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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

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# 20 Abstract:

Integrated multi-trophic aquaculture (IMTA) systems are a promising solution for sustainable 21 22 aquaculture combining nutrient recycling with increased biomass production. An innovative land-based recirculating aquaculture system (RAS) was studied in France for a 60-day 23 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 for consumption in the oyster compartment of the IMTA. Silicate was added every week after 29 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 NO<sub>3</sub>-N, 98.0  $\pm$  0.4 % for NO<sub>2</sub>-N, 97.3  $\pm$  0.7 % for NH<sub>4</sub>-N and 96.1  $\pm$  0.6 % for PO<sub>4</sub>-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 mg m<sup>-2</sup> d<sup>-1</sup>, 33 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 mg TSS L<sup>-1</sup> and 0.42  $\pm$  0.03 mg chl a L<sup>-1</sup>) after which the microalgae collapsed due to a CO<sub>2</sub> 36 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 ovsters 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 throughout the centuries, with combinations of different products (terrestrial and aquatic, 58 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

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

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 120 121 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 classical RAS fish tanks (triplicate), as described by Blancheton (2000), B) outdoor HRAP 123 124 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.

European sea bass (Dicentrarchus labrax) were stocked at an initial density of 30 kg m<sup>-3</sup> 129 130 (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  $(180 \text{ L} \text{ h}^{-1})$  flew directly towards three algal raceways (area = 12 m<sup>2</sup>, depth = 0.50 m; volume 134 = 6 m<sup>3</sup>). Each algal raceway (water depth = 0.40 m, working volume = 4.8 m<sup>3</sup>, hydraulic 135 136 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 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 139 140 bloom. After 15 days, the fish effluent (from RAS) was diverted through the three algal 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 141 achieve a N:Si:P molar ratio of 10:5:1 as suggested for marine diatom dominance by Lefebvre 142 143 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 distributed to six oyster tanks (volume =  $0.5 \text{ m}^3$ ) at a continuous flow rate of 5 L.h<sup>-1</sup>; all oyster 145 tanks were also fed with 500 L.h<sup>-1</sup> of filtered seawater in order to approach a suitable 146 147 concentration of microalgae for the ovsters according to Rico-Villa et al. (2009) (around 1.5- $3.7 \times 10^6$  cells/ml) and to reduce pH variability. After 20 days of acclimatization, 300 smaller 148 149 juvenile oysters (0.05  $\pm$  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  $\pm$  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.).

For the RAS-IMTA system, water temperature, salinity, pH and dissolved oxygen concentrations (DO) were monitored daily (9:00 a.m.) in the fish rearing tanks and twice a day (9:00 a.m. and 3:00 p.m.) in the algal raceways and the oyster tanks using a YSI  $\circledast$  probe. CO<sub>2</sub> concentrations in the algal raceways were recorded every 10 minutes using a CO<sub>2</sub> OxyGuard $\circledast$  probe. In the natural lagoon, where oyster controls were stored, environmental 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.

Based on these results, 50 ml water samples were taken twice a week at 2:00 p.m. at three points in the IMTA system (fish tanks, RAS effluent and algal raceways), monthly in the natural lagoon. Water samples were filtered (GF/F, Whatman<sup>TM</sup>), and stored it at -25 °C for 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 
$$RE(\%) = \frac{C_{eff} - C_{AR}}{C_{eff}} \times 100\%$$
 (1)

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

173 Where  $C_{eff}$  is the nutrient concentration in the RAS effluent (mg L<sup>-1</sup>),  $C_{AR}$  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**

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

and of all the larger juvenile oysters were measured. They were compared against the oystersgrown 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 precombusted GF/F filters (Whatman<sup>TM</sup>). We freeze-dried the filters and packed them in tin 206 capsules for carbon isotope analysis. The isotopic ratio  $({}^{13}C/{}^{12}C)$  of the samples were 207 208 analyzed by continuous-flow elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) 209 using an Isoprime GVI IRMS (Elementar, Langenselbold, Germany) coupled to and EuroEA 3000 elemental analyzer (Eurovector, Pavia, Italia). The  ${}^{13}C/{}^{12}C$  ratio was expressed as  $\delta^{13}C$ 210 211 per mille ( $\%_0$ ) relative to Vienna Pee Dee Belemnite, according to the following equation:

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

213 Where R is the ratio of heavy to light isotope  $({}^{13}C/{}^{12}C)$ , 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  $\pm 0.16$  per mille points for  $\delta^{13}$ C, 216 and  $\pm 1.05\%$  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 multipoint219 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**

Over the study period, air temperature ranged from 9.5 to 28.9 °C (17.7  $\pm$  3.9 °C), and 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.

In the indoor fish tanks (RAS), the water temperature ranged from 16.3 to 25.2 °C (19.4  $\pm$  2.3 °C). Temperature was much more variable at the outdoor facilities, ranging from 7.9 to 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
- ranged from 4.1 to 17.0 mg  $L^{-1}$  (8.6 ± 2.6 mg  $L^{-1}$ ) and in the outdoor algal raceways from 1.1
- to 22.1 mg  $L^{-1}$  (12.2 ± 4.1 mg  $L^{-1}$ ). In the oyster tanks DO was in a much narrower range from

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

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

# 251 **3.2. Nutrient bioremediation**

The nutrient concentrations in the RAS effluent and algal raceways are presented in Fig.3. 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 mg N L<sup>-1</sup> on day 43, and then remained stable. The NO<sub>2</sub> concentrations increased from 0.29 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 the experiment (day 60). The NH<sub>4</sub> concentration in RAS effluents averaged 0.43 ± 0.07 mg N L<sup>-1</sup> and the PO<sub>4</sub> concentration averaged 0.31 ± 0.06 mg P L<sup>-1</sup>.

Nutrient concentrations in the algal raceways were significantly lower than those in the RAS effluents. Overall average concentrations in the algal raceways were  $0.11 \pm 0.22$  mg NO<sub>3</sub>-N  $L^{-1}$ ,  $0.02 \pm 0.03$  mg NO<sub>2</sub>-N  $L^{-1}$ ,  $0.01 \pm 0.01$  mg NH<sub>4</sub>-N  $L^{-1}$ , and  $0.03 \pm 0.06$  mg PO<sub>4</sub>-P  $L^{-1}$ . After day 32, no NO<sub>3</sub>-N and NO<sub>2</sub>-N was detected in the algal raceways. The nutrient concentrations in the natural lagoon (control) were very low,  $0.61 \pm 0.55 \mu$ M NO<sub>3</sub>-N,  $0.13 \pm$  $0.11 \mu$ M NO<sub>2</sub>-N,  $2.37 \pm 0.40 \mu$ M NH<sub>4</sub>-N and  $0.05 \pm 0.03 \mu$ M PO<sub>4</sub>-P.

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

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

The total suspended solids (TSS) and chlorophyll *a* concentration (Chl *a*) in the algal raceways, two proxies of microalgal biomass, are presented in Fig.4. TSS and Chl *a* showed similar trend, with concentrations increasing slowly, reaching maxima from 107 to 174 mg L<sup>-1</sup> 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, the microalgal biomass collapsed in the three raceways. During summer, the natural lagoon Chl *a* median value is around 12.7-15.2  $\mu$ g L<sup>-1</sup> (Lerustre et al, 2015).

The structure of the microalgal community in the four sampling days (day 1, 16, 30 and 43) is shown in Fig.5. There was a shift in algal population that occurred between day 16 and day 30. During the first part of the experiment (at least until day 16), *Tetraselmis sp.* was dominant (45.7% to 73.8%) and the diatom *Stauroneis sp.* was a major component (11.5 – 16.9 %) in all raceways. Samples from day 30 and after showed that the diatom *Phaeodactylum sp.* was 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. However, oysters cultivated in natural conditions grew from  $4.76 \pm 0.15$  g ind<sup>-1</sup> on day 1 to 282  $11.4 \pm 4.3$  g ind<sup>-1</sup> on day 57 for larger oysters and from  $0.05 \pm 0.00$  g ind<sup>-1</sup> on day 1 to  $2.5 \pm$ 283 1.0 g ind<sup>-1</sup> on day 57 for smaller oysters. At the end of the experiment the smaller oysters 284 285 growing in natural conditions were 12.5 times heavier  $(2.5 \pm 1.0 \text{ g ind}^{-1})$  than those in the 286 IMTA system (0.20  $\pm$  0.10 g ind<sup>-1</sup>). The same effect was observed for larger oysters, with a weight of  $11.4 \pm 4.3$  g for those in natural conditions against 5.3 g to 5.5 g for those in the 287 IMTA system. 288

The  $\delta^{13}$ C signatures for the microalgae and juvenile oysters in the IMTA system and the control oysters are shown in Fig.7. The  $\delta^{13}$ C signature of the control oysters remained stable (-22.37 ± 0.99) during the experiment, while the  $\delta^{13}$ C signature of the oysters in the IMTA system increased from -24.00  $\pm$  0.14, at day 2, up to -15.02  $\pm$  0.23 at the end of the experiment, close to the  $\delta^{13}$ C signature of the microalgae in the raceways (-15.36  $\pm$  2.63).

#### 294 **4. Discussion**

One of the objectives of this study was to evaluate the nutrient removal efficiency of 295 microalgae in an RAS-IMTA system. More than 96 % of nitrogen and phosphorus were 296 297 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 seaweed where the algae removed approximatively 20.0 mg N m<sup>-2</sup> d<sup>-1</sup> and 2.5 mg P m<sup>-2</sup> d<sup>-1</sup> 300 301 (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 supplied with fish tank effluents (NH<sub>4</sub>-N, 1.27 mg L<sup>-1</sup> and PO<sub>4</sub>-P, 0.24 mg L<sup>-1</sup>) to feed oysters 304 305 (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 concentrations; the microalgae in raceways removed 67% (21.3 mg N m<sup>-2</sup> d<sup>-1</sup>) NH<sub>4</sub>-N and 307 46.6 % (2.8 mg P m<sup>-2</sup> d<sup>-1</sup>) PO<sub>4</sub>-P. In a recent study using microalgae photo-bioreactors to treat 308 309 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 PO<sub>4</sub>-P, keeping the nutrients at low levels (i.e.,  $C_{DIN}$  < 0.2 mg L<sup>-1</sup> and C<sub>P</sub> < 0.02 mg L<sup>-1</sup>) (Ju et al., 2015). IMTA system was indoors and the *Stvela* 314 clava concentration was kept at low level, around 200-300 ind. m<sup>-3</sup>. For these studies, the N 315 316 and P concentrations were lower than concentrations we obtained, RAS effluents producing

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

319 The nutrient bioremediation rates obtained in our IMTA systems were close to or greater than previous macroalgae-based IMTA systems. In a 15-month study of Pagand et al. (2000), 320 green macroalgae were used to treat sea bass RAS effluents containing 10 mg L<sup>-1</sup> of DIN and 321 1.3 mg  $L^{-1}$  of P; they obtained DIN removal efficiencies between 30% and 88% (removal rate: 322 from 420 to 1220 mg m<sup>-2</sup> d<sup>-1</sup>) and PO<sub>4</sub>-P removal efficiencies between 0% and 82% (removal 323 rate: from 0 to 148 mg m<sup>-2</sup> d<sup>-1</sup>). Similarly, a 12-month-study treating sea bass RAS effluent in 324 macroalgae raceways, achieved  $360 \pm 140 \text{ mg m}^{-2} \text{ d}^{-1} \text{ DIN}$  and  $8 \pm 16 \text{ mg m}^{-2} \text{ d}^{-1} \text{ P}$  removal, 325 326 close to our results (Deviller et al., 2004; Metaxa et al., 2006). The highest N and P removal rates in their studies occurred during summer. Although our results suggest microalgae as a 327 328 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

The proxy used to evaluate the microalgal biomass in the RAS-IMTA system reached maximal values of 107 - 174 mg TSS L<sup>-1</sup> and 0.36 - 0.48 mg Chl *a* L<sup>-1</sup>. Previous studies have demonstrated the microalgal wastewater treatment (municipal, agricultural and industrial wastewater) with production rates varying from 74 to 5530 mg TSS L<sup>-1</sup> d<sup>-1</sup>, most of which were much higher than our study (Wilkie & Mulbry, 2002; Sacristán de Alva et al., 2013; Dahmani et al., 2016) probably because much higher nutrient concentration in their effluents.

The microalgal biomass yield was calculated based on the N consumed (Table 4) and the results showed that biomass production in the raceways was in the range of those measured in previous studies using various types of wastewater (i.e. aquaculture, domestic, municipal and 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 42.6 to 520.0 mg  $L^{-1} d^{-1}$  and from 0.35 to 1.95 mg  $L^{-1} d^{-1}$ , respectively (Borges et al., 2005; 344 Michels et al., 2014; Gao et al., 2016). Microalgal production could be affected by various 345 346 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  $CO_2$  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 addition of  $CO_2$  is mandatory to maintain the system working as long as necessary for oyster to grow. In future experiments, the system could be improved, using a  $CO_2$  low cost regulation and further investigations will be conducted using enriched  $CO_2$  air from RAS compartment.

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

378

The change in the oyster's  $\delta^{13}$ C signature in the IMTA system towards values close to the 379 380 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 phytoplancton. 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.

- Microalgae-based RAS-IMTAs have potential for nutrient bioremediation. Nutrient
   removal rates were close to those obtained in similar macroalgae-based IMTA systems.
- The RAS-IMTA system produced microalgae with the maximal yield of 20.5 33.3 g Algae gN<sup>-1</sup> and 0.07 - 0.09 g Chla gN<sup>-1</sup>. Adding silicate shifted the dominant 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.
- Further experiments should be carried out to improve the overall production and resilience of the IMTA system by: (a) adding an extra low-cost CO<sub>2</sub> source in order to stabilize pH and to maintain a high CO2 level in algal raceways, (b) optimizing the food access to oysters.

406

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- 532



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

539 1: Fish tank, 4 m<sup>3</sup>; 2: Particle trap; 3: Mechanical filter, 30-μm 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 m<sup>2</sup>; 11: Large juvenile oyster tank, 0.5 m<sup>3</sup>; 12: Juvenile oyster tank, 0.5 m<sup>3</sup>.

542



Time (h)



545

Time (h)



547 Fig.2 Nitrate (A), nitrite (B), ammonia (C) and phosphate (D) concentration curves in the
548 RAS-IMTA system, measured along the experiment during a period 24 hours





Fig.3 Nitrate (A), nitrite (B), ammonia (C) and phosphate (D) concentrations in RAS effluents
(RAS EFF) (fish tanks) and microalgal raceways over the experimental period; dashed arrows
represent the addition of silicates in raceways



Time (d)



Fig.4 Microalgal biomass concentration based on TSS (A) and chlorophyll a (B) over the
experimental period; dashed arrows represent the dates of addition of silicates in raceways



559 Fig.5 The microalgal community structure composition at genus level (Rx-Dy indicates the560 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.

563

558



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





575 control smaller juvenile oysters (Oyst-Juv-Nature)

# 577 Tables

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

581 Table 2 Mean ± SD of water properties in each compartment of RAS-IMTA and in natural

582 lagoon

	Т	Dissolved Oxygen	0.1				
	(°C)	$(mg L^{-1})$	Salinity	рн			
RAS-IMTA							
Fish RAS $(n = 60)$	$19.4 \pm 2.3$	8.6 ± 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 $(n = 120)$	$16.4 \pm 3.6$	$7.5 \pm 0.8$	$38.2 \pm 1.7$	$8.7 \pm 0.3$			
Natural lagoon (control) (n=2)							
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			

583

585 Table 3 Mean ± 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	NH4-N	PO <sub>4</sub> -P
Removal efficiency (%)	98.6 ± 0.2	$98.0 \pm 0.4$	$97.3 \pm 0.7$	96.1 ± 0.6
Removal rate $(mg m^{-2} d^{-1})$	335.8 ± 0.8	$23.6 \pm 0.2$	$30.9 \pm 0.2$	$22.3 \pm 0.2$

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

Table 4 Microalgae production and yield based on N and P consumed compared withprevious literature results based on microalgae bioremediation

						(2002)
Aquaculture	107-174	5.2	0.3	20.5-33.3		Present stud
Aquaculture	0.36-0.48 mg	5.2	0.3	0.07-0.09	g ]	Present study
	Chl $a L^{-1}$			Chl a / gN		