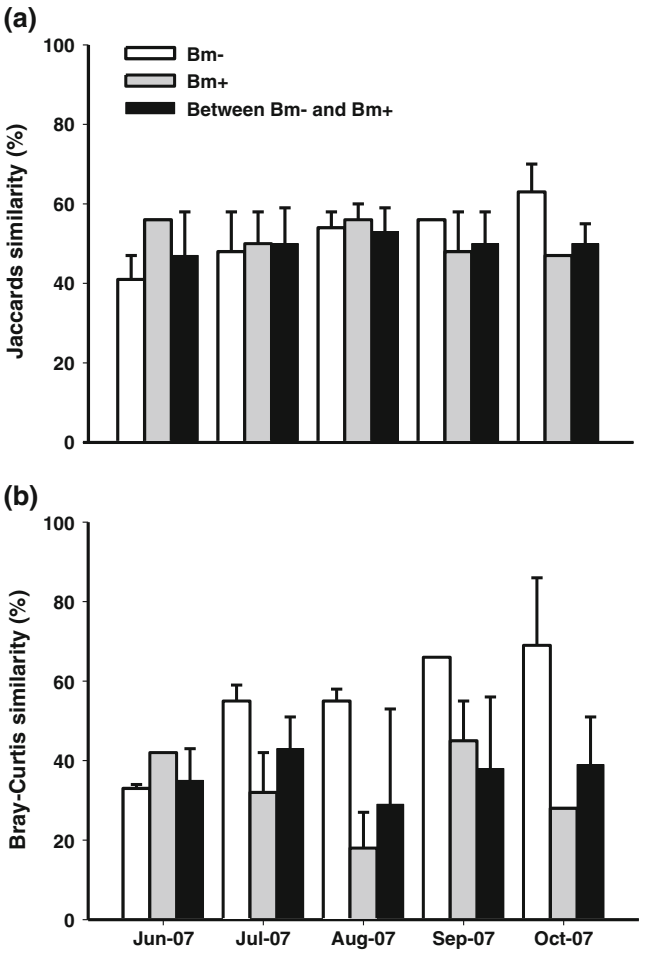

Figure 6 Comparison of similarities between bacterial communities in cockles sampled in the Banc d'Arguin (Arcachon Bay) from June 2007 to October 2007 according to their parasitic state. *Histogram bars with error bars* (*error bars* represent SD) correspond to similarities of cockle bacterial communities between individuals sampled on the same month. Absence of error bars is due to a lack of replicates. **a** Similarity based on the presence/absence of the ITS; **b** similarity based on the relative abundance of ITS (peak relative fluorescence)

individuals. The maximum *B. minimus* prevalence recorded during this survey (5%) agreed with previous observations [16] suggesting that the studied set of individual cockles experienced the usual parasitic infection pressure.

On average during the 10-month survey, *B. minimus*-free cockles presented a $14\pm2\times10^6$ cells g^{-1} [FIL] bacterial load composed of a quite stable ITS number of 112±26 ITS per individual. Given the typical range between culture-dependent and culture-independent numerations of bacteria [11], our data agreed with bacterial densities up to 10^5 MPN g^{-1}·[FIL] reported in Blanchet et al. [5]. Comparable OTU richness was reported for lobster gut [39]. The inter-individual variability of CBCD attested by Jaccard or Bray–Curtis similarities averaging 53±7% and 53±12%, respectively, indicated a relative homology between bacterial communities of individuals sampled within a given month. This result was expected because cohort individuals were sampled on the same sampling site, were recruited on the same period (same age) and have thus experienced similar environmental conditions such as, for instance, being submitted to the same bacterial contamination events. Despite ITS binning, rare ITS (= ITS recorded in less than 10% of the studied cases) have represented 33% of the 284 detected ITS. The rare ITS marginally contributed to the ITS richness; hence, the CBCD was satisfactorily described. That no distinctive temporal pattern could be evidenced even when higher bacterial abundances were found in cockles during the summer period suggested a relative stability of the bacterial taxa harboured by

cockles (CBCD). Changes in cockles' bacterial load (CBCA) during the summer period could be due to an increase in gut microflora caused by (a) enhanced seawater bacterioplankton densities during the productive period [1] or (b) enhanced filtration rates linked to individual growth [12] or (c) the reproductive life cycle affecting the FIL mass during the spawning period [12]. The gut microflora hypothesis is emphasized by the probable importance of gut bacterial numbers (e.g. 90% of the cultivable heterotrophic bacteria are in oyster gut [33]). Considering the different organs (e.g. gut, gills, muscle) separately would allow this hypothesis to be tested in further studies.

The cockle size is an important factor contributing to *B. minimus* infestation as *B. minimus* is known to parasitize cockles having reached at least a 16-mm shell length [16]. In the present study, *B. minimus* was first detected later than expected (in June) when cockles were 13 months old and had a shell length of 29 mm. The fact that parasitized and not parasitized cockles exhibited comparable condition indexes suggested that parasitized individuals were not affected physiologically [36]. However, the parasite flesh can represent more than 25% of the total flesh of the host–parasite system [18], and a similar condition index between parasitized and non-parasitized cockles can signify a deficit of host flesh in parasitized individuals. The only indication of an interaction between CBC and *B. minimus* infestation was the enhanced CBCA recorded in cockles parasitized by *B. minimus* during the summer period. It might indicate a secondary bacterial infection linked to bacterial invasive development in damaged cockle tissues (gonads, gills and digestive gland). Another indication of secondary infection is the change in the individual bacterial community composition as observed for coral by Sunagawa et al. [50] and Pereira de Castro et al. [45]. Lysis of the tissues due to the primary infestation may lead to development of new niches (organic matter release, sites to be colonized) for saprophytic bacteria. These niches could be settled by exogenous and/or commensal opportunistic bacteria leading to changes in bacterial community composition. In the present study, no change in CBCD between Bm− and Bm+ individuals could be evidenced by comparing Jaccard and Bray–Curtis similarity indexes. Given that no difference was found in the physiological state of the cockles (condition index) with respect to the parasitic state, the CBCA increase still needs to be explained. It could be hypothesized that sampled individuals were in an early stage of the secondary infection, when major changes in CBCD have not occurred yet.

A rarefaction curve based on Michaelis–Menten equation was designed a posteriori from the CBC presence/absence matrix of all samples (Fig. 7). According to Magurran [37], this curve tentatively documented the maximum ITS richness (S_{max}) and the sampling strategy

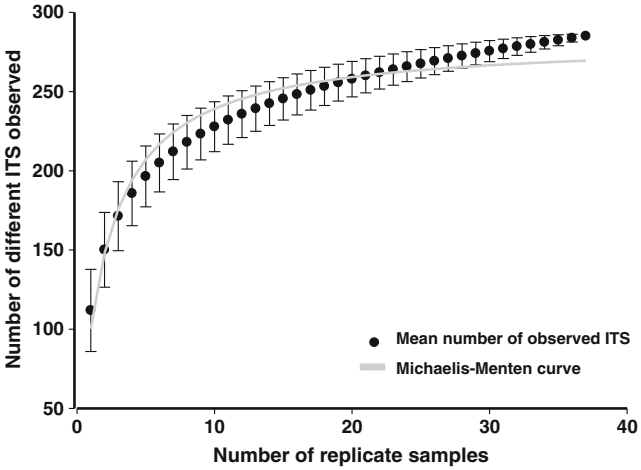

Figure 7 Rarefaction curve. *Black points with error bars* represent the averages and SD of observed ITS as the samples are accumulated. This average was obtained from 100,000 permutations of 37 presence/absence matrices of ITS. *Grey curve* represents the curve regression based on Michaelis–Menten equation (NumberofITS = (283 × numberofreplicates)/(1.8 + numberofreplicates)

(optimal number of replicates for an exhaustive diversity analysis). For S_{max}, the best fit of the Michaelis–Menten equation indicated a 283 ITS per cockle value. The S_{max} value is probably an overestimation of the actual CBCD at the individual scale although it is likely to be an underestimate at the population scale. On the other hand, taxa–area relationships were shown for bacteria being mainly supported by the habitat heterogeneity [30]. Cockle tissues and particularly the gut could be possibly seen as diversified habitats for bacterial settlement. Accordingly, high bacterial diversity was reported in oyster gut, gills and gonads [28]. Moreover, comparable orders of magnitude (hundreds of OTUs) were reported in lobsters [39] and in corals [45]. For the sampling strategy, the a posteriori test indicated that working on three replicates allowed only 62% of the theoretical total CBCD to be taken into account. That may explain the high rate of rare ITS. According to our estimation, 16 replicates would be required to base analyses from 90% of theoretical total CBCD. This is a challenge in such a field experiment since collecting 16 parasitized cockles could require collecting hundreds of cockles where cockle densities range from 100 to 200 ind m^{-2}. Further studies should obviously consider a trade-off in the number of replicate individuals per condition for a more accurate analysis of the cockle bacterial community dynamics.

Conclusion

This study investigated the dynamics of the symbiotic bacterial communities during cockle growth or infestation

by *B. minimus*. Some ITS (93 out of 284) occurred in less than 10% of individuals sampled during the monitoring, but bacterial community compositions of cockle individuals that had been submitted to the same bacterial contamination events were similar at a 52±6% level (mean Jaccard similarity). Further studies should consider the number of replicate individuals per condition and may take advantage of considering separately the different organs (e.g. gut, gills, muscle) for a more accurate analysis of the cockle bacterial community dynamics. In the present case study, however, typical cockle growth patterns and parasite prevalence were observed. Apart from transient changes in cockle bacterial loads during summer, marked changes in the bacterial community composition were not linked to changes in individual physiological state (attested by condition index) during the 10-month cohort monitoring. Likewise, apart from enhancement of parasitized cockle bacterial loads during summer, *B. minimus* infestation of cockles did not affect the individual physiological state and bacterial community composition. These data suggested that bacterial community composition, in contrast with community abundances, could be consistently linked to cockle fitness descriptors highlighting the need to consider bacterial diversity when studying their association with marine invertebrates.

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