Effect of the Carbon Source on Nitrifying in an Activated Sludge System Treating Aquaculture Wastewater

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Abstract
The nitrogen in the aquaculture wastewater can have significant effects on receiving water bodies like eutrophication and ammonia toxicity to fish communities. Removing nitrogen by nitrification-denitrification can reduce the potential impact of aquaculture wastewater discharge. Nitrification is affected by different factors including dissolved oxygen, temperature, pH, alkalinity, toxicity, unionized ammonia and substrate concentration. All these parameters affect ammonia-oxidizing and nitrite-oxidizing bacterial activity.

The aim of this work is to study the effect of the carbon source on nitrifying bacterial activity in an activated sludge treating aquaculture wastewater.

An activated sludge (AS) system was set up and operated continuously for 180 days. The operation was divided into two phases. During Phase I a source of organic carbon (CH₃COONa) with a C/N level of 2.4 (operation days 1 to 101) was fed into the system, while in Phase II a source of inorganic carbon (NaHCO₃) with a C/N level of 16.2 was fed into the system (operation days: 102 to 180).

The maximum NH₄⁺-N removal efficiency was 49.7% during the Phase I, during which the NO₃⁻-N and NH₃⁺-N concentrations were 37.1 ± 14.0 and 2.9 ± 1.1 mg/L, respectively. In Phase II, the maximum NH₄⁺-N removal efficiency was 45% and NO₃⁻-N and NH₃⁺-N effluent concentrations were 2.8 ± 0.3 mg/L and 210 ± 49 mg/L, respectively. Ammonia- and nitrite-oxidation decreased in Phase I from 0.231 ± 0.005 mg NH₄⁺-N/gVSS min to 0.018 ± 0.004 mg NH₄⁺-N/gVSS min and in Phase II from 0.049 ± 0.011 mgNO₂⁻-N/gVSS min to 0.010 ± 0.002 mgNO₂⁻-N/gVSS min.

Keywords: nitrification, carbon source, nitrifying activity, activated sludge, aquaculture

1. Introduction
Aquaculture activities discharge effluents into the receiving aquatic ecosystem with pathogenic bacteria, therapeutic chemicals, antibiotics, metabolic products and food wastes (Cripps & Bergheim, 2000; Michael, 2003; Stewart et al., 2006). These effluents are rich in solids, organic matter and dissolved metabolites such as ammonia, urea and carbon dioxide. In particular, one kilogram of fish production discharges approximately 577 g of BOD₅ (biological oxygen demand), 90.4 g of nitrogen and 10.5 g of phosphorus (Jegatheesan & Shu, 2011). Aquaculture wastewaters rich in nitrogen content may have a significant effect on the receiving water bodies such as eutrophication and ammonia toxicity to fish communities (Boaventura et al., 1996; Jegatheesan & Shu 2011). Consequently, biological nitrification-denitrification treatment is employed to remove nitrogen from wastewater (Campos et al., 2007). In this biological process the nitrification stage can determine the performance of the entire process (Shammas, 1986). Nitrification consists of the biological oxidation of NH₄⁺ to NO₃⁻ under aerobic conditions. First, NH₄⁺ is oxidized into NO₂⁻ by autotrophic ammonia-oxidizing bacteria (e.g. Nitrosomonas). NO₂⁻ is then oxidized to NO₃⁻ by autotrophic nitrite-oxidizing bacteria (e.g. Nitrobacter) (Ahn, 2006; Chen et al., 2006). Equations (1) and (2) show the basic chemical conversions that occur in nitrification (Chen et al., 2006).

\[
\text{NH}_4^+ + 1.5\text{O}_2 \rightarrow 2\text{H}^+ + \text{H}_2\text{O} + \text{NO}_2^- \quad (1)
\]

\[
\text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^- \quad (2)
\]
Conventional biological treatment, like activated sludge (AS), is used to remove nitrogen (Ahn, 2006; Leu et al., 2010). Such systems can remove up to 99% of NH\textsubscript{4}\textsuperscript{+}-N operated under an ammonia loading rate (ALR) of 4 gNH\textsubscript{4}\textsuperscript{+}-N/L·d (Antileo et al., 2002; Campos et al., 2002). Although AS systems are used for nitrification, they are not ideal for this purpose because they generate large and rapidly growing populations of heterotrophs compared to small and slow-growing populations of nitrifying bacteria (Gerardi, 2002; Campos et al., 2007). As well, nitrification is affected by a number of environmental factors including dissolved oxygen, temperature, pH, alkalinity, toxicity, unionized ammonia and substrate concentrations. Consequently, nitrification takes place with dissolved oxygen concentrations of > 2 mg/L and at temperatures between 4 and 45 °C. The temperature range determined for \textit{Nitrosomonas} is 5 to 30 °C, while for \textit{Nitrobacter} it is 5 to 40 °C (Gerardi, 2002). The optimal pH for nitrification is between 7.5 and 8.5. Specifically the optimal pH range for ammonium-oxidizing bacteria is 7.5-8.0, while for nitrite-oxidizing bacteria it is 7.2-7.8 (Chen et al., 2006). Complete ammonium oxidation consumes medium alkalinity and reduces pH. The alkalinity required is 7.1 mg CaCO\textsubscript{3}/mg NH\textsubscript{4}\textsuperscript{+}-oxidized (Ahn, 2006; Li & Irvin, 2007). Autotrophic nitrifying bacteria use CaCO\textsubscript{3} (CO\textsubscript{2} or HCO\textsubscript{3}) as an external carbon source for growth, which is useful as a buffer to maintain medium pH (Ahn, 2006). On the other hand NH\textsubscript{3} concentrations of 10-150 mg/L and 0.1-1 mg/L inhibit ammonia- and nitrite-oxidizing bacteria, respectively (Anthonisen et al., 1976; Gerardi, 2002). Consequently, simultaneous growth of nitrifying and heterotrophic organisms in a single reactor results in low rates of nitrification due to the sensitivity of organic matter. Nitrification can only be successfully carried out under low chemical oxygen demand (COD) (Li & Irvin, 2007) because of which industrial operations produce wastewater with low C/N ratios to support the growth of nitrifying bacteria favored by a C/N level of < 10:1 (Campos et al., 2007; Xia et al., 2008). As well, a substrate with organic carbon (CH\textsubscript{3}COONa) promotes the growth of heterotrophic bacteria in the system, as opposed to a substrate of inorganic carbon (NaHCO\textsubscript{3}), which favors the growth of nitrifying bacteria (Austin, 1980; Gerardi, 2002).

The objective of this work is to study the effect of the carbon source on nitrifying bacterial activity in an activated sludge treating aquaculture wastewater.

2. Materials and Methods

2.1 Inoculum

The inoculum consisted of 5 g/L of volatile suspended solids (VSS) of sludge obtained from the nitrifying reactor of a fish farm in southern Chile.

2.2 Synthetic Wastewater

The influent was synthetic medium aquaculture wastewater composed of NH\textsubscript{4}Cl - 700 mg/L, MgSO\textsubscript{4} - 0.06 g/L, NaCl - 1.0 g/L, KH\textsubscript{2}PO\textsubscript{4} - 0.025 g/L, CH\textsubscript{3}COONa - 1.5 g/L was used to study the effects of organic and inorganic carbon sources in Phase I, while NaHCO\textsubscript{3} - 20 g/L was used as a carbon source in Phase II.

2.3 Activated Sludge System

A laboratory scale system of activated sludge (AS) was set up that included an aerobic reactor (1.0 L) and a settling unit (0.4 L), both made of glass agrees with Vidal et al. (2004). Synthetic wastewater was fed into the aerobic reactor by a peristaltic pump with the flow rate adjusted to the desired hydraulic retention time (HRT) based on the net liquid volume of the reactor. The system was operated at a temperature of 18.0 ± 2.0 °C. The dissolved oxygen (DO) concentration was maintained at > 2 mg/L with an air diffusion system. The sludge was periodically recycled from the settling unit to the aerobic reactor to maintain approximately 3.0 gVSS/L. Figure 1 shows a scheme of the activated sludge system and its components.
2.4 Operational Conditions

The system was operated continuously for 180 days. The operation was divided into two phases. In Phase I a source of organic carbon (CH3COONa) was fed into the system from days 1 to 101 at a C/N ratio of 2.4. In Phase II a source of inorganic carbon (NaHCO3) was fed into the system from days 102 to 189 at a C/N ratio of 16.2. The ammonium loading rate (ALR) in the influent was maintained throughout the operation at 0.33 gNH4+/L·d, with a hydraulic retention time of 2.05 ± 0.19 d. Table 1 shows the characteristics of the system during the two phases of operation.

Table 1. Characteristics of influent fed to the activated sludge system during the two operating conditions

<table>
<thead>
<tr>
<th>Characteristics of influent</th>
<th>Phase I</th>
<th>Phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>CH3COONa</td>
<td>NaHCO3</td>
</tr>
<tr>
<td>Operating time</td>
<td>0-101</td>
<td>102-180</td>
</tr>
<tr>
<td>C:N ratio</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>ALR gNH4+/L·d</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>OLR gCOD/L·d</td>
<td>0.36 ± 0.04</td>
<td>-</td>
</tr>
<tr>
<td>HRT d</td>
<td>2.09 ± 0.18</td>
<td>2.01 ± 0.20</td>
</tr>
<tr>
<td>Sludge concentration gVSS/L</td>
<td>7.22 ± 3.81</td>
<td>3.47 ± 1.20</td>
</tr>
<tr>
<td>DO mg/L</td>
<td>6.75 ± 1.18</td>
<td>5.55 ± 0.87</td>
</tr>
<tr>
<td>pH influent</td>
<td>7.11 ± 0.21</td>
<td>7.40 ± 0.65</td>
</tr>
</tbody>
</table>

Note. ALR: Ammonium Loading Rate, OLR: Organic Loading Rate, HRT: Hydraulic Retention Time, DO: Dissolved Oxygen.

The efficiency of NH4+-N removal was calculated throughout the operation. COD and BOD5 removal efficiencies were evaluated only in Phase I. Efficiencies were calculated using Equation (3):

\[ E(\%) = \frac{(Q_i \times C_i - Q_o \times C_o)/(Q_i \times C_i \times 100)} {1} \]  

Where, E (%) is the removal percentage; Q is the flow rate (L/d); C is the parameter concentration (mg/L); and subindex “i” and “o” are the inflow and outflow, respectively.

NO2--N, NO3--N and NH3 concentrations in the effluent were determined weekly and alkalinity, the oxidation reduction potential (ORP), temperature, pH, and DO were monitored daily.

Ammonium- and nitrite-oxidation were analyzed by the oxygen uptake rate (OUR) and the specific oxygen
uptake rate (SOUR) during both operating phases on days 29, 62, 118, 139, 162 and 168. As well, microorganisms were studied microscopically.

2.5 Analytical methods

Chemical oxygen demand (COD), biological oxygen demand (BOD₅), total suspended solids (TSS), volatile suspended solids (VSS) and alkalinity were measured according to standard methods (APHA-AWWA-WPCF, 1998). NH₄⁺-N, NO₂⁻-N and NO₃⁻-N content were measured in filtered samples (0.45 µm) with a Merck specific spectrophotometric NOVA-60 kit. Free ammonia content in the effluent was estimated according the Equation (4) (Hansen et al., 1998):

\[
\frac{[\text{NH}_3]}{[\text{TNH}_3]} = \left(1 + \frac{10^{-pH}}{10^0.002729[\text{NH}_3]} \right)^{-1}
\]  

Where, [NH₃] is the concentration of free ammonia, [TNH₃] is the total ammonia concentration and T (K) is the temperature (Kelvin).

Temperature and DO were measured using a HQ-10 oxygen meter with an LDO sensor. pH and ORP were measured by a multiparameter (Oakton PC650).

Ammonium- and nitrite-oxidation were evaluated with a respirometry using a biological oxygen monitor (BOM) YSI 5,300. The system was operated with an air-tight respiration vessel fitted with a DO probe YSI 5,231. The vessel was continuously stirred and thermally controlled according to procedure of López-Fiuza et al. (2002). (NH₄)₂SO₄ 0.25 M and NaNO₂ 0.25 M were used as a degradation substrate for ammonium- and nitrite-oxidizing bacteria, respectively. The OUR was determined by linear regression from the slope obtained by plotting dissolved oxygen concentration versus time and SOUR with the SSV value used in the assay.

Microscopic examination was performed within 1 h of collection, using a Leica Microsystems microscope (model DM500).

3. Results and Discussion

Figure 2 shows the evolution of the oxidation reduction potential (ORP) and alkalinity during Phases I and II in the AS. During Phase I the C/N ratio was 2.4 with an organic carbon source meanwhile during Phase II the C/N was 16.2 with an inorganic carbon source. The average ORP value was 105.2 ± 38.7 mV and the DO concentration was 6.2 ± 1.2 mg/L. During Phase I pH in the influent was 7.1 ± 0.2 and in Phase II it was 6.9 ± 0.1, while in the reactor it was 7.7 ± 0.6 and 8.9 ± 0.4 for Phases I and II, respectively. Alkalinity increased from 81 mgCaCO₃/L during Phase I to a maximum of 12,000 mgCaCO₃/L during Phase II. Under these conditions the microorganisms observed in the reactor in Phase I were principally stalked ciliates (e.g. *Vorticella* sp). According to Gerardi (2002), these protozoa are present in relatively large numbers during rapid nitrification. In Phase II this type of protozoa was not observed.
Figure 2. Evolution of alkalinity (▼), pH (■) and oxidation-reduction potential (ORP) (○) in the activated sludge system for different operating conditions.

Figure 3 shows COD and NH$_4^+$-N removal efficiencies and Figure 4 shows the NO$_2^-$-N, NO$_3^-$-N and NH$_3^+$-N concentrations in the effluent. In Phase I COD and NH$_4^+$-N removal efficiencies were 84 ± 3 and 37 ± 8%, respectively, while NO$_2^-$-N and NO$_3^-$-N concentrations in the effluent were 27.5 ± 13.1 and 37.1 ± 14.0 mg/L, respectively. Nitrite accumulation was detected during transformation. The estimated NH$_3$-N concentration was 2.9 ± 1.1 mg/L. Wang et al. (2010) reported a maximum NH$_4^+$-N removal of 99.8% in a batch system with an organic carbon source as substrate and a C/N ratio of 4 to 10. In a simultaneous nitrification-denitrification process, nitrification was dominant at a low C/N. With a C/N ratio of 6.3 and an organic carbon source, NH$_4^+$-N removal was 41%, with nitrite accumulation present. The highest nitrification rate was observed with C/N at 19.7 (Chiu et al., 2007).

In Phase II, the NH$_4^+$-N removal was 33 ± 9%. NO$_2^-$-N and NO$_3^-$-N concentrations were 1.01 ± 0.02 and 3.84 ± 0.81 mg/L, respectively, and the value of NH$_3$-N was 210 ± 49 mg/L. In an activated sludge system with a C/N ratio of 5.7 and NaHCO$_3$ as the carbon source, full ammonia removal to nitrate was observed (Campos et al., 2007).

The efficiency of NH$_4^+$-N removal decreased by 4% with the switch to an inorganic substrate. Decreases of up to 99% in NO$_2^-$-N and NO$_3^-$-N concentrations were observed, while the value of NH$_3$-N increased by two orders of magnitude in Phase II.
Table 2 shows ammonia- and nitrite-oxidation and concentrations of NH$_4^+$-N on operation days 29 and 62 during Phase I and days 118, 139, 162 and 168 during Phase II. There was no variation in activity during the two phases.

During Phase I, ammonia-oxidation was $0.231 \pm 0.005$ mgNH$_4^+$-N/gVSS·min, nitrite-oxidation was $0.049 \pm 0.001$ mgNO$_2^-$-N/gVSS·min, and NH$_4^+$-N removal efficiency was $31 \pm 1\%$. During Phase II, when the carbonaceous substrate was NaHCO$_3$, ammonia-oxidation decreased by 90% to $0.018 \pm 0.002$ mgNH$_4^+$-N/gVSS·min, nitrite-oxidation decreased 76% and $0.010 \pm 0.002$ mgNO$_2^-$-N/gVSS·min, and NH$_4^+$-N removal efficiency was $28 \pm 4\%$.

The pH values ($9.22 \pm 0.29$) and ammonia concentrations (208-331 mg/L) may be the main factors for the lower ammonia- and nitrite-oxidation values. Shammas (1986) showed that a pH level between 8 and 9 is optimal for nitrification and that when pH increases to 9.6 nitrification drops almost zero. In this study, during the Phase II pH values reached over 9.6, at which level nitrite was not detected in the process. These results concur with
those of Ruiz et al. (2003), who found a complete inhibition of nitrification in an AS system without nitrite accumulation at a pH level higher than 8.95. Campos et al. (2007) found a reduction from 0.4 to 0.2 mgNH₄⁺-N/gVSS-min of ammonia-oxidation when the system was subjected to a pH level of 11. However, specific nitrite-oxidation remained constant at around 0.7 g NO₂⁻-N/gVSS d when pH was recovered. Moreover, nitrate accumulation and NH₃ were in the effluent. Willke and Vorlop (1996) found a nitrite-oxidizing bacteria resistant to pH variations, indicating that activity is affected when pH is in the range 4.5 and 10, among others. In this way Suthersand and Ganczarczyk (1986) found that a pH shock reduces nitrite-oxidation by 14% less than ammonia-oxidation.

While ammonia and nitrite serve as energy sources for the microorganisms responsible for oxidation, they can inhibit biological activity in their unionized forms, NH₃ and HNO₂, respectively. The concentrations of free nitrous acid and free ammonia are functions of temperature, pH and ammonia and nitrite concentrations, respectively (Villaverde et al., 1997). The free ammonia concentration increases to basic pH while the nitrous acid concentration increases to acid pH (Gerardi, 2002).

NH₃ concentrations increase with higher temperatures (Emerson et al., 1975). From Phase I to II, the pH level increased by 1.2 points (from 7.7 to 8.9) and temperature increased by 2 degree °C (from 14.9 to 16.9 °C). Under these conditions 20% of the total ammonia in the system is in the form of NH₃ in the system. The maximum pH was 9.75 and under these conditions 64% of ammonia was NH₃ (Hansen et al., 1997). At these levels of NH₃, ammonia- and nitrite-oxidation are inhibited (Fontenot et al., 2007).

Table 2. Ammonium-oxidation in sludge, influent and effluent ammonia concentration (mg/L), and percentage reduction of total ammonia during operation of the activated sludge system

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time (d)</th>
<th>Ammonium-oxidation (mgNH₄⁺-N/gVSS·min)</th>
<th>Nitrite-oxidation (mgNO₂⁻-N/gVSS·min)</th>
<th>NH₄⁺-N concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Influent (mg/L)</td>
<td>Effluent (mg/L)</td>
<td>Reduction (%)</td>
</tr>
<tr>
<td>I</td>
<td>29</td>
<td>0.233 ± 0.008</td>
<td>0.049 ± 0.001</td>
<td>695 ± 10</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>0.229 ± 0.002</td>
<td>0.049 ± 0.001</td>
<td>700 ± 10</td>
</tr>
<tr>
<td>II</td>
<td>118</td>
<td>0.013 ± 0.003</td>
<td>N. D.</td>
<td>750 ± 20</td>
</tr>
<tr>
<td></td>
<td>139</td>
<td>0.017 ± 0.001</td>
<td>N. D.</td>
<td>702 ± 10</td>
</tr>
<tr>
<td></td>
<td>162</td>
<td>0.020 ± 0.002</td>
<td>0.008 ± 0.002</td>
<td>695 ± 5</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>0.023 ± 0.001</td>
<td>0.012 ± 0.001</td>
<td>685 ± 5</td>
</tr>
</tbody>
</table>

Note. N.D.: Not determined.

Table 3 shows the nitrogen mass balance. The tendency of the nitrogen balance evolution concurs with the analysis of the ammonia-oxidizing and nitrate-oxidizing bacteria. The NH₄⁺-N load in the influent of the AS system during Phase I and II was 0.355 ± 0.012 g/d, while the NH₄⁺-N load in the effluent was 0.502 ± 0.039 g/d. The effluent loads of NO₂⁻-N and NO₃⁻-N decreased from Phase I to Phase II, to a non-detectable level for NO₂⁻-N and to 92% for NO₃⁻-N. The effluent load increased from Phase I (0.001 g/d) to Phase II (0.135 ±0.018 g/d). NH₄⁺-N removal was over 40%, in both phases.

Table 3. Evolution of nitrogen matter balance in an activated sludge system during the two operating phases

<table>
<thead>
<tr>
<th>Phase</th>
<th>Time (d)</th>
<th>Input (g/d)</th>
<th>Reactor (gO₂/d)</th>
<th>Output (g/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NH₄⁺-N</td>
<td>NO₂⁻-N</td>
<td>NO₃⁻-N</td>
</tr>
<tr>
<td>I</td>
<td>29</td>
<td>0.347</td>
<td>0.596</td>
<td>0.242</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>0.350</td>
<td>1.440</td>
<td>0.237</td>
</tr>
<tr>
<td>II</td>
<td>118</td>
<td>0.375</td>
<td>0.010</td>
<td>0.295</td>
</tr>
<tr>
<td></td>
<td>139</td>
<td>0.351</td>
<td>0.006</td>
<td>0.256</td>
</tr>
<tr>
<td></td>
<td>161</td>
<td>0.348</td>
<td>0.045</td>
<td>0.238</td>
</tr>
<tr>
<td></td>
<td>168</td>
<td>0.343</td>
<td>0.018</td>
<td>0.233</td>
</tr>
</tbody>
</table>

Note. N.D.: Not Detected.
4. Conclusions
The maximum NH$_4$\textsuperscript{+}-N removal efficiency was 49.7% in Phase I, during which NO$_3$-N and NH$_3$-N concentrations were 37.1 ± 14.0 and 2.9 ± 1.1 mg/L, respectively. In Phase II, the maximum NH$_4$\textsuperscript{+}-N removal efficiency was 45% and NO$_3$-N and NH$_3$-N concentrations were 2.8 ± 0.3 mg/L and 210 ± 49 mg/L, respectively. Ammonia-oxidizing and nitrite-oxidation decreased from 0.231 ± 0.005 mgNH$_4$\textsuperscript{+}/gVSS·min to 0.018 ± 0.004 mgNH$_4$\textsuperscript{+}-N/gVSS·min in Phase I and from 0.049 ± 0.011 mgNO$_2$-N/gVSS·min to 0.010 ± 0.002 mgNO$_2$-N/gVSS·min in Phase II.

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