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Chaetomorpha linum in the bioremediation of aquaculture wastewater: Optimization of nutrient removal efficiency at the laboratory scale

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ABSTRACT

Marine pollution from aquaculture wastewater is a widespread and increasing ecological problem. Algae, with their ability to remove surplus nutrients from wastewater, are a good tool for achieving more sustainable aquaculture. In this study, the capability of different biomasses of *Chaetomorpha linum* and *Cladophora prolifera* for the bioremediation of nutrient-rich (ammonium, nitrate and phosphate) seawater was compared. The results suggest that 10 g L^{-1} *C. linum* is an excellent candidate for aquaculture wastewater bioremediation. However, the bioremediation efficiency of *C. linum* was significantly affected by seasonality, with the greatest performance in nutrient removal exhibited by algae harvested in summer. *C. linum* harvested in winter and acclimated to lab conditions for two months, significantly improved the removal efficiency of both ammonium and nitrate, while worsening that of phosphate. Irrespective of season and acclimation, the simultaneous presence of ammonium and nitrate in seawater strongly inhibited nitrate removal. Thus, we propose the use of a two-step system, tested at the laboratory scale, in which nutrient-enriched seawater can pass through two different algal ponds. *C. linum* was able to achieve almost complete removal of ammonium in 24 h in the first step, while the second step improved both nitrate and phosphate removal efficiency. The two-step system is an effective innovation for the use of algae in bioremediation of aquaculture wastewaters.

1. Introduction

The rapid global expansion of aquaculture requires careful attention to wastewater treatment to avoid environmental damage and to ensure sustainable management of natural resources (Claudet and Fraschetti, 2010; FAO, 2018). Indeed, aquaculture wastewater can constitute a serious environmental hazard in marine habitats since it can effect changes in the physical-chemical characteristics of seawater, such as the reduction of dissolved oxygen and excess concentrations of nutrients, in turn causing alterations in both animal and plant communities (Claudet and Fraschetti, 2010). In marine environments, especially in those with low hydro-dynamism, increases in nutrients such as phosphorous and nitrogen can lead to diverse and often poorly understood and dangerous consequences, leading to eutrophication, with profound alterations of the delicate trophic balance (Mee, 2006; Chan et al., 2008; Li et al., 2010; Wallace et al., 2016). Excess nutrients in the Mediterranean basin have caused negative effects on *Posidonia oceanica* meadows, leading to reductions in leaf density and in the growth rates of rhizomes and to increases in death rates (Cancemi et al., 2003; Diaz-Almela et al., 2008). Thus, the mitigation of the environmental impact of nutrient-rich effluents from aquaculture farms is an important and urgent challenge (Chopin et al., 2001; Neori et al., 2004). The effectiveness of bioremediation of surplus nutrients in aquaculture wastewater has been widely demonstrated (Chung et al., 2002; Turcios and Papenbrock, 2014). Various studies emphasize the importance of integrated aquaculture, in which seaweeds, as photoautotrophic organisms, can counteract the environmental effects of heterotrophic fed-organisms and restore water quality (Chopin et al., 2001; Troell et al., 2003; Neori, 2008). The integration of macroalgae, good assimilators of nutrients, in aquaculture is an ecological and sustainable practice for the remediation of wastewater (Sivakumar et al., 2012). Moreover, the use of macroalgae for wastewater bioremediation offers the advantage of possible biomass recovery, since macroalgae are potential producers of substances useful in the pharmaceutical and cosmetic fields and are good candidates for production of fertilizers and biofuels (Zhou et al., 2010; Plaza et al., 2010; Shilton et al., 2012; Sivakumar et al., 2012; Bohutskyi et al., 2016; Ben Yahmed et al., 2016; Nhat et al., 2018).

The choice of algae to be utilized in aquaculture wastewater bioremediation requires careful discrimination since efficiency in nutri-

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ent removal may significantly change depending on the algal species and environmental conditions. An optimal candidate for aquaculture wastewater bioremediation should have rapid growth, permitting a high rate of nutrient absorption, have adaptability to different environmental conditions and be sourced from local communities to avoid the introduction of non-indigenous species (Kang et al., 2013; Sindelar et al., 2015; Ge and Champagne, 2017; Nhat et al., 2018).

Chaetomorpha linum (O.F. Muller) Kutzing and *Cladophora prolifera* (Roth) Kützing are two filamentous green algae with worldwide distributions (Guiry and Guiry, 2019). These two algal species are characterized by rapid growth, with the ability to completely cover a small basin in a short time, forming mats that can persist from a few months up to a year (Guiry and Guiry, 2019). *C. linum* and *C. prolifera* are highly tolerant to a variety of environmental stressors such as changes in salinity, temperature, light intensity, pH, dissolved oxygen and nutrient concentration (Gordon et al., 1980; Krause-Jensen et al., 1996, 1999; McGlathery et al., 1997, 1999; Taylor et al., 2001; Xu and Lin, 2008; Tsutsui et al., 2015; Sorce et al., 2018). These ecological features make both species particularly suitable for seawater bioremediation purposes as they have already been proposed for the remediation of heavy metals in polluted sites and for the removal of nitrogen and phosphorus in nutrient-rich seawater (Lapointe and Oconnell, 1989; Krause-Jensen et al., 1996; Pedersen and Borum, 1996; McGlathery et al., 1997; Menendez et al., 2002; Pierri et al., 2006; Taylor et al., 2001; Giangrande et al., 2007; Ajjabi and Chouba, 2009; Lee and Chang, 2011; Liu and Vyverman, 2015). However, despite considerable data in the literature, the nutrient removal efficiency of these two algal species cannot be accurately estimated and compared, principally due to the great diversity of experimental conditions assayed.

In this study, the nutrient removal efficiency of different densities of *Chaetomorpha linum* and *Cladophora prolifera* is compared under the same experimental conditions in order to select the most suitable species for bioremediation of nutrient-rich seawater. This comparison supported a density of 10 g L^{-1}*C. linum* as the best choice for nutrient removal. Based on this result, seasonal changes in the bioremediation capability of this alga have been studied. Finally, the optimal algal culture conditions to maximize nutrient removal efficiency have been investigated on a laboratory scale.

2. Materials and methods

2.1. Algae sampling and experimental design

Chaetomorpha linum (O.F. Müller) Kützing, 1845 (Chlorophyta, Ulvophyceae) and *Cladophora prolifera* (Roth) Kützing, 1843 (Chlorophyta, Ulvophyceae) are green algae with macroscopic siphonocladal thalli consisting of linear (*C. linum*) or branched (*C. prolifera*) uniseriated filaments. Both species were collected in the second inlet of the basin of Mar Piccolo, a semi-enclosed sea basin in the Taranto Gulf, southeastern Italy, during 8 sessions between July 2014 and July 2016. Within the basin, both species are very abundant and persistent all year (Cecere and Petrocelli, 2009). After the removal of epiphytes, algal thalli (Fig. S1) were rinsed briefly and grouped in 5, 10 and 20 g (fresh weight) samples. To avoid cell breakage and consequent physiological stress for the thalli, filaments were carefully gathered to reach the selected weight without cutting them. Algae were maintained in filtered seawater in glass jars with gentle aeration, without addition of nutrients, and under controlled environmental conditions (natural photoperiod and air temperature of 20–22 °C) for 3 to 5 days before the experiments (non-acclimated algae). On the other hand, acclimated algae, used in experiments 2 and 3, were maintained under the same culture conditions, without nutrient addition, for 60 days before the experiments.

2.1.1. Experiment 1: selection of the effective algal density and species

C. prolifera and *C. linum* were tested for their ability to remove nitrogen and phosphate in nutrient enriched seawater. To select effective species and optimal densities, 5, 10 and 20 g (fresh weight) of *C. prolifera* and *C. linum* were placed in 3 L glass jars filled with 1 L of filtered seawater under gentle aeration and subjected to two different treatments for 48 h: (A) ammonium + phosphate (10 mg L^{-1} NH_4Cl and 5 mg L^{-1} KH_2PO_4); (B) nitrate + phosphate (10 mg L^{-1} KNO_3 and 5 mg L^{-1} KH_2PO_4). The nutrient concentrations were selected as comparable to average levels detectable in aquaculture wastewaters and close to the maximum levels allowed by national law D.Lgs. 152/2006.

Algae (5, 10 and 20 g L^{-1}) maintained in filtered seawater without nutrient addition were used as a control. All treatments and controls were conducted in triplicate and each experiment was repeated twice, with algae harvested in July 2014 and June 2015.

Seawater samples (25 mL) were taken at the beginning of the experiments (0 h) and after 1, 4, 24 and 48 h to determine nutrient concentrations. Algae, weighed (fresh weight) at the end of the experiment, did not show significant changes in growth.

Chlorophyll content was measured in algae grown at 10 and 20 g L^{-1} densities for 24 and 48 h under control conditions (without the addition of nutrients) according to Garcıa-Sanchez et al. (2014).

2.1.2. Experiment 2: nutrient removal efficiency of Chaetomorpha linum collected in different seasons

C. linum, harvested in winter (December 2015 and January 2016), spring (April 2015 and May 2016) and summer (July 2015 and July 2016) was subjected to treatments (A) and (B) as described above, at a density of 10 g L^{-1}.

Since aquaculture wastewater may simultaneously contain nitrate and ammonium, acclimated and non-acclimated *C. linum* (10 g L^{-1}), harvested in winter and spring, were subjected to treatment with ammonium, nitrate and phosphate as follows: (C) ammonium + nitrate + phosphate (10 mg L^{-1} NH_4Cl, 10 mg L^{-1} KNO_3 and 5 mg L^{-1} KH_2PO_4). Each treatment was conducted in triplicate.

Seawater samples (25 mL) were taken at the beginning of the experiments (0 h) and after 1, 4, 24 and 48 h to determine nutrient concentrations.

2.1.3. Experiment 3: setup of the two-step system

Experiment 3 (two-step system) was conducted with acclimated spring algae and with non-acclimated winter algae, which have been previously shown to possess the highest and the lowest nutrient removal efficiencies, respectively.

In the two-step system, seawater enriched with ammonium, nitrate and phosphate, as reported in (C), was maintained for 24 h in a jar with *C. linum* at a density of 10 g L^{-1}. Afterwards, the seawater was moved to a second jar containing a new *C. linum* biomass (10 g L^{-1}) for an additional 48 h period. In addition, a one-step system was tested in which seawater enriched with the three nutrients, as reported in (C), was maintained in the presence of the same concentration of algal biomass for 72 h. Each treatment was conducted in triplicate.

Seawater samples (25 mL) were collected at the beginning of the experiments (0 h) and after 24, 48 and 72 h to determine nutrient concentrations. Algae, weighed (fresh weight) at the end of the experiment, did not exhibit significant changes in growth.

2.2. Nutrient removal efficiency and uptake rate

In all experiments, algal nutrient removal efficiency was calculated using the concentration of each nutrient in water samples collected after different incubation times:

$$\textit{Nutrient removal efficiency } (\%) = 100 - \left(\frac{100 * C_t}{C_0}\right)$$

where C_0 and C_t are nutrient concentrations (mg L^{-1}) at the beginning and end of the sampling period, respectively.

Nutrient uptake rate, expressed as μg per gram (FW) per hour, was calculated according to Xu et al. (2011):

$$\textit{Nutrient Uptake Rate} = \frac{\left[(C_0 - C_t) * V\right]}{m * \Delta t}$$

where C_0 and C_t are nutrient concentrations (mg L^{-1}) at the beginning and end of the sampling period, respectively, V is the incubation volume (L), m is the algae fresh weight (g) and Δt (h) is the incubation time at the time of sampling.

2.3. Determination of nitrate, ammonium and phosphate in seawater

Ammonium content was monitored with a pH meter (HI 5222, Hanna Instruments, Woonsocket, USA) equipped with an ammonium ion selective electrode (HI 4101, Hanna Instruments, Woonsocket, USA), calibrated in the range 0–10 mg L^{-1} according to the manufacturer's instructions and expressed as mg NH_3 L^{-1} (Neori et al., 2000; Sanz-Lazaro and Marin, 2006).

Nitrate content was determined spectrophotometrically with a Beckman DU 6400 spectrophotometer according to Carvalho et al. (1998) with minor modifications. Seawater samples (1.25 mL) were treated with 0.025 mL HCl and specific absorbance, measured at 220 nm, was adjusted by subtracting nonspecific absorbance at 275 nm due to the interference of organic compounds. Nitrate concentration was determined by referring to a standard curve with a range of 0–10 mg L^{-1} and expressed as mg NO_3^- L^{-1}.

Phosphate content was determined according to Strickland and Parsons (1972) by the spectrophotometric determination of a blue phosphomolybdic complex that specifically absorbs at 882 nm. Phosphate concentration was determined by referring to a standard curve with a range of 0–5 mg PO_4^{3-} L^{-1}.

2.4. Statistical analysis

All experimental data were computed as dependent variables using PERMANOVA in an approach similar to parametric ANOVA. Univariate PERMANOVA tests were run on Bray-Curtis similarity matrices with 9999 permutations (Anderson, 2001). Species (S, 2 levels), Biomass concentration (Biomass, 3 levels) and Time (T, 4 levels) factors were used to detect differences in removal efficiency and uptake rate in Species x Biomass interactions. Each interaction was individually analysed using Univariate PERMANOVA tests with the same experimental design. Season (Season, 4 levels) and Time (T, 4 levels) were used to detect seasonal changes in nutrient removal efficiency. If necessary, square root transformed data in a Bray–Curtis similarity matrix with 9999 permutations was used to perform the analyses (Anderson, 2001). If it was impossible to obtain enough permutations for PERMANOVA analysis, the reference p was obtained using a permutation simulation test (MONTECARLO test). The PAIR-WISE test was applied to discover statistically significant differences in each pair of factor levels based on the significance value of PERMANOVA/MONTECARLO tests. All analyses were conducted using PRIMER v6 + PERMANOVA software (Anderson et al., 2008).

3. Results

3.1. Efficiency of *Chaetomorpha linum* and *Cladophora prolifera* in the removal of excess nutrients from seawater

Three different densities (5, 10 and 20 g L^{-1}) of *C. prolifera* and *C. linum* were tested for their ability to remove excess nitrogen and phosphate from seawater (Figs. 1, 2).

When ammonium and phosphate ($NH_4^+ + PO_4^{3-}$) were added to seawater, both species were able to remove ammonium efficiently, proportionate to the biomass utilized (Fig. 1a; Table S1a). However, *C. linum* exhibited a significantly higher ammonium removal efficiency than *C. prolifera*, reaching at a density of 20 g L^{-1}, a 92% ammonium removal, after 48 h. The difference in in ammonium removal efficiency was confirmed by analysis of the uptake rate, which was significantly greater in *C. linum* (Fig. 1b; Table S1b) than in *C. prolifera,* both at 4 and 24 h and at all densities assayed. Ammonium removal by both species was very fast and the uptake rate decreased significantly with time; moreover, a lower biomass/volume ratio permitted a higher uptake rate of ammonium (Fig. 1b).

In the $NH_4^+ + PO_4^{3-}$ treatment both species exhibited a very different phosphate removal efficiency (Fig. 1c; Table S1c). At 10 and 20 g L^{-1} densities, *C. linum* was able to completely remove phosphate in 48 h. In the same incubation time, a phosphate removal efficiency of 58% was reached at the 5 g L^{-1} density. On the other hand, *C. prolifera* failed to efficiently remove phosphate, at the highest density (20 g L^{-1}) reaching a removal of only 20% after 48 h. The phosphate uptake rate, determined at 4 and 24 h, was significantly lower in *C. prolifera* than in *C. linum*. Moreover, phosphate removal by *C. linum* decreased very quickly over time. In contrast to the ammonium uptake rate, an increase in biomass/volume permitted a higher uptake rate of phosphate (Fig. 1d; Table S1d).

When nitrate and phosphate ($NO_3^- + PO_4^{3-}$) were added to seawater, a significant increase in nitrate removal efficiency occurred only after 24 h. For ammonium, the removal efficiency of *C. linum* was greater than that of *C. prolifera* (Fig. 2a; Table S2a). The uptake rate of nitrate was constant over time and significantly lower in *C. prolifera* than in *C. linum* (Fig. 2b; Table S2b). In the $NO_3^- + PO_4^{3-}$ treatment, *C linum* exhibited a high phosphate depletion capacity, increasing proportionally with biomass density. After 4 h, 8%, 27% and 48% of phosphate was taken up by 5, 10 and 20 g L-1 biomasses, respectively, and at 48 h, 10 and 20 g L^{-1} of *C linum* almost completely removed excess phosphate from seawater. On the other hand, *C. prolifera* at the highest density removed approximately 45% of excess phosphate within 48 h (Fig. 2c; Table S2c). The phosphate uptake rate for *C. prolifera* in the presence of nitrate was higher than that occurring in presence of ammonium (Fig. 1d; 2d).

Based on the comparative results, *C. linum* most efficiently removed nitrogen and phosphate and was therefore selected as the most suitable species for subsequent experiments on wastewater bioremediation. Although in some cases the best nutrient removal performance was observed at the highest densities, *C. linum* at 10 g L^{-1} was selected because, while maintaining good nitrogen and phosphate removal efficiency, this concentration avoided saturation conditions, safeguarding algal fitness. This was confirmed by the chlorophyll content, which was, after 48 h, higher in algae at a density of 10 g L^{-1} than in algae at a density of 20 g L^{-1} (Fig. S2).

3.2. Seasonal changes in the capability of *C. linum* for seawater bioremediation

To investigate the possibility of using *C. linum* in aquaculture wastewater bioremediation throughout the year, algae collected in differ-

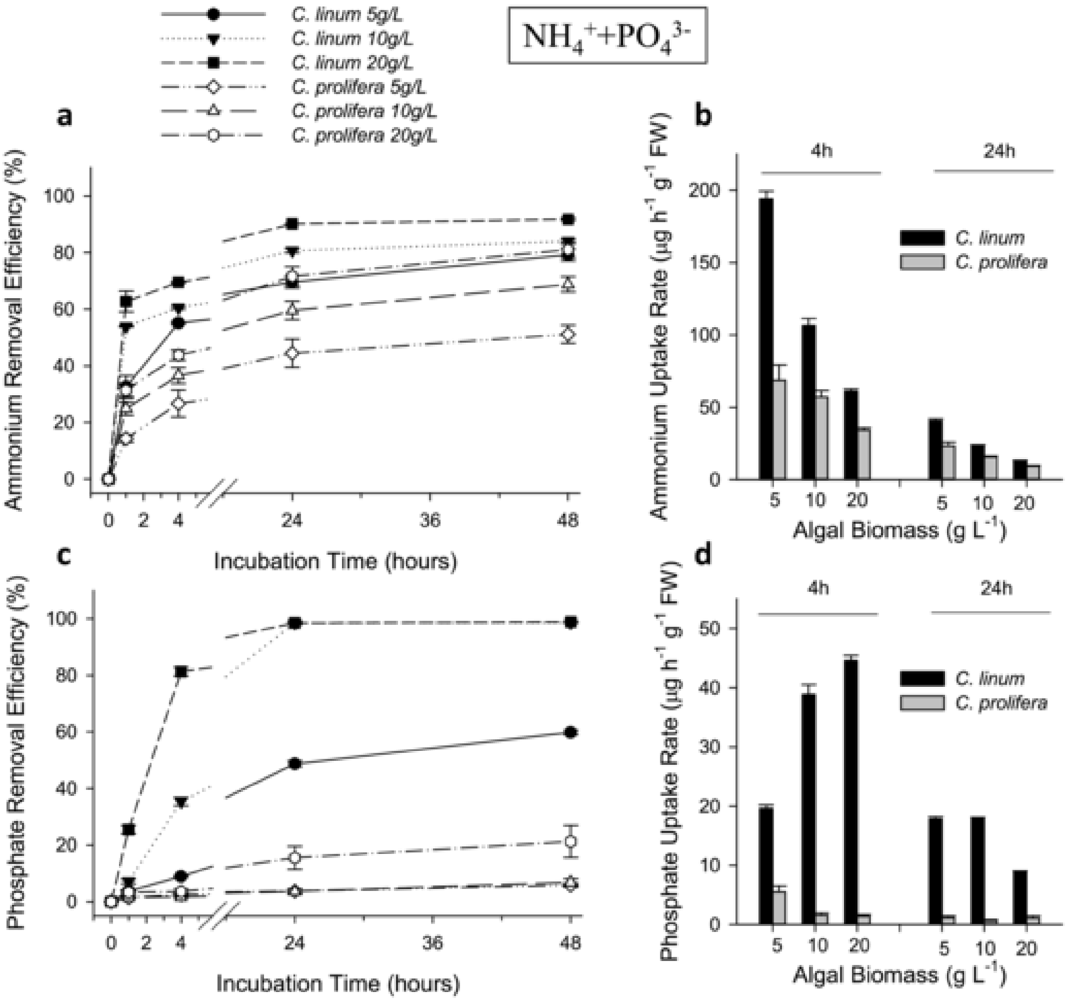

Fig. 1. Time-dependent removal efficiency (a, c) and uptake rate (b, d) of ammonium (a, b) and phosphate (c, d) by different biomasses of *C. linum* and *C. prolifera* in seawater enriched with 10 mg L^{-1} NH_4Cl and 5 mg L^{-1} KH_2PO_4. The values are the means of six different experiments ± SE. Statistical analyses are reported in Table S1.

ent seasons were subjected to treatment with ammonium and phosphate, and with nitrate and phosphate (Fig. 3).

When subjected to the ammonium/phosphate treatment, algae harvested during the summer had a significantly higher ammonium uptake capacity than those collected in spring and winter, removing almost all the ammonium excess (82%) in the first 24 h. On the other hand, *C. linum* harvested in winter and spring removed approximately 60% of excess ammonium within 48 h, without any significant difference between them. Interestingly, 24 h of acclimation permitted winter algae to reach the same performance levels for ammonium uptake as summer algae (Fig. 3a; Table S3a). In the ammonium/phosphate treatment, algae collected in summer and spring almost completely removed excess phosphate in the first 24 h, while winter algae took 48 h to achieve the same result. In contrast to the case of ammonium removal, phosphate uptake of winter algae was significantly lower after acclimation (Fig. 3b; Table S3b).

C. linum harvested in summer showed the highest efficiency in nitrate uptake during the nitrate/phosphate treatment, removing almost all excess nutrient in 48 h. Nitrate removal efficiency was very low in spring and winter algae, reaching 20% after 48 h. Acclimation of winter algae significantly improved its nitrate uptake capability, permitting a better performance than summer algae; indeed, acclimated winter algae were able to completely remove excess nitrate in the first 24 h of treatment (Fig. 3c; Table S3c). Seasonal changes in the phosphate removal efficiency of *C linum* in the nitrate/phosphate treatment was similar to that described for the ammonium/nitrate treatment, with good performance by summer and spring algae and a significant decrease in winter algae, especially when they were subjected to acclimation (Fig. 3d; Table S3d).

When algae were grown in seawater with the contemporaneous presence of all three nutrients, ammonium and phosphate removal efficiencies were not significantly different from those observed in the paired treatments previously described (Figs. 4a, b, 3a, c). Acclimation permitted a significant improvement in ammonium uptake (Fig. 4a; Table S4a) and a decline in phosphate removal (Fig. 4b; Table S4b) in both spring and winter algae. With ammonium, nitrate and phosphate present in the seawater, nitrate uptake efficiency was completely inhibited in winter and spring in non-acclimated algae. Acclimation improved nitrate uptake efficiency only in spring algae, which were able to remove approximately 40% of excess nitrate within 48 h.

3.3. Development of a two-step system for full aquaculture wastewater bioremediation

Considering the different rates of ammonium and nitrate uptake by *C. linum*, we tested a two-step system using acclimated spring algae and non-acclimated winter algae, which previously showed the highest and the lowest nutrient removal efficiency, respectively.

Acclimated spring algae did not show a different ammonium removal efficiency in one-step and two-step systems, since they were

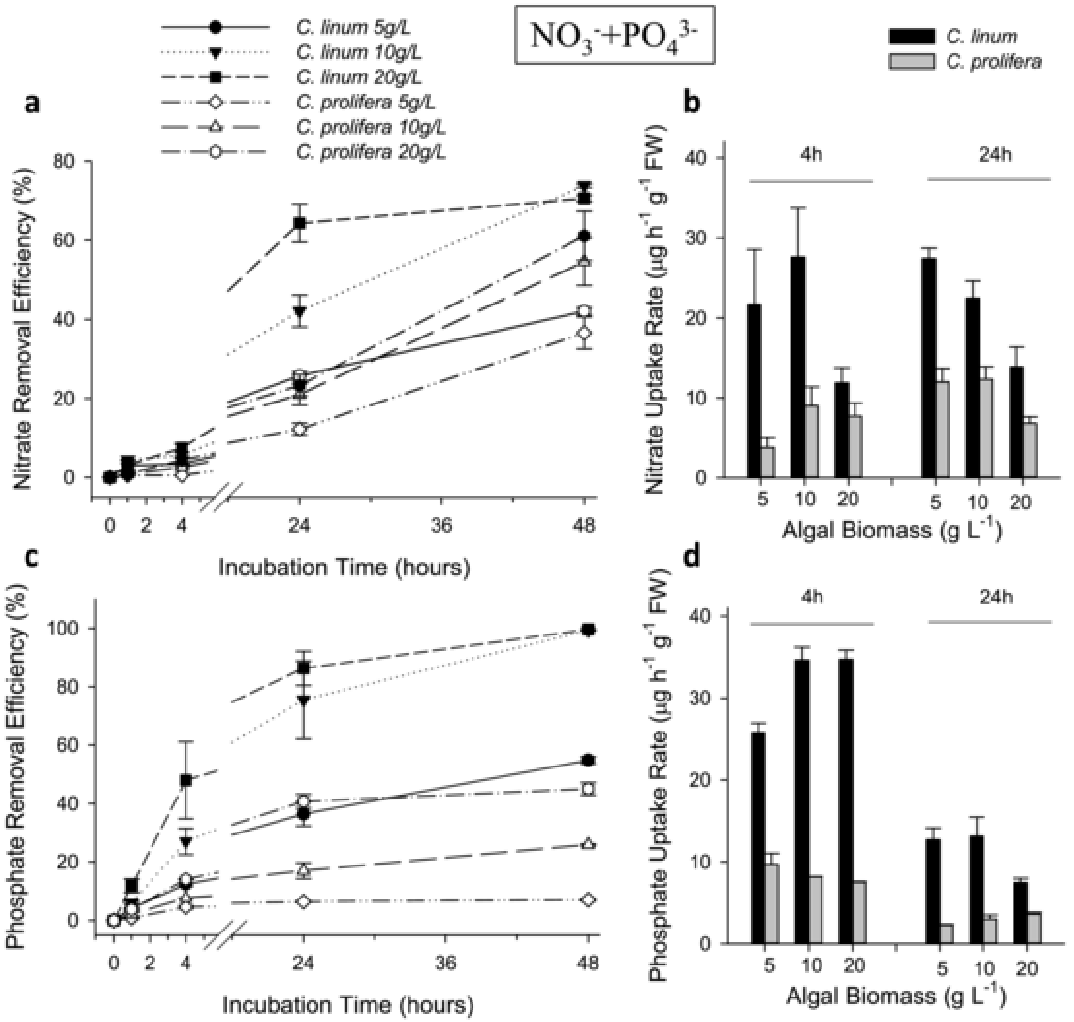

Fig. 2. Time-dependent removal efficiency (a, c) and uptake rate (b, d) of nitrate (a, b) and phosphate (c, d) by different biomasses of *C. linum* and *C. prolifera* in seawater enriched with 10 mg L^{-1} KNO_3 and 5 mg L^{-1} KH_2PO_4. The values are the means of six different experiments ± SE. Statistical analyses are reported in Table S2.

able to almost completely remove ammonium in the first 24 h. On the other hand, the two-step system improved ammonium uptake when winter algae were used, with removal efficiencies after 72 h of 72% and 85% in the one- and two-step systems, respectively (Fig. 5a; Table S5a). The two-step system slightly increased the nitrate uptake of winter algae: a 15% nitrate removal efficiency was observed after 72 h. The improvement in nitrate removal was very high when acclimated spring algae were used. While in the one-step system 48% of excess nitrate was removed in 72 h, the two-step system resulted in removal of almost all excess nitrate after 48 h (Fig. 5b; Table S5b). Interestingly, the two-step system also permitted an improvement in phosphate removal efficiency in both acclimated spring and winter algae. In the one-step system, after 72 h, winter and acclimated spring algae removed 58% and 87% of excess phosphate, respectively; on the other hand, in the two-step system both winter and spring acclimated algae showed a complete removal of phosphate in the first 48 h (Fig. 5c; Table S5c).

4. Discussion

The success of aquaculture wastewater bioremediation using algae strongly depends on the selection of specific algal species (Xu et al., 2011; Kang et al., 2013; Lawton et al., 2013; Anibal et al., 2014). *Chaetomorpha linum* and *Cladophora prolifera*, the two algal species that are the objects of this study, have previously been proposed as candidates for the removal of excess nutrients from seawater (Lapointe and Oconnell, 1989; Krause-Jensen et al., 1996; Pedersen and Borum, 1996; McGlathery et al., 1997; Krause-Jensen et al., 1999; Taylor et al., 2001; Giangrande et al., 2007). The results obtained in this study, using three algal experimental densities that were supplied with ammonium, nitrate and phosphate in the range of nutrient concentrations observed in aquaculture wastewater (Ghaly et al., 2005; Hayashi et al., 2008; Ross et al., 2018), permitted comparison of the nutrient removal efficiency of the two algal species and showed that *C. linum* has a higher bioremediation capability than *C. prolifera*. Both species increase ammonium and nitrate removal from seawater in proportion to the utilized biomass; however, with the same biomass the efficiency of *C. linum* is always higher compared with that of *C. prolifera*. In both the algal species, ammonium uptake was efficient and very fast, as shown by the decrease in the uptake rate with the time. Rapid intake of ammonium has been observed in various algal species, especially in species with annual development, which are not capable of long-term storage of this nutrient (Dy and Yap, 2001; Harrison and Hurd, 2001; Phillips and Hurd, 2003; Cohen and Fong, 2004; Liu et al., 2010; Kennison et al., 2011; Anibal et al., 2014; Ross et al., 2018). Nitrate removal efficiency differs significantly between the two algal species; indeed, 10 g L^{-1} of *C. linum* completely removes surplus nitrate after 48 h, while in the same time period, an equal biomass of *C. prolifera* eliminates only 55% of nitrate. In both species, the nitrate uptake rate was significantly lower than

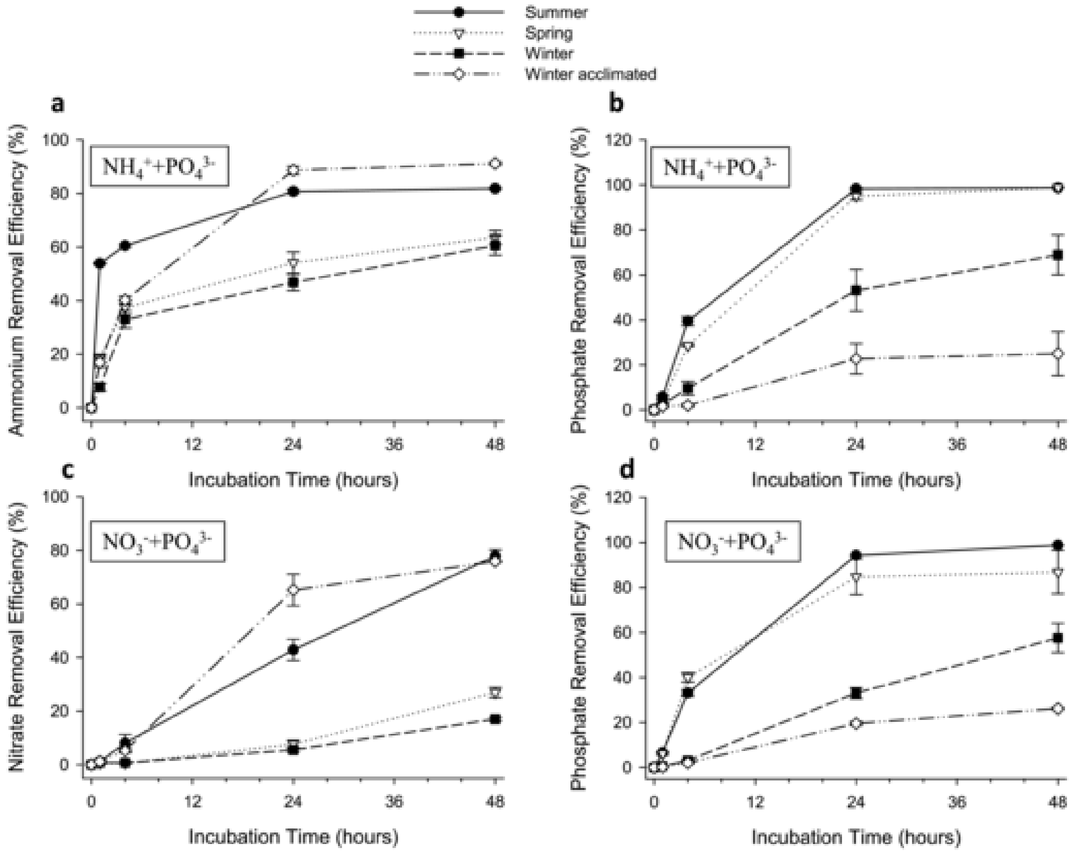

Fig. 3. Influence of seasonality and acclimation on nutrient removal efficiency by *C. linum* (10 g L^{-1}) Ammonium (a) and phosphate (b) removal efficiency in seawater enriched with 10 mg L^{-1} NH_4Cl and 5 mg L^{-1} KH_2PO_4. Nitrate (c) and phosphate (d) removal efficiency in seawater enriched with 10 mg L^{-1} KNO_3 and 5 mg L^{-1} KH_2PO_4. The values are the means of six different experiments ± SE. Statistical analyses are reported in Table S3.

that of ammonium. The higher uptake rate of ammonium compared to nitrate is a common feature in macroalgae (Hurd et al., 1995; Pedersen and Borum, 1996; Naldi and Wheeler, 2002; Runcie et al., 2003; Cohen and Fong, 2004; Teichberg et al., 2007; He et al., 2008; Liu et al., 2010; Luo et al., 2012; Lawton et al., 2013; Anibal et al., 2014). The difference in uptake rate between the two nitrogen species seems to be dependent on the different type of transport of the two nutrients (Campbell, 1999; Taylor and Rees, 1999; Harrison and Hurd, 2001), as well as on the energetic demand for nitrate reduction prior to amino acid incorporation (Harrison and Hurd, 2001; Jones et al., 2001; Teichberg et al., 2007; He et al., 2008; Liu et al., 2010; Luo et al., 2012).

Macroalgal capability for removal of phosphate from wastewater is variable but generally exhibits low efficiency (Garcia et al., 2000; El Hamouri, 2009; Shilton et al., 2012). The N/P ratio of wastewater can heavily influence nutrient uptake (Leonardos and Geider, 2004; Arbib et al., 2013; Perini and Bracken, 2014), and algae require more N than P, with an optimal N/P ratio varying in a wide range from 10:1 to 80:1 (Harrison and Hurd, 2001 and references therein). *Cladophora* sp. has been reported to have a good capacity to remove phosphate from wastewater with a low N/P ratio (Liu and Vyverman, 2015). However, in accordance with our results, the data reported by Liu and Vyverman (2015) show that in 48 h, *Cladophora* sp. removes approximately 15% of phosphate in wastewater with a N/P ratio of 2. Our data demonstrate that the phosphate removal capability of *C. linum* is significantly higher, with algal densities of 10 and 20 g L^{-1} able to remove excess phosphate excess from wastewater almost completely in 24 h in the presence of ammonium and in 48 h in the presence of nitrate. The high efficiency of phosphate removal by *C. linum* observed in our experimental conditions confirm that this species is capable of high P uptake (Lavery and McComb, 1991; Menendez et al., 2002). The high capability of *C. linum* to remove both nitrogen and phosphorus has also been demonstrated in municipal wastewater treatment, in which nitrogen and phosphorus removal efficiencies are approximately 87% and 93%, respectively (Ge and Champagne, 2017).

Data obtained in this work allowed us to select 10 g L^{-1}*C. linum* as an excellent candidate for aquaculture wastewater bioremediation. Indeed, although 20 g L^{-1} in some cases showed a higher nutrient removal efficiency, in bioremediation, it is desirable to use the smallest biomass that can ensure good nutrient removal efficiency and avoid competition for nutrients and light within the mats, which may be limiting for algal growth and health (Krause-Jensen et al., 1996; McGlathery et al., 1997; McGlathery and Pedersen, 1999; Krause-Jensen et al., 1999). Our results consistently show a lower chlorophyll content in *C. linum* grown at 20 g L^{-1} density compared to that grown at 10 g L^{-1}.

Changes in environmental conditions, such as light, temperature, salinity and nutrient availability, which are season-dependent, can heavily influence not only growth but also nutrient removal efficiency of macroalgae (Lavery and McComb, 1991; Pedersen and Borum, 1996; Harrison and Hurd, 2001; Taylor et al., 2001; Raven and Taylor, 2003; Marinho-Soriano et al., 2009; de Paula Silva et al., 2012; Sorce et al., 2018; Gao et al., 2018). Our results show that the bioremediation efficiency of *C. linum* is significantly affected by seasonality and algae harvested in the summer has the highest performance in nutrient removal. Consistent with data reported for other macroalgae species (Harrison and Hurd, 2001; Phillips and Hurd, 2003; Taheri and Shariati, 2014), a decrease in removal efficiency of nitrogenous species, with higher rates for nitrate than for ammonium, has been observed in *C. linum* harvested in spring and win-

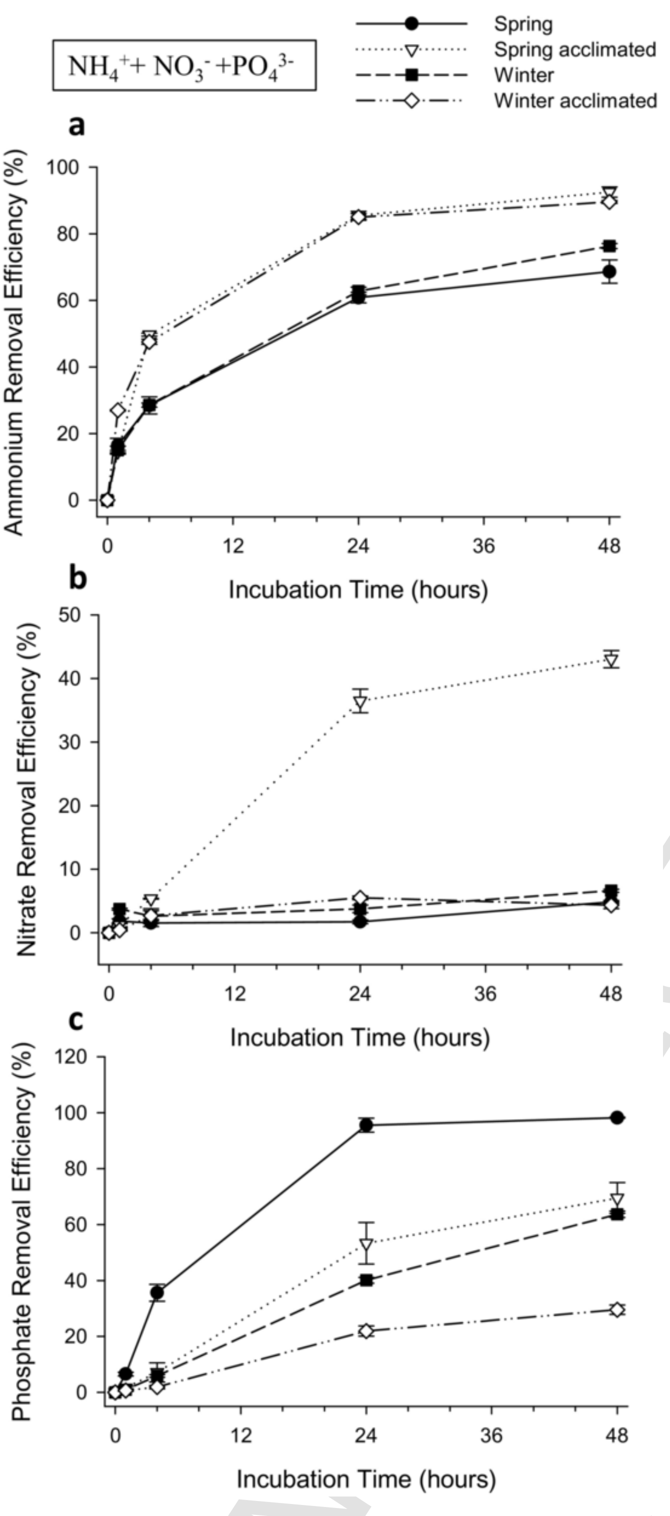

Fig. 4. Inhibitory effect of ammonium on nitrate removal efficiency by *C. linum* (10 g L^{-1}). Ammonium (a), nitrate (b) and phosphate (c) removal efficiency of spring and winter *C. linum*, acclimated and not, in seawater enriched with 10 mg L^{-1} NH_4Cl, 10 mg L^{-1} KNO_3 and 5 mg L^{-1} KH_2PO_4. The values are the means of six different experiments ± SE. Statistical analyses are reported in Table S4.

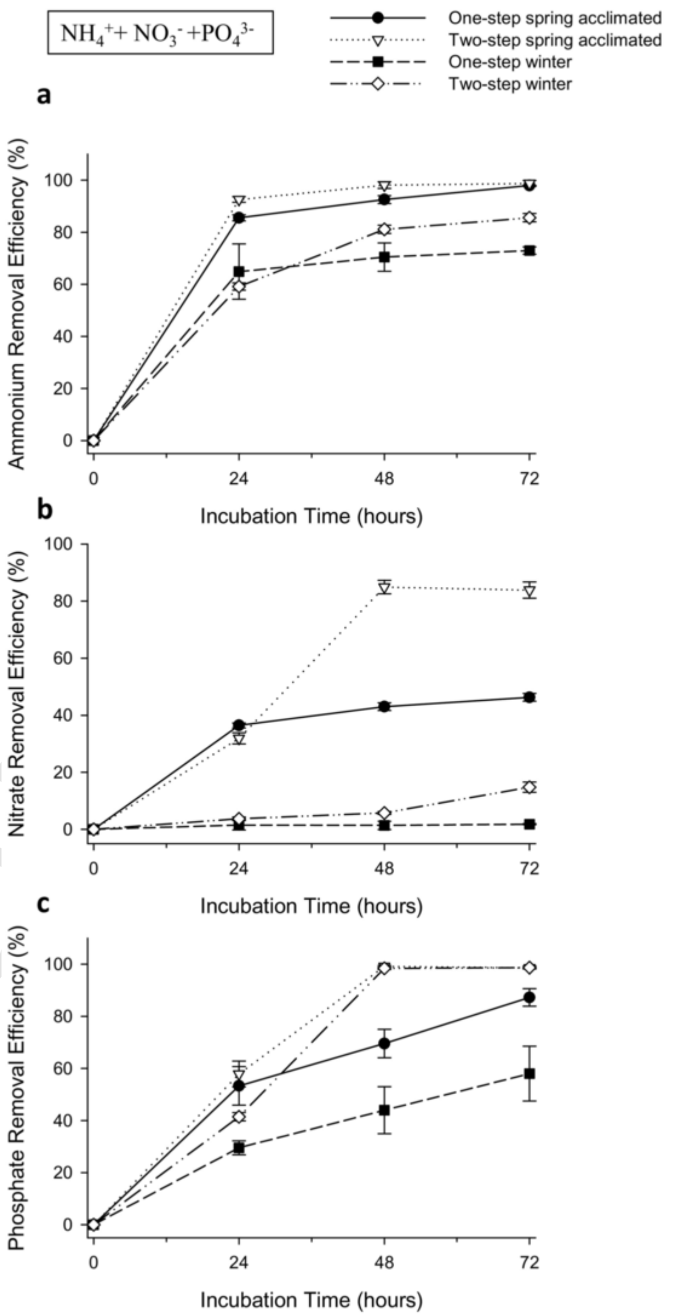

Fig. 5. Improvement of nitrate and phosphate removal by the two-step system. Ammonium (a), nitrate (b) and phosphate (c) removal efficiency by spring acclimated and winter *C. linum* in seawater enriched with 10 mg L^{-1} NH_4Cl, 10 mg L^{-1} KNO_3 and 5 mg L^{-1} KH_2PO_4 in a single jar (one-step) and with transfer to a second jar after 24 h (two-step). The values are the means of six different experiments ± SE. Statistical analyses are reported in Table S5.

ter. The higher inhibition of nitrate removal efficiency can be due to the light-dependent uptake of this molecule, which occurs at maximum rates in the summertime when light is not a limiting parameter (Harrison and Hurd, 2001). A higher nitrate removal efficiency in summer than in other seasons, not directly dependent on temperature, has been recently reported for *Chaetomorpha crassa* (Gao et al., 2018).

Interestingly, *C. linum* harvested in winter and acclimated to lab conditions for two months has a significantly improved ability to remove both ammonium and nitrate. Previous studies show that acclimation of *C. linum* as well as other species such as *Ulva* spp. in seawater with low nitrogen concentrations significantly improves the removal efficiency of nitrogenous species (Lavery and McComb, 1991; Krause-Jensen et al., 1999; Menendez et al., 2002; Menendez, 2005; Kennison et al., 2011). *C. linum* and other Ulvophyceae are able, for a limited period, to sustain their growth in seawater using only their reserves stored at the intracellular level (McGlathery et al., 1996; Taylor et al., 2001). After acclimation, the improvement in nutrient removal efficiency, reaching an uptake rate higher than that normally required for growth, would allow algae to cope with these previous deficiencies (Harrison and Hurd, 2001). However, acclimation of *C. linum*, while improving nitrogen uptake, significantly worsens phosphate removal efficiency. Therefore, for an effective use of *C. linum* in the bioremediation of aquaculture wastewater, it is necessary to consider the influence of seasonality and to evaluate the opportunity to subject the algal biomass to a period of acclimation, according to the presence and the relative concentration of the different nutrients that must be removed.

The data presented in this work show that, irrespective of season and acclimation, when ammonium and nitrate are simultaneously present in seawater, the nitrate removal by *C. linum* is strongly inhibited. Suppression of nitrate uptake in the presence of ammonium has been reported for several algal species (Raven et al., 1992; Navarro-Angulo and Robledo, 1999; Carmona et al., 2006; Ross et al., 2018) and could be a serious problem for the use of algae in the bioremediation of aquaculture wastewater. The use of biofilters with denitrifying bacteria, which favour the oxidation of ammonium to nitrate, has been proposed as a possible solution to permit subsequent seawater bioremediation by algae (de Paula Silva et al., 2012). As an alternative, we propose the use of a two-step system for the complete removal of excess nutrients from seawater. In the two-step system nutrient-enriched seawater passes through two different algal ponds to eliminate ammonium in the first and nitrate in the second. *C. linum* can remove ammonium almost completely in 24 h in the first step and efficiently remove nitrate in the second step, at the same time improving phosphate removal efficiency.

5. Conclusions

The results obtained in this work demonstrate that *C. linum* more efficiently removes nitrogen and phosphorus from seawater than *C. prolifera*, suggesting that *C. linum* at a density of 10 g L^{-1} is an excellent candidate for aquaculture wastewater bioremediation. Although the bioremediation efficiency of *C. linum* is significantly affected by seasonality, with a maximum nutrient removal capability in summer growing algae, acclimation in laboratory conditions can significantly improve nitrogen uptake. Suppression of nitrate uptake in the presence of ammonium, which occurs irrespective of season and acclimation, can be avoided by the use of a two-step system. In this system the flow of nutrient-enriched seawater through two algal ponds improves nitrate and phosphate removal efficiency. The two-step system represents an innovative process to improve the use of algae in the bioremediation of aquaculture wastewater.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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