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1 **Bioremediation of fishpond effluent and production of microalgae for an oyster farm in**  
2 **an innovative recirculating integrated multi-trophic aquaculture system**

3

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19

20 **Abstract:**

21 Integrated multi-trophic aquaculture (IMTA) systems are a promising solution for sustainable  
22 aquaculture combining nutrient recycling with increased biomass production. An innovative  
23 land-based recirculating aquaculture system (RAS) was studied in France for a 60-day  
24 experiment. It combined a European sea bass (*Dicentrarchus labrax*) RAS with two other  
25 production systems: high rate algal ponds (HRAP) with natural marine polyspecific algal  
26 assemblages, and oysters in separate open tanks. The objective was the assessment of: 1) the  
27 efficiency and the stability of the microalgae bioremediation of the effluent from a fish RAS  
28 in spring and summer, 2) the abundance and the diversity patterns of the microalgae biomass  
29 for consumption in the oyster compartment of the IMTA. Silicate was added every week after  
30 the beginning of the experiment for maintaining a Si:N:P molar ratio of 10:5:1 in the HRAP  
31 to encourage the growth of diatoms. The HRAP have an overall removal efficiency of  $98.6 \pm$   
32  $0.2 \%$  for  $\text{NO}_3\text{-N}$ ,  $98.0 \pm 0.4 \%$  for  $\text{NO}_2\text{-N}$ ,  $97.3 \pm 0.7 \%$  for  $\text{NH}_4\text{-N}$  and  $96.1 \pm 0.6 \%$  for  
33  $\text{PO}_4\text{-P}$ , with removal rates of  $335.8 \pm 0.8$ ,  $23.6 \pm 0.2$ ,  $30.9 \pm 0.2$ , and  $22.3 \pm 0.2 \text{ mg m}^{-2} \text{ d}^{-1}$ ,  
34 respectively. The concentration of total suspended solid (TSS) and chlorophyll *a* (chl *a*)  
35 increased during the experiment and reached maximum values on day 46 ( $135.3 \pm 34.7 \text{ mg}$   
36  $\text{TSS L}^{-1}$  and  $0.42 \pm 0.03 \text{ mg chl } a \text{ L}^{-1}$ ) after which the microalgae collapsed due to a  $\text{CO}_2$   
37 limitation (pH *ca.* 10). Sequencing analysis revealed that the microalgae community was  
38 dominated by *Tetraselmis sp.* from day 1 to day 16 (45.7 % to 73.8 % relative abundance).  
39 From day 30 to day 43 the culture was dominated by diatoms, *Phaeodactylum sp.* (83.4 % to  
40 98.1 % relative abundance). Although the stable carbon isotope signatures confirmed that the  
41 microalgae were consumed, oysters' growth was limited in the RAS-IMTA, suggesting that  
42 oysters were under stress or not fed enough.

43 **Keywords:** Microalgae; IMTA; Nutrient bioremediation; Community structure; oysters

44

## 45 **1. Introduction**

46 The global aquaculture industry is one of the fastest growing food production sectors, with an  
47 average annual increase of 5.8% (FAO, 2016). With the expansion and intensification of land-  
48 based marine farms, a large quantity of wastewater is released (Webb et al., 2012). Unless  
49 care is taken to select a suitable production area (with adequate effluent dispersal capacity),  
50 the discharged nutrients (e.g. nitrogen and phosphorus) could cause eutrophication of the  
51 aquatic system and degrade benthic and pelagic habitats (Nasir et al., 2015; Wuang et al.,  
52 2016). Aquaculture effluents need, therefore, to be treated properly to avoid environmental  
53 hazards.

54 Integrated Multi-Trophic Aquaculture (IMTA) is considered as one of the promising method  
55 for improving aquacultural performance while minimizing the environmental footprint and it  
56 offers an alternative approach for the long-term sustainability and profitability of the  
57 aquaculture industry (Le Gouvello et al., 2017). Integrated aquaculture has been used  
58 throughout the centuries, with combinations of different products (terrestrial and aquatic,  
59 vegetable and animal). For marine products, IMTA combines complementary biological  
60 compartments, at different trophic levels, in a single farm to optimize the nutrient utilization,  
61 to reduce environmental impact and to increase the overall biomass production. The  
62 wastewater from the first fed product (usually fish) is used by extractive species (macro or  
63 microalgae) which can be either turned into a marketable co-product or used as a feed  
64 resource for primary consumers (e.g. bivalves, sea-cucumbers) considered as a second  
65 product (Troell et al., 2009; Milhazes-Cunha & Otero, 2017). Each biological compartment of  
66 the IMTA, either in a single structure or in separate units, is connected with the others by  
67 water streams carrying nutrients and energy (Barrington et al., 2009). During the past 15 years,  
68 the inclusion of algae in IMTAs has been widely investigated and developed all over the  
69 world, in Asia, Canada, Chile, France, New Zealand and the US, in both open water systems

70 and land-based systems (Granada et al., 2016). *Ulva* and *Gracilaria* are the most common  
71 seaweeds used to treat mariculture effluent while oxygenating the water during day-time  
72 (Neori et al., 2004).

73 Numerous studies have evaluated the potential of macroalgae as biofilter, but the use of  
74 microalgae for IMTAs has been less studied (FAO, 2009). Microalgae present many  
75 advantages, including higher photosynthesis and nutrient remediation potentials due to a  
76 higher specific surface area, and provide high value biomass and compounds for fuel,  
77 chemicals, cosmetics and animal feed (Hein et al., 1995). Coupling microalgal biomass  
78 production with wastewater treatment was first proposed by Oswald and Golueke (1960), and  
79 now it is considered as a sustainable process successfully used for various water treatment  
80 plants (e.g. agricultural, industrial, aquacultural and domestic wastewater treatment) (Muñoz  
81 & Guieysse, 2006; Ansari et al., 2017; Gonçalves et al., 2017). Microalgae can be cultivated  
82 in wastewater in both open (e.g. raceways and ponds) and closed systems (e.g.  
83 photobioreactors, microalgae turf scrubbers and hybrid systems). Of these, open ponds  
84 account for more than 80% of global algal biomass production (Moreno-Garcia et al., 2017).  
85 Open raceway ponds for microalgal biomass production have the advantage of simple design,  
86 low operating and maintenance costs, high production volumes and the ability to capture  
87 atmospheric CO<sub>2</sub> (Lam & Lee, 2012). On the other hand, microalgal biomass production  
88 using raceway ponds is strongly influenced by several parameters, including environmental  
89 factors (i.e. temperature, solar radiation, pH, dissolved oxygen, salinity), engineering design  
90 (e.g. water dynamics, hydraulic retention time, CO<sub>2</sub> injection, mixing and depth), nutrient  
91 availability and ratios (e.g. N, P, Si and N:P), contamination (e.g. competing microalgae) and  
92 predation by zooplankton (Das et al., 2015; Kumar et al., 2015). The environmental  
93 conditions and wastewater composition influence the community structure, stability and the  
94 population density of the microalgae, which in turn affects the wastewater treatment capacity

95 and the potential of the microalgae as food for other compartments, such as filter-feeders (Li  
96 et al., 2017). The use of Pacific oysters *Crassostrea gigas*, may be considered as a logical first  
97 choice as it is one of the main bivalve species farmed in Europe. However, assessment of the  
98 oyster's ability to assimilate microalgae produced in an IMTA system is required to optimize  
99 the growth of the oysters and to improve the overall IMTA production efficiency (Lefebvre et  
100 al., 2000). The microalgal community can be guided towards suitable species for oysters by  
101 controlling the nutrient ratios (Lefebvre et al., 1996).

102 To date, very few studies on IMTA using microalgae for bioremediation have been published  
103 and they have focused mainly on the removal of key nutrients, microalgal biomass production  
104 or the dominant algae produced (Milhazes-Cunha & Otero, 2017). Very little information is  
105 available on the microalgal community structure and its dynamics as a function of biotic and  
106 abiotic parameters, its ability to clean wastewater from fish ponds and its suitability for  
107 feeding oysters. **This** study used a land-based IMTA system with a recirculating aquaculture  
108 system (RAS) for European sea bass (*Dicentrarchus labrax*) coupled to open raceway ponds  
109 with natural microalgae populations cultivated using the High Rate Algal Pond (HRAP)  
110 system (Deviller et al., 2004; Metaxa et al., 2006). The microalgae population structure was  
111 biased towards diatoms by the addition of silicate, and the microalgae produced was fed to  
112 **smaller and larger juvenile** oysters (*Crassostrea gigas*). **Main objectives of this experiment**  
113 were: 1) to assess the nutrient removal efficiency, and microalgal biomass production of this  
114 innovative system 2) to characterize the dynamics of the microalgal diversity using genomics  
115 and 3) to evaluate the assimilation of the microalgae by **smaller and larger juvenile** oysters  
116 using stable carbon isotope signatures.

117

## 118 **2. Materials and methods**

### 119 **2.1. RAS-IMTA system facilities**

120 The experiment ran over 60 days, from April 3<sup>rd</sup> 2017 (day 1) to June 1<sup>st</sup> 2017 (day 60), at the  
121 Ifremer Station at Palavas-les-Flots, southern France in a Mediterranean climate. The  
122 experimental RAS-IMTA system (Fig.1) presented three different units in series: A) indoor  
123 classical RAS fish tanks (triplicate), as described by Blancheton (2000), B) outdoor HRAP  
124 microalgal units (algal raceways, triplicate), as described by Deviller et al. (2004); C) outdoor  
125 oyster units with larger juveniles (triplicate) and smaller juveniles (triplicate). The wastewater  
126 from the three RAS tanks was pooled and then distributed into three algal raceways. The  
127 outflow of the raceways was then pooled in a mixing tank, and distributed with air stones to  
128 feed the oyster units.

129 European sea bass (*Dicentrarchus labrax*) were stocked at an initial density of 30 kg m<sup>-3</sup>  
130 (1000 fish per tank, 120 g ind<sup>-1</sup>). A commercial diet (Neo Grower Extra Marin 5<sup>®</sup>, Gouessant)  
131 was supplied *ad libitum* using self-feeders, and oxygen was supplied in fish tanks to saturated  
132 concentration. The water from the fish tanks passed through mechanical filter (30µm mesh),  
133 UV-treatment and bacterial biofilter; a part was recirculated into the fish unit and another part  
134 (180 L h<sup>-1</sup>) flew directly towards three algal raceways (area = 12 m<sup>2</sup>, depth = 0.50 m; volume  
135 = 6 m<sup>3</sup>). Each algal raceway (water depth = 0.40 m, working volume = 4.8 m<sup>3</sup>, hydraulic  
136 retention time = 4 days) was equipped with air-diffuser and circulating pump to homogenize  
137 the water column. Each raceway was initially filled with natural seawater pumped from the  
138 shore. One month before the experiment, ammonia and phosphate (5 mmol L<sup>-1</sup> NH<sub>4</sub>Cl and 0.5  
139 mmol L<sup>-1</sup> HK<sub>2</sub>O<sub>4</sub>P) were added to each of the three algal raceways to initiate a microalgal  
140 bloom. After 15 days, the fish effluent (from RAS) was diverted through the three algal  
141 raceways. On March 24<sup>th</sup> 2017, silicate (Na<sub>2</sub>SiO<sub>3</sub>•5H<sub>2</sub>O) was added to the three raceways to  
142 achieve a N:Si:P molar ratio of 10:5:1 as suggested for marine diatom dominance by Lefebvre  
143 et al. (1996), and weekly added during the experiment to maintain this molar ratio.

144 From day 1, the cultured algae were pooled in a mixing tank (equipped with air-stones), then  
145 distributed to six oyster tanks (volume = 0.5 m<sup>3</sup>) at a continuous flow rate of 5 L.h<sup>-1</sup>; all oyster  
146 tanks were also fed with 500 L.h<sup>-1</sup> of filtered seawater in order to approach a suitable  
147 concentration of microalgae for the oysters according to Rico-Villa et al. (2009) (around 1.5-  
148 3.7 × 10<sup>6</sup> cells/ml) and to reduce pH variability. After 20 days of acclimatization, 300 smaller  
149 juvenile oysters (0.05 ± 0.00 g ind<sup>-1</sup>, 7-month-old) were placed in each of 3 of the tanks (i.e.  
150 900 ind.) and 45 larger juvenile oysters (4.76 ± 0.15 g ind<sup>-1</sup>, 19-month-old) were placed in  
151 each of the other three tanks (i.e. 135 larger ind.). A similar weight of smaller (nb= 300) and  
152 larger (nb = 45) juvenile oysters was reared nearby in natural conditions as controls.

## 153 2.2. Environmental parameters

154 Throughout the experiment, local climatic conditions including air temperature and solar  
155 irradiance were recorded twice a day (9:00 a.m. and 3:00 p.m.).

156 For the RAS-IMTA system, water temperature, salinity, pH and dissolved oxygen  
157 concentrations (DO) were monitored daily (9:00 a.m.) in the fish rearing tanks and twice a  
158 day (9:00 a.m. and 3:00 p.m.) in the algal raceways and the oyster tanks using a YSI ® probe.  
159 CO<sub>2</sub> concentrations in the algal raceways were recorded every 10 minutes using a CO<sub>2</sub>  
160 OxyGuard® probe. In the natural lagoon, where oyster controls were stored, environmental  
161 data was monthly measured (temperature, salinity and nutrients).

## 162 2.3. Nutrient concentrations

163 Nutrient concentrations were first measured over two 24-h periods (Fig.2) to evaluate the  
164 fluctuations and determinate the sampling time (2:00) of the water samples.

165 Based on these results, 50 ml water samples were taken twice a week at 2:00 p.m. at three  
166 points in the IMTA system (fish tanks, RAS effluent and algal raceways), monthly in the  
167 natural lagoon. Water samples were filtered (GF/F, Whatman<sup>TM</sup>), and stored it at -25 °C for  
168 NO<sub>3</sub>-N, NO<sub>2</sub>-N, NH<sub>4</sub>-N and PO<sub>4</sub>-P analysis (Alliance® auto-analyzer).



169 **Nutrient** removal efficiency (RE, %) and removal rate (RR, mg m<sup>-2</sup> d<sup>-1</sup>) were calculated for  
170 each microalgal raceway using the following equations:

$$171 \quad RE (\%) = \frac{C_{eff} - C_{AR}}{C_{eff}} \times 100\% \quad (1)$$

$$172 \quad RR (mg \, m^{-2} \, d^{-1}) = \frac{RE \times C_{eff} \times Q \times 24}{S \times 100} \quad (2)$$

173 Where C<sub>eff</sub> is the nutrient concentration in the RAS effluent (mg L<sup>-1</sup>), C<sub>AR</sub> is the nutrient  
174 concentration in the algal raceways, Q is the water flow through the algal raceway (L h<sup>-1</sup>), and  
175 S is the surface area of the algal raceway (12 m<sup>2</sup>).

#### 176 **2.4. Microalgal production and community structure**

177 The microalgal growth was monitored twice a week at 2:00 p.m. by estimating the total  
178 suspended solids (TSS) and chlorophyll *a* (Chl *a*). TSS was determined gravimetrically from  
179 the solids retained on GF/F filters (Whatman<sup>TM</sup>) (Association, 1995). For Chl *a*, the water  
180 samples were filtered onto GF/F filters (Whatman<sup>TM</sup>) and the pigments were extracted with  
181 methanol (Ritchie, 2006). The Chl *a* concentration was calculated from spectrophotometer  
182 measurements using the equation provided by Ritchie (2006).

183 The microalgal community structure was determined on day 1, day 16, day 30 and day 43  
184 using 18S rRNA gene analysis. 10mL samples were filtered onto 0.2µm membranes (PALL  
185 Supor® 200 PES) and stored at -20 °C for subsequent DNA extraction. The DNA was  
186 extracted using the DNeasy PowerWater Kit (Qiagen) according to the manufacturer's  
187 instructions. The V4 region of the 18S rRNA gene was amplified over 30 amplification cycles  
188 at an annealing temperature of 65 °C, with forward and reverse primers (Table 1) with their  
189 associated linkers. The resulting products were purified and loaded onto an Illumina MiSeq  
190 cartridge for sequencing paired 300 bp reads following manufacturer's instructions (v3  
191 chemistry). Sequencing and library preparation were carried out at the Genotoul Lifescience  
192 Network Genome and Transcriptome Core Facility in Toulouse, France (get.genotoul.fr). A  
193 modified version of the standard operation procedure for MiSeq data (Kozich, 2013) in

194 Mothur version 1.35.0 (Schloss, 2009) was used for alignment and taxonomic outline. Mothur  
195 was also used to identify representative sequences of operational taxonomic units (OTUs).

## 196 **2.5. Oyster growth rates and assimilation capacity**

197 **Every two weeks, the length and weight of the smaller juvenile oysters (60 randomly sampled)**  
198 **and of all the larger juvenile oysters were measured.** They were compared against the oysters  
199 grown in the natural conditions.

200 The carbon stable isotopes ratios were measured to determine the capacity of oysters to  
201 assimilate the microalgae. Oysters and microalgae were sampled once a week from day 1 to  
202 day 16 and then every two weeks from day 16 to day 60. For oysters, all the flesh was  
203 separated from the shells, rinsed with milliQ water, freeze-dried and ground to a fine powder.  
204 Approximately 1 mg of each dried sample was weighed and packed in a tin capsule for carbon  
205 isotope analysis. Microalgae was sampled by filtering 10 to 30 ml of water onto pre-  
206 combusted GF/F filters (Whatman<sup>TM</sup>). We freeze-dried the filters and packed them in tin  
207 capsules for carbon isotope analysis. The isotopic ratio (<sup>13</sup>C/<sup>12</sup>C) of the samples were  
208 analyzed by continuous-flow elemental analyzer/isotope ratio mass spectrometry (EA/IRMS)  
209 using an Isoprime GVI IRMS (Elementar, Langensfeld, Germany) coupled to and EuroEA  
210 3000 elemental analyzer (Eurovector, Pavia, Italia). The <sup>13</sup>C/<sup>12</sup>C ratio was expressed as δ<sup>13</sup>C  
211 per mille (‰) relative to Vienna Pee Dee Belemnite, according to the following equation:

$$212 \quad \delta^{13}\text{C} = \left( \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000$$

213 Where R is the ratio of heavy to light isotope (<sup>13</sup>C/<sup>12</sup>C), R<sub>sample</sub> is the ratio of oyster samples,  
214 R<sub>standard</sub> is the ratio of standard substance.

215 Repeated measurements on glycine showed a precision of ± 0.16 per mille points for δ<sup>13</sup>C,  
216 and ± 1.05% for C. Alanine, wheat flour and corn flour from IsoAnalytical Lab (Crew, United  
217 Kingdom), IAEA-N-1, IAEA-N-2 and IAEA-CH3 cellulose and USGS24 graphite from

218 National Institute of Standard and Technology (Gaithersburg, USA) were used for multipoint  
219 calibration.

## 220 **2.6. Statistical analysis**

221 **Mean** and standard deviation of replicated experimental variables (environmental parameters,  
222 microalgal biomass, nutrient removal efficiency and oyster biomass) **were calculated** using  
223 Microsoft Excel 2016. SPSS 21.0 for one-way analysis of variance (LSD post-hoc test,  $p =$   
224 0.05) **was used** to assess differences between raceways triplicates and between oyster biomass  
225 in the IMTA and natural conditions.

226

## 227 **3. Results**

### 228 **3.1. Environmental parameters**

229 Over the study period, **air** temperature ranged from 9.5 to 28.9 °C ( $17.7 \pm 3.9$  °C), and  
230 irradiance varied between 0.2 to 20.6 W m<sup>-2</sup> ( $11.7 \pm 5.7$  W m<sup>-2</sup>).

231 Mean values ( $\pm$  SD) of the temperature, dissolved oxygen, salinity and pH of the water in the  
232 IMTA and in the natural conditions (temperature and salinity) are given in Table 2. There was  
233 no significant difference ( $p > 0.05$ ) between the RAS tanks, algal raceways and oyster tanks  
234 replicates.

235 In the indoor **fish tanks (RAS)**, the water temperature ranged from 16.3 to 25.2 °C ( $19.4 \pm$   
236 2.3 °C). **Temperature** was much more variable at the outdoor facilities, ranging from 7.9 to  
237 26.9 °C ( $17.3 \pm 4.3$  °C) **in the algal raceways** and from 7.3 to 24.2 °C ( $16.4 \pm 3.6$  °C) **in the**  
238 **oyster tanks**.

239 During the 60 days' experiment, the dissolved oxygen concentrations (DO) in the **indoor** RAS  
240 ranged from 4.1 to 17.0 mg L<sup>-1</sup> ( $8.6 \pm 2.6$  mg L<sup>-1</sup>) **and in the outdoor algal** raceways from 1.1  
241 to 22.1 mg L<sup>-1</sup> ( $12.2 \pm 4.1$  mg L<sup>-1</sup>). **In the oyster tanks DO was in** a much narrower range from

242 5.0 and 9.5 mg L<sup>-1</sup> (7.5 ± 0.8 mg L<sup>-1</sup>). No CO<sub>2</sub> was detected in the algal raceways throughout  
243 the experiment.

244 The salinity in the RAS ranged from 34.1 to 38.8 (37.8 ± 1.3) but was higher in the outdoor  
245 algal raceways and oyster tanks. PH in the RAS ranged from 6.3 to 7.7 (7.2 ± 0.2), in optimal  
246 range for seabass rearing (Mladineo et al, 2010). It was higher in the algal raceways, ranging,  
247 from 8.8 to 10.5 (9.9 ± 0.4), and in the oyster tanks, ranging from 8.2 to 9.6 (8.7 ± 0.3).  
248 Differences between algal raceways and oyster tanks are mainly due to a dilution factor (= 10),  
249 with water from the raceways being diluted with natural seawater by new seawater in oyster  
250 tanks, to maintain temperature and pH.

### 251 3.2. Nutrient bioremediation

252 The nutrient concentrations in the RAS effluent and algal raceways are presented in Fig.3.  
253 The NO<sub>3</sub> concentrations in the RAS effluent increased from 1.87 mg N L<sup>-1</sup> on day 3 to 5.12  
254 mg N L<sup>-1</sup> on day 43, and then remained stable. The NO<sub>2</sub> concentrations increased from 0.29  
255 mg N L<sup>-1</sup> on day 3 to 0.50 mg N L<sup>-1</sup> on day 16 before falling to 0.20 mg N L<sup>-1</sup> at the end of  
256 the experiment (day 60). The NH<sub>4</sub> concentration in RAS effluents averaged 0.43 ± 0.07 mg N  
257 L<sup>-1</sup> and the PO<sub>4</sub> concentration averaged 0.31 ± 0.06 mg P L<sup>-1</sup>.

258 Nutrient concentrations in the algal raceways were significantly lower than those in the RAS  
259 effluents. Overall average concentrations in the algal raceways were 0.11 ± 0.22 mg NO<sub>3</sub>-N  
260 L<sup>-1</sup>, 0.02 ± 0.03 mg NO<sub>2</sub>-N L<sup>-1</sup>, 0.01 ± 0.01 mg NH<sub>4</sub>-N L<sup>-1</sup>, and 0.03 ± 0.06 mg PO<sub>4</sub>-P L<sup>-1</sup>.  
261 After day 32, no NO<sub>3</sub>-N and NO<sub>2</sub>-N was detected in the algal raceways. The nutrient  
262 concentrations in the natural lagoon (control) were very low, 0.61 ± 0.55 μM NO<sub>3</sub>-N, 0.13 ±  
263 0.11 μM NO<sub>2</sub>-N, 2.37 ± 0.40 μM NH<sub>4</sub>-N and 0.05 ± 0.03 μM PO<sub>4</sub>-P.

264 Overall nutrient removal efficiencies and removal rates for the algal raceways are given in  
265 Table 3. More than 96% of N and P were removed at rates of 391.1 ± 0.6 mg N m<sup>-2</sup> d<sup>-1</sup> and  
266 22.3 ± 0.2 mg P m<sup>-2</sup> d<sup>-1</sup>, respectively (Table 3).

### 267 **3.3. Microalgae production and community structure**

268 The total suspended solids (TSS) and chlorophyll *a* concentration (Chl *a*) in the algal  
269 raceways, two proxies of microalgal biomass, are presented in Fig.4. TSS and Chl *a* showed  
270 similar trend, with concentrations increasing slowly, reaching maxima from 107 to 174 mg L<sup>-1</sup>  
271 for TSS and from 0.36 to 0.48 mg L<sup>-1</sup> for Chl *a*, between day 43 and day 50. After day 50,  
272 the microalgal biomass collapsed in the three raceways. During summer, the natural lagoon  
273 Chl *a* median value is around 12.7-15.2 µg L<sup>-1</sup> (Lerustre et al, 2015).

274 The structure of the microalgal community in the four sampling days (day 1, 16, 30 and 43) is  
275 shown in Fig.5. There was a shift in algal population that occurred between day 16 and day 30.  
276 During the first part of the experiment (at least until day 16), *Tetraselmis sp.* was dominant  
277 (45.7% to 73.8%) and the diatom *Stauroneis sp.* was a major component (11.5 – 16.9 %) in all  
278 raceways. Samples from day 30 and after showed that the diatom *Phaeodactylum sp.* was  
279 strongly dominant (83.4% to 98.1%) in all the raceways.

### 280 **3.4. Oyster growth and assimilation capacity**

281 The oyster growth is shown in Fig. 6. Oysters in the RAS-IMTA system did not seem to grow.  
282 However, oysters cultivated in natural conditions grew from 4.76 ± 0.15 g ind<sup>-1</sup> on day 1 to  
283 11.4 ± 4.3 g ind<sup>-1</sup> on day 57 for larger oysters and from 0.05 ± 0.00 g ind<sup>-1</sup> on day 1 to 2.5 ±  
284 1.0 g ind<sup>-1</sup> on day 57 for smaller oysters. At the end of the experiment the smaller oysters  
285 growing in natural conditions were 12.5 times heavier (2.5 ± 1.0 g ind<sup>-1</sup>) than those in the  
286 IMTA system (0.20 ± 0.10 g ind<sup>-1</sup>). The same effect was observed for larger oysters, with a  
287 weight of 11.4 ± 4.3 g for those in natural conditions against 5.3 g to 5.5 g for those in the  
288 IMTA system.

289 The δ<sup>13</sup>C signatures for the microalgae and juvenile oysters in the IMTA system and the  
290 control oysters are shown in Fig.7. The δ<sup>13</sup>C signature of the control oysters remained stable  
291 (-22.37 ± 0.99) during the experiment, while the δ<sup>13</sup>C signature of the oysters in the IMTA

292 system increased from  $-24.00 \pm 0.14$ , at day 2, up to  $-15.02 \pm 0.23$  at the end of the  
293 experiment, close to the  $\delta^{13}\text{C}$  signature of the microalgae in the raceways ( $-15.36 \pm 2.63$ ).

#### 294 4. Discussion

295 One of the objectives of this study was to evaluate the nutrient removal efficiency of  
296 microalgae in an RAS-IMTA system. More than 96 % of nitrogen and phosphorus were  
297 removed, **indicating nutrient bioremediation of the studied IMTA system**. This compares  
298 favorably with the small number of published studies of microalgae-based IMTA systems.  
299 Goldman et al. (1974), for instance, described a system with marine microalgae, oysters and  
300 seaweed where the algae removed approximately  $20.0 \text{ mg N m}^{-2} \text{ d}^{-1}$  and  $2.5 \text{ mg P m}^{-2} \text{ d}^{-1}$   
301 **(corresponding to removal efficiency of 95% N and 45% to 60% P)**. **According to these**  
302 **authors**, the main reason for the high nitrogen and low phosphorus removal was the low N/P  
303 atomic ratio of 4.9:1. **Likewise, Hussenot et al. (1998) reared microalgae in a raceway**  
304 **supplied with fish tank effluents ( $\text{NH}_4\text{-N}$ ,  $1.27 \text{ mg L}^{-1}$  and  $\text{PO}_4\text{-P}$ ,  $0.24 \text{ mg L}^{-1}$ ) to feed oysters**  
305 **(*Crassostrea gigas*). In that study, fish effluents in microalgae raceways were diluted with a**  
306 **dilution rate of  $70\% \pm 10\%$  per day (much higher than our study) to reduce total ammonia**  
307 **concentrations; the microalgae in raceways removed 67% ( $21.3 \text{ mg N m}^{-2} \text{ d}^{-1}$ )  $\text{NH}_4\text{-N}$  and**  
308 **46.6 % ( $2.8 \text{ mg P m}^{-2} \text{ d}^{-1}$ )  $\text{PO}_4\text{-P}$ . In a recent study using microalgae photo-bioreactors to treat**  
309 **tilapia RAS effluent, Michels et al. (2014) found** when extra phosphate was added into the  
310 wastewater, the N removal efficiency was improved from 49.4 % to 99.7 % (the P removal  
311 efficiency was always more than 99 %). More recently, a IMTA system combining the  
312 tunicate *Styela clava* and the sea cucumber *Stichopus japonicus* removed 54% of dissolved  
313 inorganic nitrogen (DIN) and 50% of  $\text{PO}_4\text{-P}$ , keeping the nutrients at low levels (i.e.,  $\text{C}_{\text{DIN}} <$   
314  $0.2 \text{ mg L}^{-1}$  and  $\text{C}_{\text{P}} < 0.02 \text{ mg L}^{-1}$ ) (Ju et al., 2015). **IMTA system was indoors** and the *Styela*  
315 *clava* concentration was kept at low level, around 200-300 ind.  $\text{m}^{-3}$ . **For these studies, the N**  
316 **and P concentrations were lower than concentrations we obtained, RAS effluents producing**

317 higher concentrations of microalgae and giving ten times higher nutrient removal rates, 391.1  
318  $\pm 0.6 \text{ mg N m}^{-2} \text{ d}^{-1}$  and  $22.3 \pm 0.2 \text{ mg P m}^{-2} \text{ d}^{-1}$ .

319 The nutrient bioremediation rates obtained in our IMTA systems were close to or greater than  
320 previous macroalgae-based IMTA systems. In a 15-month study of Pagand et al. (2000),  
321 green macroalgae were used to treat sea bass RAS effluents containing  $10 \text{ mg L}^{-1}$  of DIN and  
322  $1.3 \text{ mg L}^{-1}$  of P; they obtained DIN removal efficiencies between 30% and 88% (removal rate:  
323 from 420 to  $1220 \text{ mg m}^{-2} \text{ d}^{-1}$ ) and  $\text{PO}_4\text{-P}$  removal efficiencies between 0% and 82% (removal  
324 rate: from 0 to  $148 \text{ mg m}^{-2} \text{ d}^{-1}$ ). Similarly, a 12-month-study treating sea bass RAS effluent in  
325 macroalgae raceways, achieved  $360 \pm 140 \text{ mg m}^{-2} \text{ d}^{-1}$  DIN and  $8 \pm 16 \text{ mg m}^{-2} \text{ d}^{-1}$  P removal,  
326 close to our results (Deviller et al., 2004; Metaxa et al., 2006). The highest N and P removal  
327 rates in their studies occurred during summer. Although our results suggest microalgae as a  
328 viable alternative to macroalgae in IMTA systems, it needs further study to assess the year-  
329 round performance as N and P removal rates since they may be strongly influenced by the  
330 season.

331  
332 The proxy used to evaluate the microalgal biomass in the RAS-IMTA system reached  
333 maximal values of 107 -  $174 \text{ mg TSS L}^{-1}$  and 0.36 –  $0.48 \text{ mg Chl } a \text{ L}^{-1}$ . Previous studies have  
334 demonstrated the microalgal wastewater treatment (municipal, agricultural and industrial  
335 wastewater) with production rates varying from 74 to  $5530 \text{ mg TSS L}^{-1} \text{ d}^{-1}$ , most of which  
336 were much higher than our study (Wilkie & Mulbry, 2002; Sacristán de Alva et al., 2013;  
337 Dahmani et al., 2016) probably because much higher nutrient concentration in their effluents.  
338 The microalgal biomass yield was calculated based on the N consumed (Table 4) and the  
339 results showed that biomass production in the raceways was in the range of those measured in  
340 previous studies using various types of wastewater (i.e. aquaculture, domestic, municipal and  
341 dairy wastewater).

342 Even in IMTA systems, regardless of culturing regimes (i.e. continuous, semi-continuous,  
343 batch), the maximum biomass expressed as TSS and Chl *a* concentrations have ranged from  
344 42.6 to 520.0 mg L<sup>-1</sup> d<sup>-1</sup> and from 0.35 to 1.95 mg L<sup>-1</sup> d<sup>-1</sup>, respectively (Borges et al., 2005;  
345 Michels et al., 2014; Gao et al., 2016). Microalgal production could be affected by various  
346 factors, both biotic and abiotic (Gonçalves et al., 2017). The temperature and light conditions  
347 during our study were adequate for algal growth (Table 2). Nutrient concentrations and ratios,  
348 especially the N:P ratio, affects the growth of microalgae by influencing the metabolic  
349 activity and growth rate (Werner, 1977; Smith & Geider, 1985). However, most of inorganic  
350 nitrogen and phosphate and all the detectable CO<sub>2</sub> in the microalgal raceways were consumed,  
351 indicating that the N, P and CO<sub>2</sub> availability were the main factors limiting the algal growth in  
352 the raceways. **Under continuous cultivation**, Borges et al. (2005) reported that high microalgal  
353 biomass and nutrients removal efficiencies would be obtained only if the molar N:P ratio in  
354 the wastewater is in balance with the ratio of the microalgal biomass. Here, the RAS effluent  
355 had an average N:P molar ratio of 38, which was **twice as high as** the Redfield ratio of 16 (the  
356 **N:P molar** ratio reported for the biomass of marine microalgal species in general) (Redfield,  
357 1958). This suggests that P **was a limiting** factor for the growth of microalgae in the raceways  
358 **under continuous effluent supply. However, because of high photosynthetic CO<sub>2</sub> fixation, pH**  
359 **reached values greater than 9 (9.9), high pH values would shift CO<sub>2</sub> to bicarbonate**  
360 **equilibrium towards bicarbonate, thereby further reduce CO<sub>2</sub> concentration.** A CO<sub>2</sub> limitation  
361 affected the duration of the experiment and probably explains the collapse of the algal culture  
362 in the raceways. The lack of CO<sub>2</sub> may not be the only factor, high pH (ca. 10), the  
363 development of viruses, parasites and predators may also affect the algal growth in HRAPs  
364 (see Flynn et al 2017). In this experiment, it has been decided to develop a landbased-IMTA  
365 system in a simplest way, minimizing energy costs, and using water mixing to get an air-  
366 water CO<sub>2</sub> equilibrium. This choice did not meet the long-term algal CO<sub>2</sub> demand, and



367 addition of CO<sub>2</sub> is mandatory to maintain the system working as long as necessary for oyster  
368 to grow. In future experiments, the system could be improved, using a CO<sub>2</sub> low cost  
369 regulation and further investigations will be conducted using enriched CO<sub>2</sub> air from RAS  
370 compartment.

371 The high *Phaeodactylum sp.* abundance in the later part of the study period confirmed the  
372 interest of adding silicate to obtain diatom dominance in microalgae populations. For example,  
373 Lefebvre et al. (1996) added silicate into mariculture effluent to achieve a Si:P ratio of 4:1,  
374 while Hussenot et al. (1998) used an Si:P ratio of 5:1 and in both cases, the diatom of  
375 *Skeletonema costatum* became dominant. With an N:Si:P ratio of 10:3.3:1 in wastewater,  
376 Lefebvre et al. (1996) found that *Chaetoceros simplex* became dominant. The dominant  
377 diatom mainly depends on the species initially present in the inoculum.

378  
379 The change in the oyster's  $\delta^{13}\text{C}$  signature in the IMTA system towards values close to the  
380 microalgal signature showed that the oysters consumed the microalgae. However, neither  
381 larger nor smaller oyster juveniles in the IMTA system showed any increase in weight during  
382 the experiment even though they were able to consume the microalgae. Two hypotheses could  
383 explain the low growth performance: 1) a biochemical composition of the microalgae did not  
384 meet the oyster growth requirements; and 2) a poor access to food probably due to an  
385 inadequate hydrodynamic in the oyster tanks and a fouling on the oyster baskets obstructing  
386 the passage of phytoplankton. Further investigations have thus required in order to study the  
387 influence of size and biochemical composition of IMTA-produced microalgae on the feeding  
388 response of oysters in different food mixing conditions.

389

## 390 **5. Conclusions**

391 Although some research has been carried out using microalgae-based RAS-IMTA systems,  
392 there is limited information on the performance of these systems. Our study used an  
393 innovative RAS-IMTA system to test the nutrient bioremediation efficiency and the ability to  
394 produce a consortium of diatoms suitable for oyster farming.

- 395     ▪ Microalgae-based RAS-IMTAs have potential for nutrient bioremediation. Nutrient  
396         removal rates were close to those obtained in similar macroalgae-based IMTA systems.
- 397     ▪ The RAS-IMTA system produced microalgae with the maximal yield of 20.5 – 33.3 g  
398         Algae gN<sup>-1</sup> and 0.07 – 0.09 g Chla gN<sup>-1</sup>. Adding silicate shifted the dominant  
399         microalgae species from *Tetraselmis sp.* to *Phaeodactylum sp.*
- 400     ▪ The oyster growth was very **poor**, although juvenile oysters **seemed to be** able to  
401         assimilate produced microalgae.

402 Further experiments should be carried out to improve the overall production and resilience  
403 of the IMTA system by: (a) adding an extra low-cost CO<sub>2</sub> source in order to stabilize pH  
404 and to maintain a high CO<sub>2</sub> level in algal raceways, (b) optimizing the food access to  
405 oysters.

406

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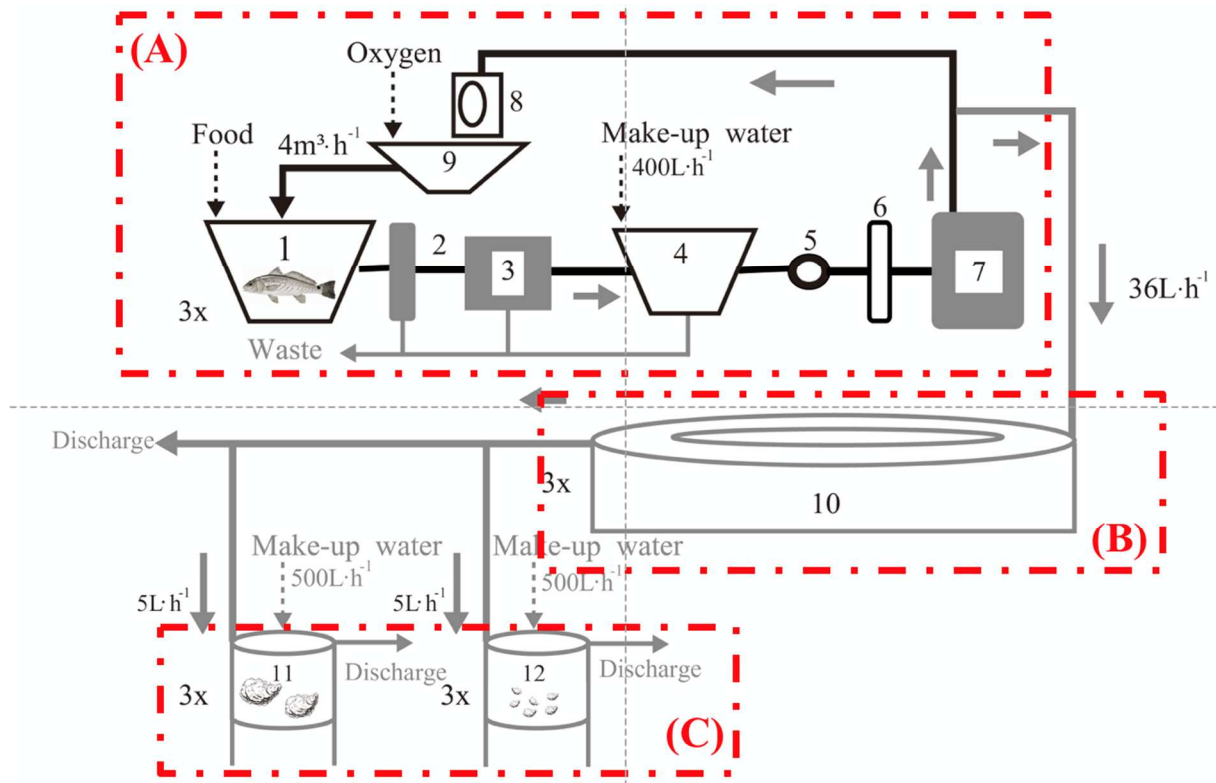
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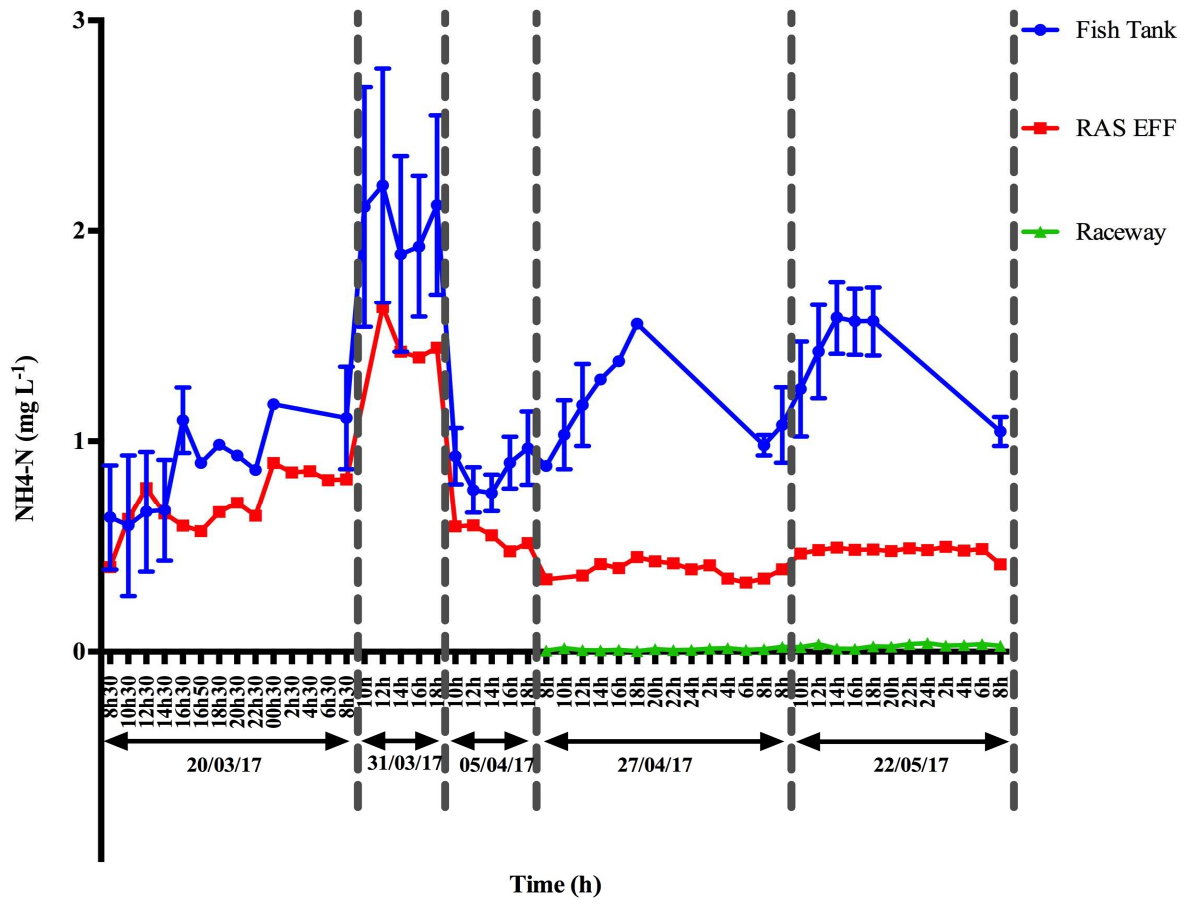
536 Fig.1 Schematic diagram of the recirculating aquaculture system / integrated multi-trophic  
 537 aquaculture (RAS-IMTA) system (A: RAS compartment; B: Microalgae raceways; C: Oyster  
 538 tanks.)

539 1: Fish tank,  $4\text{ m}^3$ ; 2: Particle trap; 3: Mechanical filter,  $30\text{-}\mu\text{m}$  mesh filter; 4: Pumping tank;  
 540 5: Pump; 6: UV lamp; 7: Biological filter; 8: Packed column; 9: Storage tank; 10: microalgal  
 541 raceway,  $12\text{ m}^2$ ; 11: Large juvenile oyster tank,  $0.5\text{ m}^3$ ; 12: Juvenile oyster tank,  $0.5\text{ m}^3$ .

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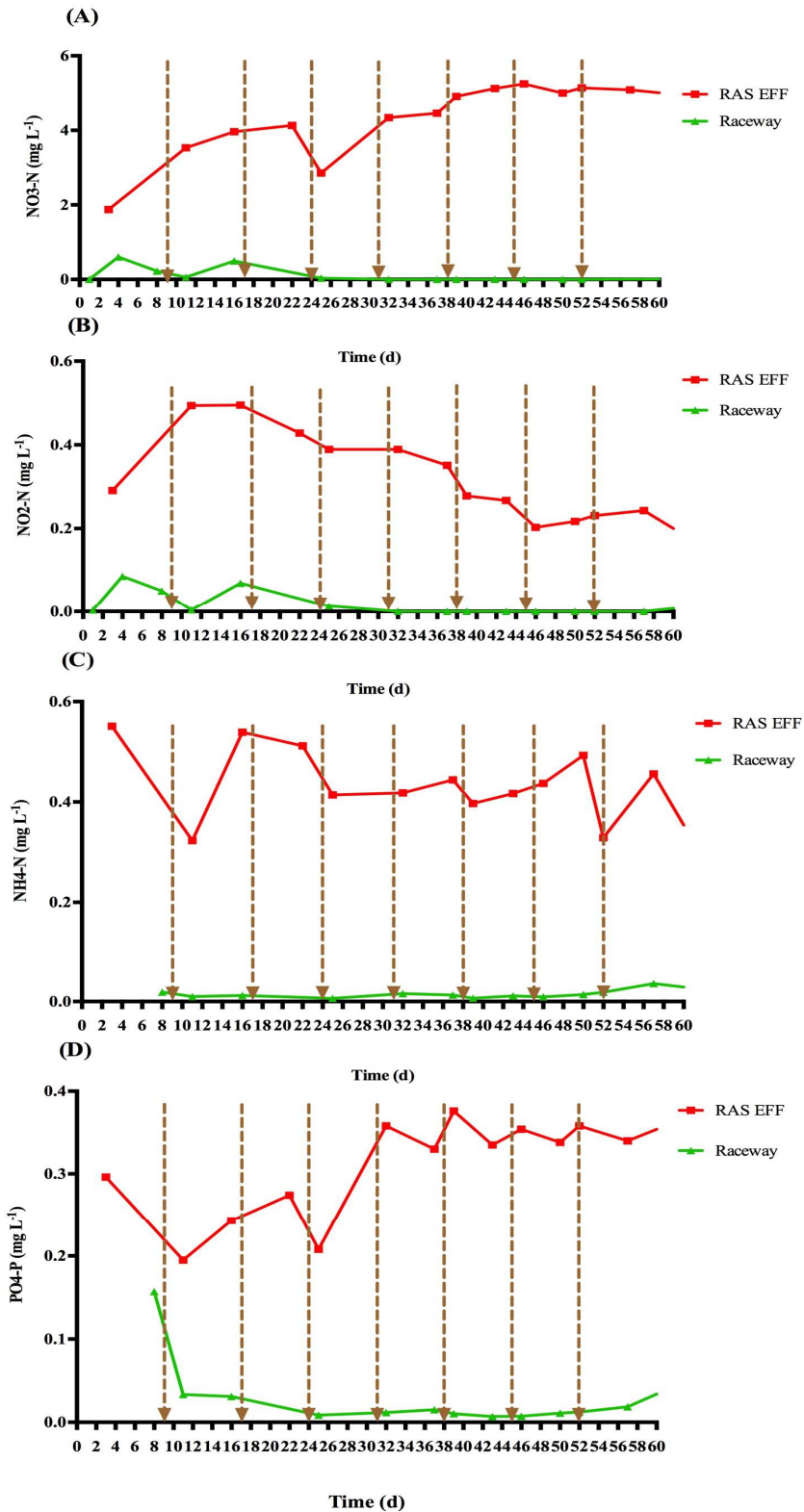


(C)







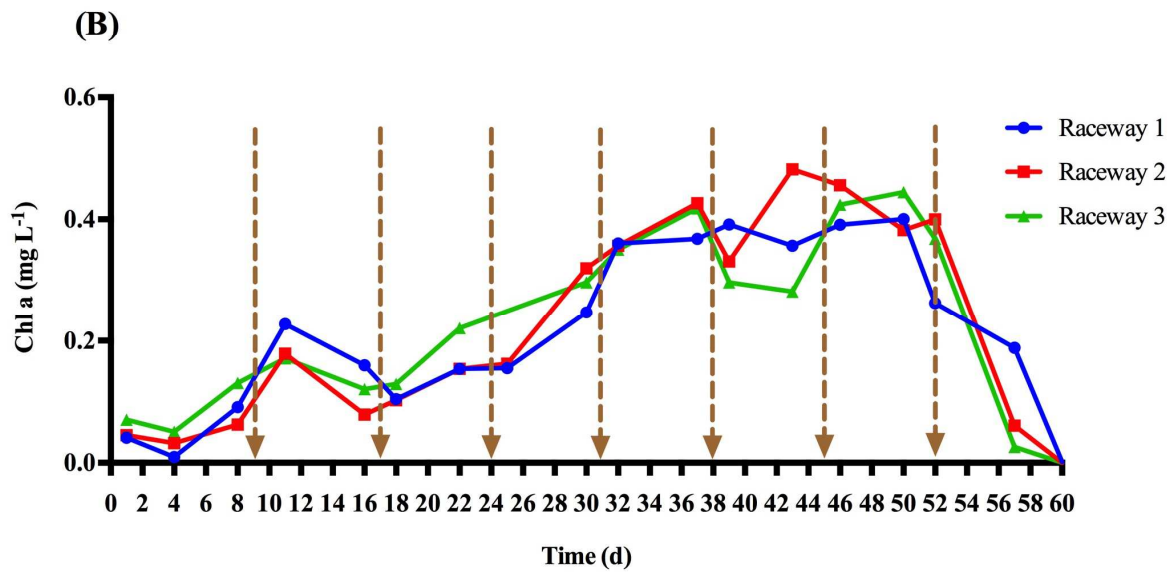
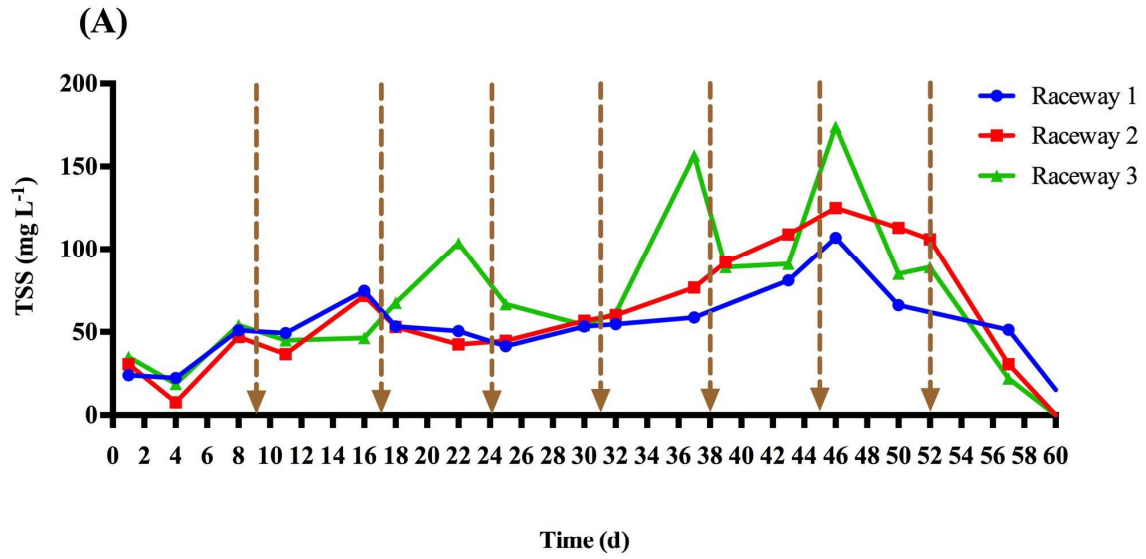


550

551 Fig.3 Nitrate (A), nitrite (B), ammonia (C) and phosphate (D) concentrations in RAS effluents

552 (RAS EFF) (fish tanks) and microalgal raceways over the experimental period; dashed arrows

553 represent the addition of silicates in raceways

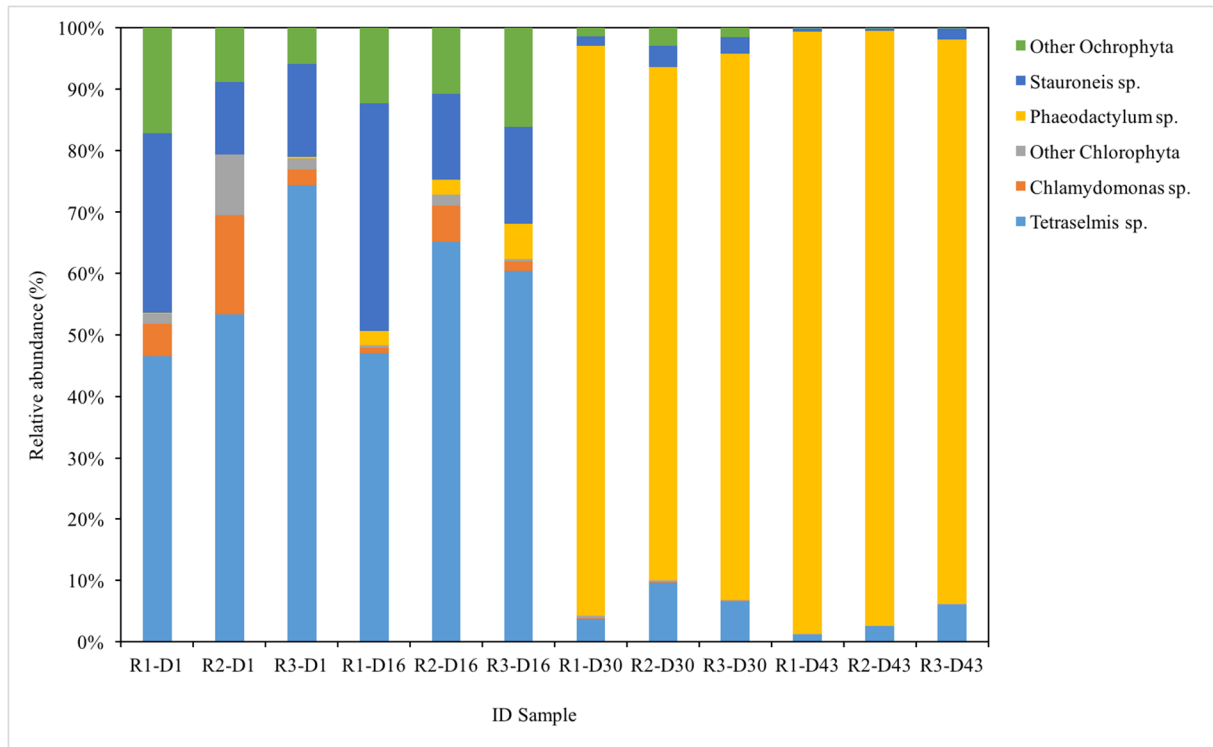


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555 Fig.4 Microalgal biomass concentration based on TSS (A) and chlorophyll a (B) over the

556 experimental period; dashed arrows represent the dates of addition of silicates in raceways

557



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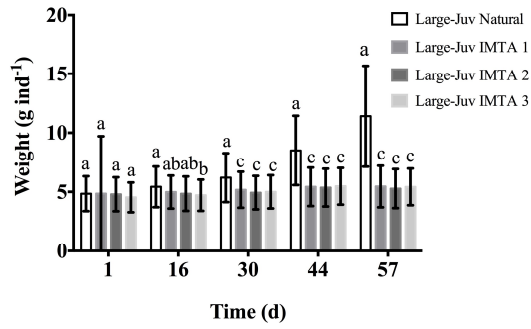
559 Fig.5 The microalgal community structure composition at genus level (Rx-Dy indicates the  
 560 samples taken in Raceway x on day y)

561 Note: the abundance was defined as the number of sequences affiliated with that genus  
 562 divided by the total number of sequences per sample.

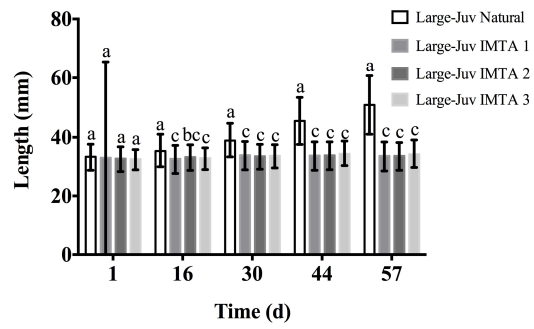
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565 (A)

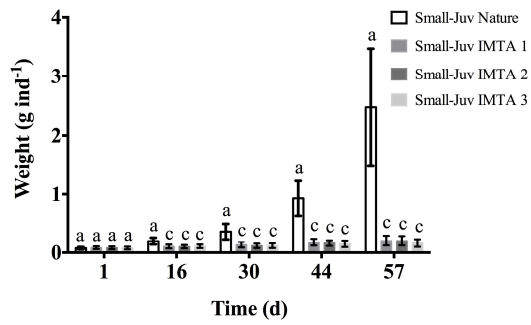


(B)

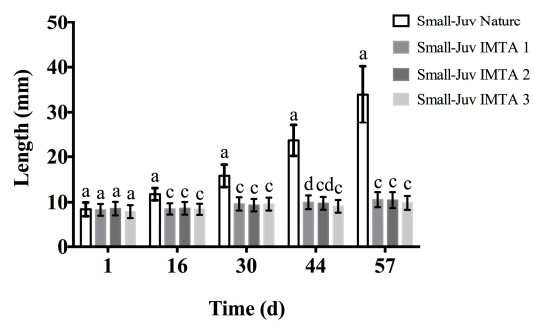


566

567 (C)



(D)



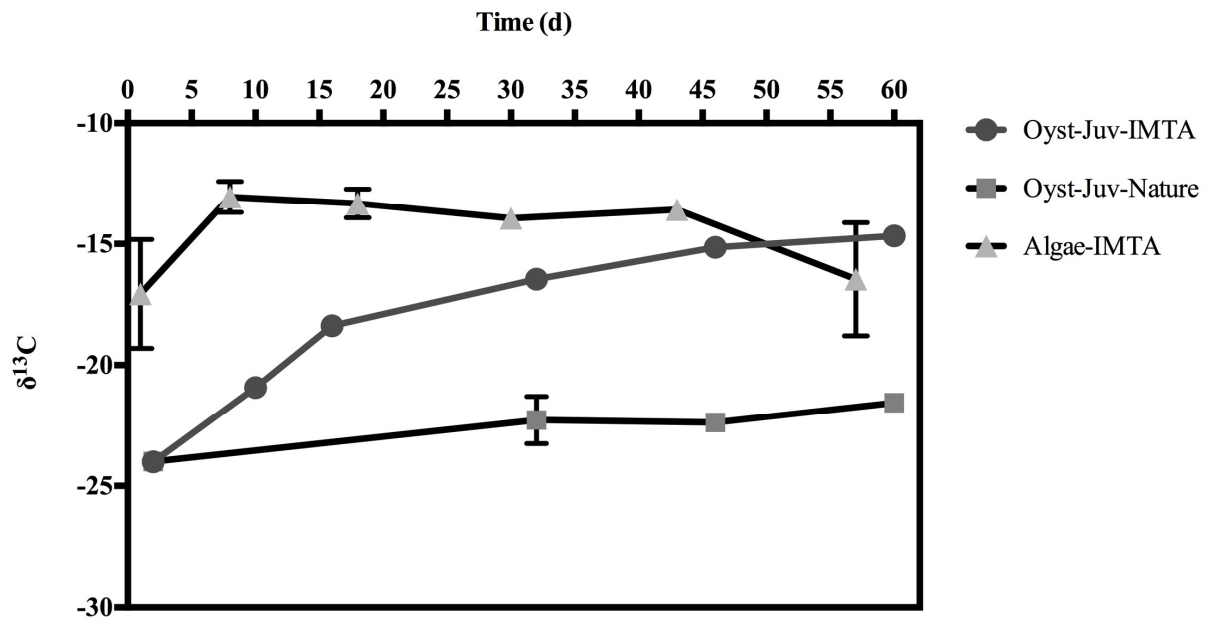
568

569 Fig.6 Weight and length of oysters over the study period (weight of larger oyster juveniles (A),

570 length of larger oyster juveniles (B), weight of smaller oyster juveniles (C) and length of

571 smaller oyster juveniles (D))

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574 Fig.7  $\delta^{13}\text{C}$  signatures of Algae-IMTA, smaller juvenile oysters-IMTA (Oyst-Juv-IMTA) and  
575 control smaller juvenile oysters (Oyst-Juv-Nature)

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577 **Tables**

578 Table 1 18S rRNA gene sequencing primers

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forward primer	5'-CTTCCCTA ACGACGCTCTTCCGATCTGCGGTAATTCCAGCTCCAA-3'
reverse primer	5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTTGGCAAATGCTTTCGC-3'

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581 Table 2 Mean  $\pm$  SD of water properties in each compartment of RAS-IMTA and in natural  
 582 lagoon

	T (°C)	Dissolved Oxygen (mg L <sup>-1</sup> )	Salinity	pH
<b>RAS-IMTA</b>				
Fish RAS (n = 60)	19.4 $\pm$ 2.3	8.6 $\pm$ 2.6	37.8 $\pm$ 1.3	7.2 $\pm$ 0.2
Algal Raceway (n = 120)	17.3 $\pm$ 4.3	12.2 $\pm$ 4.1	39.4 $\pm$ 1.4	9.9 $\pm$ 0.4
Oyster Tank (n = 120)	16.4 $\pm$ 3.6	7.5 $\pm$ 0.8	38.2 $\pm$ 1.7	8.7 $\pm$ 0.3
<b>Natural lagoon (control) (n=2)</b>				
Beginning of experiment	15.1 $\pm$ 0.05	N.A	18.7 $\pm$ 0.4	N.A
End of experiment	23.0 $\pm$ 0.05	N.A	37.1 $\pm$ 0.1	N.A

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585 Table 3 Mean  $\pm$  SD of nutrient removal efficiencies and removal rates of the algal raceways  
586 over the period of the study (n = 19)

	NO <sub>3</sub> -N	NO <sub>2</sub> -N	NH <sub>4</sub> -N	PO <sub>4</sub> -P
Removal efficiency (%)	98.6 $\pm$ 0.2	98.0 $\pm$ 0.4	97.3 $\pm$ 0.7	96.1 $\pm$ 0.6
Removal rate (mg m <sup>-2</sup> d <sup>-1</sup> )	335.8 $\pm$ 0.8	23.6 $\pm$ 0.2	30.9 $\pm$ 0.2	22.3 $\pm$ 0.2

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589 Table 4 Microalgae production and yield based on N and P consumed compared with  
 590 previous literature results based on microalgae bioremediation

Type of water	Biomass produced (mgTSS L <sup>-1</sup> )	N consumed (mg L <sup>-1</sup> )	P consumed (mg L <sup>-1</sup> )	Yield based on N (g Algae / gN)	Reference
Aquaculture	42.6	5.86	0.35	7.3	Gao et al. (2016)
Aquaculture	107.85	36.0-37.5	8.82	3.0-4.3	Ansari et al. (2017)
Aquaculture	150-262	4.96	1.47	30.2-52.8	Borges et al. (2005)
Aquaculture	0.35-1.95 mg Chl <i>a</i> L <sup>-1</sup>	4.96	1.47	0.07-0.39 g Chl <i>a</i> / gN	Borges et al. (2005)
Aquaculture	520	39.5	4.95	13.2	Michels et al. (2014)
Domestic	160	43.9	2.6	36	Dahmani et al. (2016)
Municipal	73.7	9.5	6.3	7.8	Sacristán de Alva et al. (2013)
Municipal	3040	54.5	6.1	55.8	Koreivienė et al. (2014)
Dairy manure	5340-5530	48	13	111.3-115.2	Wilkie and Mulbry

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					(2002)
Aquaculture	107-174	5.2	0.3	20.5-33.3	Present study
Aquaculture	0.36-0.48 mg Chl <i>a</i> L <sup>-1</sup>	5.2	0.3	0.07-0.09 g Chl <i>a</i> / gN	Present study

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