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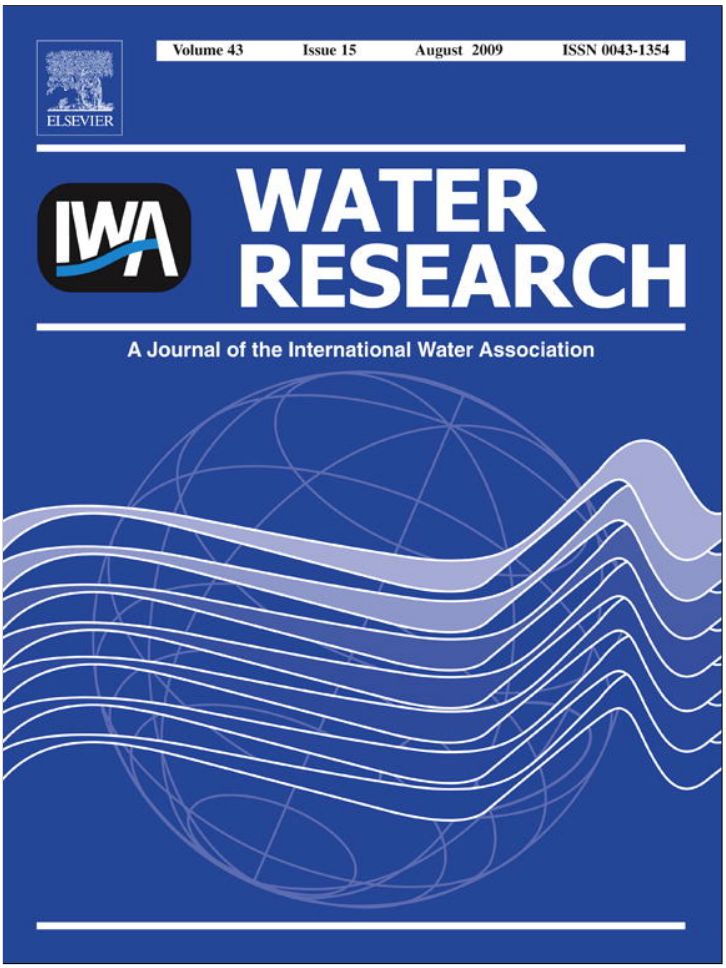

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Azolla–Anabaena's behaviour in urban wastewater and artificial media – Influence of combined nitrogen

M.L. Costa[a,1], M.C.R. Santos[b,2], F. Carrapiço[c,*], A.L. Pereira[c,3]

[a]Departamento de Ciências Exactas e do Ambiente, CERNAS, Escola Superior Agrária de Coimbra, I.P.C., Bencanta, 3040-316 Coimbra, Portugal

[b]Departamento de Ciências e Engenharia de Ambiente, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Caparica, Portugal

[c]Universidade de Lisboa, Faculdade de Ciências, Departamento de Biologia Vegetal, Centro de Biologia Ambiental, Bloco C2, Campo Grande, 1749-016 Lisboa, Portugal

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ABSTRACT

The results of using the nitrogen fixing symbiotic system *Azolla–Anabaena* to improve the quality of treated urban wastewater, particularly on what concerns phosphorus removal efficiencies (40–65%), obtained in continuous assays performed during the past few years and presented earlier, were very promising. Nevertheless, the presence of combined nitrogen in some wastewaters can compromise the treatment efficiency. The main goal of this work was to compare plants behaviour in wastewater and in mineral media with and without added nitrogen.

Azolla filiculoides's specific growth rates in wastewater and in mineral media without added nitrogen or with low nitrate concentration were very similar (0.122 d^{-1}–0.126 d^{-1}), but decreased in the presence of ammonium (0.100 d^{-1}). The orthophosphate removal rate coefficients were similar in all the growth media (0.210 d^{-1}–0.232 d^{-1}), but ammonium removal rate coefficient in wastewater was higher (0.117 d^{-1}) than in mineral medium using that source of nitrogen (0.077 d^{-1}).

The ammonium present in wastewater, despite its high concentration (34 mg N L^{-1}), didn't seem to inhibit growth and nitrogen fixation, however, in mineral media, ammonium (40 mg N L^{-1}) was found to induce, respectively, 18% and 46% of inhibition.

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1. Introduction

The use of different floating macrophytes for the removal of nutrients in wastewater treatment, particularly *Eichhornia crassipes* (Metcalf and Eddy, Inc., 1995; Crites and Tchobanoglous, 1998), duckweeds (*Lemna* spp., *Spirodela* spp., *Wolffia* spp.; Reddy and DeBusk, 1985; Metcalf and Eddy, Inc., 1995; Crites and Tchobanoglous, 1998; Körner and Vermaat, 1998; Körner et al., 2003) and pennywort (*Hydrocotyle umbellate*, *Hydrocotyle ranunculoides*; Metcalf and Eddy, Inc., 1995) has already been reported and discussed. Wastewater treatment facilities using macrophytes present some advantages such as: low costs of installation, easy maintenance and good landscape integration. The success of the treatments using

* *Corresponding author*. Tel.: +351 217500381x22145; fax: +351 217500048.
E-mail addresses: mlcosta@esac.pt (M.L. Costa), mcrs@fct.unl.pt (M.C.R. Santos), fcarrapico@fc.ul.pt (F. Carrapiço).
[1] Tel.: +351 239802940; fax: +351 239802979.
[2] Tel.: +351 212948500; fax: +351 212948300.
[3] Tel.: +351 217500381x22145; fax: +351 217500048.
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these plants depends on adequate macrophyte growth throughout the year and on the rate of nutrient uptake.

The use of *Azolla* in wastewater treatment to remove contaminants, particularly phosphorus, was already suggested by several authors (Reddy and DeBusk, 1985) and began to be studied for this purpose, a few years ago (Kitoh et al., 1993; Costa et al., 1993; Vermaat and Hanif, 1998; Costa et al., 1999; Forni et al., 2001; Costa et al., 2002). *Azolla* spp. is unique among floating macrophytes, because it can grow in waters with low nitrogen content, due to a symbiosis with an N_2 fixing cyanobacterium, *Anabaena azollae*, which lives in the dorsal lobe cavity of plant's leaves. The plant can even grow in effluents devoid of combined nitrogen, if their phosphorus content is high enough.

It is already very well known that many eutrophication problems all over the world are due to the discharge of domestic or industrial wastewaters subjected only to secondary biological treatment and which still contain high levels of nutrients, particularly phosphorus. After secondary biological treatment, many wastewaters still maintain high levels of phosphorus but their nitrogen concentrations may limit the growth of most floating macrophytes. Indeed, aquatic plants, other than *Azolla*, need nitrogen and phosphorus present in growth medium in molar ratios between 10:1 and 16:1 to have a good development (Heathwaite, 1993; Kitoh et al., 1993). When nitrogen is not enough, only *Azolla* can improve the removal of phosphorus, due to its ability to fix N_2 from the atmosphere. Also *Azolla*'s biomass produced in wastewater treatment plants can easily be harvested to be used as a fertilizer in sustainable agriculture practices, such as it is done in the rice fields of Asia and Africa (Lumpkin and Plucknett, 1980; Lejeune et al., 1999; Carrapiço et al., 2000).

Thus, *Azolla filiculoides–Anabaena* biosystem can be used as a biofilter for phosphorus removal from urban wastewaters treated by conventional processes. As wastewater is a complex medium, the main goal of this work was to study and compare the behaviour of this water fern in wastewater and in mineral media (Hoagland H-40 basis) with and without added nitrogen. The objective was to search *Azolla*'s ability to remove not only phosphorus but also nitrogen and, at the same time, to study the effect of combined nitrogen present in wastewater on nitrogen fixation by the cyanobacteria *Anabaena* that lives in symbiosis with *Azolla*.

2. Material and methods

A. filiculoides, the only *Azolla* species identified in Portugal (Pereira et al., 2001), was cultivated under batch and controlled conditions, in a urban wastewater stabilization pond effluent (pH = 7.4, COD = 154 mg $O_2\,L^{-1}$, 5.68 mg P–$PO_4^{3-}\,L^{-1}$, 8.85 mg Pt L^{-1}, 34.1 mg N–$NH_4^+\,L^{-1}$, 2.9 mg N–$NO_3^-\,L^{-1}$ and N_{Kj} = 49 mg N–$NO_3^-\,L^{-1}$) and in Hoagland H-40 media (10 mg P L^{-1}) without nitrogen (control) and with nitrogen concentrations similar to those present in the treated wastewater (40 mg N–$NH_4^+\,L^{-1}$ and 5 mg N–$NO_3^-\,L^{-1}$). Another assay was performed with Hoagland H-40 medium with 40 mg N–$NO_3^-\,L^{-1}$, to assess the influence of two different sources of nitrogen on *A. filiculoides*'s growth and nitrogen fixation. The cultures were carried out in PVC boxes (17.0 × 17.0 × 7.5 cm) with 2 L of growth media, placed in a Fitoclima 750E culture chamber illuminated by a combination of incandescent and white fluorescent lamps (Osram L-21-84; Lumilux Plus ECO-18 W) at a photon fluency rate of 88 μmol $m^{-2}\,s^{-1}$ (7 klux). The culture chamber stays under 25 ± 0.5 °C during 14 h/day and 18 ± 0.5 °C, during 10 h/night and illumination was measured with a luximeter (Delta OHM-HD-8366). The relative humidity inside the chamber was kept between 70 and 75% (Hygrometer Delta OHM-HD-8901). At the beginning of the assays the density of *Azolla* was 69 $g_{w.wt.}\,m^{-2}$. Media pH was verified and adjusted to 6.2 every three days and distilled water was added to compensate evaporation and evapotranspiration. Water samples were analysed according to standard methods (APHA, 1998), and the analyses of biomass were performed at the end of the assays, according to AOAC (1997). Biomass composition was expressed on a dry weight basis. To evaluate the specific growth rate *Azolla*'s fresh weight was measured three times a week. The plants were harvested and gently blotted between absorbent papers, to remove adhering liquid, and let to dry during half an hour on filter paper (Whatman no 1). Specific growth rate (admitting an exponential growth as reported by Vermaat and Hanif (1998) and Rai et al. (2006)) and maximum plant density (considering biomass at the end of the assays and the area of PVC boxes) were evaluated. Productivities were calculated based on *Azolla*'s densities and specific growth rates for each time interval. Phosphorus and nitrogen removal rates coefficients were calculated using first-order kinetics equations, as referred by Körner and Vermaat (1998) and Sooknah and Wilkie (2004). The phosphorus and nitrogen uptake rates by *A. filiculoides*'s biomass were determined based on productivity values and biomass phosphorus and nitrogen contents.

The nitrogen fixation was evaluated by the acetylene reduction activity (ARA) method. Exact quantities of *A. filiculoides*, corresponding to five to seven plants, previously adapted to every growth media, were placed in 35 mL serum vials, containing 10 mL growth medium and the flasks were sealed with rubber and metal stoppers. Before beginning the assays, 10% of air was withdrawn and replaced by an identical volume of acetylene. After acetylene addition, 0.5 mL samples were taken at several time intervals (~1 h) with gas-tight syringes and the ethylene produced from acetylene reduction was measured by gas chromatography (Carlo Erba-GC 6000 Vega series 2), using a N-Porapack column (Supelco, 80–100 mesh, 2 m) and a flame ionisation detector. A control assay to evaluate nitrogenase activity in wastewater growth medium (without *Azolla*) was performed. The results, expressed on a dry weight basis, are the average of three independent experiments.

Samples of plant's specimens from the different assays were fixed, using a methodology according to Pereira et al. (2001), and observed by scanning electron microscopy (SEM) (Jeol JSM 5200 LV).

3. Results and discussion

Azolla's specific growth rates in wastewater (34.1 mg N–$NH_4^+\,L^{-1}$), and in mineral media without added nitrogen or

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Table 1 – Growth rates, maximum densities and productivities and phosphorus and nitrogen content of *Azolla* grown in media with different compositions.

Assays	Growth rate (d^{-1})	Density ($g_{d.wt.}\,m^{-2}$)	Productivity ($g_{d.wt.}\,m^{-2}\,d^{-1}$)	Phosphorus (%)	Nitrogen (%)
Wastewater	0.124**	50.2	3.4	0.68	5.71
Hoagland H-40	0.122**	54.4	3.9	0.91	5.22
H-40 + N–NO_3^--5	0.126 ± 0.003**	62.3 ± 6.1	4.6	0.70	5.49
H-40 + N–NO_3^--40	0.119 ± 0.020*	53.0 ± 7.2	3.6	0.43	4.38
H-40 + N–NH_4^+-40	0.100 ± 0.006**	44.4 ± 5.0	2.8	0.60	6.09

Significance level of fitted curves was set at: $^*p < 0.05$; $^{**}p < 0.01$.

with low nitrate concentration were very similar (0.122 d^{-1}–0.126 d^{-1}) (Table 1). Indeed, in Hoagland medium with the lowest dose of nitrates, a slight increase of *Azolla*'s specific growth rate was observed (3.3%). Maejima et al. (2002) also reported that low levels of combined nitrogen in the medium increased the specific growth rate of *Azolla pinnata* strains.

When *Azolla* grew in Hoagland medium with 40 mg N–$NH_4^+\,L^{-1}$, an 18.0% specific growth rate reduction was observed, when compared with the control (Hoagland H-40). This can be explained by the inhibition due to nitrogen in excess or to pH drops to values of 3.4, despite previous adjustment to 6.2 (Uheda and Kitoh, 1992). In wastewater and Hoagland H-40 with 40 mg N–$NO_3^-\,L^{-1}$ media, were always observed pH values higher than in mineral media with ammonium nitrogen. Though in wastewater it was also registered a pH decrease ($pH_{min} = 3.8$), this effect was very limited in time and did not seem to affect the specific growth rate. In fact, during other wastewater assays performed under continuous conditions (including the pH adjustment to 6.2), it was even observed a pH increase (up to 7.5, data not yet published).

Maximum densities achieved by the plant in these assays (Table 1) were in accordance with operational plant density referred by Reddy and DeBusk (1985) and similar to the result obtained in assays performed under continuous regimen (about 60 $g_{d.wt.}\,m^{-2}$, data not yet published). Plant grown in wastewater presented good productivity when results are compared with those obtained in the other media (Table 1), reflecting an adequate growth of *Azolla*, despite the presence of combined nitrogen. The lowest plant productivity was obtained in mineral medium with 40 mg N–$NH_4^+\,L^{-1}$ and presented the same order of magnitude of the results reported by Reddy and DeBusk (1985) for *Azolla* grown in mineral media without nitrogen.

The lowest phosphorus and nitrogen content *of Azolla*'s biomass was observed in mineral medium with 40 mg N–$NO_3^-\,L^{-1}$ assay (Table 1). In general, the phosphorus content of *Azolla*'s biomass grown in this medium was similar to the phosphorus content of the biomass of plants grown in natural ecosystems, but nitrogen content was higher (Lumpkin and Plucknett, 1980; Costa et al., 1999).

The presence of nitrates in the mineral media didn't seem to affect phosphorus removal rate coefficients (KPt and KPO_4^{3-}) (Table 2). Maximum total phosphorus removal (95.3%) was observed in H-40 medium with 40 mg N–$NO_3^-\,L^{-1}$ (Fig. 1b). On the contrary, the total phosphorus removal rate coefficient decreased 52.7% in wastewater and 24.6% in H-40 with 40 mg N–$NH_4^+\,L^{-1}$, when results are compared with those obtained in H-40 medium without added nitrogen. However, orthophosphate removal rate coefficients were higher than those of total phosphorus in all media, particularly in wastewater, and presented orders of magnitude similar to those reported by Körner and Vermaat (1998) for *Lemna gibba* grown in domestic wastewater. The development of algae and other organisms in wastewater promoted additional orthophosphate removal and contributed to a higher total phosphorus concentration in this media and to greater differences between orthophosphate and total phosphorus removal efficiencies (Fig. 1a and b).

The highest phosphorus uptake rate was observed in mineral media without added nitrogen, where specific growth rate, productivity and phosphorus biomass were high (Tables 1 and 2). Nevertheless, the phosphorus uptake rates presented here were statistically correlated only with phosphorus

Table 2 – Total phosphorus and orthophosphate removal rates coefficients and maximum phosphorus uptake rates of *Azolla* grown in media with different compositions.

Assays	KPt (d^{-1})	KPO_4^{3-} (d^{-1})	Phosphorus uptake rate ($mg\,P\,m^{-2}\,d^{-1}$)
Wastewater	0.052**	0.222**	23.1
Hoagland H-40	0.110**	0.213**	35.1
H-40 + N–NO_3^--5	0.108 ± 0.001**	0.232**	32.2
H-40 + N–NO_3^--40	0.117 ± 0.010**	0.210**	15.3
H-40 + N–NH_4^+-40	0.083 ± 0.010**	0.210**	16.9

Significance level of fitted curves was set at: $^*p < 0.05$; $^{**}p < 0.01$.

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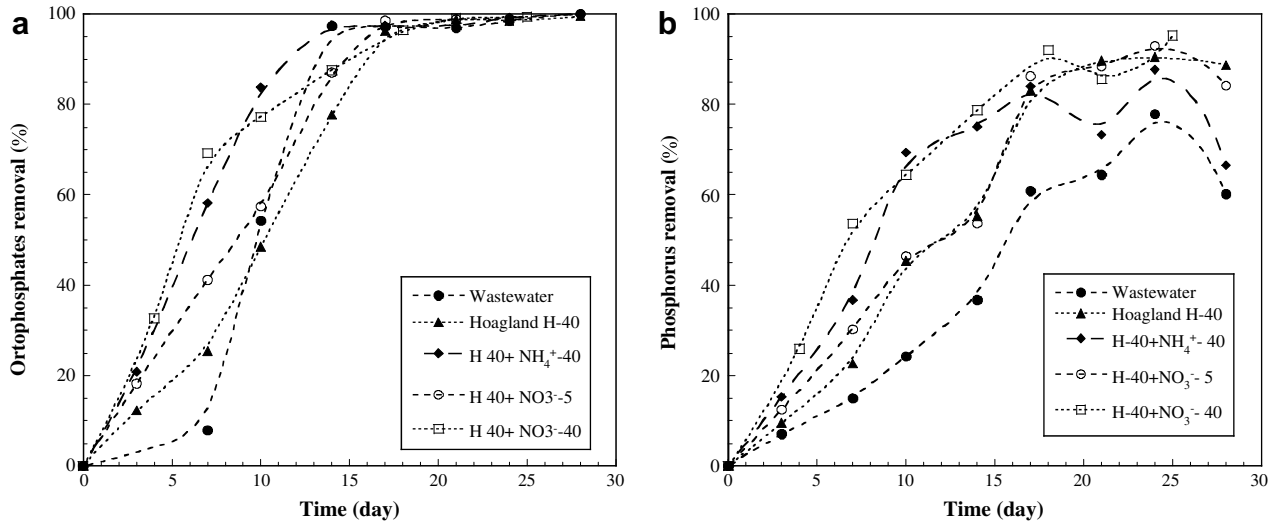

Fig. 1 – Phosphorus removal efficiencies obtained in different media utilised for *Azolla*'s culture: (a) Orthophosphate; (b) Total phosphorus.

contents in the biomass ($r = 0.890$; $p < 0.05$). The values obtained in these assays presented generally the same order of magnitude of those reported by Reddy and DeBusk (1985) for an outdoor culture of *Azolla* in mineral medium with 3 mg P L^{-1} and were higher than those reported by Körner and Vermaat (1998) for *L. gibba* grown in domestic wastewater (12–15 mg P m^{-2} d^{-1}).

In wastewater, the maximum ammonium removal efficiency was 99.7% (Fig. 2b) and the ammonium removal rate coefficient (KNH_4^+) was 51.9% higher than the one observed in mineral medium with added ammonium (Table 3). These results were due, not only to higher plant specific growth rate, but also to algae and bacteria activities, more prominent in this medium. Nevertheless, the ammonium removal rate coefficient observed in wastewater assay was lower than the one reported by Körner and Vermaat (1998) for *L. gibba* grown in domestic wastewater, since *Azolla–Anabaena* is a nitrogen fixing biosystem.

The nitrate removal rate coefficients (KNO_3^-) obtained in wastewater and in H-40 with 5 mg N–NO_3^- L^{-1} were greater than in H-40 with 40 mg N–NO_3^- L^{-1}, as it was observed for plant specific growth rates (Tables 1 and 3). The decrease of nitrate removal rate coefficients observed when this nutrient concentration increased in the mineral medium was also expected, as the reported by Costa et al. (1993) for *Azolla*'s phosphorus removal rate coefficients. However, nitrification, which normally occurs in the type of wastewater (2.9 mg N–NO_3^- L^{-1}) used in these assays (as was observed in ongoing assays under continuous steady state conditions, data not yet published), could have contributed to the decrease of nitrate removal rate coefficient in this medium if compared with the value obtained in H-40 with 5 mg N–NO_3^- L^{-1} growth medium.

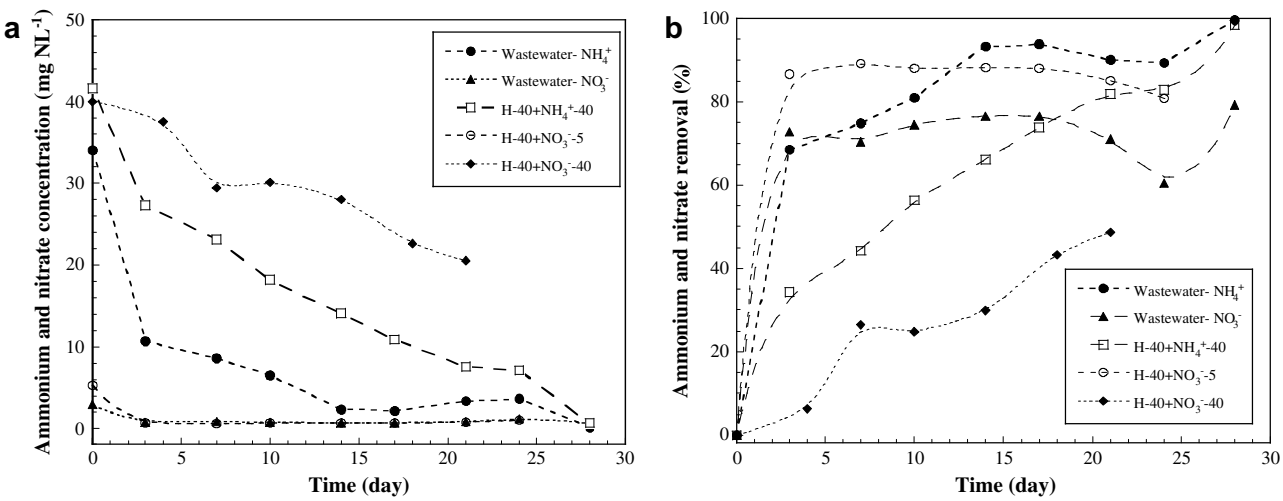

Fig. 2 – Evolution of ammonium and nitrate in *Azolla*'s culture media with different compositions: (a) concentration; (b) removal efficiencies.

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Table 3 – Ammonium and nitrate removal rate coefficients and nitrogen uptake rate of *Azolla* grown in media with different compositions.

Assays	KNH_4^+ (d^{-1})	KNO_3^- (d^{-1})	Nitrogen uptake rate (mg N m^{-2} d^{-1})
Wastewater	0.117**	0.043 n.s.	194.1
Hoagland H-40	–	–	201.5
H-40 + N–NO_3^--5	–	0.058 ± 0.013 n.s.	252.5
H-40 + N–NO_3^--40	–	0.034 ± 0.004**	155.9
H-40 + N–NH_4^+-40	0.077 ± 0.001**	–	171.1

Significance level of fitted curves was set at: *$p < 0.05$; **$p < 0.01$; n.s. – not significant.

As it can be observed in mineral media with the same nitrogen concentrations, nitrate removal rate coefficient was about half of the ammonium removal rate coefficient. This fact might have resulted from a preferential assimilation of ammonium by *Azolla*, but the growth of algae observed in H-40 with 40 mg N–NH_4^+ L^{-1} also may have contributed to an increase in ammonium removal from the medium.

The nitrogen uptake data presented here (155.9–252.5 mg N m^{-2} d^{-1}) were higher than those reported by Reddy and DeBusk (1985) (99 ± 34 mg N m^{-2} d^{-1}), especially due to higher nitrogen biomass contents. Nevertheless, the values are lower than those reported by Vicenzini et al. (1985) (370–540 mg N m^{-2} d^{-1}) for *A. filiculoides* grown in outdoor culture and mineral medium with 12 mg P L^{-1}, where the nitrogen biomass contents were similar to those reported here. In wastewater, *Azolla*'s nitrogen uptake rate presented an order of magnitude similar to the one reported by Körner and Vermaat (1998) for *L. gibba* grown in domestic wastewater (120–209 mg N m^{-2} d^{-1}).

As it was not possible to assess which nitrogen existing in the *Azolla*'s biomass came from assimilation and from nitrogen fixation, the influence of the nitrogen contents of the media on nitrogen fixation was studied. Globally, nitrogen fixation rates obtained (Table 4) were lower than those referred by Lumpkin and Plucknett (1980) in a study with different light intensities, but presented the same order of magnitude of those reported by Singh and Singh (1989) for *A. pinnata* and *Azolla caroliniana* in flooded rice fields. The values reported here are also similar to those reported by Forni et al. (2001) for *A. filiculoides* Lam. grown under natural conditions in April, but lower than those obtained when the plant was grown in July. The low light intensity used in these laboratory assays may be an explanation for this fact. Indeed, a low light intensity (<10–13 klux = 125–162 μmol photon m^{-2} s^{-1}) can decrease nitrogen fixation, as observed in *A. pinnata* cultures by Bar et al. (1991) and Tel-Or et al. (1991).

The nitrogen fixation by *Azolla*'s specimens grown in wastewater and in mineral media without nitrogen was similar (Table 4). Thus, the high ammonium concentration in wastewater (34.1 mg L^{-1}) didn't seem to have affected nitrogen fixation. In H-40 media, an increasing inhibition of the nitrogen fixation rate was observed as the media nitrogen content increased, with a maximum (45.8%) for plants grown in H-40 medium with 40 mg N–NH_4^+ L^{-1}. This level of inhibition is between 20% and 70% as referred by Watanabe et al. (1991) for the same level of ammonium concentration. The nitrogen fixation inhibition due to the same concentration of nitrogen but this one in the nitrate form was only 30.3%. These results allow concluding that ammonium has an inhibitory effect on nitrogen fixation greater than nitrates. Others authors, like Kitoh and Shiomi (1991), already reported identical conclusions for *A. filiculoides*. The pH decrease observed in the mineral medium with added ammonium might have been relevant in nitrogenase activity inhibition. In fact, according to Uheda and Kitoh (1992), inhibition of plant growth and of nitrogen fixation is more relevant at pH 3.5 than at pH 6.5, even at higher ammonium concentrations (280 mg L^{-1}). In that study, the apical symbionts were significantly damaged or disappeared when they were exposed simultaneously to high ammonium concentrations and low pH. The host plants seemed to be more resistant to these conditions, although growing more slowly. The increase of ammonium ions toxicity at low pH may result from an increase in ammonium absorption rate, without the corresponding conversion to other nitrogenous compounds inside the cells, leading to an accumulation of toxic levels of ammonium (Uheda and Kitoh,

Table 4 – Maximum nitrogenase activities, nitrogen fixation rates, nitrogen uptake rates and N_2 fixing efficiency of *Azolla* grown in media with different compositions.

Assays	Max. Nitrogenase activity* (nmol C_2H_4 $mg_{d.wt.}^{-1}$)	Nitrogen fixation rate (nmol C_2H_4 $mg_{d.wt.}^{-1}$ h^{-1})	Nitrogen uptake rate (nmol N_2 $mg_{d.wt.}^{-1}$ h^{-1})	N_2 fixing efficiency (nmol C_2H_4/nmol N_2)
Wastewater	255.3 ± 17.8	19.5 ± 0.8	10.7	1.8
Hoagland H-40	244.8 ± 11.3	19.3 ± 1.8	6.2	3.1
H-40 + N–NO_3^--5	252.4 ± 29.1	18.6 ± 1.8	7.7	2.4
H-40 + N–NO_3^--40	162.0 ± 1.8	13.5 ± 0.3	5.2	2.6
H-40 + N–NH_4^+-40	139.1 ± 10.7	10.5 ± 1.0	8.7	1.2

*After about 24 h.

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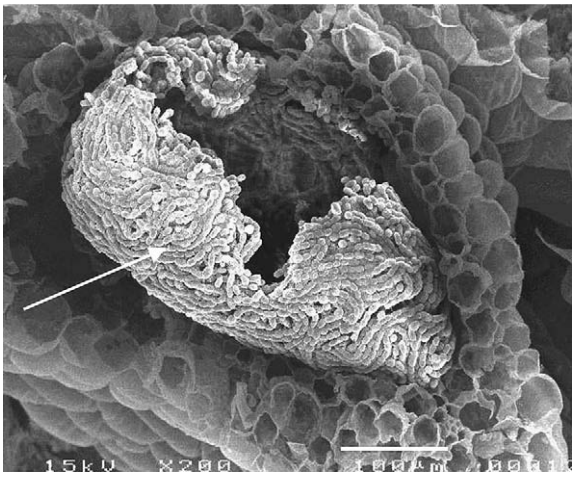

Fig. 3 – SEM observations of the mature cavities of *Azolla filiculoides* grown in the wastewater. General view of the high density of *A. azollae* filaments (arrow).

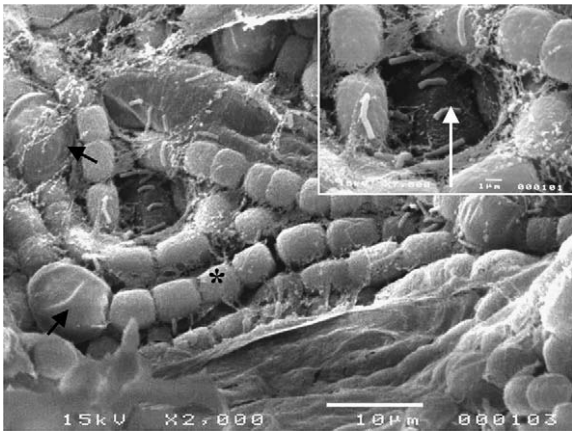

Fig. 5 – SEM observations of the mature cavities of *Azolla filiculoides* grown in the wastewater. The filaments have many vegetative cells (*) and heterocysts (arrowhead) and also many *Arthrobacter* (arrow) in the upper right of the photograph.

1992). Nevertheless, the concentration of ammonium (34.1 mg N–NH_4^+ L^{-1}) in the wastewater used in this work didn't seem to have affected *Azolla–Anabaena*'s nitrogen fixation or the plant specific growth rate, probably due to a better buffer capacity existing in this type of media (a less pH decrease), resulting from bacterial metabolism. This could also result in higher concentrations of nitrogen in the NH_3 form and in lower ammonium assimilation, as admitted by Uheda and Kitoh (1992). On the other hand, the ionised ammonium may lead to an increase in proton intracellular concentration. The removal of protons in excess consumes ATP, which compromises the plant growth and nitrogen fixation by *A. azollae*, since both processes need high amounts of energy (about 16 mole ATP/mole of reduced N_2 in nitrogen fixation) (Stanier et al., 1990; Fonseca and Teixeira, 2007). Accordingly, if a lower assimilation of ammonium in the ionised form was observed, the *pool* of intracellular protons would be less, leading to more energy available for growth and nitrogen fixation.

The nitrogen fixation rate was found to be significantly correlated with plant specific growth rate ($r = 0.886$; $p < 0.05$), what might mean that *Azolla*'s growth depends on nitrogen fixation, even when combined nitrogen is present. The results showed that *Azolla* could grow and accumulate nitrogen obtained from both the growth medium and nitrogen fixation.

The C_2H_4 produced/N_2 uptake ratio, observed in Hoagland medium without nitrogen (Table 4), was similar to the *in vitro* theoretical value of 3 nmol C_2H_4 produced/nmol N_2, assuming the absence of H_2 evolution in air atmosphere, as reported by Vicenzini et al. (1985) and Stanier et al. (1990). In containing nitrogen media, the ratios decreased and therefore the underestimation of nitrogen uptake rate, based on ARA analysis (a simple and sensitive method to evaluate nitrogenase activity), would be more significant. Especially in these cases,

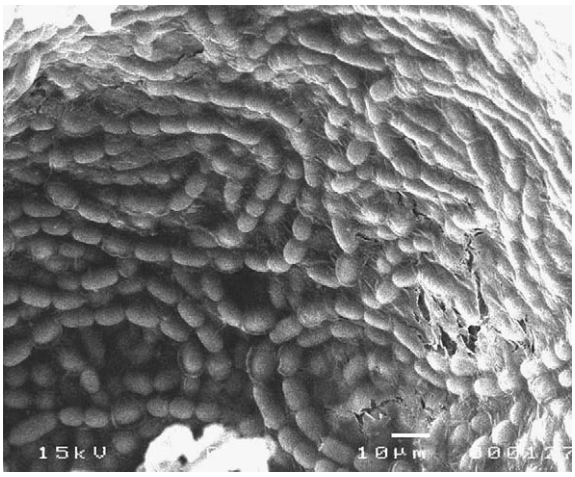

Fig. 4 – SEM observations of the mature cavities of *Azolla filiculoides* grown in the wastewater. General view of the high density of *A. azollae* filaments with an envelope covering the cyanobacteria.

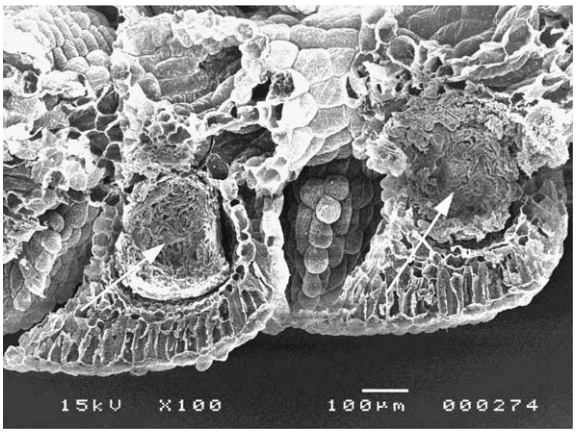

Fig. 6 – SEM observations of the mature cavities of *Azolla filiculoides* grown in H-40 culture media. The cavities are completely filled with filaments of the cyanobacteria (arrow).

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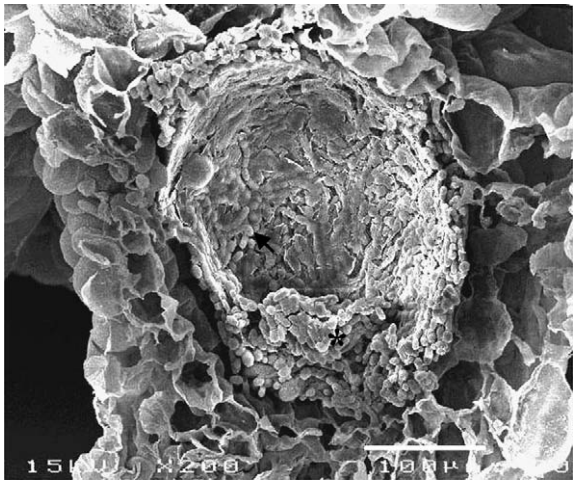

Fig. 7 – SEM observations of the mature cavities of *Azolla filiculoides* grown in H-40 culture media. The cavities are completely filled with filaments of the cyanobacteria with heterocysts (arrowhead) and vegetative cells (*).

the nitrogen uptake rate must be based on biomass nitrogen. Using this last method, the nitrogen uptake rate by *Azolla–Anabaena* grown in H-40 medium without nitrogen was 6.2 nmol $mg^{-1}_{d.wt.}\,h^{-1}$, a value lower than the nitrogen fixing capacity observed by Vicenzini et al. (1985) in *A. filiculoides* cultivated in mineral medium and outdoor conditions (10.0 nmol $mg^{-1}_{d.wt.}\,h^{-1}$–21.3 nmol $mg^{-1}_{d.wt.}\,h^{-1}$). The maximum plant's nitrogen uptake rate (10.7 nmol $mg^{-1}_{d.wt.}\,h^{-1}$) determined, based on biomass nitrogen, was found in plants grown in wastewater, where nitrogen was provided both by assimilation from the growth media and from nitrogen fixation. This fact can be of importance if it is intended to use the plant on wastewater treatment, because its value as fertilizer could be greater than that of *Azolla* grown in natural ecosystems.

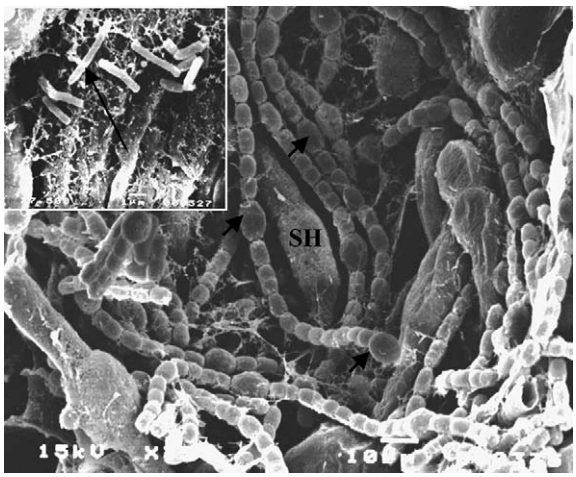

Fig. 8 – SEM observation of the mature cavities of *Azolla filiculoides* grown in culture media H-40 with added N–NH_4^+. The filaments near the simple hairs (SH) have many heterocysts (arrowhead) and bacteria (arrow) in the upper left of the photography.

Indeed, as well as the reported here, in continuous experiments with wastewater from stabilization pond, under controlled conditions or field conditions (data not yet published), the nitrogen accumulation rate by *Azolla* was higher (6.3 ± 2.2 nmol $mg^{-1}_{d.wt.}\,h^{-1}$ and 10.3 nmol $mg^{-1}_{d.wt.}\,h^{-1}$, respectively) than the observed in *Azolla* grown in natural aquatic ecosystems (1.3–3.7 nmol $mg^{-1}_{d.wt.}\,h^{-1}$), what increases its fertilizer value. Also, the nitrogen and phosphorus contents of *Azolla*'s biomass grown in wastewater will be greater than those of *Azolla* from natural aquatic ecosystems.

Observations of mature leaf cavities of *A. filiculoides* specimens grown on all studied media were performed on SEM, to assess the morphological variations that could be related with different rates of nitrogen fixation (Figs. 3–8).

In all specimens there were large quantities of cyanobacteria filaments (Figs. 3, 6 and 8), which occupied a narrow space near the epidermal cells of the cavity and were surrounded by simple hairs (Fig. 8). Several vegetative cells formed the filaments of *A. azollae* with heterocysts inserted between them (Figs. 5, 7 and 8).

Inside all *Azolla*'s leaves cavities it could also be observed a high quantity of bacteria, namely belonging to the genus *Arthrobacter* (Fig. 5 upper right and Fig. 8 upper left).

The observations made with SEM in our experiments indicated that neither *A. azollae* nor bacteria were affected by the levels of combined nitrogen existing in mineral culture media and in wastewater. The presence of an envelope covering endosymbionts in *Azolla* grown in wastewater (Fig. 4) suggests a defence mechanism that protects the prokaryote colony.

4. Conclusions

Results obtained showed that:

- *Azolla* can grow well in effluents from wastewater stabilization ponds (with a doubling time of 5.6 d), despite the high ammonium content of the medium;
- The orthophosphate removal rate coefficient and phosphorus uptake rates observed for *Azolla–Anabaena* symbiotic system could be even higher than those obtained with others small aquatic plants, what confirmed its promising ability to remove this nutrient from wastewaters;
- Despite *Azolla–Anabaena* is a nitrogen fixing biosystem, the ammonium removal efficiency observed in wastewater was good. The nitrogen uptake rate by *Azolla* was similar to the one reported to *Lemna*;
- Although there was a high concentration of ammonium in the wastewater, it didn't seem to inhibit nitrogen fixation, in contrast with the nitrogen fixation inhibition observed in H-40 medium with an identical concentration of ammonium. These findings can be related to a best buffer capacity of the wastewater, which can also be implicated in a decrease of the ammonium absorption rate what will then lead to the existence of higher quantities of ATP available for plant growth and nitrogen fixation. The presence of an envelope covering endosymbionts in the cavities of *Azolla* grown in wastewater also suggests that this can be a defence mechanism to protect the prokaryote colony.

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The results here presented (and those obtained in assays performed in continuous reactors with the same wastewater and that are prepared to be published), enhance the possibility of using *Azolla* as a biofilter not only for phosphorus but also for nitrogen removal. The ability of this macrophyte for nutrient removal, namely phosphorus, can improve the quality of previously treated wastewater, mainly when nitrogen becomes a limiting growth factor, what is sometimes the case of urban wastewaters treated by conventional processes. This can be an advantage of using *Azolla* instead of other small macrophytes (Lemnaceae) also used to upgrade wastewater quality. Furthermore, *Azolla*'s biomass can be used as a biofertiliser, contributing to the decrease the exploitation costs of phosphorus removal in wastewaters, especially from small communities.

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