The marine intertidal zone shapes oyster and clam digestive bacterial microbiota

Clément Offret, Sauvann Paulino, Olivier Gauthier, Kevin Château, Adeline Bidault, Charlotte Corporeau, Philippe Miner, Bruno Petton, Fabrice Pernet, Caroline Fabioux, et al.

The marine intertidal zone shapes oyster and clam digestive bacterial microbiota.

Clément Offret[1], Sauvann Paulino[1], Olivier Gauthier[1], Kevin Château[1], Adeline Bidault[1], Charlotte Corporeau[2], Philippe Miner[2], Bruno Petton[2], Fabrice Pernet[2], Caroline Fabioux[1], Christine Paillard[1], Gwenaelle Le Blay[1*]

[1]Univ Brest, CNRS, IRD, Ifremer, LEMAR, F-29280 Plouzané, France

[2]Ifremer, Univ Brest, CNRS, IRD, LEMAR, F-29280 Plouzané, France

*** Corresponding author:** F-29280 Plouzané, France. Tel: +33 (0)2 90 91 55 65; E-mail: gwenaelle.leblay@univ-brest.fr

One sentence summary: structuration of bivalve digestive microbiota is shaped by intertidal position after a four months field implantation.

Abstract

Digestive microbiota provides a wide range of beneficial effects on host physiology and are therefore likely to play a key role in marine intertidal bivalve ability to acclimatize to the intertidal zone. This study investigated the effect of intertidal levels on the digestive bacterial microbiota of oysters *Crassostrea gigas* and clams *Ruditapes philippinarum,* two bivalves with different ecological niches. Based on the 16S rRNA region sequencing, digestive glands, seawater and sediments harbored specific bacterial communities, dominated by OTUs assigned to the *Mycoplasmatales*, *Desulfobacterales* and *Rhodobacterales* orders, respectively. Field implantation modified digestive bacterial microbiota of both bivalve species according to their intertidal position. *Rhodospirillales* and *Legionellales* abundances increased in oysters and clams from low intertidal level, respectively. After a 14-day depuration process, these effects were still observed especially for clams, while digestive bacterial microbiota of oysters were more subjected to short-term environmental changes. Nevertheless, 3.5 months stay on intertidal zone was enough to leave an environmental footprint on the digestive bacterial microbiota, suggesting the existence of autochthonous bivalve bacteria. When comparing clams from the three intertidal levels, 20% of the bacterial assemblage was shared among the levels and it was dominated by OTU affiliated to the *Mycoplasmataceae* and *Spirochaetaceae* families.

Keywords: microbiota; oysters; clams; intertidal zone; digestive gland; metabarcoding

INTRODUCTION

Host-associated microbiota play a key role in host homeostasis and health, by (i) promoting development (McFall-Ngai 2002), (ii) providing protection against pathogens (Offret *et al.* 2018) and/or (iii) improving adaptation to environmental modifications (Torda *et al.* 2017). It is hypothesized that microbiota modification may strongly impact its host in terms of physiology, immunology and nutrient uptake (McFall-Ngai *et al.* 2013; Baker *et al.* 2018; Clerissi *et al.* 2018; Dubé *et al.* 2019). Host-associated microbiota consist of more or less complex communities of microorganisms, some of which are more adapted to their host, others generalist, or transient, representing a wide range of potential contributions (Shapira 2017). It is well known that bivalves harbor their own microbiota (as for other organisms), whose characteristics and functions are still poorly understood, but cannot be ignored (Desriac *et al.* 2014; Offret *et al.* 2019).

Microbial community composition and diversity associated with oysters (Trabal *et al.* 2012; Trabal Fernández *et al.* 2014; King *et al.* 2019b) and clams (Romalde *et al.* 2013; Meisterhans *et al.* 2015) are beginning to be described with culture-independent methods from different tissues, such as hemolymph (Lokmer and Wegner 2014; Lokmer *et al.* 2016b, 2016a), mantle (Lokmer *et al.* 2016b; King *et al.* 2020), gills (Wegner *et al.* 2013; Lokmer *et al.* 2016b; King *et al.* 2020), adductor muscle (King *et al.* 2019c, 2020) or digestive gland (King *et al.* 2012, 2019a, 2020; Lokmer *et al.* 2016b; Milan *et al.* 2018; Vezzulli *et al.* 2018). The digestive gland (DG) is one of the most colonized tissue of bivalves with the highest concentrations of bacteria (Kueh and Chan 1985). Digestive microbiota generally supplies the host with exogenous nutrients and extracellular enzymes, fatty acids and vitamins (Dhanasiri *et al.* 2011), thus contributing to nutrient degradation and uptake (Harris 1993; Simon *et al.* 2019). The establishment and structuring of the DG microbiota depend on physiological, genetic and immune characteristics of the host, the environment, the type of food ingested, as well as the interactions between microorganisms (Hacquard and Schadt 2015). For that reason, structuring of DG microbiota may play a key role in the metabolic condition of bivalves by influencing their growth capacity, immunity, energy load, nutrition process and digestive enzyme activities (Harris 1993; Rőszer 2014).

Microbiota structuration and composition are affected by both host and habitat factors (Kvennefors *et al.* 2010), such as intertidal position. Marine intertidal zones represent a heterogeneous environment (Harley *et al.* 2006) structured by different gradients of biotic and abiotic factors including temperature, salinity, nutrients, UV and rainfall variations (Connell 1972; Helmuth and Hofmann 2001). Marine organisms are facing different physiological challenges based on their position within

the intertidal zone, which further result in physiological differences (Soudant *et al.* 2004; Fernández-Reiriz, Irisarri and Labarta 2016; Yin *et al.* 2017). The Pacific oyster, *Crassostrea gigas*, and the Manilla clam, *Ruditapes philippinarum*, are epifaunal and infaunal bivalves, respectively, with important economic value worldwide. Both species are subject to different biotic and abiotic factors due to their distribution within the marine tidal zone. Oysters living in the intertidal zone, attach and feed on planktonic microalgae, while clams burrowing in sediment mainly ingest benthic microalgae and sedimented phytoplankton (Simons et *al.* 2018). To date, previous studies have shown that the oyster microbiota could change under a multitude of different stressful treatments, such as translocation, starvation, temperature, infection and antibiotic treatment (Green and Barnes 2010; Wegner *et al.* 2013; Lokmer and Wegner 2014; Lokmer *et al.* 2016b, 2016a; Green *et al.* 2019). However, to our knowledge no study has ever investigated the effect of different intertidal levels on bivalve microbiota along a transect between the upper and lower limits of their distribution from shore. The aim of this study was to investigate consequences of intertidal position on DG microbiota in two bivalve species with different ecology, the Pacific oyster *C. gigas* and the Manilla clam *R. philippinarum*. To this end, individuals of both species were deployed at one site in the Bay of Brest (Brittany, France) at three contrasted intertidal levels (high, middle and low) for 3.5 months. Bacterial microbiota from the DG were explored for composition and structuration by metabarcoding analysis. To evaluate the environmental footprint on the DG microbiota, a cohort of bivalves was placed in depurated conditions to analyze the intertidal level-specific microbiota that remains in the animals.

MATERIALS AND METHODS

Biological samples

C. gigas oysters' families were produced in February 2017 using a developed methodology that allowed the production of pathogen-free juveniles, called "Naissain Standardisé Ifremer" (NSI). In larval and post-larval stages, the oysters were maintained in controlled condition at the laboratory (Argenton, France). The clams *R. philippinarum* were provided by a commercial exploitation (SATMAR, France). They were descendants of clams families (around 1000 families) born in April 2016. Before deployment in the field, mean shell length was 47.1 ± 5.2 mm for oysters and 20.3 ± 2.3 mm for clams.

Experimental design on intertidal site

Oysters and clams were transferred in mid-October 2017 to a farming area (surface of ca. 200m^2) located in the Bay of Brest at Pointe du Chateau (48° 20' 06.19" N, 4° 19' 06.37" W, Britany, France). The chosen implantation period (water temperature < 16°C) is a period without oyster mortality events (Petton *et al.* 2015), without phytoplanktonic bloom (Lessin *et al.* 2019), with low growth rates (Menzel 2018) and no breeding, which facilitated access to the DG. Animals were deployed at three rearing heights (1, 2.8 and 4 meters above sea level) corresponding to 20%, 56% and 80% of exondation time (Fig. 1). Similar to cultivation practices, animals were placed in two duplicated mesh bags of 190 individuals for oysters (2 ×190 = 380) and 250 individuals for clams (2 × 250 = 500). Oyster bags were attached to an iron table, whereas clam bags were directly placed in the sediment. Sediment was collected next to the clams in October at each level in triplicate to evaluate the bacterial community.

An initial sampling was performed just before deployment in the field. Digestive gland (DG) of oysters (n=15) and clams (n=15) were sampled in RNase-DNase free conditions. Dissected DG were rinsed using sterilized filtered (0.22µm) and autoclaved seawater and were frozen in liquid nitrogen before being stored separately in cryotubes at -80°C. Sediments were collected next to the clams in October at each level in triplicate to evaluate the bacterial community.

In February 2018, oysters and clams were removed from the three intertidal levels over three consecutive days at spring low tides (31/01, 01/02 and 02/02). For each level, collected animals were either directly dissected (n=15; clams 19.6 ± 3.4 mm; oysters 49.1 ± 7.1 mm) or brought to the laboratory to be placed in depuration (n=15; clams 18.9 ± 4.1 mm ; oysters 45.7 ± 5.7 mm). In the present study, the purpose of depuration was to empty the digestive glands and to reduce the environmental microorganisms (Romero *et al.* 2002; Lee *et al.* 2008), in order to evaluate the persistence of environmental conditioning on bivalve DG microbiota.

Depurated oysters and clams were grouped by sampling day in a bag and placed for 14 days in 30L-tanks (one by intertidal level) containing filtered seawater (10- and 5-µm sand filters and UV treatment before two 1-µm filters and a second UV treatment) renewed at 3L minutes^{-1}. Temperature of seawater in tanks was similar to temperature variations of the natural seawater. Tanks were cleaned every second day to avoid biofilm formation and no feed was added. Sediments (25g on triplicate) were collected at each level, while seawater was sampled (1 L in triplicate) 2 hours before low tide, close to animals. Sediments collected next to clams were directly stored at -80°C, while the seawater samples were successively passed through 8- and 0.22-µm polycarbonate filters (Whatman, USA),

before being stored at -80°C until DNA extraction. During this period, no mortality events occurred for both bivalves, no brown ring disease nor *Vibrio tapetis* were detected in clams, suggesting that this study was realized on healthy bivalves.

DNA extraction

The extraction of bacterial genomic DNA (gDNA) from the DG of oysters and clams combined the use of phenol-chloroform-isoamyl alcohol (PCI) extraction with a DNA extraction kit (PowerLyser Powersoil DNA Isolation, Qiagen, USA). Briefly, after homogenization of the DG, 40 mg were collected to be digested at 45°C for 30 minutes in a lysis buffer (178 µL) consisting in TNE (Tris-HCl 1M at pH 8, NaCl 5M, EDTA 0.5M at pH 8), SDS 20% and proteinase K (20 mg mL^{-1}). After centrifugation (10 min, 10,000 g), supernatant was recovered and stored at 4°C. A second digestion was carried out on the pellet by adding 100 µL of lysis buffer at 45°C for 1 hour. After centrifugation (10 min, 10,000 g) the recovered digestate was then pooled with the first one. This digestion product (200 µL) was then mechanically lysed in PowerBead tubes (0.1 mm) from the PowerLyser kit, to which Beads (650 µL) and the C1 solution (60 µL) were added, before being shaken in the FastPrep24TM (2 x 45 seconds). Supernatant (750 µL) was transferred to a new tube. One volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added. After mixing (45 sec) and centrifugation (15 min, 16,000 *g* at 4°C), 1 vol of chloroform was added to the supernatant, mixed again (45 sec) and centrifuged (15 min, 16,000 *g* at 4°C). Isopropanol (0.7 vol, -20°C) was added to the supernatant to precipitate DNA. The tubes were placed overnight at -20°C. Precipitated DNA was centrifuged (30 min, 16,000 g at 4°C). Pellet was solubilized in 500 µL of TNE buffer and PowerLyser Powersoil DNA Isolation kit was used according to manufacturer's protocol. Finally, DNA was precipitated by centrifugation (30 sec, 10,000 g at room temperature), eluted in 100 µL ultra-pure water (Gentrox, UK) and stored at -80°C.

gDNA from sediment samples (250 mg) was extracted using PowerLyser Powersoil DNA Isolation kit (Qiagen, USA), exclusively, according to the manufacturer's instructions. gDNA from seawater filters (0.22 µm and 8µm) were extracted using PCI extraction according the same steps used for tissue samples as described above. After precipitation, DNA was washed with ethanol 75% (500 µL) and dried before being hydrated with 100 µL ultra-pure water (Gentrox, UK).

To check for bacterial contamination of reagents, additional blank extractions were included. gDNA concentrations from tissues and environmental samples were determined by spectrofluorometric quantification using Quantifluor kit (Promega, USA) according to manufacturer's protocol.

Microbiota analyses

For each sample, 16S rRNA amplicon libraries were generated using the 341F (5'-CCTACGGGNGGCWGCAG -3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') primers targeting the variable V3V4 region (Herlemann *et al.* 2011). Paired-end sequencing with a 300-pb read length was performed at McGill University (Génome Quebec Innovation Centre, Montréal, Qc, Canada) on a MiSeq system (Illumina).

The sequencing data obtained were processed via the FROGS pipeline (Find Rapidly OTU with Galaxy Solution, v2.0.0) developed in the Galaxy environment (http: //sigenae-workbench.toulouse.inra.fr/galaxy/). This pipeline groups sequences by similarities into OTUs (Operational Taxonomic Units) and calculates taxonomic affiliations (Escudié *et al.* 2018). Briefly, the "pre-process" step allowed to join the paired ended reads together using FLASH with a mismatch of 0.1 (Magoč and Salzberg 2011) and to remove both primers and adapters using cutadapt (Martin 2011). A *de novo* clustering was carried out using the SWARM method, which groups the sequences into clusters from a local clustering threshold with an aggregation distance d = 3 (Mahé *et al.* 2014). Chimeras were removed using VSEARCH, a method dividing each sequence into four fragments and then searching for possible parent sequences in all OTUs (Rognes *et al.* 2016). An abundance filter with an optimal threshold of 0.005% was applied on OTUs (Bokulich *et al.* 2013), except for α-diversity. Finally, the OTUs were assigned using Blast+ and the Silva 132 16S database containing known sequences of bacterial 16S rRNA. The multi-affiliated sequences were corrected by indicating for each of them an affiliation at a higher taxonomic rank. A phylogenetic tree of the OTUs and a table of abundances of affiliated OTUs were then produced in the standard BIOM format.

Statistical analyses

Niche-wise (oyster, clam, sediment, seawater fractions) microbial communities α-diversity was assessed at the OTU level after assigning OTUs to the lowest possible taxonomic level using Simpson's inverse and Shannon entropy. Indices were computed for each individual bivalve microbiota and Kruskal-Wallis tests were used to investigate mean differences between experimental conditions (*ecological niche*, *sampling period*, *depuration impact*, *intertidal level effect* and *sampling day*).

Variation in microbiota composition and structure between individual bivalves, β-diversity, was first visualized with principal component analysis (PCA) of Hellinger transformed OTU abundances. The Hellinger transformation does not give excessive weight to rare categories and may therefore help to overcome differences in sequencing depth (Legendre and Gallagher 2001). The effects of *ecological*

niche, sampling period, depuration, intertidal level and *sampling day* (and their interactions) were tested using permanova (McArdle and Anderson 2001). Homogeneity of multivariate dispersion to group medoid was first assessed in order to satisfy assumptions.

To assess whether presence/absence based β-diversity in DG microbiota between tidal levels was predominantly driven by changes in species identity or fluctuations in species richness, Jaccard dissimilarity between each pair of samples was partitioned in species replacement ($\beta_{Replacement}$) and richness difference ($\beta_{RichDiff}$) following protocols described by Legendre 2014. Calculation and decomposition of the Jaccard dissimilarity was performed for each DG microbiota from non-depurated or depurated clams (separately), between levels (pairwise comparisons). Venn diagrams based on the Jaccard dissimilarity (presence/absence of OTUs) gave access to shared or specific OTUs of the different intertidal levels. All analyses were carried out using R3.5.2 (Team 2018), with all β-diversity analysis conducted with functions from the *vegan* package (Oksanen *et al.* 2019).

RESULTS

Data analysis information

A total of 3,193,963 sequences were kept from all samples (n=245) after processing via the FROGS pipeline (Supplementary Data). These sequences represented about 51% of all reads sequenced from the V3V4 hypervariable region of the 16S rRNA gene. The average Quality Score of amplicons was 33 (Sogin *et al.* 2006). After removing clusters representing less than 0.005% of all sequences, the swarm clustering produced 1,322 different OTUs divided into seawater fractions (987 OTUs), sediments (705 OTUs), DG of oysters (1129 OTUs) and clams (1197 OTUs).

α-diversity of OTUs from bivalve DG and their environment

The number of most abundant OTUs was higher in DG than in seawater or sediments. Shannon and inverse Simpson's indices (Supplementary Table 1) indicated that total bacterial diversity (considering singletons and rare OTUs) was lower in the DG of both bivalve species (Kruskal Wallis, Shannon: d.f. = 3; p = 9.65e-13 and Simpson's inverse: d.f. = 3; p = 4.20e-08) compared to their immediate environment (Supplementary Figure 1). Bivalve digestive microbiota were dominated by few OTUs accounting for the majority of reads, whereas rare OTUs were determinant for structuring bacterial community composition of environmental samples. The implantation on the intertidal zone led to a significant increase of both α-diversity indices (Kruskal Wallis, $p < 0.001$) for oysters and

clams. Depuration significantly reduced the α-diversity of clams DG microbiota (Kruskal Wallis, Shannon: d.f. = 1; p = 2.53e-11 and Simpson's inverse: d.f. = 1; p = 7.68e-05). In depurated oysters, only the Shannon index was significantly reduced, indicating a loss of rare OTUs. On the other hand, whatever indices, α-diversity were not impacted by intertidal position for both bivalve species.

Microbiota specific structure according to the host ecological niche

Principal component analysis (PCA) of the Hellinger-transformed abundances (Fig.2) identified a separation of microbiota which were significantly different between sample types (permanova test, R^2 = 27%, F = 30.28, p = 0.001), in terms of both OTUs richness and relative abundances (Fig. 3). Bacterial community composition was represented on two principal component (PC) axes, separating DG microbiota of bivalve species on PC1 (15.2%) and environmental from animal samples on PC2 (10.4%). PCA showed that environmental microbiota were closer to DG microbiota of oysters compared to that of clams. The observed patterns were mostly associated to the contribution of OTUs affiliated to the orders of *Mycoplasmatales* and *Rhodospirillales* for oysters, *Spirochaetales*, *Rickettsiales* and *Oceanospirillales* for clams, and *Rhodobacterales* for environmental samples (Supplementary Figure 2). Seawater fractions appeared to be clearly separated on both axes, with the 8-0.22µm fraction closer to animals than the >8µm fraction which was confounded with bacterial communities from sediments. Interestingly, a lower similarity of bacterial communities was established between clams DG and their closest environment, the sediment.

Taxonomical composition of DG microbiota and environmental samples

Microbiota (OTUs > 0.5% of total sequences), whatever from bivalve or environmental samples, were dominated by 8 phyla, *Proteobacteria, Tenericutes, Actinobacteria, Spirochaetae, Chlamydiae*, *Fusobacteria*, *Bacteroidetes* and *Planctomycetes* (Fig. 3). Before implantation in the field in October, oyster DG microbiota were mainly dominated by *Mycoplasmatales* (87%), while *Mycoplasmatales* (43%), *Chlamydiales* (23%), *Rickettsiales* (21%) and *Spirochaetales* (10%) were dominant in clams. After 4 months of implantation in the intertidal zone, the DG microbiota diversity of both bivalves was significantly modified (Fig.4). Indeed, the relative abundance of *Mycoplasmatales* decreased in non-depurated oysters, while that of *Rhodospirillales* and *Campylobacterales* increased. Moreover, *Chlamydiales*, *Legionellales* and *Planctomycetales* were detected in February but not in October. The same trend was observed in clams, with a decreased abundance of *Mycoplasmatales* and *Chlamydiales*

in February compared to October in favor of *Rickettsiales*, *Spirochaetales* and *Oceanospirillales* as well as, to a lesser extent, *Legionellales*, *Corynebacteriales* and *Planctomycetales*. Bacterial communities from bivalves DG microbiota were clearly different from those in environmental samples (Fig. 3), which were mainly dominated by *Desulfobacterales* (36%), *Campylobacterales* (30%), *Acidimicrobiales* (25%) and *Fusobacteriales* (7%) for sediments, and *Rhodobacterales* (99%) for the seawater small particles fraction (8-0.22µm); and *Rhodobacterales* (66%), *Campylobacterales* (14%), *Desulfobacterales* (10%), *Acidimicrobiales (5%)* and *Fusobacteriales* (3%) for the large particles fraction (> 8 µm). This later fraction harbored several taxa similar to those found in sediments (Supplementary Data).

For both bivalves, depuration decreased the abundance of *Mycoplasmatales*. This decrease, in favor of *Spirochaetales* in clams and *Chlamydiales* in oysters, was associated with a decrease of less abundant OTUs, such as *Desulfobacterales* and *Rhodobacterales* in both bivalves. In non-depurated oysters, a negative correlation (Pearson correlation: -0.34, $p = 0.020$) was observed between the three intertidal levels and relative abundance of *Rhodospirillales*, which were more abundant at the lowest level. In non-depurated clams, the relative abundance of *Oceanospirillales* was positively correlated (Pearson correlation: 0.33, $p = 0.025$) with a higher position on the intertidal zone, whereas this correlation was negative for *Legionellales* (Pearson correlation: -0.39, $p = 0.008$).

Consequences of implantation on digestive microbiota and inter-individual variability

OTU abundances from non-depurated oysters or clams highlighted two separate groups (Fig. 4) according to the sampling period (permanova test, oyster: $R^2 = 17\%$, $F = 11.48$, $p = 0.001$ and clam: $R^2 = 15\%$, $F = 15.59$, $p = 0.001$). These results demonstrated that bivalve DG microbiota were drastically modified after three-and-a-half-month implantation in the intertidal zone. These changes in DG microbiota were mainly explained by OTUs affiliated to *Mycoplasmatales* and *Rhodobacterales* for oysters (Supplementary Figure 4A), and to *Chlamydiales*, *Legionellales*, *Mycoplasmatales*, *Oceanospirillales*, *Rhodospirillales* and *Rickettsiales* for clams (Supplementary Figure 4B). Additionally, sample dispersion, measured by average of the distances to the median, was significantly higher (ANOVA, d.f. = 1, $p = 2$, 51e- 05) for oysters sampled in February (d = 0, 73) than in October (d = 0, 59). These results reflected an increase in the inter-individual variability during implantation. Unlike oysters, clams' inter-individual variability was not modified during implantation, indicating different responses in both species.

Short spatial and temporal scales as shapers of bacterial community of bivalve DG microbiota and environmental samples

Bivalve position on the intertidal zone significantly modified their DG microbiota (Table 1). The "*Sampling day*" factor only affected DG microbiota composition in oysters (permanova test, $p = 0.013$). No interaction between "*level*" and "*sampling day*" factors was observed, regardless of the species. Oysters DG microbiota were impacted both by short spatial and temporal scales, while that of clams was only impacted by the position on the intertidal zone. Bacterial communities from sediments differed significantly regarding the intertidal level (permanova test, $p = 0.001$). Bacterial community found in the 8-0.22µm seawater fraction was the only water fraction impacted by the sampling day (permanova test, $p = 0.01$).

In order to evaluate the persistence of intertidal level impact on bivalve DG microbiota, oysters and clams were placed in controlled laboratory conditions for depuration. After 14 days of depuration, significant dissimilarities were observed between DG microbiota of depurated and non-depurated bivalves for both species (Supplementary Figure 5). Moreover, depuration induced an increase of the inter-individual variability in both species, compared to non-depurated bivalves (ANOVA, oysters: d.f. = 1, $p = 1.624e-10$ and clams: d.f. = 1, $p = 0.017$). Both species DG microbiota were still significantly impacted after depuration by their intertidal levels (Table 1). Moreover, even if the sampling day did not significantly impact oysters DG microbiota after depuration, this factor significantly interacted with the intertidal levels factor (permanova test, $p = 0.005$), suggesting that oysters DG microbiota were fairly unstable from one day to the next.

Decomposition of OTU variations in DG microbiota of clams placed at three different intertidal levels

Calculation and decomposition of the Jaccard dissimilarity between the three intertidal levels provided information on the percentage of inter-level similarity (Fig. 5). Inter-levels comparisons highlighted that non-depurated clams DG microbiota shared on average 25% of their OTUs between the three levels (Fig. 5A), with the most pairwise similarities lying between 20% and 40% of shared OTUs. For all level comparisons, dissimilarities between clams DG microbiota were mainly the consequence of OTU replacement (43%-48%), while richness difference ranged from 26% to 32%. Compared to similarity, values of the two dissimilarity components were much less uniform, with replacement and difference richness lying respectively between 10%-75% and 5%-80%. If dissimilarity seemed to be distributed in a consistent manner in high-middle and low-middle level

312 comparisons, replacement was higher (48% vs 43%) and richness difference lower (27% vs 33%) for
313 high-low level comparison. These results indicated a global microbiota composition shifting (led by
314 OTUs replacement) between DG microbiota from clams placed on these two extreme levels.
315 Comparatively to non-depurated clams, depuration led to a drastically decrease of richness
316 difference (19% on average) for all inter-level comparisons, while similarity (22% on average) was
317 also reduced by 3% (Fig. 5B). These richness-difference and similarity decrease were offset by a higher
318 replacement (59% on average), indicating that some OTUs were removed and that depurated clams
319 DG microbiota were more homogeneous in term of OTUs numbers. Compared to non-depurated
320 bivalves, amplitudes of pairwise comparison values (for all intertidal levels comparisons) of both
321 replacement and richness difference were lower, ranging respectively between 25%-80% and 5%-60%.
322 This lower amplitude of pairwise comparisons induced by depuration highlighted that dissimilarities
323 of DG microbiota between intertidal levels were mainly explained by a replacement of OTUs rather
324 than a richness difference.

325

326 **Identification of shared and specific OTUs among clams DG microbiota according to**
327 **their intertidal level**

328 Non-depurated clams from the all three intertidal levels shared a total of 737 common OTUs out
329 of 1102 identified (Fig. 6A and Supplementary Material). These common OTUs were mainly affiliated
330 to the orders *Planctomycetales* (13%), *Legionellales* (9%) and *Rhodobacterales* (8%), with the most
331 abundant OTU affiliated to the *Mycoplasmataceae* family, representing 11% of total sequences. Clams
332 from the high and middle levels shared the highest number of OTUs (110) compared to high-low (66)
333 and middle-low (32) levels. For level-specific OTUs, clams placed on high and middle intertidal levels
334 exhibited respectively 68 and 63 specific OTUs. OTUs specifically found in clams placed on the high
335 level were affiliated to the orders *Flavobacteriales* (18%) and *Rhodobacterales* (15%), while those
336 from the middle level were affiliated to the orders *Planctomycetales* (16%) and *Rhodobacterales* (8%).
337 By contrast, only 23 OTUs were specific to clams placed on the low intertidal level and were dominated
338 by *Planctomycetales* (12%).
339 Depuration led to a major overhaul of the specific and common clams DG microbiota (Fig. 6B).
340 Compared to non-depurated clams, depuration decreased by 42% the number of total identified OTUs
341 (636). Common OTUs (240) were mainly affiliated to *Legionellales* (16%), *Chlamydiales* (11%),
342 *Rickettsiales* (7%) with the most abundant OTU affiliated to the *Spirochaetaceae* family representing
343 19% of total sequences. Nevertheless, the general pattern remained the same as for non-depurated

clams, with DG microbiota originating from high and middle levels sharing the highest number of OTUs (88), and DG microbiota from low intertidal level showing the lowest number of specific OTUs (55), which were mainly affiliated to *Flavobacteriales* (15%) and *Planctomycetales* (15%). Clams from the high-low and middle-low levels shared respectively 44 and 37 OTUs. Clams placed on high and middle intertidal levels exhibited respectively 91 and 81 specific OTUs, those placed on the high level were dominated by *Rhodobacterales* (16%) and *Planctomycetales* (11%), while those from the middle level were mainly affiliated to *Planctomycetales* (12%). At the same time both absolute and proportional values of level-specific OTUs increased for all intertidal levels after depuration compared to non-depurated clams (high: 6% to 14%, middle: 6% to 12% and low: 2% to 9%). In that respect, the depuration emphasized clams DG microbiota differences observed between the three intertidal levels.

DISCUSSION

In the present study, we investigated the structuration of the DG microbiota of the Pacific oyster *C. gigas* and the Manilla clam *R. philippinarum,* in response to their location on the intertidal zone during a three-and-a-half-months period (October 2017 to February 2018). We found that location on the intertidal zone shaped DG microbiota of both bivalve species, in terms of taxonomical composition and structuration. The footprint of the intertidal position on bivalve DG microbiota persisted after depuration at the laboratory. The DG microbiota of oysters were unstable and fluctuated on a daily basis, while that of clams appeared to be more stable in the short-term.

DG microbiota specificity depends on its ecological niche

OTUs present in the DG microbiota of oysters were mainly associated to the orders *Mycoplasmatales, Rhodospirillales, Campylobacterales* and *Chlamydiales,* while those of clams were associated to *Mycoplasmatales, Chlamydiales, Rickettsiales, Spirochaetales* and *Oceanospirillales.* These taxa, including the predominance of *Mycoplasmatales,* are commonly described in oysters (King *et al.* 2012; Lokmer *et al.* 2016b) and clams (Milan *et al.* 2018) DG microbiota, as well as in the gut of other invertebrates (Tanaka *et al.* 2004; Meziti *et al.* 2010; Hollants *et al.* 2011; King *et al.* 2012; Cleary *et al.* 2015). Bacteria assigned to the *Spirochaetales* order have often been associated to the crystalline style of bivalves (Bernard 1970), whereas *Chlamydiales* and *Rickettsiales* are known as intracellular bacteria found in digestive cells of oysters and clams (Harshbarger and Chang 1977; Fryer and Lannan 1994). OTUs belonging to the orders *Mycoplasmatales* and *Rickettsiales*, and OTUs assigned to the family *Spirochaetaceae*, were recently identified as core members of the Manila clam

and Pacific oyster microbiota, respectively (Milan *et al.* 2018; King *et al.* 2020). Although large intra-species differences for relative abundances of different taxa have previously been described in bivalves, mostly associated to location, age and sampling period, microbiome host-specificity is widely accepted (Pierce and Ward 2018).

As in all filter feeding bivalves, DG of these two bivalve species are indirectly linked to their surrounding environment (seawater, sediment) through the gills that pump water into the pallial cavity to capture, process and transport food particles (Rosa *et al.* 2018). Nevertheless, DG microbiota of both bivalves were clearly different from the bacterial communities of sediments and seawater, confirming the existence of a gut-specific microbiota in clams (Meisterhans *et al.* 2015; Milan *et al.* 2018) and oysters (Lokmer *et al.* 2016b; Vezzulli *et al.* 2018; Dubé *et al.* 2019). The majority of microorganisms present in seawater were affiliated to the orders *Rhodobacterales*, *Campylobacterales*, and *Desulfobacterales* previously found in seawater from the Atlantic ocean (Celikkol-Aydin *et al.* 2016; Papadatou and Harder 2016). Bacterial communities from the two seawater fractions presented dissimilarities that could be linked to the presence of free-living bacteria in one fraction (0.22-8 µm fraction) and particle associated bacteria in the second one (> 8 µm) as previously described by Milici *et al.* (2017). This later fraction (> 8 µm) showed a high similarity with sediment bacterial communities, suggesting a sinking capability of the bigger particles which may be also found on the seabed. Bacterial communities from environmental samples, including sediments and water; and more specifically the 0.22-8 µm seawater fraction, were closest from oyster DG microbiota than clams. This is probably the result of the differences in feeding behavior between oysters and clams, an important factor that can contribute to specific microbial differences across bivalve species (Murphy *et al.* 2019). Oysters filter particulate matter from pelagic zone, while clams use two siphons to ingest deposition (Rosa *et al.* 2018). Despite the absence of a clear trend in the composition of major taxa, several OTUs belonging to the major orders observed in oysters such as *Mycoplasmatales (*genus *Mycoplasma)*, *Campylobacterales* (genus *Arcobacter*), and *Planctomycetales* (genera *Blastopirellula* and *Singulisphaera*) were also present in the water fraction (0.22-8 µm fraction). This proximity suggests that oysters may have preferentially fed on small particles during the sampling period, as previously described by Wisely and Reid (1978) where they identified an optimal particle ingestion size (< 5µm) in the oyster *Saccostrea glomerata.* The daily changes in seawater bacterial community (0.22-8µm fraction) were previously observed and expected here (Yung *et al.* 2016). Similarities between the oysters DG and seawater (0.22-8µm fraction) bacterial communities, suggest a direct relationship between environmental changes (mostly seawater) and oysters DG microbiota. Lokmer *et al.* (2016a)

previously showed the impact of short-term environmental fluctuations on oysters hemolymph microbiota. This study emphasizes this point showing the same tendency for the DG, an external tissue, which is more likely to reflect short-term environmental fluctuations.

Clams DG microbiota and their surrounding environment exhibited low similarities, especially for sediments, which were closer to oysters DG microbiota. OTUs present in the sediments belonged to the orders *Desulfobacterales (Desulfosarcina, Desulfobulbus, Desulfococcus, Desulforhapalus, Desulfovibrio)* and *Campylobacterales (Sulfurovum, Sulfurimonas, Arcobacter*) that are common sulphure cycle-associated bacteria present in marine sediments (de Wit 2008; Colin *et al.* 2013). *Acidimicrobiales* are generally observed in marine sediments with low salinity (Wu *et al.* 2009), and *Fusobacteriales* (*Psychrilyobacter* & *Propionigenium*) are involved in denitrification processes (Otte *et al.* 2019). Oysters possess a higher filtration rate (3.92 µg carbon consumed L^{-1} g^{-1}) than clams (3.03 µg carbon consumed L^{-1} g^{-1}) with a lower trophic efficiency (18.38% for oysters and 23.69% for clams) (Tenore, Goldman and Clarner 1973). This suggests that oysters ingested more bacteria from the environment than clams, and that the transit of these microorganisms through the digestive gland was therefore more important.

Spatial trends for non-depurated DG microbiota

The 3.5 months spent on the intertidal zone led to significant and differential changes in the DG microbiota of both bivalves, confirming that they were highly influenced by site of implantation, as already observed in oysters (Clerissi *et al.* 2018). However, in this study, microbiota of oysters DG seemed to be highly sensitive to small-scale environmental fluctuations, whereas that of clams was more stable at the same scale of observation. Implantation on the intertidal zone led to an increase of inter-individual heterogeneity of DG microbiota in non-depurated oysters but not in clams. This may be explained either by a different environmental impact on each individual due to genetics (Wegner *et al.* 2013; Clerissi *et al.* 2018) and/or the presence of micro-environmental heterogeneity (Lokmer *et al.* 2016a). The different intertidal positions, localized within a small area, impacted the relative abundance of major OTUs of DG microbiota, with a predominance of OTUs related to *Rhodospirillales* and *Legionellales* orders for oysters and clams, respectively, placed at the low level on the intertidal zone. Previous studies have shown that the oyster microbiota are influenced by large and small spatial location (< 1m), engendering heterogeneity in microbial composition (Wegner *et al.* 2013; Lokmer *et al.* 2016a; King *et al.* 2019a).

Environmental footprint on depurated DG microbiota

In order to evaluate how deeply the implantation in different intertidal positions influenced their DG microbiota, bivalves were placed in depuration for 14 days without feeding. Mostly, depuration is used to remove environmental contaminants such as microplastics (Paul-Pont *et al.* 2016), heavy-metal (Freitas *et al.* 2012) or human pathogens (El-Shenawy 2004), during a short period (few hours) (Vezzulli *et al.* 2018). In the present study, it was used to empty the DG and to reduce transient environmental microorganisms (Romero *et al.* 2002; Lee *et al*. 2008).

The α-diversity of the DG microbiota was reduced in clams and oysters following depuration, and a restructuring of OTU abundances was observed, as it was highlighted in oysters and mussels tissues (Lokmer *et al.* 2016a; Vezzulli *et al.* 2018). These modifications were probably related to the new environmental niches made available for other bacteria following depuration. Depuration also induced a drastic OTU reduction, which mostly affected OTUs common to all the three levels, leading to a strong increase in level-specific OTUs for clams, and inter-individual variability for both species. In oysters, the intertidal position effect observed after depuration was in interaction with the sampling day, supporting the hypothesis that oysters DG microbiota were susceptible to seawater variations on a daily basis.

For clams, persistence of intertidal position effect on depurated animals could be related to bacteria closely associated to the clams DG. This environmental footprint coupled with the increase of level-specific OTUs (belonging to *Rhodobacterales*, *Planctomycetales* and *Flavobacteriales*), suggest the existence of autochthonous bacteria in clams DG microbiota. The notion of autochthonous and allochthonous microorganisms has already been evocated for the hemolymph microbiota of Pacific oysters, where seawater-associated OTUs are transient within the microbial community (Lokmer and Wegner 2014). Based on similarities comparisons between depurated or non-depurated clams placed on the three intertidal levels, autochthonous bacteria of the DG represented around 20% of bacteria present in the microbiota. Although it has not been possible yet to make a clear distinction between non-native and indigenous microorganisms in bivalves (King *et al.* 2012), microbiota associated with clams internal organs, may have been made up of indigenous populations despite the strong environmental influence (Meisterhans *et al.* 2015).

CONCLUSION

The present study confirmed that the DG bacterial microbiota of the Pacific oyster and the Manilla clam clearly differed from their surrounding environments. It highlighted that small differences in the spatial distribution of oysters and clams, along the intertidal zone, induced significant changes in their DG bacterial microbiota after three-and-a-half-month of winter implantation. We currently do not know whether these changes, which persisted after 14 days of depuration, were likely to affect the nutrient absorption capacity or other physiological traits of the two bivalves. While the DG microbiota of oysters were unstable and fluctuated on a daily basis, that of clams seemed to be more stable in the short-term, suggesting a better ability to regulate its DG microbiota. The depuration process revealed the presence of 20% identical OTUs shared among the three intertidal levels in clams. The exact roles of these resident bacteria on clams physiology are currently unknown, but they may play a key role by maintaining specific metabolic functions within the DG bacterial microbiota that was otherwise subjected to a wide influence of transient bacteria.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

AUTHOR CONTRIBUTIONS

Experiments, microbiota analyses, results interpretation, writing-original draft preparation, C.O.; microbiota analyses, results interpretation, S.P.; Experiment preparations, K.C.; Expertise for statistical analyses, O.G.; Project and experimental design, oyster and clam dissections, A.B., P.M., B.P., C.C., F.P., C.F., and C.P.; Project supervision and experimental design, field monitoring, oyster and clam dissections, results interpretation, writing-review and funding acquisition, G.L.B.

FUNDINGS

This work was supported by ISblue project, Interdisciplinary graduate school for the blue planet (ANR-17-EURE-0015) and co-funded by a grant from the French government under the program "Investissements d'Avenir". The Region Bretagne SAD (2017, "Stratégie d'Attractivité Durable") contributed to this study through postdoctoral fellowship of Clément Offret. This work was also supported by the HORIZON2020 project “Preventing and mitigating farmed bivalve disease—VIVALDI (grant number 678589)”.

ACKNOWLEDGMENTS

The authors wish to thank Elodie Fleury, Christophe Lambert, Nelly Le Goic, Morgan Perennou, Christine Dubreuil, Valérian Le Roy, Claudie Quere, Amélie Segarra, Alexandra Rahmani, Elyne Dugény, Morgan Smith, Lizenn Delisle, Véronique Loiseau and Amandine Morot for their help with sampling preparation and dissections of bivalves, and Dominique Hervio for her help with water filtration.

Conflict of Interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

Baker DM, Freeman CJ, Wong JCY *et al.* Climate change promotes parasitism in a coral symbiosis. *ISME J* 2018;**12**:921–30.

Barnes RD, Ruppert EE. *Invertebrate Zoology*. Fort Worth [Tex.: Saunders College Pub., 1996.

Bernard FR. Occurrence of the spirochaete genus Cristispira in western Canadian marine bivalves. *Veliger* 1970;**13**:33–36.

Bokulich NA, Subramanian S, Faith JJ *et al.* Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods* 2013;**10**:57–9.

Celikkol-Aydin S, Gaylarde CC, Lee T *et al.* 16S rRNA gene profiling of planktonic and biofilm microbial populations in the Gulf of Guinea using Illumina NGS. *Marine Environmental Research* 2016;**122**:105–12.

Cleary DFR, Becking LE, Polónia ARM *et al.* Composition and predicted functional ecology of mussel-associated bacteria in Indonesian marine lakes. *Antonie Van Leeuwenhoek* 2015;**107**:821–34.

Clerissi C, de Lorgeril J, Petton B *et al.* Diversity and stability of microbiota are key factors associated to healthy and diseased Crassostrea gigas oysters. *bioRxiv* 2018:378125.

Colin Y, Goñi-Urriza M, Caumette P *et al.* Combination of high throughput cultivation and dsrA sequencing for assessment of sulfate-reducing bacteria diversity in sediments. *FEMS Microbiol Ecol* 2013;**83**:26–37.

Connell JH. Community interactions on marine rocky intertidal shores. *Annual review of ecology and systematics* 1972;**3**:169–192.

Desriac F, Le Chevalier P, Brillet B *et al.* Exploring the hologenome concept in marine bivalvia: haemolymph microbiota as a pertinent source of probiotics for aquaculture. *FEMS Microbiology Letters* 2014;**350**:107–16.

Dhanasiri AKS, Brunvold L, Brinchmann MF *et al.* Changes in the Intestinal Microbiota of Wild Atlantic cod Gadus morhua L. Upon Captive Rearing. *Microb Ecol* 2011;**61**:20–30.

Dubé CE, Ky C-L, Planes S. Microbiome of the Black-Lipped Pearl Oyster Pinctada margaritifera, a Multi-Tissue Description With Functional Profiling. *Front Microbiol* 2019;**10**, DOI: 10.3389/fmicb.2019.01548.

El-Shenawy NS. Heavy-metal and microbial depuration of the clam Ruditapes decussatus and its effect on bivalve behavior and physiology. *Environmental Toxicology* 2004;**19**:143–53.

Escudié F, Auer L, Bernard M *et al.* FROGS: Find, Rapidly, OTUs with Galaxy Solution. *Bioinformatics* 2018;**34**:1287–94.

Fernández-Reiriz MJ, Irisarri J, Labarta U. Flexibility of Physiological Traits Underlying Inter-Individual Growth Differences in Intertidal and Subtidal Mussels Mytilusgalloprovincialis. *PLOS ONE* 2016;**11**:e0148245.

Freitas R, Ramos Pinto L, Sampaio M *et al.* Effects of depuration on the element concentration in bivalves: Comparison between sympatric Ruditapes decussatus and Ruditapes philippinarum. *Estuarine, Coastal and Shelf Science* 2012;**110**:43–53.

Fryer JL, Lannan CN. Rickettsial and chlamydial infections of freshwater and marine fishes, bivalves, and crustaceans. *Zoological studies* 1994;**33**:95–107.

Green TJ, Barnes AC. Bacterial diversity of the digestive gland of Sydney rock oysters, Saccostrea glomerata infected with the paramyxean parasite, Marteilia sydneyi. *Journal of Applied Microbiology* 2010;**109**:613–22.

Green TJ, Siboni N, King WL *et al.* Simulated marine heat wave alters abundance and structure of Vibrio populations associated with the Pacific Oyster resulting in a mass mortality event. *Microbial ecology* 2019;**77**:736–747.

Hacquard S, Schadt CW. Towards a holistic understanding of the beneficial interactions across the Populus microbiome. *New Phytologist* 2015;**205**:1424–1430.

Harley CDG, Hughes AR, Hultgren KM *et al.* The impacts of climate change in coastal marine systems. *Ecology Letters* 2006;**9**:228–41.

Harris JM. The presence, nature, and role of gut microflora in aquatic invertebrates: A synthesis. *Microb Ecol* 1993;**25**:195–231.

Harshbarger JC, Chang SC. Chlamydiae (with phages), mycoplasmas, and richettsiae in Chesapeake Bay bivalves. *Science* 1977;**196**:666–668.

Helmuth BST, Hofmann GE. Microhabitats, Thermal Heterogeneity, and Patterns of Physiological Stress in the Rocky Intertidal Zone. *The Biological Bulletin* 2001;**201**:374–84.

Herlemann DP, Labrenz M, Jürgens K *et al.* Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J* 2011;**5**:1571–9.

572 Hollants J, Leroux O, Leliaert F *et al.* Who Is in There? Exploration of Endophytic Bacteria within
573 the Siphonous Green Seaweed Bryopsis (Bryopsidales, Chlorophyta). *PLOS ONE* 2011;**6**:e26458.

574 King GM, Judd C, Kuske CR *et al.* Analysis of Stomach and Gut Microbiomes of the Eastern Oyster
575 (Crassostrea virginica) from Coastal Louisiana, USA. *PLoS ONE* 2012;**7**:e51475.

576 King WL, Jenkins C, Go J *et al.* Characterisation of the Pacific Oyster Microbiome During a Summer
577 Mortality Event. *Microb Ecol* 2019a;**77**:502–12.

578 King WL, Jenkins C, Seymour JR *et al.* Oyster disease in a changing environment: decrypting the
579 link between pathogen, microbiome and environment. *Marine environmental research*
580 2019b;**143**:124–140.

581 King WL, Siboni N, Kahlke T *et al.* Regional and oyster microenvironmental scale heterogeneity in
582 the Pacific oyster bacterial community. *FEMS Microbiol Ecol* 2020;**96**, DOI:
583 10.1093/femsec/fiaa054.

584 King WL, Siboni N, Williams NLR *et al.* Variability in the Composition of Pacific Oyster
585 Microbiomes Across Oyster Families Exhibiting Different Levels of Susceptibility to OsHV-1 µvar
586 Disease. *Front Microbiol* 2019c;**10**, DOI: 10.3389/fmicb.2019.00473.

587 Kueh CSW, Chan K. Bacteria in bivalve shellfish with special reference to the oyster. *Journal of*
588 *Applied Bacteriology* 1985;**59**:41–7.

589 Kvennefors ECE, Sampayo E, Ridgway T *et al.* Bacterial Communities of Two Ubiquitous Great
590 Barrier Reef Corals Reveals Both Site- and Species-Specificity of Common Bacterial Associates.
591 *PLOS ONE* 2010;**5**:e10401.

592 Lee R, Lovatelli A, Ababouch L. *Bivalve Depuration: Fundamental and Practical Aspects*. Food and
593 Agriculture Organization of the United Nations, 2008.

594 Legendre P. Interpreting the replacement and richness difference components of beta diversity.
595 *Global Ecology and Biogeography* 2014;**23**:1324–34.

596 Legendre P, Gallagher ED. Ecologically meaningful transformations for ordination of species data.
597 *Oecologia* 2001;**129**:271–80.

598 Lessin G, Bruggeman J, McNeill CL *et al.* Time Scales of Benthic Macrofaunal Response to Pelagic
599 Production Differ Between Major Feeding Groups. *Front Mar Sci* 2019;**6**, DOI:
600 10.3389/fmars.2019.00015.

601 Lokmer A, Goedknegt MA, Thieltges DW *et al.* Spatial and Temporal Dynamics of Pacific Oyster
602 Hemolymph Microbiota across Multiple Scales. *Front Microbiol* 2016a;**7**, DOI:
603 10.3389/fmicb.2016.01367.

604 Lokmer A, Kuenzel S, Baines JF *et al.* The role of tissue-specific microbiota in initial establishment
605 success of Pacific oysters. *Environ Microbiol* 2016b;**18**:970–87.

606 Lokmer A, Wegner KM. Hemolymph microbiome of Pacific oysters in response to temperature,
607 temperature stress and infection. *ISME J* 2014;**9**:670–82.

Magoč T, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 2011;**27**:2957–63.

Mahé F, Rognes T, Quince C *et al.* Swarm: robust and fast clustering method for amplicon-based studies. *PeerJ* 2014;**2**:e593.

Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet journal* 2011;**17**:10–12.

McArdle BH, Anderson MJ. Fitting Multivariate Models to Community Data: A Comment on Distance-Based Redundancy Analysis. *Ecology* 2001;**82**:290–7.

McFall-Ngai M, Hadfield MG, Bosch TCG *et al.* Animals in a bacterial world, a new imperative for the life sciences. *Proc Natl Acad Sci USA* 2013;**110**:3229–36.

McFall-Ngai MJ. Unseen forces: the influence of bacteria on animal development. *Developmental biology* 2002;**242**:1–14.

Meisterhans G, Raymond N, Girault E *et al.* Structure of Manila Clam (Ruditapes philippinarum) Microbiota at the Organ Scale in Contrasting Sets of Individuals. *Microb Ecol* 2015:1–13.

Menzel W. *Estuarine and Marine Bivalve Mollusk Culture*. CRC Press, 2018.

Meziti A, Ramette A, Mente E *et al.* Temporal shifts of the Norway lobster (Nephrops norvegicus) gut bacterial communities. *FEMS Microbiol Ecol* 2010;**74**:472–84.

Milan M, Carraro L, Fariselli P *et al.* Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. *Aquatic Toxicology* 2018;**194**:195–207.

Milici M, Vital M, Tomasch J *et al.* Diversity and community composition of particle-associated and free-living bacteria in mesopelagic and bathypelagic Southern Ocean water masses: Evidence of dispersal limitation in the Bransfield Strait. *Limnology and Oceanography* 2017;**62**:1080–95.

Murphy AE, Kolkmeyer R, Song B *et al.* Bioreactivity and Microbiome of Biodeposits from Filter-Feeding Bivalves. *Microb Ecol* 2019;**77**:343–57.

Offret C, Jégou C, Mounier J *et al.* New insights into the haemo- and coelo-microbiota with antimicrobial activities from Echinodermata and Mollusca. *Journal of Applied Microbiology* 2019, DOI: 10.1111/jam.14184.

Offret C, Rochard V, Laguerre H *et al.* Protective Efficacy of a Pseudoalteromonas Strain in European Abalone, Haliotis tuberculata, Infected with Vibrio harveyi ORM4. *Probiotics Antimicrob Proteins* 2018, DOI: 10.1007/s12602-018-9389-8.

Oksanen J, Blanchet FG, Friendly M *et al. Vegan: Community Ecology Package*., 2019.

Otte JM, Blackwell N, Ruser R *et al.* N2O formation by nitrite-induced (chemo)denitrification in coastal marine sediment. *Sci Rep* 2019;**9**, DOI: 10.1038/s41598-019-47172-x.

Papadatou M, Harder J. Bacterial diversity associated with anthropogenic particles and neuston in the

surface layer of the East Atlantic Ocean. 2016.

Paul-Pont I, Lacroix C, González Fernández C *et al.* Exposure of marine mussels Mytilus spp. to polystyrene microplastics: Toxicity and influence on fluoranthene bioaccumulation. *Environmental Pollution* 2016;**216**:724–37.

Petton B, Boudry P, Alunno-Bruscia M *et al.* Factors influencing disease-induced mortality of Pacific oysters Crassostrea gigas. *Aquaculture Environment Interactions* 2015;**6**:205–22.

Pierce ML, Ward JE. Microbial Ecology of the Bivalvia, with an Emphasis on the Family Ostreidae. *shre* 2018;**37**:793–806.

Rognes T, Flouri T, Nichols B *et al.* VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016;**4**:e2584.

Romalde JL, Diéguez AL, Doce A *et al.* Advances in the knowledge of the microbiota associated with clams from natural beds. *Clam Fisheries and Aquaculture* 2013:163–190.

Romero J, García-Varela M, Laclette JP *et al.* Bacterial 16S rRNA Gene Analysis Revealed That Bacteria Related to Arcobacter spp. Constitute an Abundant and Common Component of the Oyster Microbiota (Tiostrea chilensis). *Microb Ecol* 2002;**44**:365–71.

Rosa M, Ward JE, Shumway SE. Selective Capture and Ingestion of Particles by Suspension-Feeding Bivalve Molluscs: A Review. *shre* 2018;**37**:727–46.

Rőszer T. The invertebrate midintestinal gland ("hepatopancreas") is an evolutionary forerunner in the integration of immunity and metabolism. *Cell Tissue Res* 2014;**358**:685–95.

Shapira M. Adaptation from Within or from Without: A Reply to Rodrigo et al. *Trends in Ecology & Evolution* 2017;**32**:85.

Simon C, Truong H, Noble T *et al.* Microbial biomass, marine invertebrate meals and feed restriction influence the biological and gut microbiota response of shrimp Penaeus monodon. *Aquaculture* 2019:734679.

Simons AL, Churches N, Nuzhdin S. High turnover of faecal microbiome from algal feedstock experimental manipulations in the Pacific oyster (*Crassostrea gigas*). *Microbial Biotechnology* 2018;**11**:848–58.

Sogin ML, Morrison HG, Huber JA *et al.* Microbial diversity in the deep sea and the underexplored "rare biosphere." *PNAS* 2006;**103**:12115–20.

Soudant P, Paillard C, Choquet G *et al.* Impact of season and rearing site on the physiological and immunological parameters of the Manila clam Venerupis (=Tapes, =Ruditapes) philippinarum. *Aquaculture* 2004;**229**:401–18.

Tanaka R, Ootsubo M, Sawabe T *et al.* Biodiversity and in situ abundance of gut microflora of abalone (Haliotis discus hannai) determined by culture-independent techniques. *Aquaculture* 2004;**241**:453–63.

Team RC. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2012. *URL http://www R-project org* 2018.

Tenore KR, Goldman JC, Clarner JP. The food chain dynamics of the oyster, clam, and mussel in an aquaculture food chain. *Journal of Experimental Marine Biology and Ecology* 1973;**12**:157–65.

Torda G, Donelson JM, Aranda M *et al.* Rapid adaptive responses to climate change in corals. *Nature Climate Change* 2017;**7**:627–36.

Trabal Fernández N, Mazón-Suástegui JM, Vázquez-Juárez R *et al.* Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis , Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiology Ecology* 2014;**88**:69–83.

Trabal N, Mazón-Suástegui JM, Vázquez-Juárez R *et al.* Molecular analysis of bacterial microbiota associated with oysters (Crassostrea gigas and Crassostrea corteziensis) in different growth phases at two cultivation sites. *Microb Ecol* 2012;**64**:555–69.

Vezzulli L, Stagnaro L, Grande C *et al.* Comparative 16SrDNA gene-based microbiota profiles of the Pacific oyster (Crassostrea gigas) and the Mediterranean mussel (Mytilus galloprovincialis) from a shellfish farm (Ligurian Sea, Italy). *Microbial ecology* 2018;**75**:495–504.

Wegner KM, Volkenborn N, Peter H *et al.* Disturbance induced decoupling between host genetics and composition of the associated microbiome. *BMC Microbiology* 2013;**13**:252.

Wisely B, Reid BL. Experimental feeding of Sydney rock oysters (Crassostrea commercialis = Saccostrea cucullata): I. Optimum particle sizes and concentrations. *Aquaculture* 1978;**15**:319–31.

de Wit R. Microbial diversity in the Bassin d'Arcachon coastal lagoon (SW France). *Hydrobiologia* 2008;**611**:5–15.

Wu J, Guan T, Jiang H *et al.* Diversity of Actinobacterial community in saline sediments from Yunnan and Xinjiang, China. *Extremophiles* 2009;**13**:623–32.

Yin X, Chen P, Chen H *et al.* Physiological performance of the intertidal Manila clam (*Ruditapes philippinarum*) to long-term daily rhythms of air exposure. *Scientific Reports* 2017;**7**:41648.

Yung C-M, Ward CS, Davis KM *et al.* Insensitivity of Diverse and Temporally Variable Particle-Associated Microbial Communities to Bulk Seawater Environmental Parameters. Schloss PD (ed.). *Appl Environ Microbiol* 2016;**82**:3431.

Figure 1. Representation of experimental design detailing the deployment of bivalves on the three intertidal levels (low, middle and high, corresponding to 20%, 56% and 80% of exondation time, respectively) and the sampling of digestive glands and environmental samples.

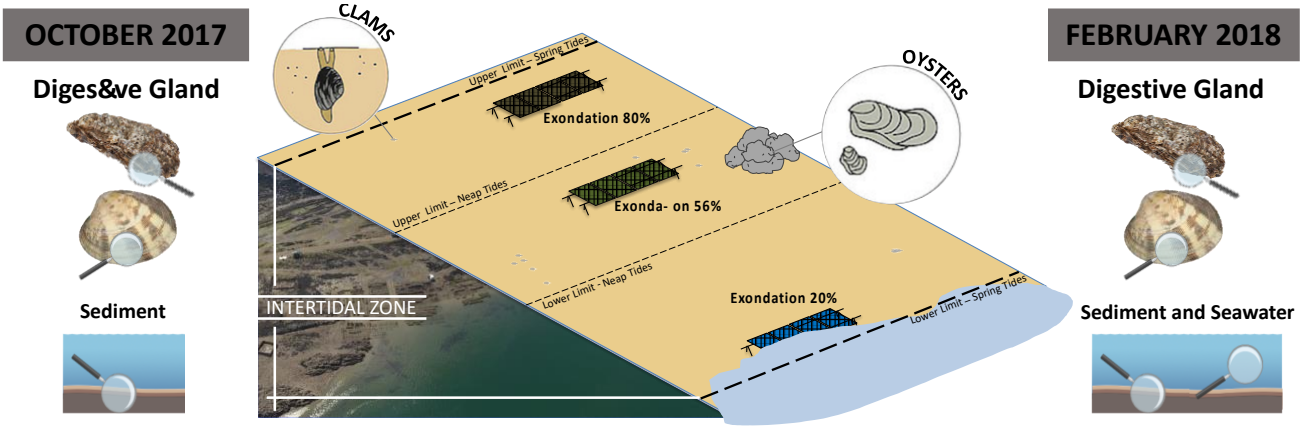
Figure 2. Principal component analysis of the Hellinger-transformed OTUs abundances for the bacterial community of sediment (yellow circles), 8µm seawater fraction (blue squares), 0.22-8µm seawater fraction (blue triangles), and DG of *C. gigas* (red circles) and *R. philippinarum* (green circles) sampled in October and February. Ellipses represent standard deviation (99%) of data.

Figure 3. Relative abundance of majoritarian OTUs (OTUs representing 0.5% of total sequences) summarized at the order taxonomic rank, found in sediments, seawater (SW), and DG of *C. gigas* (OYSTER) and *R. philippinarum* (CLAM) sampled in October (OCT) or February (FEB) from non-depurated (ND) or depurated (D) animals placed at different intertidal levels (H, high ; M, middle ; L, low).

Figure 4. Principal component analysis of the Hellinger-transformed OTUs abundances for non-depurated DG bacterial communities of *C. gigas* (A) and *R. philippinarum* (B) sampled in October (violet) and February (yellow). The first two axes of PCA explain 28.4% and 27.4% of total variation of bacterial communities for oyster and clam respectively. Ellipses represent standard deviation (99%) of data.

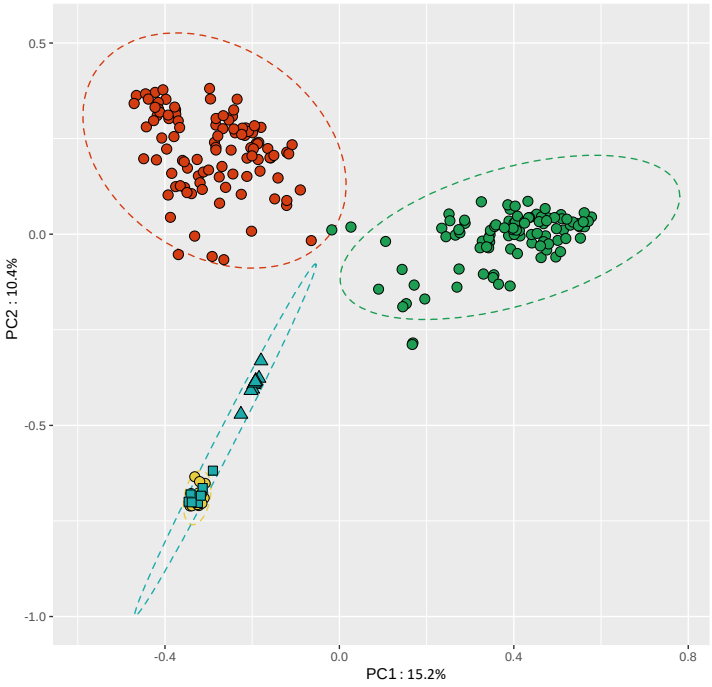
Figure 5. Triangular plots illustrating the variations of the Jaccard dissimilarity between OTU composition (presence/absence data) of non-depurated (**A**) and depurated (**B**) *R. philippinarum* sampled on the three intertidal levels (high, middle, low), and its decomposition into similarity, richness difference (variation in OTU richness) and OTU replacement (variation in OTU identity). Legend information are provided in the box. Contributions were calculated for each group of depurated or non-depurated animals separately, and for pairwise comparisons between each sample belonging to one level with all samples from a different level. Due to the high number of pairwise comparisons, the density of points was estimated by two-dimensional kernel estimations and was represented with dark blue for higher numbers of comparisons. Red lines indicate the centroid value for each graph with its associated mean values for the three components of dissimilarity.

Figure 6. Venn diagrams representing shared OTUs (based on presence/absence data) between DG bacterial communities of non-depurated (A) and depurated (B) *R. philippinarum* that had been placed on high (red), middle (yellow) or low (blue) intertidal level.


OCTOBER 2017
Diges&ve Gland
Sediment
CLAMS
Upper Limit – Spring Tides
Exondation 80%
Upper Limit – Neap Tides
Exonda- on 56%
Lower Limit - Neap Tides
INTERTIDAL ZONE
Exondation 20%
Lower Limit – Spring Tides
OYSTERS
FEBRUARY 2018
Digestive Gland
Sediment and Seawater

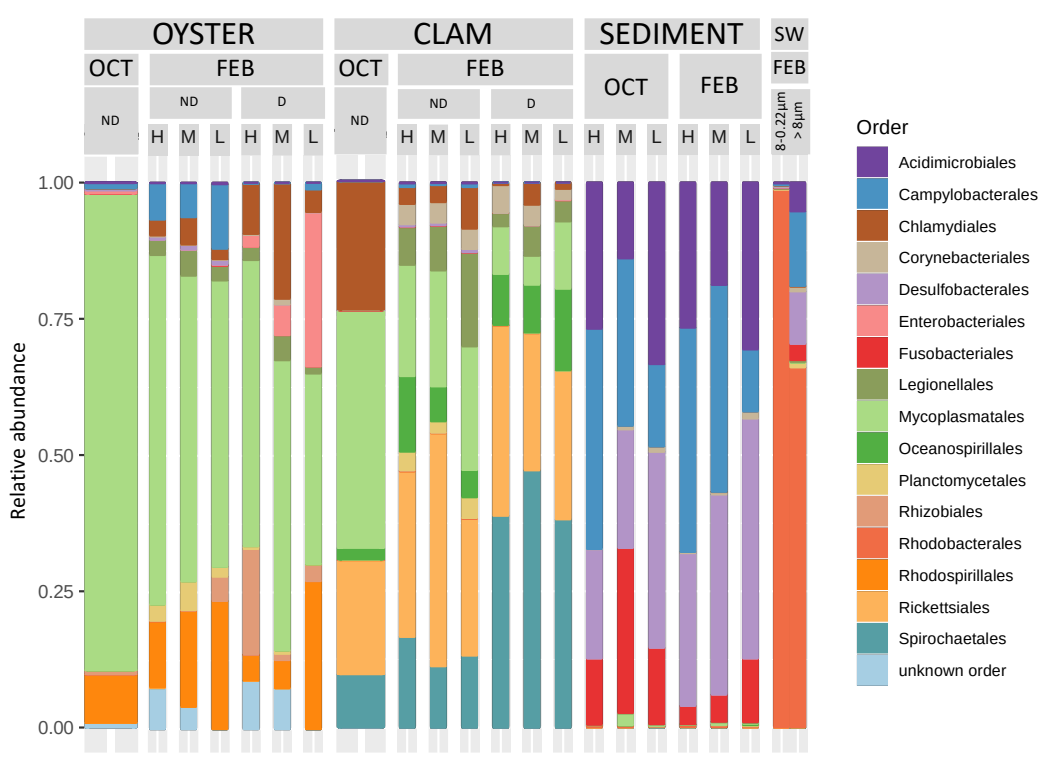
740

741



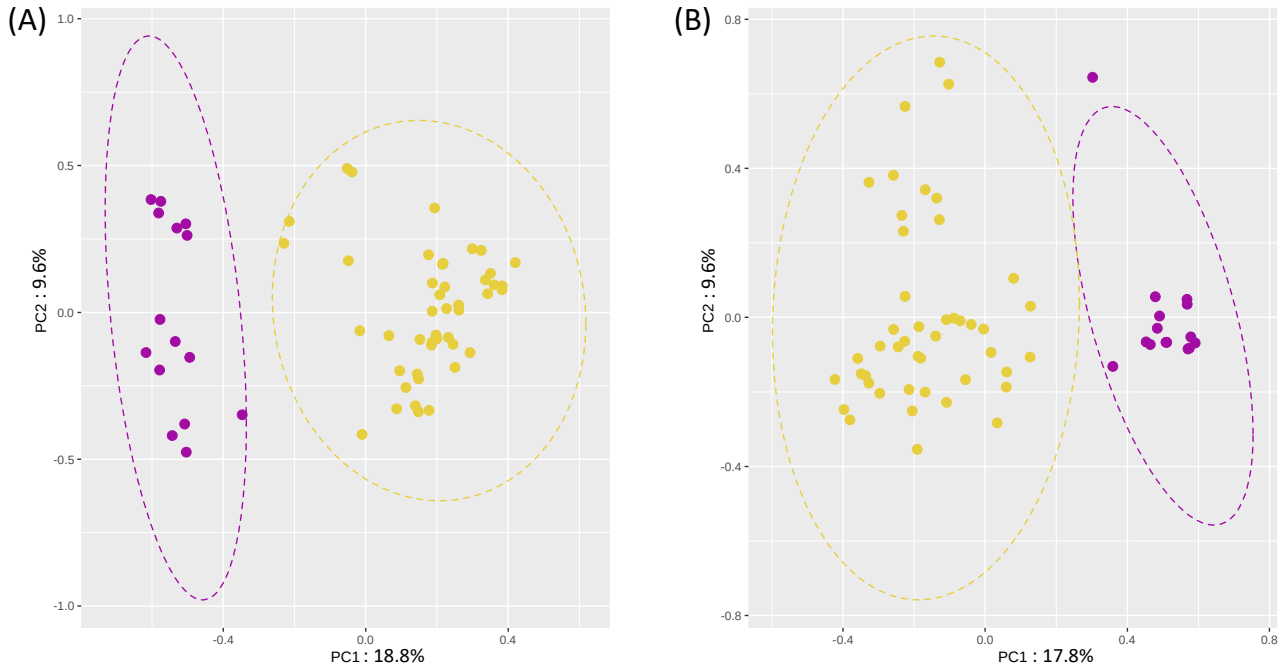
742

743

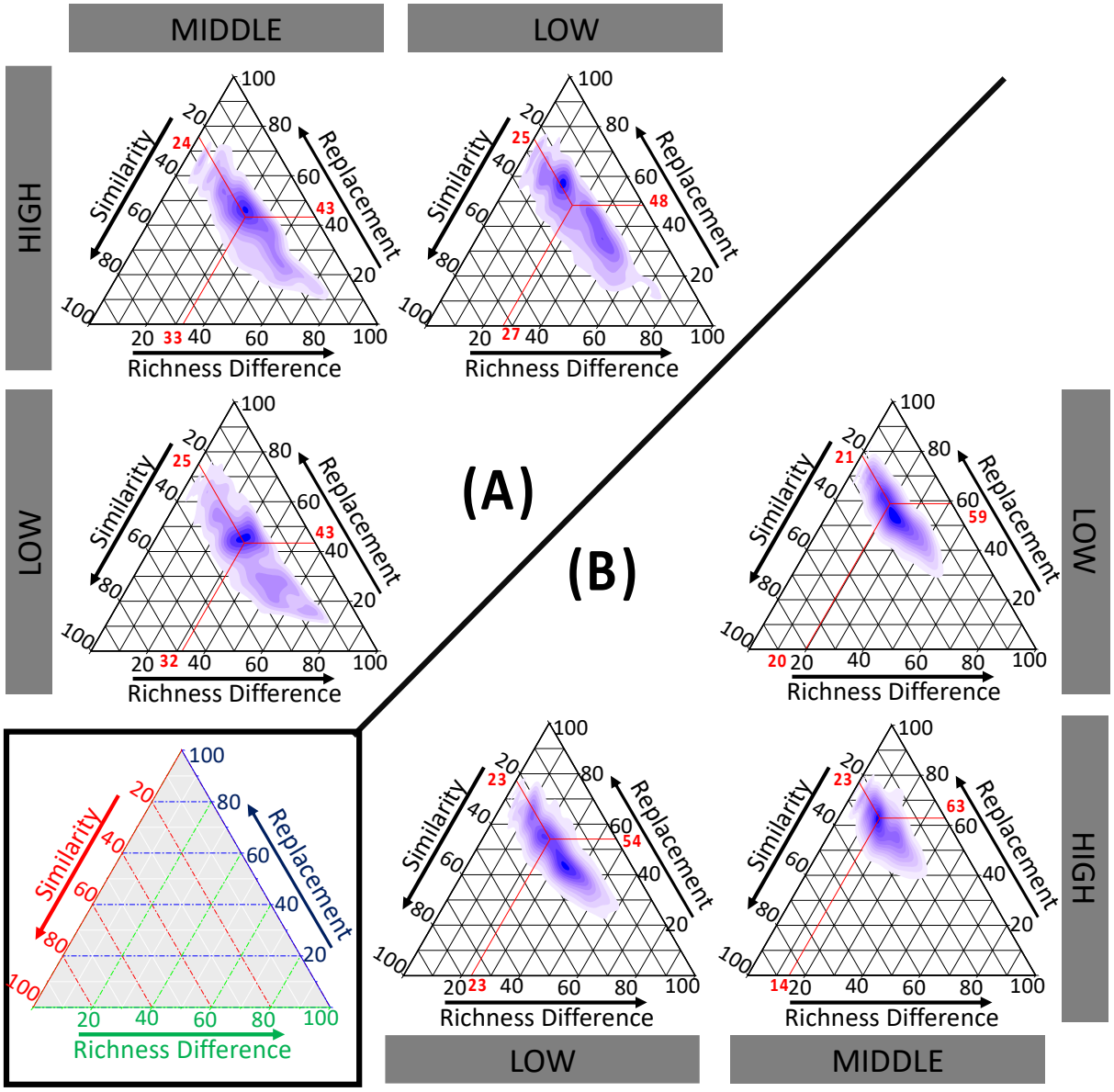

OYSTER
CLAM
SEDIMENT
SW
OCT
FEB
ND
D
H
M
L
8-0.22µm
>8µm
Relative abundance
1.00
0.75
0.50
0.25
0.00
Order
Acidimicrobiales
Campylobacterales
Chlamydiales
Corynebacteriales
Desulfobacterales
Enterobacteriales
Fusobacteriales
Legionellales
Mycoplasmatales
Oceanospirillales
Planctomycetales
Rhizobiales
Rhodobacterales
Rhodospirillales
Rickettsiales
Spirochaetales
unknown order

744

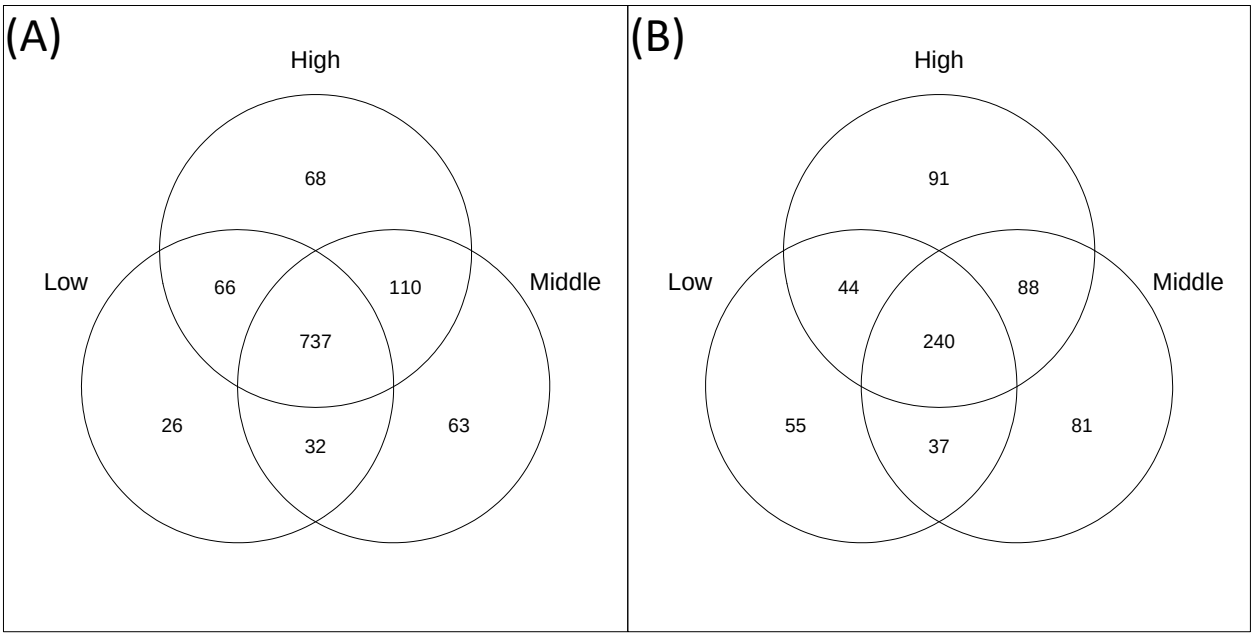
745


(A)
1.0
0.5
0.0
-0.5
-1.0
PC2 : 9.6%
-0.4
0.0
0.4
PC1 : 18.8%
(B)
0.8
0.4
0.0
-0.4
-0.8
PC2 : 9.6%
-0.4
0.0
0.4
0.8
PC1 : 17.8%

746


MIDDLE
LOW
HIGH
LOW
LOW
HIGH
LOW
MIDDLE
(A)
(B)
Similarity
Replacement
Richness Difference
24
43
33
25
48
27
25
43
32
21
59
20
23
54
23
23
63
14

747


(A)
High
68
Low
66
110
Middle
737
26
32
63
(B)
High
91
Low
44
88
Middle
240
55
37
81

748