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

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Abstract :

Integrated multi-trophic aquaculture (IMTA) systems are a promising solution for sustainable aquaculture combining nutrient recycling with increased biomass production. An innovative land-based recirculating aquaculture system (RAS) was studied in France for a 60-day experiment. It combined a European sea bass (Dicentrarchus labrax) RAS with two other production systems: high rate algal ponds (HRAP) with natural marine polyspecific algal assemblages, and oysters in separate open tanks. The objective was the assessment of: 1) the efficiency and the stability of the microalgae bioremediation of the effluent from a fish RAS 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 the beginning of the experiment for maintaining a Si:N:P molar ratio of 10:5:1 in the HRAP to encourage the growth of diatoms. The HRAP have an overall removal efficiency of 98.6 ± 0.2% for NO3-N, 98.0 ± 0.4% for NO2-N, 97.3 ± 0.7% for NH4-N and 96.1 ± 0.6% for PO4-P, with removal rates of 335.8 ± 0.8, 23.6 ± 0.2, 30.9 ± 0.2, and 22.3 ± 0.2 mg m−2 d−1, respectively. The concentration of total suspended solid (TSS) and chlorophyll a (chl a) increased during the experiment and reached maximum values on day 46 (135.3 ± 34.7 mg TSS L−1 and 0.42 ± 0.03 mg chl a L−1) after which the microalgae collapsed due to a CO2 limitation (pH ca. 10). Sequencing analysis revealed that the microalgae community was dominated by Tetraselmis sp. from day 1 to day 16 (45.7% to 73.8% relative abundance). From day 30 to day 43 the culture was dominated by diatoms, Phaeodactylum sp. (83.4% to 98.1% relative abundance). Although the stable carbon isotope signatures confirmed that the microalgae were consumed, oysters' growth was limited in the RAS-IMTA, suggesting that oysters were under stress or not fed enough.

Highlights

► Microalgae-based -IMTA has potential for N/P bioremediation. ► Adding silicate shifted algae to diatom of interest for oysters.

Keywords : Microalgae, IMTA, Nutrient bioremediation, Community structure, oysters

1. Introduction

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

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

Numerous studies have evaluated the potential of macroalgae as biofilter, but the use of microalgae for IMTAs has been less studied (FAO, 2009). Microalgae present many advantages, including higher photosynthesis and nutrient remediation potentials due to a higher specific surface area, and provide high value biomass and compounds for fuel, chemicals, cosmetics and animal feed (Hein et al., 1995). Coupling microalgal biomass production with wastewater treatment was first proposed by Oswald and Golueke (1960), and now it is considered as a sustainable process successfully used for various water treatment plants (e.g. agricultural, industrial, aquacultural and domestic wastewater treatment) (Muñoz & Guieysse, 2006; Ansari et al., 2017; Gonçalves et al., 2017). Microalgae can be cultivated in wastewater in both open (e.g. raceways and ponds) and closed systems (e.g. photobioreactors, microalgae turf scrubbers and hybrid systems). Of these, open ponds account for more than 80% of global algal biomass production (Moreno-Garcia et al., 2017). Open raceway ponds for microalgal biomass production have the advantage of simple design, low operating and maintenance costs, high production volumes and the ability to capture atmospheric CO_2 (Lam & Lee, 2012). On the other hand, microalgal biomass production using raceway ponds is strongly influenced by several parameters, including environmental factors (i.e. temperature, solar radiation, pH, dissolved oxygen, salinity), engineering design (e.g. water dynamics, hydraulic retention time, CO_2 injection, mixing and depth), nutrient availability and ratios (e.g. N, P, Si and N:P), contamination (e.g. competing microalgae) and predation by zooplankton (Das et al., 2015; Kumar et al., 2015). The environmental conditions and wastewater composition influence the community structure, stability and the population density of the microalgae, which in turn affects the wastewater treatment capacity

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

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

2. Materials and methods

2.1. RAS-IMTA system facilities

The experiment ran over 60 days, from April 3rd 2017 (day 1) to June 1st 2017 (day 60), at the Ifremer Station at Palavas-les-Flots, southern France in a Mediterranean climate. The 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 microalgal units (algal raceways, triplicate), as described by Deviller et al. (2004); C) outdoor oyster units with larger juveniles (triplicate) and smaller juveniles (triplicate). The wastewater from the three RAS tanks was pooled and then distributed into three algal raceways. The outflow of the raceways was then pooled in a mixing tank, and distributed with air stones to feed the oyster units.

European sea bass (*Dicentrarchus labrax)* were stocked at an initial density of 30 kg m^{-3} (1000 fish per tank, 120 g ind^{-1}). A commercial diet (Neo Grower Extra Marin 5®, Gouessant) was supplied *ad libitum* using self-feeders, and oxygen was supplied in fish tanks to saturated concentration. The water from the fish tanks passed through mechanical filter (30µm mesh), UV-treatment and bacterial biofilter; a part was recirculated into the fish unit and another part (180 L h^{-1}) flew directly towards three algal raceways (area = 12 m^2, depth = 0.50 m; volume = 6 m^3). Each algal raceway (water depth = 0.40 m, working volume = 4.8 m^3, hydraulic retention time = 4 days) was equipped with air-diffuser and circulating pump to homogenize the water column. Each raceway was initially filled with natural seawater pumped from the shore. One month before the experiment, ammonia and phosphate (5 mmol L^{-1} NH_4Cl and 0.5 mmol L^{-1} HK_2O_4P) were added to each of the three algal raceways to initiate a microalgal bloom. After 15 days, the fish effluent (from RAS) was diverted through the three algal raceways. On March 24th 2017, silicate ($Na_2SiO_3 \bullet 5H_2O$) was added to the three raceways to achieve a N:Si:P molar ratio of 10:5:1 as suggested for marine diatom dominance by Lefebvre et al. (1996), and weekly added during the experiment to maintain this molar ratio.

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 m^3) at a continuous flow rate of 5 $L.h^{-1}$; all oyster tanks were also fed with 500 $L.h^{-1}$ of filtered seawater in order to approach a suitable concentration of microalgae for the oysters according to Rico-Villa et al. (2009) (around 1.5-3.7 × 10^6 cells/ml) and to reduce pH variability. After 20 days of acclimatization, 300 smaller juvenile oysters (0.05 ± 0.00 g ind^{-1}, 7-month-old) were placed in each of 3 of the tanks (i.e. 900 ind.) and 45 larger juvenile oysters (4.76 ± 0.15 g ind^{-1}, 19-month-old) were placed in each of the other three tanks (i.e. 135 larger ind.). A similar weight of smaller (nb= 300) and larger (nb = 45) juvenile oysters was reared nearby in natural conditions as controls.

2.2. Environmental parameters

Throughout the experiment, local climatic conditions including air temperature and solar 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 ® probe. CO_2 concentrations in the algal raceways were recorded every 10 minutes using a CO_2 OxyGuard® probe. In the natural lagoon, where oyster controls were stored, environmental data was monthly measured (temperature, salinity and nutrients).

2.3. Nutrient concentrations

Nutrient concentrations were first measured over two 24-h periods (Fig.2) to evaluate the 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™), and stored it at -25 °C for NO_3-N, NO_2-N, NH_4-N and PO_4-P analysis (Alliance® auto-analyzer).

Nutrient removal efficiency (RE, %) and removal rate (RR, mg m^{-2} d^{-1}) were calculated for each microalgal raceway using the following equations:

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

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

Where C_{eff} is the nutrient concentration in the RAS effluent (mg L^{-1}), C_{AR} is the nutrient concentration in the algal raceways, Q is the water flow through the algal raceway (L h^{-1}), and S is the surface area of the algal raceway (12 m^2).

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™) (Association, 1995). For Chl *a*, the water samples were filtered onto GF/F filters (Whatman™) 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).

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

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

2.5. Oyster growth rates and assimilation capacity

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 oysters grown in the natural conditions.

The carbon stable isotopes ratios were measured to determine the capacity of oysters to assimilate the microalgae. Oysters and microalgae were sampled once a week from day 1 to day 16 and then every two weeks from day 16 to day 60. For oysters, all the flesh was separated from the shells, rinsed with milliQ water, freeze-dried and ground to a fine powder. Approximately 1 mg of each dried sample was weighed and packed in a tin capsule for carbon isotope analysis. Microalgae was sampled by filtering 10 to 30 ml of water onto pre-combusted GF/F filters (Whatman^{TM}). We freeze-dried the filters and packed them in tin capsules for carbon isotope analysis. The isotopic ratio ($^{13}C/^{12}C$) of the samples were analyzed by continuous-flow elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) 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 $\delta^{13}C$ per mille (‰) relative to Vienna Pee Dee Belemnite, according to the following equation:

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

Where R is the ratio of heavy to light isotope ($^{13}C/^{12}C$), R_{sample} is the ratio of oyster samples, $R_{standard}$ is the ratio of standard substance.

Repeated measurements on glycine showed a precision of ± 0.16 per mille points for $\delta^{13}C$, and ± 1.05% for C. Alanine, wheat flour and corn flour from IsoAnalytical Lab (Crew, United Kingdom), IAEA-N-1, IAEA-N-2 and IAEA-CH3 cellulose and USGS24 graphite from

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

2.6. Statistical analysis

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

3. Results

3.1. Environmental parameters

Over the study period, air temperature ranged from 9.5 to 28.9 °C (17.7 ± 3.9 °C), and irradiance varied between 0.2 to 20.6 W m^{-2} (11.7 ± 5.7 W m^{-2}).

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

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

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^{-1} (7.5 ± 0.8 mg L^{-1}). No CO_2 was detected in the algal raceways throughout the experiment.

The salinity in the RAS ranged from 34.1 to 38.8 (37.8 ± 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 ± 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 ± 0.4), and in the oyster tanks, ranging from 8.2 to 9.6 (8.7 ± 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.

3.2. Nutrient bioremediation

The nutrient concentrations in the RAS effluent and algal raceways are presented in Fig.3. The NO_3 concentrations in the RAS effluent increased from 1.87 mg N L^{-1} on day 3 to 5.12 mg N L^{-1} on day 43, and then remained stable. The NO_2 concentrations increased from 0.29 mg N L^{-1} on day 3 to 0.50 mg N L^{-1} on day 16 before falling to 0.20 mg N L^{-1} at the end of the experiment (day 60). The NH_4 concentration in RAS effluents averaged 0.43 ± 0.07 mg N L^{-1} and the PO_4 concentration averaged 0.31 ± 0.06 mg P L^{-1}.

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 ± 0.22 mg NO_3-N L^{-1}, 0.02 ± 0.03 mg NO_2-N L^{-1}, 0.01 ± 0.01 mg NH_4-N L^{-1}, and 0.03 ± 0.06 mg PO_4-P L^{-1}. After day 32, no NO_3-N and NO_2-N was detected in the algal raceways. The nutrient concentrations in the natural lagoon (control) were very low, 0.61 ± 0.55 µM NO_3-N, 0.13 ± 0.11 µM NO_2-N, 2.37 ± 0.40 µM NH_4-N and 0.05 ± 0.03 µM PO_4-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 ± 0.6 mg N m^{-2} d^{-1} and 22.3 ± 0.2 mg P m^{-2} d^{-1}, respectively (Table 3).

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^{-1} for TSS and from 0.36 to 0.48 mg L^{-1} 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 µg L^{-1} (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.

3.4. Oyster growth and assimilation capacity

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 ± 0.15 g ind^{-1} on day 1 to 11.4 ± 4.3 g ind^{-1} on day 57 for larger oysters and from 0.05 ± 0.00 g ind^{-1} on day 1 to 2.5 ± 1.0 g ind^{-1} on day 57 for smaller oysters. At the end of the experiment the smaller oysters growing in natural conditions were 12.5 times heavier (2.5 ± 1.0 g ind^{-1}) than those in the IMTA system (0.20 ± 0.10 g ind^{-1}). The same effect was observed for larger oysters, with a weight of 11.4 ± 4.3 g for those in natural conditions against 5.3 g to 5.5 g for those in the IMTA system.

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 ± 0.14, at day 2, up to -15.02 ± 0.23 at the end of the experiment, close to the $\delta^{13}C$ signature of the microalgae in the raceways (-15.36 ± 2.63).

4. Discussion

One of the objectives of this study was to evaluate the nutrient removal efficiency of microalgae in an RAS-IMTA system. More than 96 % of nitrogen and phosphorus were removed, indicating nutrient bioremediation of the studied IMTA system. This compares favorably with the small number of published studies of microalgae-based IMTA systems. 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^{-2} d^{-1} and 2.5 mg P m^{-2} d^{-1} (corresponding to removal efficiency of 95% N and 45% to 60% P). According to these authors, the main reason for the high nitrogen and low phosphorus removal was the low N/P atomic ratio of 4.9:1. Likewise, Hussenot et al. (1998) reared microalgae in a raceway supplied with fish tank effluents (NH_4-N, 1.27 mg L^{-1} and PO_4-P, 0.24 mg L^{-1}) to feed oysters (*Crassostrea gigas*). In that study, fish effluents in microalgae raceways were diluted with a dilution rate of 70% ± 10% per day (much higher than our study) to reduce total ammonia concentrations; the microalgae in raceways removed 67% (21.3 mg N m^{-2} d^{-1}) NH_4-N and 46.6 % (2.8 mg P m^{-2} d^{-1}) PO_4-P. In a recent study using microalgae photo-bioreactors to treat tilapia RAS effluent, Michels et al. (2014) found when extra phosphate was added into the wastewater, the N removal efficiency was improved from 49.4 % to 99.7 % (the P removal efficiency was always more than 99 %). More recently, a IMTA system combining the tunicate *Styela clava* and the sea cucumber *Stichopus japonicus* removed 54% of dissolved inorganic nitrogen (DIN) and 50% of PO_4-P, keeping the nutrients at low levels (i.e., C_{DIN} < 0.2 mg L^{-1} and C_P < 0.02 mg L^{-1}) (Ju et al., 2015). IMTA system was indoors and the *Styela clava* concentration was kept at low level, around 200-300 ind. m^{-3}. For these studies, the N 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 ± 0.6 mg N m^{-2} d^{-1} and 22.3 ± 0.2 mg P m^{-2} d^{-1}.

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), green macroalgae were used to treat sea bass RAS effluents containing 10 mg L^{-1} of DIN and 1.3 mg L^{-1} of P; they obtained DIN removal efficiencies between 30% and 88% (removal rate: from 420 to 1220 mg m^{-2} d^{-1}) and PO_4-P removal efficiencies between 0% and 82% (removal rate: from 0 to 148 mg m^{-2} d^{-1}). Similarly, a 12-month-study treating sea bass RAS effluent in macroalgae raceways, achieved 360 ± 140 mg m^{-2} d^{-1} DIN and 8 ± 16 mg m^{-2} d^{-1} P removal, 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 viable alternative to macroalgae in IMTA systems, it needs further study to assess the year-round performance as N and P removal rates since they may be strongly influenced by the season.

The proxy used to evaluate the microalgal biomass in the RAS-IMTA system reached maximal values of 107 - 174 mg TSS L^{-1} and 0.36 – 0.48 mg Chl *a* L^{-1}. Previous studies have demonstrated the microalgal wastewater treatment (municipal, agricultural and industrial wastewater) with production rates varying from 74 to 5530 mg TSS L^{-1} d^{-1}, 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).

Even in IMTA systems, regardless of culturing regimes (i.e. continuous, semi-continuous, 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; Michels et al., 2014; Gao et al., 2016). Microalgal production could be affected by various factors, both biotic and abiotic (Gonçalves et al., 2017). The temperature and light conditions during our study were adequate for algal growth (Table 2). Nutrient concentrations and ratios, especially the N:P ratio, affects the growth of microalgae by influencing the metabolic activity and growth rate (Werner, 1977; Smith & Geider, 1985). However, most of inorganic nitrogen and phosphate and all the detectable CO_2 in the microalgal raceways were consumed, indicating that the N, P and CO_2 availability were the main factors limiting the algal growth in the raceways. Under continuous cultivation, Borges et al. (2005) reported that high microalgal biomass and nutrients removal efficiencies would be obtained only if the molar N:P ratio in the wastewater is in balance with the ratio of the microalgal biomass. Here, the RAS effluent had an average N:P molar ratio of 38, which was twice as high as the Redfield ratio of 16 (the N:P molar ratio reported for the biomass of marine microalgal species in general) (Redfield, 1958). This suggests that P was a limiting factor for the growth of microalgae in the raceways under continuous effluent supply. However, because of high photosynthetic CO_2 fixation, pH reached values greater than 9 (9.9), high pH values would shift CO_2 to bicarbonate equilibrium towards bicarbonate, thereby further reduce CO_2 concentration. A CO_2 limitation affected the duration of the experiment and probably explains the collapse of the algal culture in the raceways. The lack of CO_2 may not be the only factor, high pH (ca. 10), the development of viruses, parasites and predators may also affect the algal growth in HRAPs (see Flynn et al 2017). In this experiment, it has been decided to develop a landbased-IMTA system in a simplest way, minimizing energy costs, and using water mixing to get an air-water CO_2 equilibrium. This choice did not meet the long-term algal CO_2 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.

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

5. Conclusions

Although some research has been carried out using microalgae-based RAS-IMTA systems, there is limited information on the performance of these systems. Our study used an innovative RAS-IMTA system to test the nutrient bioremediation efficiency and the ability to 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^{-1} and 0.07 – 0.09 g Chl*a* gN^{-1}. Adding silicate shifted the dominant microalgae species from *Tetraselmis sp.* to *Phaeodactylum sp.*
- The oyster growth was very poor, although juvenile oysters seemed to be able to 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_2 source in order to stabilize pH and to maintain a high CO2 level in algal raceways, (b) optimizing the food access to oysters.

Acknowledgements

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Figures & Tables

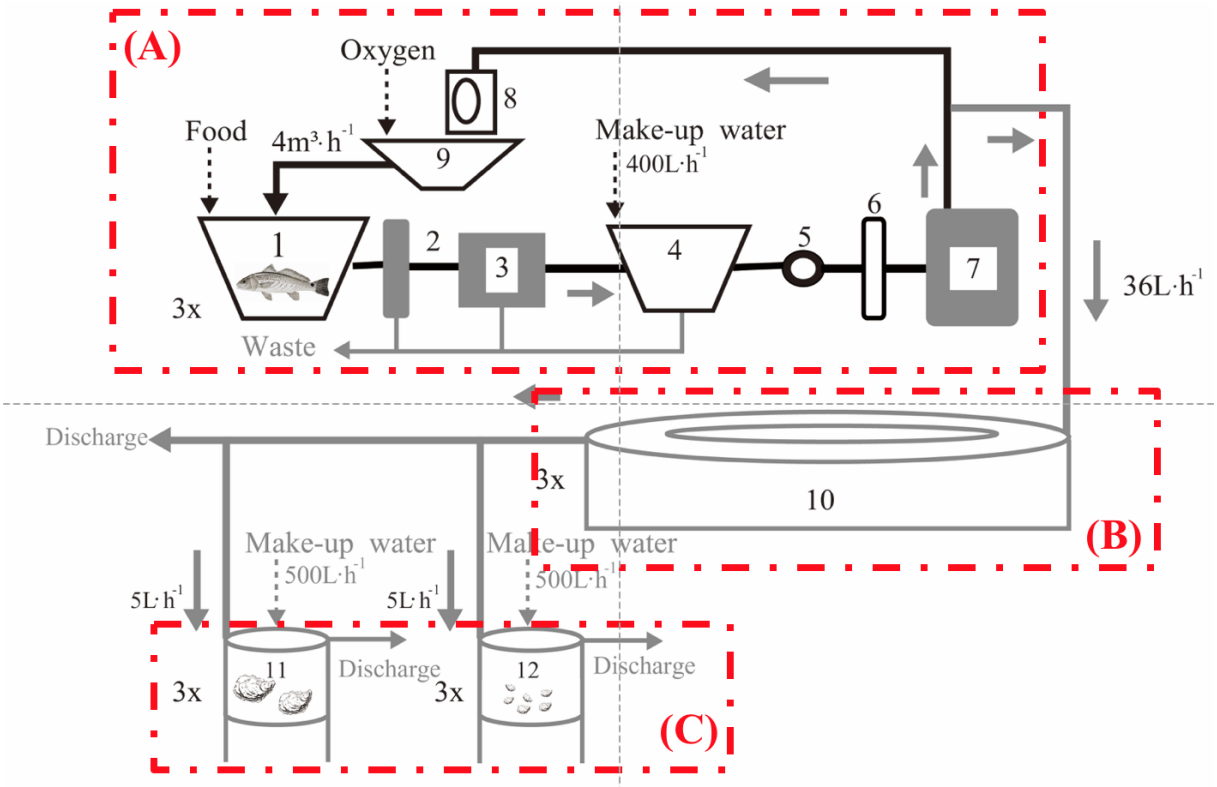
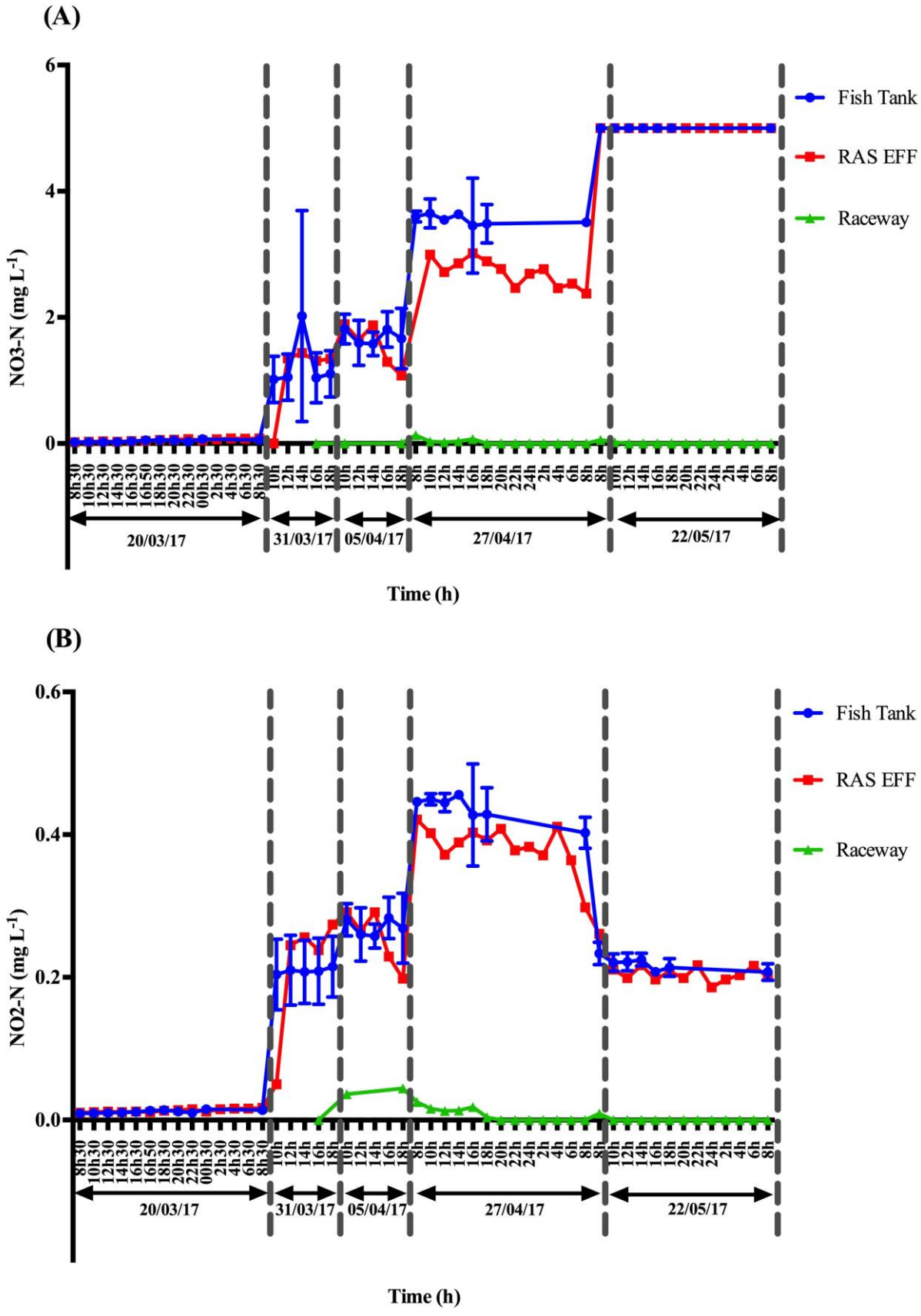
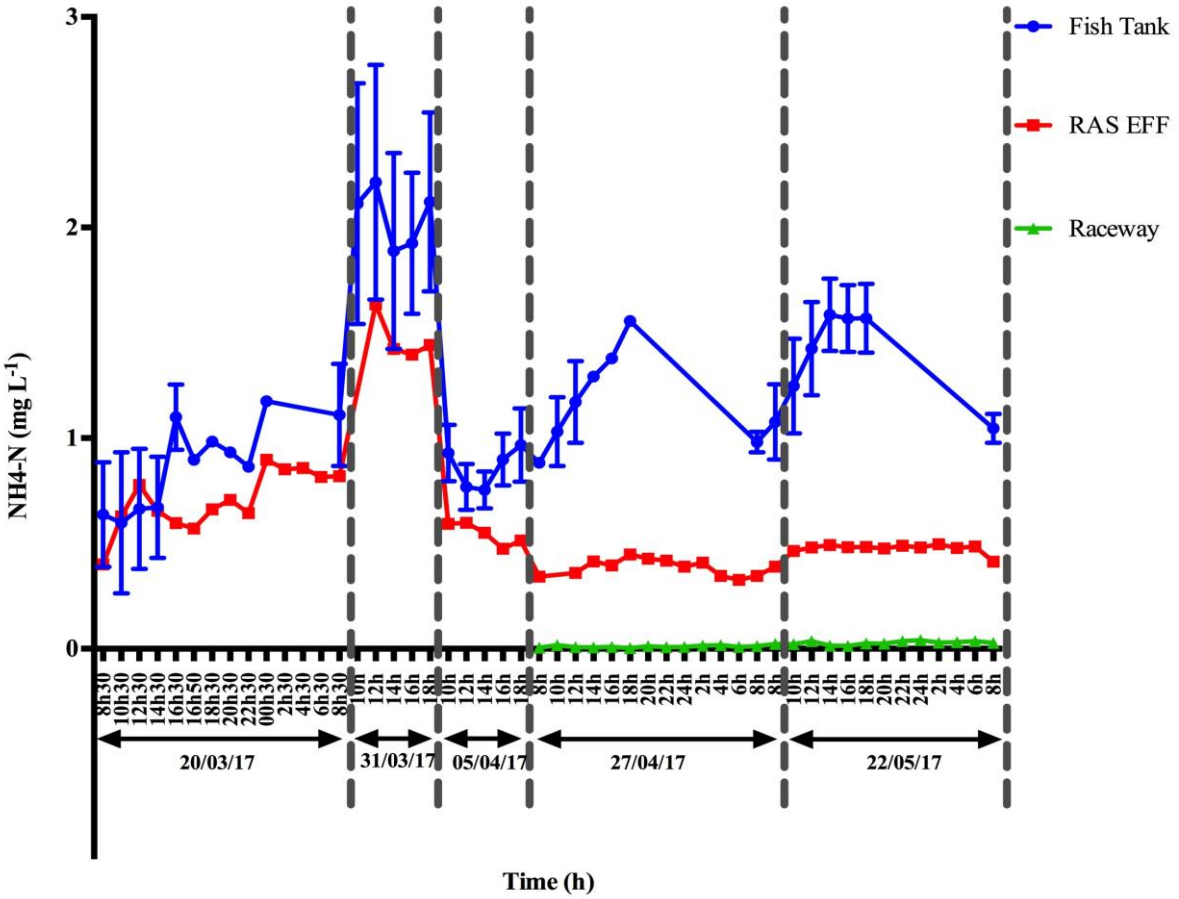


Fig.1 Schematic diagram of the recirculating aquaculture system / integrated multi-trophic aquaculture (RAS-IMTA) system (A: RAS compartment; B: Microalgae raceways; C: Oyster tanks.)

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



(C)



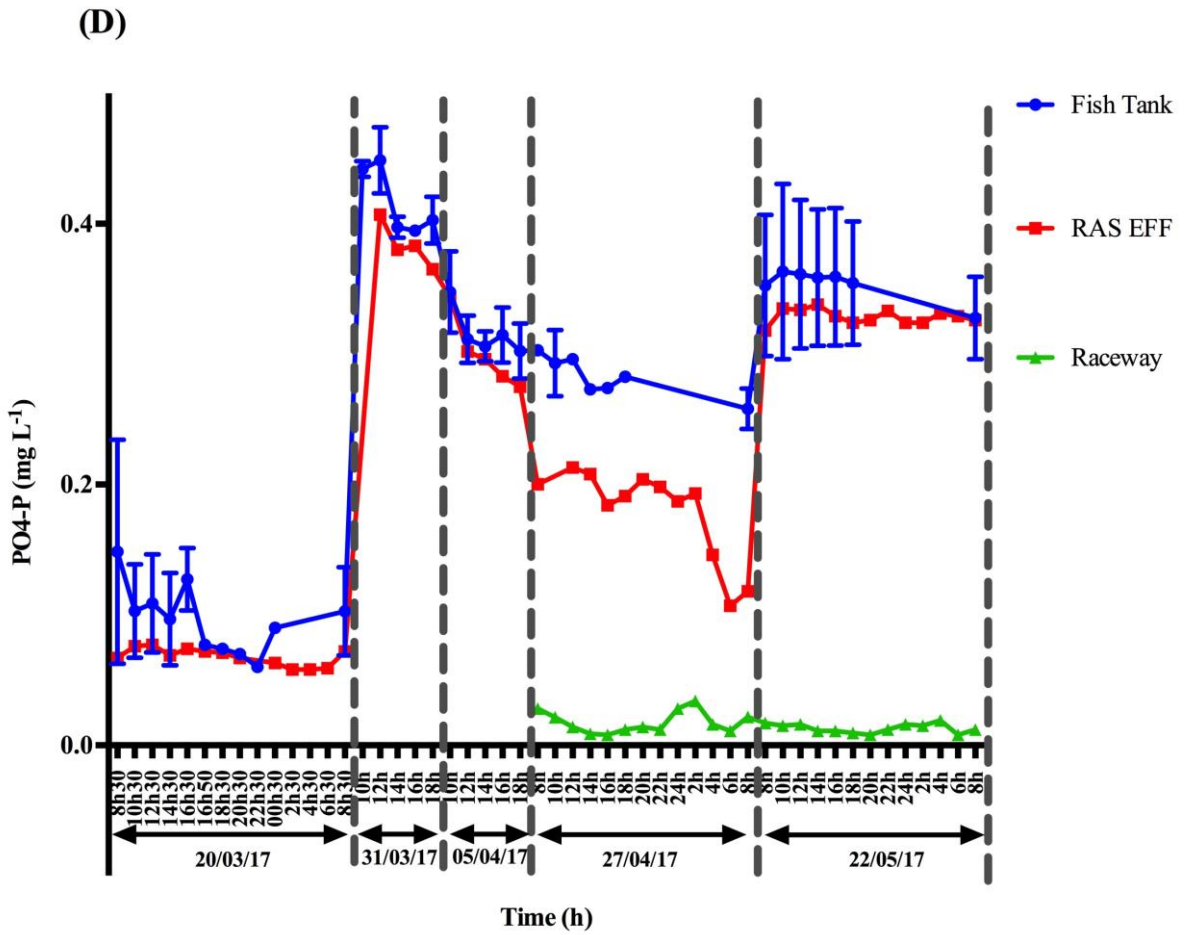


Fig.2 Nitrate (A), nitrite (B), ammonia (C) and phosphate (D) concentration curves in the RAS-IMTA system, measured along the experiment during a period 24 hours

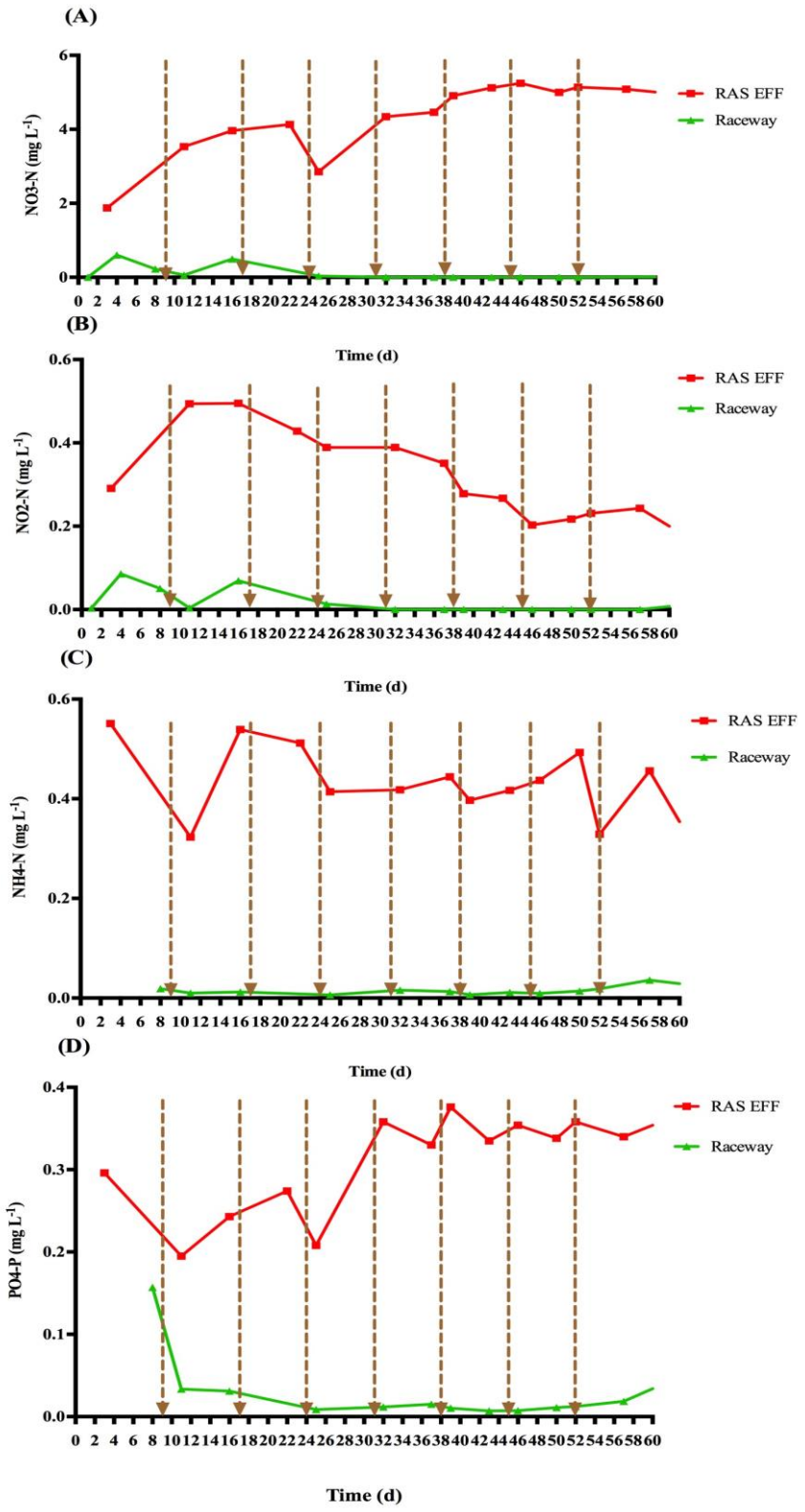


Fig.3 Nitrate (A), nitrite (B), ammonia (C) and phosphate (D) concentrations in RAS effluents (RAS EFF) (fish tanks) and microalgal raceways over the experimental period; dashed arrows represent the addition of silicates in raceways

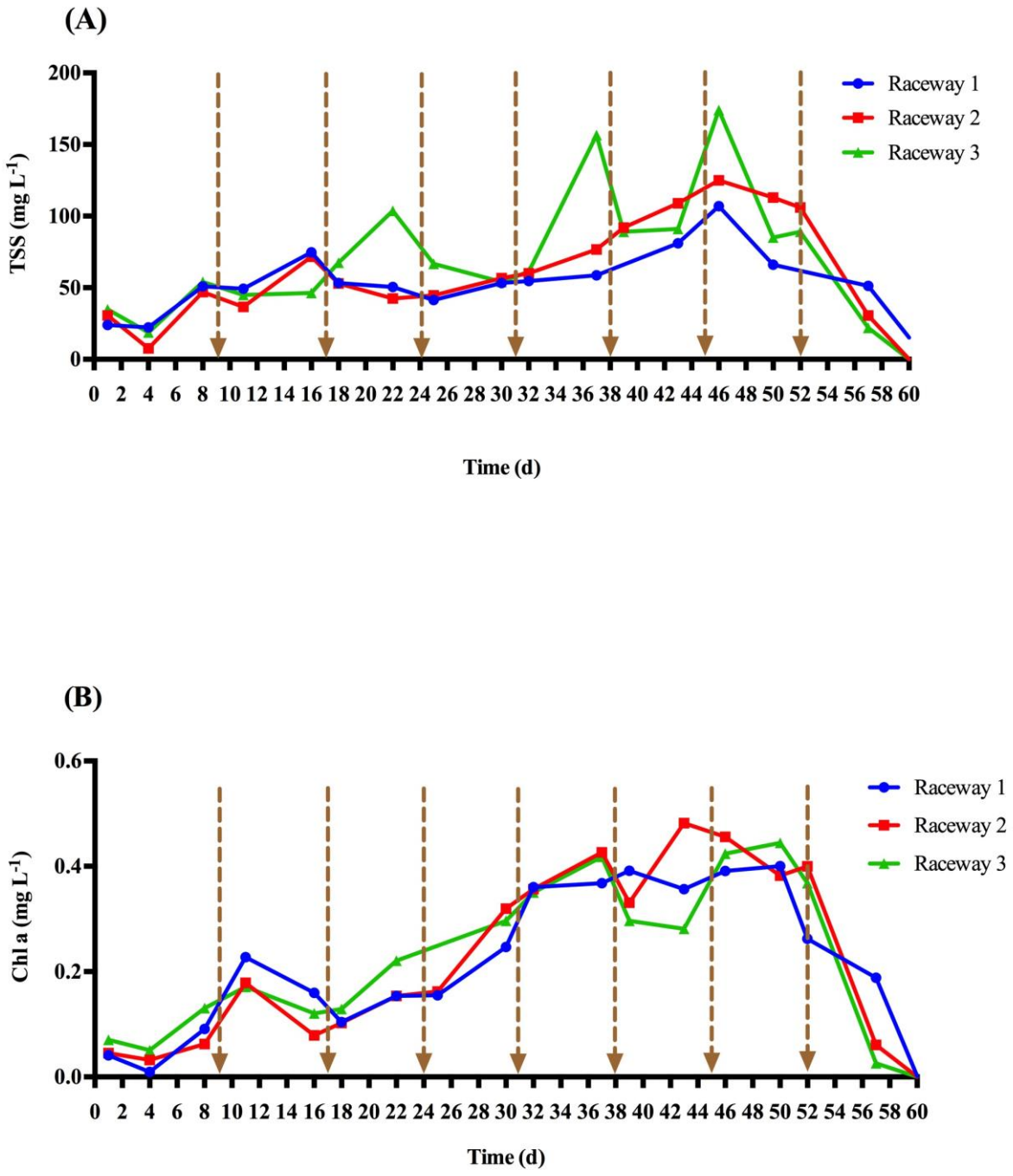


Fig.4 Microalgal biomass concentration based on TSS (A) and chlorophyll a (B) over the experimental period; dashed arrows represent the dates of addition of silicates in raceways

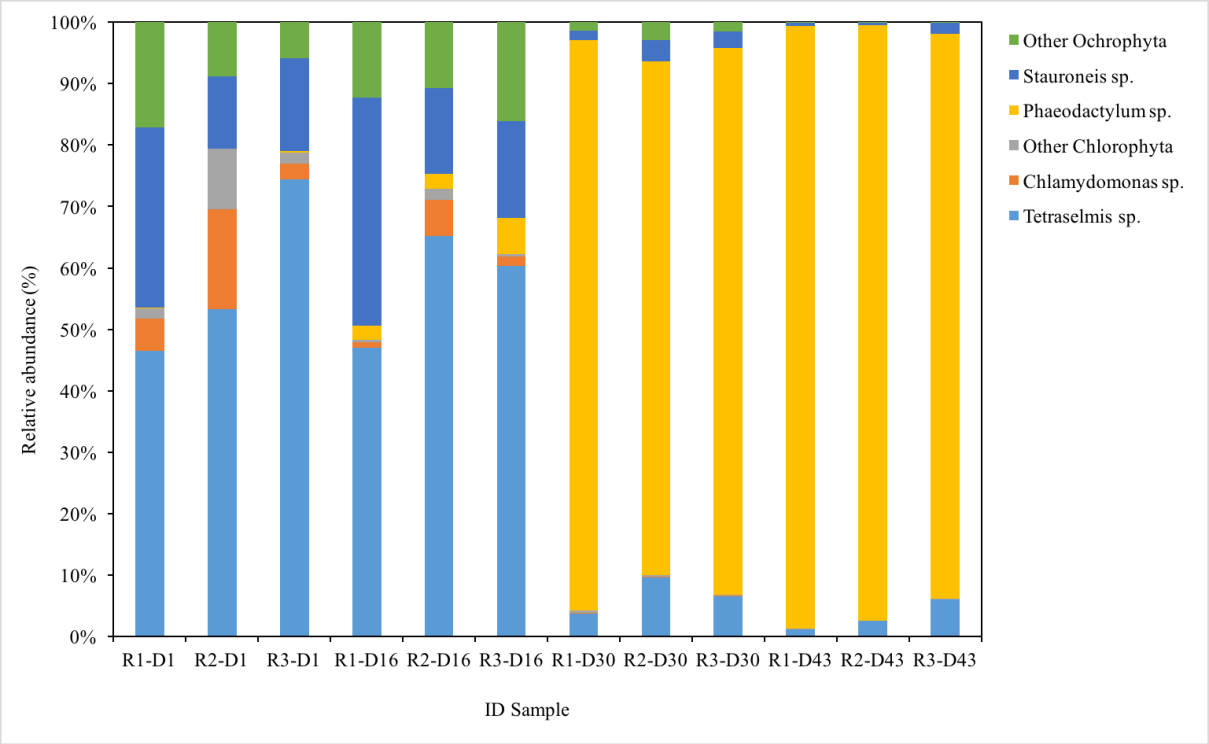


Fig.5 The microalgal community structure composition at genus level (Rx-Dy indicates the samples taken in Raceway x on day y)

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

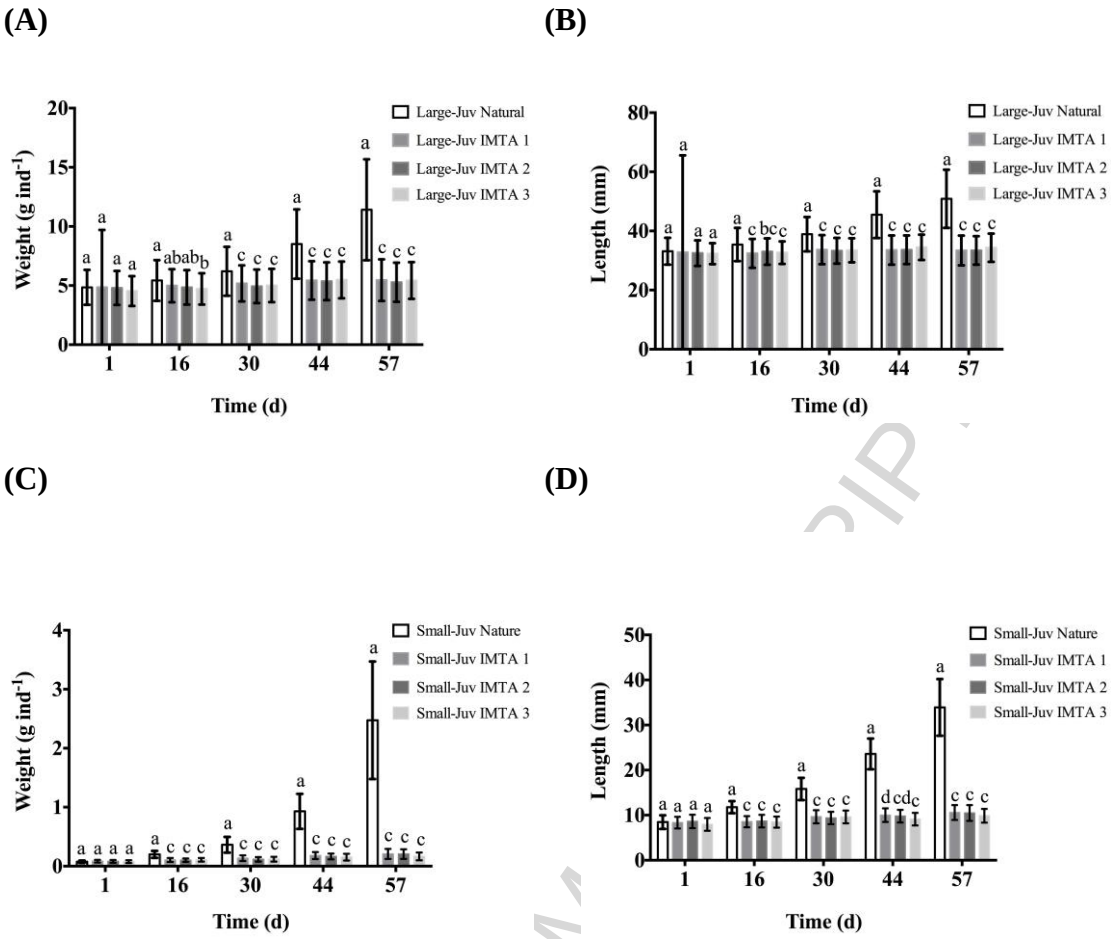


Fig.6 Weight and length of oysters over the study period (weight of larger oyster juveniles (A), length of larger oyster juveniles (B), weight of smaller oyster juveniles (C) and length of smaller oyster juveniles (D))

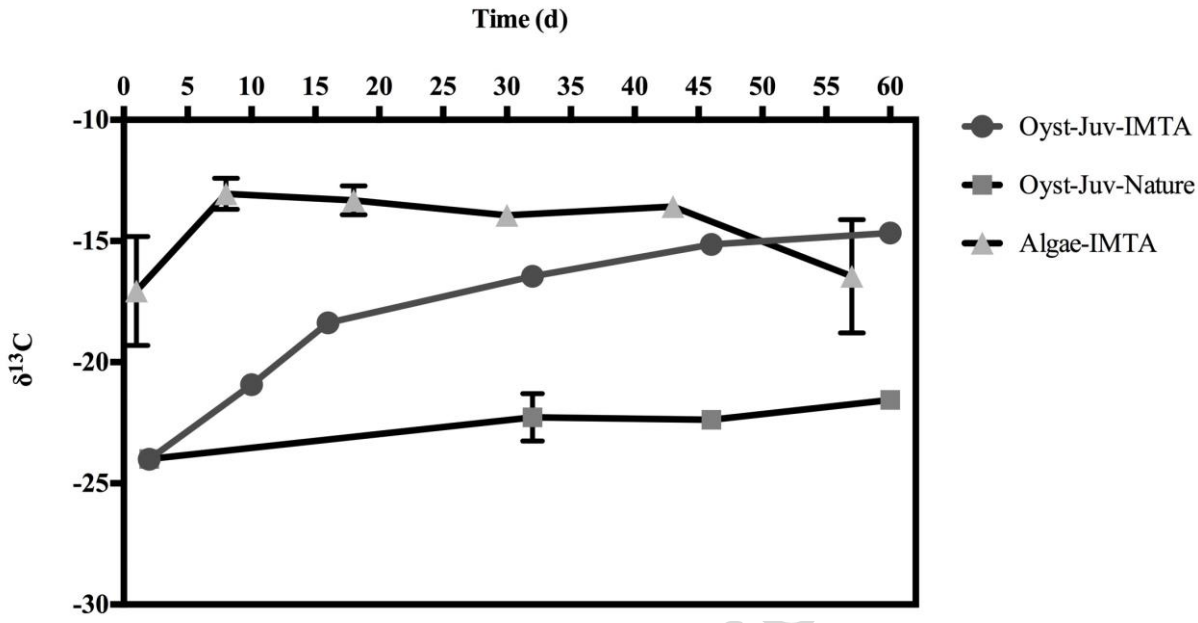


Fig.7 $\delta^{13}C$ signatures of Algae-IMTA, smaller juvenile oysters-IMTA (Oyst-Juv-IMTA) and control smaller juvenile oysters (Oyst-Juv-Nature)

Tables

Table 1 18S rRNA gene sequencing primers

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

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

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

Table 3 Mean ± SD of nutrient removal efficiencies and removal rates of the algal raceways over the period of the study (n = 19)

	NO_3-N	NO_2-N	NH_4-N	PO_4-P
Removal efficiency (%)	98.6 ± 0.2	98.0 ± 0.4	97.3 ± 0.7	96.1 ± 0.6
Removal rate (mg m^{-2} d^{-1})	335.8 ± 0.8	23.6 ± 0.2	30.9 ± 0.2	22.3 ± 0.2

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

Type of water	Biomass produced (mgTSS L^{-1})	N consumed (mg L^{-1})	P consumed (mg L^{-1})	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 *a* L^{-1}	4.96	1.47	0.07-0.39 g Chl *a* / 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

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

In this work, there are some highlights of our manuscript

- Microalgae-based RAS-IMTAs have potential for nutrient bioremediation.
- The RAS-IMTA system produced microalgae. Adding silicate shifted the dominant microalgae species from *Tetraselmis sp.* to *Phaeodactylum sp.*
- The oyster growth was very low although juvenile oysters were able to assimilate the microalgae.