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Diversity and predation capacities of Amoebozoa against opportunistic vibrios in contrasting Mediterranean coastal environments.

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Abstract

Free living amoebae (FLA) are ubiquitous and found in many types of environments including soil, freshwater, air and marine environments. They feed on various microorganisms, and can play a key central role in the food web and its dynamic. We described previously that FLA belonging to the *Vannella* genus from the oyster farming area of the Thau lagoon in France could establish stable interactions with *Vibrionaceae* and could play a role in the selection of some virulence factors and potentially affect pathogen dynamics. To investigate further the ecological interactions between FLA populations and *Vibrionaceae* in Mediterranean coastal waters, we conducted a monthly sampling for one year in three contrasted sites. Free-living amoebae populations were isolated by culturing water and sediment samples on different bacterial lawns including *E. coli* SBS363 or *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi A01*. Amoebozoa diversity was analyzed using v4-18S barcoding and revealed distinct communities of Amoebozoa between the sediment and the water column, with Vannellidae significantly enriched in the water column whereas Paramoebidae were significantly enriched in the sediments. Selection of grazers on different bacterial lawns revealed that *V. tasmaniensis* LGP32 inhibited the growth of most Vannellidae whereas *V. crassostreae* J2-9 inhibited the growth of a large part of Paramoebidae. These differences were further confirmed in functional grazing assays using isolates belonging to each Amoebozoa taxonomic group. Altogether, our results highlight that Amoebozoa diversity in marine waters and population dynamics still need to be studied in a more comprehensive manner and the role of these diversified grazers in shaping vibrio communities is complex and still poorly characterized in the environment.

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Introduction

Grazing protists, such as free-living amoebae (FLA), are found in many diverse environments. Free-living amoebae are mainly found in freshwater and marine environments but also in soil, air and associated with different hosts (Rodríguez-Zaragoza, 1994; Samba-Louaka et al., 2019). The diversity of FLA has been thoroughly studied in freshwater environments compared to marine environments especially because amoebae pathogenic for humans are mostly found in freshwaters (Visvesvara et al., 2007). Amoebae are a polyphyletic group branching along the eukaryotic tree and belonging to 4 supergroups such as Amoebozoa, which is the only one to gather amoebae only, Opisthokonta, Excavata and SAR (Lahr et al., 2011). Free-living amoebae feed on various microorganisms present in their environment and digests them by phagocytosis. They can feed on bacteria, yeasts, fungi, algae or other protists (De Moraes and Alfieri, 2008; Radosa et al., 2019; Salt, 1968; Smirnov et al., 2011). Heterotrophic protists are pivotal members of the food web in environment and their predation activity over bacteria is a major driver that shapes microbial communities (Batani et al., 2016; Corno and Jürgens, 2008; Gao et al., 2019; Jürgens et al., 1999; Pernthaler, 2005). Protist dynamics (e.g. abundance and diversity) is influenced by many environmental factors like type of preys and abiotic factors such as salinity, temperature and oxygen availability (Amaro et al., 2015; Guillou et al., 2001; Kim et al., 2014; Orsi et al., 2011; Smirnov, 2007). Recently we observed that the amoebae diversity in Mediterranean Thau lagoon close to oyster farming area is relatively low with mainly amoebae belonging to the *Vannella* genus, and some of these *Vannella* could establish stable interactions with Vibrionaceae (Robino et al., 2020). Vibrios are γ-proteobacteria living in aquatic environments both freshwater and marine. They are ubiquitous and found associated with many hosts from protozoans to metazoans, and these associations range from symbiosis to pathogenesis (Mandel and Dunn, 2016; Takemura et al., 2014). Among them, the best known and described example is the Human pathogen *Vibrio cholerae* (Clemens et al., 2017). Vibrios are also involved in vibriosis in aquaculture including oyster-farming (Dubert et al., 2017; Le Roux et al., 2016). As for example, they can be involved in mortality events of juvenile oysters *Crassostrea gigas* (De Lorgeril et al., 2018). Immunosuppressed oysters upon OsHV-1 infection are colonized by opportunistic vibrios causing septicemia leading to animal death. These opportunistic vibrios that have been implicated in mortalities during the Pacific Oyster Syndrome belong to *V. crassostreae*, *V. tasmaniensis* and *V. harveyi* species (Gay et al., 2004; Lemire et al., 2015; Oyanedel et al., 2023). Interestingly, the cytotoxicity against hemocytes by *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi* A01 has been shown to be a major driver of their virulence but relies on species-specific mechanism (Oyanedel et al., 2023; Rubio et al., 2019). The acquisition of such anti-eucaryotic activities can result from the long co-evolution history between bacteria and FLA. Some bacteria have evolved resistance against amoeba

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predation through extracellular and intracellular defense mechanisms, which can play a role in virulence against animal hosts (Matz and Kjelleberg, n.d.; Pernthaler, 2005). Hence, amoebae are considered as evolutionary precursors of interactions and act as training ground for intracellular pathogens bacteria, by favoring selection of virulence factors through coincidental evolution (Diard and Hardt, 2017; Molmeret et al., 2005). In the case of vibrios, *Vibrio cholerae* has been shown to be able to survive within *Acanthamoeba castellanii* amoebae and use some virulence genes which play a minor role during the interaction with the human incidental host (Van Der Henst et al., 2018). Still, when Vibrio cholerae exit *Acanthamoeba castellanii* through exocytosis of vibrio-containing food vacuole this can increase its virulence against mammal hosts (Espinoza-Vergara et al., 2019). In the case of *V. vulnificus*, it was shown that the virulence factor MARTX type III can be involved in resistance against grazing by *Neoparamoeba permaquidensis* isolated from fish gills (Lee et al., 2013). By comparative cellular biology, we previously showed that the oyster opportunistic pathogen *V. tasmaniensis* LGP32 is able to resist phagocytosis by environmental marine amoeba *Vannella* sp. AP1411 using some virulence factors also involved in pathogenesis in oysters, in particular the secreted metalloprotéase Vsm and the copper efflux pump CopA (Robino et al., 2020). Finally, lipopolysaccharide O-antigen variations in *Vibrio splendidus* strains were shown to determine resistance to grazing by *Vannella* sp. AP1411 (Oyanedel et al., 2020). Altogether, these different reports suggest that amoeba-vibrios interactions are diverse and complex and need to be further studied to better evaluate their role in vibrios dynamics and pathogen emergence.

To investigate the ecology and the interactions between FLA populations and *Vibrionaceae* in Mediterranean coastal waters, in the present report, we conducted a monthly sampling for one year in three contrasted environments. In the Thau lagoon, used for oyster farming and where oyster mortalities due to POMS occur annually (Richard et al., 2021), but also in the Mediterranean Sea outside of the Sète harbor, and 3) near the marine protected area of Banyuls-sur-Mer, both serving as reference sites. Free-living amoebae populations were isolated by culturing water and sediment samples on different bacterial lawns including *E. coli* or *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi A01*. By 18S barcoding, we found that Amoebozoa dominated the FLA populations with a higher diversity in the sediment than in the water column with distinct communities and site specific diversity in the sediments. One of the most striking differences was that Vannellidae are abundant and specifically enriched in the water column whereas Paramoebidae are abundant and specifically enriched in the sediments. Moreover, these two families of Amoebozoa harbor contrasted predation capacities against two different species of vibrios of the Splendidus clade *V. tasmaniensis* LGP32 or *V. crassostrae* J2-9, which was further confirmed functionally using amoeba isolates belonging to each family. Altogether, our results highlight that Amaoeboza diversity is still understudied and need to be characterized further in varied marine

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environments, and the nature of amoeba-vibrio interactions can take place at very different taxonomic levels, and may participate in the dynamics of opportunistic pathogens in marine coastal waters.

Materials and methods

Bacterial strains, amoeba strain and growth conditions

E. coli strain SBS363 was grown in Luria-Bertani (LB) or LB-agar (LBA) at 37°C, for 24 hours prior experiments. *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi* A01 were grown in LB + NaCl 0.5M or LBA + NaCl 0.5M at 20°C, for 24 hours prior experiments. Vibrios strains carrying the pMRB-GFP plasmid were grown in LB + NaCl 0.5M supplemented with chloramphenicol (10µg.mL^{-1}) at 20°C, for 24 hours prior experiments. *Vannella sp*. AP1411 (isolated in a previous study, (Robino et al., 2020) and *Paramoeba atlantica* strain CCAP1560/9 (purchased from the CCAP collection, Scotland, UK) were cultivated at 18-20°C in 3ml of 70% sterile seawater (SSW) supplemented with 200µL of an *Escherichia coli* SBS363 suspension (OD600=20).

Sampling

Sampling was performed monthly during one year between 2017 and 2018 at three contrasted environments in south of France. Water and sediment were collected at Thau lagoon next to oyster tables at the Bouzigues station Ifremer-REPHY (GPS: N 43°26’.058' - E 03°39’.878’), at open sea close to Sète harbor (GPS: N 43°23’.539' - E 03°41’.933’) and near the marine protected area of Banyuls-sur-Mer at SOLA station (GPS: N 42°29'300’ - E 03°08'700’) (Figure S1). New juvenile oysters were placed each month and sampled only at oyster tables at the Bouzigues station of the Ifremer REPHY observatory. Surface water (1 meter depth) was collected using a hydrobios bottle at the three sites and first centimeters of sediments with cores taken by scuba divers at 9, 10 and 30 meters depth respectively at Thau lagoon, Sète and Banyuls-sur-Mer. The sediments from Thau and Sète were respectively muddy and sandy while sediments from Banyuls-sur-Mer had intermediate composition. Water was filtered on the boat with a 63 µm pore size nylon filter then re-filtered at the lab using a 5 µm pore size MF-Millipore membrane. The 5 µm pore size membrane was then cut in four pieces and three quarters were put upside down on a lawn of *E. coli* SBS363 seeded on 70% SSW-agar while one other entire filter was cryopreserved at minus 80°C. One gram of sediment and one piece of oyster gill of roughly 0.5 cm^2 were deposited in the center of a lawn of *E. coli* SBS363 seeded on 70% SSW-agar in triplicate and incubated at 20°C while the same type of samples were cryopreserved at minus 80°C. Briefly, after 2 weeks, amoebae were flushed by 70% SSW and one milliliter of flushed solution was cryopreserved at minus 80°C for each condition. Same steps were performed with *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi* A01 in

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May and October 2017 during mortality events of oysters and January and February without mortality events. A total of 76 samples from *E. coli* lawn and 84 samples from vibrios lawns were recovered for diversity analyses by 18S barcoding.

18S barcoding data processing

Total DNA from flushed grazing plates were analyzed by performing barcoding on the v4 loop of the 18S rRNA coding gene using universal primers at an annealing temperature of 53°C (TAReuk454FWD-illumina: 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG YRC CAG CAS CYG CGG TAA TTCC-3' and TAReukRev3-illumina : 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GYR ACT TTC GTT CTT GAT YRA-3') (Stoeck et al., 2010). Paired-end sequencing (300 bp read length) was performed by the GenSeq platform (Labex CEMEB, Montpellier, France) on the MiSeq system (Illumina). Then, SAMBA v3.0.1 (https://gitlab.ifremer.fr/bioinfo/workflows/samba) pipeline was used to process raw data, a Standardized and Automated MetaBarcoding Analyses workflow. This workflow developed by the SeBiMER (Ifremer's Bioinformatics Core Facility) is an open-source modular workflow to process eDNA metabarcoding data. SAMBA developed by the SeBiMER (Ifremer's Bioinformatics Core Facility) is an open-source modular workflow to process eDNA metabarcoding data. SAMBA was developed using the Nextflow workflow manager (Di Tommaso et al., 2017) and enables the execution of three main components: data integrity verification, bioinformatics processes and statistical analyses.

Amoebozoa diversity analysis on different nutritive sources.

For the analysis of all the samples acquired on E. coli agar plates, on the 72 samples was performed, 6 samples that recovered too few sequences were removed, with less than 1000 sequences (10-17-Ba-S-E-coli, 11-17-Th-S-E-coli, 06-17-Th-S-E-coli, 09-17-Th-S-E-coli, 12-17-Ba-S-E-coli and 11-17-Ba-S-E-coli) as well as 2 samples considered as outliers due to the species composition that was completely different from the rest of the samples (08-17-Th-W-E-coli and 09-17-Ba-W-E-coli). New analysis on the 64 samples resulted in a total of 2,780,405 sequences after data integrity checking. QIIME 2 was used for primers removing using the Cutadapt plugin, sequences quality checking using the DADA2 R package (removing low-quality sequences, assembling the forward and reverse sequences, and removing chimeras), and ASV clustering using dbOTU3 algorithm, to obtain a total of 2556 ASVs (Bolyen et al., 2019; Callahan et al., 2016; Hedjazi et al., 2011; Olesen et al., 2017). Taxonomic affiliation was performed using the PR2 database (4.13.0 version) with taxonomy filtering to select only ASVs belonging to Amoebozoa phylum which is composed only of amoeboid protists (Guillou et al., 2012). A total of 465 ASVs

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were regrouped in a table comprising ASVs' names with their taxonomic affiliations as well as the names of the samples including the ASVs and the quantities identified for each sample (Table S2).

For the analysis of samples acquired on the four different nutritive sources (*E. coli* SBS363, *V. harveyi* A01, *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9) was performed on the 112 samples (i.e 84 samples from vibrios lawn and 28 samples from *E. coli* lawn), and 6 samples with less than 900 sequences were removed (02-18-Ba-S-LGP32, 01-18-Th-S-A01, 10-17-Ba-S-E-coli, 05-17-Th-S-LGP32, 05-17-Ba-S-J2-9 and 05-17-Se-S-J2-9). New analysis on the 106 samples resulted in a total of 5,298,844 sequences after data integrity checking. Through QIIME 2, we performed primers removing using the Cutadapt plugin, sequences quality checking using the DADA2 R package, and ASV clustering using dbOTU3 algorithm, to obtain a total of 3084 ASVs (Bolyen et al., 2019; Callahan et al., 2016; Hedjazi et al., 2011; Olesen et al., 2017).Taxonomic affiliation was performed using the PR2 database with taxonomy filtering to select only ASVs belonging to Amoebozoa phylum which is composed only of amoeboid protists (Guillou et al., 2012). We obtained a total of 474 ASVs regrouped in a table comprising ASVs' names with their taxonomic affiliations as well as the names of the samples including the ASVs and the quantities identified for each sample (Table S5).

Then SAMBA performed extensive analyses of the alpha- and beta-diversity using homemade R scripts (R Core Team, 2020). Alpha diversity was studied using Chao1, Shannon and InvSimpson indexes. Beta diversity was investigated using principal coordinate analysis (PCoA) with the DEseq2 normalization method and Unifrac distance matrix. Repartition of the specifics and shared ASVs to the various conditions have highlighted the taxonomy for each variable or combined variables and were plotted with UpsetR V1.4.0. The phylogenetic tree was performed using MAFFT and FastTree (Maximum Likelihood tree) and annotated using iTOL software highlighting fraction, sites and season variables (Letunic and Bork, 2021).

Grazing assay

To prepare the co-culture of vibrios and amoebae, 1mL of vibrio overnight culture (3.10^9 bacteria. mL^{-1}) was mixed with 100µL of three days old *Vannella sp.* AP1411 or *Paramoeba atlantica* culture (5.10^5 cells. mL^{-1}) or with 100µL of 70% SSW for control condition. A volume of 50 µL per well of the mixed culture was seeded on top of 500µL of 1% SSW-agar, in a 24-well plate with transparent flat bottom. Amoebae and bacteria lawn were carefully homogenized in the wells and let dry, for 4 hours at room temperature under the flow of a sterile hood, and then incubated at 18 °C in a humidified atmosphere. GFP fluorescence intensity was measured every day over 7 days using a TECAN plate reader (λex 480 nm/λem 520 nm). Then, to estimate the effect of the amoebae grazing activity on the abundance of living vibrios expressing GFP, the fluorescence intensity of the wells containing amoebae was compared to the fluorescence of vibrios lawn without amoebae, and

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expressed as a ratio. Each condition was performed in technical triplicates and the results shown are the average of three independent experiments. Error bars represent the standard error of the mean (±SEM). Statistical analysis was performed using RM-ANOVA over the independent experiments.

Results

Amoebozoa diversity strongly differs between the water column and the sediments

Grazers' dynamics remain poorly described in the Mediterranean environments and even less in lagoons close to oyster farms. Therefore, we attempted to describe cultivable grazer's diversity and distribution in Mediterranean coastal environments by studying 3 contrasted environments. A monthly sampling survey was performed during one full year in the environment close to oyster farming tables in Thau lagoon (Bouzigues), in an open sea site outside of the Sète harbor and a last site more distant and more protected in Banyuls-sur-Mer area next to the marine protected area (Figure S1). We sampled sediments and water at the 3 sites every month over an entire year from March 2017 to March 2018. In order to recover cultivable amoebae samples were put on non-nutrient plates with a layer of *E. coli* SBS363 as a non-pathogenic and permissive nutritive food source to isolate the highest diversity possible in these conditions. Briefly, after 15 days incubation at 20°C, the total cultivable diversity was estimated by sequencing the v4 hypervariable region of the 18S rRNA using universal primers. Then, we used the SAMBA pipeline to process sequencing data. Due the lack of taxonomic references in 18S SSU databases for some protists groups, we chose to focus on ASVs affiliated to Amoebozoa only, as they represented the majority of the ASVs with a taxonomic affiliation and this taxonomic group contains FLA only. First analyses showed that the alpha-diversity and beta-diversity of Amoebozoa significantly differs between the two different sampling fractions (e.g. sediments or water column) independently of the sampling site (Figure 1A and B). The Chao1 index (estimator of species richness) was found to differ significantly, with the Chao1 median in the water column of 6.5 ASVs as opposed to a median of 18 ASVs for the sediments, showing that the alpha diversity of the water column tend to be lower than in the sediments (ANOVA test; $p = 0.0138$, Table S2). The two other alpha-diversity indexes Shannon and InvSimpson did not significantly differ suggesting that the differences between fractions were mostly due to species of low abundance. For the beta-diversity, PCoA revealed distinct Amoebozoa communities between water and sediment samples (Adonis test; Adonis $R^2 = 0.19$, $p = 0.0001$, Table S3, Figure 1A). Moreover, the samples from sediment appeared more grouped than those from the water column suggesting a more stable community over the sampling months (Figure 1A). The repartition of ASVs grouped by fractions showed that out of

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the 465 ASVs, 312 ASVs (67.1%) were found only in the sediments whereas 122 ASVs (26.2%) were found only in the water column (Figure 1B). In addition, very few ASVs were shared by both fractions (31 ASVs representing 6.7% of total ASVs). This ASVs distribution illustrates the statistical differences found in beta-diversity analyses, highlighting a higher richness in the sediments than in the water samples.

In line with the differences of beta-diversity observed between the two different habitats (water column versus sediments), the distribution of two abundant families of Amoebozoa appeared in very contrasted ASVs between water samples and sediments. ASVs belonging to Paramoebidae were found specifically enriched in the sediments whereas ASVs belonging to Vannellidae were found specifically enriched in the water column (ANCOM analyses; Table S4), which was not the case for the other Amoebozoa family that tended to be found more evenly distributed between the two habitats independently of the sampling site.

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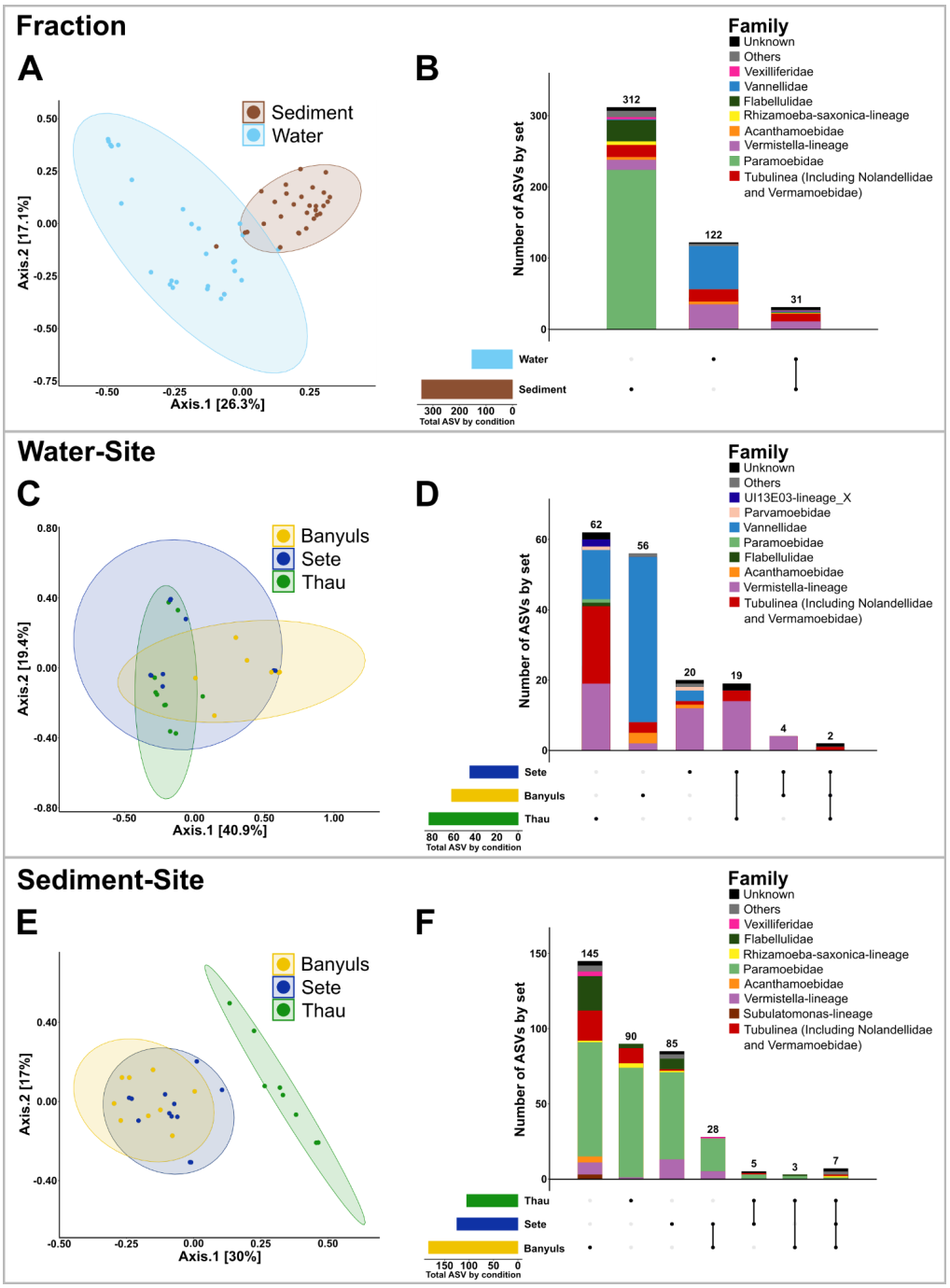

Figure 1. Amoebozoa diversity is structured according to the sampling fraction and the sampling site. PCoA of the unweighted Unifrac matrix distance revealed that the beta-diversity of Amoebozoa in the sediment is different from the beta-diversity in the water samples (A). ASVs distribution revealed that ASVs belonging to the Paramoebidae are particularly enriched in the sediment whereas ASVs belonging to Vannellidae are particularly enriched in the water samples (B). PCoA of the unweighted Unifrac matrix distance between water samples from the different sampling sites revealed that the beta-diversity of Amoebozoa in the water is highly variable and not significantly different between sampling sites (C). ASVs distribution highlights that most ASVs found in water samples are specific to each site and the community structure is variable but ASVs of each family are found in all sites (D). PCoA of the unweighted Unifrac matrix distance between sediment samples from the different sampling sites revealed that the beta-diversity of Amoebozoa in the sediments is more homogeneous and the beta-diversity found in Thau sediments is different from the beta-diversity in the sediments of Sète and Banyuls that are more similar (E). ASVs distribution highlight that most ASVs found in the sediments are specific to each site and the community structure is variable but ASVs of each family are found in all sites (D).

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Cultivable marine Amoebozoa diversity depends on the geographical site.

Secondly, we studied the differences in Amoebozoa diversity between the three contrasted sampling sites Banyuls-sur-Mer, Sète and Thau. No significant differences in alpha diversity were observed between sites, however beta-diversity using PCoA with the DEseq2 normalization method and Unifrac distance matrix revealed that beta-diversity of each site was specific and significantly different from others (Table S3). Due to the strong fraction effect, the taxonomic level of the beta-diversity differences between sites were hard to evaluate independently of the fraction. So we compared the Amoebozoa beta-diversity between sites among the water samples on the one hand, and the sediments from each site on the other hand. Although grazer's communities were all significantly different (Table S3), the three groups of samples overlapped (Figure 1C and 1D). These results suggest that the variance between samples belonging to the same site was big and in a similar range for each site. Indeed, the communities composition between the three sites was similar with intra-family abundance variations (Figure 1D). The repartition of ASVs by sites in water column showed that out of the 163 ASVs, 62 ASVs (38%) and 56 ASVs (34.4%) were specific to Thau and Banyuls-sur-Mer respectively while only 20 ASVs (12.3%) were specific to Sète (Figure 1D). For the sediment samples, PCoA and Unifrac distance matrix showed that the beta-diversity was significantly different between each site, with a bit more homogeneity between samples from the same site than what was observed in the water samples (Figure 1E and 1F). The group of samples from Banyuls-sur-Mer and Sète overlapped while the group of samples from Thau were very distinct suggesting that Amoebozoa communities in the sediments from Banyuls-sur-Mer and Sète are more similar between them than the community from Thau. The repartition of ASVs by site in sediments showed that out of the 363 ASVs, 145 ASVs (39.9%) were specific to Banyuls-sur-Mer while only 90 ASVs (24.8%) and 85 ASVs (23.4%) were specific to Thau and Sète respectively (Figure 1F). All of these results showed that Amoebozoa beta-diversity was not stable and different communities between contrasted sites are observed in the water column and in the sediments. While the communities seem more stable in sediments than in the water fraction, the Amoebozoa community assemblages are more specific to each site in the sediments. Altogether, these results suggest that most of the taxonomic diversity specific to each environment appears to be at the sub-family taxonomic level as a large majority of ASVs belonging to each family were found in all the sampling sites, whereas a very limited number of ASVs (only 9 ASVs) belonging to different families were present ubiquitously and common to all the sampling sites. This was best illustrated by a phylogenetic analysis of all the ASVs which highlighted intra-family branches of ASVs sharing similar ecological characteristics like sampling sites and fraction (Figure 3).

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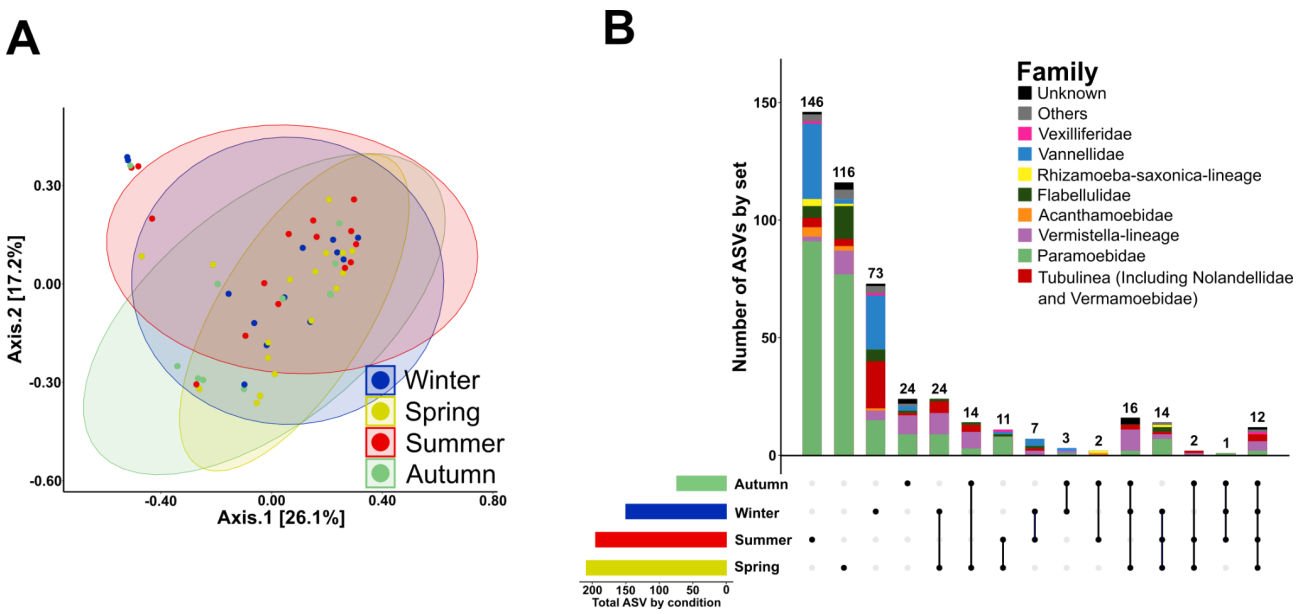

Figure 2. Seasonal variations of Amoebozoa diversity are very heterogeneous, but more different ASVs are found in Spring and Summer than in Winter and Autumn. PCoA of the unweighted Unifrac matrix distance revealed that the beta-diversity of Amoebozoa found at the different seasons is highly variable and overlaps between seasons (A). ASVs distribution revealed that the total number of different ASVs is higher during Spring and Summer than during Winter and Autumn, ASVs belonging to each family are found at all seasons in varying numbers (B).

Seasonal variations of amoebozoa diversity are present at the sub-genus level

We next attempted to see if our dataset would allow us to evaluate seasonal variations of Amoebozoa diversity regarding both fractions and the three sites. No significant differences for the alpha-diversity or beta-diversity (Table S2 and S3). The repartition of ASVs between seasons highlighted some noticeable differences with a higher number of ASVs found in Summer and Spring and lower number of ASVs found in Winter and Autumn (Figure 2A and 2B). Indeed, out of the 465 ASVs, 146 ASVs (31.4%) and 116 ASVs (24.9%) were specific to Summer and Spring respectively while only 73 ASVs (15.7%) and 24 ASVs (5.2%) were specific to Winter and Autumn respectively (Figure 2B). The specific ASVs identified during Summer and Spring are very similar with some abundance differences. They comprise ASVs belonging to Rhizamoeba-saxonica lineage, Vermistella lineage, Tubulinea as well as Flabellulidae, Acanthamoebidae, Vannellidae and Paramoebidae families (Figure 2B). Representatives of most Amoebozoa genera were present in Winter and Fall as in Summer and Spring but overall diversity was less dominated by ASVs belonging to the Paramoebidae family as seen in Spring and Summer (Figure 2B). Such sub-genus level variations were clearly apparent in the phylogenetic classification of ASVs, with some intra-genus clades that were particularly present at one particular season (Figure 3). As for example, among Paramoebidae, ASVs of clade 11 were found only during summer in the Thau lagoon,

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whereas among Vannellidae, ASVs of clade 2 were found in Winter in the Thau Lagoon, and ASVs belonging to Clade 1 and Clade 3 were found during Summer in Banyuls (Figure 3). All of these results suggest the presence of contrasted seasonal variations at the sub-genus level, but our dataset and the lack of taxonomic information did not allow to highlight statistically significant seasonal variations of the Amoebozoa diversity.

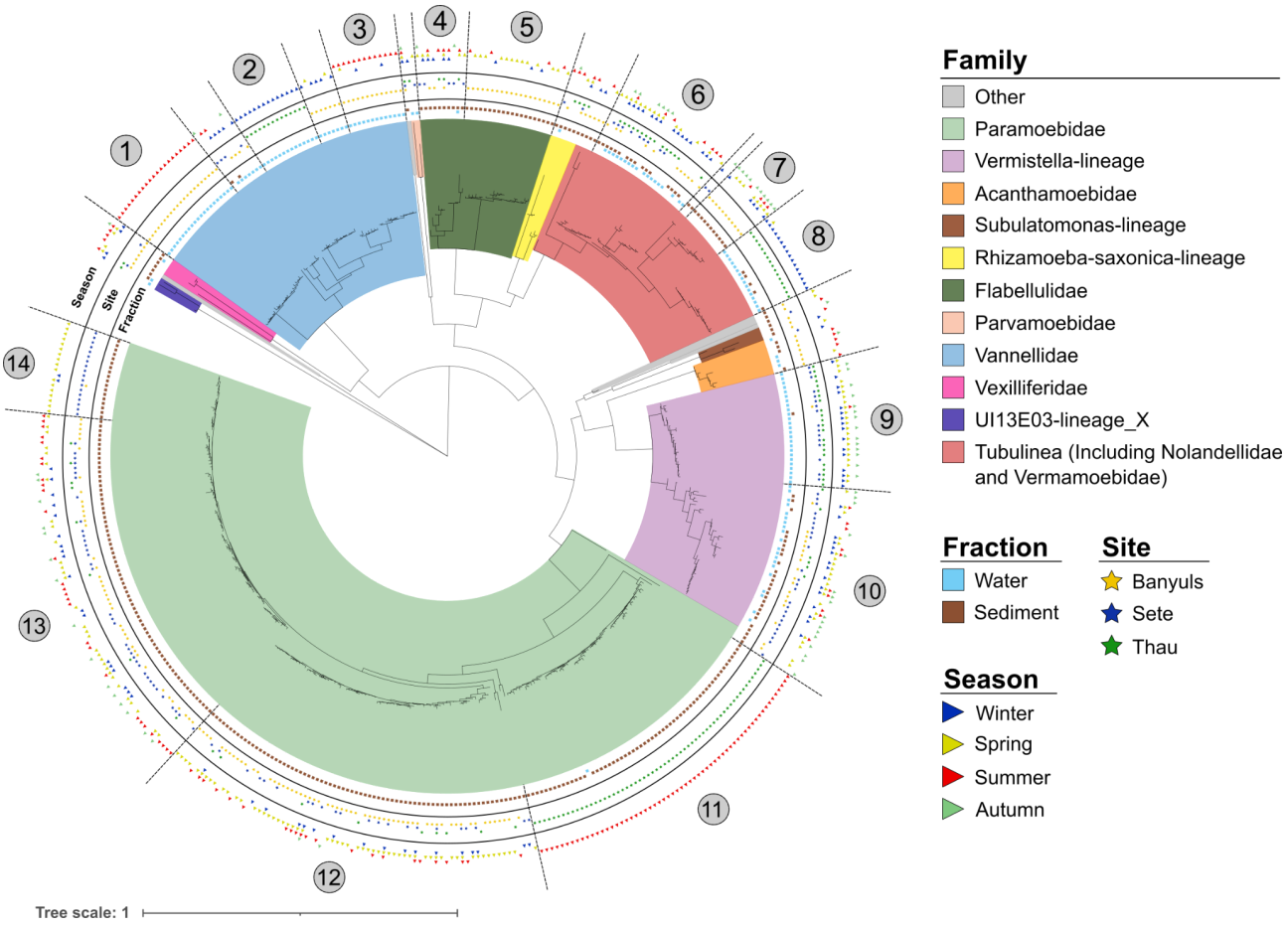

Figure 3. Phylogeny of ASVs highlights intra-family clades of Amoebozoa that are present in varying habitats. The phylogenetic classification of the ASVs was performed using MAFFT and FastTree (Maximum Likelihood tree) and annotated using iTOL software highlighting fraction, sites and season variables.

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Diverse Amoebozoa taxonomic groups harbor varying predation capacities against different opportunistic vibrios.

As the diversity of predation-prey interactions and their specificity remain poorly studied regarding Amoebozoa diversity. We wondered whether different opportunistic vibrios that harbor different anti-eucaryotic virulence mechanisms would select for different predators. Therefore, at four different months of the sampling campaign, *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi* A01 were used as nutritive sources in addition to the regular *E. coli* SBS363 (Figure S1). As pathogenic vibrios abundance in the two different fractions, water column and sediments, differ during oyster mortalities (Lopez-Joven et al., 2018), we chose 2 months during oyster mortality events (May 2017 and October 2017) and 2 months without mortality events (January 2018 and February 2018). The highest contrast of alpha diversity was observed between *V. harveyi* A01 lawn compared to alpha diversity observed with *V. tasmaniensis* LGP32 lawn as revealed by all the alpha-diversity indexes (Table S6), while the alpha diversity observed with *E. coli* SBS363 and *V. crassostrea*e J2-9 lawns were intermediate. Beta diversity didn't show significant differences between the four kinds of preys (Figure 4A and Table S6). The repartition of ASVs confirms results from alpha and beta diversity. Indeed, we identified the maximum of specific ASVs and the maximum diversity with A01 lawn (140 ASVs representing 29.5% of the 474 total ASVs) with specific ASVs belonging to the *Paramoebidae* family that dominated the observed diversity and contrasted between the four different preys (Figure 4B). Among all the detected ASVs 58,7% were found only on one type of bacterial lawn whereas 41,3% were found on at least two different types of bacterial lawn. Interestingly, 39 ASVs, representing 8,2% of the total ASVs, and belonging to diverse Amoebozoa families had the capacity to feed and grow on the four different types of bacterial lawn suggesting a less specific predatory activity (Figure 4B).

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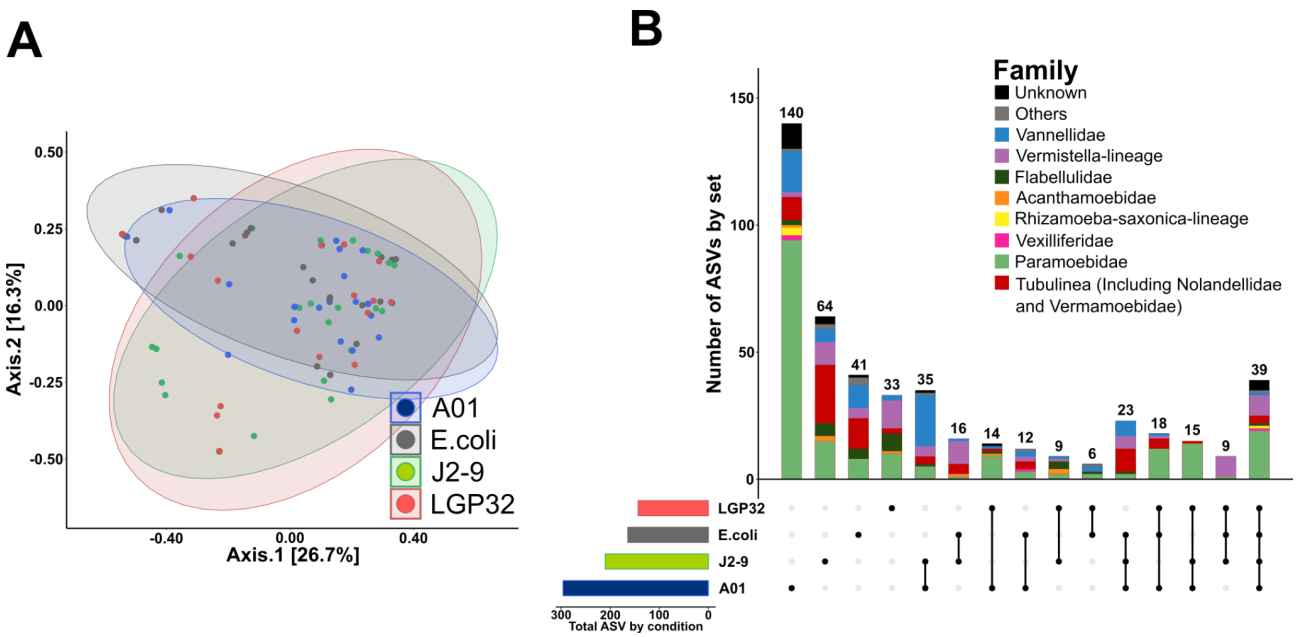

Figure 4. Selection on different bacterial prey revealed intra-family variations in predation capacity of Amoebozoa against different opportunistic vibrios. PCoA of the unweighted Unifrac matrix distance revealed that the beta-diversity of Amoebozoa that grew on the different bacterial prey is highly variable and mostly overlaps between conditions (A). ASVs distribution revealed that the total number of different ASVs is higher on *V. harveyi* A01 compared to the three other bacterial lawn, but 39 ASVs belonging to the different Amoebozoa families were able to grow on the four different bacterial lawn (B).

Vibrio tasmaniensis LGP32 strongly inhibited the growth of Vannellidae

As the two most contrasted families appeared to be the Paramoebidae and Vannellidae as a matter of sampling fraction, sampling sites and predatory capacities we analyzed in greater detail these two families. Phylogenetic classification of Vannellidae ASVs revealed three distinct clades with different characteristics. Clade 1 was composed of relatively distant ASVs identified on the four bacterial lawns, mainly from the water samples with three ASVs from sediments at the 3 sites during the four seasons (Figure 5). Clade 2 gathers more closely related ASVs mainly found on the SBS363 lawn with 2 ASVs and 4 ASVs from LGP32 and A01 lawns, only in the water column, at Thau and during February (Figure 5). Clade 3 is composed of ASVs found mainly on A01 and J2-9 lawns with some ASVs found on SBS363 lawn, only in the water column and at Banyuls-sur-Mer and mainly during January and February with 2 ASVs identified during May (Figure 5). These observations illustrate well the existence of geographical and seasonal specificities as a matter of phylogenetic diversity of Vannellidae. Additionally, amoebae belonging to the Vannellidae appeared much less prone to grow on LGP32 lawn than on the other bacterial lawns, only few ASVs were identified from samples grown on LGP32 lawn. These results are reminiscent of our previous study showing that LGP32 is resistant to predation by the amoeba *Vannella* sp. AP1411 (Robino et al., 2020).

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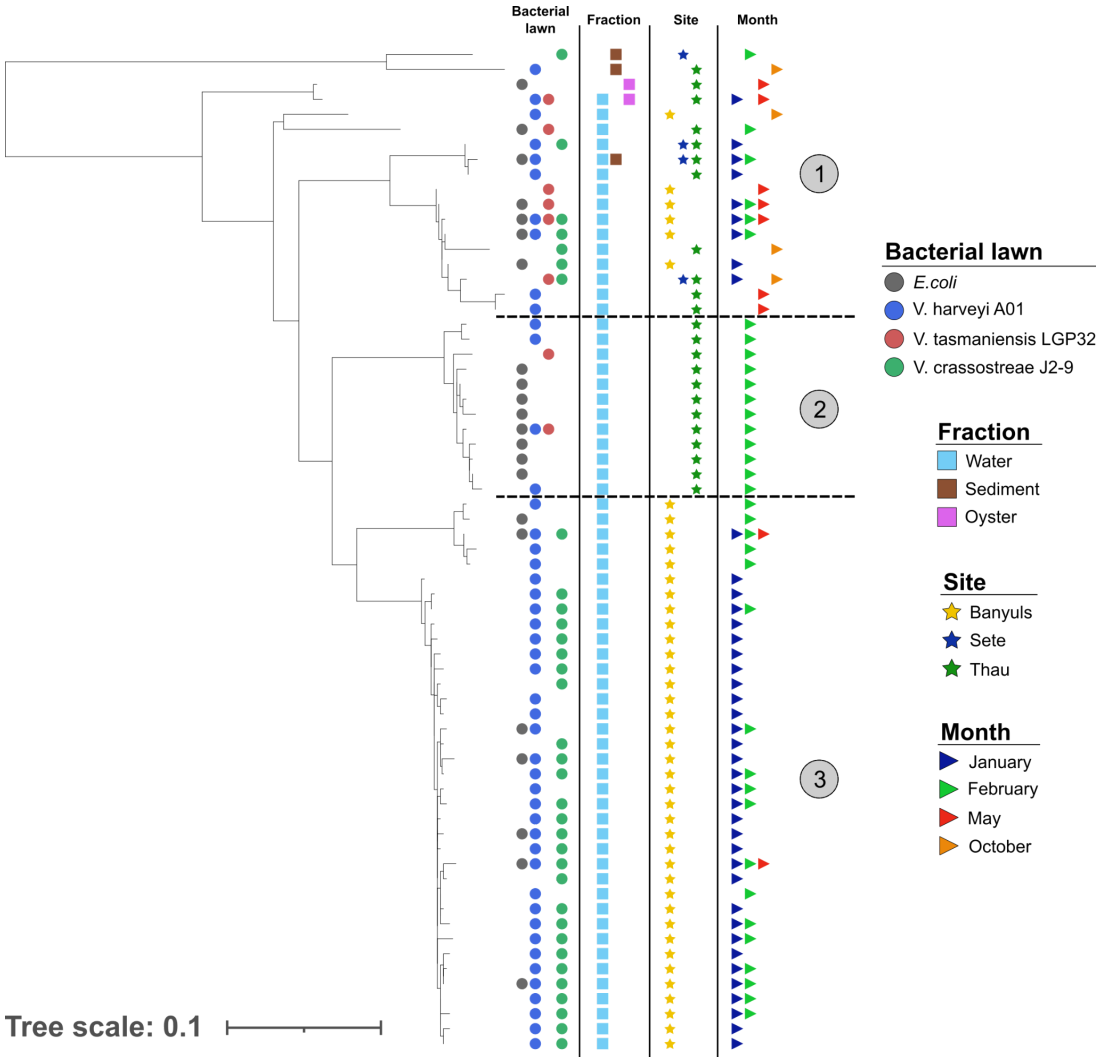

Figure 5. Phylogeny of ASVs highlights clades of Vannellidae that were found in different environments and harbor different predation capacities. The phylogenetic classification of the ASVs was performed using FastTree (Maximum Likelihood tree) and annotated using iTOL software highlighting fraction, sites and season variables. Clade 1 was heterogenous and found in varying conditions but was able to grow using any of the bacterial preys, clade 2 was found in Thau in February and grew mostly on E.coli, and clade 3 was found in Banyuls in January and February mostly and could not grow on LGP32.

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Paramoebidae represent a highly diversified taxonomic group with varying predation capacities.

The Paramoebidae represented the amoebozoa family with the highest number of identified ASVs (Figure 6). Moreover, this family appeared to contain ASVs with some harboring a tendency toward generalist predator capacity and some other appearing more specialized but most of them showed a high capacity to feed on *V. harveyi* A01. There were two clades (Clade 3 and 5) able to feed on the four nutritive sources, mainly found in the sediments, at the three sites for the clade 3 with a majority at Banyuls-sur-Mer and Sète for the clade 5, during the four months sampled with less ASVs during October. In addition, there were four more specialized clades identified (Clade 1, 2, 4 and 6) showing some differences. Clade 1 and 4 were similarly found in the water column, at Banyuls-sur-Mer and in October. These two clades are interesting because out of the total ASVs, very few have been identified during October. Clade 2 and 6 are similar and mainly identified at Sète and during May. However, clade 2 was found principally in the water column and clade 6 in the sediments. Thus, these results highlight that Paramoebidae were the largest family of Amoebozoa that was sampled during our survey and their ecological dynamics as well as their predatory capacities can vary greatly.

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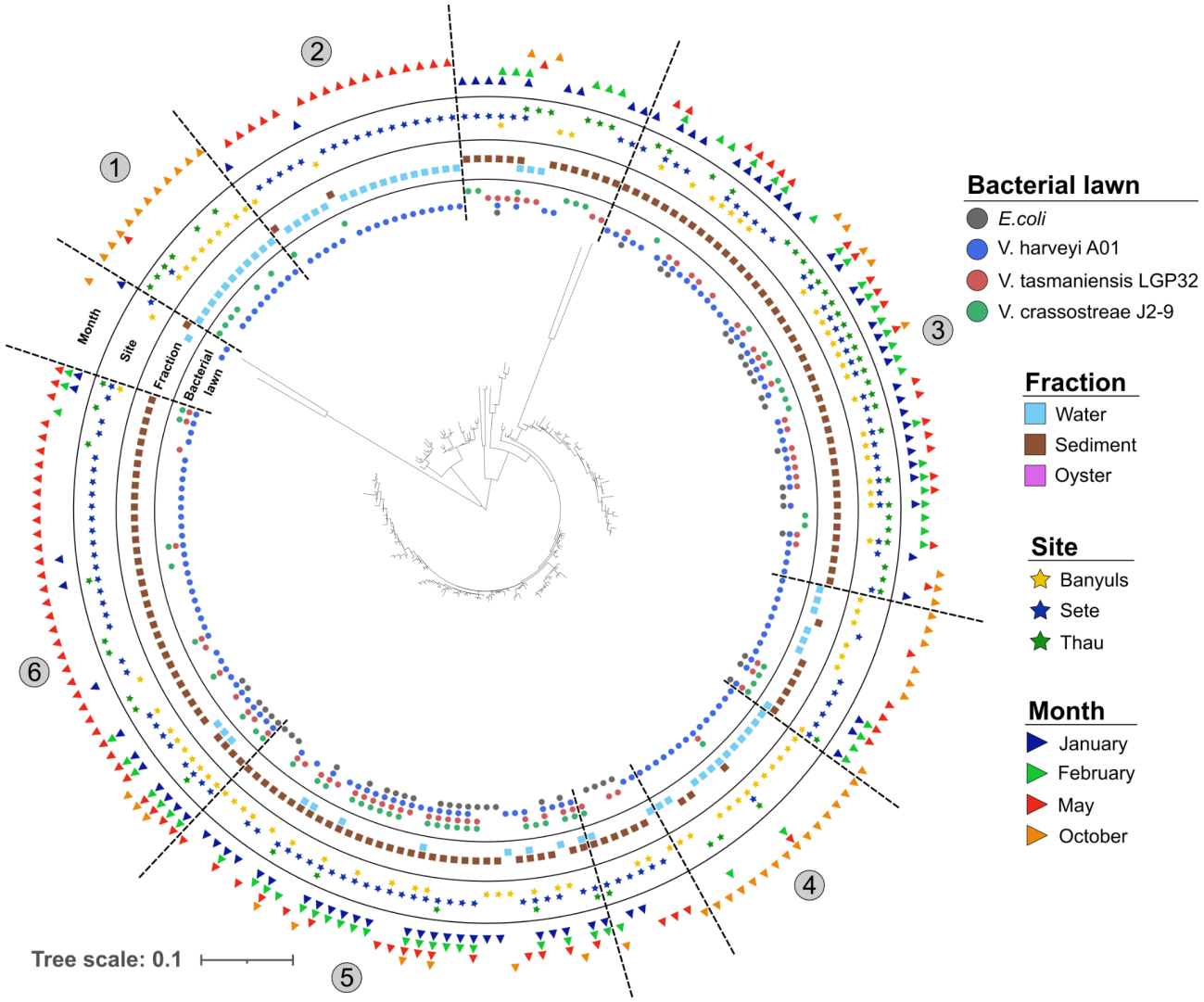

Figure 6. Phylogeny of ASVs highlights different clades of Paramoebidae that were found in different environments and harbor different predation capacities. The phylogenetic classification of the ASVs was performed using MAFFT and FastTREE FastTree (Maximum Likelihood tree) and annotated using iTOL software highlighting fraction, sites and season variables. Some clades appeared ubiquitous and mostly generalist as they could grow on the four bacterial lawns like clade 3 and 5, whereas other clades appeared to have more restricted habitats with a more restricted predation capacity.

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Different predation capacities against phylogenetically related vibrios of the Splendidus clade were functionally validated using Vannellidae and Paramoebidae isolates.

In order to confirm further that Vannellidae growth was inhibited by *V. tasmaniensis* LGP32 and Paramoebidae growth was inhibited by *V. crassostrae* J2-9, we first compared the total abundance of the ASVs belonging to these two families among all our samples obtained on LGP32 lawns and J2-9 lawns. The relative abundance of Paramoebidae was found to be significantly superior to the abundance of Vannellidae on LGP32 lawn (Fisher Test $p<0.05$; Figure 7A). On the contrary, the abundance of ASVs belonging to Paramoebidae obtained on J2-9 lawn was found to be significantly lower than the ASVs abundance of Vannellidae (Fisher Test $p<0.05$). These results suggested that LGP32 inhibits growth of Vannellidae whereas Paramoebidae can feed on this vibrio strain, and on the contrary, J2-9 tends to inhibit growth of Paramoebidae while Vannelidae grows better using LGP32 strain as a food source. To validate functionally these results, we performed grazing experiments using LGP32-GFP and J2-9-GFP strains to quantify the predation activity of *Vannella sp.* AP1411 and *Paramoeba atlantica* strain CCAP1560/9 over time. Results showed that *Vannella sp.* AP1411 could not graze on LGP32 (as publish previously Robino *et al*. 2019) whereas J2-9 was rapidly eliminated has shown by the fast decrease of the relative fluorescence of GFP that was reduced down to 0.4 ratio after five days (ANOVA, $p<0.001$; Figure 7B). In contrast, grazing experiments using *Paramoeba atlantica* showed that LGP32 was grazed faster than J2-9 by *Paramoeba atlantica,* although the differences between the two strains of vibrios were less contrasted than *Vannella sp.* AP1411 (ANOVA, $p<0.05$; Figure 7C). Altogether, these functional data confirmed that the two genera of Vannellidae and Paramoebidae can harbor distinct predation capacity against different pathogenic vibrios belonging to the Splendidus clade.

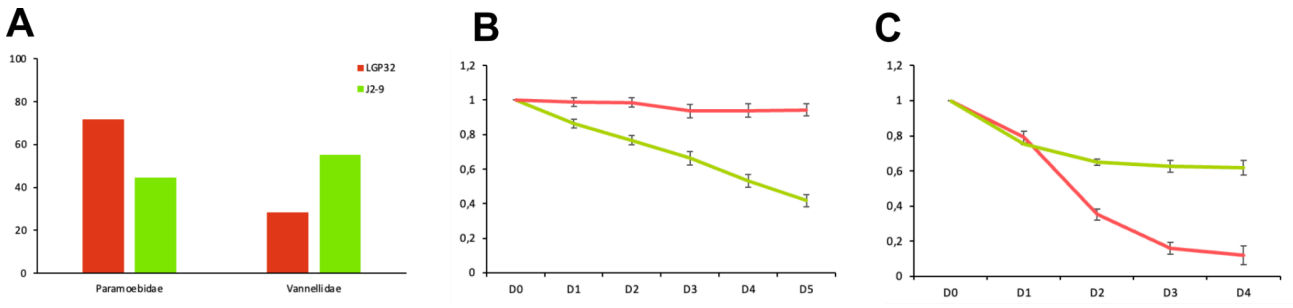

Figure 7. *V. tasmaniensis* LGP32 and *V. crassostreae* J2-9 harbor contrasted resistance to the predation by Vannellidae and Paramoebidae. Abundance of ASVs belonging to Paramoebidae or Vannellidae that grew on LGP32 or J2-9 lawns was significantly different (Fisher test; $p<0.05$) (A). Grazing experiments using *Vannella sp.* AP1411 showed that J2-9 was more sensitive than LGP32 (one experiment representative of two independent experiments, RM-ANOVA test; $p<0.01$). Grazing experiments using *Paramoeba atlantica* showed that J2-9 was more resistant than LGP32 (one experiment representative of two independent experiments, RM-ANOVA test; $p<0.001$).

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Discussion

To investigate the ecology of FLA populations and their interactions with *Vibrionaceae* in Mediterranean coastal waters, we conducted a monthly sampling for one year in three contrasted environments. Free-living amoebae populations were isolated by culturing water and sediment samples on different bacterial lawns including *E. coli* SBS363 or *V. tasmaniensis* LGP32, *V. crassostreae* J2-9 and *V. harveyi A01*. Analysis of protist diversity in the different samples by v4-18S rRNA barcoding revealed distinct communities of Amoebozoa between the sediments and the water column, with Vannellidae significantly enriched in the water column whereas Paramoebidae were significantly enriched in the sediments. Moreover, the diversity of Amoebozoa in the sediments was more specific to the sampling sites than in the water column. Selection of grazers on different bacterial lawns revealed that *V. tasmaniensis* LGP32 inhibited the growth of most Vannellidae whereas *V. crassostreae* J2-9 tended to inhibit growth of Paramoebidae. These differences were further confirmed in functional grazing assays using isolates belonging to each Amoebozoa taxonomic group. Altogether, our results highlight that Amoebozoa diversity in marine waters and population dynamics still need to be studied in a more comprehensive manner and the role of these diversified grazers in shaping vibrio communities is still poorly characterized.

In contrast with our previous observations, by performing a more extensive field survey, in addition to Vannellidae, we found many other Amoebozoa belonging to Paramoebidae, Tubulinea (regrouping Vermamoebidae and Nollandelidae), Rhizamoeba-saxonica, Vermistellae, Flabellulidae, Vexilliferidae and Paravmoebidae families. Although some were more prevalent than others including Paramoebidae, Vannellidae, Vermistellae, Tubulineae and Flabellulidae, most had already been reported from diverse marine environments previously (Garstecki and Arndt, 2000; Latifi et al., 2020; Mohd Hussain et al., 2022; Munson, 1992; Page, 1983; Samba-Louaka et al., 2019; Sousa-Ramos et al., 2022). One limitation of our study was the use of universal v4-18S primers to analyze FLA communities. They are very diverse and paraphyletic and the use universal v4-18S primers may have precluded to identify the full diversity in our samples, especially in other taxonomic phylum than Amoebozoa like Heterolobosea for example (Delafont et al., 2022). Beside Amoebozoa the other phylum of grazers that was detected were ASVs belonging to Cercozoa, however the taxonomic affiliation was limited due to the lack of reference sequences in 18S databases. Even for Amaoebozoa, the taxonomic affiliation based on 18S rRNA partial sequences can be inconsistent at the species level and in some cases even at the genus level, this is the reason why we analysed the diversity of Amoebozoa at the family level.

A limited number of studies have performed a systematic sampling survey over time to compare the diversity of FLA diversity between sites and between different fractions in marine environments has reported here. Overall, the higher diversity of Amoebozoa observed in the

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sediments compared to the water samples is consistent with the benthic grazer lifestyle. Distinct grazers communities between sediments and the water column were also reported by other studies that found different protist communities between surface and deep waters from the same location as well as between various depths of sediments (Countway et al., 2007; Orsi et al., 2011; Smirnov, 2004). Here ASVs belonging to the different families were found in the different environments, so most of the diversity differences appeared to be at the intra-family and probably intra-genera levels. However, the ASVs distribution between the two different habitats was particularly contrasted for the Vannelliadae specifically enriched in the water column and the Paramoebidae specifically enriched in the sediments. This could relate to a particular lifestyle and adaptations as Vannellidae are well known for forming characteristic elongated filopodia in their planktonic shape, which may represent an advantage to floating and moving using water flows (Smirnov et al., 2007). In contrast, Paramoebidae have often been reported in marine sediments and are rare in freshwaters suggesting a specialized benthic lifestyle (Page, 1983; Kudryavtsev et al., 2011; Volkova et al., 2019; Volkova and Kudryavtsev, 2017). Amoebozoa communities in the sediments were found here to be more stable over the sampling months but contrasted between sites whereas the Amoebozoa communities from the water samples were found more variable and less distinct between sampling sites. This may be explained by the differences in physical characteristics and fluctuations of the two fractions. The water column is subject to significant variations and rapid mixing through water movements and currents which can increase connectivity between environments, unlike the sediments which are more preserved from mixing events. Moreover the sediment physico-chemical composition between sites (depth, sand/mud ratio, and oxygen concentration for example) are likely to be different which could have a strong impact on the niche characteristics and the assemblage of microorganism communities (Kim et al., 2014; Smirnov, 2004). More efforts in exhaustive comparative analysis between contrasted environments are still needed to decipher the major environmental factors shaping FLA communities.

Unfortunately, analyzes of seasonal variations were impaired by the heterogeneity between samples over the sampling period. Still, ASVs distribution between seasons revealed that the number of different ASVs was higher during spring and summer and lower during autumn and winter (Figure 4B). These observed trends are in agreement with other studies showing seasonality in protist diversity (Berdjeb et al., 2018; Fu et al., 2020; Kim et al., 2014). Phylogenetic analysis highlighted that inside each family some clade of ASVs appeared either ubiquitous and found in all fractions, at all sites and all seasons, while other ASVs clade seemed more restricted. The Tubulinea taxon is the best example to illustrate this as it contains three completely different taxonomic groups of ASVs (Figure S6). One of them tends to be ubiquitous and found in both fractions, at the 3 sites and during the four seasons (Clade 6). The two other clades (Clade 7 and 8) seem to be more

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specialized showing several differences suggesting two different lifestyles. A more resolutive sampling strategy may be required to fully capture the seasonal variations of the different taxonomic groups.

Biotic factors such as preys can also influence assemblages of heterotrophic protist communities. Some preys have acquired the ability to resist predation using various extracellular and intracellular mechanisms and even sometimes kill them (Matz and Kjelleberg, n.d.; Pernthaler, 2005; Robino et al., 2020). Amaro and his collaborators showed that *Legionella pneumophila* can shape protist communities in microcosm experiments, having significant effects on the abundances of Cercozoa, Amoebozoa and Heterolobosea phyla (Amaro et al., 2015). Here we wondered whether three different strains of opportunistic vibrios with different anti-eukaryotic virulence mechanisms could influence the diversity of FLA communities. Surprisingly, the highest diversity was observed when using *V. harveyi* A01 as a food source compared to the other strains showing three times more specific ASVs than *E. coli* SBS363 (Figure 4B). On the contrary, *V. tasmaniensis* LGP32 and to a lower extant *V. crassostreae* J2-9 were the most selective bacterial lawns as we found a lower amount of specific ASVs suggesting that their virulence mechanisms could be efficient against a larger diversity of Amoebozoa. We observed more generalist or more specialist clades of ASVs in most Amoebozoa families with some ASVs that could grow using any of the prey suggesting the existence of generalist species with a wide range of prey and habitats. However their number was limited and a lot of ASVs harbored more specialized grazing capacity towards the different preys. Among them, a majority of Vannellidae and a large part of Tubulinea could not grow in the presence of LGP32. This is reminiscent of our previous study showing that LGP32 is resistant to predation by *Vannella sp*. AP1411 (Robino et al., 2020). Paramoebidae represented the family that contained the highest number of different ASVs but their growth was particularly inhibited by J2-9. The contrasts of Vannellidae and Paramoebidae prey specificity was functionally confirmed using *Vannella sp.* AP1411 and *Paramoeba atlantica* with both vibrios. These results underline that although intra-genera variation of predation capacities are observed the resistance to grazing of some vibrios, like LGP32 and J2-9, can impact large taxonomic groups of Amoebozoa. Habitat seasonal variations of *V. tasmaniensis* and *V. crassostreae* have been reported previously in the Thau lagoon. They are more abundant in the water column during the warmer seasons and mostly found in the sediment during the colder seasons (Lopez-Joven et al., 2018). As Vannellidae are more prevalent in the water column and Paramoebidae are more prevalent in the sediments, the differences of Amoebozoa diversity in the two different habitats could have opposite effects in shaping vibrio communities and differentially impact the dynamics of different pathogens over the seasons. In a similar manner, *in vitro* functional grazing assays were used by others to highlight varying predation capacity, prey specificity and to predict potential impact on bacterial communities

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in the plant rhizosphere (Amacker et al., 2022). Interestingly, some interactions between vibrios and Paramoebidae were reported previously, their role in vibrios population dynamics and as a potential intracellular niche need to be studied further (Lee et al., 2013; MacPhail et al., 2021).

In conclusion, our study provides a better understanding of Amoebozoa grazers communities in the Mediterranean coastal environments and sets the ground for further in depth functional studies between different Amoebozoa taxonomic groups and the bacterial communities present in those environments. By using diverse marine vibrios, we bring new evidence that Amoebozoa-vibrios interactions are highly diverse and underline the need to study further Amoebozoa diversity and their role in vibrio communities dynamics and pathogen emergence.

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Conflict of interest

The authors declare that there are no conflict of interests related to this work.

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Supplementary Figures

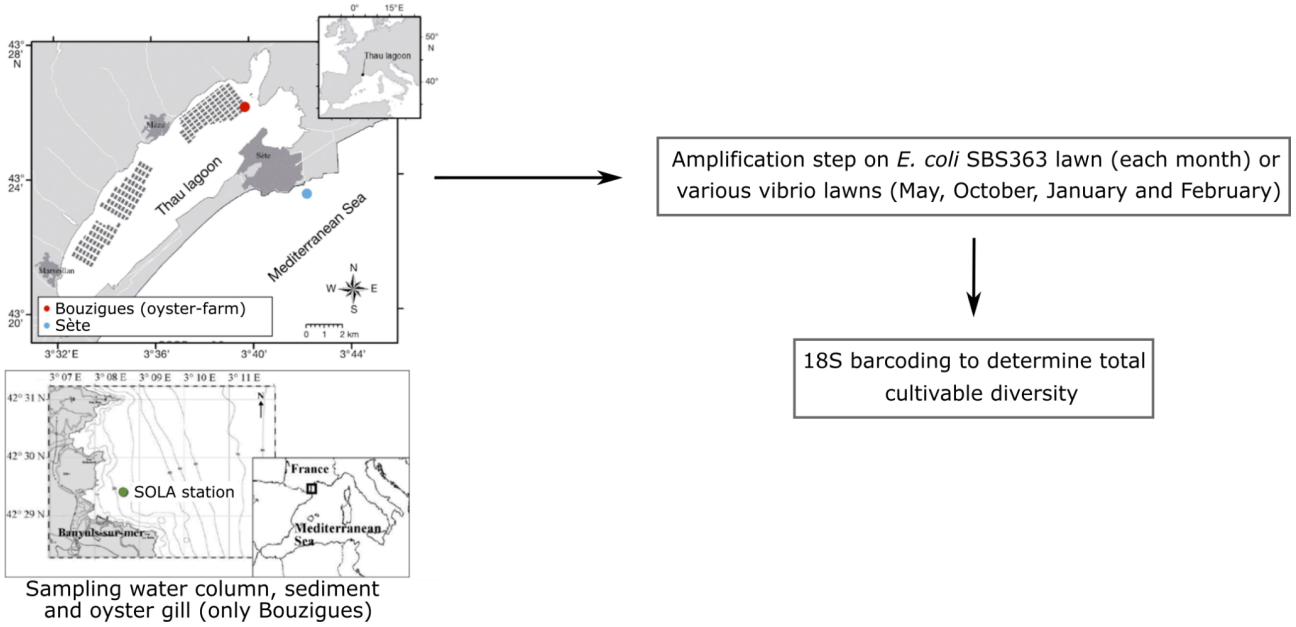

Figure S1. Simplified diagram of the sampling strategy. Briefly, water column and sediment were sampled monthly during one year at Sète, Banyuls-sur-Mer and Thau lagoon near the Bouzigues oyster farming area. Isolation of grazers was performed by selective growth and migration on agar plates covered with E. coli SBS363 lawns. In May, October, January and February, isolation of grazers from the same samples was performed additionally on vibrios lawns (V. harveyi A01, V. tasmaniensis LGP32 and V. crassostreae J2-9). All the grazers that grew and migrated out of the initial sample were recovered and total DNA extracted for v4-18S barcoding.

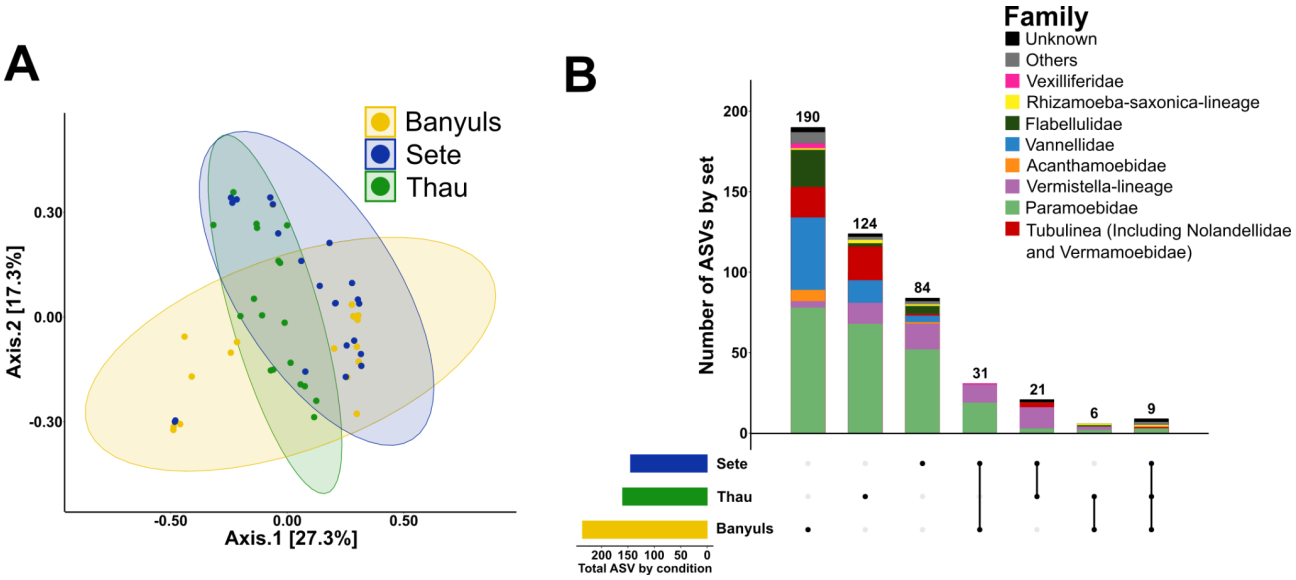

Figure S2. Amoebozoa diversity according to the sampling site. PCoA of the unweighted Unifrac matrix distance showing the beta-diversity of Amoebozoa in all the samples and fractions between the three sampling sites (A). ASVs distribution revealed that more ASVs were identified in Banyuls-sur-Mer compared to the two other sites, very few ASVs were present in more than one site, and only 9 ASVs were found in all sites (B).

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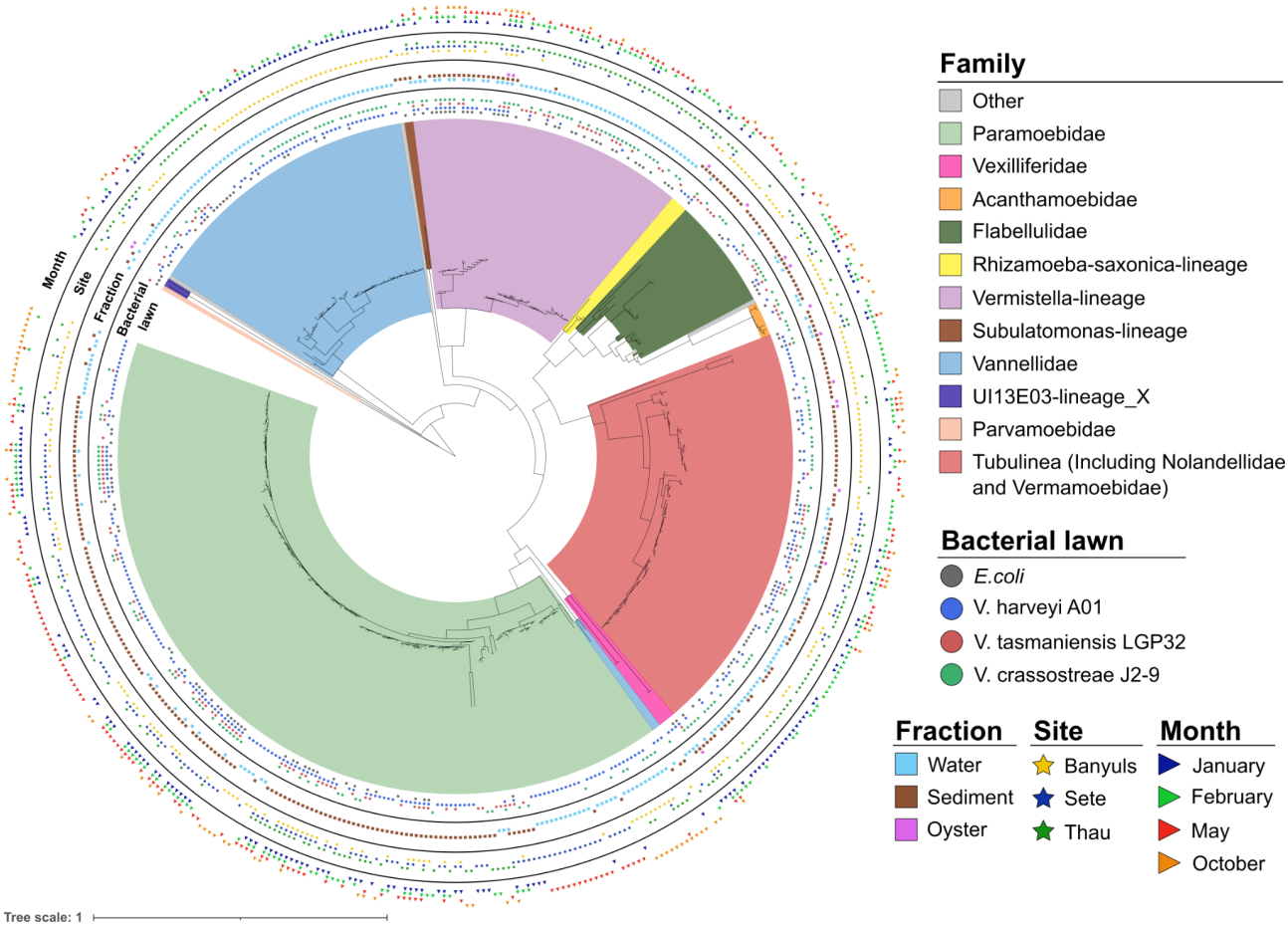

Figure S3. Phylogeny of all the ASVs identified on the four different bacterial lawns. The phylogenetic classification of the ASVs was performed using MAFFT and FastTREE FastTree (Maximum Likelihood tree) and annotated using iTOL software highlighting fraction, sites and season variables.

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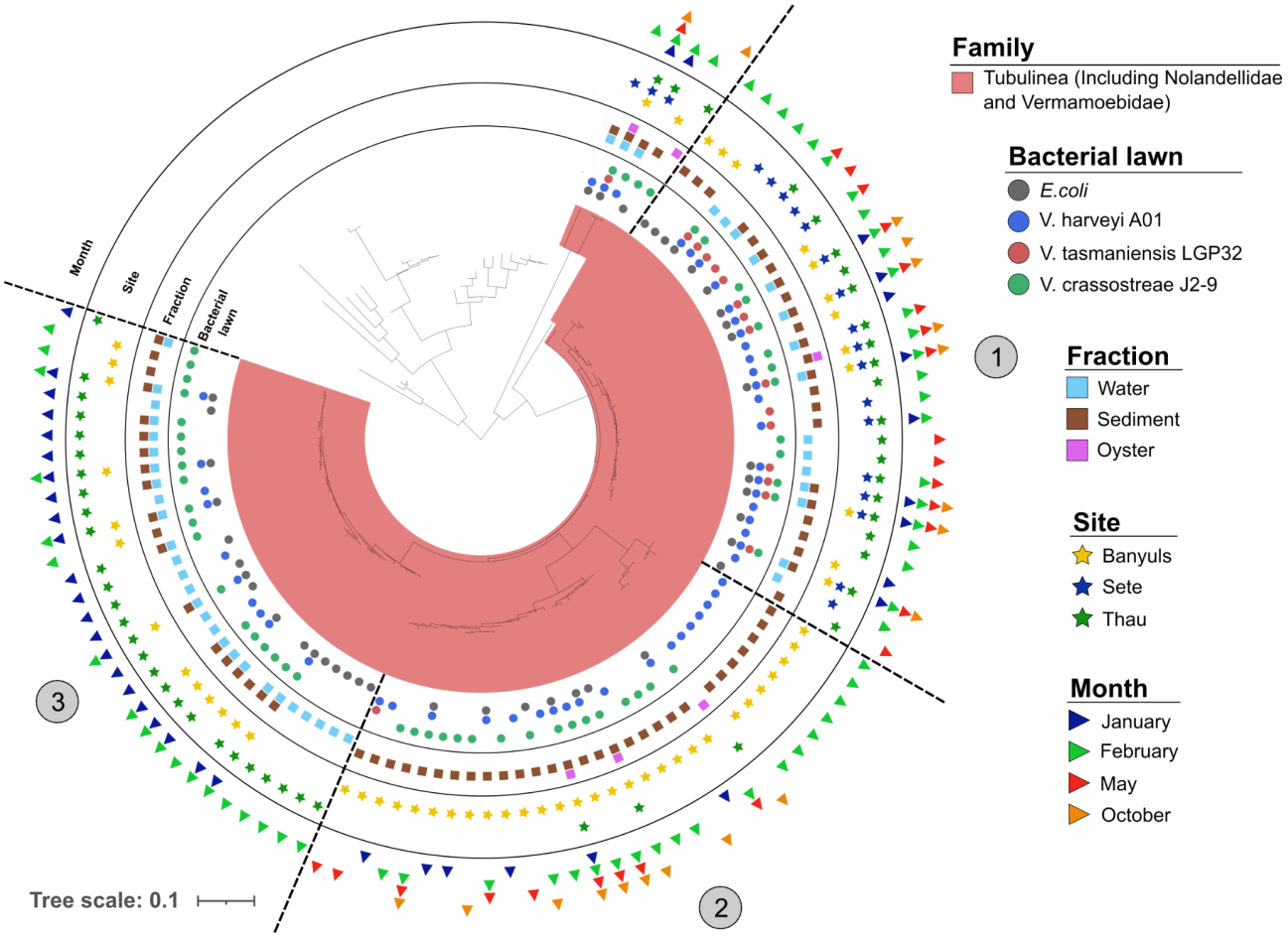

Figure S4. Phylogeny of ASVs highlights different clades of Tubulinea that were found in different environments and harbor different predation capacities. The phylogenetic classification of the ASVs was performed using MAFFT and FastTREE FastTree (Maximum Likelihood tree) and annotated using iTOL software highlighting fraction, sites and season variables. Some clades appeared ubiquitous and mostly generalist as they could grow on the four bacterial lawns like clade 1, whereas clade 2 and 3 appeared to have more restricted habitats with a more restricted predation capacity.

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Table S2

(A) Statistics of the alpha diversity using the Chao1, Shannon and InvSimpson diversity indexes on Fraction, Site and Season variables.

ANOVA on Chao1, Shannon and InvSimpson diversity indexes

		Chao1		Shannon		InvSimpson	
		F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
Fraction	Water column vs Sediment	6.525	0.0138 (*)	3.734	0.0591	2.349	0.132
Site	Banyuls vs Sète	0.698	0.40912	0.002	0.961	0.025	0.875
	Banyuls vs Thau	0.978	0.330	0.010	0.922	0.967	0.333
	Sète vs Thau	0.078	0.782	0.189	0.667	1.061	0.310
Season	Winter vs Autumn	1.027	0.32244	1.984	0.174	0.133	0.719
	Winter vs Spring	0.114	0.738	0.113	0.7394	0.466	0.500
	Winter vs Summer	0.383	0.5415	0.199	0.659	0.020	0.890
	Autumn vs Spring	1.432	0.242	3.023	0.0939	0.712	0.406
	Autumn vs Summer	0.551	0.466	0.097	0.75809	0.010	0.921
	Spring vs Summer	0.000	0.990	0.599	0.445	0.231	0.634

(B) Statistics of the alpha diversity using the Chao1, Shannon and InvSimpson diversity indexes Site and Season variables within Water samples.

ANOVA on Chao1, Shannon and InvSimpson diversity indexes

		Chao1		Shannon		InvSimpson	
		F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
Water	Banyuls vs Sète	1.09	0.313	0.426	0.523895	0.469	0.504
	Banyuls vs Thau	0.439	0.518	0.483	0.4977	1.562	0.2305
	Sète vs Thau	2.807	0.112	1.413	0.25093	2.589	0.1260

(C) Statistics of the alpha diversity using the Chao1, Shannon and InvSimpson diversity indexes Site and Season variables within Sediment samples.

ANOVA on Chao1, Shannon and InvSimpson diversity indexes

		Chao1		Shannon		InvSimpson	
		F value	Pr (>F)	F value	Pr (>F)	F value	Pr (>F)
Sediment	Banyuls vs Sète	1.110	0.306	0.054	0.819	0.000	0.997
	Banyuls vs Thau	1.770	0.205	0.325	0.5775	0.055	0.817
	Sète vs Thau	0.023	0.881	0.122	0.73094	0.054	0.8182

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Table S3

(A) Statistics of the beta diversity using the DEseq2 normalization method and Unifrac distance matrix depending on Fraction, Site and Season variables.

Adonis test on multiple variables

Permutation: free

Number of permutations: 9999

		R^2	Pr (>F)
Fraction	Water column vs Sediment	0.19817	0.0001 (***)
Site	Banyuls vs Sète	0.05936	0.0396 (*)
	Banyuls vs Thau	0.11076	0.0001 (***)
	Sète vs Thau	0.12165	0.0002 (***)
Season	Winter vs Autumn	0.04621	0.385
	Winter vs Spring	0.02893	0.5399
	Winter vs Summer	0.02488	0.7264
	Autumn vs Spring	0.04115	0.2992
	Autumn vs Summer	0.04445	0.3417
	Spring vs Summer	0.05616	0.065

(B) Statistics of the beta diversity using the DEseq2 normalization and Unifrac distance matrix depending Site variable within Water samples.

Adonis test on multiple variables

Permutation: free

Number of permutations: 9999

		R^2	Pr (>F)
Water	Banyuls vs Sète	0.1262	0.0138 (*)
	Banyuls vs Thau	0.25348	0.0012 (**)
	Sète vs Thau	0.11507	0.0213 (*)

(C) Statistics of the beta diversity using the DEseq2 normalization and Unifrac distance matrix depending Site variable within Sediment samples.

Adonis test on multiple variables

Permutation: free

Number of permutations: 9999

		R^2	Pr (>F)
Sediment	Banyuls vs Sète	0.11439	0.0044 (**)
	Banyuls vs Thau	0.27517	0.0001 (***)
	Sète vs Thau	0.25265	0.0001 (***)

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Table S6

(A) Statistics of the alpha diversity using the Chao1, Shannon and InvSimpson diversity indexes on Strain variable.

ANOVA on Chao1, Shannon and InvSimpson diversity index

		Chao1		Shannon		InvSimpson	
		F value	**Pr (>F)**	**F value**	**Pr (>F)**	**F value**	**Pr (>F)**
Strain	SBS363 vs A01	4.964	0.0313 (*)	1.063	0.308	2.056	0.159
	SBS363 vs LGP32	0.275	0.602953	3.293	0.0769	3.279	0.0775
	SBS363 vs J2-9	0.237	0.62847	0.176	0.677	0.036	0.850
	A01 vs LGP32	6.951	0.011692 (*)	7.632	0.00847 (**)	9.680	0.00334 (**)
	A01 vs J2-9	2.646	0.11076	1.523	0.224	1.183	0.282
	LGP32 vs J2-9	0.792	0.378344	1.652	0.205	2.397	0.129

(B) Statistics of the beta diversity using the DEseq2 normalization method and Unifrac distance matrix depending on Strain variable.

Adonis test on multiple variables

Permutation: free

Number of permutations: 9999

		R^2	Pr (>F)
Strain	SBS363 vs A01	0.01926	0.5398
	SBS363 vs LGP32	0.02299	0.4333
	SBS363 vs J2-9	0.02015	0.481
	A01 vs LGP32	0.02948	0.22
	A01 vs J2-9	0.03109	0.1376
	LGP32 vs J2-9	0.0178	0.61