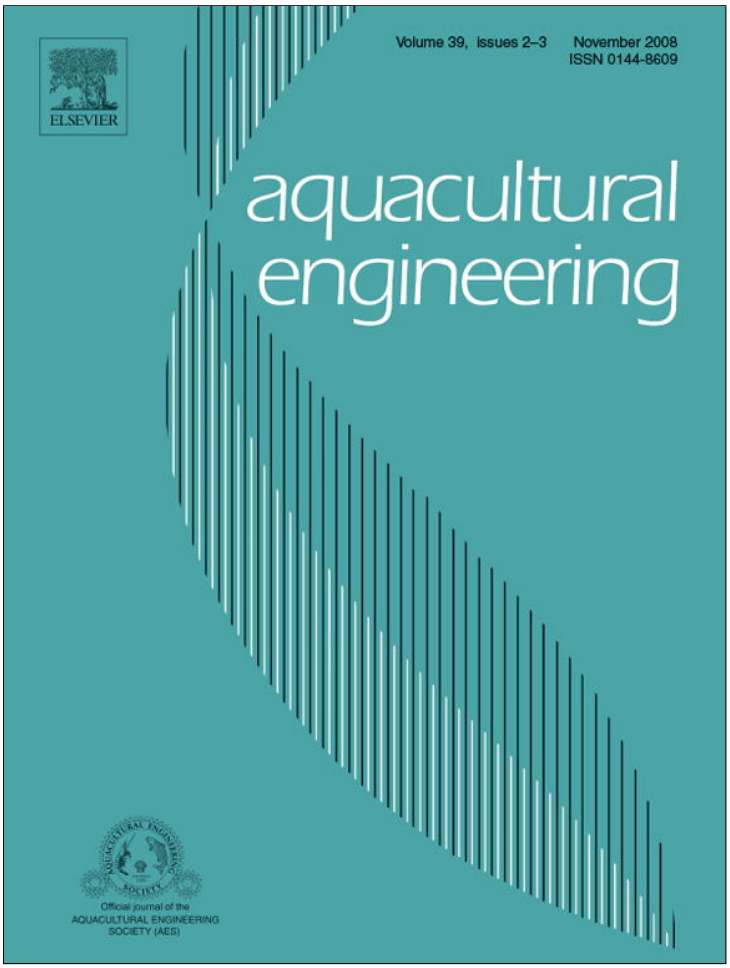

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Mutualism between euryhaline tilapia *Sarotherodon melanotheron heudelotii* and *Chlorella* sp.—Implications for nano-algal production in warmwater phytoplankton-based recirculating systems

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ABSTRACT

The West-African euryhaline tilapia, *Sarotherodon melanotheron heudelotii* shift from visually feeding on zooplankton when juveniles to mostly filter feeding on phytoplankton when adults. When reared using an appropriate ration in intensive aquaculture systems, *S. m. heudelotii* also consume algal-based detritus, and contribute to sediment mineralization, clean up their environment, and ultimately stimulate and sustain algal growth. We analysed such practical advantages for phytoplankton-based recirculating systems, using *S. m. heudelotii* and *Chlorella* sp. as biological material originating from the prototype of such a system operated in Senegal. We performed a 24-h factorial design experiment in 36 tubs, cross-classifying three levels of *S. m. heudelotii* (fishless control, unfed fish, and fed fish) with four levels of *Chlorella* initial density.

Chlorella overall mean density increased significantly from fishless, to unfed fish, and fed fish treatments, and with *Chlorella* initial density. *S. m. heudelotii* did not alter nitrogen nor phosphorus concentrations, only affected by algal initial densities. Most ammonia excreted by fish was probably uptaken by *Chlorella*. Bacteria-mediated diurnal nitrification was possibly an alternative ammonium loss mechanism at highest oxygen concentrations. Algae were not limited by nitrogen or phosphorus but most likely by low dissolved organic carbon availability. *Chlorella* differential responses with fed vs. unfed *Sarotherodon* suggest that CO_2 supplied by heterotrophic *S. m. heudelotii* respiration played a key role. Observed *Chlorella* growth rates were similar to the highest rates obtained in algal mass cultures, enriched with CO_2, nitrate and phosphate, under artificial lighting.

Our results suggest the existence of a *Sarotherodon-Chlorella* mutualism in our systems, where *S. m. heudelotii* provide CO_2, the major limiting factor of *Chlorella* growth, whereas *Chlorella* oxygenate and detoxify the water media from ammonia, promoting *S. m. heudelotii* production. This mutualism could be used to optimize photosynthetic suspended-growth aquaculture systems, particularly in the Tropics where light is abundant and temperature is continuously high.

1. Introduction

Recirculating systems (reviews in van Rijn, 1996; Crab et al., 2007), partitioned systems (namely PAS, Drapcho and Brune, 2000), phytoplankton-based systems or photosynthetic suspended-growth systems (review in Hargreaves, 2000), and zero-exchange systems (Burford et al., 2003) are some current eco-technological solutions designed to intensify aquaculture, while preserving water

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quality (i.e., adequate oxygen concentration, low organic matter accumulation, and weak production of toxic metabolites) and minimizing nutrient pollution from effluent discharge. In such green-water aquaculture systems, phytoplankton plays a major double role by purifying and oxygenating the water column (Smith, 1988; Drapcho and Brune, 2000; Brune et al., 2003). Ammonia, the main metabolic waste product from fish, primarily actively excreted across the gill membrane, is extremely toxic at high concentrations (Sayer and Davenport, 1987). By directly taking up ammonia, phytoplankton replaces bio-filters in recirculating aquaculture systems (Hargreaves, 2006). Moreover, filter-feeding omnivorous fishes, such as tilapias, are capable of enhancing nanophytoplankton (algae size <20 μm) production via their size-selective grazing on microphytoplankton (≥20 μm), predation on herbivorous zooplankton (reviews in Lazzaro, 1987; Northcote, 1988), and cycling of nutrients (Avnimelech, 1998; Vanni et al., 2006). Apart from removing excessive nutrients from effluent wastes, phytoplankton-based recirculating aquaculture systems (RAS) produce micro- or nano-algae that can be used to reduce the need for artificial feed of the target cultured fish or to produce a second crop that generates supplemental income. Most research and development are dedicated to bacteria-based systems (e.g., active suspension ponds, ASP). In contrast, phytoplankton-based recirculating systems are less studied due to the complexity to sustain the key algal species in an integrated open out-door continuous-production system operated under steady state, i.e. where nutrient uptake by phytoplankton and waste discharge by fish remain constant.

In the semi-arid and arid conditions of tropical and subtropical countries, progress in aquaculture is limited by the shortage of freshwater resources. Yet, environment-friendly but technically less complex and low-cost versions of conventional RAS developed in temperate countries are needed. Helpfully, there, solar radiation is intense all year long, water temperature is high and little variable, algal growth is typically not limited by nutrients, and filter-feeding planktivorous clupeids, cichlids, and/or cyprinids are frequently prevalent.

In the Sahelian zone, the West-African euryhaline tilapia, *Sarotherodon melanotheron heudelotii* Duméril 1861, a subspecies native from Senegal and Guinea (Trewavas, 1983; Falk et al., 2000), colonizes estuaries, rivers, and lakes. Fry visually feed on zooplankton, and insect larvae, while adults filter feed on phytoplankton (Kone and Teugels, 2003). At intermediate sizes, *S. m. heudelotii* can shift between feeding modes, as described for other cichlids (Lazzaro, 1991). Filtering is the most energetically efficient feeding mode where high densities (or patches) of small-sized particles prevail. Weight-specific costs of filter feeding are highest for smaller particles, but decrease exponentially with fish size, e.g., in blue tilapia, *Oreochromis aureus* Steindachner (Yowell and Vinyard, 1993). Yet, in nature, tilapias cannot maintain positive growth when feeding exclusively on small particles (Dempster et al., 1995), and must substantially complement their diet with algal-based detritus and periphyton (Dempster et al., 1993). In accordance, *S. m. heudelotii* is also illiophagous, consuming settled organic matter from bottom sediments (Pauly, 1976; Ugwumba and Adebisi, 1992). Thus, *S. m. heudelotii*, when reared with an appropriate ration, contribute to nutrient mineralization and clean up their environment. Non-utilized fraction of the feed, settled algae, bacteria, protozoa, and other microorganisms, and associated grazers tend to aggregate into microbial flocs. The ability of tilapias to graze on microbial flocs plus the recycling of excreted nitrogen into utilizable microbial proteins are currently used to optimize feed utilization in intensive production systems (Avnimelech, 2006). Hence, taking advantage of the herbivorous, illiophagous, and euryhaline characteristics of tilapias, such as *S. m. heudelotii,* in aquaculture engineering represents a promising perspective in brackish warmwater systems.

In the current experiment, we addressed some short-term engineering aspects of phytoplankton-based recirculating systems. We quantified herein the net diel algal production mediated by the metabolic activities of fed and unfed *Sarotherodon* in relation with algal initial density, in order to evaluate the effects of artificial feeding. We used *S. m. heudelotii* and the nano-alga *Chlorella* (Ø 3–4 μm) as biological material coming from a prototype of a recycling aquaculture system setup by IRD in Mbour, Senegal. This prototype is a brackish, warmwater, photosynthetic suspended-growth system that combines intensive fish-production in tanks, with a recirculating, partitioned, zero-exchange, integral system. Its food web is composed of *Chlorella* sp. as primary producer, rotifer *Brachionus plicatilis* O.F. Muller as intermediate consumer, both spontaneously seeded, and *S. m. heudelotii* as omnivorous top-consumer.

In intensive aquaculture recirculating systems, physico-chemical and biological parameters vary along diel cycles related to fish digestive metabolism. In photosynthetic-based aquaculture systems, algal growth varies within hours following fish excretion, and with fish respiration based on foraging and swimming rhythms, in relation with diel variation in solar irradiance. This conducted us to perform a 24-h experiment. Specifically, we tested, over this short-term cycle, whether nitrogen and phosphorus not retained by *S. m. heudelotii*, particularly the potentially toxic ammonia excreted by the gills, could be efficiently uptaken by *Chlorella* sp. (Witt et al., 1981). We tested how *Chlorella* uptake efficiency depends on *Chlorella* initial density, and the effects of fed and unfed *Sarotherodon* on *Chlorella* growth. The outcome of the *Sarotherodon–Chlorella* relationship is not straightforward. Indeed, *S. m. heudelotii* may not efficiently consume *Chlorella,* since *Chlorella* cell diameter is smaller than the size threshold of *S. m. heudelotii* selective filtering rate. The practical implications of our results about the *Sarotherodon–Chlorella* relationships are discussed in the context of warmwater green-water recirculating systems.

2. Materials and methods

2.1. Experimental design

We performed a 24-h factorial design experiment in thirty-six 20-L tubs (0.36-m diameter, 0.20 m in depth) filled with stagnant water (previously filtered through a 60-μm mesh net, in order to remove rotifers) collected from the prototype 'phytoplankton recycle pond'. Tubs were organized in two rows under a greenhouse to avoid possible dilution by rain, and minimize contamination by falling particles (e.g., leaves, insects). We cross-classified a 3-level tilapia treatment (C, fishless control; F, unfed fish; Ff, fed fish) with a 4-level *Chlorella* treatment (10%, 50%, 80%, and 100% of routine algal density in the prototype 'phytoplankton recycle pond'). Treatments were conducted in triplicates and randomly organized between tubs. To achieve initial levels of *Chlorella* density, prototype water (salinity 15 g L^{-1}, i.e., 15‰; routine algal density = 100% = 33×10^6 cells mL^{-1}) was mixed with water from a well dug 30 m from the ocean shoreline (same salinity, no phytoplankton). Prototype water was richer in NO_2+NO_3 than water from the well (means ± S.E., 76.50 ± 2.81 and 51.41 ± 2.68 mg L^{-1}, respectively; $P = 0.008$). On an average, prototype water was poorer in total ammonia–N (TAN = NH_3+NH_4^+; 142.33 ± 14.44 vs. 258.33 ± 55.74 μg L^{-1}) and PO_4 (298.67 ± 53.14 vs. 342.00 ± 60.14 μg L^{-1}), 1), yet concentrations were not statistically distinct due to high data variability. We filled the first tub at 08:00 (t_0).

We used 24 sub-adult *S. m. heudelotii* females (60.1 ± 6.9 g) from the same cohort, and stocked one individual per tub according to the design. Resulting biomass (3 kg m^{-3}) was consistent with biomass values prevailing in semi-intensive aquaculture ponds. Fish were starved for 48 h prior to the experiment. They were maintained in well water renewed several times so as to avoid faeces consumption and to ensure empty digestive tracts. The sampling chronology started half an hour after the fish stocking. When stocked (t_0), fed fish received a single 2.00 ± 0.01 g ration in pellets (Ø 1.5 mm), containing 52% protein, i.e., 8.32% total nitrogen (TN). This dose was computed using an estimated feeding rate of 3.33% body weight day^{-1}.

2.2. Physico-chemical analyses

Dissolved oxygen (DO), temperature and pH were simultaneously measured at 15:00 (t_7) and 07:00 (t_{23}), using a Cyberscan DO 310 oxymeter (Eutech Instruments, Singapore), and a Hanna HI 9025 pHmeter (Hanna Instruments Inc., Rhode Island, USA). Samples for nutrient determinations, i.e., total ammonia, nitrite–nitrate and phosphate, were obtained in two steps: (a) at $t_{0.5}$ three prototype water samples were filtered under GF/F membrane filter, and three water samples from the well were each (40 mL) preserved with 0.3 mL of chloroform in order to estimate the initial nutrient concentrations in relation to dilutions; (b) at t_{24}, one sample was collected from each of the 36 tubs, filtered and preserved as previously described.

Total ammonia–N (TAN) (Koroleff, 1969) and orthophosphate (PO_4–P) (Murphy and Riley, 1962) were measured using a Helios UV–visible spectro-photometer (Thermo Electron Corp., Winsford, Cheshire, UK) with 1- and 5-cm width vessels, respectively. Nitrate–N (N–NO_3) (Grasshoff, 1976) and nitrite–N (N–NO_2) (Bendschneider and Robinson, 1952) were measured using a Bran & Luebbe Technicon II auto-analyzer (Bran & Luebbe Analyzing Technologies, Inc., Elmsford, NY, USA).

2.3. Phytoplankton analyses

We performed five phytoplankton samplings successively at 8:30 ($t_{0.5}$), 10:00 (t_2), 14:00 (t_6), 20:00 (t_{12}), and 8:00 (t_{24}) the following morning. We used the mean *Chlorella* density at $t_{0.5}$ resulting from all C-treatment levels combined ($n = 12$) as mean algal pre-treatment density. At each sampling time, water column was gently mixed by hand without disturbing bottom sediment, and tubs were successively sampled at 1-min intervals, always following the same random order. For each sample, water was collected at mid-depth with a 30-mL container, and phytoplankton immediately fixed with three drops of 4% formaldehyde. *Chlorella* concentrations were determined by colorimetry with a Hanna C203 photometer (Hanna Instruments Inc., Rhode Island, USA) at 420-nm wavelength (tungsten lamp source). From five dilution levels we computed the relationship between the optical density (OD) obtained with the photometer and the algal density (AD) assessed from counts: AD (cells mL^{-1}) = 6.62 × 10^6 OD + 127.90, $r^2 = 0.9995$. Counts were performed using a Bürker cell under an OLYMPUS CX41 stereomicroscope (40× magnification). In order to compute the algal mean density, each sample counting was achieved on 12 optical fields, of which the ones with highest and lowest algal densities were omitted. The dilution gradient between waters from the prototype and from the well was the same as for the experiment. To control for possible effects of dissolved substances on OD, measurements were also performed after removing phytoplankton by filtration onto a Whatman GF/F membrane filter (0.7-μm pore size, Ø 47 mm; Florham Park, NJ, USA). No significant effect was observed (OD < 0.2 at 0% dilution). We computed net algal production, as the difference between the final and initial concentrations, in relation to nutrients for the overall 24-h period.

2.4. Statistical analyses

We used two-way repeated-measures ANOVA (RMA) to analyze for main effects of fish (F; three levels: C, F and Ff), algal initial density (D; four levels: 10%, 50%, 80%, and 100%), and their interaction effects (F × D), with time as a trial factor (5 samplings for phytoplankton: $t_{0.5}$, t_2, t_6, t_{12}, and t_{24}). We used two-way ANOVA to analyze for the possibility of differences between treatment levels at the start of the experiment. We performed all ANOVA on raw data, using SuperAnova® v.1.11 (Abacus Concepts, Berkeley, CA, USA), and with $\alpha < 0.05$ as significance level. We used Scheffé and Tukey post hoc tests to identify significant differences between treatment levels.

3. Results

3.1. Effects of fish, dilution, time, and their combinations on Chlorella density

The mean (±S.E.) *Chlorella* routine initial density in the prototype system, computed at $t_{0.5}$ from the triplicates of the C treatment at 100% algal initial density, was 33.0 ± 0.3 × 10^6 cells mL^{-1}. Over the course of the experiment (5 samplings), and for all algal initial densities, the main fish effect was highly significant (Fig. 1a, $P = 0.001$). The overall mean *Chlorella* density increased in presence of tilapia in relation to control (112%), and all the more when tilapia were fed (124%). These net density increases were substantial on a daily basis. The interaction effects between fish and algal initial density were highly significant, i.e., the algal initial density altered the fish effects (Fig. 1b). These effects were antagonistic since the relative magnitude of the positive fish effects on *Chlorella* density decreased, in relation to the control, with increasing *Chlorella* initial density (157–176% vs. 104–121% increase for unfed–fed fish, from 10% to 100% *Chlorella* initial density, respectively). The highly significant fish × time interaction demonstrated that time effects were altered by the fish treatment level (Fig. 1c). In the fishless control *Chlorella* density increased slowly during the first 6 h (i.e., 14:00) then declined, dropping below the initial level. With unfed tilapia, *Chlorella* density only slightly decreased at night. Conversely, with fed *Sarotherodon*, algal density continued to rise slowly.

The significant fish × algal initial density × time interaction reveals that the temporal response pattern of algal density, distinct for each fish treatment level, differed in relation to algal initial density (Fig. 1d). After 14:00, there was no temporal decline of algal density at the lowest algal initial density (10%). At the initial prototype routine density (100%), *Chlorella* densities did not differ between the control and unfed fish treatment levels, whereas at lower algal initial density levels the two fish treatment levels were identical but significantly different from the control (Scheffé, $P < 0.05$). Note that, at low algal initial densities, *Chlorella* density slightly increased at night in presence of fed fish. This arose from significant differences in algal densities between 20:00 and 08:00 only for the 10% and 50% algal initial density levels ($P = 0.01$, and $P = 0.02$, respectively; contrast tests). This pattern probably illustrates the known process of prokaryote growth in which carbohydrate production and DNA replication during daytime are followed by cell divisions at night (Hama et al., 1988; Vaulot et al., 1995).

At first sampling ($t_{0.5}$), i.e., 30 min after fish were stocked in tubs, for the highest 80–100% algal initial densities, *Chlorella* densities were significantly lower in the presence of unfed fish (i.e.,

roughly −9%) as compared to the fishless and fed fish treatments ($P = 0.049$ and 0.001, respectively; post hoc Tukey tests) (Fig. 2a). However, the unfed *Sarotherodon* did not affect significantly algal densities at the lowest *Chlorella* initial densities (50% and 10%).

3.2. Chlorella net diel production

In absence of *Sarotherodon*, the mean net diel *Chlorella* production rate (i.e., (final − initial)/initial densities) ranged from +0.29 to −0.26 day^{-1}, at the lowest and the highest *Chlorella* initial densities, respectively. Rough estimates of maximal algal death rates (i.e., (maximum − final)/maximum densities) reached about 0.30–0.40 day^{-1}.

Both the fish and the algal initial density treatments significantly affected *Chlorella* net diel production (i.e., final–initial densities over 24 h); *S. m. heudelotii* affected the algal density resulting in a significant F × D interaction (Fig. 2b). Mean net production significantly increased from fishless, to unfed *Sarotherodon*, and fed *Sarotherodon* (-3.7 ± 1.3, to 1.5 ± 2.0, and $7.1 \pm 0.7 \times 10^6$ cells mL^{-1}, respectively), with treatment means being significantly different between each others (C vs. F, $P = 0.003$; C vs. Ff, $P = 0.0001$; F vs. Ff, $P = 0.001$; Scheffé post hoc tests). At the highest algal initial density (100%), the net production of the fed fish treatment was significantly higher than the two other treatments that did not differ. For algal initial density levels of 10–80%, the two fish treatments responded similarly, but still the production of the fed fish treatment was significantly higher ($P = 0.02$, Scheffé post hoc test). They were significantly different from the fishless treatment, where production was negative above 50% algal initial density. At the highest algal initial density, the net productions of the unfed fish and fishless treatments were clearly negative and did not differ.

The algal initial density significantly altered the diel percent change in *Chlorella* mean density over the course of the experiment. The net effect switches from positive to negative with increasing *Chlorella* initial density, except for fed *Sarotherodon* for which the net change remained positive (Fig. 2c). It was always significantly lower in the fishless treatment than in presence of fish, regardless of fish being unfed or fed. At the lowest algal initial density (10%), the relative increases in *Chlorella* densities were significantly higher in the fish treatments, regardless of whether *Sarotherodon* were unfed or fed ($215 \pm 25\%$ and $245 \pm 32\%$, respectively), as compared to the fishless treatment ($29 \pm 14\%$). This difference shrunk with increasing algal initial density, starting at 50%. Note that the relative increase in *Chlorella* density was significantly higher (6–10 times) in presence of fish (either unfed or fed) at the lowest algal initial density (10%) than at higher algal initial densities.

3.3. Water temperature and chemical variables

Mean water temperature varied between 30.2 ± 0.2 °C at 07:00 and 40.6 ± 0.2 °C at 15:00. Concentrations of dissolved oxygen could not be recorded at 15:00, as they exceeded the oxymeter scale (>20 mg O_2 L^{-1}) within all tubs. At sunrise (07:00), i.e., 1 h before the experiment end, O_2 concentrations were the lowest and differed

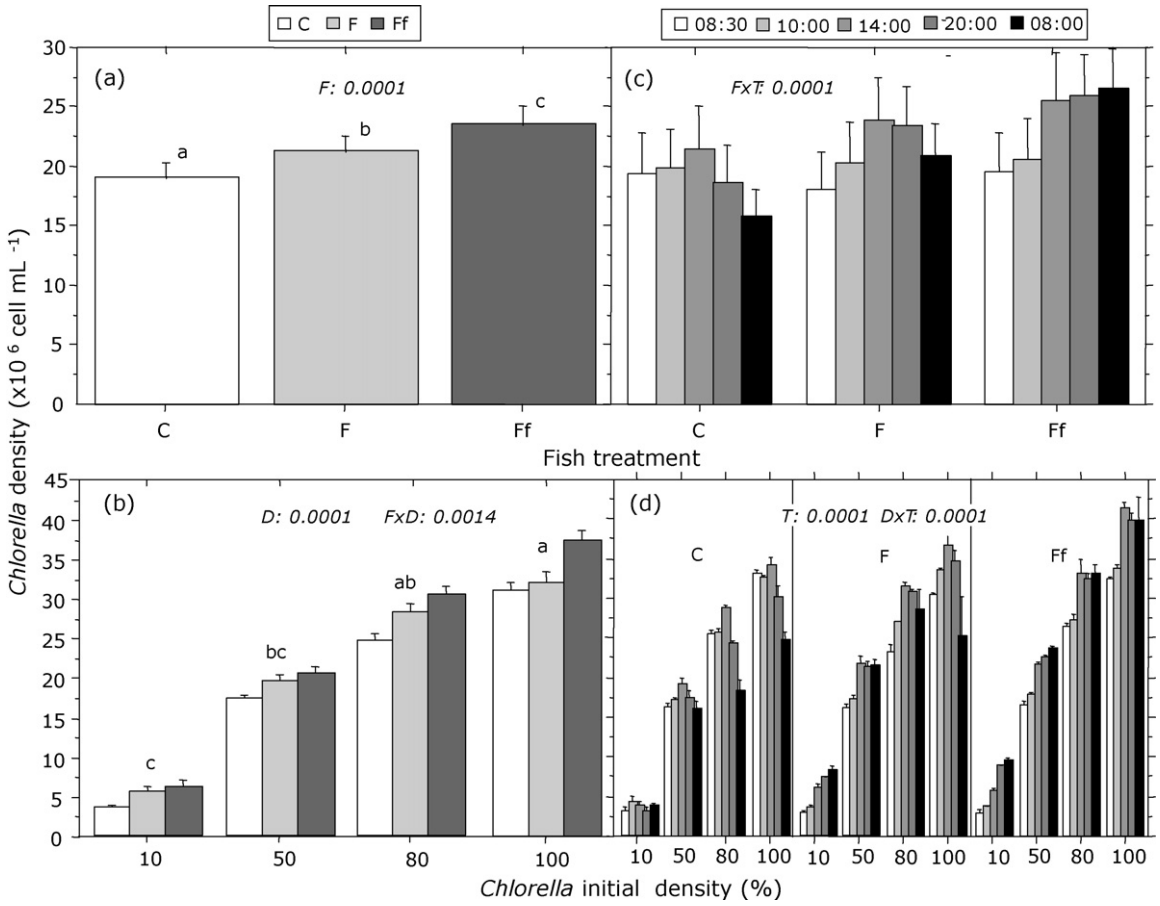

Fig. 1. Mean responses of *Chlorella* sp. density to (a) fish treatment levels, (b) interactions of fish treatment × initial *Chlorella* density as percent of routine density reached in the algal tank of the recirculating-system prototype, (c) interactions of fish treatment × time (hours of day), and (d) interactions of fish treatment × initial *Chlorella* density × time. Probabilities of RMA of fish effects (F), *Chlorella* density effects (D), time (T), and their interactions (F × D and F × T) indicated on the top of graphs. Significant values ($P < 0.05$) shown in italics. Significant post hoc Tukey tests ($P < 0.05$) indicated by letters above bars.

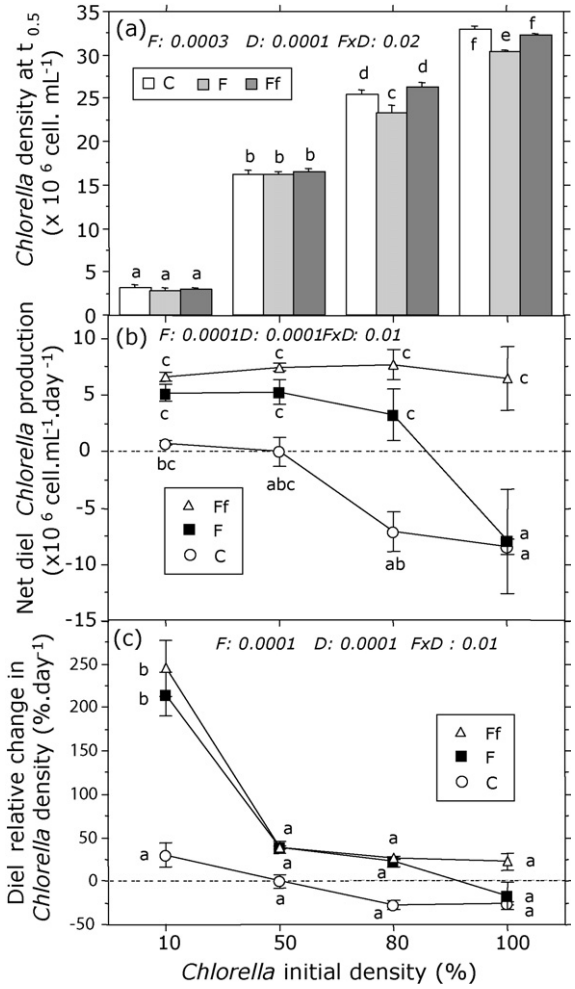

Fig. 2. (a) Initial *Chlorella* density ($t_{0.5}$) for the different treatment combinations. Mean net production of *Chlorella* in relation to fish treatment and initial *Chlorella* density as (b) difference between initial and final samplings (08:30 to 08:00, 24-h duration), and (c) percent increase. Statistics and legends as in Fig. 1. Significant Tukey tests indicated by letters above bars or close to symbols.

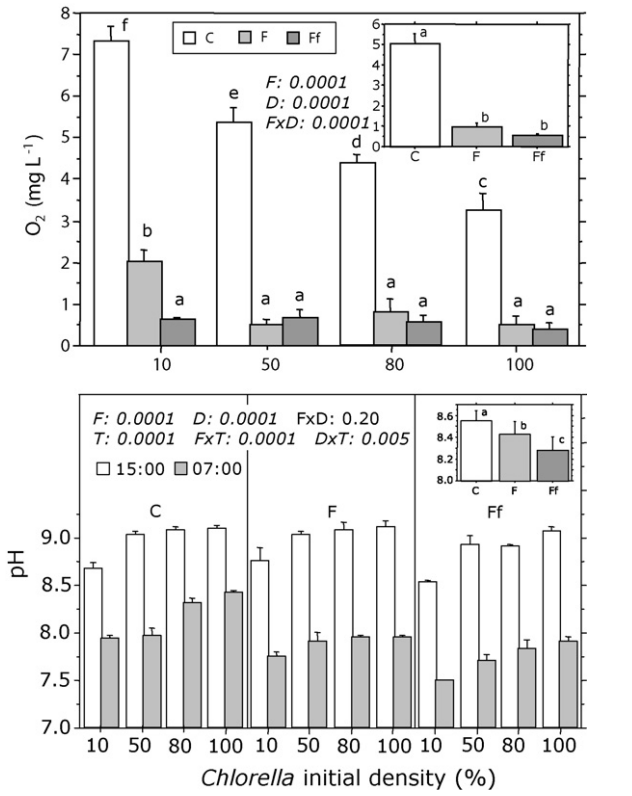

Fig. 3. Mean responses of dissolved oxygen concentration (O_2) at 07:00, and pH, in response to fish treatment (F), initial *Chlorella* density (D), and time (T, for pH only). Bar graphs of mean fish responses inserted at the top of each graph. Statistics and legends as in Fig. 1.

between fish treatments, being significantly lower in presence of fish (Fig. 3). Oxygen concentrations did not differ between fed fish and unfed fish treatments except for the 10% algal initial density level, for which the O_2 concentration was higher with unfed than with fed fish, probably related to the decomposition of the artificial feed. The significant interaction between the fish and algal initial density resulted from the decrease in O_2 concentrations with increasing algal density in the fishless treatment, as compared to the lack of response in the two treatments with fish.

Overall mean pH was significantly higher at 15:00 (8.92 ± 0.03) than at 07:00 (7.91 ± 0.04). Effects of fish and algal initial density were highly significant (RMA at 15:00 and 07:00) (Fig. 3). Overall mean pH values differed statistically over a narrow range: 8.3 (C), 8.4 (F), and 8.6 (Ff). They increased significantly with increasing algal initial densities both in the afternoon and at sunset.

Overall mean TAN concentrations were 322 ± 3 μg $N\text{–}NH_3\text{–}NH_4^+$ L^{-1} at the start ($t_{0.5}$) and 235 ± 19 μg $N\text{–}NH_3\text{–}NH_4^+$ L^{-1} at the end of the experiment (t_{24}) (RMA, $P = 0.0001$) (Fig. 4). TAN concentrations were not affected by fish (RMA, $P = 0.22$) but significantly decreased linearly with algal initial density (RMA, $P = 0.01$). Nitrate–nitrite concentrations decreased drastically and significantly over time, with overall means of 53.0 ± 0.2 and 8.4 ± 2.0 mg $(N\text{–}NO_2\text{+}N\text{–}NO_3)$ L^{-1} at the start and end of the experiment, respectively (Fig. 4). Nitrate–nitrite concentrations were not affected by fish, but responded significantly to algal initial density. We observed a highly significant interaction effect between algal initial density and time: Initial nitrate–nitrite concentrations tended to increase with algal initial density while the inverse pattern was observed at the end of the experiment. Orthophosphate concentrations significantly decreased between the start and the end of the experiment (RMA, $P = 0.0001$), from 204.20 ± 7.10 to 73.93 ± 11.95 μg $P\text{–}PO_4$ L^{-1}, respectively. As for nitrate–nitrite, orthophosphate concentrations were not affected by fish, but they significantly decreased with increasing algal initial density (RMA, $P = 0.04$). This effect mainly reflected the initial $P\text{–}PO_4$ pattern imposed by the dilution between the prototype and well waters (Fig. 4).

4. Discussion

4.1. General algal and nutrient responses

We obtained several major results concerning the effects of fish treatment and algal initial density on *Chlorella* growth.

(i) In absence of *Sarotherodon*, the mean net diel *Chlorella* production rate switch from positive to negative, at the lowest and the highest *Chlorella* initial densities, respectively. Our rough estimates of maximal algal death rates (0.30–0.40 day^{-1}) are consistent with observations of phytoplankton lysis rates in Pacific Ocean (0.12–0.67 day^{-1}; Hawakawa et al., 2008), in Mediterranean coastal waters during summer (0.41 day^{-1}; Agusti and Duarte, 2000), and in Lake Kinneret (yearly mean 0.91 day^{-1}; Berman and Wynne, 2005). These results

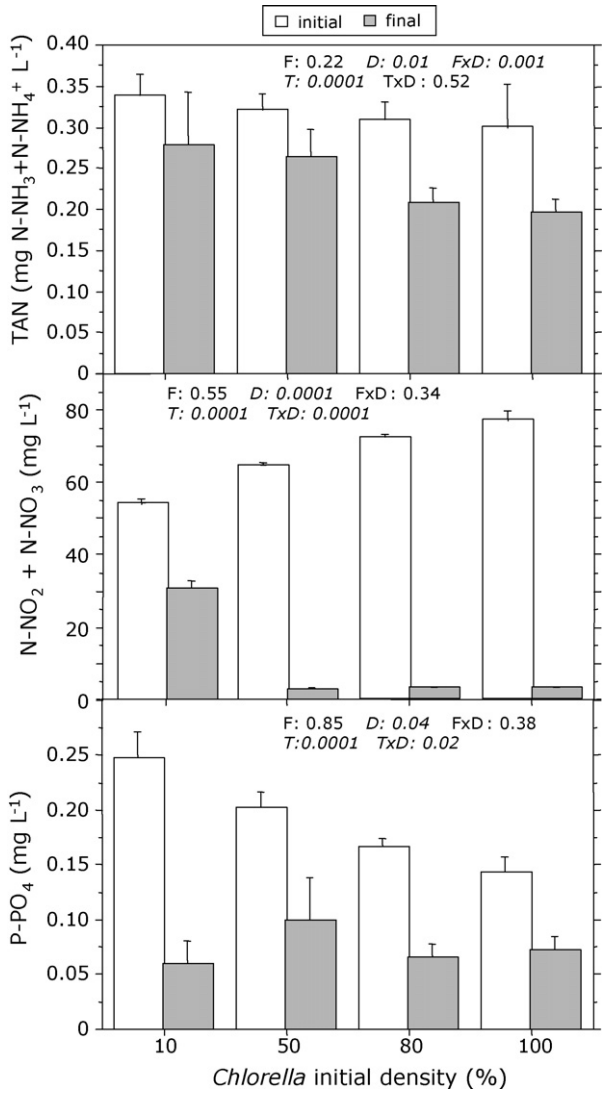

Fig. 4. Mean responses of TAN (i.e., NH_3+NH_4^+, upper graph), NO_2+NO_3 (middle graph), and PO_4 (lower graph) concentrations to fish treatment (F), initial *Chlorella* density (D), and time (T). Statistics and legends as in Fig. 1.

confirm the importance of phytoplankton lysis, which can reach a rate equivalent to that of zooplankton grazing, in both marine and freshwater algal systems.

(ii) On average, the net effect of unfed fish was an increase of *Chlorella* mean density as compared to the fishless mean. This enhancing fish effect on *Chlorella* density was not a result of the external nutrient inputs since the stocked fish were starved prior to the experiment and had empty guts. Thus, the positive indirect effect of *S. m. heudelotii* was greater than the negative direct effect of *S. m. heudelotii* grazing.

(iii) Mean *Chlorella* density increased from the fishless control to the unfed fish and fed fish treatments.

(iv) The relative algal enhancement decreased with the increasing *Chlorella* initial density, illustrating a density-dependent effect. At the highest initial density (100%), over the course of the experiment *Chlorella* reached the same mean density level whether tilapia where absent or unfed. Between 50% and 100% algal initial density, unfed *Sarotherodon* was able to keep *Chlorella* density constant. In different experimental conditions we observed that unfed *Sarotherodon* isolated in a small volume could maintain *Chlorella* density constant over several weeks (unpubl. data). With fed *Sarotherodon*, net phytoplankton production was constant in absolute value across the *Chlorella* initial density gradient, but decreased by ten times in relative value, above the 10% algal initial density. At the highest algal initial densities (80% and 100%), fed *Sarotherodon* enhanced more *Chlorella* density than unfed *Sarotherodon* did.

(v) *S. m. heudelotii* did not affect significantly nutrient final concentrations, which were only affected by algal initial density.

In the next sections, we will examine the mechanisms potentially involved in the observed nutrient and algal responses, and their practical implications for phytoplankton-based recirculating aquaculture.

4.2. Were nutrients limiting algal growth?

Initial nitrogen concentrations were very high in the tubs with a N:P mass ratio ranging between 200:1 and 387:1 according to the algal dilution rate. In such circumstances, we can rule out an overall nitrogen limitation of *Chlorella*, which is known to grow faster in a medium of low N:P ratio (11:1 in mass; Ryther, 1954). Such unbalanced ratio might have favoured P-limitation. However, mean final phosphorus concentrations were not negligible (74 ± 12 μg $P–PO_4\ L^{-1}$), demonstrating that factors other than nutrients limited algal growth. These limiting factors acted even at the lowest algal initial density as highlighted by the absence of *Chlorella* growth in the control. Yet, our experimental nutrient conditions were atypical, tubs being initially very rich in nitrogen (mean 55–75 mg $N–NO_2$+$N–NO_3\ L^{-1}$). Whereas routine concentrations in the recirculating prototype system typically did not exceed 0.2 mg $N–NO_2$+$N–NO_3\ L^{-1}$ (2007 period, Gilles and Fargier, unpubl. data), N-limitation could possibly occur, and thus the compensatory role of *S. m. heudelotii* N-excretion may occasionally be substantial.

Filter-feeding omnivores are often considered to enhance algal reproduction via nutrient regeneration (Colman and Edwards, 1987; Milstein, 1992). This was not the case in our experiment. Indeed, we observed the highest relative positive fish effect on *Chlorella* at the lowest algal initial density, whereas excretion was probably minimal because of weak *S. m. heudelotii* grazing, and dissolved nutrients were very abundant. Thus, *S. m. heudelotii* enhanced algal growth by other ways rather than nutrient release.

4.3. Nutrient losses throughout the experiment

We observed a great loss of NO_2+NO_3 over the course of the experiment, in particular at highest algal densities. Part of this N-removal may be explained by *Chlorella* uptake, since algae typically accumulate non-limiting nutrients as luxury consumption (Danger et al., 2007b). However, assuming optimum growth conditions of tropical systems (Hargreaves, 1998) and an uptake proportional to the Redfield mass ratio (C:N:P = 40:7:1), potential N-immobilization in algal cells was clearly insufficient to account for the observed N-loss pattern. Therefore, other mechanisms necessarily co-occurred, such as bacterial immobilization, coupled nitrification–denitrification, and organic matter sedimentation (Hargreaves, 1998, 2006; Brune et al., 2003).

Moreover, we did not observe any significant increase of TAN in the presence of fish excretion. We assessed the excretion rate of *S. m. heudelotii* on the basis of Kobayashi et al.'s (2007) study on Nile tilapia, a species close to *S. m. heudelotii*. For that, we assumed there was a 16-h daily feeding-excretion in our tubs because excretion rate is related to ingestion rate and declines quickly after feeding ceases (Vanni, 2002). The estimated value (5.1 mg $N–NH_4\ L^{-1}\ day^{-1}$) is consistent with that (4.4 mg $N–NH_4\ L^{-1}\ day^{-1}$) obtained by supposing that *S. m. heudelotii* totally consumed the given 2 g of feed, which contained 8.32% or 166 mg of nitrogen,

and considering that 53% of consumed nitrogen was excreted (Beveridge et al., 1991). Two mechanisms probably contributed to the disappearance of excreted NH_4. First, diurnal nitrification at the highest oxygen concentrations may have occurred, as suggested above. Second, algae may have preferentially uptaken NH_4. This is consistent with Witt et al.'s (1981) results indicating that *Chlorella* typically uptake NH_4 over NO_3. In presence of fish, the latter mechanism might have also induced an increase of algal growth.

While algal uptake could not account for the global N-loss, it could have explained a substantial part of P-sequestration. Assuming the same growth conditions as previously, and an uptake proportional to the Redfield ratio, algae would have immobilized all the initial orthophosphate.

4.4. pH, O_2 and CO_2

We observed high pH values (around 9.0) at 15:00. Situations of intensive photosynthetic activity, typical of our experimental systems at midday, are characterized by a high level of consumption of dissolved inorganic carbon, leading to an increase in pH. High pH values are indicative of low dissolved inorganic carbon (DIC) availability with a predominant form of CO_3^{2-}, which is a low efficiency carbon source for primary production. Conversely, low pH values are typical of high DIC availability with predominantly HCO_3^- and dissolved CO_2 forms, which are quite easily usable for primary production (Beardall et al., 1998). The diffusion of CO_2 in the water being much slower than its utilization by phytoplankton may cause inorganic carbon depletion that in turn decreases the photosynthesis efficiency in eutrophic conditions (Mouget et al., 1995).

The temporal dynamics of algae in absence of fish (C) and in presence of unfed *Sarotherodon* (F) seem to illustrate well the above-mentioned situation. Indeed, in these conditions, *Chlorella* densities decreased 6 h after the start of the experiment (14:00), after the onset of the photosynthetic activity. Conversely, in the presence of fed *Sarotherodon* (Ff) we observed a continuous positive algal growth throughout the entire experiment. This suggests that heterotrophic CO_2 production (fish respiration plus pellet and faeces decomposition) compensated for CO_2 consumption by algae. At the end of the experiment, early morning (07:00) O_2 concentrations were significantly lower in presence of *Sarotherodon*, either unfed or fed, than in the fishless control. The strong difference in O_2 concentration between the two categories of tubs strongly supports that global respiration was important in presence of fish. This is also reflected by the significantly lower pH values in presence of *Sarotherodon*, and even all the more when they were fed. Accordingly, fish presence contributed to increasing CO_2 concentration via respiration. Therefore, although we did not measured CO_2 fluxes within the tubs, CO_2 was certainly more available in presence of fish, and particularly when fed. Thus, indirect mutualism between fish and algae, due to the CO_2 supplied by heterotrophic respiration to the inorganic carbon-limited algae, probably played a key role in the observed algal enhancement along the C–F–Ff treatment gradient. Danger et al. (2007a) already demonstrated such an indirect mutualism between heterotrophs (bacteria) and autotrophs (green alga *Scenedesmus obliquus*) in laboratory eutrophic conditions.

4.5. Water mixing by fish

Via their swimming activities, fish may enhance water mixing (Rasmussen et al., 2005), and thus decrease algal settling. However, our experimental conditions suggest that such mechanism did not play a major role in the observed differences in *Chlorella* densities between fishless and fish treatments. Based on Reynolds' (1984) compilation using data on experimentally killed cells in quiescent water, we estimated maximum settling speed for 4-μm Ø unicells to be roughly 2.5 cm h^{-1}. Actual settling rates were probably much smaller, as Weissman et al. (1988) observed that live *Chlorella* did not settle even in absence of mixing. Moreover, our gentle water-column mixing prior to sampling considerably reduced the potential effect of settling during daytime, even though the bottom sediment was not disturbed. Thus, algal settling was probably low in all tubs regardless of fish presence. Besides, the absence of settled organic matter when starting the experiment probably prevented subsequent fish bioturbation on bottom sediment from playing an important role in algal resuspension. The suggested weak importance of hydrodynamic forces in explaining algal responses to fish treatments is supported by their visible diel patterns (see Section 4.3).

4.6. Chlorella consumption by Sarotherodon

Unfed *Sarotherodon* started to consume *Chlorella* during the 30 min preceding the first sampling ($t_{0.5}$). As a result, *Chlorella* density at $t_{0.5}$ was not only significantly the lowest in the presence of unfed fish, but also similar whether in the presence of fed fish or in the absence of fish. This suggests that *S. m. heudelotii* prefers artificial feed, even when nanophytoplankton unicells are abundant.

The observed algal net reduction in presence of unfed fish can be converted into grazing rates of $1.4–1.9 \times 10^6$ cells g^{-1} h^{-1}, and a clearance rate of 60 mL g^{-1} h^{-1} (i.e., 3.5 L h^{-1} per experimental unit). Surprisingly, this clearance rate is comparable to the highest clearance rate (30–70 mL g^{-1} h^{-1}) measured by Northcott et al. (1991) for 85-mm SL Nile tilapia foraging on large filamentous cyanobacteria *Anabaena cylindrica*. Potentially, by maintaining this maximum clearance rate during 12 h, unfed *Sarotherodon* would have been able to daily clear *Chlorella* from 42 L (i.e., roughly twofold the volume of a tub). However, decrease in *Chlorella* density was not observed, suggesting that the effective mean clearance rate was lower. Accordingly, tilapias can only sustain high filtering rates for a few hours, typically at dawn and dusk (Caulton, 1982). In addition, *Chlorella* diameter is clearly below the minimum size threshold for particle efficient filtration known for microphagous tilapias (e.g., 25 μm for *O. aureus*, Drenner et al., 1984; 10 μm for *O. aureus*, Drenner et al., 1987; 8 μm for *O. niloticus* subadults, Robinson et al., 1995).

As the effect of foraging behaviour by unfed *Sarotherodon* was not observed at the lowest *Chlorella initial densities* (10% and 50%), a density range of $15–25 \times 10^6$ cells mL^{-1}, equivalent to $5–8 \times 10^8$ μm^3 mL^{-1} (assuming a mean cell volume of 33 μm^3, based on a 4-μm diameter), may represent a size threshold below which filter feeding becomes energetically unprofitable. As a comparison, ingestion rate of 85-mm SL Nile tilapia filter-feeding on cyanobacterial two-celled colonies of *Microcystis aeruginosa* (biovolume 61 μm^3) was maximum at concentrations lower than ours (around 1.5×10^8 μm^3 mL^{-1}, equivalent to 2.4×10^6 colonies mL^{-1}; Northcott et al., 1991). According to these authors, this maximum ingestion rate would fail to support tilapia growth. This would cause weight loss, unless fish would have access to larger colonial or filamentous algae, periphyton, or sediment organic matter. Our observation of fed *Sarotherodon* consuming preferentially artificial feed over phytoplankton during the first thirty minutes is consistent with this authors' contention.

4.7. Aquacultural implications

High algal production. Herein, *Chlorella* diel growth rates in presence of fed *Sarotherodon* (Ff) at the highest *Chlorella* density

($32–40 \times 10^6$ cells mL^{-1}), and in presence of unfed or fed *Sarotherodon*, at 50% initial density ($16–22 \times 10^6$ cells mL^{-1}) are remarkably similar to *Chlorella maculata* growth rates in mass culture (up to 100×10^6 cell mL^{-1}) enriched with both CO_2, nitrate and phosphate, under artificial lighting (Huertas et al., 2000). The initial concentrations of nitrogen and phosphorus used by Huertas et al. were 1700 μM NO_3 L^{-1} and 50 μM PO_4 L^{-1}, respectively, as compared to the 3800 μM L^{-1} of inorganic nitrogen and 6.5 μM PO_4 L^{-1} at 50% algal initial density in our experiment. Thus, most probably by enhancing the CO_2 release via respiration, *S. m. heudelotii* increased algal growth to a level equivalent to that obtained in an optimized algal-production system using water mixing, and enrichment with dissolved inorganic carbon.

Efficient ammonia removal. In marine conditions, *Chlorella* quickly uptakes ammonium when exposed to ephemeral patches (Goldman and Dennett, 1985; Sunda and Hardison, 2007). Most ammonium excreted by *S. m. heudelotii* was removed in our systems. Such removal (4–5 mg N–NH_4 L^{-1} day^{-1}) fits within the upper range of uptake rates estimated for various photosynthetic suspended-growth systems (Hargreaves, 2006). This removal rate also matches the maximum rate estimated for the Partitioned Aquaculture System (PAS, 1.8–4.2 mg N L^{-1} day^{-1}; Brune et al., 2003), and the Belize Aquaculture Ltd. (BAL, 1.9–3.6 mg N L^{-1} day^{-1}; Burford et al., 2003) in tropical conditions.

Constancy of algal production. The net *Chlorella* production remained constant across algal initial densities, which resulted in constant nitrogen uptake (particularly, toxic NH_4 removal) and oxygenation by *Chlorella*. At dawn (07:00), the algal initial density had no influence on the dissolved oxygen concentration in presence of fed *Sarotherodon*. This characteristic is advantageous since it allows one to use a wide range of algal densities in rearing conditions. From an aquacultural engineering point of view, in a recirculating system under intensive rearing conditions, it is particularly advantageous to maximize the diel net production of algae (7×10^6 *Chlorella* cells mL^{-1} day^{-1} herein), while reducing their average equilibrium density down to a very low level (e.g., 10% of the routine *Chlorella* density in our experiment). The observed wide functional range of *Chlorella* densities represents a buffer against excessive algal densities, which may cause massive algal senescence, toxic conditions, and ultimately fish kill. Because of scale-transfer issues, the optimum range of *Chlorella* densities, taking into account *S. m. heudelotii* grazing, still remains to be assessed for the prototype.

Optimal control of Chorella *density*. In a recirculating system, *S. m. heudelotii* is not a good candidate for controlling *Chlorella* density about an equilibrium state. Indeed, *Chlorella* is too small to be efficiently grazed upon. In contrast, herbivorous zooplankton, and particularly rotifers might be the fittest herbivores for such system. Single-celled nano-algae represent an ideal food for rotifers, and rotifer parthenogenetic reproduction allows for fast functional response. In addition, rotifers are quality food for *S. m. heudelotii* fry and juveniles.

Optimizing the phytoplankton recycle pond. Stocking detritivorous tilapia in the recirculating ponds may not only control the development of microbial flocs, enhancing N-retention from added feed (reviews in Avnimelech, 2006, 2007; Crab et al., 2007), but above all prevent the prevalence of larger micro-algae that can provoke the collapse of the working recycle food web. Moreover, by enhancing CO_2 concentrations in the phytoplankton recycle pond, fish might favour advantageously green algae over cyanobacteria, as observed in the PAS in high CO_2 conditions (Drapcho and Brune, 2000). In ponds or recirculating systems at equilibrium, the role of fish may become important, not only via CO_2 release, but also via nutrient recycling, reduction in sedimentation, and bioturbation. Water mixing, due to fish swimming and foraging on settled organic matter, improves algal buoyancy and induces resuspension. Resuspension may contribute to maintain cells in the water column, in particular algae with relatively large settling velocity, as experimentally demonstrated by Roozen et al. (2007). In presence of fish, algae are kept in the upper layer, under improved light climate. This mechanism, in addition to nutrient resuspension, may contribute to enhance algal photosynthesis (e.g., Havens, 1991).

5. Conclusion

Using mutualism between *Sarotherodon* and nano-algae for phytoplankton mass production is promising for optimizing photosynthetic suspended-growth systems. This mutualism would also be valuable in wastewater treatment systems, for removing nutrients from effluents originating from aquacultural farms, municipal sewages, or agricultural drainage waters in semi-arid environments. In brackish warmwaters, we suggest that production of *S. m. heudelotii*, nanophytoplankton, and small herbivorous zooplankton could be combined in an integral recirculating system in order to take advantage of the fish-algae indirect mutualism. Such aquacultural activity is sustainable within the economic framework of tropical countries, where sunlight is intense all year long and water temperature is high and fairly constant. Moreover, such practice would be economically profitable owing to the triple production (*Sarotherodon, Chlorella,* herbivorous zooplankton), in addition to savings on water, feed, fertilizers, CO_2 enrichment, as well as on energy for water mixing and algae removal. Yet, additional, more full-scaled, studies are required.

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