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Cockle response to stress – Microbiota

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Contents

1. EXECUTIVE SUMMARY

Background

Restoring cockle (*Cerastoderma edule*) shellfisheries and its ecosystem services in the Atlantic Area requires a good knowledge of the drivers of cockle health and fitness. Cockle-associated microorganisms are one of them. Recent studies have demonstrated the beneficial role of the microbial communities associated with macro-species, namely the microbiota, providing their host with metabolic adds-on (e.g. detoxification or nutrient acquisition) or antimicrobial defenses. To our knowledge, the present study is the first assessment of the cockle microbiota.

Methodology

Adult cockles and sediment cores were collected in 2018 from most of the southern part of the species' distribution area: Burry Inlet (Wales), Baie de Somme and Bassin d'Arcachon (France), Ria de Noia and Ria de Arousa (Spain), Ria de Aveiro and Ria Formosa (Portugal). Cockles (n = 7 x 15) were dissected to separately characterize the microbiota of the intestinal tract, digestive glands and gills. After extraction, the sediment and cockle organ DNAs were analysed by next generation sequencing (MiSEQ Illumina) of the V3V4 region of the 16S rRNA gene to characterize bacterial community structures based on molecular taxonomic units (ASV) and their affiliated bacterial taxa.

Results

- There were significant differences in bacterial community structure between the cockle and sediment in all cockle beds, suggesting selection of certain taxa by the cockle host.
- There were significant differences in bacterial community structure between intestinal tract, digestive glands and gills. There were significant differences in bacterial community structure of the intestinal tract amongst cockle beds but not for that of gills and digestive glands. The organ-dependence of the microbiota structure is likely explained by organs forming distinct systems of microbiota assembly with regard to selection and migration processes.
- Sets of 27, 55 and 57 ASVs, with cumulative relative abundance ≥ 64% each, were respectively found in gills, digestive glands and intestinal tract of cockles from all the studied beds. These ASVs likely formed the core microbiota i.e. the stable and permanent members of the community of cockle associated bacteria.

- In gills, 4 out of the 6 top dominant ASV were affiliated to endosymbiotic taxa supporting the hypothesis that gills are preferred organs for endosymbiosis or infection.
- In gills, no ASVs affiliated with chemosynthetic sufur-oxidising ecto- or endosymbionts were abundant making such an hypothetical association in the cockle unlikely, in contrast to what has been reported for other marine invertebrates inhabiting sulfidic environments.
- In gills, the most abundant ASV (21% on average) was affiliated with *Endoizoicomonas*. This bacterial taxa was detected in all beds, with high prevalence (except in Ria Formosa) pointing to the probable importance of its association with *C. edule.*

Conclusions

This study demonstrated the occurrence of favored associations between selected bacteria and the cockle identifying putatively essential taxa whose contribution to the functioning, health and fitness of the *C. edule* holobiont has to be investigated.

2. INTRODUCTION

Current losses in marine biodiversity mainly occur in coastal ecosystems simultaneously affected by the cumulative effects of global changes and increasing anthropogenic stresses and disturbances due to coastalisation (Bindoff et al., 2019; IPBES, 2019). Overexploitation and non-optimal management practices worsen this situation for exploited species. Accordingly, the frequency and intensity of periodic mass mortalities increased in the last 50 years with severe impacts on the natural stocks of a valued bivalve species, the common cockle *Ceratoderma edule* (Burdon et al., 2014).

The distribution of this common species stretches from the Barents Sea to Mauritania along the northeastern coastline of the Atlantic Ocean (Malham et al., 2012; FAO, 2020; see also COCKLES Deliverable 4.2 report). In intertidal mud and sandflats of sheltered bay and estuaries, this burrowing bivalve can thrive in dense populations of more than 1 000 individuals per m² (i) contributing to sediment reworking (Li et al., 2017) and pollutant remobilization (Ciutat et al., 2006), food web (Malham et al., 2012) and (ii) providing European fisheries with up to 26 000 tons of halieutic resources in 2015, the most productive year of the last decade (FAO, 2019). Some of the most productive populations in Spain were affected by dramatic marteiliosis outbreaks with 100% mortality rate, in 2012 (Villalba et al., 2014). Other mortality episodes were related to disseminated neoplasia (Díaz et al., 2011). In addition more than 18 Digenean species, 5 Bacteria including *Vibrio tapetis*, and 19 Protists including *Perkinsus* form an extended list of *C. edule* parasites and pathogens (Longshaw and Malham, 2013) suggesting that cockles are facing biotic challenges in natural conditions.

As a larger number of studies focuses on organisms' microbiota, the hologenome concept has emerged defining the host and its associated microbiota (holobiont) as the actual evolutionary entity (Zilber-Rosenberg and Rosenberg, 2008). Whereas the negative effect of pathogens on their host physiology and fitness has long been documented, the relationship between the microbiota and its host is regarded as a long-term positive interaction (Dittami et al., 2019). Metabolic add-ons (e.g. detoxification or nutrient acquisition) and the contribution to host defense are the two major assets of the beneficial microbiota. For bivalves, sulfur oxidizing ecto- or endosymbiotic bacteria were described in gills of common benthic species (*Codakia orbicularis*, *Solemya velum*, *Loripes lucinalis*, *Thyasira gouldi*) providing their host with sulfide detoxification together with carbon and nitrogen supply (Eisen et al., 1992; Caro et al., 2009; Dmytrenko et al., 2014; König et al., 2016; Petersen et al., 2016). In oysters (*Crassostrea gigas*) only 2.2% of bacterial strains isolated from the tissues of healthy individuals exhibited antimicrobial properties however bacterial antimicrobial peptides were detected in the

hemolymph of the tested oysters (Defer et al., 2013). In clams (*Ruditapes philippinarum*), the fraction of isolated bacterial strains exhibiting antimicrobial properties shifted from 13% to 71% in supposedly stressed individuals collected in a formerly Hg-contaminated site (Leite et al., 2017).

In addition, competitive exclusion of new comers (i.e. pathogens) occurring in a diversified and saturated community (i.e. the microbiota) could be, for the host, a positive side – effect of a regular and balanced microbiota as previously hypothesized for fish skin microbiota (Chiarello et al., 2015) and as previously stated in the colonization resistance concept of the human microbiome (e.g. Britton and Young, 2012). This highlights the relevance of considering the microbiota at the community level.

Recent studies have provided insights into the dynamics of microbiota associated with marine bivalves in the host-interaction perspective and in response to biotic and abiotic factors. Short term changes of bacterial Operational Taxonomic Unit (OTU) composition of the haemolymph microbiota of Pacific oyster (*C. gigas*) were observed in response to a non-abrupt 14°C cooling and/or warming of warm- and/or cold-acclimated individual batches (Lokmer and Wegner, 2015). Such changes were related to an increase in the abundance of putative bacterial pathogens in microbiota of Pacific oyster exposed to simulated marine heat waves (Green et al., 2019). In the mussel *Mytilus galloprovincialis*, increased bacterial diversity of gut microbiota associated with a proliferation of opportunistic pathogen genus such as *Vibrio* and *Arcobacter* was induced by a 6°C warming (Li et al., 2019). Likewise, hypoxia enhanced bacterial abundance and diversity in the digestive gland of the eastern oyster *Crassostrea virginica* (Khan et al., 2018). Stressful conditions provided by exposure to TiO_2 nanoparticles and/or metal contamination respectively altered haemolymph microbiota of the Mediterranean mussel *M. galloprovincialis* (Auguste et al., 2019) and digestive gland microbiota of the Manila clam *R. philippinarum* (Milan et al., 2018). Parasite infestation by the paramyxean parasite, *Marteilia sydneyi*, reduced the diversity of the digestive gland microbiota of the Sydney rock oyster *Saccostrea glomerata* (Green and Barnes, 2010) whereas *Bucephalus minimus* infection induced enhanced bacterial abundances but no changes in bacterial community composition of whole flesh homogenate and intervalvular liquid in cockles (*C. edule*) (Meisterhans et al., 2011). Major shift in the haemolymph microbiota of Pacific oyster only occurred in moribund and dead individuals, not in survivors suggesting that the *Vibrio* challenge barely affected their microbiota diversity (Lokmer and Wegner, 2015).

Two points suggest that the microbiota is at least partially composed of microorganisms strongly associated to host, sometimes referred as resident microorganisms. Firstly, the difference between the host microbiota composition and that of the biotope microbial communities as observed in clam, oyster

or mussel when compared to their surrounding waters (Meisterhans et al., 2016; Vezzulli et al., 2018; Pierce and Ward, 2019). Secondly, the difference in the microbiota of individuals belonging to distinct species living in the same zone (i.e. specimens of sympatric species) as observed in oyster and mussel individuals co-cultivated (Vezzulli et al., 2018; Pierce and Ward, 2019). Furthermore, the importance of considering the microbiota tissue-dependence was respectively highlighted in Pacific oyster and Manila clam microbiota (Lokmer et al., 2016; Meisterhans et al., 2016). The different organs are not under the same pressure of lateral contamination since they are not equally exposed to the environment (i.e. the water or sediments where the organism lives). Therefore, the microbiota tissue-dependence should be regarded as a special case of the processes that shape the tissue or organ microbiota by means of lateral contamination from the pool of microorganisms present in the biotope.

Microbiology of oyster (Pierce and Ward, 2018) and mussel (Rubiolo et al., 2019) has been quite extensively studied. In contrast, information related to the microbiology of *C. edule* and especially its bacterial component is scarce with one review (Longshaw and Malham, 2013) primarily focusing on bacterial pathogens listing Mycoplasma-like microorganisms (Azevedo, 1993), *Vibrio tapetis* (the causing agent of brown disease occurring in clams) (Paillard, 2004), symbiotic bacteria in the epithelial cells of gills and digestive glands, with respectively Rickettsia- and Chlamydia-like bacteria (Carballal et al., 2001), an unclassified bacteria enclosed in branchial extracellular large cysts (Carballal et al., 2001), and the occurrence of food-borne pathEogens and faecal indicator bacteria (Martínez et al., 2009). As far as we know, the only study based on a microbial ecology approach to assess the dynamics of bacterial communities in *C. edule*, was the one questioning the link between bacteria, macroparasite occurrence and individual cockle fitness (Meisterhans et al., 2011). However this study was performed on whole flesh homogenates and intervalvular liquid, therefore not providing an accurate analysis of *C. edule* microbiota at the tissue-level.

The present study addresses the structure of the cockle microbiota, at the organ level, along 7 sites from 5 countries of the Atlantic Area. The aims were (i) to compare the cockle microbiota to the bacterial communities of the sediments where cockle live; (ii) to compare the microbiota of cockle gills, intestinal tract and digestive glands; (iii) to define and describe a core microbiota.

3. MATERIALS AND METHODS

3.1. Sample set

Cockles from seven *C. edule* cockle populations in the Atlantic Area were investigated (**Table 1** and **Appendix 9.1**). Samples i.e. cockles (at least 15 + 5 individuals) and associated sediments (3 cores) were provided by partners in the COCKLES project (see **Acknowledgements**). The total of 105 individuals provided a unique sampling set across this latitudinal range within the species range. According to genotypic data available when sampling was designed, all the sampled populations of cockle belong to the southern or central – southern genotypes (Krakau et al., 2012; Martinez et al., 2015). The Baie de Somme population was not investigated in those studies and therefore, it is unknown whether the cockles of this bed belongs to the southern, central or northern genotype. Recent outcomes from the COCKLES project, showed that Baie de Somme population is closer to northern groups (Vera et al., 2021). To characterize the sampled cockles, shell length measurement was the unique non-invasive measurement compatible with subsequent dissection for microbiota analyses. In each bed, the largest cockles were collected. On average, largest cockles (> 30 mm in shell length) were from Bassin d'Arcachon and Ria de Noia while smallest ones (< 26 mm in shell length) were from Ria de Arousa and Ria Formosa. Sampling date ranged from January to May. In most beds, cockles were sampled prior to major water warming in a pre-spawning stage. Indeed, no visible sign of gonadic maturation were noticed during dissection. However, cockles sampled from Galician beds, in May, showed rip gonads or even could have spawned. In Ria de Arousa, the prevalence of *M. cochillia* have ranged from 67% in November 2018 to 80% in February 2018 (A. Villalba, comm. pers). On average, the Bassin d'Arcachon cockles showed the lowest condition index (44.9 mg g^{-1}), the Baie de Somme ones showed the highest (78.8 mg g^{-1}) whereas both sets were sampled within the same 2-week timeframe in the mid-winter.

3.2. Sample treatment, preservation and storage to prevent cross contamination

Cockles and sediment cores shipped in cool boxes under wet atmosphere were treated immediately upon arrival at the laboratory i.e. 3 to 4 days after sampling because of shipment time. Only the samples from Bassin d'Arcachon were processed 24h after collection. Before dissection, cockles were flushed thoroughly with sterilized seawater in order to remove sediment particles and transient microorganisms. All tissues were removed aseptically with sterilised scissor, scalpel and dissecting forceps. Gills were washed three times in sterilized seawater. Intestinal tract and digestive glands dissection were performed under a stereo-microscope. Dissected organs were put in sterile CTAB

preservative buffer and ground using a tissue homogenizer (OMNI TH, Omni International, USA). Ground samples were stored deeply frozen at -78°C until their analyses.

Table 1. Cockle beds analysed for microbiota of *Cerastoderma edule* across the Atlantic Area ecosystems, arranged in decreasing latitudinal coordinates. Shell length (mean ± standard deviation in mm) of the 15 individuals whom microbiota is analysed. Condition index of 5 spare individuals collected in the same bed on the same sampling date (dry flesh mass / dry shell mass, in mg g^{-1}, according to Walne and Mann, 1975).

Country	Ecosystem	Sampling date	Coordinates	Shell length	Condition index	Genotypes
Wales	Burry Inlet (Bur)	30/03/2018	51°40'N 4°12'W	26.3 ± 1.1	57.7 ± 3.1	KS, MSC
France	Baie de Somme (Som)	15/02/2018	50°14'N 1°33'W	27.8 ± 1.3	78.8 ± 7.1	KS, n.a.
France	Bassin d'Arcachon (Arc)	30/01/2018	44°39'N 1°08'W	31.6 ± 1.2	44.9 ± 3.2	KS, MSS
Spain	Ria de Noia (Noi)	14/05/2018	42°47'N 8°55'W	30.2 ± 1.6	60.7 ± 3.5	KS, MSS
Spain	Ria de Arousa (Aro)	14/05/2018	42°30'N 8°49'W	25.3 ± 1.7	n.a.	KS, MSS
Portugal	Ria de Aveiro (Ave)	19/02/2018	40°38'N 8°44'W	26.8 ± 1.6	54.8 ± 6.5	KS, MSS
Portugal	Ria Formosa (For)	19/02/2018	37°01'N 7°48'W	25.9 ± 2.4	66.2 ± 7.8	KS, MSS

n.a. = not available

KS: southern genotype according to Krakau et al., 2012

MSC: southern mitochondrial and central nuclear genotype according to Martinez et al., 2015

MSS: southern mitochondrial and nuclear genotypes according to Martinez et al., 2015

3.3. Metabarcoding analysis of the cockle microbiota at the organ - level

DNA from 25 to 30 mg of cockle tissues and 200 mg of sediments was respectively extracted using the QIAgen QIAamp DNA Mini Kit and the DNeasy PowerSoil Kit (QIAGEN, Courtaboeuf, France). Before extraction, an additional bead-beating lysing step was performed on cockle tissue and sediment using Lysing Matrix A or Lysing Matrix E (MP Biomedicals, Illkirch-Graffenstaden, France; 6 m s^{-1}, 40 s), respectively.

A total of 378 samples were extracted: 315 corresponding to the *C. edule* tissues, 42 to the sediments and 21 negative controls to assess for DNA extraction kits and lab contaminations. Additional controls were performed to assess for the PCR and sequencing steps, including amplifications (n = 2) of the ZymoBIOMICS commercial microbial mock community DNA standard (Zymo research, Irvine, CA, USA), and 8 negative control for the subsequent PCRs.

DNA concentration and quality were checked with a QUANT iT spectrofluorometer (Invitrogen).

For each sample, 16S rDNA amplicon libraries were generated using the Bakt_341F-CCTACGGGNGGCWGCAG and Bakt_805R-GACTACHVGGGTATCTAATCC primers which amplify a 460 bp fragment corresponding the variable V3V4 regions of the bacterial 16S rRNA genes (Klindworth et al., 2013). Samples were amplified from 30ng of DNA for gills, intestinal tract and sediment, and 90ng for digestive glands using the hotstart readymix KAPA HiFi DNA Polymerase (Roche).The PCR mixture was incubated at 95°C for 5 min. The mixture was then subjected to 30 cycles of PCR consisting of 30 s at 98°C, 30 s at 60°C, and 30 s at 72°C, followed by a final extension step at 72°C for 5 min.

Paired-end sequencing with a 250-bp read length was performed at The Genome Transcriptome Facility of Bordeaux (PGTB, Bordeaux, France) on a MiSeq Illumina using the v2 chemistry according to the manufacturer's protocol.

Sequence analysis was performed using the QIIME2 platform (Bolyen et al., 2019) (release 2019.10). The pipeline is detailed in **Appendix 9.2**. Briefly, amplicon sequence variants (ASV) were generated from raw sequences after denoising and removal of chimeric sequences using the Divisive Amplicon Denoising Algorithm 2 (Callahan et al., 2016) implemented into QIIME2. Taxonomy was assigned to ASVs using a scikit-learn Naïve Bayes classifier trained against the SILVA v132 database (Quast et al., 2013). The resultant ASV table was filtered to remove rare ASVs (frequency of less than 0.1% of the mean sample depth), contaminants ASVs assigned to mitochondrial, chloroplast, archaeal and eukaryotic 16S sequences, as well as bacterial ASV unclassified at the phylum level and ASVs retrieved in control samples. Sequences from these control samples clustered into 200 ASVs which were also filtered out from our biological samples dataset. A total of 5 865 097 sequences were obtained corresponding to 7 937 ASVs for 345 samples, with a median and an interquartile range of 16 273 and 10 314 sequences per samples, respectively. Finally, sequences were rarefied to the lowest number of sequences per sample, namely 5 912 sequences for alpha diversity analyses and ordination plots, resulting in 7 834 ASVs. A phylogenetic tree was generated using the SEPP QIIME 2 plugin (Janssen et al., 2018).

The complete data set was deposited in the NCBI Sequence Read Archive (SRA) database under study accession no (under submission).

3.4. Data treatment and statistical analysis

Alpha diversity analysis

The Faith's phylogenetic diversity was calculated using QIIME2 (https://qiime2.org/). As conditions were not met to use a parametric test, differences between sites and tissues were tested using the non-parametric Scheirer-Ray-Hare test or Kruskal-Wallis test both followed by Dunn post-hoc tests to test for pairwise multiple-paired comparisons at the level of significance $p < 0.05$. All statistical analysis was performed using R version 3.6.2 (https://www.r-project.org/).

Beta diversity analysis

For statistical analysis ASV matrices of microbial communities, were log-transformed. Non metric multidimensional scaling (NMDS) plot based on Bray-Curtis dissimilarities were performed using R package vegan (version 2.5-6) (Oksanen et al., 2019). As conditions were not met to use a parametric test, the non-parametric Multiple Response Permutation Procedure (MRPP) was used to test for the significance of differences between groups of samples based on bed and/or organs.

3.5. Supplementary actions

Haemolymph has been sampled for each of the 105 cockles studied but several attempts failed to amplify the bacterial DNA extracted from different samples although the *coxB* gene of cockle could be amplified based on those sample DNA extracts. Given the work package deadlines, no further test was conducted and this option was abandoned although haemolymph microbiota has proven to be an interesting target to study the oyster microbiota (Lokmer and Wegner, 2015; Lokmer et al., 2016).

Seasonal sampling of the cockle population (15 individuals per sampling season) was performed in January, May, July and October 2018 in the French bed of Bassin d'Arcachon. In addition, 3 sediment cores were collected so as to match with the spatial experiment conducted on the 7 beds of the Atlantic Area. When available, the results will be credited to the COCKLES project.

4. RESULTS

4.1. Cockle associated bacteria vs sediment bacterial communities

A total of 5 865 097 sequences of the 16S rRNA v3v4 loop that clustered in 7 834 ASVs were recovered from 343 samples including 40 sediment samples and 305 cockle samples consisting in gills (102), digestive glands (100) or intestinal tract samples (101). The overall cockle microbiota yielded 5 990 ASVs (intestinal tract, digestive glands and gills tissues combined). Of these, 3 144 (52.5%) were exclusively retrieved in cockle organs and 2 846 (47.5%) were present in at least one of the organs and shared with the sediment bacterial communities. The sediment bacterial communities had a total of 4 691 ASVs. 60% of those ASVs were also detected in the cockle organs.

The Faith's phylogenetic index was significantly higher in the bacterial communities of the sediment than in the microbiota of cockles (i.e. combined organ microbiota) of the same bed (Figure 1). As a measure of the α-diversity, the Faith's phylogenetic diversity data indicated that the cockle microbiota was less diversified than sediment bacterial communities. As for the β-diversity, a two-factor MRPP test was performed to compare Bray-Curtis similarities between the bacterial communities of gills, digestive glands, intestinal tract and sediments (factor 1) of the different beds (factor 2). Both factors and their interaction were significant (p-value = 0.001). In addition, NMDS plots of the Bray-Curtis similarities showed that cockle microbiota (pooled organ microbiota) and sediment bacterial communities clearly differed in the different beds (Figure 2). Gill microbiota, on the one side, and digestive gland and intestinal tract microbiota, on the other one, also clearly differed on the NMDS plot with stress value ≤ 0.14 (figure not presented).

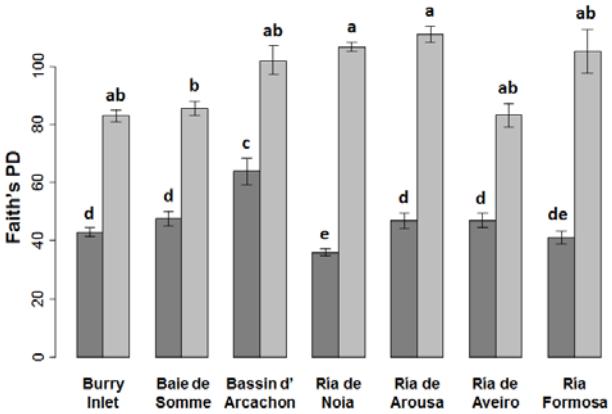

Figure 1. Faith's phylogenetic diversity (Faith's PD) of cockles (dark gray) and sediments (light gray) microbiota across the studied beds. Errors bars represent standard error. Different letters (in superscript) indicate significant differences at the 5% level (Kruskal-Wallis and Dunn post hoc tests).

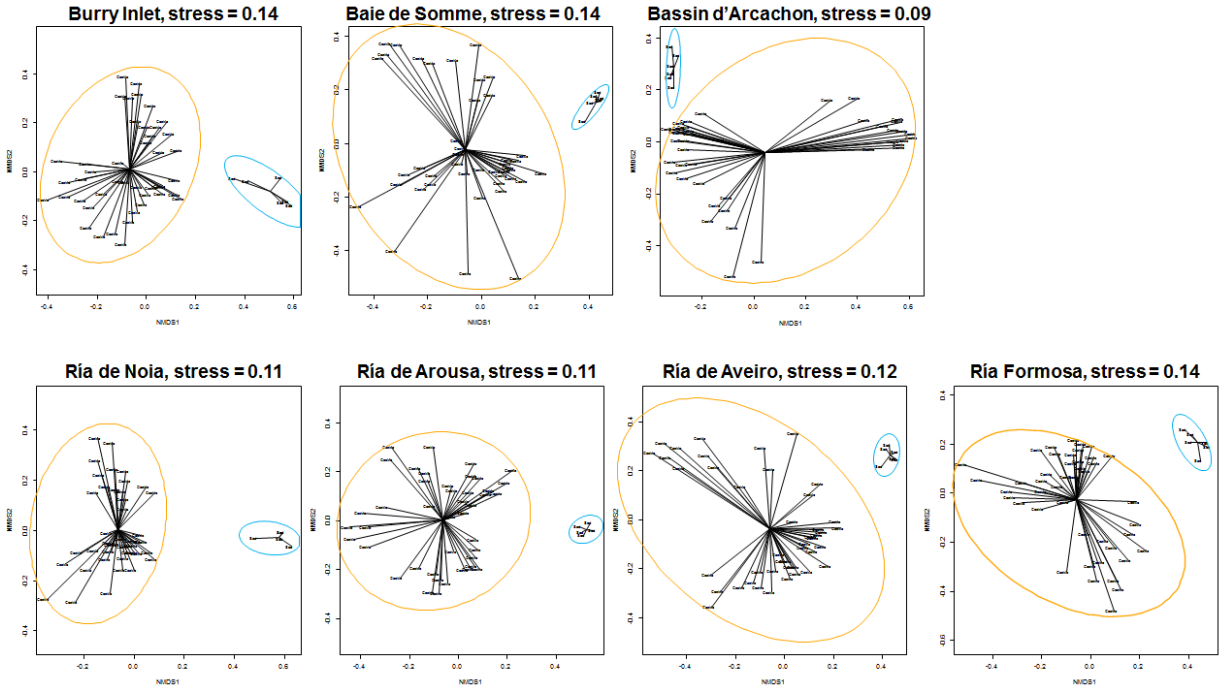

Figure 2. Non metric dimensional scaling plots based on Bray-Curtis similarities between microbiota associated with cockles (orange ring) to those living in the sediment (blue ring) of the different beds along the Atlantic Area. Stress values around or below 0.1 are usually considered fair

4.2. Cockle associated bacteria at the organ level

Apart from sediments, the Faith's phylogenetic diversity of pooled gill, digestive gland and intestinal tract microbiota did not show significant differences between beds (**Figure 1**). Only the α-diversity of the Ria de Noia and Baie d'Arcachon cockles' microbiota significantly differed from each other and from those of the other beds. As for the organ-level microbiota, NMDS plots of the Bray-Curtis similarities showed that unlike the intestinal tract microbiota, the digestive gland microbiota and that of gills hardly differed between the different beds (**Figure 3**).

Gills

In the 104 analysed samples, 6 out of 30 phyla i.e. *Gammaproteobacteria*, *Spirochaetes*, *Margulisbacteria*, *Epsilonproteobacteria*, *Bacteroidetes* and *Patescibacteria*, gathered more than 94% of the 3 334 ASVs in the gill microbiota. Other phyla encompassed less than 2.5% of the ASVs (**Figure 4**). The two main taxa were the genus *Endozoicomonas* (*Gammaproteobacteria*) and a bacterial endosymbiont of *Ridgeia piscesae* (*Epsilonproteobacteria*) (**Table 2**). Unclassified ASVs at the genus

level, ranged from 34% (at Burry inlet) to 67% of sequences (at Ria de Noia). Twenty-five out of 3 334 ASVs (0.75%) were detected in the 7 beds (ubiquitous ASVs) while 2 340 (70.2%) were detected in only one bed (**Figure 5**). The 25 ubiquitous ASVs accounted for 1 261 249 sequences, representing 64 % of the 1 941 103 sequences retrieved in the gill microbiota of the cockles from the 7 studied beds along the Atlantic Area (**Figure 6**).

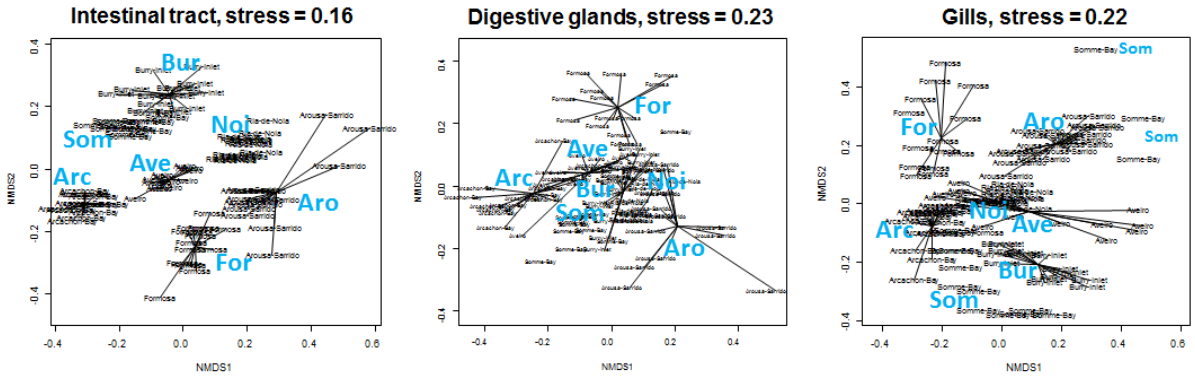

Figure 3. Non metric dimensional scaling plots based on Bray-Curtis similarities between microbiota associated with intestinal tract, digestive glands and gills of the different beds along the Atlantic Area: Burry Inlet (Bur), Baie de Somme (Som), Bassin d'Arcachon Bay (Arc), Ria de Noia (Noi), Ria de Arousa (Aro), Ria de Aveiro (Ave) and Ria Formosa (For). Stress values around or below 0.1 are usually considered fair, those around or above 0.2 suspect.

Table 2. Cockle gill microbiota. Relative abundance (%) of bacterial genera associated with cockle gills of the 7 beds along the Atlantic Area. Only genera with relative abundance ≥ 2% in at least one bed are listed. "Unclassified" include summed relative abundances of taxa that could not been classified to the genus level. "Others" include summed relative abundances of all other taxa out of 584 bacterial taxa. Relative abundance = ratio of the number of sequences affiliated to the taxa to the total number of sequences in the sample set.

Bacterial genus	Burry Inlet	Baie de Somme	Bassin d' Arcachon	Ria de Noia	Ria de Arousa	Ria de Aveiro	Ria Formosa
Endozoicomonas	46.8	5.7	10.4	12.4	1.8	37.3	32.6
Endosymbiont_of_*Ridgeia_piscesae*	4.4	7.8	27.1	9.6	9.4	4.7	0.2
Arcobacter	1.7	11.1	0.1	0.6	4.7	2.8	0.9
Candidatus_*Megaira*	0.2	4.0	2.8	0.6	1.0	0.8	0.0
Shewanella	0.1	1.2	0.0	0.0	4.4	0.1	0.1
Candidatus_*Endoecteinascidia*	0.0	0.3	3.6	0.2	0.0	0.0	0.0
Lentibacter	0.1	0.1	0.0	0.0	2.9	0.5	0.3
Unclassified	33.8	53.7	52.4	66.8	60.9	40.1	51.7
Others	12.8	16.0	3.5	9.5	14.8	13.9	14.2

Intestinal tract

In the 101 analysed samples, 4 phyla i.e. *Epsilonproteobacteria*, *Tenericutes*, *Gammeproteobacteria* and *Bacteroidetes*, gathered more than 80% of the 4 391 ASV. Other encompassed less than 3% of ASVs except in Ria de Arousa cockles where *Fusobacteria* (genus *Psychrilyobacter*) accounted for 23.3% of the ASVs (**Figure 7**). The two main genera were *Arcobacter* (*Epsilonproteobacteria*) and *Mycoplasma* (*Tenericutes*) with relative abundance >40% in each beds (**Table 3**). Fifty-five ASVs (1.25%) were detected in the 7 beds (ubiquitous ASVs) while 2 725 (62.05%) were detected in only one bed (upset plot not presented). The 55 ubiquitous ASVs accounted for 1 421 302 sequences, representing 74.1 % of the 1 918 896 sequences retrieved in the intestinal tract microbiota of the cockles from the 7 studied beds along the Atlantic Area (upset plot not presented).

Digestive glands

In the 100 analysed samples, 5 phyla i.e. *Gammaproteobacteria*, *Tenericutes*, *Spirochaetes*, *Epsilonproteobacteria* and *Bacteroidetes*, gathered more than 85% of the 3 780 ASV in the digestive gland microbiota. Other phyla encompassed less than 3% of ASV except for *Fusobacteria* in Ria de Arousa (8.7%) and Ria de Noia (3.5%) and *Chlamydiae* in Ria de Noia (7.2%) and Ria Formosa (13.8%) cockles (**Figure 8**). The three main genera were *Mycoplasma* (*Tenericutes*), *Arcobacter* (*Epsilonproteobacteria*) as found in the intestinal tract microbiota, and *Endozoicomonas* as found in the gill microbiota (**Table 4**). Most dominant genera were close to those found in the intestinal tract microbiota. Fifty-seven ASVs (1.5%) were detected in the 7 beds (ubiquitous ASVs) while 2 501 (66.1%) were detected in only one bed (upset plot not presented). The 57 ubiquitous ASVs accounted for 1 147 519 sequences, representing 72.4 % of the 1 585 578 sequences retrieved in the digestive gland microbiota of the cockles from the 7 studied beds along the Atlantic Area (upset plot not presented).

Case of *Vibrio*

In the whole sample set, 60 ASVs were affiliated to the genus *Vibrio* of which 3 were affiliated to *V. rumoiensis*, 1 to *V. anguillarum* and 1 to *V. haioticoli*. *Vibrio* were detected in 88 out of the 105 studied cockles, with a prevalence of 100% in the Galician and Portuguese beds, 73.3% in both the French beds and 40% in the welsh bed. There were significant differences in the *Vibrio* relative abundance between organs (Kruskal-Wallis test, p-value < 0.001). This bacterial genus was more abundant in the intestinal tract microbiota (median = 0.23%) than in that of digestive glands (median = 0.03%) and/or gills (median = 0%; 3rd quartile = 0.03%) (Dunn post hoc test at the p = 0.05 level).

Table 3. Cockle intestinal tract microbiota. Relative abundance (%) of bacterial genera associated with cockle intestinal tract of the 7 beds along the Atlantic Area. Only genera with relative abundance ≥ 2% in at least one bed are listed. "Unclassified" include summed relative abundances of taxa that could not been classified to the genus level. "Others" include summed relative abundances of all other taxa out of 635 bacterial taxa. Relative abundance = ratio of the number of sequences affiliated to the taxa to the total number of sequences in the sample set.

Bacterial genus	Burry Inlet	Baie de Somme	Bassin d' Arcachon	Ria de Noia	Ria de Arousa	Ria de Aveiro	Ria Formosa
Arcobacter	45.6	26.5	25.6	52.0	30.2	58.2	48.5
Mycoplasma	20.1	29.2	15.5	12.0	13.7	18.6	20.2
Psychrilyobacter	0.3	0.6	1.1	5.5	22.9	0.7	0.9
Polaribacter_1	5.7	2.2	0.3	5.5	0.3	1.2	0.0
Psychromonas	1.0	4.0	0.4	0.6	1.4	0.8	0.2
Colwellia	1.8	3.8	0.2	0.8	0.1	0.9	1.3
Shewanella	0.8	1.1	0.0	0.2	4.2	0.4	0.2
Ilumatobacter	0.4	0.7	3.9	0.1	0.2	0.6	0.4
Candidatus_*Megaira*	0.2	1.7	3.0	0.1	0.1	1.9	6.7
*Synechococcus*_CC9902	0.0	0.3	4.7	0.0	0.0	0.3	0.2
Endozoicomonas	2.0	0.4	0.2	1.8	0.0	0.5	0.1
*Chitinivibrionaceae*_possible_genus_03	2.6	0.5	0.1	0.2	0.1	0.5	0.0
Pseudoalteromonas	0.1	1.1	0.1	0.1	0.8	2.2	2.3
Unclassified	4.3	8.2	4.6	10.9	9.7	2.9	6.4
Others	15.0	19.7	40.1	10.4	16.3	10.2	12.5

Table 4. Cockle digestive gland microbiota. Relative abundance (%) of bacterial genera associated with cockle digestive glands of the 7 beds along the Atlantic Area. Only genera with relative abundance ≥ 2% in at least one bed are listed. "Unclassified" include summed relative abundances of taxa that could not been classified to the genus level. "Others" include summed relative abundances of all other taxa out of 597 bacterial taxa. Relative abundance = ratio of the number of sequences affiliated to the taxa to the total number of sequences in the sample set.

Bacterial genus	Burry Inlet	Baie de Somme	Bassin d' Arcachon	Ria de Noia	Ria de Arousa	Ria de Aveiro	Ria Formosa
Mycoplasma	38.5	29.1	40.3	17.9	23.7	37.2	45.1
Arcobacter	5.7	1.8	4.2	13.4	10.7	11.8	11.9
Endozoicomonas	8.9	4.7	4.1	6.6	0.1	3.6	2.1
Psychrilyobacter	0.1	0.0	0.1	3.5	8.7	0.1	0.1
Shewanella	0.1	1.4	0.1	0.4	6.1	0.5	0.0
Polaribacter_1	2.2	0.4	0.1	4.0	0.1	0.4	0.0
Coxiella	0.2	5.4	0.0	0.0	0.0	0.0	0.0
Marinomonas	0.1	0.2	0.0	0.0	3.4	0.1	0.0
Kistimonas	2.7	0.0	0.1	0.0	0.0	0.0	0.1
Unclassified	30.3	45.2	28.9	39.0	32.5	31.5	34.4
Other	11.0	11.7	21.9	15.0	14.6	14.8	6.2

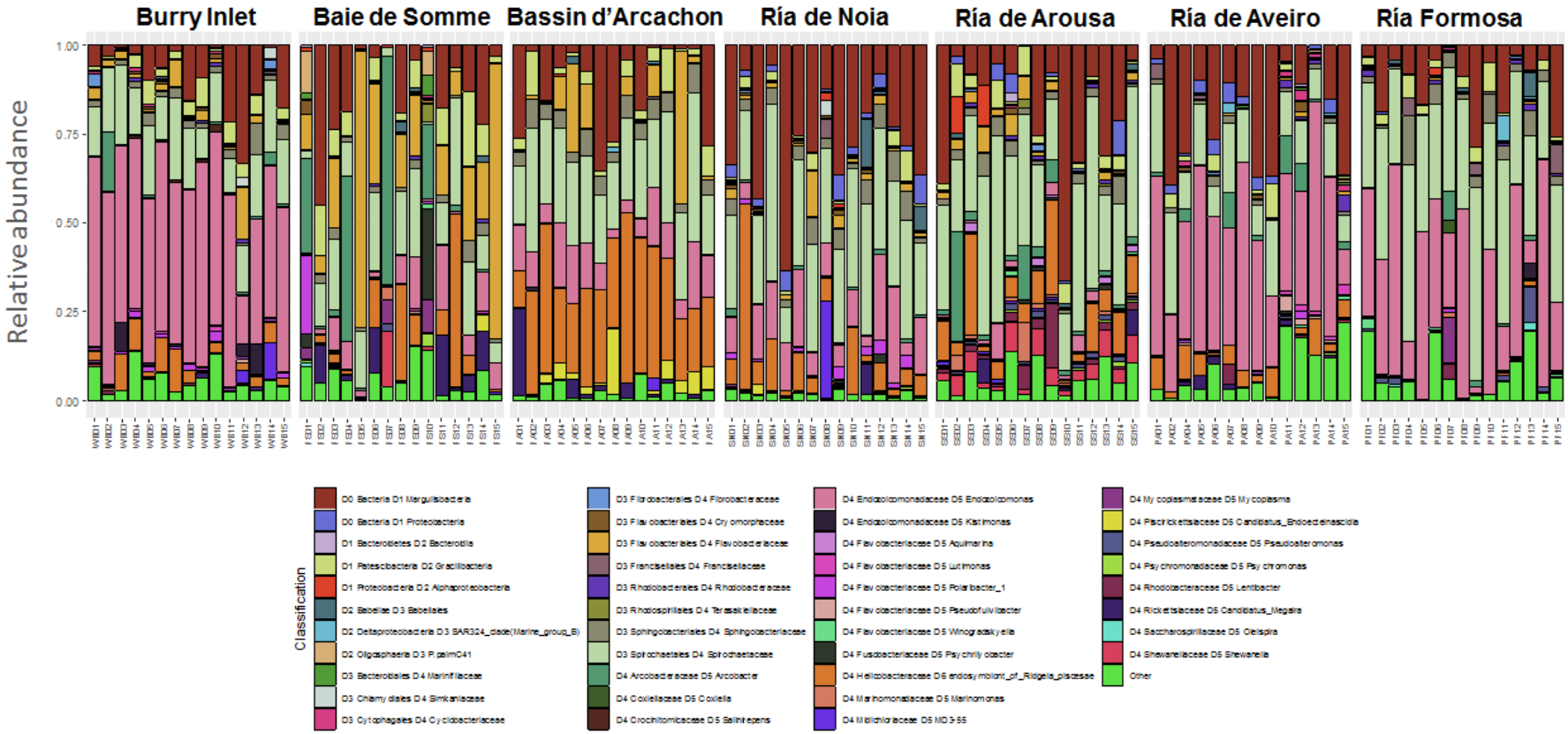

Figure 4. Cockle gill microbiota. Taxa bar plot at the "Genus" taxonomic rank level (when identification was possible at this taxonomic level) of the 15 cockle individuals from Burry Inlet, Baie de Somme, Bassin d'Arcachon, Ria de Noia, Ria de Arousa and Ria Formosa. In Ria de Aveiro only 14 individuals were analysed. Relative abundance = ratio of the number of sequence affiliated to the taxa to the total number of sequences in the individual.

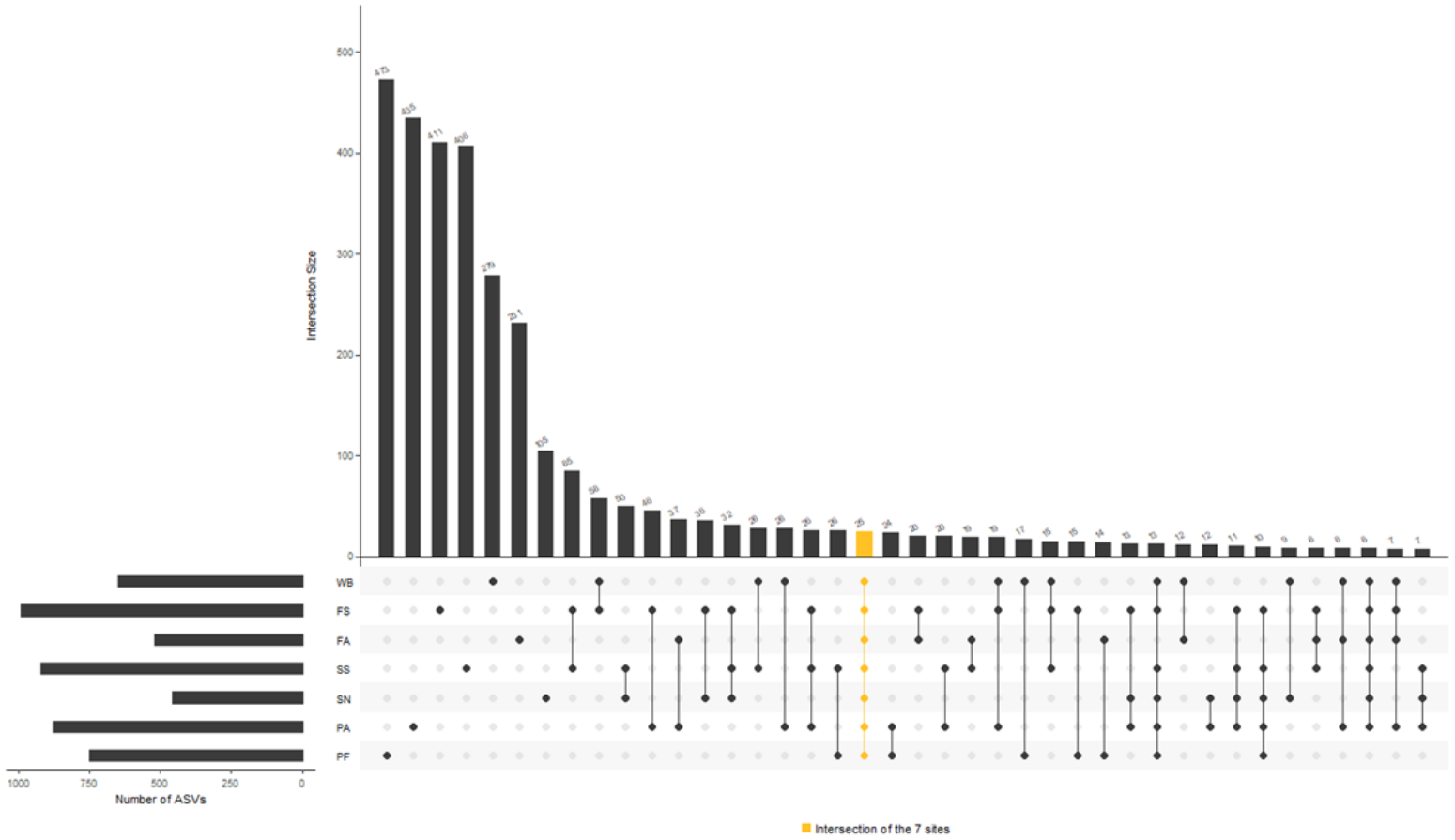

Figure 5. Cockle gill microbiota. Upset plot showing all the unique and common ASV across all beds: The majority of ASV are observed only at one bed. However there are 25 ASVs out of 3 334 (0.75 %) which are observed at the 7 beds (orange drawings).

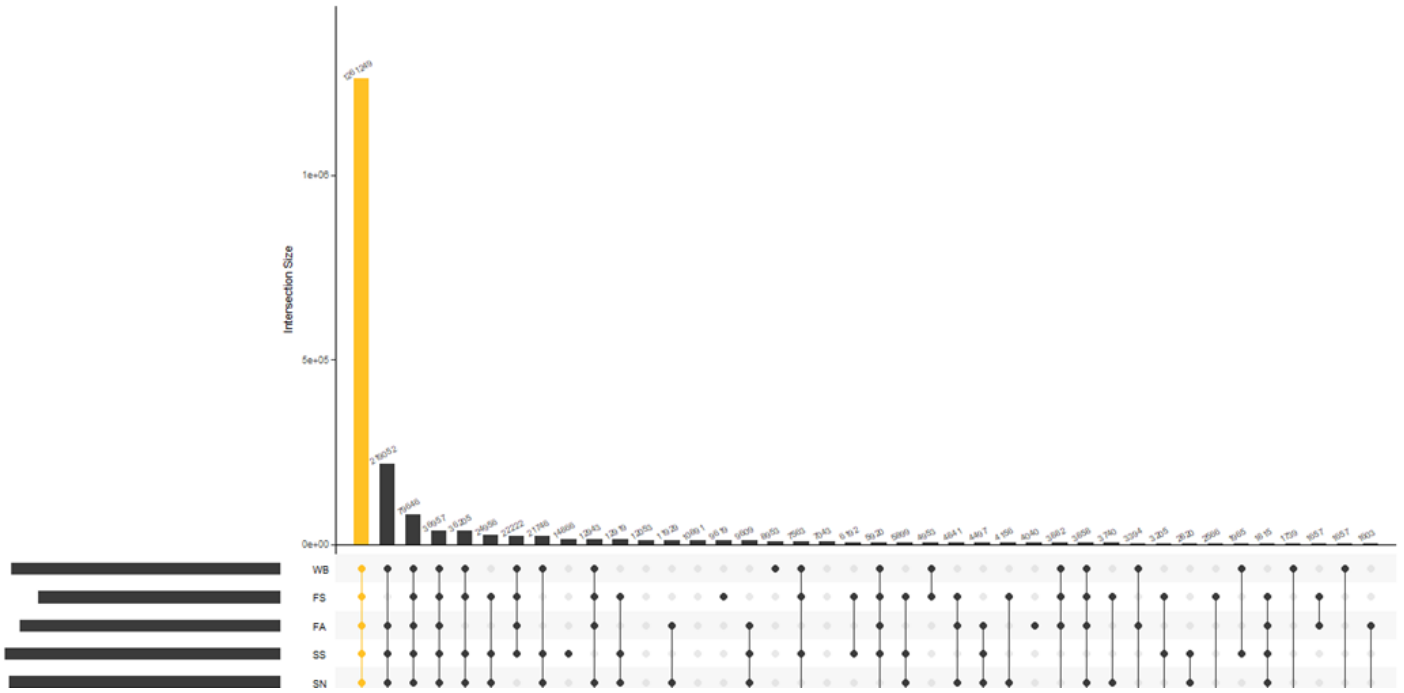

Figure 6. Cockle gill microbiota. Upset plot showing all the unique and common sequences across all beds: The majority of ASV are observed only at one bed. However there are 25 ASVs out of 3 334 (0.75 %) which are observed at the 7 beds (orange drawings).

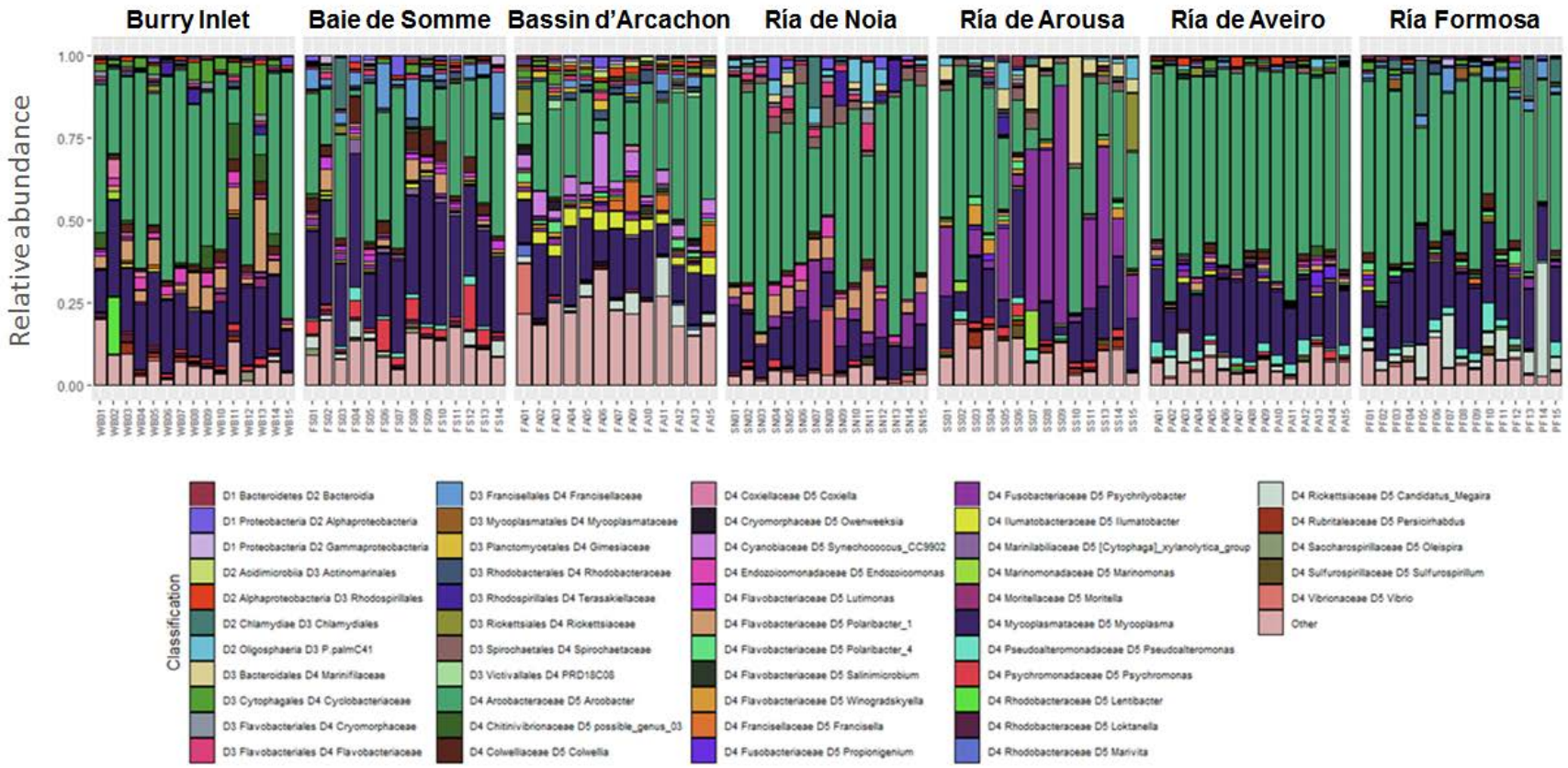

Figure 7. Cockle intestinal tract microbiota. Taxa bar plot at the "Genus" taxonomic rank level (when identification was possible at this taxonomic level) of the 101 cockle individuals from Burry Inlet (15 individuals), Baie de Somme (14), Bassin d'Arcachon (13), Ria de Noia (15), Ria de Arousa (14), Ria de Aveiro (15) and Ria Formosa (15). Relative abundance = ratio of the number of sequence affiliated to the taxa to the total number of recorded sequences in the individual.

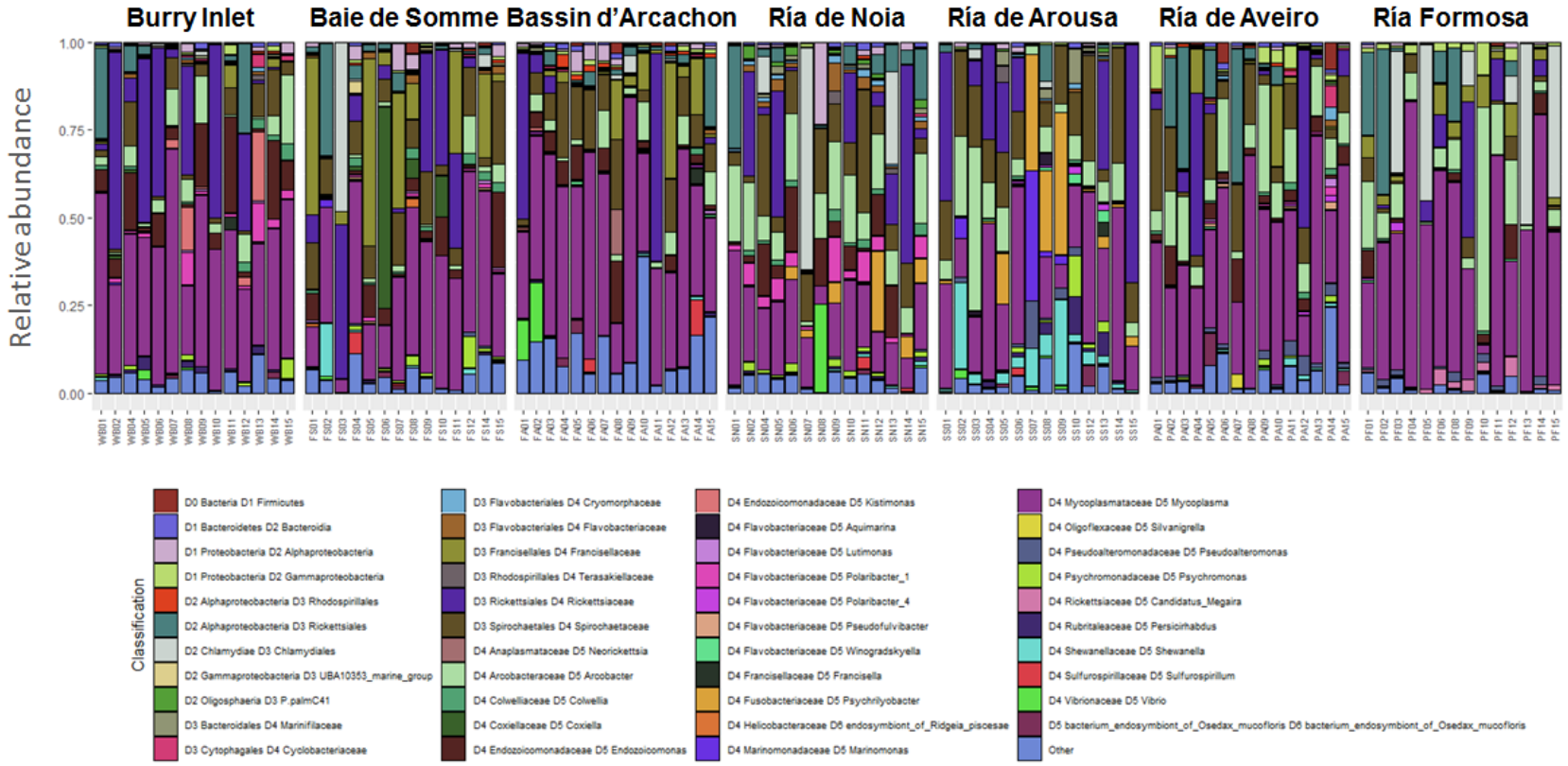

Figure 8. Cockle digestive gland microbiota. Taxa bar plot at the "Genus" taxonomic rank level (when identification was possible at this taxonomic level) of the 100 cockle individuals from Burry Inlet (14 individuals), Baie de Somme (14), Bassin d'Arcachon (15), Ria de Noia (14), Ria de Arousa (14), Ria de Aveiro (15) and Ria Formosa (14). Relative abundance = ratio of the number of sequence affiliated to the taxa to the total number of recorded sequences in the individual.

Table 6. Sulfoxidizing taxa identified in the cockle microbiota

Endosymbiotic		**Genus**	**ASV**	**References**
Gammaproteobacteria	Sedimenticolaceae	Candidatus *Thiodiazotropha*	4	König et al., 2016
	Thiomicrospiraceae	Uncultured endosymbiont	9	
	Thiotrichaceae	*Cocleiomonas*	3	Tanaka et al., 2011
Ectosymbiotic				
Gammaproteobacteria	Chromatiaceae	*Thiobos*	18	Rinke et al., 2006
Free living				
Epsilonproteobacteria	Thiovulaceae	*Sulfurimonas*	74	Inagaki et al., 2003; Takai et al., 2006; Labrenz et al., 2013
	Sulfurovaceae	*Sulfovorum*	24	Inagaki et al., 2004; Mino et al., 2014; Giovannelli et al., 2016; Mori et al., 2018
Gammaproteobacteria	Woeseiaceae	*Woeseia*	34	Du et al., 2016
	Ectothiorhodospiraceae	*Thiogranum*	12	Mori et al., 2015
	Sedimenticolaceae	*Sedimenticola*	11	Flood et al., 2015
	Thiotricaceae	*Thiotrix*	4	Boden and Scott, 2018
	Not identified	*Thioalkalivibrio*	4	Sorokin et al., 2001; Sorokin et al., 2002
	Thiotricaceae	Not identified	29	Boden and Scott, 2018
	Thioalkalispiraceae	Not identified	4	Mori et al 2011
	Thioglobaceae	Not identified	1	Bertagnolli et al., 2020
	Thiohalorhabdaceae	Not identified	10	Sorokin et al., 2008; Sorokin et al., 2010 ; Kumar et al., 2009 while all genera would not be SOB (Baek et al., 2014)

Case of some dominant taxa

Four taxa were dominant in the cockle organ studied (**Table 5**). ASVs classified as *Endozoicomonas* were dominant in gills and digestive glands. In gills, their cumulative relative abundance (median value = 13.6%) was significantly higher than that in both the other studied organs. In the digestive glands, their cumulative relative abundance (2.02%) was significantly higher than that in the intestinal tract (0.26%). ASVs classified as Endosymbiont of *Ridgeia piscea* were dominant in gills (4.80%) with a significantly higher cumulative relative abundance than in both the other studied organs. No ASV affiliated to this taxa was detected in half of the 101 samples of intestinal tract (median value = 0%). ASVs classified as *Arcobacter* were dominant in the three organs. Their cumulative relative abundance was significantly higher in the intestinal tract (44.1%) as compared to the digestive glands (6.48%) and gills (0.51%). ASVs classified as *Mycoplasma* were dominant in the digestive glands and intestinal tract. In the digestive glands, their cumulative relative abundance (31.6%) was significantly higher than that in both the other studied organs. In the intestinal tract, their cumulative relative abundance (17.9%) was significantly higher than that in the gills (0.04%).

Table 5. Median value of the cumulative relative abundance (%) of ASVs affiliated to *Endoizoicomonas*, Endosymbiont of *Rigeia piscesae, Arcobacter* and *Mycoplasma* in cockle digestive glands, intestinal tract and gills. The relative abundance of the taxa is the sum of the relative abundances of the ASVs affiliated to the considered taxa, i.e. 40, 6, 91 and 72 ASVs for *Endoizoicomonas*, Endosymbiont of *Rigeia piscesae, Arcobacter* and *Mycoplasma*, respectively. The relative abundance of one ASV is the ratio between the number of sequences associated with the ASV and the total number of sequences in the sample. Samples of the 7 studied cockle beds were pooled (n = number of pooled samples) to test for differences between organs (Kruskal-Wallis test, $p < 0.05$), then post-hoc pairwise multiple comparison tests were conducted using post–hoc Dunn test. Different letters indicate significant differences at the p = 0.05 level.

	n	*Endozoicomonas*		Endosymbiont *Rigeia piscesae*		*Arcobacter*		*Mycoplasma*	
		Median	Dunn test	Median	Dunn test	Median	Dunn test	Median	Dunn test
Digestive glands	100	2.02	a	0.15	a	6.48	a	31.6	a
Intestinal tract	101	0.26	b	0.00	a	44.10	b	17.9	b
Gills	102	13.60	c	4.80	b	0.51	c	0.04	c

Case of bacteria involved in sulfur cycling

Seventy - eight out of 3 144 ASVs recovered in cockle organs were affiliated to taxa belonging to sulfate reducing bacteria (SRB) according to Rabus et al. (2015) and Wasmund et al. (2017): *Nitrospirae* – *Thermodesulfovibrionia* (2 taxa, 8 ASVs) and *Deltaproteobacteria* (19 taxa, 70 ASVs) amongst which *Desulfobacterales* (11 taxa, 45 ASVs), *Desulfuromonadales* (5 taxa, 22 ASVs), *Desulfarculales* (1 taxa, 2 ASVs) and 1 *Desulfovibrionales* (1 taxa, 1 ASVs). The median value of the SRB relative abundance was less than 0.015% in all the studied organs. There were significant differences in the relative abundance of the SRB between the digestive glands and intestinal tract, on the one hand, the gills on the other one (Kruskal-Wallis and Dunn Post hoc tests) (**Figure 9**). Sulfur oxidizing bacteria (SOB) were considered according to their lifestyle discriminating endosymbiotic and ectosymbiotic taxa from free living ones (**Table 6**). There were no significant differences in the relative abundance of free living SOB amongst the different organs. Median value ranged from 0.0045% in the gills to 0.35% in the intestinal tract. No ASV affiliated to ecto- or endosymbiotic SOB were detected in 75% of the gill samples. Symbiotic SOB were significantly more abundant in digestive glands than in gills whereas their relative abundance was very low with a 3rd quartile of 0.024%.

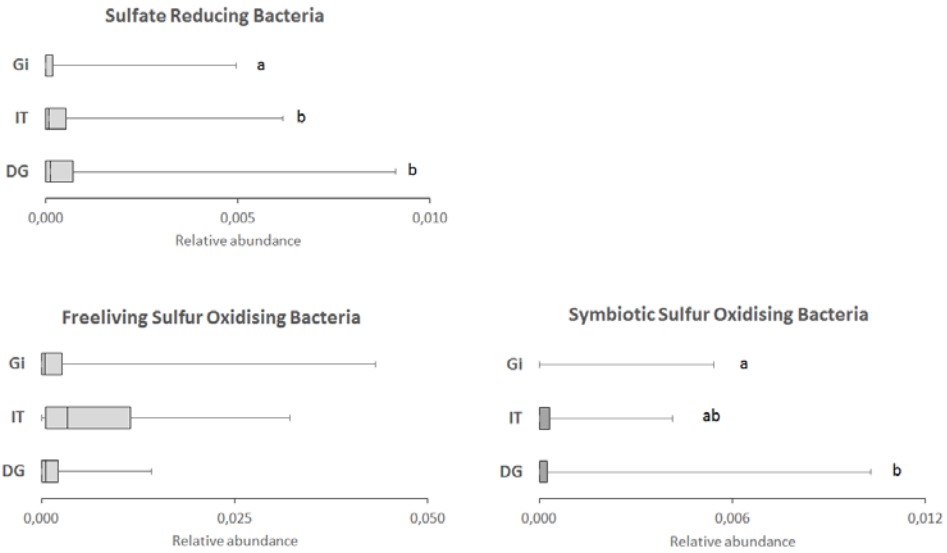

Figure 9. Distribution of the relative abundance of sulfate reducing bacteria, free living sulfur oxidising bacteria, and endo- or ectosymbiotic sulfur oxidising bacteria and in cockle gills (Gi, n = 102), intestinal tract (IT, n = 101) and digestive glands (DG, n = 100). Boxes present the 1st quartile, median and 3rd quartile, whiskers present the min and max values. Samples of the 7 studied cockle beds were pooled to test for differences between organs based on the Kruskal-Wallis test. Post-hoc pairwise multiple comparison tests were conducted using post–hoc Dunn test. Different letters indicate significant differences at the p = 0.05 level. No letters indicate not significant differences between organs at the p = 0.05 level.

4.3. The core microbiota of cockle

By organ

Different classifiers for profiling the core microbiota were tested (**Table 7**). The least restrictive classifier would be to look for ASVs detected in at least one individual per site. Less than 1.5% of the 4 391, 3 780 and 3 334 ASVs recovered from the intestinal tract, digestive glands or gills, respectively, were accounting for ≥ 65% of the relative abundance and would then constitute the core microbiota. On the other hand, the most restrictive classifier would be to look for the ASVs detected in all individuals studied. None of the thousands of ASVs detected in the intestinal tract, digestive glands or gills would meet this latter requirement. A compromise would be to look for ASVs detected in a representative proportion of the individuals collected in each site so as to take into account to both the ubiquity of the ASVs and their prevalence. The higher the threshold (i.e. 50 or 75% of the individuals per site), the lower the number of ASVs (down to 2 ASVs) and their relative abundance (< 45%).

Table 7. Summary of the classifiers that would allow to define experimentally the core microbiota of the cockle at the organ level. Relative abundance = ratio of the number of sequences affiliated to the taxa to the total number of sequences in the sample set.

	Intestinal tract	**Digestive glands**	**Gills**
Number of samples	101	100	104
Number of sequences	1 918 896	1 585 578	1 941 103
Number of ASV	4 391	3 780	3 334
Number of ASV (their summed relative abundances)			
• seen in at least 1 samples at each bed	55 (74%)	57 (72%)	25 (65%)
• seen in 100% of tested samples	0	0	0
• seen in 75% of replicate samples at each bed	2 (43%)	2 (20%)	4 (23%)
• seen in 50% of replicate samples at each bed	9 (61%)	10 (35%)	5 (36%)

Focus on ASV "06fd8c4955ddc4d8ae5929f22ad51144"

This ubiquitous ASV (with an easy-to-memorize nickname!) was detected in the seven studied beds along the Atlantic Area (**Table 8**). It was also detected in the 3 studied organs, with higher prevalence in gills as compared to intestinal tract and digestive glands. In Ria de Formosa, its prevalence was under the 50% threshold set to define the core microbiota, unlike the other beds where its prevalence was above the 75% threshold in most the organs particularly in gills.

Table 8. Prevalence of the ASV "06fd8c4955ddc4d8ae5929f22ad51144" in the digestive glands, intestinal tract and gills of cockles samples in the 7 beds along the Atlantic Area. Prevalence = percentage of samples where the ASV was detected.

	Burry Inlet	Baie de Somme	Bassin d' Arcachon	Ria de Noia	Ria de Arousa	Ria de Aveiro	Ria Formosa
Digestive glands	100%	71%	100%	93%	57%	100%	7%
Intestinal tract	100%	93%	93%	100%	57%	100%	21%
Gills	100%	93%	100%	100%	93%	100%	40%

According to the 16S v3v4 SILVA classifier, the ASV 06fd8c4955ddc4d8ae5929f22ad51144 was one of the 60 ASVs affiliated to the Genus *Endozoicomonas* (*Gammaproteobacteria*; *Oceanospirillales*; *Endozoicomonadaceae*). *Endozoicomonas*-related ASV have been detected in all studied organs or beds with variable relative abundances (**Table 9**). The mean cumulative relative abundance values of *Endozoicomonas* – related ASV were highest in the gills (from 1.8 to 47.2%), then in the digestive glands (from 0.05% to 9.5%) and then in the intestinal tract (from 0.04 to 2%). As for the beds, the lowest values were found in Ria de Arousa (e.g. 1.8% in gills), the highest in Burry Inlet (e.g. 47.2% in gills).

Table 9. Mean of cumulative relative abundances of the 60 ASVs (including ASV "06fd8c4955ddc4d8ae5929f22ad51144") affiliated to the genus *Endozoicomonas* in the digestive glands, intestinal tract and gills of cockle samples in the 7 beds along the Atlantic Area.

	Burry Inlet	Baie de Somme	Bassin d' Arcachon	Ria de Noia	Ria de Arousa	Ria de Aveiro	Ria Formosa
Digestive glands	9.5%	4.7%	4.1%	6.6%	0.05%	3.6%	2.1%
Intestinal tract	2.0%	0.4%	0.2%	1.8%	0.04%	0.5%	0.1%
Gills	47.2%	5.7%	10.6%	12.4%	1.8%	37.2%	32.6%

5. DISCUSSION

The present study of bacterial communities associated with cockles is the first high-throughput sequencing culture-independent survey of the microbial diversity associated to *C. edule*. Thanks to the consortium of partners brought together in the COCKLES project, batches of 15 cockles from 7 different beds could be analysed. The sampled beds expand along a wide latitudinal range in the southern part of the overall *C. edule* species' distribution area. The combined analysis of cockle genomes and larvae dispersal showed that French Brittany delimited a major genetic division between the cockle populations from Burry inlet and Baie de Somme, on the one hand, those from Bassin d'Arcachon, Ria de Noia, Ria de Arousa, Ria de Aveiro and Ria Formosa, on the other one (Vera et al., 2020). In addition, this study showed a clear genetic subdivision amongst the 7 studied beds (belonging to 5 different groups). Hence, together with other parameters such as local environmental conditions, genetic variations between the studied cockle batches could have contributed to differences in the cockle microbiota composition, as previously reported for plants, human and other animals. The logistical constraints inherent to the processing of such samples did not allow seasonal sampling nor the collection of a range of individuals covering the entire population (e.g. juvenile or mature stages). Instead, the present study focussed on batches of comparable individuals within and between beds. It provided a snapshot of cockles' microbiota (mostly in the pre-spawning stage except for Galician beds) and at their maximum size (in the sampled bed and on the sampling date). The mean shell length of the studied batches was consistent with the characteristics of the cockle population in each bed (see COCKLES Deliverable 4.2 report) suggesting that the studied individuals were representative.

The three paths for microbiota acquisition have long been described (Harris, 1993). In vertical transmission, bacteria shift from parents to offspring, through the incorporation of bacteria in or on gametes. In horizontal transmission, bacteria acquisition is independent of host reproduction and bacteria are taken up through the spread of bacteria between contemporary hosts. Lateral transmission concerns the microflora acquired directly from the environment. Burrowing suspension feeders such as *C. edule* live in a highly septic environment and are exposed to lateral transmission as evidenced by the harmful occurrences of e.g. norovirus or fecal bacteria contamination sometimes recorded during sanitary surveys (e.g. French microbiological monitoring network for shellfish growing areas – REMI; http://envlit.ifremer.fr/surveillance/microbiologie_sanitaire).To characterize the cockle microbiota, the starting point was thus to address the similarities - dissimilarities between the microbial communities associated to the cockle and those present in the biotope. Comparisons between oyster or mussel

microbiota composition and that of the planktonic microbial communities of their surrounding waters was relevant for such suspension feeders living in the water column above the sediment – water interface (e.g. Vezzulli et al., 2018; Pierce and Ward, 2019; Stevic et al., 2021) or even more so for their fully planktonic larvae (Arfken et al. 2021). For burrowing organisms such as cockles or clams, comparison of the bivalve microbiota composition to that of the sediment seemed more appropriate (Meisterhans et al., 2016). Expectedly, the bacterial community composition of cockle digestive glands, intestinal tract or gills significantly differed from that of the sediment in bed. As compared to the sediments, the Faith phylogenetic diversity of the cockle microbiota was lower in each of the 7 beds studied. The Faith's phylogenic diversity is a measure of the α-diversity which is fully suitable for molecular-based characterization of community composition as it exploits the different degrees of similarity between sequences (Faith et al., 2009). The lower α-diversity of cockle microbiota as compared the sediment bacterial communities might be explained by cockle selecting of a subset of bacterial species among a larger pool available in the cockle biotope. Only those bacteria escaping the host defenses and finding suitable conditions could settle in the cockle microbiota. Nevertheless, cockle organs and sediment shared 47% of the total bacterial ASVs supporting the hypothesis of a partial structuration of the cockle microbiota by lateral transmission.

As for the comparison of cockle microbiota among the different beds, only the Ria de Noia and Bassin d'Arcachon samples showed a significantly different Faith's Phylogenetic diversity. Apart from these two sites, a relative stability of the cockle microbiota phylogenetic diversity was observed from the southern Portugal to Wales's beds. In Ria de Noia, the lowest phylogenetic diversity could not be explained. Likewise for Bassin d'Arcachon where the highest phylogenic diversity was observed despite a known strong parasitism pressure (de Montaudouin et al., 2000). First, these observations would need to be replicated. Second, a dedicated experiment would be necessary to test if biotic challenges such as marteiliosis or parasitism would similarly or distinctly affect the cockle microbiota. Finally, in such a multi-stress environment as coastal ecosystems where cockles live (Paul Pont, 2010), biotic challenges most probably interact with other drivers, e.g. metallic contamination (Paul Pont, 2010; Leite et al., 2017) or warming (Lokmer and Wegner, 2015; Li et al., 2019), which should be taken into account.

Organ or tissue-dependence of microbiota is now well acknowledged for bivalves (Lokmer et al., 2016; Meisterhans et al., 2016; King et al., 2020). Organs form distinct systems of microbiota assembly, specifically in regard to migration and selection processes (sensu Nermergut et al., 2013). According to these authors, migration is the process "in which a new organism is incorporated into a community from

outside". Selection deals with the deterministic theory that a population will settle and thrive if matching a niche (i.e. a unique set of suitable biotic and abiotic factors). The intestinal tract, where migration could be a major driver particularly through lateral transmission based on ingested particles by feeding, would have a temporally or spatially variable microbiota in relation with the temporal or spatial variation of the ingested particles composition. Indeed, the gut microbiota of the Norway lobster was shown to reflect the seasonal shifts in planktonic community on which the crustacean relies for feeding (Meziti et al., 2010). In line with this, the composition of the cockle intestinal tract microbiota was found to be spatially variable (transient populations?) among cockle beds studied whereas no significant differences were found in the composition of both the digestive gland and the gill microbiota along the Atlantic area. This suggested that the proportion of selected populations (resident populations?) is larger in the microbiota of the digestive glands and gills than in the microbiota of the intestinal tract where a higher proportion of transient populations related to ingested particles would be found. In agreement with this hypothesis, infectious bacterial taxa known to form intra – tissue microbial colonies such as *Mycoplasma* and *Endoizoicomonas* (Cano et al. 2020; Neave et al., 2016) and a putative endosymbiotic taxa (i.e. the Endosymbiont of *Ridgeia piscea*) were found to be significantly more abundant in the microbiota of the digestive glands or gills as compared to the intestinal tract. In addition, it was found that the microbiota of the cockle intestinal tract was (i) dominated by *Arcobacter*, and (ii) significantly enriched in SRB as compared to gills. *Arcobacter* is a much diversified bacterial genus. Two species of this genus were described as free living SOB of deep sea hydrothermal vents (Moussard et al., 2006; Sievert et al., 2007) whereas *A. peruensis* was another SOB recently isolated form the highly productive Peruvian coastal waters (Callbeck et al. 2019). Surprisingly, other species of this genus were filed as emerging human food–borne pathogens. In coastal exploited shellfishes, *A. butzleri, A. cryaerophilus and A. skirrowii* were found to be highly prevalent (from 15 to 88%) (Collado and Figueras, 2011; Collado et al., 2014). Those bacteria were generally considered to accumulate as a result of fecal contamination by humans or livestock (Leoni et al. 2017), suggesting that the occurrence of *Arcobacter* in the intestinal tract of the studied cockles could have resulted from lateral contamination of the bivalves. Unfortunately, none of the retrieved sequences allowed affiliation to the species level to confirm this hypothesis by assessing whether the retrieved ASVs were affiliated to SOB species (which is unlikely) or to allochtonous species of the *Arcobacter* genus (which is more likely). The status of the SRB was also debatable. Hydrogen gas (H_2) produced by fermentation in the intestinal tract was hypothesized to support methanogenesis and the occurrence of methanogen archaeal genes (i.e. cells?) in the bivalve *Limecola baltica* (Bonaglia et al., 2017). SRBs also rely on dihydrogen and anaerobic conditions and could therefore flourish if such

conditions are met in the intestinal tract of *C. edule*. However, previous studies have shown that mimicking the effects of an antimicrobial agent added experimentally, the bivalve respiratory activity and subsequent tissue oxygenation controlled the deleterious invasion of the bivalve tissues by anaerobic bacteria in *C. edule* (Babarro and de Zwaan, 2008). In the present study, the SRBs were detected in all the studied organs whereas they were more abundant in the intestinal tract and digestive glands. The possibility cannot be excluded that these bacteria started to invade the cockle organs during shipping from the sampling site to the laboratory, except for the Bassin d'Arcachon cockles which were sampled and treated the same day. Another technical concern is the rinsing baths carried out on the gills (see materials and method section) which may have emphasized the recorded differences between gills on the one side, and digestive glands and intestinal tract on the other one. Cockle gills, as many other bivalves, could harbor endosymbiotic bacteria. Discarding the particles and bacteria loosely attached to the gill surface would favor the detection of those tightly attached or internalized. Enhancing the detection of tightly and internalized bacteria was intentionally carried out in the present study which aimed at focusing on the core microbiota of *C. edule* discarding the transient part when possible.

The core microbiota encompasses the stable and permanent members of the community and its study may be beneficial to identify host-microbe partnerships in holobiont associations (Astudillo-Garcia et al., 2017). In common practice, the core microbiota is approached by identifying persistent or ubiquitous (i.e. taxa found on all the sampling occasions of spatial or temporal surveys) and, sometimes, abundant taxa in the host microbiota (i.e. taxa whose affiliated ASVs represent a significant cumulative relative abundance). To assess the cockle core microbiota in the present study, we primarily considered both the percentage of cockle individual where the taxa was detected, i.e. its prevalence, and the occurrence of the bacterial taxa in most or all studied beds, i.e. its ubiquity. This resulted in a severe reduction of the number of bacterial taxa considered in each organ microbiota as more than 95% of the ASVs were thereby discarded. For instance, no bacterial taxa was detected in all the cockle individuals studied and only a few tens of ASVs (out of thousands) were detected in at least one individual of each bed. The (few) ASVs identified as core microbiota nevertheless met the criterion of abundance with cumulative relative abundance ranging from to 20 to 74%. No Vibrio ASV was in this short list despite the genus prevalence (> 70%, except in Burry inlet) in the study set. Vibrios are abundant in coastal waters and sediments (Thompson et al., 2004). With the exception of *V. tapetis*, vibrios were not filed as major cockle pathogens (Longshaw and Malham, 2013; Burdon, 2015) until *V. aesterianus* was recently involved in cockle mass mortality in Baie de Somme (Garcia et al., 2021). The most abundant ASVs were affiliated to the genera *Mycoplasma* and *Arcobacter* in the digestive glands and intestinal tract. The case

of *Arcobacter* has been discussed above. The *Mycoplasma* genus has long been detected in bivalves (Harshbarger and Chang, 1977; Stevick et al., 2021) and was recently involved in intracellular micro-colonies of bacteria in marine mollusks (Cano et al., 2020). No current information on these two bacterial genera allowed to conclude that they contributed positively to the cockle fitness as assumed in the working hypothesis on the core microbiota. In gills, 4 ASVs identified as possible core microbiota were affiliated to endosymbiotic bacteria i.e. *Endozoicomonas*, Endosymbiont of *Ridgeia piscesae*, Candidatus *Megaira* (a member of *Rickettsiaceae*) and Candidatus *Endoecteinascidia*.

Sulfur oxidation, carbon dioxide and dinitrogen fixation have been acknowledged as metabolic adds-on provided by gill endosymbiotic bacteria to bivalves (e.g. Caro et al., 2009 ; Dmytrenko et al., 2014 ; König et al., 2016; Petersen et al., 2016). A quick analysis of the data set showed that ecto- and endosymbiotic SOB accounted for a negligible fraction of the microbiota (< 0.1% on average). Their relative abundance was higher in the intestinal tract and digestive glands than in the gills which was not expected. In addition, free living SOB were more abundant than symbiotic SOB, equally distributed in all organs and, for some of them, affiliated to uncultured sediment taxa. Together these observations suggested that SOBs have only marginal relevance in the cockle microbiota especially in gills where no ecto- or endosymbiotic-SOB has been clearly detected.

The ASV affiliated to *Endozoicomonas* was the most abundant ASV in gills, with up to 46.8% of relative abundance at Burry Inlet. This ASV was present in cockles of all the studied beds with high prevalence, (generally > 55%) except in cockles from Ria Formosa, in all studied organs. According to Neave et al. (2016), this endosymbiotic bacteria of marine invertebrates is a « prevalent symbiotic marine genus » found ubiquitously in a very large diversity of hosts (sponges, corals, tubeworms, mollusks, jellyfishes, fishes). Neave et al. (2016), state that “all *Endozoicomonas* microscopy studies have found aggregations in host tissues, suggesting that these formations are an important part of *Endozoicomonas* function and colonization”. The list of the suggested functions resumes as “nutrient acquisition and provision, structuring of the host microbiome, and roles in host health or disease”. *Endozoicomonas* genomes revealed high genomic plasticity (transposable elements) and various metabolic pathways suggesting they could be involved in sugar and protein delivery to their host. In essence, Neave et al. (2016) presented *Endozoicomonas* as rather beneficial endosymbionts although they mention these bacteria could be responsible for diseases. Recently, Cano et al. (2020) clearly identified *Endozoicomonas*-like organisms as cells forming intracellular micro-colonies of bacteria. In cockles, the colonies were observed in the gills and in the digestive glands. The study reports that intracellular micro-colonies were

demonstrated to induce lesions and were associated with mass mortality events (but not in cockle). Although Cano et al. (2020) acknowledged the need for "information to establish the type of symbiosis they maintain with their host (mutualisms, commensalism or parasitism) and whether they can act as true pathogens", they primarily considered *Endozoicomonas* as a pathogen. *Endozoicomonas* has been included in the list of *C. edule* pathogens and their description in census book established in the COCKLES project (See COCKLES deliverable 5.1). The individual pathogenicity of *Endozoicomonas* was filed as "unknown", the populational pathogenicity as "not reported". The present data do not permit to support either of the half-empty or half-full glass visions of the role of *Endozoicomonas* proposed by Cano et al. (2020) and Neave et al. (2016), respectively. Neave et al. (2016) thoroughly detailed how the novel techniques such as RNA-seq on isolated single cells or secondary ion mass spectrometry will allow to address the functional role of *Endozoicomonas* bacteria within their hosts. It's worth it to develop such experimental studies. The present study confirmed the high prevalence of *Endozoicomonas* in *C. edule* from 6 out of 7 beds along the Atlantic Area, in agreement with Cano et al. (2020) reporting prevalence ranging from 40 to 100% in one Welsh and one Galician beds. Such prevalent, ubiquitous and abundant bacteria must, indeed, be pivotal for the *C. edule* holobiont.

6. CONCLUSIONS

An original dataset on the *C. edule* microbiota was newly acquired thanks to the COCKLES project. The COCKLES consortium permitted the collection of cockles from 7 cockle beds covering most of the distribution area of *C. edule* southern genotype. Adults at the maximum local size were sampled and treated as quickly as possible to ensure reliable comparisons between the populations along the Atlantic area. Three organs were targeted: gills for possible endosymbiosis, digestive glands for exposure to infections and intestinal tract for strong exposure to lateral contamination via the feeding activity. Bacterial communities were analysed by next generation sequencing based on the MiSEQ Illumina technics which, at that moment, represented the best trade – off between analysis costs and sequence lengths to ensure optimal affiliation to bacterial taxa.

In summary, the analysis of the present dataset showed that:

- In all the cockle beds, the community structure of cockle associated bacteria was significantly different than that of sediment bacterial community, suggesting selection of certain taxa by the cockle host.
- The structure of the bacterial community was significantly different between gills, intestinal tract and digestive glands. There were also significant differences in bacterial community structure of the intestinal tract amongst cockle beds but not for that of gills and digestive glands. The organ-dependence of the microbiota structure suggested organs form distinct systems of microbiota assembly with regard to selection (e.g. bacteria – host association) and migration (e.g. lateral acquisition of the microbiota) processes.
- The core microbiota of cockle i.e. the stable and permanent members of the community of cockle associated bacteria was made of 3 sets of 27, 55 and 57 ASV that were respectively found in gills, digestive glands and intestinal tract of cockles from all the studied beds. Each set had cumulative relative abundance $\geq$ 64% indicating dominance of the related bacterial populations.
- In gills, 4 out the 6 top dominant ASV were affiliated to endosymbiotic taxa supporting the hypothesis that gills are preferred organs for endosymbiosis or infection.
- In gills, no ASVs affiliated with chemosynthetic sufur-oxidising ecto- or endosymbionts were abundant making such an hypothetical association in the cockle unlikely, in contrast to what has been reported for other marine invertebrates inhabiting sulfidic environments.

- In gills, the most abundant ASV (21% on average) was affiliated to *Endoizoicomonas*. This bacterial taxa was detected in all beds, with high prevalence (except in Ria Formosa) pointing to the probable importance of its association with *C. edule.*

Supplementary analyses should still be conducted. The Faith's phylogenetic diversity has proven to be powerful to address the α-diversity of both sediment and cockle. Accordingly, the UniFrac distance (Lozupone and Knight, 2005) could provide an improved assessment of the β-diversity to compare the bacterial community structure in 2-factors ANOSIM with beds (factor 1) and organs (factor 2). This metric should be tested and compared to the Bray-Curtis distance used in the present study. The different work packages of the COCKLES project synthetized information on the cockle populations and beds along the Atlantic Area. This will be useful to search for possible links between environmental parameter, cockle population dynamics and bacterial communities of the cockle microbiota. Indeed, we could possibly explain some unsolved results such as e.g. differences in the phylogenetic diversity of the bacterial communities associated to cockles from Ria de Noia, from Bassin d'Arcachon or low prevalence of *Endoizoicomonas* in the gills of cockle from Ria Formosa.

More generally, the functional role of the cockle microbiota has still to be defined. Different options exist for different research strategies and perspectives. First, the functional role of the cockle microbiota could be deciphered using bioinformatics approach to predict function from taxonomic sequences based on available software such as PICRUSt (Langille et al., 2013) or Tax4Fun (Aßhauer et al., 2015). This is a relevant perspective to value the dataset acquired in the present study. The functional role of the cockle microbiota could be studied based on laboratory or field experiments dedicated to manipulating the cockle microbiota and monitor subsequent effects on the cockle physiology (respiration, reproduction) or ethology as conducted e.g. with parasites (Dairain et al., 2020). Finally, the functional role of the cockle microbiota could also be addressed by targeting typical cockle – bacteria associations based on novel molecular technics such as RNA-seq on isolated single cells or secondary ion mass spectrometry. According to our results, the *Endoizoicomonas* – *C. edule* association would be a highly relevant candidate and the timeliest scope regarding the functioning, health and fitness of the *C. edule* holobiont.

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9. APPENDIX

9.1. Cockle sample set

Ecosystem	Cockle bed	GPS coordinates	Sampling Date	Treatment Date	Cockle ID	Shell Length(1)	Cond. index(2)	Gi(3)	DG(3)	IT(3)
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB1	24		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB2	27		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB3	27		x		x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB4	28		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB5	26		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB6	27		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB7	27		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB8	27		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	30/03/2018	5WB9	25		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB10	25		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB11	25		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB12	26		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB13	27		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB14	27		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB15	26		x	x	x
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB16	26	57			
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB17	25	57			
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB18	24	62			
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB19	24	59			
Burry Inlet	**Burry South point**	51°38'33"N 04°09'58"W	27/03/2018	31/03/2018	5WB20	24	53			
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS1	28		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS2	28		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS3	29		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS4	29		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS5	30		x	x	x

Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS6	26		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS7	29		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS8	29		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS9	28		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS10	27		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS11	28		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS12	26		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS13	26		x		x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS14	27		x	x	x
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS15	27		x	x	
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS16	24	84			
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS17	25	76			
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS18	27	88			
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS19	28	75			
Baie de Somme	**Crotoy - Station CH4**	50°14'49"N 1°33'12"E	12/02/2018	15/02/2018	5FS20	27	70			
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA1	29		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA2	33		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA3	33			x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA4	31		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA5	32		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA6	31			x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA7	31		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA8	33		x	x	
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA9	33		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA10	32		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA11	31		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA12	32		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA13	33		x	x	x
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA14	31		x	x	
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA15	33		x	x	x

Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA16	32	44			
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA17	31	46			
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA18	30	40			
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA19	32	47			
Bassin d'Arcachon	**Arguin**	44°35'04"N 1°14'32"W	30/01/2018	30/01/2018	5FA20	32	48			
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN1	28		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN2	31		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN3	34		x		x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN4	29		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN5	31		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN6	30		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN7	30		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN8	30		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN9	32		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	17/05/2018	5SN10	31		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN11	30		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN12	29		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN13	29		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN14	31		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN15	28		x	x	x
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN16	28	65			
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN17	29	26			
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN18	27	62			
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN19	30	58			
Ria de Noia	**Misela**	42°47'25"N 8°55' 21"W	14/05/2018	18/05/2018	5SN20	28	57			
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	16/05/2018	5SS1	27		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	16/05/2018	5SS2	24		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	16/05/2018	5SS3	23		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	16/05/2018	5SS4	23		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS5	28		x	x	x

Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS6	23		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS7	26		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS8	24		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS9	27		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS10	27		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS11	25		x		x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS12	27		x	x	
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS13	24		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS14	26		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS15	25		x	x	x
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS16	17	102			
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS17	17	130			
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS18	19	116			
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS19	20	182			
Ria de Arousa	**Sarrido**	42°30'24"N 8°49'32"W	14/05/2018	17/05/2018	5SS20	20	171			
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA1	29		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA2	24		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA3	28			x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA4	25		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA5	24		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA6	27		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA7	28		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA8	27		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA9	27		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA10	28		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA11	27		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA12	25		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA13	27		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA14	28		x	x	x
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA15	28		x	x	x

Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA16	31	55			
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA17	25	53			
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA18	26	62			
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA19	27	45			
Ria de Aveiro	**Mira - Station 25**	40°38'34"N 8°44'7"W	1902/2018	23/02/2018	5PA20	25	59			
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF1	28		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF2	25		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF3	24		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF4	29		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF5	28		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF6	28		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF7	22		x		x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF8	22		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF9	28		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF10	27		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF11	25		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF12	28		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF13	25		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF14	27		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF15	23		x	x	x
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF16	26	67			
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF17	25	53			
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF18	25	73			
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF19	28	65			
Ria Formosa	**Faro**	36°59'51'N 7°49'48"W	19/02/2018	22/02/2018	5PF20	25	72			

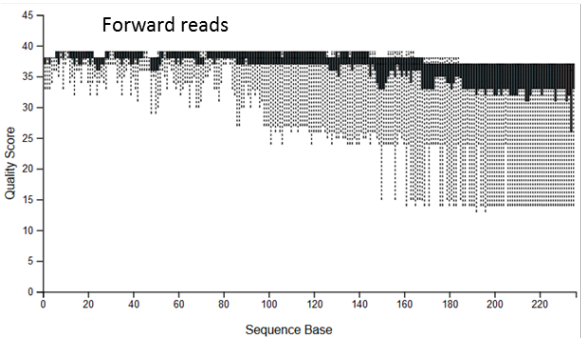
(1) Shell length in mm

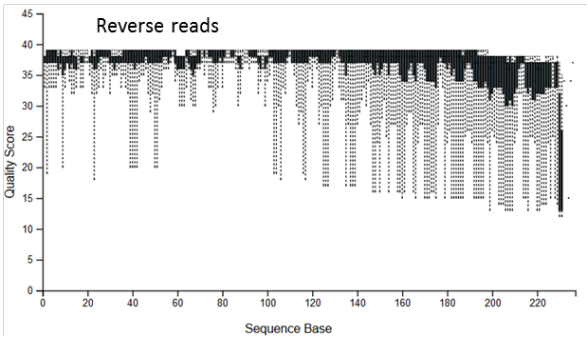
(2) Condition index as mg dry flesh mass per g dry shell mass

(3) Samples of cockle gills (Gi), digestive glands (DG) and intestinal tact (IT) studied after discarding those with improper amplification or low sequence frequency

9.2. Pipeline (QIIME2 - DADA2 - SILVA)

1. **Import FASTQs files** *(2*250 Illumina MiSeq reads)* **as QIIME 2 artifact format**
   CasavaOneEightSinglelanePerSampleDirFmt





2. **Trim primers with cutadapt: (341F and 785R)**

Size of the V3V4 amplicon without primers = 427 pb in *E. coli* 16S rRNA

Demultiplexed sequence length summary

Forward Reads

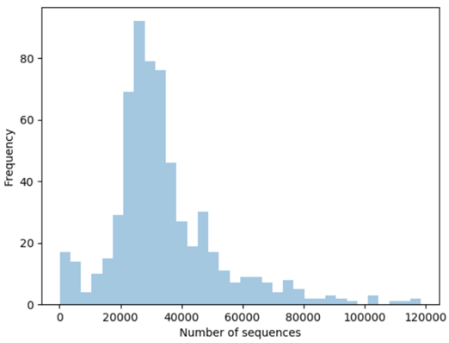
Total Sequences Sampled	10000
2%	232 nts
9%	232 nts
25%	234 nts
50% (Median)	234 nts
75%	234 nts
91%	234 nts
98%	234 nts

Reverse Reads

Total Sequences Sampled	10000
2%	229 nts
9%	230 nts
25%	230 nts
50% (Median)	230 nts
75%	230 nts
91%	230 nts
98%	230 nts

Demultiplexed sequence counts summary
(621 samples including controls)

Minimum	18
Median	30 667
Mean	34 178 64
Maximum	118 598
Total	21 224 939



3. Denoise the reads into Amplicon Sequence Variants (ASV) : DADA2 method

i. **Filter reads:** parameters maxN=0 (DADA2 requires no Ns); rm.phix=TRUE; maxEE=3

ii. **Trim reads**: parameters truncQ=2 and trunc lengh (forward reads = 234; reverse reads =229)

iii. **Learn the Error Rates:** 1000000 reads used for training the error model (parameter --p-n-reads-learn)

iv. **Dereplicate reads:** to combine all identical sequencing reads into "unique sequences" with a corresponding "abundance" equal to the number of reads with that unique sequence.

v. **Merge paired reads**: merged sequences are only output if the forward and reverse reads overlap by at least 12 bases and are identical to each other in the overlap region

vi. **Remove chimeras** consensus methods: chimera detected in samples individually, then sequences found chimeric in a sufficient fraction of samples are removed).

	All samples	**Controls removed**
input	21 224 939	21 044 891
filtered	14 140 560	14 058 580
percentage of input passed filter	67%	68%
denoised	13 592 426	13 511 210
merged	12 583 819	12 510 569
percentage of input merged	61%	62%
non-chimeric	11 645 022	11 571 796
percentage of input non-chimeric	56%	57%

Dada2 output summary

		Frequency per sample		Frequency per ASV	
Number of samples	616	Minimum	2	Minimum	19
Number of ASV	9 738	1st quartile	13 091	1st quartile	28
Total frequency	11 207 550	Median	17 972	Median	51
		3rd quartile	22 795	3rd quartile	141
		Maximum	51 327	Maximum	1 302 302
		Mean	18 194	Mean	1 151

Mean frequency per sample *= mean sample depth: define the cut-off for how frequent an ASV needs to be for it to be retained during the filter out rare ASV step.*
Remove all ASVs that have a frequency of less than 0,1% of the mean sample depth

4. Assign taxonomy to ASVs

i. **Acquire the 16S v3v4 SILVA taxonomic classifier** *from the Microbiome Helper website (Comeau et al. 2017)*

https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2019.10)

This trained Naive Bayes classifier was created from the SILVA_132_99_QIIME_release sequences file.

Sequences were sliced at the bacterial V3/V4 regions (341F/805R primers respectively CCTACGGGNGGCWGCAG and GACTACHVGGGTATCTAATCC) and trained with the SILVA_132_QIIME_release/taxonomy/16S_only/99/majority_taxonomy_7_levels.txt taxonomy file (scikit-learn version 0.21.2).

ii. **Classifiy COCKLES reads by taxon against the 16S v3v4 SILVA classifier**

with the auto parameter of plugin QIIME Pre-fitted sklearn-based taxonomy classifier to autodetect orientation based on the confidence estimates for the first 100 reads

5. Filter resultant table

i. **Filter out rare ASVs**

ASVs with frequency < 19 sequences removed

ii. **Filter out the control samples**

- Control samples = DNA extraction controls, negative and positive PCR control and internal MiSeq control
- Remove 28 control samples and their 200 associated ASV (total frequency of 87 060 sequences)

		Frequency per sample		Frequency per ASV	
Number of samples	588	Minimum	2	Minimum	3
Number of ASV	9 650	1st quartile	13 879	1st quartile	28
Total frequency	11 120 490	Median	18 428	Median	50
		3rd quartile	23 124	3rd quartile	137
		Maximum	51 327	Maximum	1 301 639
		Mean	18 912	Mean	1 152

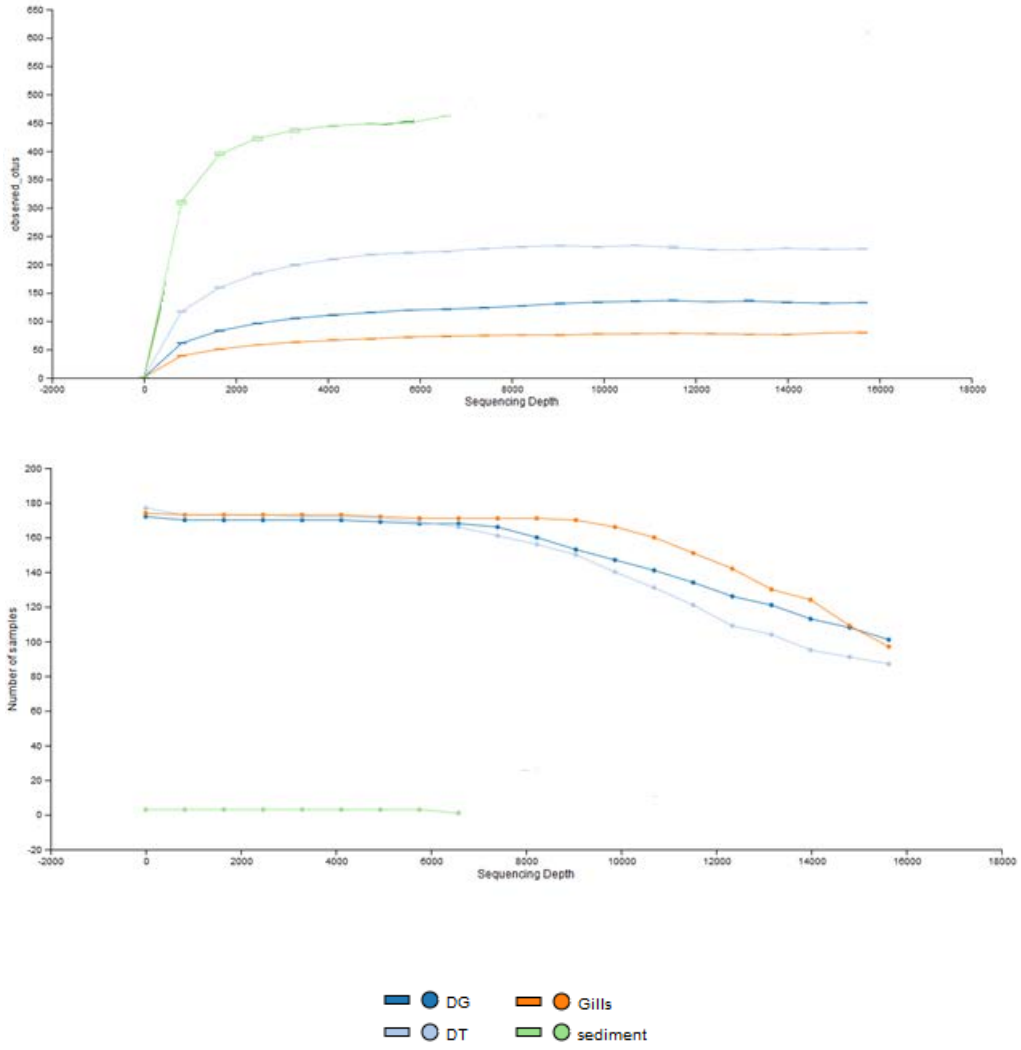
iii. **Filter out contaminant and unclassified sequences**

- Contaminant =mitochondria, chloroplast, archaea and eukaryota sequences
- Unclassified = unclassified ASVs at the phylum level since these sequences are more likely to be noise (e.g. possible chimeric sequences)

		Frequency per sample		Frequency per ASV	
Number of samples	587	Minimum	2	Minimum	3
Number of ASV	9 018	1st quartile	11 083	1st quartile	28
Total frequency	9 497 597	Median	15 631*	Median	49
		3rd quartile	20 326	3rd quartile	128
		Maximum	47 764	Maximum	1 301 639
		Mean	16 179	Mean	1 053

* --p-max-depth for QIIME diversity alpha-rarefaction

iv. **Rarefaction curves**



v. **Exclude low-depth samples**

- Samples rarefaction at **5 912 sequences per sample**
- FINAL COCKLES dataset

		Frequency per sample		Frequency per ASV	
Number of samples	569	Minimum	5 912	Minimum	3
Number of ASV	9 015	1st quartile	11 530	1st quartile	27
Total frequency	9 447 589	Median	15 845	Median	48
		3rd quartile	20 452	3rd quartile	127
		Maximum	47 764	Maximum	1 298 880
		Mean	16 603	Mean	1 047

6. **Build tree with SEPP QIIME 2 plugin**

   - SEPP is one method for placing short sequences into a reference phylogenetic tree (here the qiime Silva128-SEPP_ref_db release)
   - Used default qiime2 parameters
   - The tree will be used for calculating phylogenetic diversity metrics

7. **Generate stacked barchart of taxa relative abundances**

8. **Calculating diversity metrics and generating ordination plots**