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**Nitrogen and organic matter removal and enzyme activities in constructed wetlands operated under different hydraulic operating regimes**

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Abstract:

Constructed wetlands (CWs) are environmentally-friendly methods for mariculture wastewater purification. The hydraulic regime is a key factor in the effectiveness of sub-surface flow CW treatment. The objectives of this study were to investigate the effects of five hydraulic operating regimes (i.e. Intermittent, Continuous, Batch I, Batch II and Batch III) on the purification performance of CW treated with mariculture wastewater and to assess the correlations between enzyme activities (i.e. urease, dehydrogenase) and purification performance of CW. Fifteen pilot sub-surface CWs with *Salicornia bigelovii* were investigated for the performance of CW as well as urease activity (UA) and dehydrogenase activity (DA). Over the experiment, removal efficiencies of TAN,  $\text{NO}_3^-$ -N, TN and COD under five hydraulic operating regimes were 26.6 to 37.2%, -6.0 to 16.5%, 9.4 to 16.8% and 33.9 to 44.6% respectively, corresponding to removal rates of 147.6 to 456.9  $\text{mg m}^{-2} \text{d}^{-1}$ , -18.1 to 229.2  $\text{mg m}^{-2} \text{d}^{-1}$ , 174.0 to 603.6  $\text{mg m}^{-2} \text{d}^{-1}$  and 501.9 to 1421.6  $\text{mg m}^{-2} \text{d}^{-1}$  respectively. CW with a Batch III operating regime had the best treatment performance, with mean removal efficiencies of TAN,  $\text{NO}_3^-$ -N, TN and COD of 37.2 %, 16.5%, 14.9 % and 34.0 % respectively, with the corresponding removal rates of 456.9, 229.2, 603.6 and 873.6  $\text{mg m}^{-2} \text{d}^{-1}$ . As for enzyme activities, the UA was significantly higher in CW under Batch II than in Intermittent and Continu-

ous operating regimes and the DA in CW with Batch I was significantly higher than under an Intermittent operating regime. UA and DA had significant positive correlations with COD concentrations but negative correlations with TAN and TN concentrations. The correlation analysis results showed that UA and DA can be an important indicator in evaluating removal performance of CW with *Salicornia bigelovii* in marine aquaculture wastewater treatment.

**Keywords:** Mariculture wastewater; constructed wetland; *Salicornia bigelovii*; enzyme activity; removal performance

## 1. Introduction

Global aquaculture production, as an alternative source of high-quality protein, expanded at an average annual rate of 5.8% during 2005-2014, to 73.8 million tons (FAO<sup>1</sup>, 2016). Marine aquaculture releases a large quantity of wastewater. Without proper treatment, the discharged dissolved nitrogenous substances (e.g.  $\text{NH}_4^+$ ,  $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ) and organic matter could have a detrimental effect on aquatic organisms and give rise to eutrophication in receiving ecosystems (Hongfang et al., 2014; Wu Haiming et al., 2015). Mariculture effluents therefore need to be treated properly to avoid environmental hazards.

Many conventional biological methods, including submerged bio-filters, trickling filters, rotating biological contactors and fluidized bed reactors, have been applied worldwide in aquaculture wastewater purification (Van Rijn, 1996). Compared with these costly approaches in terms of installation and operation as well as energy consumption (Papenbrock & Turcios, 2014), constructed wetlands (CW) are now attracting more attention and are considered promising for being environmentally-friendly and for their cost and efficiency features (Shpigel et al., 2013; Wu Haiming et al., 2015). Over the past few decades, CW have proved to be functional in treating domestic municipal wastewater (Kivaisi, 2001), agricultural effluents (Wood et al., 2007), landfill leachates (Justin & Zupančič, 2009) and industrial sewage water

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<sup>1</sup> Food and Alimentation Organization

(Guittonny-Philippe et al., 2015). CW can also effectively remove nitrogen and organic matter from catfish, shrimp and milkfish pond effluents (Schwartz & Boyd, 1995; Sansanayuth et al., 1996; Lin et al., 2002). Recently, mariculture effluent purification has become increasingly important with the expansion of aquaculture and the development of more intensive land-based marine farms (Webb et al., 2012).

The CW purification performance is mostly attributed to the combined functions of microbes and filtering materials, which could be complemented by hydrophyte (Truu Marika et al., 2009). As an effective way to describe the general conditions of CW substrate microbial populations (Margesin et al., 2000a; Margesin et al., 2000b), substrate enzyme activity (EA) plays a vital role in nitrogen and organic matter transformations (Zhou et al., 2005; Reboreda & Caçador, 2008). Urease, for example, catalyzes the hydrolysis of urea into ammonia and carbon dioxide; dehydrogenase is an indicator of microbial activity in CW beds, considering its function in oxidizing organic matter by transferring electrons and protons from substrates to acceptors (Zhang et al., 2013; Baddam et al., 2016). Consequently, measuring enzyme activities in CW helps to provide useful information in understanding and controlling the purification activity of the microorganisms.

According to the hydrology and flow paths, CW can be divided into three categories: surface flow constructed wetlands (SFCW), horizontal sub-surface-flow constructed wetlands (HSSF-CW) and vertical-flow constructed wetlands (VFCW) (Vymazal, 2007). Previous studies have shown that a single-stage CW is unable to achieve adequate total nitrogen (TN) removal due to its inability to provide favorable conditions simultaneously for nitrification and denitrification (Zhi et al., 2015). One common way to enhance TN removal is to configure the hybrid systems using different categories of CW or to couple CW with other types of treatment system (i.e. the electrolysis system and the microbial fuel cell system) (Ayaz et al., 2012; Zhao et al., 2013; Ju et al., 2014). However, it is difficult to popularize these systems, because they will inevitably bring extra land occupation and capital investment or require complex operating conditions. An alternative to improve the purification capacity of single-stage CW is to adjust the hydraulic operating regime, which may affect the substrate microorganisms, thus enhancing the wastewater purification performance of sub-surface flow CW without additional cost (Zhi & Ji, 2014). Batch flow (also called tidal flow), Continuous flow and Intermittent flow are three common hydraulic operating regimes used in sub-surface CW (Faulwetter et al., 2009). CW with Batch operating regimes generate a rhythmic sequential cycle of “flooding” and “draining/resting” phases: wetlands are filled with wastewater for a determined period



and completely drained before injection of the next batch of wastewater. In Intermittent flow operation, wetlands are not drained before refilling with wastewater (Caselles-Osorio & García, 2007). To date, only a few studies focusing on the three operating regimes and treatment performance have been published (Faulwetter et al., 2009), and, in particular, little information is available about relating the removal performance of CW under different hydraulic operating regimes to substrate enzyme activities. The relationships between enzyme activities and aquaculture effluent concentrations would promote our ability to understand the treatment processes and better monitor the wetland management in order to control the effluent quality reliably.

CW for marine aquaculture wastewater treatment necessitate the selection and use of hydrophytes with a high salinity tolerance. As illustrated in previous studies, reed, cattail (*Typha angustifolia* L.), *P. australis*, mangroves and *Salicornia* spp. are common halophytes used in mariculture effluent purification (Alongi, 2002; Lin et al., 2002; Lin et al., 2005; Shpigel et al., 2013). Among these, *Salicornia bigelovii*, is often used due to its high nutrient removal capacity at seawater salinity, as well as its promising commercial value (potential use in the health, cosmetic and nutraceutical industries) (Brown et al., 1999; Manhee et al., 2009; Quintã et al., 2015).

This paper presents the results of a 78-day experiment using pilot-scale CW planted with *Salicornia bigelovii* and fed with artificial mariculture wastewater under different hydraulic operating regimes. An original feature of the study is that it focuses at the same time on 1), evaluating the effects of hydraulic operating regimes (i.e. Intermittent, Continuous, Batch I, Batch II, Batch III) on CW performance, considering the nitrogen and organic matter removal efficiencies and removal rates and plant growth and 2), investigating the potential of urease activity (UA) and dehydrogenase activity (DA) as indicators to describe CW performance with *Salicornia bigelovii* in mariculture wastewater treatment.

## 2. Material and Methods

The 78-day experiment was conducted using five triplicate pilot CW systems on an intensive recirculating Atlantic salmon farm (i.e. Oriental Ocean Ltd., located in Shandong Province, China) from 28 July 2016 to 13 October 2016. The five triplicates were performed under the five hydraulic operating regimes described in 2.2. Each CW system included one barrel and three square tanks. Plants in the CW (i.e. *Salicornia bigelovii*) were grown initially in Weifang City, Shandong Province, China. They went through thirty-day salt acclimation to adapt to the fish-rearing salinity before being planted in the CW.

Artificial wastewater was made of uneaten feed and faeces collected from an Atlantic salmon RAS following the steps described by Zhang et al. (2011). Uneaten feed and salmon faeces were firstly oven dried (105°C for 48 h). After grinding to powder, they were then mixed with seawater (i.e. 250 g powder in 1 L seawater) and transferred into a closed container for seven days to carry out anaerobic fermentation; finally, the “mother liquid” was obtained after filtration through bolting-silk (Mesh: 100). During the experimental period, artificial wastewater was treated separately in the fifteen CW under different hydraulic operating regimes. *Salicornia* production, nitrogen content in plants and four water quality parameters in each CW were monitored. Principal components analysis (PCA) was used to identify the optimal regime.

### 2.1. Filter construction and operation

The pilot CW system is shown in Fig. 1 (A). Artificial wastewater was stored in a tank (4.65 × 2.25 × 0.60 m, W × L × H), and distributed into five barrels (D, 0.90 m; H, 0.67m); wastewater was pumped from each barrel to the three parallel CW (0.30 × 0.30 × 0.30 m, W × L × H) using peristaltic pumps. After purification by CW, wastewater drained out of the cylindrical barrel by gravity. During the experimental period, each CW had twelve individual *Salicornia bigelovii* plants and the flow rate was maintained at 100 ml.min<sup>-1</sup>.

The fifteen identical sequencing Batch sub-surface flow (SSF) CW microcosms (five sets × three replicates) were built as shown in Fig. 1 (B): Their filter beds were constructed of polypropylene random (PPR) frames, placed on a stainless steel stand above the cylindrical barrels and filled to 80 mm of clean, graded smooth cobblestone (Ø 30-50 mm) to allow a smooth sub-surface flow. The cobblestone was overlaid with a sheet of plastic mesh (0.2 mm<sup>2</sup> pore size) to separate the two layers and a 100 mm layer of clean haydites (Ø 5-8 mm). The first layer of haydites was covered with plastic mesh (0.074 mm<sup>2</sup> pore size) and a second layer of smaller haydites (120 mm, Ø 3-5 mm).

## 2.2. Experimental design

Five trials were performed simultaneously under different flow regimes, including Mode 1 (Intermittent: hydrostatic test for 6 h, four batches per day); Mode 2 (Continuous); Mode 3 (Batch I : 12 h flooding, 12 h resting); Mode 4 (Batch II : 16 h flooding, 8 h resting); Mode 5 (Batch III: 19 h flooding, 5 h resting). The different flow regimes were controlled manually throughout the experiment. The CW were fed with Atlantic salmon wastewater for one year before the experiment started.

## 2.3. Sampling and analysis

Water samples were taken every week from the inlet and outlet of each CW. The pH and water temperature were measured and recorded using a YSI pH 100. The samples were refrigerated at 4°C in labelled polystyrene bottles for chemical analysis. Water samples were analyzed for total ammonium nitrogen (TAN), nitrite ( $\text{NO}_2^-$ -N), nitrate nitrogen ( $\text{NO}_3^-$ -N), total nitrogen (TN) and chemical oxygen demand (COD). TAN and  $\text{NO}_2^-$ -N measurements were performed following the seawater quality standard protocol (China, 2002);  $\text{NO}_3^-$ -N was measured using ultraviolet spectrophotometry according to Gong Z J (2005); TN concentration was analyzed through the combined digestive method described by Xing D L (2006) and COD concentration was determined by the oven-heating method according to Shen et al. (2011).

The activity of N and organic matter removal related to substrate enzymes (i.e. urease and dehydrogenase) was determined five times over the 78 days. The substrate samples (100 g haydites) were taken randomly at six points from each CW upper layer (3-7 cm depth), considering the higher enzyme activity in sediment upper layers (Niemi et al., 2005; Baddam et al., 2016). The samples were then mixed and air dried in the laboratory for 24 hours. UA was determined using the procedure described by Klose and Tabatabai (2000). DA was measured using the TTC-method according to Zhu et al. (1995).

#### 2.4. Statistical analysis

The mean, standard deviation, one-way analysis of variance (ANOVA), principal components analysis (PCA) and correlation analysis were calculated using Microsoft Excel and SPSS 21.0.

Over the study, plant growth rate, nutrient removal efficiency, nutrient removal rate and enzyme activity under different hydraulic operating regimes were analyzed by ANOVA for LSD tests. The statistical analysis was considered significant below 0.05 ( $p < 0.05$ ).

In the experiment, various parameters could change widely even with similar flow conditions. Hence, in order to understand better the effects of different flow regimes, PCA was used as a multivariate data reduction technique to generate scores representing trends observed between simultaneously-measured indicators (Turnbull et al., 2005). PCA reduces the dimensionality of possibly correlated variables by using orthogonal transformation into a set of values of linearly uncorrelated variables (principal components) (Zhao & Tan, 2015). The variables addressed in the PCA were water quality index (four data sets of TAN, Nitrate, TN and COD), *Salicornia* growth as well as nitrogen retention in plants. The relative contribution of each variable within the different principal components (PC) was obtained along with factor scores (FS) for each of the valid PC (Eigenvalues > 1) for each treatment within the data set.

### 3. Results

### 3.1. Water temperature and pH values

Over the entire experiment, no significant difference ( $P > 0.05$ ) was observed in water temperatures and pH values in the various flow regime treatments. Water temperature varied from 17.8 to 27.0°C ( $23.4 \pm 3.3^\circ\text{C}$ ), and pH values from 7.8 to 8.6 ( $8.1 \pm 0.1$ ).

### 3.2. Salicornia production

Plant growth rate and nitrogen retention in plants under different flow regimes are shown in Table 1. At the end of the experiment, the average Salicornia fresh weights under the five operating regimes (from Mode 1 to Mode 5) were  $16.9 \pm 2.7$ ,  $25.9 \pm 4.5$ ,  $17.6 \pm 3.3$ ,  $17.0 \pm 1.3$  and  $16.7 \pm 3.0$  g ind<sup>-1</sup> respectively. Plants in CW with Continuous operating regimes grew faster ( $P < 0.05$ ) than in any other regimes, with a growth rate of  $40.4 \pm 9.9$  g m<sup>-2</sup> d<sup>-1</sup>. There is no significant difference between the Salicornia growth rates under the other four operating regimes. The values for Intermittent and Batch modes were  $25.7 \pm 2.8$  (Intermittent),  $27.0 \pm 3.9$  (Batch I),  $26.0 \pm 2.3$  (Batch II) and  $25.3 \pm 4.6$  g m<sup>-2</sup> d<sup>-1</sup> (Batch III) respectively. Over the experiment (78 days in total), Salicornia retained 2792.3, 2521.8 and 1935.3 mg N m<sup>-2</sup> in Batch I, Batch II and Batch III operating regimes respectively, while Salicornia under Intermittent flow retained only 633.3 mg N m<sup>-2</sup>.

### 3.3. Mariculture effluent purification

The influent and effluent concentrations, as well as the removal performance of TAN,  $\text{NO}_2^-$ -N,  $\text{NO}_3^-$ -N and TN are shown in Fig. 2a-d and Table 2. Relatively constant reductions ( $26.6 \pm 14.2$  to  $37.2 \pm 13.7$  %) of TAN were achieved in all CW throughout the experiment without significant difference ( $p > 0.05$ ). CW in Continuous and Batch III modes had a higher removal rate ( $432.8 \pm 219.3$  and  $456.9 \pm 198.8$   $\text{mg m}^{-2} \text{d}^{-1}$  respectively) while CW with Intermittent flow had the lowest ( $147.6 \pm 85.3$   $\text{mg m}^{-2} \text{d}^{-1}$ ). No significant accumulation of  $\text{NO}_2^-$ -N (less than  $0.025$   $\text{mg L}^{-1}$ ) was observed in the effluent of the CW under five operating regimes. Also, no significant difference was observed for  $\text{NO}_3^-$ -N removal efficiency between the five CW hydraulic regime treatments ( $p > 0.05$ ). However, CW with Intermittent operating regimes had an  $\text{NO}_3^-$ -N accumulation (negative removal efficiency and removal rate) and CW in the three Batch operations had higher  $\text{NO}_3^-$ -N removal rates (average of  $126.6$ - $229.2$   $\text{mg m}^{-2} \text{d}^{-1}$ ) compared with Intermittent and Continuous treatments ( $-18.1$  and  $14.7$   $\text{mg m}^{-2} \text{d}^{-1}$  respectively). CW in Intermittent flow operation had also significantly lower ( $p < 0.05$ ) TN removal efficiency and removal rate over the experiment ( $9.4 \pm 5.8$  % and  $174.0 \pm 104.9$   $\text{mg m}^{-2} \text{d}^{-1}$  respectively).

The COD concentrations over the sampling time as well as the overall removal performance are shown in Fig. 2e and Table 2. Average COD removal efficiency was not significantly dif-



ferent ( $p > 0.05$ ) between the five hydraulic operating treatments and was maintained in the range of 33.9 - 44.6 %. However, the average COD removal rates of the CW under the five hydraulic regimes were significantly different. CW in Continuous flow had far higher average COD removal rates ( $1421.6 \pm 365.4 \text{ mg m}^{-2} \text{ d}^{-1}$ ) ( $p < 0.01$ ) than CW in the other flow regimes (501.9 to  $873.6 \text{ mg m}^{-2} \text{ d}^{-1}$ ).

#### 3.4. Principle component analysis (PCA) of CW treatment performance

The PCA extracted two principle components (i.e. PC1 and PC2) from ten observations of potentially correlated variables and received a single factor score (FS) reflecting the CW performance. The ten observations of variables included plant growth and nitrogen retention, waste removal efficiency and removal rate. Based on Eigenvalues ( $> 1$ ), two variable PC were extracted, accounting together for 90.4% of the observed variability (Fig. 3). Nitrogen retention,  $\text{NO}_3^-$ -N removal rate and removal efficiency of TAN,  $\text{NO}_3^-$ -N and TN had high positive loadings on the PC1 axis and negative loading on the COD removal efficiency. On the PC2 axis, there were high positive loadings for plant growth rate and the TAN and COD removal rates.

Eigenvalues for PC1 and PC2 were 5.883 and 3.159 respectively. Based on these values, the expressions to calculate FS1, FS2 and FS are as below:

$$\begin{aligned}
 FS1 = & 0.409NitraterE + 0.405NitraterR + 0.376TNRE - 0.362CODRE \\
 & + 0.356TANRE + 0.332NR + 0.302TNRR - 0.047CODRR + 0.188TANRR \\
 & - 0.176PGR
 \end{aligned}$$

$$\begin{aligned}
 FS2 = & 0.037NitraterE + 0.004NitraterR - 0.126TNRE + 0.249CODRE \\
 & + 0.086TANRE - 0.017NR + 0.378TNRR + 0.558CODRR + 0.485TANRR \\
 & + 0.474PGR
 \end{aligned}$$

$$FS = \frac{PC1}{PC1 + PC2} FS1 + \frac{PC2}{PC1 + PC2} FS2$$

Where, FS1 means the factor score for PC1, FS2 means the factor score for PC2 and the FS means the factor score.

Table 3 presents the principal component scores and sorting. Batch operating regimes scored higher in FS1 while Continuous flow operation scored the highest in FS2. Batch III ranked first for the FS scores. CW with Intermittent flow had the lowest score for FS1, FS2 and FS.

### 3.5. Substrate enzyme activity

UA and DA throughout the experiment and their correlations to nitrogen (i.e. TAN, NO<sub>3</sub><sup>-</sup>-N and TN) and organic matter (COD) concentrations are shown in Fig. 4 and Table 4 respectively. Significant differences in UA and DA were observed between Batch, Continuous and Intermittent operating treatments. With regard to the correlations, UA and DA had a significant

positive correlation with COD concentrations, but a negative correlation with TAN and TN concentrations ( $p < 0.05$ ).

#### 4. Discussion

##### 4.1. CW with a *Salicornia bigelovii* bed as the bioreactor unit

One main objective of this study was to assess the ability of CW planted with *Salicornia bigelovii* to recycle mariculture wastewater under different hydraulic operating regimes. Over the experiment, *Salicornia* grew well in CW and the amount of nitrogen retained in plant tissues (Table 1) is comparable with that measured by Webb et al. (2012), who found a 1.09 mol N m<sup>-2</sup> (i.e. 173.4 mg N m<sup>-2</sup> d<sup>-1</sup>) retention in *Salicornia europaea* agg. in an 88-day experiment with wastewater from marine fish and shrimp in RAS. Over the same period, in the present study, the CW also removed TN (174.0 to 603.6 mg N m<sup>-2</sup> d<sup>-1</sup>), which is in line with the TN removal rates of CW with *Salicornia* (144.2, 203.6 and 868.0 mg N m<sup>-2</sup> d<sup>-1</sup>) recorded by Webb et al. (2012); Shpigel et al. (2013); Webb et al. (2013).

The COD removal performance obtained in our study (0.5 to 1.4 g m<sup>-2</sup> d<sup>-1</sup> of removal rates, 33.9-44.6% of removal efficiency) is in line with previous studies using sub-surface flow wetlands for fresh water aquaculture wastewater purification (Naylor et al., 2003; Sindilariu et al., 2007). Sindilariu *et al.* (2007) used sub-surface flow CW to treat flow-through trout

wastewater in Bavaria, Germany and obtained a COD removal rate of 0.4 to 1.4 g m<sup>-2</sup> d<sup>-1</sup>, with a removal efficiency of 24.3 to 52.2%. Naylor et al. (2003) used synthetic aquaculture wastewater and found a COD removal efficiency ranging from 52.8% to 91.1% in six sub-surface wetlands with different filtering materials.

#### 4.2. Effects of hydraulic operating regimes on CW removal performance

In this study, the Batch operating regime was more efficient in removing TAN, NO<sub>3</sub><sup>-</sup>-N and TN than the Continuous and Intermittent regimes. Clearly, Batch loadings enhanced N removal, which agrees with previous CW investigations, e.g. for the treatment of piggery sewage and domestic wastewater (Sun et al., 2005; Chan et al., 2008; Zhang et al., 2012; Jong & Tang, 2016). In Batch modes, the oxygen transportation and consumption rate in the CW filter beds could be greatly promoted by the alternating “ponding” and “resting” phrases (Sun et al., 1999). This is closely related to sustaining aerobic conditions within CW beds for the growth of attached microorganisms biodegrading nitrogen wastes (Chan et al., 2008). In Intermittent operation, CW were filled with wastewater immediately after draining, with no “resting” phase, which limited the oxygen addition compared with the Batch modes.

In the present study, the three Batch operating regimes had different TN removal, considering the different “ponding” and “resting” period: Batch III > Batch II > Batch I . As illustrated by

Lin et al. (2002) and Hadad et al. (2006), nitrification and denitrification are the main microbial processes responsible for nitrogen removal in wetlands. TN removal would be enhanced with a proper anaerobic environment and sufficient carbon source for denitrification process (Jong & Tang, 2016). Therefore, the ponding and resting periods are both important for TN removal: the ponding phase promotes an oxygen-deficient microenvironment in the filter beds and ensures a sufficient wastewater residence time for denitrification, while the resting phase provides re-oxygenation conditions for nitrification after the ponding period (Jong & Tang, 2016). Furthermore, the increased ponding period from Batch I to Batch III allows more nutrients to be supplied for microorganisms under aerobic (early period of ponding after a sufficient rest period) and anaerobic (extended period of ponding) conditions enhancing the N removal (Sun et al., 2005).

When examining the three Batch regimes, we can conclude that Batch III (19 h ponding, 5 h resting) showed a higher nitrogen removal through allowing maximum wastewater-substrate (i.e. biofilm) contact and promoting the oxygen transfer by drawing air from the atmosphere into filter beds when “ponding” and “resting” periods were alternating. It indicates that CW performed well with mariculture wastewater, considering *Salicornia* growth and organic and nitrogen removal efficiency.

However, the effects of hydraulic operating regimes on COD removal efficiency were different from those on N removal. Our results showed that in Intermittent or Batch operating regimes, removal efficiencies of COD were not improved compared with a Continuous flow regime. This agrees with the investigations of Caselles-Osorio and García (2007) and Zhang et al. (2012), who found no difference in COD removal of CW at different feeding strategies with synthetic and urban wastewater. However, more oxidized conditions are generally observed in Batch-fed wetlands, which therefore encourages COD removal compared with a Continuous flow (Faulwetter et al., 2009). Considering this, a study will be conducted to understand further the COD removal mechanisms, e.g. to relate the redox conditions and the substrate microbial community in CW treating mariculture wastewater under different hydraulic operating regimes to COD removal capacity.

#### 4.3. Enzymatic activity response to mariculture effluents

Oxygen availability, organic matter and nutrient availability are common factors affecting enzyme activities in wetlands (Baddam et al., 2016). In our study, correlations between substrate enzyme activity and nutrient concentrations (i.e. TAN,  $\text{NO}_3^-$ -N, TN and COD) varied greatly (Table 4). UA had a significant negative correlation with TAN and TN concentrations, which agrees with previous investigations (Kong et al., 2009; Cui et al., 2013; Baddam et al.,

2016). From an ecological point of view, inverse relationships were observed between enzyme activity and nutrient availability (Sinsabaugh et al., 1993) considering the repression-depression and end-product inhibition of enzymes (Chrost, 1991). As an N resource for nitrification (Dong & Reddy, 2010) as well as the end product of urea hydrolysis (Thorén, 2007), decreased  $\text{NH}_4^+$  concentration stimulates urease enzyme microbial production for urea hydrolyze. This also explains the higher UA in CW in a Batch operating regime: during the “resting” period, filter beds drain completely enhancing oxygen transfer to improve nitrification (Ni et al., 2016). This is in line with the better N removal performance in Batch operation evidenced by the higher PC1 scores. Consequently, UA in the rhizosphere can be used as the major indicator to estimate N removal performance of CW in treating mariculture wastewater.

Dehydrogenase, expressing the biological oxidation processes of substrate microorganisms (Nannipieri, 1994), had the highest activity in CW with Batch operating regimes (Batch I). It is known to promote the oxidation conditions in CW (Jong & Tang, 2016). Dehydrogenase, which is sensitive to oxygen levels, has been proposed to measure global soil microbial activity in aerobic conditions (Caravaca et al., 2005). Furthermore, it has been shown that the activity and decomposition ability of dehydrogenase is dependent on organic substance availability (Baddam et al., 2016). A more accurate representation and analysis of the relations between

DA and oxygen levels, as well as the organic substance availability in CW under different operating regimes, necessitate further research. The correlation analysis in the present research suggests that DA has the potential to be an indicator in estimating the removal performance of CW in mariculture wastewater treatment.

## 5. Conclusion

This study on the nitrogen and COD removal capacity of a single-stage HFCW under different operating regimes (i.e. Intermittent, Continuous, Batch I, Batch II and Batch III) has produced the following new elements:

- CW with *Salicornia* have the potential to remove nitrogen and organic matters in mariculture wastewater, with nitrogen removal rates of 174.0 to 603.6  $\text{mg}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ , COD removal rates of 501.9 to 1421.6  $\text{mg}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$ ; and 9.0 to 39.8  $\text{mg N m}^{-2}\cdot\text{d}^{-1}$  retained in *Salicornia* tissues.
- Batch III operating regime (19 h ponding, 5 h resting) gives the best performance considering removal efficiency and the removal rate of nitrogen and organic matter as well as *Salicornia* growth.



- UA and DA are good potential indicators in evaluating CW performance with *Salicornia* in mariculture wastewater treatment: UA and DA have a negative correlation with TAN and TN concentrations, but a positive correlation with COD concentration.

To conclude, CW with a *Salicornia bigelovii* bed can act as a bioreactor unit to recycle mariculture nitrogen and COD effluents with the same efficiency as found in previous studies. The Batch regime showed higher nitrogen removal promoting the oxygen transfer. However, COD operating regimes did not differ. In our study, correlations between substrate enzyme activity and nutrient concentrations varied greatly, in line with the nutrient removal performance. Our study suggests that UA in the rhizosphere can be used as an indicator to estimate N removal performance and that UA can potentially be an indicator in estimating the removal performance of CW in mariculture wastewater treatment. Further investigations are necessary to develop complex and precise expressions using UA and DA with more predictive power, and to understand the COD removal mechanisms, e.g. to relate the redox conditions, the microbial community and diversity role in CW treating mariculture wastewater. Besides, the pilot CWs in our experiment give a proof of concept, but are specific to the tested experimental conditions. Other experiments at a

farm level taking the seasonal variations (i.e. temperature and solar energy) into account

have to be conducted and will permit to validate our results.

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**Tables**

Table 1 *Salicornia bigelovii* growth and nitrogen retention (n=3) in different operating hydraulic regimes

Operating regime	Initial fresh weight (g ind <sup>-1</sup> )	Final fresh weight (g ind <sup>-1</sup> )	Plant growth rate (g m <sup>-2</sup> d <sup>-1</sup> )	Nitrogen retention rate (mg N m <sup>-2</sup> d <sup>-1</sup> )
Intermittent	1.8 ± 0.1 <sup>a</sup>	16.9 ± 2.7 <sup>c</sup>	25.7 ± 2.8 <sup>c</sup>	9.0
Continuous	2.2 ± 0.2 <sup>a</sup>	25.9 ± 4.5 <sup>a</sup>	40.4 ± 9.9 <sup>a</sup>	21.5
Batch I	1.8 ± 0.1 <sup>a</sup>	17.6 ± 3.3 <sup>bc</sup>	27.0 ± 3.9 <sup>bc</sup>	39.8
Batch II	1.8 ± 0.1 <sup>a</sup>	17.0 ± 1.3 <sup>c</sup>	26.0 ± 2.3 <sup>c</sup>	35.9
Batch III	1.9 ± 0.1 <sup>a</sup>	16.7 ± 3.0 <sup>c</sup>	25.3 ± 4.6 <sup>c</sup>	27.6



Table 2 Influent concentration (IN), effluent concentration (OUT), removal efficiency (RE) and removal rate (RR) of CW under five operating regimes.

Parameters	Operating regime	IN (mg L <sup>-1</sup> )	OUT (mg L <sup>-1</sup> )	RE (%)	RR (mg m <sup>-2</sup> d <sup>-1</sup> )
TAN	Intermittent	0.95 ± 0.10	0.69 ± 0.13	26.6 ± 14.2 <sup>a</sup>	147.6 ± 85.3 <sup>d</sup>
	Continuous		0.68 ± 0.11	28.0 ± 11.9 <sup>a</sup>	432.8 ± 219.3 <sup>ab</sup>
	Batch I		0.65 ± 0.12	30.4 ± 13.5 <sup>a</sup>	234.8 ± 116.7 <sup>d</sup>
	Batch II		0.66 ± 0.10	30.1 ± 11.5 <sup>a</sup>	309.1 ± 134.0 <sup>bc</sup>
	Batch III		0.59 ± 0.10	37.2 ± 13.7 <sup>a</sup>	456.9 ± 198.8 <sup>a</sup>
NO <sub>3</sub> <sup>-</sup> -N	Intermittent	1.14 ± 0.22	1.15 ± 0.27	-6.0 ± 35.8 <sup>a</sup>	-18.1 ± 240.7 <sup>a</sup>
	Continuous		1.11 ± 0.42	1.1 ± 33.6 <sup>a</sup>	14.7 ± 705.2 <sup>a</sup>
	Batch I		0.96 ± 0.35	12.7 ± 34.8 <sup>a</sup>	126.6 ± 364.6 <sup>a</sup>
	Batch II		0.99 ± 0.33	10.5 ± 29.4 <sup>a</sup>	133.6 ± 437.6 <sup>a</sup>
	Batch III		0.94 ± 0.34	16.5 ± 30.8 <sup>a</sup>	229.2 ± 477.3 <sup>a</sup>
TN	Intermittent	3.12 ± 0.43	2.82 ± 0.40	9.4 ± 5.8 <sup>b</sup>	174.0 ± 104.9 <sup>c</sup>
	Continuous		2.80 ± 0.47	10.4 ± 7.6 <sup>b</sup>	520.4 ± 393.2 <sup>a</sup>
	Batch I		2.60 ± 0.49	16.8 ± 9.8 <sup>a</sup>	415.7 ± 218.2 <sup>ab</sup>
	Batch II		2.65 ± 0.46	15.2 ± 7.4 <sup>ab</sup>	500.0 ± 203.4 <sup>a</sup>
	Batch III		2.64 ± 0.38	14.9 ± 7.7 <sup>ab</sup>	603.6 ± 315.5 <sup>a</sup>
COD	Intermittent	2.14 ± 0.57	1.27 ± 0.41	41.2 ± 9.9 <sup>a</sup>	501.9 ± 174.2 <sup>e</sup>
	Continuous		1.25 ± 0.59	44.6 ± 16.4 <sup>a</sup>	1421.6 ± 365.4 <sup>a</sup>
	Batch I		1.44 ± 0.64	35.7 ± 20.5 <sup>a</sup>	560.7 ± 260.7 <sup>de</sup>
	Batch II		1.46 ± 0.60	33.9 ± 16.1 <sup>a</sup>	726.0 ± 332.3 <sup>cde</sup>
	Batch III		1.45 ± 0.61	34.0 ± 18.1 <sup>a</sup>	873.6 ± 481.1 <sup>c</sup>

Table 3 Principal component scores and sorting

Hydraulic operating regimes	CF1	CF1 sequence	CF2	CF2 sequence	CF	CF sequence
Intermittent	-3.181	5	-1.741	5	-2.678	5
Continuous	-1.882	4	2.763	1	-0.259	4
Batch I	1.210	3	-1.131	4	0.392	3
Batch II	1.224	2	-0.531	3	0.611	2
Batch III	2.629	1	0.640	2	1.934	1

Table 4 Correlation of nitrogen and COD concentrations to the activity of urease and dehydrogenase over the experimental phase

Concentration Enzyme	TAN	NO <sub>3</sub> <sup>-</sup> -N	TN	COD
Urease	-0.293 <sup>*</sup>	-0.163	-0.459 <sup>**</sup>	0.379 <sup>**</sup>
Dehydrogenase	-0.350 <sup>**</sup>	-0.145	-0.457 <sup>**</sup>	0.476 <sup>**</sup>

<sup>\*</sup>Correlation was significant at the 0.05 level.

<sup>\*\*</sup>Correlation was significant at the 0.01 level.

**Figures**

Fig. 1 The pilot CW system (A) and a single CW unit (B)

(a, settlement tank: 4.65 m×2.25 m×0.60 m, W×L×H; b, cylindrical barrel: D= 0.90 m, H= 0.67 m; c, pilot wetland: 0.30 m× 0.30 m× 0.30 m, W×L×H. Mode 1: Intermittent flow operation; Mode 2: Continuous flow operation; Mode 3: Batch I; Mode 4: Batch II; Mode 5: Batch III)

Fig. 2 Dynamics of TAN (a),  $\text{NO}_2^-$ -N (b),  $\text{NO}_3^-$ -N (c), TN (d) and COD (e) concentrations in the influent and effluent of five hydraulic operating regimes.

Fig. 3 Factor loading plots for principal component analysis in the experimental phase (PGR – Plant growth rate; NR – Nitrogen retention in plant; TANRR – TAN removal rate, NitrateRR – Nitrate removal rate, TNRR – TN removal rate, CODRR- COD removal rate; TANRE – TAN removal efficiency, NitrateRE – Nitrate removal efficiency, TNRE – TN removal efficiency, CODRE – COD removal efficiency)

Fig. 4 Temporal values of urease (UA) and dehydrogenase (DA) activities in CW under different hydraulic operating regimes

## Highlights

- Constructed wetlands with *Salicornia bigelovii* have the potential to remove nitrogen and organic matters from mariculture wastewater
- The batch operating regime (19 h ponding, 5 h resting) gives the best performance for nitrogen and organic matter removal and *Salicornia* growth in wetlands
- Substrate enzyme activities, i.e. urease activity (UA) and dehydrogenase activity (DA), have the potential to be indicators of nitrogen and organic matter removal performance in CWs for mariculture wastewater treatment

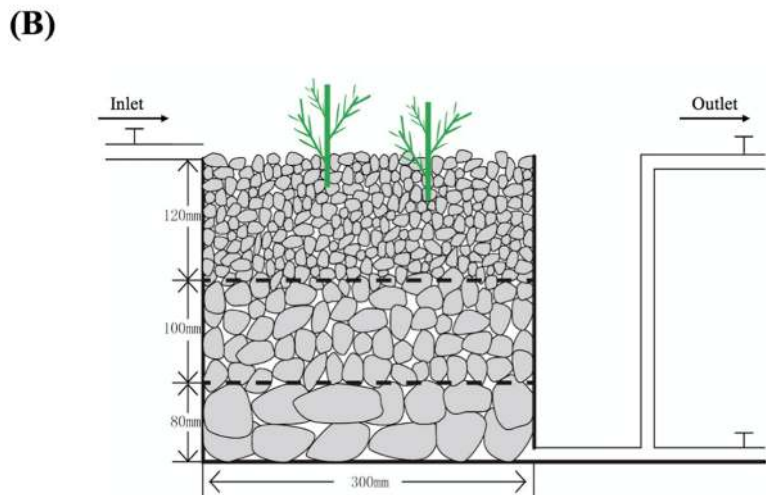
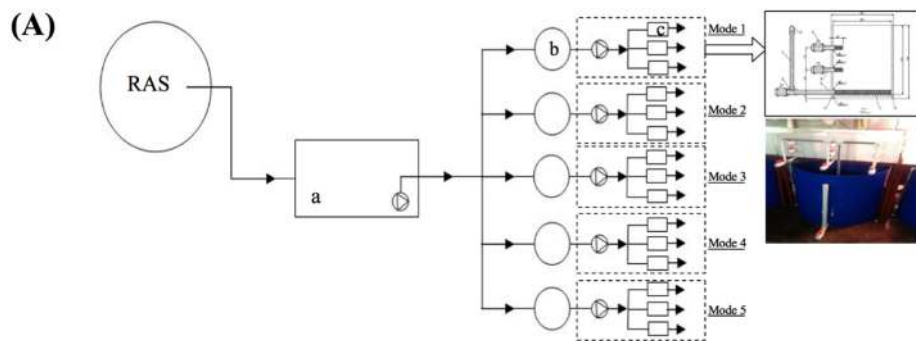


Figure 1

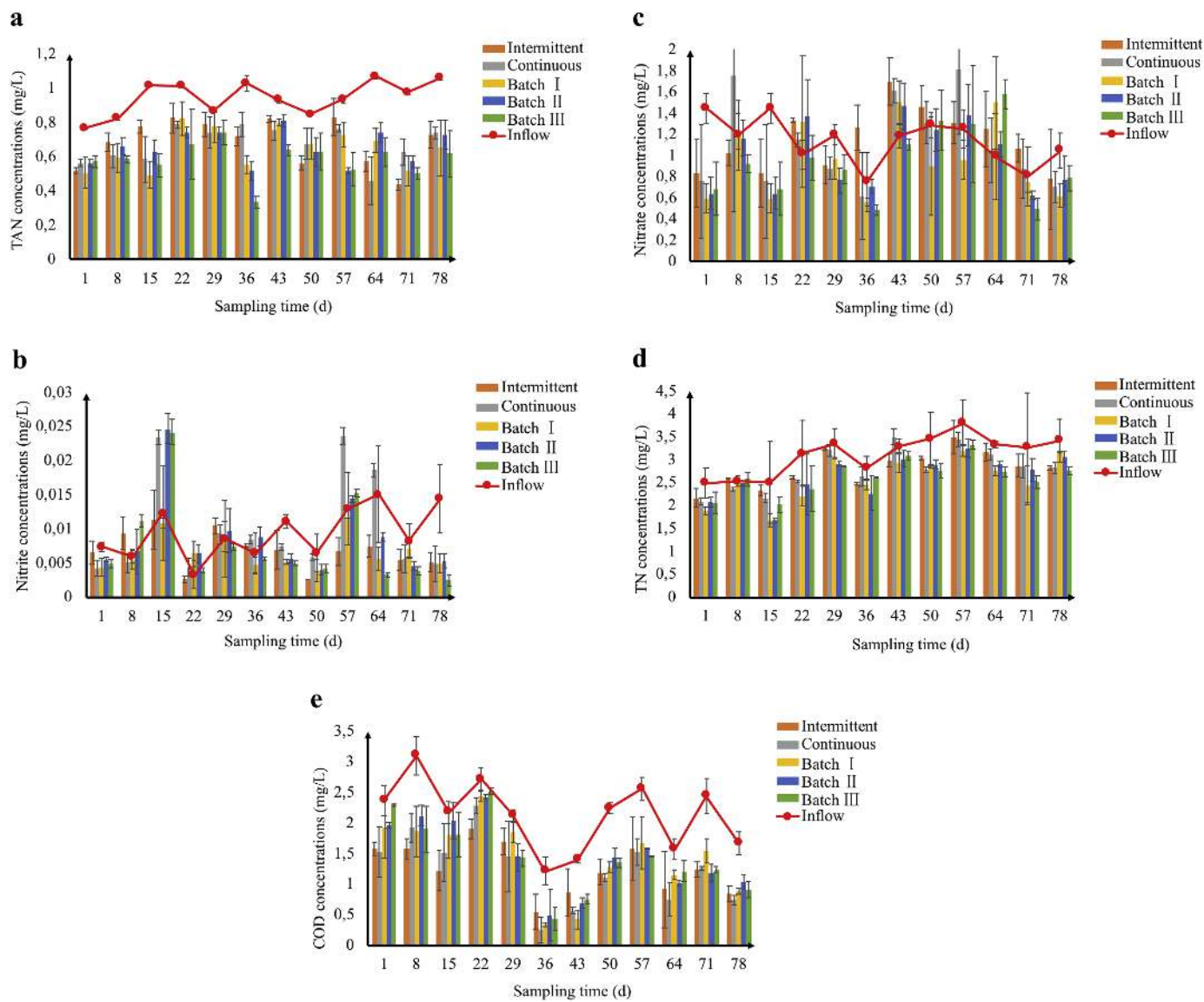


Figure 2

**Component Plot in Rotated Space**

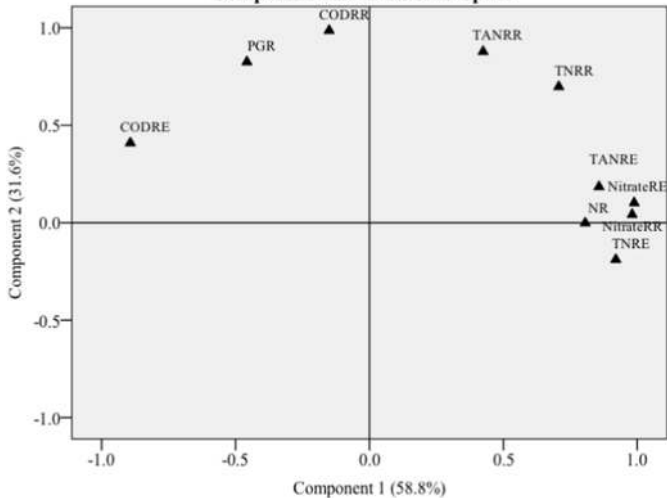
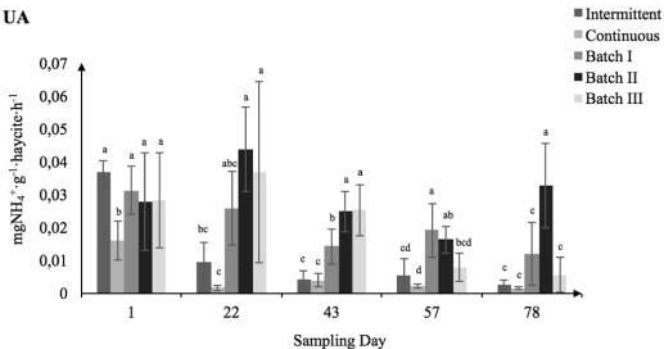


Figure 3



UA



DA

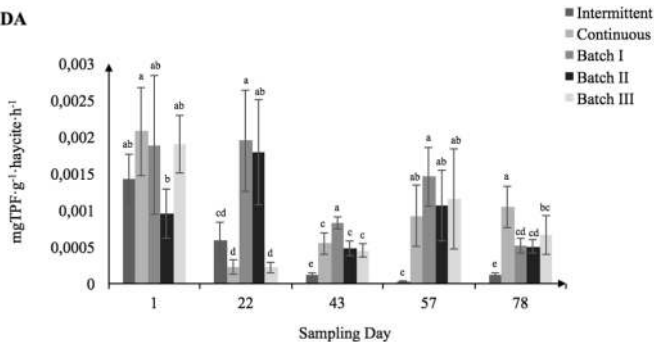


Figure 4