**Dual purpose system for water treatment from a polluted river and the production of Pistia stratiotes biomass within a biorefinery**

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**Abbreviations:** **ANOVA**, analysis of variance; **BOD**, biochemical oxygen demand; **COD**, chemical oxygen demand; **dw**, dry weight; **RGR**, relative growth rate; **SWW**, synthetic wastewater; **TKN**, total Kjeldhal nitrogen; **WSR**, water from the Sordo River; **WSR + F**, WSR amended with fertilizer

Keywords Aquatic phytoremediation, Biomass production, Nutrient removal, Pollution, Urban river

**Abstract**

The use of lagoons with floating aquatic plants for the treatment of a polluted urban river with recovery of *Pistia stratiotes* biomass was investigated. A first group of experiments was performed during spring, comparing three different media: synthetic wastewater (SWW), water from the Sordo River (WSR) and WSR amended with fertilizer (WSR + F). The second and third experiments were performed during summer and winter, respectively, using only WSR. During spring, the productivity in WSR and WSR + F was similar between them and significantly higher compared to the one observed in the SWW: 0.949 vs. 0.379 g dry weight (dw) m--2 day--1, respectively. During summer, the productivity in the WSR was similar to the one observed in spring but significantly different to the one registered in winter: 0.946 vs. 0.347 g dw m--2 day--1. During spring and summer, the uptake rate of ammonium nitrogen was significantly higher compared to the one registered for nitrate, although in winter there were no differences between such rates. For phosphates, the removal percentage was similar in spring and winter (96.25 and 99.1%, respectively). It was concluded that this system serves a dual purpose, treating water from a polluted river effectively and producing high biomass yield during spring and summer.

# 1 Introduction

The use of floating aquatic plants as a phytofiltration system for removing nutrients and other pollutants is a potential alternative for wastewater treatment, especially in tropical and sub-tropical regions. Their ability to remove pollutants depends on the species, type and composition of the wastewater and weather conditions, among other factors [1]. Within this context, diverse floating plant species have been used to treat different kind of wastewaters [2--5].

*Pistia stratiotes* has been studied under various conditions and different purposes. The dynamics of nitrogen uptake and storage in the plant tissues has been previously studied by [6]. Its effectiveness for removing nutrients [2, 7--10] and metals [11--13] has been demonstrated in the treatment of different types of wastewaters. It has been also used to treat domestic wastewater [14--16]. The biomass produced during the wastewater treatment has been used as the added value products such as fish or cattle food [17], fodder [18] and biogas feed [19]. The anaerobic digestion of this species has resulted in an average biogas production between 0.53--0.70 m3 kg--1 SV--1 [20] with a methane composition between 58--68% [21].

In the context of a biorefinery strategy, a phytofiltration system for wastewater treatment and production of biogas (methane and carbon dioxide) from the harvested macrophyte biomass seems to be an appropriate alternative. The water from an urban river polluted with untreated domestic wastewater could be effectively used as a nutrient source for *P. stratiotes* growth and this application has not been reported so far. There is only one report using *P. stratiotes*, in which domestic wastewater diluted with tap water 1:2 (v/v) was called “polluted river water” and experiments were performed only for 18 days [16]. The harvested biomass could be processed through anaerobic digestion. The treated water and the CO2 generated (mixed with methane), could be used for oleaginous microalgae cultivation as it has been previously described [22]. Therefore, the aim of the present work was to assess the use of lagoons with *P. stratiotes* as a treatment system for removing nutrients from water of an urban polluted river, evaluating productivity and nutrient removal at different seasons within the context of a dual purpose system for the treatment of water and for the production of plant biomass.

## 2 Materials and Methods

## 2.1 Plant collection and preparation

*Pistia stratiotes* was collected in a natural wetland located in Actopan (19°30’N; 96°37’W), in Veracruz, Mexico. The plants were washed with tap water and for acclimatization, were grown in water from the Sordo River for 20 days in greenhouse conditions, in the city of Xalapa, Veracruz, Mexico. The Sordo River is located at the Western side of the City of Xalapa (19°32’2’’N; 96°55’3’’W) and it is part of the “La Antigua” watershed which drains into the Gulf of Mexico.

**2.2 Chemical characterization of water from the Sordo River and control wastewater**

The water quality of the Sordo River has been evaluated previously [23], and the results indicated that in most of the sampling points, the BOD5/COD ratio was nearly 1, indicating that most of the contaminants were biodegradable. For the current experiments, a partial chemical characterization of the various tested waters was performed (Table 1), including a synthetic wastewater (SWW) which was considered as a control media, with a similar composition to that found in a weak domestic wastewater (this medium was prepared by dissolving the following components in tap water: 100 mg L--1 glucose, 15 mg L--1 NaH2PO4, 1.5 mg L--1 KH2PO4, 4 mg L--1 CaCl2, 2 mg L--1 MgSO4) [24], water from the Sordo River (WSR) and WSR amended with fertilizer (Tricel-20© and urea) (WSR + F), considered as a second control media. WSR was analyzed for Cu2+, Cd2+, Pb2+, Zn2+, and Al3+ using spectrophotometry (Shimadzu© Mod. AA-460-13).

**2.3 Experimental treatments**

The experiments were performed during 2012 under greenhouse conditions at Xalapa City in Mexico. Temperature and light intensity were registered daily at 9:00 am, 2:00 and 5:00 pm to obtain daily average values.

At the beginning of each experiment 400 g (fresh weight) of *P. stratiotes* healthy plants were cultivated in 40-L plastic containers filled with different types of water, depending on the season. The experiments were carried out in triplicate.

During spring (May), three treatments were tested: a) SWW, b) WSR, and c) WSR + F. The experiment was carried out for a period of 28 days.

Based on the results from the experiments performed in spring (May), it was decided that during summer (August) and winter (December), all experiments would be carried out only in WSR, and that the duration of each experiment would be only ten days.

**2.4 Evaluation ofgrowth**

The biomass fresh weight was registered every 48 h after removing the water in excess. This process was performed by placing the plants over filter paper during 15 min (fresh weight). In order to determine the dry weight of the biomass, it was first grounded with a food processor (Hamilton Beach, Mod. 70610) to release particles of 7 mm. Afterwards, a homogeneous mixture of already grounded plant material (roots and leaves), was placed into porcelain capsules (triplicates) into an oven at a temperature of 105 °C until reaching constant weight. The relative growth rate (RGR) and productivity were calculated by using the following equations according to Olguín et al. [3]:

RGR (day--1) = (1)

where *w*1 is the dry weight at *t*1: initial (g), w2 is the dry weight at *t*2: final (g) and *t* is the time (days).

The biomass density (g m−2) is the biomass dry weight (g) by surface units (m2).

Productivity (g m--2 day--1) = (2)

**2.5 Sampling and treatments**

Water column samples were taken from the treatments at the beginning of the experimental period and afterwards, every 48 h. Samples were filtered using no. 4 Whatman® filter paper to separate plant debris. Clear water samples were analyzed as described in the section below.

**2.6 Water chemical analysis**

Chemical oxygen demand (COD) was determined according to standard methods [25] whereas parameters as the total Kjeldhal nitrogen content (TKN), ammonium nitrogen (N-NH4+), nitrates (N-NO3--), and *ortho*-phosphates (P-PO43--) were quantified using spectrophotometric methods (HACH© Mod. DR-5000) [26]. pH was determined using a multi-parametric analyzer (HANNA© Mod. HI9828).

**2.7 Statistical analysis**

One-way ANOVA was performed to compare data among the different treatments and Turkey’s comparison test was conducted to determine differences between means using the PAST statistical software (version 2.17b). A 95% confidence level was applied for all analysis.

**3 Results**

**3.1 Environmental conditions**

The average temperature registered inside the greenhouse was 25.8 ± 2.9, 25.2 ± 1.2 and 21.2 ± 3.2 °C, during spring, summer and winter, respectively (Fig. 1). The average temperature during winter was significantly lower to that registered during spring (*p* = 0.0005) and summer (*p* = 0.002). The average light intensity registered during the three seasons was 292, 522 and 527 µmol m--2 s--1 at 10:00 am; 1095, 697 and 602 µmol m--2 s--1 at 2:00 pm and 148, 62 and 26 µmol m--2 s--1 at 5:00 pm, respectively.

**3.2 *P. stratiotes* growth**

Biomass density registered in the cultures containing WSR and WSR + F, during spring, was significantly higher (*p* = 0.003 and *p* = 0.015, respectively) in comparison to that registered in cultures containing synthetic wastewater (SWW) after 28 days of cultivation. On the other hand, biomass density registered during summer was similar (*p* = 0.659) to the one obtained in WSR in spring in the first ten days of experiment (Fig. 2).

During spring, the pH decreased in all treatments. The mean pH values maintained neutral during summer and winter (Fig. 3).

Productivity at day 28 did not show significant differences between WSR and WSR + F (*p* = 0.509), but it was significantly higher to that obtained in SWW (*p* = 0.003) (Table 2). During spring, at day 10 of the cultivation productivity in treatment, WSR was similar (*p* = 0.537) to the one obtained at day 28. The productivity obtained in WSR during summer did not show significant differences between the one obtained during spring at day 10 (*p* = 0.659). The growth obtained in WSR and WRS + F during spring showed a triauxic pattern; thus, there was the need of calculating three RGRs. On the other hand, in SWW and WSR during summer, a diauxic pattern was observed and only two RGRs were calculated (Table 2).

**3.3 Nutrients removal**

The organic matter and nutrient removal varied according to the type of water tested and the season after 10 days (Table 3). In the case of the COD, the highest removal percentage (82.54 ± 0.051 %) was observed during May for WSR (initial concentration = 101.7 ± 2.6 mg L--1; final concentration = 12 ± 1.05 mg L--1).

During spring the TKN concentration decreased 87% (from 11.4 ± 0.4 to 1.5 ± 0.1 mg L--1) and 91% (from 34.2 ± 7.3 to 3.2 ± 1.2 mg L--1) at day 18 in WSR and WSR + F, respectively, meanwhile for the summer and winter, TKN was almost completely eliminated on day 10 (Fig. 4a).

During spring, ammonium nitrogen was totally removed on day 4 in WSR treatment and on day 11 in treatment WSR + F, whereas for summer and winter it was totally removed on days 2 and 6, respectively (Fig. 4b).

The nitrates concentration was reduced to <0.4 mg L--1 in all treatments and seasons (Fig. 4c). However, the removal percentage was very different in summer (16.67%) compared to that obtained in winter (98.30%), due to the higher initial concentration observed in winter (3.9 ± 0.2 mg L--1) compared to the one registered in summer (0.2 ± 0.1 mg L--1) as a consequence of a dilution effect by rain in summer.

During spring, phosphates were removed from the WSR+F treatment to a higher rate during the first 14 days, in contrast to those from SWW (*p* = 0.006). Meanwhile, the P-PO43-- initial concentration in WSR treatment was very low and it was totally removed after seven days. During winter, a similar pattern was observed (Fig. 4d).

In relation to the COD, a decrease in the first four days was observed in all treatments and seasons. Thereafter, a high variation in the COD concentration was obtained (Fig. 5).

In spring, the P-PO43--, N-NH4+ and TKN (having initial concentrations of 3.6 ± 0.1, 9.8 ± 0.2 and 11.4 ± 0.4 mg L--1, respectively for WSR and 22.1 ± 0.1, 10.8 ± 0.6 and 34.2 ± 7.3 mg L--1, respectively, for WSR + F) removal was very high in WSR and WSR + F: >96 % (having final concentrations of 0.2 ± 0.14, 0.4 ± 0.18 and 5.2 ± 1.86 mg L--1, respectively, for WSR and 10.4 ± 0.52, 1.0 ± 0.1 and 5.6 ± 1.45 mg L--1, respectively, for WSR + F) after 28 days of cultivation. On the other hand, at day 10 of cultivation, for P-PO43-- and N-NH4+, the removal percentages were very high: >95 %, in all seasons (Table 3).

An elevated consumption of N-NH4+ from WSR during the first two days was observed in all seasons and especially during spring and winter (with initial-to-final concentrations from 9.8 ± 0.16 to 5.2 ± 0.1 mg L--1, and from 11.1 ± 0.1 to 6.7 ± 2.1 mg L--1, respectively). However, the consumption rate of N-NO3-- was positive only during spring and winter (Table 4).

**4 Discussion**

It has been previously pointed out thatthe selection of a particular plant species to be applied for a specific phytofiltration purpose should be a function of several factors [27]. Among the most relevant are: (1) its productivity under the particular climatic conditions of application; (2) its efficiency for nutrient or pollutant removal from a given type of wastewater, throughout different seasons; (3) its capacity to overgrow other aquatic macrophytes in the same environment; (4) the cost of harvesting; (5) the possible use of the harvested biomass. Thus, in this work, factors 1 and 2 have been evaluated for a treatment lagoon system using *P. stratiotes* as the only macrophyte in batch operated lagoons treating water from an urban polluted river.

In relation to the water quality of the Sordo River, a variation on the nutrients concentration was observed throughout the different seasons of the year. This was also reported during the rainy and dry season in a previous work [23]. Despite all of these differences, the biomass density of *P. stratiotes* was not significantly different during spring and summer. Furthermore, the productivity found during these two seasons was similar to the one reported for *P. stratiotes* treating domestic wastewater [15]. The difference observed in the *P. stratiotes* biomass density between the winter season and the rest of the year might be attributed to the temperature decrease during such season, an effect already reported by the pioneer work of Odum [28] and then by Perdomo et al. [29].

In this work, the RGR of *P. stratiotes* registered in the Sordo River water during summer was of 0.064 day--1, a value that is higher than the one previously reported for this species grown in other type of wastewater. Sooknah and Wilkie [2], evaluated the growth of *Pistia* in a 1:2 dilution of dairy manure anaerobically digested during 30 days, observing a RGR of 0.011 day--1. In a study that was performed in Brazil by Henry-Silva et al. [7], the *P. stratiotes* growth was monthly evaluated in effluents from a fish farm culture and the maximum values of RGR were 0.031 and 0.016 day--1. Perdomo et al. [29] evaluated *Pistia* growth weekly during four years under temperate climate using a synthetic medium and obtained RGR values between 0.006 and 0.039 day--1. Thus, the results presented in this work indicate that this plant was able to uptake nutrients at a fast rate (see below) and that accelerated growth during summer was the result of availability of nutrients in the polluted river, high temperature, high light intensity and absence of toxic compounds.

The growth curve using WSR and WSR + F, during the month of May, presented a triauxic growth pattern. Meanwhile, a diauxic pattern was observed in the growth curves using the SWW, as well as in the curves with WSR, performed during August and December. This kind of growth was initially observed and described in bacteria [30]; nevertheless it has been also studied in yeasts [31], fungi [32] and vegetal cells [33]. Diauxic growth is due to the sequential usage of two sources of carbon or nitrogen. In aquatic plants it has been reported that *Salvinia minima* also presented diauxic growth when it was cultured in synthetic medium (Hutner medium) under controlled pH [3]. The authors mentioned that this kind of growth was possible due to the sequential use of two sources of nitrogen, being the first one the nitrogen taken from the medium and the second one the use of an intracellular source of nitrogen such as a reserve compound. However, further research is needed to elucidate in depth the nature of the nitrogen sources used sequentially in the case of the growth of *P. stratiotes* in polluted water containing several organic compounds which could serve as nitrogen sources. The formation of nitrogen rich reserve compounds which could be used in the second exponential or third exponential phase is feasible taking into account that it has been reported that the accumulation of nitrogen in the tissues of *P. stratiotes* was five to 15 fold higher than the concentration of nitrogen in the water and that the accumulation in the tissues was higher during summer compared to winter [6].

Concerning the removal capacity of *P. stratiotes* for various nutrients from the WSR during all seasons tested, the values of nutrient removal observed after ten days of experimentation (TKN = 22--91%, N-NH4+ = 96--99%, P-PO43-- = 95--99%, N-NO3-- = 17--98%) are similar or even higher to those that have been reported in other studies performed with this species [8, 9, 34].

Nitrogen forms that are mostly used by aquatic plants are N-NH4+ and N-NO3--, from these, N-NH4+ is preferred as source of nitrogen as it is energetically more efficient [35--37]. The preference of N-NH4+ consumption over N-NO3-- in different species of aquatic plants has been determined in other studies. In a study carried out by Nelson et al. [35], it was concluded that *P. stratiotes* consumes N-NH4+ more rapidly than N-NO3--. Reddy and Tucker [36] reported that *Eichhornia crassipes* was more efficient in using N-NH4+ in comparison to N-NO3--, when the two forms of nitrogen were available in the same proportion in the culture medium. For *P. stratiotes*, Wang et al. [38] demonstrated that in different media containing N-NH4+ and N-NO3-- in the same proportion as nitrogen source, the *V*max velocities were higher for NH4+ and the values of *K*m were lower for N-NH4+, indicating greater affinity for this type of nitrogen source. *Salvinia natans* also presented this consumption preference [36]. In this study, the consumption rate of N-NH4+ was significantly superior to the one of N-NO3-- during spring and summer, but not in winter. It has been shown previously that light has an effect upon the uptake rate of N-NO3-- but not on the uptake rate of N-NH4+ [35]. It is possible that light intensity and temperature were responsible for such differences found in this study in relation to the uptake rate of these two forms of nitrogen source and under the influence of seasons and further research is needed in order to understand better the effect of such environmental factors.

**5 Conclusions**

The results indicated that the water of a polluted urban river provided enough nutrients for obtaining a high productivity of *P. stratiotes* during spring and summer. During all seasons, at day 10 of the treatment, the percentage removal of N-NH4+ and P-PO43-- was >90. Comparing the three periods evaluated, at day 10 of the treatment, the highest percentage of nutrient removal was obtained during December, indicating that the systems operate effectively even at the lowest temperature tested. Thus, the use of lagoons with *P. stratiotes* serves a dual-purpose, to treat water from a polluted river and to produce a high biomass yield during spring and summer. Future studies should be performed to understand, in depth, nutrient and organic carbon uptake and the role of the dissolved oxygen consumption and of the microbial populations attached to the plants and also present in the water column.

**Acknowledgements**

The authors thank the Energy Ministry and the National Council of Science and Technology (SENER-CONACYT by its Spanish acronym) for financing the project 152931 entitled “Biorefinery for Biogas, Biodiesel and Hydrogen production from microalgae and wastewater”. They also thank the financial support from the National Council of Science and Technology (CONACYT) through the student grant 322493.

The authors have declared no conflict of interest.

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**Figure 1.** Daily variation of mean (•), maximum (■) and minimum (▲) temperature and average light intensity at three times of the day (♦10:00 am, ■2:00 pm and ▲5:00 pm) during (a) spring, (b) summer and (c) winter.

**Figure 2.** Growth curve of *P. stratiotes* in three types of wastewater during spring (May) and in WSR during summer (August) and winter (December).

**Figure 3.** pH profile in three types of wastewater during spring (May) and in WSR during summer (August) and winter (December).

**Figure 4.** Temporal changes in TKN (a), N-NH4+ (b), N-NO3-- (c) and P-PO43-- (d) concentrations in three different types of wastewater during spring (May) and in WSR during summer (August) and winter (December).

**Figure 5.** Temporal changes in COD concentration in three different types of wastewater during spring (May) and in WSR during summer (August) and winter (December).

## Table 1. Chemical characterization of synthetic wastewater (SWW), water from the Sordo River (WSR) and WSR amended with fertilizer (WSR + F)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Parameter | Type of wastewater | | | | |
| **SWW** | **WSR** | | | **WSR + F** |
| **Spring**  **(May)** | **Summer**  **(August)** | **Winter**  **(December)** |
| COD | 64.3 ± 2.7 | 101.7 ± 2.6 | 34.0 ± 4.9 | 63.7 ± 5.0 | 83.7 ± 0.5 |
| TKN | -- | 11.4 ± 0.4 | 10.2 ± 1.1 | 12.8 ± 0.2 | 34.2 ± 7.3 |
| N-NH4+ | -- | 9.8 ± 0.2 | 2.3 ± 0.1 | 11.1 ± 0.1 | 10.8 ± 0.6 |
| N-NO3-- | 1.6 ± 0.1 | 0.8 ± 0.2 | 0.2 ± 0.1 | 3.9 ± 0.2 | 0.8 ± 0.1 |
| P-PO43-- | 10.2 ± 0.1 | 3.6 ± 0.1 | 1.2 ± 0.1 | 3.3 ± 1.7 | 22.1 ± 0.1 |
| pH | 7.09 ± 0.09 | 7.16 ± 0.02 | 7.18 ± 0.02 | 7.05 ± 0.01 | 7.17 ± 0.02 |
| Trace metals (Cu2+, Cd2+, Pb2+, Zn2+, Al3+) | -- | ND | ND | ND | -- |

All values (except pH) expressed in mg L-1. Values shown as mean ± standard deviation.

ND, not detected. (Undetectable at a sensibility limit = 0.008 mg L--1)

**Table 2.** RGR and productivity of *P. stratiotes* evaluated during three seasons.

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Spring (May)** | | | | **Summer (August)** | | | **Winter (December)** | | | |
|  | **RGR (day--1)** | | **Productivity**  **(g dw m--2 day--1)** | | **RGR**  **(day--1)** | | **Productivity\***  **(g dw m--2 day--1)** | **RGR**  **(day--1)** | | **Productivity\***  **(g dw m--2 day--1)** | |
| **SWW** | µ0--4 | 0.063 | Day 10 | 0.668 ± 0.262ª | -- | | -- | -- | | | -- |
| µ7--16 | 0.023 | Day 28 | 0.379 ± 0.088x |
| **WSR** | µ0--16 | 0.046 | Day 10 | 1.042 ± 0.287b | µ0--2 | 0.064 | 0.946 ± 0.176b | µ0--10 | 0.017 | | 0.347 ± 0.102c |
| µ18--23 | 0.022 | Day 28 | 0.921 ± 0.106y | µ4--10 | 0.043 |
| µ25--18 | 0.015 |
| **WSR + F** | µ0--9 | 0.038 | Day 10 | 0.728 ± 0.287a | -- | | -- | -- | | | -- |
| µ11--21 | 0.042 | Day 28 | 0.949 ± 0.224y |
| µ23--28 | 0.027 |

\*Calculated at day 10.

Sub-index in μ indicates the period of days in which every RGR was calculated.

Different letters indicate significant differences (*p* < 0.05) in productivity. a, b, c were used for comparison between data calculated at day 10 and x, y for comparison between data calculated at day 28.

**Table 3.** Removal efficiency (%) of organic matter and nutrients in lagoons with *P. stratiotes* in three types of wastewater during spring (May), summer (August) and winter (December).

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  |  | **COD** | **P-PO4-3** | **N-NH4+** | **N-NO3-** | **TKN** |
| **SWW( May)** | Day 10 | 61.66 ± 0.080a | 11.62 ± 0.064a | ND | 92.47 ± 0.081a | ND |
| **WSR + F (May)** | 38.35 ± 0.175a | 47.11 ± 0.024c | 66.49 ± 0.016a | 62.0 ± 0.035b | 67.99 ± 0.039a |
| **WSR (May)** | 82.54 ± 0.051b | 95.05 ± 0.035b | 96.13 ± 0.010b | 69.57 ± 0.038b | 22.41 ± 0.326a |
| **WSR (August)** | --38.8 ± 0.350d | 97.69 ± 0.017b | 97.69 ± 0.017b | 16.67 ± 0.167c | 90.86 ± 0.016b |
| **WSR (December)** | 43.85 ± 0.080a | 99.10 ± 0.005b | 99.25 ± 0.002c | 98.30 ± 0.015a | 86.5 ± 0.031b |
|  |  |  |  |  |  |  |
| **SWW(May)** | Day 28 | 60.10 ± 0.074x | 61.29 ± 0.029x | -- | 100.0x | -- |
| **WSR + F (May)** | 73.71 ± 0.057x,y | 96.10 ± 0.012y | 97.83 ± 0.002x | 60.00 ± 0.202y | 99.44 ± 0.001y |
| **WSR (May)** | 78.03 ± 0.037y | 96.25 ± 0.034y | 97.92 ± 0.003x | 86.96 ± 0.052y | 98.37 ± 0.002x |

ND, not detected.

Different letters in each column indicate significant differences between treatments (*p* < 0.05). a, b, c were used for comparison between data calculated at day 10 and x, y for comparison between data calculated at day 28.

ND, not determined

**Table 4.** Ammonium nitrogen and nitrate consumption rate in WSR during three seasons.

|  |  |  |  |
| --- | --- | --- | --- |
| **WSR** | **N-NH4+ consumption rate (mg L--1 day--1)** |  | **N-NO3- consumption rate (mg L--1 day--1)** |
| May (Spring) | 2.306 ± 0.095a |  | 0.325 ± 0.147b |
| August (Summer) | 1.063 ± 0.118a |  | --0.033 ± 0.029b |
| December (Winter) | 2.21 ± 1.10a |  | 1.77 ± 0.03a |

Different letters in each row indicate significant differences between the two data (*p* < 0.05).