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Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*

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Abstract :

This study has two main objectives: (1) to implement a recycling aquaculture system (RS) for the larvae of the oyster Crassostrea gigas, and (2) to characterise the bacterial communities established in different compartments of this system. An RS with 25% fresh seawater addition per hour and another with no addition (0%) were compared with a flow-through system (FT). Larval survival was equivalent in RS and FT, but growth rate was 17% slower in RS than in FT. The physical chemical parameters remained stable, except for pH that decreased to 7.75 and salinity that increased to 37.5‰ in the RS 0%. In both systems, the cultivable bacteria were present in similar numbers in seawater (around 105 ml− 1) and in larvae (103 larva− 1) on day 15.

Bacterial assemblages, characterised by 454 pyrosequencing of the V1–V3 region of 16S rRNA, were highly similar (50–65%) for compartments, regardless of rearing system and sampling time, but the compartments were clearly different from one another. At the beginning of rearing, larval microbiota was mostly composed of Proteobacteria (~ 90%), with 47% Rhodobacteraceae (α-Proteobacteria). γ-Proteobacteria, including *Pseudoalteromonas*, *Alteromonas* and a few vibrios, declined in the rearing period (25% on day 7 to 9% on day 15). At the end of rearing, colonisation by two members of the Burkholderiales (β-Proteobacteria), 45% on average on day 15, had decreased overall diversity. Seawater microbiota was more stable with in all batches as one unclassified bacterium present in all batches (27 ± 7%), 42 OTUs of α-Proteobacteria (19 ± 7%) and 26 of γ-Proteobacteria (14%). Change was due notably to a species of Cryomorphaceae (Flavobacteria) that reached 15 ± 7% on day 15. Predatory bacteria, *Bdellovivrio* spp. and *Bacteriovorax* spp. were present (3–12%) and could participate in the regulation of bacterial populations. Bacterial assemblages in RS bioreactors remained stable and were mainly composed of Rhodobacteraceae, Rhizobiales and Planctomycetes. Only a few nitrite oxidisers were detected and no ammonia oxidisers, although nitrification was efficient in RS without addition of new water. The larval microbiota was made up of bacteria growing in seawater, but some such as the Burkholderiales could have come from the broodstock. Several bacteria predominant in seawater were first harboured in the algal culture.

Finally, despite sanitary measures in the hatchery (UV treatment and frequent cleaning), the diversity of

microbiota remained high, and it did not contain pathogenic bacteria. The presence of this microbiota might even be indispensable to ensure normal ontogenesis, as suggested by the holobiont concept.

Statement of relevance

RAS and control of microbiota should improve bivalve larval culture

Highlights

► Oyster larval survival was equivalent between recycling and flow-through rearing systems. ► Larval growth was slower in the recycling system than in the flow-through system. ► A specific and diverse microbiota was established in each system compartment: seawater, bioreactor and larvae. ► The type of rearing system did not greatly influence the microbiota, which seemed to be favourable to larval culture, with a very low occurrence of vibrios

Keywords : RAS, Bivalve larvae, Microbiota, Pyrosequencing, Hatchery

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1. Introduction

The main bacteria associated with bivalves and the influence of these bacteria on the health of their hosts have not been investigated a great deal until now, although many studies have been published on the microbiota of other marine invertebrates, especially shrimps, corals and sponges (Erwin et al., 2012; Schulze et al., 2006; Rungrassamee et al., 2013). These studies reported that gut-associated bacteria are under strong selection to constitute a resident microbiota that can perform essential functions for the host's health. The bacteria associated with bivalves were originally investigated to determine the cause of larval and post-larval mortality (Elston et al., 1981; Nicolas et al., 1996; Sainz-Hernández and Maeda-Martínez, 2005) and to detect bacteria that are harmful to human health (Charles et al., 1992). Initial studies on the microbiota harboured by larval and adult bivalves began in the 1980s, using cultural methods to isolate the bacteria and phenotypic methods to identify them. They showed that bivalves could easily filter and ingest exogenous bacteria from seawater (Prieur, 1976, 1982; Prieur et al., 1990) even though their ingestion and nutritional value were much lower than those of phytoplankton (Moal et al., 1996). According to these reports, the microbiota of larvae is mainly composed of bacteria from the surrounding seawater, with a higher prevalence of some bacterial groups, including Vibrionaceae, which presumably grow inside the gut (Prieur, 1976, 1982). In adults, these bacteria are replaced in the lower digestive tract by a more indigenous population after selective digestion and multiplication (Kueh and Chan, 1985). The existence of specific microbiota in bivalve larvae and adults has already been determined by a culture-dependent approach, although transient bacteria were frequently identified. Using a cultural approach combined with in situ hybridisation, Pujalte et al. (1999) observed a high occurrence of vibrios in the oyster *Ostrea edulis* collected from the Mediterranean, while the seawater contained mainly α-Proteobacteria. With the adoption of the metagenomic approach, the description of microbiota in bivalves has become more

accurate and exhaustive. Authors have found oyster microbiota to be very diverse, without a predominance of vibrios. Bacteria present in the digestive tract belong to a broad diversity of phyla, such as Firmicutes, Proteobacteria, Cyanobacteria and Spirocheates (Green and Barnes, 2010), as well as Planctomycetes, Verrucomicrobia, Fusobacteria, Tenericutes and Bacteroidetes (Fernandez-Piquer et al., 2012). Conversely, other authors reported that the dominant bacteria were limited to some bacterial groups, including some γ-Proteobacteria and Gram-positive bacteria (Hernandez-Zarate and Olmos-Soto, 2006). The dominant bacteria could also be restricted to particular bacterial species, such as *Burkholderia cepacia* with some Firmicutes and Spirochaetes in *C. gigas* and *C. corteziensis* (Trabal et al., 2012), or Planctomyces and Mollicutes in the stomach of *C. virginica* (King et al., 2012). In this case, the bacterial assemblages were distinct from those established in the lower gut, indicating a high specialisation by compartment. The microbiota of oyster gills was dominated by a few abundant taxa (*Sphingomonas* and *Mycoplasma*), and the rare taxa disappeared in disturbed conditions (Wegner et al., 2013). A correlation was also found between individual variations in gill microbiota and the genetics of the host, demonstrating that a strong interaction could exist between the host and its microbiota.

Although these studies assessed the occurrence of a specific microbiota, drawing conclusions about the core of resident bacteria, the stability of these consortia and their possible role in bivalve health is premature. The primary colonisation could be crucial in the establishment of microbiota in bivalves. Indeed, pioneer bacteria may possess an ecological advantage that enables them to persist at subsequent stages, as reported by Trabal et al. (2012). Nevertheless, these authors did not confirm these initial results in subsequent investigations (Trabal Fernandez et al., 2014). In this last case, the microbiota of postlarvae and adult oysters appeared more complex and versatile than previously reported. These bacteria, both outside and inside the larvae, could also be a key parameter for the success of larval culture (Powell et

al., 2013) and it is recommended to maintain a high diversity, notably to avoid unwanted proliferation of detrimental bacteria such as vibrios (Schulze et al., 2006). In an attempt to fill in the gaps in this domain, we undertook a study on the bacterial communities established in oyster larval cultures (*Crassostrea gigas*) as part of an experiment on a recycling system (RS) for larval rearing. Recent experiments have shown flow-through (FT) systems to be well adapted to larvae of both Pacific oyster (Rico-Villa et al., 2008) and flat oyster *Ostrea edulis* (Araya et al., 2012). The next step to improving larval culture is the implementation of a recycling aquaculture system to save energy (heating) and to protect larvae from variability in seawater quality. The outflow seawater system is recycled through a biological filter (bioreactor), as used in fish aquaculture (Blancheton, 2000). The present experiment compared larval performance in three rearing conditions: two RS, at 25% and 0% seawater renewal, and an FT system.

We analysed the bacterial communities in *C. gigas* larvae, seawater and bioreactors over a larval culture period of two weeks. These analyses were performed by a culture-independent method using pyrosequencing targeting the V1–V3 region of the 16S rRNA gene after direct extraction of DNA and amplification. A comparison was made with the culture-dependent method for the microbiota of larvae.

2. Materials and methods

2.1.Experimental conditions

The experiment was performed using two different rearing systems, "Flow-Through" (FT) and "Recycling System" (RS), at the IFREMER experimental station in Argenton (Brittany, France). These systems enabled the comparison of different seawater renewal rates: 100% (FT), 25% (RS-25) and 0% (RS-0) per hour (Fig. 1).

The FT consisted of four replicates of a 5-l cylinder containing larvae, as described by Rico-Villa et al. (2008), and was considered as the control. In this system, inlet seawater was filtered at 1 µm, passed through an ultraviolet (UV) lamp to be decontaminated and renewed at 100% per hour.

A similar unit of four cylinders was used for the RS, which was connected to a recycling loop to treat the outflow seawater (Fig. 1). The remaining algae and particles were trapped on a 1-µm cotton bag filter, and the dissolved organic matter was partially eliminated by a skimmer. The seawater was then pumped to circulate several times through the skimmer (9 × 5.9 cm, Ø × H) and a nitrifying bioreactor (30 × 13 × 28 cm, L × W × H) filled with plastic beads of 55 mm diameter with a total surface of 300m^3/m^2. Then a second pump distributed a small amount of treated seawater to the unit of cylinders, after UV disinfection to prevent bacterial contamination and limit bacterial proliferation. The rest of the water was returned to the bioreactor. The total volume of the system, including the four cylinders and treatment unit, was 40 l. The rate of seawater circulation through the RS rearing tank was the same as through the FT system described above, i.e., 100% or 5 L per hour with 25% of fresh seawater and 75% recycled seawater for RS-25, and 100% recycled seawater for RS-0. The fresh water was treated in the same way as for the FT system.

To enable the establishment of nitrifying autotrophic bacteria in the bioreactor prior to larval culture, unsterilized seawater enriched once per week with ~10 mg L^{-1} ammonium hydroxide was circulated through the bioreactors for 1.5 month. Nitrification was verified by regular ammonium, nitrite and nitrate measurements. Before the larvae were added to the system, the bioreactor was rinsed with UV-treated seawater.

Gametes were obtained by stripping oyster broodstock conditioned in the hatchery. After fertilisation and incubation at 25°C for 48 h, larvae were introduced into the four replicates of

each system (cylinders) at a density of 50 larvae ml^{-1} and reared according to Rico-Villa et al. (2008): 25°C; mean salinity 34.5 ppm; gentle aeration to homogenise the larvae in the tank, provide oxygen and remove CO_2; and continuous distribution of two microalgae, *Tisochrysis lutea* (strain CCAP 927/14) and *Chaetoceros gracilis* (Utex LB 2375), at 1500 μm^3 μl^{-1} (at 1:1 equivalent volume). The volume of algal culture continuously supplied to the systems was adjusted to the requirement of larvae estimated by the algal cells left in the outflow seawater. It varied from around 50 ml day^{-1} on day 4 to 800 ml day^{-1} on day 15. The total volume added during a 15-day run was around 24 l. Some seawater evaporated or was discarded. To reduce the salinity in RS-0, tap water was sometimes added.

2.2.Rearing parameters

Larval rearing lasted 15 days, from day 2 to day 16 after fertilisation. On day 16, larval survival was determined from a sample of at least 200 larvae after draining and homogenising the total larval population in each tank. Larval growth was estimated by measuring the shell length of 100 larvae per replicate every two to three days using image analysis techniques (Rico-Villa et al., 2008).

Physical and chemical parameters were measured in the three rearing systems. Nitrate, nitrite and ammonium were measured with a Multiparameter Bench Photometer HI 83200 using cadmium reduction, iron sulphate and Nessler methods, respectively. Water temperature, salinity, pH, dissolved oxygen and redox potential were recorded using a multi-parameter system connected to a multi-sensor probe (HI 9828).

2.3.Evolution of cultivable bacteria, isolation and identification

On days 2, 7 and 12, the total cultivable bacteria and vibrio concentrations in outflow seawater and larvae were estimated by the plate counting method using Marine Agar (Difco) and thiosulfate–citrate–bile salts–sucrose medium (TCBS, Difco), respectively. The seawater

was diluted 10-fold in sterile seawater, and about 1000 sampled larvae were crushed using a Potter tissue grinder in sterile conditions and diluted 10-fold. An aliquot of 100 µl per dilution was plated on each medium. The plates were incubated aerobically at 25°C for 4 (Marine Agar) or 2 days (TCBS).

2.4.Detection of ammonia monooxygenase (amoA) gene

To verify if the ammonia-oxidising bacteria (AOB) and archaea (AOA) were present in the bioreactors, the *amoA* genes of both types of microorganisms were tentatively amplified from the DNA of the samples collected from the bioreactor on days 2, 7 and 15. DNA was extracted from the bioreactor of a fish aquarium to obtain a positive control. The protocols used were those described by Han et al. (2013) for the primers of archaea *amoA* gene (forward Arch-amoA gene F 5′-STAATGGTCTGGCTTAGACG-3′ and reverse Arch-amoA-R 5′-GCGGCCATCCATCTGTATGT-3′) and for bacterial *amoA* (forward amoA-1F 5′-GGGGTTTCTACTGGTGGT-3′ and reverse amoA-2R 5′-CCCCTCKGSAAAGCCTTCTTC-3). Positive controls consisted of DNA extracted from the biofilter of the aquarium for which the primers were being used. The amplification mix contained 10 units of Pfu polymerase (BioVision, Mountain View, CA, USA), 5 ml Pfu reaction buffer and 200 mM dNTPs (Amersham, Piscataway, NJ, USA). A 50 ml volume of each primer at 0.2 mM concentration (10 ng) was added to each amplification mix. Cycling conditions were as follows: an initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 30 s and a final 10 min extension at 72°C.

2.5.Bacterial community analysis by 454 pyrosequencing

To analyse the bacterial community structure and composition, samples were collected on days 7 and 15 from the different compartments of the systems (outflow seawater, larvae, bioreactors and algal culture mixtures before they were supplied to the larvae) and on day 2

for seawater and the bioreactor. In both FT and RS, 500 ml of outflow seawater was taken and filtered on sterile 0.22 µm pore size polyethersulfone membranes (Pall) to collect bacteria. Ten ml of algal culture were collected and passed through a 3 µm membrane to separate algal cells from the medium; this was then filtered in the same way as seawater to collect bacteria. For the larvae, about 1000 individuals were collected, rinsed three times with filtered sterile seawater and then crushed. To examine the biofilm of the bioreactor, small pieces of the CPVC (Chlorinated polyvinyl chloride) used to fill the bioreactor and constitute a substrate for nitrifying bacteria were sampled and treated with an extraction buffer containing Tris-EDTA-SDS: 40 mM EDTA, 50 mM Tris (TRIZMA Base, pH 8.3) and sodium dodecyl sulfate (SDS) 1%. All the samples were subsequently frozen at –80°C until DNA extraction. All samples were then lysed using a Tris-EDTA-SDS extraction buffer and 20 µl proteinase K (20 mg ml^{-1}) (Sigma, France) and incubated for 1 h at 65°C. The total DNA was subsequently extracted with phenol/chloroform/isoamyl alcohol and precipitated with ethanol, as described by Romero et al. (2002). The concentration and quality of the DNA were determined at A260 and A280 nm, respectively, using a Nanodrop spectrophotometer (ND-1000 Nanodrop technologies, Wilmington, DE).

To investigate the composition of the microbial communities, the variable regions V1–V3 of the 16S rRNA gene were amplified from template DNA using primers tailed on each side with the Roche multiplex identifiers, 27F (5'-AGA GTT TGA TCC TGG CTC AG-3', corresponding to *Escherichia coli* positions 8–27) and 533R (5'-TTA CCG CGG CTG CTG GCA C 3', *E. coli* positions 515–533), which were modified by adding unique barcode sequences to discriminate the different samples. Polymerase chain reaction (PCR) mixtures (50 µl) were prepared in triplicate, with each containing 1 µl DNA template, 5 µl PCR buffer, 200 µM dNTP, 0.2 µm of each primer and 2.5 U Taq polymerase. PCR reactions were performed with the following program: 94°C for 5 min, 20 cycles at 56°C for 45 s, 56°C for

45 s, 72°C for 50 s, and 72°C for 10 min. The PCR products were purified with Agencourt AMPure XP (Beckman, USA) and then sequenced by the pyrosequencing method on a 454 Life Sciences Genome Sequencer FLX (Roche Diagnostics, USA). Reads were generated from both ends, with a read length of ~500 bp.

2.6.DNA sequence processing and analysis

The composition and diversity of microbial communities of 16S rRNA sequence data were determined using the Quantitative Insights Into Microbial Ecology (QIIME, version 1.7.0) pipeline (Caporaso et al., 2010). Raw sequences with an average quality score below 25 and containing more than 6 ambiguous bases were discarded, as were sequences below 200 bp or greater than 1000 bp in length. No primer mismatches were accepted. Operational taxonomic units (OTUs) were chosen using the UCLUST method and the greengenes 16S rRNA gene database (version 13.5) as reference, with a similarity threshold of over 97% that resulted from the selection of 12094 OTUs. Core diversity analysis was performed and rarefaction curves were generated using the Shannon and Simpson diversity indexes. For the rest of the study, only OTUs that had a relative abundance were included for further analysis, represented by 50 sequences in all samples. The overall OTU table was divided by sample to determine the separate phylogenetic trees. The resulting pyrosequencing data were deposited at the European Nucleotide Archive (Study accessionnumber PRJEB12894. and sample accession numbers ERS1183264 toERS1183283).

2.7.Statistical analyses

Only one sample could be analysed per compartment and sampling time. A previous analysis by denaturing high-performance chromatography on two replicates (two cylinders) per rearing unit showed that these replicates had the same molecular fingerprint, which could be explained by the fact that the same inflow of seawater was distributed to each cylinder of the

same rearing unit. The proximity of the different units and the UV seawater treatment could favour the homogeneity of bacterial communities. Nevertheless, it was possible to apply some statistical tests to compare the bacterial community structures according to the sampling date, type of system (FT or RS) and compartment. Cluster analyses were made from the Bray–Curtis similarities and ANOSIM tests done using PRIMER 6 software (PRIMER-E, Plymouth, UK). A one-way crossed SIMPER test was also performed to determine which taxa contributed most to the dissimilarity among the treatments.

ANOVA was used to test the effects of the rearing system on larval length and survival. When necessary, data transformation (arcsin[sqr(χ/100)]) was conducted to maintain the homogeneity of the residual distribution. Differences in means were assessed using a posteriori Tukey tests. The correlation coefficient was calculated between larval growth and consumption of algal cells.

3. **Results**

3.1. Larval development

Larval survival at the end of rearing (day 16) was 77% in FT, 80% in RS-25 and 85% in RS-0, without any significant difference (Fig. 2A). Mean larval length in FT reached 271 ± 7 µm, which was significantly higher than that in RS, where the mean was 225 ± 15 µm ($p<0.01$) (Fig. 2B). Total algal consumption was significantly lower in RS than in FT (Fig. 2C), and larval growth was correlated with algal consumption per larva ($R^2 = 0.76$, $p = 0.038$).

Physico-chemical parameters

From days 2 to 15, salinity remained stable in the outflow of FT and RS-25 but increased up to 37.50 g l^{-1} in RS-0 (Table 1). The pH remained stable in FT, with extreme values ranging

from 8.10 to 8.30. By contrast, pH decreased to 7.75 in RS-0 and varied in RS-25 (7.82–8.03), with the lowest values recorded during the first days. The oxygen level could be maintained close to saturation despite a slight decrease at the end of the experiment, probably because of the increase in larval need (Table 1).

Ammonium-nitrogen concentration (NH_4-N) in the inflow seawater never exceeded 1 mg l^{-1} throughout the whole experiment (Table 1). It was mainly contained in the fresh seawater and was not produced as a result of the larval rearing. In RS-0, nitrite-nitrogen concentration (NO_2-N) reached 0.12 mg l^{-1}, whereas it remained below 0.10 mg l^{-1} in the other batches. Nitrate-nitrogen accumulated progressively in RS-0 to reach 1.51 mg l^{-1} on day 15.

3.2. Cultivable bacteria

The number of colony forming units (CFU) in outflow seawater that would grow on marine agar stabilised at around 8×10^4 cell ml^{-1} in all treatments (Fig. 3A). Vibrios were commonly undetectable but some peaks occurred, in FT on days 9 and 12 with 5 and 30 CFU ml^{-1}. Only a peak of 5 CFU ml^{-1} on day 12 was detected in RS-25 on day 12 and RS-0 on day 6. The bacterial concentrations of the larvae in FT were significantly higher than those in RS (Fig. 3B). Bacterial concentrations in larvae on day 16 were significantly higher in FT (2.1×10^3 CFU $larva^{-1}$) than in RS-0 (8.9×10^2 CFU $larva^{-1}$) while they were intermediate in RS-25 (Fig. 3B). Vibrio concentrations tended to be higher in FT (67 CFU $larva^{-1}$) than in RS-0 (24 CFU $larva^{-1}$) and RS-25 (8 CFU $larva^{-1}$).

3.1.Composition of bacterial assemblages

The mean number of sequences per sample was 5370, ranging from 2536 to 9613. The number of OTUs that were represented by at least 50 sequences in all samples varied from 72 to 191.

The bacterial populations were clearly different between: larvae, seawater, bioreactor and algae as assessed by ANOSIM tests (Table 3).

The presence of algal cells distributed to the larvae was revealed by 2 OTUs affiliated with Haptophyceae (T-iso) and Stramenopiles (*Chaetoceros gracilis*). These OTUs reached up to 6% in the algal culture and 5% in the bioreactor samples but only <1% in the larvae and seawater samples.

The bacterial communities of seawater were comparable in the three rearing conditions on days 7 and 15. The similarity reached ≥69.2 % between the two RS at any date, whereas the communities were more distinct between FT and RS, with only 56.8 ± 5.1% similarity, as shown by the dendrogram of the Cluster analysis (Fig. 4). Thus RS was more stable than FT. These system types were significantly different according to the ANOSIM test (Table 3). They were mainly differentiated by 11 OTUs affiliated with *Phaeobacter* sp., *Rickettsia* sp., *Alteromonas* sp., *Arcobacter* sp., Flavobacteriales, Cytophagales, Kordiimonadaceae and unclassified γ-Proteobacteria (Table 5). Most of these bacteria were only present in RS, where they accounted for up to 30.3%. The Shannon index indicated a decrease in diversity over the rearing period, mainly in FT and RS-25 (Table 2).

On day 2, seawater was found to be colonised by some predominant OTUs (Fig. 5). Several of these OTUs were mainly affiliated with the Actinobacterium *Canditatus aquiluna rubra*. They constituted one-third of the microbiota on day 2 (29.4%) but decreased to 3% in FT and were negligible in RS by day 7. Another OTU, a member of the Pelagibacteraceae family (Rickettsiales), also accounted for a large part of the initial microbiota (26.2%). However, it decreased to 4% at later sampling times. On days 7 and 15, some OTUs belonging to environmental bacteria (GN02 and OD1-ZB2) colonised seawater (34.3 ± 6.9%). One of them accounted for 18.2% to 36.4%, with an increase between days 7 and 15 regardless of the

larval batch considered. It should be noted that these OTUs were already present in seawater on day 2 and algal mixtures but at a low level (Table 4). Flavobacteriales (Bacteroidetes) were represented by 11 OTUs representing from 5.1% to 12.4% on day 7, with an increase in all batches on day 15 to reach 11.5% to 24.5%notably due to a member of the Cryomorphaceae. On average, Proteobacteria were the most abundant phylum in all seawater samples, representing 45% of the total sequences. α-Proteobacteria were diverse, with more than 50 OTUs, principally affiliated with the unclassified bacteria BD7-3, Kordiimonadales, Hyphomicrobiaceae and Rhodobacteraceae (Table 5). They accounted for ~50% in terms of abundance on day 2, decreasing thereafter to stabilise at ~27% in RS-25 and ~16% in RS-0 and FT.

The OTUs related to δ-Proteobacteria were affiliated to the genera *Bdellovibrio* and *Bacteriovorax*. They occurred at a substantial level (3.3%–12.4%) in all batches, despite a diminishing trend on day 15 in FT (5.8%) and RS-25 (3.3%). γ-Proteobacteria were represented by several OTUs mainly affiliated with Alteromonadales, Legionellades, Oceanospirillales and unclassified γ-Proteobacteria. They were particularly numerous in RS-0 on day 15 (20.5%) compared with the other batches (10.9% for FT and 12.7% for RS-25). *Vibrio* spp. were not detected, except in RS-0 on day 15 (0.33%).

The microbiota of larvae (Fig. 6, Table 4) was similar in all batches sampled on the same date (~73% and 67% on average on days 7 and 15, respectively) but changed between days 7 to 15, as revealed by the cluster analysis (Fig. 4) and ANOSIM test (Table 3). No well-represented OTU could differentiate the larvae from the different batches. The microbiota was composed of ~90% Proteobacteria, including α, β and γ classes (Fig. 7). In this phylum, changes occurred between day 7 and day 15.

On day 7, α-Proteobacteria (61% to 70%), were composed of 31 OTUs belonging to Rhizobiales (6) and Rhodobacteraceae (25), of which 5 were affiliated with the genus *Phaeobacter* (Table 4). These OTUs accounted for 27, 12 and 38% in FT, RS-25 and RS-0, respectively. γ-Proteobacteria ranged from 14.1 to 33.7%. They were diverse, with 17 OTUs belonging mainly to *Alteromonas* and *Pseudoalteromonas*.

On day 15, the overall diversity decreased, especially in RS-0 (Table 2). The main changes revealed by Simper analyses (Table 5) were due to the increase of two members of the Burkholderiales (β-Proteobacteria): one belonging to the genus *Delftia* and the other to the family Oxalobacteraceae. These two bacteria constituted 30% (FT), 61% (RS-25) and 55% (RS-0) of microbiota. Consequently, the predominant OTUs (>1%) diminished from 23 to 8 in RS-25, from 19 to 11 in RS-0 and from 18 to 12 in FT, coinciding with the decrease in α- and γ-Proteobacteria. However the larvae of FT retained more α-Proteobacteria,(47% versus 20% and 26 % in RS-25 and RS-0, respectively) One OTU (Rhodoacteraceae) of FT reached 24%, while it accounted for less than 5% in RS. The other OTUs (about 10%) were mainly related to Bacteroidetes and some to Planctomycetes. Some *Vibrio* spp. were found in FT and RS-0 (0.08% and 0.72%, respectively).

The composition of bacterial communities in the bioreactor samples remained very stable from days 2 to 15 in both RS-25 and RS-0. Indeed, these bacterial populations had 65.8–78.8% similarity between them (Fig. 4). Proteobacteria were predominant and constituted 64.3–84.2% of the bacterial sequences, with a majority of α-Proteobacteria (46.3–62.6%) (Table 4). They mainly belonged to Rhizobiales (10 OTUs) and Rhodobacteraceae (39 OTUs), or were unclassified (19 OTUs). No bacterium was consistently predominant. However, some were occasionally invasive, such as one OTU (Rhodobacteraceae) that reached up to 31% in RS-25 on day 7, but decreased thereafter. γ-Proteobacteria were

represented by 12 OTUs affiliated mainly with the Alteromonadales, Oceanospirillaceae or unclassified bacteria.

Interestingly, the Planctomycetes reached up to 10.5% in RS-25 (6.7% on average). The other characteristic OTUs were one member of the Acidobacteria that increased up to 4.6% in both RS from days 2 to 15, and a member of Acidimicrobia that varied from 1.5% to 3.2%. The main characteristics distinguishing RS-25 from RS-0 were a higher abundance of Rhodobacteraceae (22% vs. 3%) and a lower abundance of γ-Proteobacteria (15.7% vs. 25.7%) in RS-25 and RS-0, respectively. The Shannon index indicated a high and stable diversity (Table 2).

Some nitrite-oxidizing bacteria, including *Nitrobacter* sp., *Nitrospira* sp., *Nitrospina* sp. and *Nitrococcus* sp. were identified in the seawater of RS-25 and in the bioreactor of RS-0, but remained at very low levels (from 2 to 6 sequences per sample). No ammonia oxidizers such as *Nitrosomonas* sp. and *Nitrosococcus* sp. were found and the detection of the *amoA* genes of AOB and AOA was negative in all samples, but positive in the control, for both genes.

The mixture of algal cultures harboured less OTUs (~70) than the other compartments. Eleven OTUs represented 98% of the microbiota on day 7, and 14 OTUs constituted 88% on day 15. Consequently, the Shannon index was low: 2.2 and 3.8 on days 7 and 15, respectively. These bacteria mainly belonged to Bacteroidetes, α-Proteobacteria and unclassified bacteria. Several OTUs in the algal cultures grew in seawater and some were also found in larvae and the bioreactor. Two Cryomorphaceae (Flavobacteriales, Bacteroidetes) and the environmental bacteria OD1- ZBD2 and GNO1 FD1, which were abundant in algal cultures, also predominated in the seawater. Some others belonging to *Candidatus Aquiluna rubra* (Actinobacteria), Flavobacteriales (3 OTUs) and Rhodobacteraceae (2 OTUs) and Rickettsiales (2 OTUs) were also common in both these compartments, although they were

sometimes rarer in the algal cultures than in the seawater. In addition, some OTU, including 3 members of Rhodobacteraceae and one of Hyphomicrobiaceae colonised all compartments. Finally, although the algal culture supplied a relatively low number of OTUs to other compartments, these constituted on average 64% (43–82%) of microbiota in seawater, 34% (12–56%) in larvae and 11% (9–16%) of bioreactor biofilm.

4. Discussion

The first objective was almost accomplished, as larval survival was not statistically different between RS and FT. However, larval growth was slower in both RS, correlated with low algal consumption, which was less noticeable in RS-0. This lesser growth reveals that the rearing conditions in RS were not optimal. Among the physico-chemical parameters in RS, some low values of pH and high salinity in RS-0 could have disturbed the larval physiology, especially calcification (Hernandez-Zarate and Olmos-Soto, 2006). The loss of calcium bicarbonate, taken up by the larvae for their calcification, could have increased the carbonic acid and, combined with ammonia excretion and respiration, decreased the pH. However, no abnormal shells were observed in RS-0. Oyster larvae are particularly resistant to pH decreases by CO_2 (Ginger et al., 2013). In RS-25, this parameter was also low but only during the first days, and it could not affect the calcification during the second week. A seawater renewal of 25% could also avoid the accumulation of any toxic compound from the system that would, by contrast, be accumulated in RS-0. No major bacterial group in any compartment could consistently differentiate the RS from FT and explain this disturbance. Other hypotheses could be investigated, such as the negative influence of the biofilm that may colonize the walls of recycling loop and/or the impoverishment in dissolved organic matter that can contribute to bivalve growth (Perez et al., 2013). Nevertheless the rate of metamorphosis for RS-0 was equivalent to that of larvae from FT (~50%) and the growth lag was rapidly caught up after the metamorphosis. A renewal rate of 10%, the one generally used for fish, could be more

suitable. It was initially planned to test this renewal rate in the present experiment but its use was prevented by a technical failure.

The cultivable bacterial concentrations in seawater remained at a moderate level, even in RS-0, which was a completely closed system. Several factors could contribute to limiting bacterial growth. Firstly, the UV treatment, which generally inactivates more 90% of bacteria, but generates an ecological vacuum that may be quickly colonized by bacteria unless the flow rate is sufficiently fast. The second factor comprises the filtration at 1 µm, which eliminates the rest of the algal cells, and the skimmer, which removes the dissolved organic matter. The third factor is the possibility that the bacterial assemblages can be antagonistic against pathogenic bacteria and the predatory bacteria that burst the bacteria as discussed below. Finally, the other sanitary measures, besides UV treatment, aim to limit the contamination of larval culture by pathogens.

This study on the bacterial communities associated with different compartments of a larval culture system by a metagenomic approach provides new insights into the microbiota of oyster larvae. Previous reports based on an independent culture method focused on postlarvae and juveniles (Trabal et al., 2012) or adults collected in the natural environment (Pujalte et al., 1999; Romero et al., 2002; Hernandez-Zarate and Olmos-Soto, 2006; Green and Barnes, 2010, King et al., 2012; Trabal Fernandez et al., 2014). These studies reported a large variation in the bacterial assemblages according to location and season, except for Trabal et al. (2012), who reported relatively stable microbiota from hatchery to open sea. In none of these studies was bacteria in the surrounding seawater analysed to determine the relationship between the microbiota of both compartments. Other marine organisms such as corals, on which numerous studies have been made, impose strong selection on seawater bacteria through their mucus as they constitute their microbiota, although their composition can still

change greatly according to season (Chen et al., 2011) and location (Morrow et al., 2012; Fernandez-Piquer et al., 2012).

One of the main findings of this study is the specificity of bacterial communities that became established in seawater and larvae, regardless of the rearing system and sampling date. Inside each niche, the bacterial communities mainly varied as a function of time. The relative proximity of the rearing units, enabling the exchange of bacteria, may homogenise the microbiota. It is possibly one of the reasons why the bacterial assemblages were similar regardless of the rearing conditions. Thus, for larvae, the differences between FT and RS were smaller than the differences between the sampling dates.

In fish aquaculture, some studies have demonstrated the beneficial effect of RAS on fish health. This was attributed notably to the mature bacterial assemblages that became established in RAS and prevented the proliferation of pathogens (Attramadal et al., 2012, 2014; Blancheton et al., 2013). However, this was not verified by this study, probably because the bacterial populations in seawater are 10 to 100 times lower than those reported in seawater of fish RAS. Indeed fish aquaculture is much more productive with a greater stocking rate of live animals and a longer run.

The relative abundance of the different taxonomic groups changed from one niche to another. The Proteobacteria ranged on average from 45% in seawater to 89% in larvae and 74% in the bioreactors. α-Proteobacteria, including numerous Rhodobacteraceae (OTUs>30), reached 50% in the larvae and in the bioreactor. In seawater, they did not exceed 24%, with less OTUs (<20). β-Proteobacteria constituted a large part of the microbiota of the larvae on day 15, but were negligible in the other compartments. The other phyla and classes such as the Bacteroidetes, mainly Flavobacteriia, were well represented (11–18%) in seawater and they were at very low levels in both the other compartments. The unclassified bacteria mainly OD1

ZB2 constituted up to 40% of microbiota in SW and 18% in algae but were 2% less in larvae and bioreactors.

Another characteristic of seawater compartment are the occurrence of *Bdellovibrio* spp. and *Bacteriovorax* spp. These predatory bacteria are frequently isolated from estuarine seawater (Pineiro et al., 2013). They can attack and burst a large variety of gram-negative bacteria (Chen et al., 2012). Therefore, they may limit bacterial proliferation in aquaculture systems (Qi et al., 2009) and, more importantly, pathogenic *Vibrio* spp. in oyster (Richards et al., 2012). They could become an important means to improve control over pathogenic bacteria and bacterial proliferation in the future (Qi et al., 2009). Ciliates and bacteriophages have been initially considered as the main microorganisms able to modulate bacterial populations in a natural environment. Ciliates cannot be used because they could compete with the bivalve larvae for food (algal cells). Bacteriophages can certainly be used, but reports on their occurrence and impact on bacterial proliferation in aquaculture are rare. Nevertheless, they have been recently tested for their ability to control pathogenic vibrios, especially in oyster (Rong et al., 2014).

As filter feeders, bivalves ingest bacteria that can be mixed with their own microbiota and could thus mask the core of resident microbiota. Therefore, the elimination of the transitory bacteria by several days of depuration in clean seawater has been proposed (Trabal et al., 2012). In our study, the clear distinction between the bacteria present in larvae and those in seawater indicates that bacteria may either have a low efficiency for penetrating the larval gut or be rapidly digested. Finally, like other aquatic organisms, bivalve larvae exert drastic selection to constitute their own resident microbiota, except if there is a large quantity of bacteria in the seawater (Pieur, 1982). The OTUs of seawater affiliated with the Cryomorphaceae family or the environmental bacteria GN02-BD1-5 and OD1-ZB2 were never detected in the larvae. The larvae were also not found to contain many chloroplasts of

either alga (T-iso and *Chaetoceros gracilis*). Ground larvae were not 1-µm filtered before DNA extraction to retain microalgae, as was done for seawater, so we can conclude that the microalgae were rapidly digested. Although the larvae shared about 42 common OTUs with seawater, specifically five *Phaeobacter* sp., two *Nautella sp.* and two other members of the Rhodobacteraceae, accounting for 60% of their microbiota, they represented less than 10% of the seawater microbiota. A substantial part of the microbiota of larvae, especially Burkholderiales, was also constituted by OTUs absent from seawater and algal cultures , but which could come from the broodstock. The same changes occurred in all three systems: a decrease in diversity of α and γ-Proteobacteria on day 15 and colonisation by two members of the Burkholderiales. However, the Burkholderiales occurrence was higher in RS than in FT. These β-Proteobacteria seem particularly well adapted to oysters. *Burkholderia cepacia* was found to colonise postlarvae and juveniles of *C. gigas* in Baja California (Trabal et al., 2012). In a more recent study, the same authors once again found none of this bacterial species in postlarvae, but observed that OTUs belonging to *Burkholderia* genus were generally seen to constitute a large part of the microbiota of adult oysters (Trabal Fernandez et al., 2014). However, other authors did not report this type of bacteria in oysters (Romero et al., 2002; King et al., 2012).

The low occurrence of vibrios was corroborated by the small number of CFU counted on TCBS. Other recent studies (e.g. Waldbusser et al., 2013) have reported a similar low concentration of these γ-Proteobacteria in oysters, in contrast to all previous studies using a culture-dependent method (Pujalte et al., 1999). Nevertheless the vibrios reached up to 75 per larva on day 16 in larvae especially in FT whereas they were poorly detected by pyrosequencing. The difference between these methods might be the result of PCR bias, leading to the preferential amplification of certain groups and lower amplification efficiencies of others. The underestimations could have resulted from the forward primer used in this

study, if it was poorly adapted to the *Vibrio* spp.. The 16S rRNA sequences of 54 out of the 78 known *Vibrio* spp. show at least two mismatches in this primer sequence. Quantification of the vibrio population could be improved by using quantitative PCR with specific primers for vibrios, as previously described by Thompson et al. (2004).

In the bioreactors, the bacterial populations were already established before the beginning of rearing, since as early as day 2 they appeared complex and did not change significantly thereafter. The OTUs affiliated with α-Proteobacteria were largely predominant. These aerobic organisms have been reported to be excellent biofilm formers and are among the first and dominant colonisers of surfaces in all marine environments (Dang et al., 2011). Members of this group are likely responsible for the biodegradability of organic matter (OM). Nonetheless, the load of particulate and dissolved OM may be weak as the remaining algal cells are retained by the 1-µm bag filter, and the skimmer may eliminate a large amount of the dissolved OM. The degradation of dissolved OM probably occurred as soon as it was released and during the circulation of seawater through the system. According to most studies, the oxidation of ammonium is realised by ammonia oxidisers belonging to *Nitrosospira* spp. and *Nitrosomonas* spp., with the *amoA* (ammonia monooxygenase) gene (Rejish Kumar et al., 2009). The negative PCR results of these *amoA* genes suggests the lack of common nitrifiers. Alternative means of nitrification have been described, such as the following: (1) Some heterotrophic bacteria can nitrify and denitrify but have only been found in cold temperatures (Yao et al., 2013). (2) One archaeabacterium *Nitrosopumilus maritimus* has a gene similar to *amoA*. Although it requires microaerophilic conditions to oxidise ammonium (Bouskill et al., 2012), it was observed at high concentrations in RAS for shrimp (Brown et al., 2013).

The role of the bacterial communities could not be properly investigated by this metagenomic approach, which was limited to bacterial identification by 16S rRNA. Such a study would require a true metagenomic analysis coupled with a metatranscriptomic or metaproteomic

analysis performed, if possible, on more contrasted microbiota. Deducing a potential function from only the affiliation of OTUs with common groups (e.g., Rhodobacteraceae, Flavobacteriales and Alteromonadaceae, among others), is risky. These groups include bacteria that can have different functions. In contrast, for the bacteria belonging to some specific groups such as Bdellovibrionales, *Nitrospira*, *Nitrospina* and Nitrococcus the functions are more easily deduced. Other groups, such as Burkholderiales, should play an important role in larvae *and* postlarvae although it would probably be difficult to elucidate this.

In conclusion, some general characteristics of the microbiota associated with bivalve larval rearing in RS and FT can, nevertheless, be drawn from this first metagenomic analysis. Bacterial assemblages are complex and specific to compartments even though some bacteria can be common to two or three compartments. Bacterial diversity remained high despite decreases in seawater and larvae in the final days of the experiment. Thus, the sanitary procedures including UV treatment and frequent cleaning did not seem to strongly affect the diversity, but could influence the type of bacteria.

Bacterial proliferation did not occur even if fresh seawater was not added to RS-0, and bacterial composition was similar to that of the other two systems. They are unlikely to be responsible for the slower larval growth observed in the two RS because they were also found in the FT. These bacterial communities can be considered as neutral, especially because they did not contain pathogenic bacteria apart from a very low level of vibrios. We need to ask if such communities are necessary to ensure normal larval development. According to the holobiont concept, their role could be primordial in ontogenesis and ulterior development. However, as for the other animals, for example corals (Sharp et al., 2012), an experiment aiming to modify microbiota, combined with a complete metagenomic approach, would be necessary to demonstrate their potential role in larval development..

It would also be interesting to confirm these first results and to pursue monitoring of the microbiota of bivalve larvae until after metamorphosis and grow-out in the intertidal zone. According to the hypothesis that pioneer bacteria remain in the microbiota of juveniles and adults or influence their establishment, this early-life microbiota could have a significant effect on the composition of microbiota in oyster seed. Hatchery seed microbiota could be more limited than that of seed from natural recruitment because selection could occur of the bacteria best adapted to the hatchery environment. As the microbiota of an organism may influence its health, the microbiota established in hatcheries could be a key factor in the improvement of seed management. Additionally, knowledge about the sources of the bacteria, especially from broodstock, would make it possible to control and possibly to optimize the microbiota.

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Figure Captions

Fig. 1. Schema of the recycling system.

Fig. 2. Larval survival (A) on day 16. Larval growth (B) and total algal consumption per larva between day 2 and day 15 (C). Significant differences are indicated by different letters (n=4. $p<0.05$). FT: Flow-Through. RS25: Recycling system with 25% new seawater. RS0: Recycling system without new seawater.

Fig. 3. Evolution of cultivable bacteria in seawater (A) and concentrations of cultivable bacteria (B) and vibrios (C) in larvae on day 16 (n=4 $p<0.05$). For other legends see Figure 2.

Fig. 4. Cluster analysis of all samples (Bray-Curtis similarity). L: larvae; SW: Seawater; BR: Bioreactor.

Fig. 5. Composition of microbiota in different compartments: seawater (SW) larvae. biofilm of bioreactor (BR) and algal mixture (algae).

Fig. 6. Evolution of bacterial communities in seawater as a function of the rearing system from day 2 (common to all systems) to day 15. For other legends. see Figure 2.

Fig. 7. Composition of bacterial communities in larvae as a function of the rearing system on days 7 and 15. For other legends. see Figure 2.

Fig. 8. Evolution of bacterial communities in the bioreactor from day 2 (common to both RS) to day 15. For other legends. see Figure 2.

Fig. 1

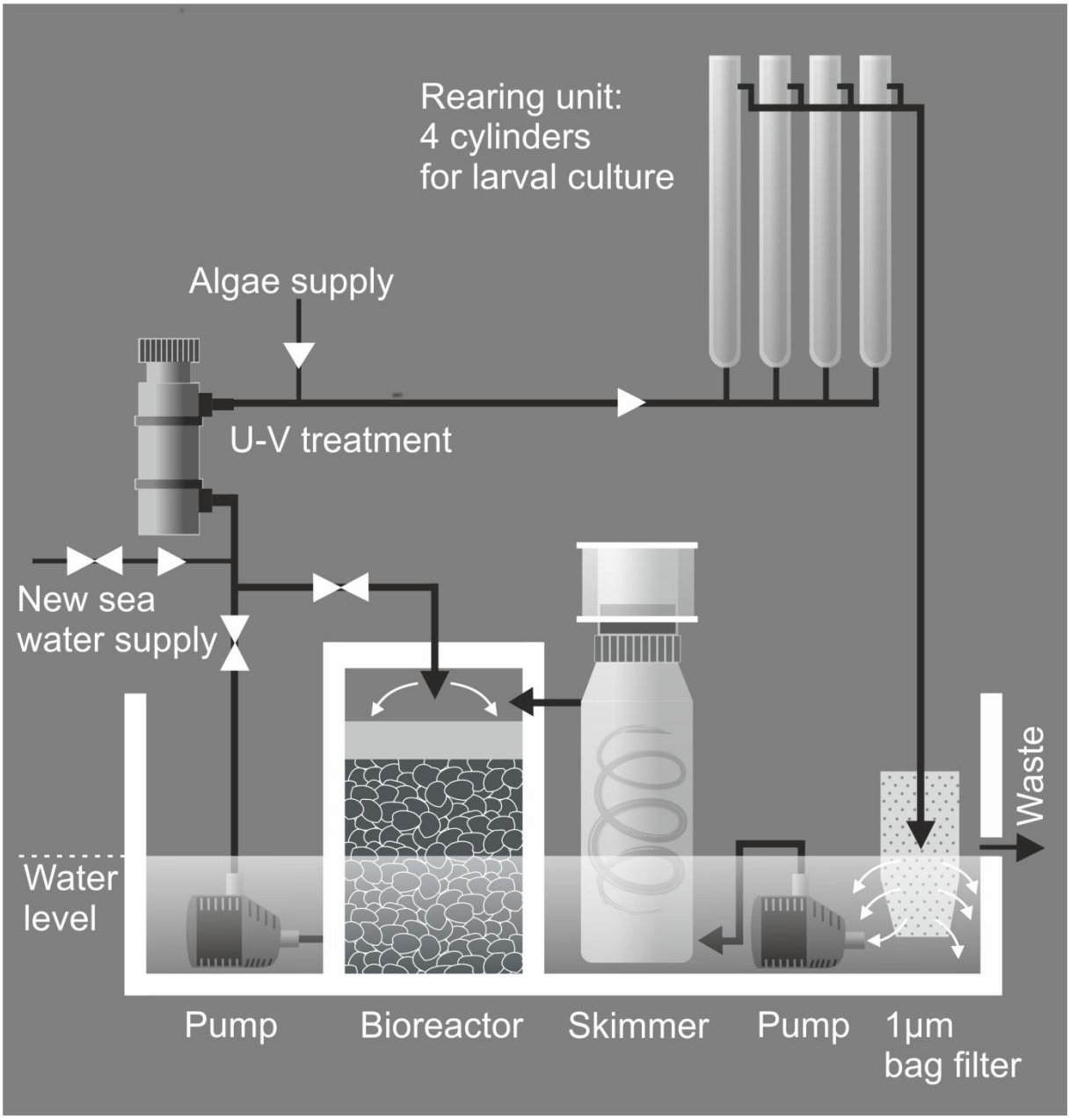

Fig. 2

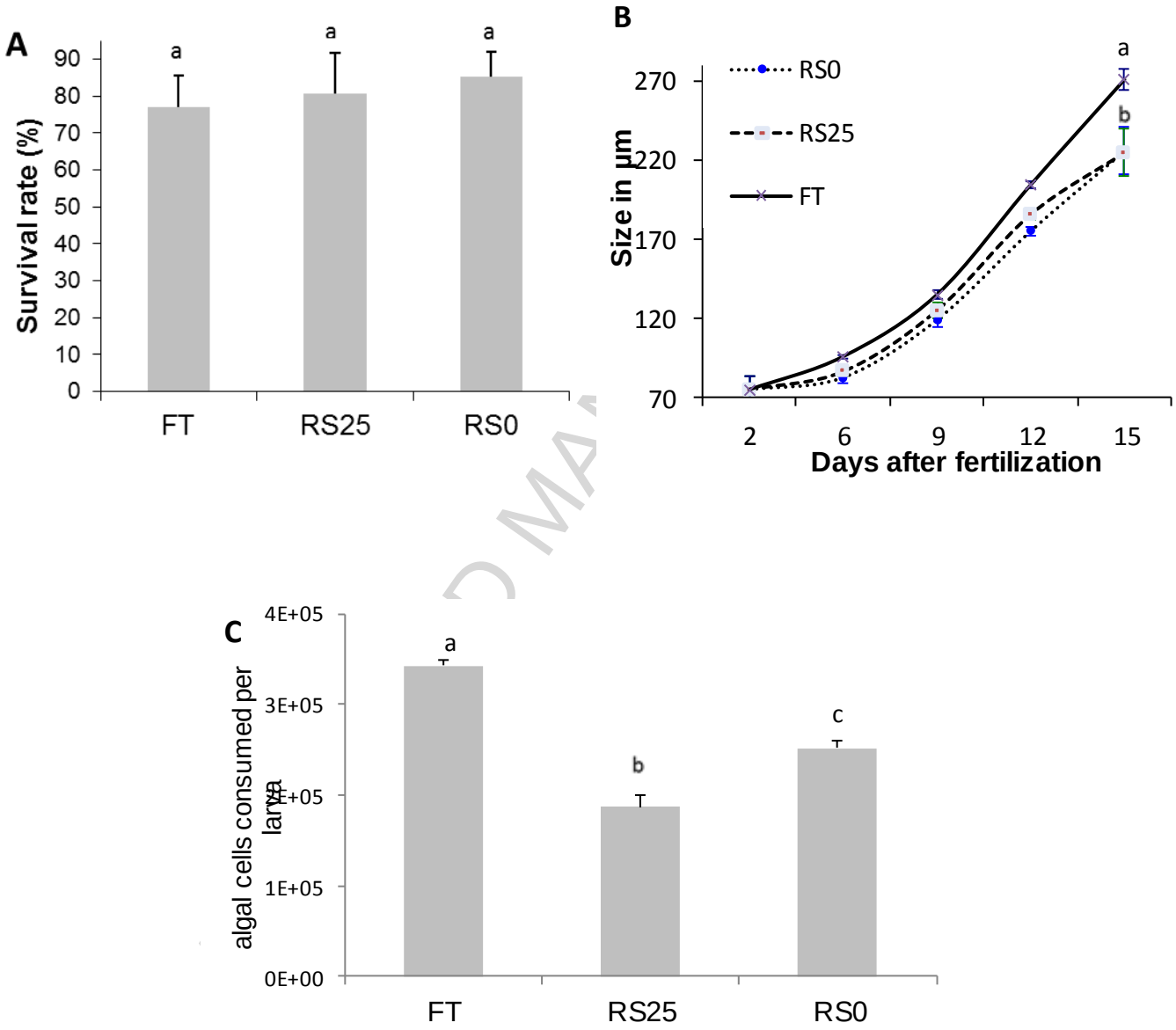

Fig. 3

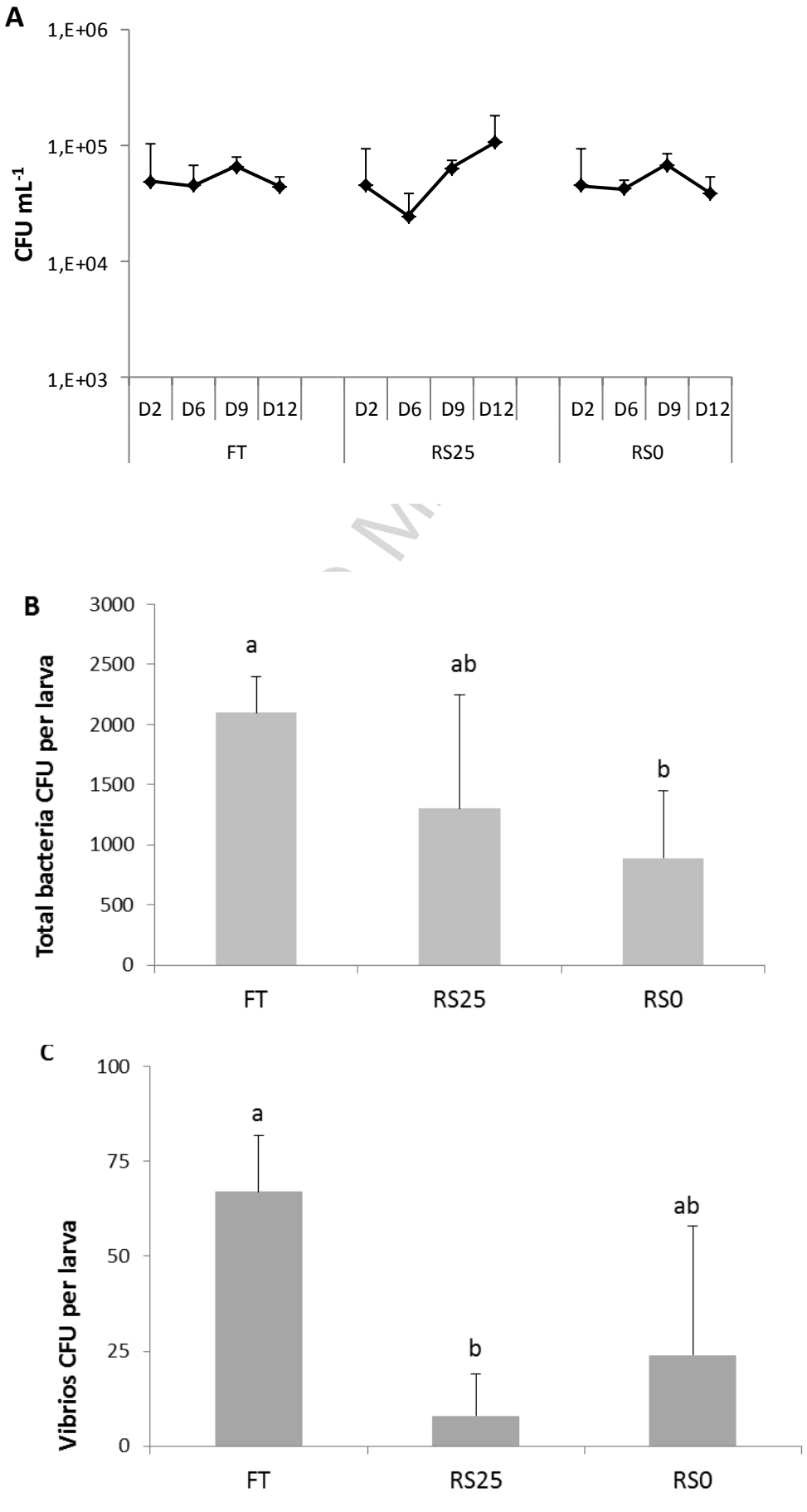

Fig. 4

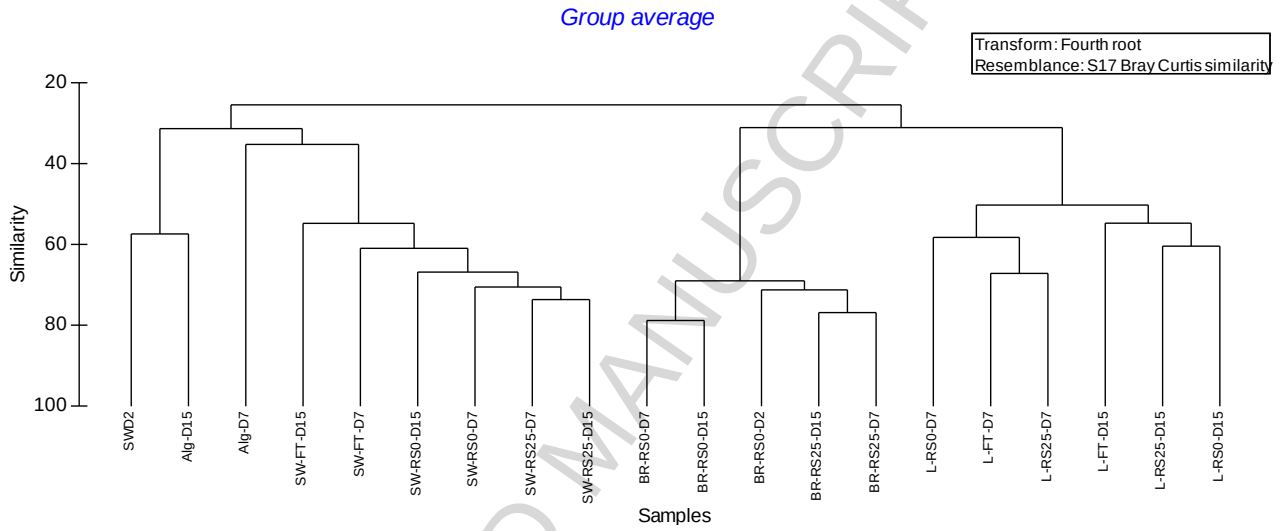

Fig. 5

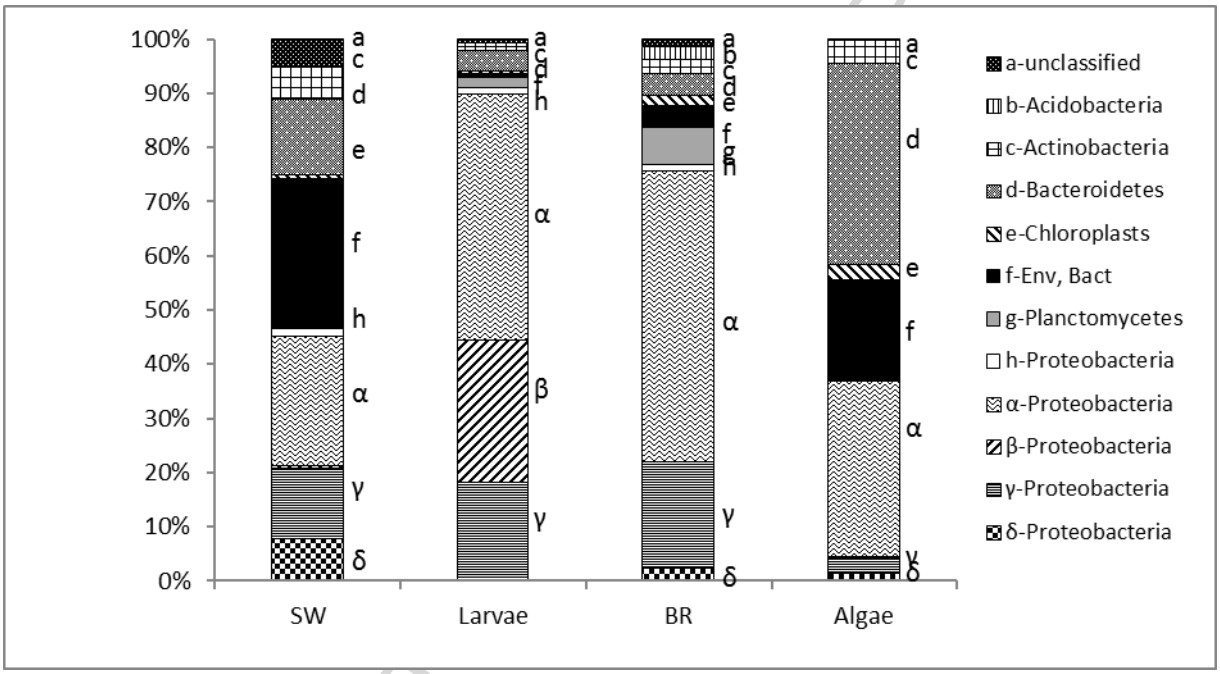

Fig. 6

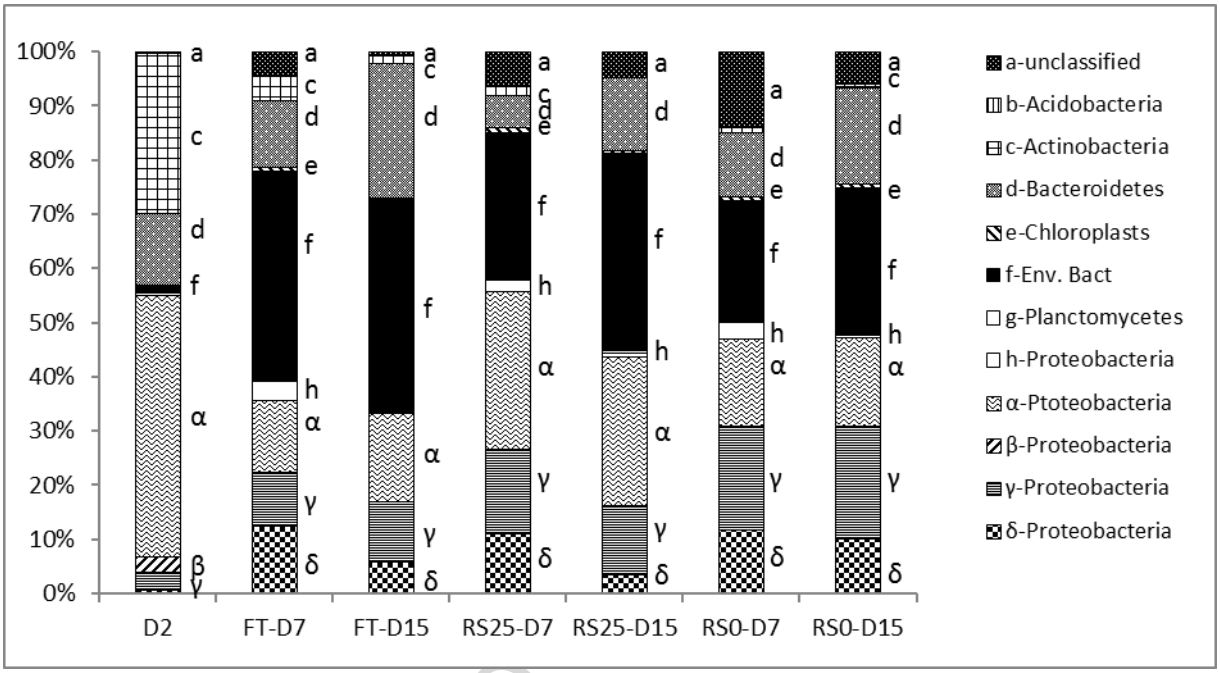

Fig. 7

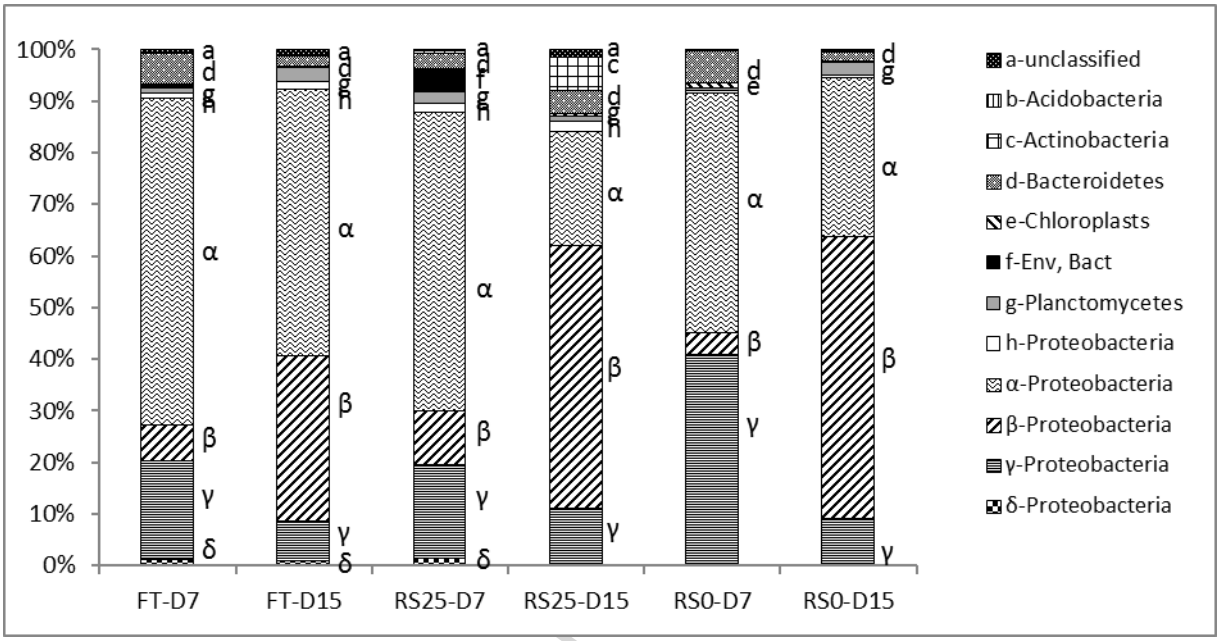

Fig. 8

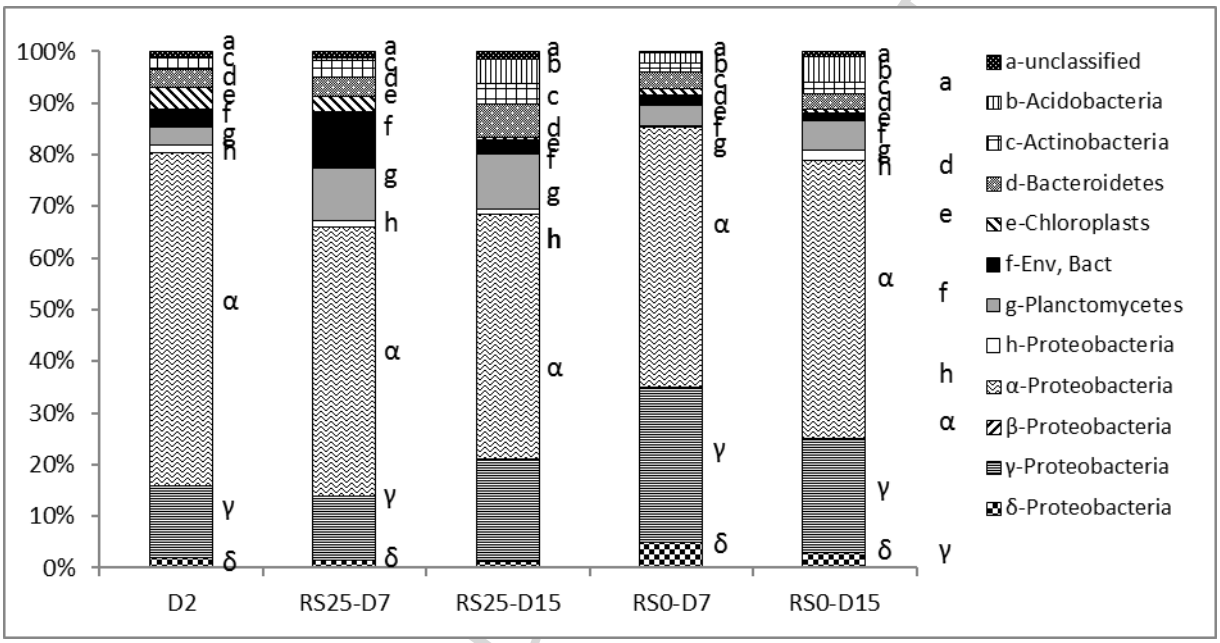

Table 1. Water quality parameters (temperature, oxygen concentration, salinity, pH and ammonium) recorded in the rearing systems. The reported values were measured at day 2 and day 15,

Rearing system	Temperature (°C)	Oxygen (mgL^{-1})	Salinity(‰)	pH
FT	24.80–26.30	6.44–6.11	35.20–35.40	8.11–8.21
RS25	25.10–27.00	7.12–5.91	35.30–35.60	7.82–8.03
RS0	25.10–27.10	6.41–5.89	35.30–37.50	7.95–7.75

Rearing system	Seawater	$N\text{-}NH_4^+$	$N\text{-}NO_2^-$	$N\text{-}NO_3^-$
FT	Inlet	0.5–0.59	0.01–0.00	0.00
	Outlet	0.5–0.74	0.01–0.02	0.00
RS25	Inlet	0.39–0.66	0.03–0.00	0.00
	Outlet	0.39–0.63	0.03–0.01	0.00
RS0	Inlet	0.54–0.68	0.02–0.12	0.00–1.20
	Outlet	0.54–0.78	0.02–0.11	0.00–1.70

Table 2. Shannon index for the estimation of bacterial diversity in the different samples

	D2	D7	D15
SEAWATER			
FT	4.63	4.71	3.56
RS25	4.63	5.15	4.26
RS0	4.63	4.99	4.50
LARVAE			
FT		5.06	4.69
RS25		5.46	4.99
RS0		5.33	4.37
BIOREACTOR			
RS25	5.23	4.88	5.70
RS0	5.23	5.54	5.55
ALGAE			
		2.23	3.85

Table 3. ANOSIM tests between samples collected on different days and in the compartments larvae, seawater (SW) and bioreactor (BR) and algae. L: larvae, SW: seawater, BR: bioreactor, D: day.

Samples	R	Level	permutations
L / SW	0.994	0.2%	462
L / BR	1	0.2%	462
L / Algae	0.948	3.6%	28
SW / BR	0.786	0.1%	462
SW / Algae	0.481	8.3%	28
BR / Algae	1	4.8%	21
L D7 / L D 15	0.813	10%	10
SW FT / SW RS	0.857	6.7%	15

Table 4. Mean percentage (standard deviation) of sequences per bacterial group. L: larvae, SW: seawater, BR: bioreactor, D: day.

	SW-D7	SW-D15	L D7	L D15	BR D7	BR D15	Algae
Bacteria unclassified	0.0%	0.0%	1.2±1.8%	0.9±1.4%	6.7±2.9%	2.9±0.6%	0.1±0.1%
GND2	6.2±3.4%	0.4±0.4%	0.8±0.9%	0.0%	1.2±1.2%	0.4±0.3%	0.0%
OD1 -ZB2 %	26.4±2.5%	32.1±5.4%	0.4±0.4%	0.0%	0.2±0.0%	0.1±0.0%	18.4±15.1%
P. Actinobacteria .	0.0%	0.0%	0.3±0.2%	1.0±0.6%	0.1±0.1%	4.7±0.2%	0.1±0.1%
P. Acidobacteria;	0.0%	0.0%	0.2±0.2%	2.3±3.6%	2.6±1.1%	3.0±1.1%	4.4±5.6%
P. Bacteroidetes.							
O. others	0.0%	0.0%	3.2±1.5%	0.8±0.1%%	1.3±0.1%	1.5±0.9%	35.9±30.4%
O. Flavobacteriales	13.8±3.8%	16±6.3%	1.8±2.0%	0.2±0.2%	3.1±0.8%	4.0±1.3%	1.11.1%
P. Cyanobacteria . O. Chloroplast	1.1±0.1%	0.4±0.3%	0.4±0.6%%	0.2±0.2%	1.6±1.1%	0.6±0.1%	3.0±3.7%
P. Planctomycetes	0.0%	0.0%	1.2±0.9%%	2.1±1.0%	7.0±4.4%	8.0±3.5%	0.0%
C. α-Proteobacteria							
unclassified	3.3±2.8%	3.4±1.8%	2.0±1.9%	0.8±0.2%	11.6±6.9%	14.5±6.8%	0.9±0.6%
O. Kordiimonadales.	3.8±4.8%	1.3±2.1%	0.00%	0.0±0.1%	0.0%	0.1±0.1%	0.0%
O. Rhizobiales	1.3±0.5%	0.9±0.5%	4.3±2.2%	5.8±2.2%	5.1±0.6%	7.5±4.5%	1.1±1.1%
O. Rhodobacteriales	9.7±1.7%	5.5±2.7%	47.5±8.3%	25.4±13.3%	32.5±8.6	26.0±5.4%	21.4±9.7%
O. Rickettsiales.	2.2±0.4%	6.1±0.1%	0.6±0.7%	0.3±0.2%	0.2±0.1%	0.4±0.1%	8.6±11.3%
O. Sphingomonadales.	0.3±0.1%	0.2±0.2%	0.2±0.3%	1.2±0.8%	0.7±0.3%	1.3±1.3%	0.4±0.2%
C. β-Proteobacteria	0.2±0.1%	0.0±0.1%	7.0±3.0%	44.7±11.3%	0.0%	0.0%	0.3±0.4%
C. δ-Proteobacteria;	15.5±0.8%	6.3±3.2%	0.9±0.5%	0.4±0.4%	3.1±2.4%	2.0±1.1%	1.6±2.3%
C. γ-Proteobacteria	14.6±4.7%	14.7±5.1%	25.6±12.4%	8.9±1.5%	20.9±12.5%	20.6±1.6%	2.9±1.6%

Table 5. Results of SIMPER (% of contribution) investigating the dissimilarity in bacterial communities inside every compartment between days 7 and 15 or FT and RS. L: larvae, SW: seawater, BR: bioreactor, D: day.

	SW -D7/D15	L D7/D15	BR D7/D15	SW FT/RS	L FT/RS
Bacteria unclassified	7.52	7.66	13.71	11.77	8.75
OD1 -ZB2	4.13	7.83			9.92
GN02	13.41	6.89	8.92	9.29	6.81
P. Actinobacteria +. Acidobacteria	8.05	4.24	9.64	6.96	4.19
P. Bacteroidetes.					
O. others	6.18	4.88		7.9	4.85
O. Flavobacteriales	5.24	6.97	5.99	5.45	8.28
P. Planctomycetes	5.02	4.54	7.22	4.62	6.52
C. α-Proteobacteria					
unclassified	4.87	4.36	6.99	4.57	4.48
O. Kordiimonadales.	14.3		19.58	17.8	
O. Rhizobiales				5.19	
O. Rhodobacteriales	4.51	5.23	4.78		5.11
O. Rickettsiales.	9.57	6.48	6.12	5.12	9.51
O. Sphingomonadales.			5.65		5.77
C. β-Proteobacteria		13.3			10.67
C. δ-Proteobacteria;	5.58	4.14	5.04	4.2	3.89
C. γ-Proteobacteria	4.06	8.6	4.35	5.95	6.22