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AMMONIUM UPTAKE OF *CLADOPHORA PROLIFERA* (CHLOROPHYTA, CLADOPHORALES) A CANDIDATE SPECIES FOR BIOREMEDIATION OF AQUACULTURE WASTES

RIASSUNTO

Vengono riportati alcuni esperimenti di laboratorio condotti per stimare la capacità di riduzione dei rifiuti azotati (azoto ammoniacale) da parte dell'alga verde *Cladophora prolifera* (Roth) Kützing, individuata quale specie estrattiva di acque reflue provenienti da impianti di acquacoltura.

Nel corso di questo lavoro sono stati condotti tre differenti esperimenti, il primo, preliminare, è stato condotto per verificare la sopravvivenza dell'alga in acque ricche di metaboliti azotati, nonché per ottenere una prima stima sui tempi e sull'efficienza di abbattimento.

Per ogni esperimento sono stati allestiti tre acquari per il trattamento e tre di controllo. La durata dei due esperimenti è stata di 240 minuti ed ogni 30 minuti sono state prelevate da ogni acquario aliquote di acqua per le analisi successive. L'abbattimento dell'ammoniaca è stato valutato come variazione della concentrazione fra due intervalli di tempo successivi, normalizzati rispetto ai controlli e rapportati ad un grammo di peso secco dell'alga.

I risultati hanno mostrato che l'alga è stata in grado di abbattere gran parte del carico ammoniacale già nella prima ora di attività (rimozione del 78% di ammoniaca) e che ad elevate concentrazioni di residui azotati, corrispondevano alti rendimenti nell'abbattimento, sottolineando un rapporto diretto fra quantità di ammoniaca ed efficienza estrattiva dell'alga.

L'uptake medio è risultato di 1.27±0.003 μmol N/g dw h^{-1} a bassa concentrazione e di 15.6±0.01 μmol N/g (dw) h^{-1} ad alta concentrazione. Questi valori sono simili a quanto riportato in letteratura per alghe già comunemente utilizzate come organismi biorimediatori in acquacoltura.

SUMMARY

The green seaweed *Cladophora prolifera* (Roth) Kützing, proposed for the treatment of waste deriving from aquaculture systems, was tested in laboratory experiments for ammonium uptake. Three experiments were conducted in different conditions. The preliminary one was performed using waste-water from a fish farm with ammonium concentration of 12 μM, in order to test viability and time efficiency of this species in removing the ammonium from the wastes. The other two experiments were conducted at both lower and higher ammonium concentrations, in order to obtain the ammonium uptake in different conditions. The ammonium uptake was very similar to that observed in literature for red seaweeds already utilized for similar purposes. When exposed to a relatively high ammonium concentration (34 μM), an overall high uptake was observed with the maximum decrease of ammonium in the water occurring during the first hour of experiment. In these conditions an uptake efficiency of about 78% was found. The mean uptake rate was of 1.27±0.003 μmol N/g (dw) h^{-1} and 15.6±0.01 μmol N/g dw h^{-1} at low concentration (2 μM) and high concentration (34 μM), respectively.

INTRODUCTION

Cladophora prolifera (Roth) Kützing, (Chlorophyta, Cladophorales), was recently considered in a large bioremediation project for ammonium reduction from the water column in an integrated aquaculture system of an area located at Porto Cesareo (North Ionian Sea, South Eastern Italy) (Giangrande *et al.*, 2003).

Among seaweeds, many species are successfully utilized to remove nutrients from aquaculture effluent waters (Van Rijn, 1996; Troell *et al.,* 1997; Hernandez *et al.*, 2002; Msuya and Neori, 2002), so that the yearly biomass produced as a by-product can feasibly be taken away from the system and utilized, resulting in both environmental and economic advantages.

Seaweeds are commonly used as sources of chemical and biological products such as food, fertilizer and the production of phycocolloids, being rich in important elements (Fujiwara-Arasaki *et al.*, 1984; Nisizawa *et al.*, 1987; Arieli *et al.*, 1993; Jurkovic *et al.*, 1995; Fleurence, 1999; Serfor-Armah *et al.*, 1999). The utilization of *C. prolifera* as fodder, was first proposed by Parenzan (1970), who observed an abundant population of floating mats in the Porto Cesareo bay. From a nutritional point of view, the protein and oligo-elements content of the alga is very high (about 25 %) (Bonotto *et al.*, 1987), especially when compared to values obtained from other green algae, utilized by the food industry (Fleurence, 1999), confirming the usefulness of its biomass as fodder. Moreover, a positive test on its utilization as fertilizer in agriculture was made by Cavallo *et al.*, 2006.

Our attention focused on this green macroalga because of its morphological and physiological features making it potentially useful for waste treatment: the species seems to have high versatility in growing and accumulating phosphorus in extreme trophic condition, but also in tolerating anaerobic and unfavourable conditions (BACH and JOSSELYN, 1978; 1979; SCHRAMM and BOOTH, 1981; BIRCH *et al.*, 1983). It grows in compact ramified cushions, which can give it a competitive advantage, improving nutrient uptake efficiency because of higher surface-volume ratio compared to less branched species. Finally the unattached thalli of this species, makes it comparatively easy to harvest.

Cladophora prolifera is an Indo-Atlantic species colonizing eutrophic environments, widespread within Mediterranean as well and recently observed at Bermuda Islands inland salt waters, where a mass development was attributed to the increased load of nutrients (BACH and JOSSELYN, 1979).

Cladophora prolifera have been well studied concerning phosphorus uptake and its accumulation, productivity, biomass, and growth (BACH and JOSSELYN, 1978; 1979, SCHRAMM and BOOTH, 1981; BIRCH *et al.*, 1983; LAPOINTE and O'CONNEL, 1989). However, no experiments have been conducted up to now to test its ability in utilizing the ammonium, the preferred N source for the growth of macroalgae (DY and YAP, 2001).

The ammonium is also the main dissolved nitrogen compound excreted by fish, and the capability to bio-filter this compound is one of the main aim in the bioremediation research. In the present paper we evaluated the ammonium uptake of the seaweed in laboratory experiments.

MATERIAL AND METHODS

Fresh healthy thalli of *Cladophora prolifera* were collected at Porto Cesareo using a handle net, in July 2002. Samples were transported to the laboratory where epiphytes were removed by successive washing with distilled seawater and rapid exposure to fresh water.

Three types of experiments were conducted at 24°C with a light intensity of 400 μE $m^2 s^{-1}$.

In a preliminary experiment we evaluated *C. prolifera* survival in wastewater from a fish farm with an ammonium concentration of 12 μM, obtaining first data on its efficiency in ammonium removal. This experiment lasted 27 hours, samples were collected after the first 3 hours and subsequently every 12 hours.

This experiment was conducted using six aerated aquaria with a capacity of 18 l. Three aquaria without alga acted as controls (C1, C2, C3) and 120 g of *C. prolifera* were introduced in each of the remaining aquaria (T1, T2, T3). The water used for the experiment was collected in the fish farm OROVIVO of Brindisi (South Eastern Italy, Adriatic Sea), sea-bass and sea-bream producing. Water samples (50 ml) were collected from each aquarium (controls and treatments) for ammonium measurement.

Difference between controls and treatments was tested using the analysis of variance.

According to the results of the first experiment, the whole sampling interval for the following experiments was reduced to 4 h, with triplicate water samples for ammonium measurement (50 ml) each 30 minutes.

These experiments were carried out at two different initial ammonium concentration: filtered seawater with lower (2 μM) and enriched (34 μM) ammonium concentration.

Triplicate transparent beakers were prepared using 1 litre of water and 5 g of alga for each of them.

Ammonium uptake was calculated from changes in ammonium concentration during each sampling interval, normalized to 1 g dw of alga. The mean ammonium uptake and standard error were calculated at each time, and time series curves evaluated using the analysis of variance. Uptake rate (U) was calculated from changes in NH_4^+ concentration at each sampling interval, utilizing the formula: $U= (S_0*V_0) - (S_t*V_t)/(t*B)$ by PEDERSEN (1994), where S_0 is the initial concentration, V_0 is the initial water volume, S_t is the NH_4^+ concentration and V_t is the water volume at the end of the sampling interval, t is the time elapsed between two successive intervals, and B is the dry weight of the biomass. Differences between uptake rates, relative to both experiments, were tested using the analysis of variance

In all the experiments samples were filtered with Wathman GF/F glass-filter previously treated in a furnace (450° C, 4 h). After filtering, all samples were utilized for ammonium determination with standard methods.

RESULTS

In the preliminary experiment (Fig 1a), after 3 h a significant decrease in ammonium concentration was observed in the treatments, dropping from 12 ± 0.7 μM to 2.5 ± 1.1 μM. After 15 hours it reached a value close to 0. By contrast, the ammonium concentration in the controls decreased from 12 ± 0.2 μM to 10 ± 3.2 μM only after 15 h. Differences between the controls and treatments were significant ($P<0,05$).

A similar ammonium concentration trend was observed in the other two experiments (Fig 1b). In the experiment where the concentration was initially lower (2 μM) a decrease in concentration to 0.68 ± 0.2 μM was observed after only 30 minutes, after which it remained constant throughout the experiment. In the experiment with initial high ammonium concentration (34.10 μM), the decrease was more gradual, reaching 9.85 ± 1.2 μM after 90 minutes and then remaining almost constant until the end of the experiment.

In the low concentration experiment an ammonium uptake value of 1.68 μM/g dw was achieved after 30 min after which no significant changes were observed (Fig 2b). In the high concentration experiment, the uptake was 31.7 μM/g dw after 90 min.

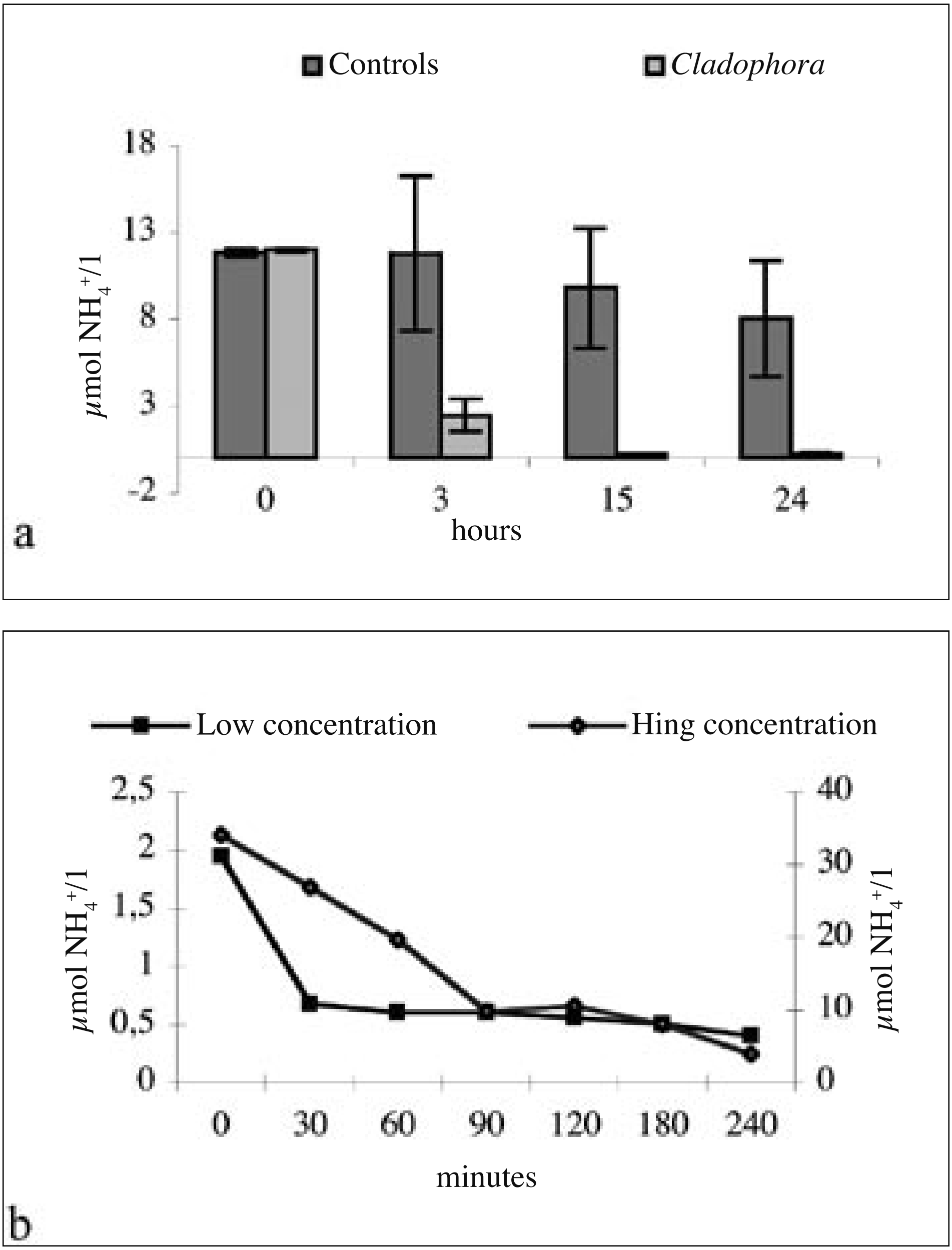

Fig. 1 - *Cladophora prolifera* ammonium depletion:

a) Trend in ammonium concentration during 27 hours of experiment; b) trend in ammonium concentration during 4 hours at different condition of initial concentration.

The values of the high concentration experiment were consistently higher than those observed in the low concentration experiment. This is best enhanced when uptake rate was computed at the different time intervals (Fig 2a). Moreover the maximum uptake rate was observed after 30 min (0.06 μM/g dw), followed by a decrease, conversely, an increase in uptake rate was observed in the second experiment from 0 to 90 min; at this time interval the uptake rate was 0.34 μM/g dw. Afterwards it decreased. Changes in time in the rate of ammonium uptake were significant only in the experiment conducted at the highest concentration ($P< 0,005$). Differences between ammonium uptake at different concentrations were significant ($P< 0,005$).

The mean uptake was 0.31± 0.002 μM/g dw at low concentrations, with an uptake efficiency of about 12%, and 27.33± 0.007 μM/g dw at high concentrations, with an uptake efficiency of about 78%. The mean uptake rate was 1.27± 0.002 μM/g dw/h at low concentrations, and 15.6 ± 0.03 μM/g (dw)h^{-1} at high concentrations.

DISCUSSION AND CONCLUSION

The results of the preliminary experiment showed a significant difference between treatments and controls, testing the algal viability in wastewater. In subsequent experimentation we observed that maximum depletion of ammonium levels occurred when the alga is exposed to a higher ammonium concentration. This is in accordance with literature data dealing with other species of macroalgae already utilized as biofilters in aquaculture (Dy and Yap, 2001; Ik Kyo Chung, 2003).

Cladophora prolifera does not seem to affect ammonium concentrations below 1 μM. In our low concentration experiment we observed that the values remained constantly below 1μM. The analysis of ammonium uptake confirmed these observations, with values stabilizing after the first sampling time. Thus post-stabilization time is not a statistically significant variable. In our high-concentration experiment, a relatively high reduction of ammonium was observed in the first 90 min. At this time the concentration was recorded as 10 μM. A change in concentration to 2.5 μM was recorded after 3 h. A similar trend was observed in our preliminary experiment, when after 3 h the concentration decreased from 12 μM to 2.5 μM.

The trend of the uptake relative to the high concentration experiment confirms a high increase until 90 minutes, followed by a slight increase until the end of the experiment.

Our conclusion is that the optimal ammonium uptake for *C. prolifera* occurs at higher ammonium concentration levels. Ammonium reduction under our high concentration conditions (32 μM) takes about 4 h. Most of the depletion, however, occurs during the initial 90 min, with a maximum rate of reduction at this time, and a lower reduction during the other 3 h. Furthermore, in comparison to the previously described low concentration experiment, the initial uptake rate was significantly higher.

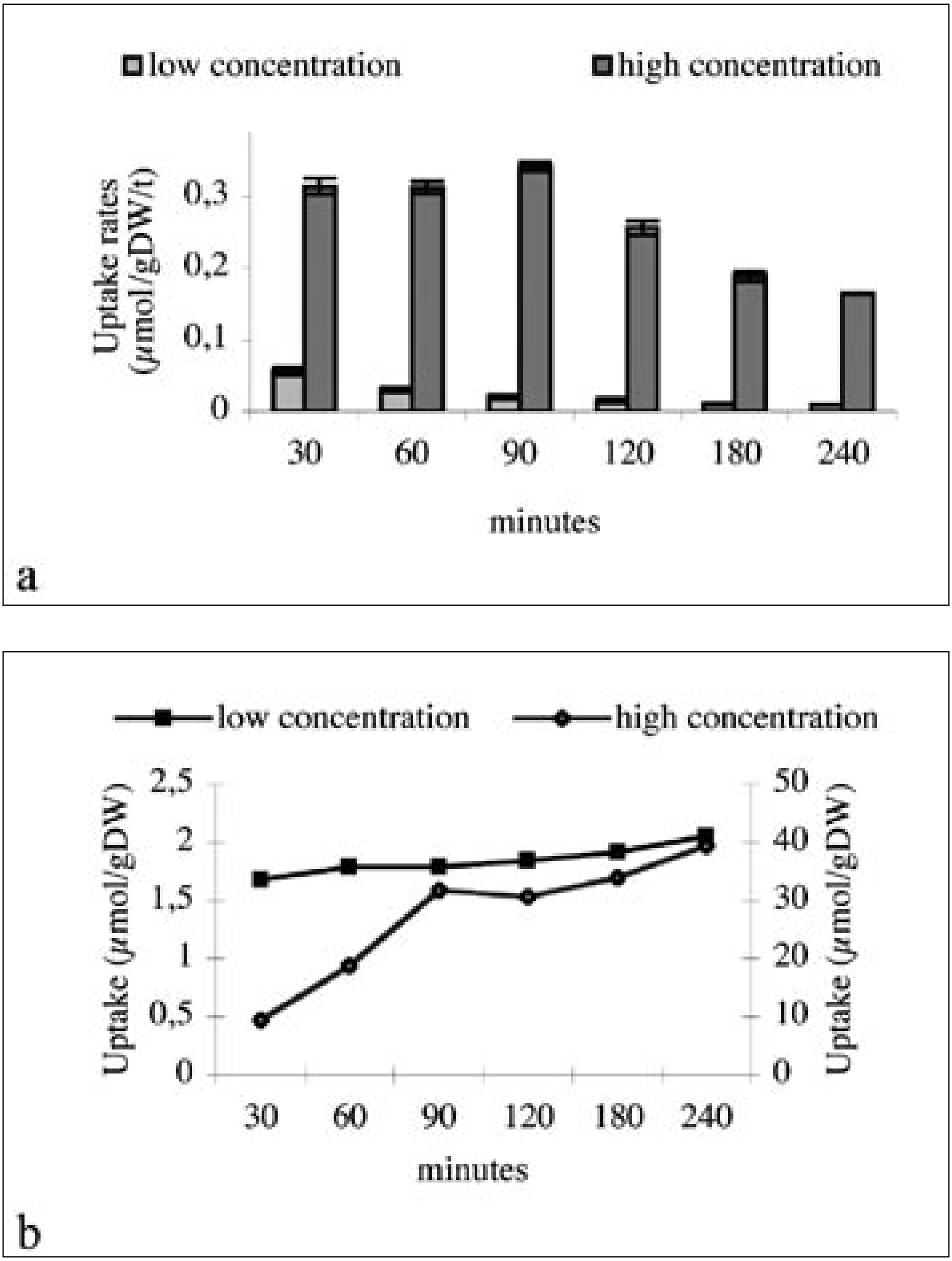

Fig. 2 - Ammonium uptake of *Cladophora prolifera*: a) comparison between uptake rates of the two experiments; b) uptake with low initial concentration and high initial concentration.

Uptake rate was always significantly higher in the experiment conducted at high concentration, from which an uptake efficiency of about 78% can be extrapolated.

A mean uptake rate of 1.27± 0.003 μmol N /g (dw) h^{-1} at low concentration and 15.6 ± 0.01 μmol N/g (dw) h^{-1} at high concentration was computed.

The mean uptake value obtained at high ammonium concentration appears similar to that observed for other macroalgae commonly utilized in waste treatment. However, it must be stressed that value comparison was possible only with those species which were investigated with comparable methods such as the Rhodophycea *Kappaphycus alvarezii*, (Dy and Yap, 2001), *Porphyra yezoensis*, and the Chlorophycea *Ulva* sp. (as *Enteromorpha* sp.) (Ik Kyo Chung, 2003).

Most of the recent papers concerning laboratory experiments in still water, in fact, deal with the use of isotopes for ammonium uptake investigation (O'Brien and Wheeler, 1987; Fujita *et al.*, 1988); Other papers refer to field investigations and only give data for filtration efficiency (Hernàndez *et al.*, 2002; Troell *et al.*, 2003) or for total nitrogen removal (Msuya and Neori, 2002).

Currently, no data about ammonium uptake were available on *C. prolifera*, and nothing is known about the ammonium accumulation in its thalli. The storage of accumulated ammonium in the thalli of some cultured macroalgae, which were observed to remove more nitrogen than they need, is suggested by Msuya and Neori (2002). The high efficiency as fertilizer of *C. prolifera* (Cavallo *et al.*, 2006) can suggest ammonium accumulation in the thalli, the analysis of which is still under investigation in our laboratory.

In a previous study Schramm and Booth (1981), showed the capacity of *C. prolifera* to accumulate phosphorus in the thalli and at the same time to growth in extremely oligotrophic conditions; enhancing phosphorous uptake and productivity even at high phosphorous concentration as occurs only as peak values in urban waste waters. These Authors pointed out that these features coupled with high tolerance to unfavourable conditions and the comparative ease of harvesting could favour the use of this alga for sea waste treatment and nutrient recovery. Present observations on ammonium uptake strengthen this opinion.

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