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Influence of oxytetracycline on the fate of nitrogen species in a recirculating aquaculture system

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Abstract. Common aquaculture practices include the use of certain pharmaceuticals such as antibiotics in avoiding diseases and promoting a healthier growth of the culture. The aim of this study is to monitor and assess the influence of different low oxytetracycline concentrations on the transformation of nitrogen compounds under aeration condition in a lab-scale recirculating aquaculture system (RAS). Over 1 mg L^{-1} dose of oxytetracycline to aquaculture had induced ammonia(NH_4-N), nitrate(NO_3-N), soluble COD accumulation in RAS. In addition, nitrous oxide (N_2O) emission from RAS was significantly reduced during the oxytetracycline dose periods. After ceasing the dose of oxytetracycline, ammonia oxidation and nitrous oxide re-emission were observed. This observation indicated that low concentrations of oxytetracycline could affect the nitrogen species in RAS. Also, the emission mechanisms of N_2O may not be only dependent on nitrification process but also dependent on denitrification process in our RAS system.

Keywords: recirculating aquaculture system (RAS); oxytetracycline; ammonia; nitrification

1. Introduction

With the growing demand for food, industries improve their practices to cater to such demand increase. This food demand is accommodated by different trades including the aquaculture industry which is one of the stable growing industries. Aquaculture systems have contributed to the total fish production from 32.4% in 2005 to 40.3% in 2010. Also, 89% of global aquaculture produce is contributed by Asia with Korea as one of its top producers by Food and Agriculture Organization (FAO 2012). Recirculating aquaculture systems (RAS) were already introduced a few decades ago for research by Carmignani, Bennett (1977) and Collins (1975). Recently, Ebeling and Timmons (2012) and Nazar (2013) have studied that fish industries and research institutes have developed this system into a commercial scale to assist the fish producing sector. The success of this system depends on its ability to be built inland and consume less water than a typical aquaculture system.

Previous studies (Lawson (1995), Losordo and Masser (1998)) showed RAS is a very intensive system which relies on different biological processes. In particular, the transformation of various nitrogenous compounds plays a vital role maintaining equilibrium in this system by Hargreaves (1998). Nitrogen is introduced regularly in the RAS through protein-rich feeds and is either converted into culture biomass or waste excreted in the form of ammonia. Its conversion to other forms happens through biological transformations by various microorganisms present in the system. Levy-Booth (2014) has studied that the biological conversion of these nitrogenous compounds is due to different enzymes produced by microorganisms through the expression of specific genes. There are implications in varying levels of nitrogenous compounds in RAS. For example, high ammonia and nitrite concentrations are harmful to the animals in an aquaculture by Rodrigues (2007), Svobodova (2005) and USEPA (2009). Further, the presence of the greenhouse gas, nitrous oxide (N_2O), has also become a design and maintenance consideration due to its high-level production in aquaculture systems which may bring environmental concerns by Datta (2009), Williams and Crutzen (2010).

Often, fish farmers use pharmaceutical products such as antibiotics to prevent diseases and promote a healthier growth of the culture. Even though many countries like USA and other European countries have stopped the use of antibiotics in aquaculture, other countries like China and other Asian countries still apply this technique. However, Lalumera (2004) was found that these antibiotics persist in the system at low concentrations. Oxytetracycline is one of the most common antibiotics used in aquaculture by Benbrook (2002). Rigos and Smith (2015) have studied this

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drug belongs to a broad-spectrum antibiotic, tetracycline family which is produced by *Streptomyces* spp. Due to its wide range of applicability against various gram-positive and gram-negative bacteria, it is often selected as the drug used in fish cultures by Elia (2014) and Nakano (2015).

Klaver and Matthews (1994) studied the effect of this antibiotic to nitrifying bacteria, stating that the drug affects nitrification. Liu (2012) and Suga (2013)'s studies have also researched on its effect on microbial communities. Nevertheless, which concentration of oxytetracycline in the RAS could inhibit the nitrogen transformation has not been clearly understood. D.J Randall's study has showed that if practically used oxytetracycline concentration in the RAS inhibits the nitrification. Randall and Tsui (2002) have studied that ammonia accumulation which is the toxic for fish and how fish could alleviate its effect. In addition, increasing the nitrous oxide (N_2O) emission by partial nitrification which is greenhouse gas was studied by Satoshi Okabe (2011).

Therefore, the aim of this study is to monitor and assess the influence of low oxytetracycline concentrations, which is used in RAS practice, on the transformation of selected nitrogenous compounds, especially ammonia (NH_4-N), nitrite (NO_2-N), nitrate (NO_3-N), nitrous oxide(N_2O), in a lab-scale RAS.

2. Material and methods

2.1 RAS study

2.1.1 RAS set-up

A 200-L rearing tank was set up with customized biofilters attached inside. Water is recirculated using a submersible pump connected to an aquarium temperature controller (DB-050D; Daeil, Korea). The air was supplied via an air pump with a maintained flow rate of 2L min^{-1}. No other material was used for filter support to avoid premature sorption of chemicals out of the water column. After set-up, 16 koi fish (*Cyprinus carpio*) were placed in the aquarium. The fish were fed with 32% protein, 15% fat feed (Gold Silk; Woosung Feedstuff, Korea) daily at 1.5% of their body weight. No significant growth of the koi was observed for the duration of the study.

Oxytetracycline HCL (Sigma-Aldrich, Switzerland) was used in dosing the aquarium. Dosing is performed during feeding to mimic actual feeding practice with antibiotic content. The experiment was performed through a three-phased pattern; Phase 1 (or the control phase) is where normal aquarium conditions (without oxytetracycline dose) were monitored while phases 2 and 3 were maintained at 1 mg L^{-1} and 2 mg L^{-1} dose of oxytetracycline respectively. Samples were regularly taken for water quality measurement. After phase 3, samples were continuously taken to check for system normalization. Water exchange was done every other day, replacing 10% of total water volume. A five-day system stabilization and conditioning were done followed by 7 days of phase 1, 7 days of phase 2, and 12 days of phase 3. The recovery phase ran for another 10 days before the experiment ended.

2.1.2 Water quality monitoring

Samples were taken regularly in duplicates. Samples were checked for NH_4-N (Nessler, Hach Co., USA), nitrite, nitrate (Ion Chromatography; Chromeleon IC-System, Dionex Corp., CA, USA), soluble chemical oxygen demand (sCOD) (Nessler, Hach Co., USA). The oxytetracycline concentration was measured using HPLC (Nanospace SI-2, Shiseido co., Japan) and a Unison UK-C18 column (2.6 x 250 mm, 3 μm; Imtakt, USA) with the method developed by Lee (2005). The isocratic mobile phase is a solution composed of LC-grade methanol, acetonitrile, and filtered 0.01 M oxalic acid in water with a ratio of 1:2:7. Standard concentrations were measured and were compared to sample measurements to compute for the accurate concentrations.

N_2O concentration in gas phase was measured using DS62000 PDHI detector Gas Chromatograph (Donam Instruments, Korea) using a Hayesep D packed column (80/100, 8' x 1/8"). All conditions were performed in an isothermal run with an oven temperature of 60^oC, injection temperature of 150^oC, and a detector temperature of 170^oC for 5 m with the N_2O peak found at 1.7 m. Triplicate water samples were placed in 10 mL gas-tight bottles with 4 mL headspace and a 20 m equilibration time in a 20^oC chamber. One milliliter of sample was injected per sample analysis. Standard concentrations were also measured and compared to the measured samples to compute for the N_2O concentration.

2.2 Pure culture study

2.2.1 Nitrosomonas europaea set-up

Nitrosomonas europaea (KCTC 12270, Daejeon, South Korea) was cultivated in 5-L ammonia-rich medium containing 300 mg L^{-1} NH_4-N and other chemicals ($MgSO_4$, CaCl, $FeSO_4$ in EDTA, $CuSO_4$, PO_4^-, and CO_3^-) as prescribed by Hyman and Arp (1992). The bacterial solution was dosed with different oxytetracycline concentrations: 0 mg L^{-1} (control), 2, 5, and10 mg L^{-1}. The containers were placed in a 20^oC shaking incubator to maintain an optimum culture growth condition. Samples were taken regularly in duplicates for a span of 8 days.

2.2.2 amoA gene expression effect by oxytetracycline

Fifty milliliters of Nitrosomonas europaea cell suspension were collected and centrifuged immediately at 4℃ and 5000g for 10 m.

The resulting pellet was reconstituted with RNA Protect™ Bacteria Reagent (Qiagen) for 5 mins. RNA isolation was done using RNeasy® Plus Mini kit (Qiagen) per Manufacturer's instructions. The expression abundance of amoA gene was quantified using RT-PCR with the primer sets presented in Table 1. Plasmids of the functional genes were prepared for the q-RT-PCR standard curves. End-point primers presented in the table (Table 1) were used in preparing the gDNA and were cloned using pGEM-T® Easy (Promega, USA) as the cloning vector and then transferred to an E. coli DH5α. The plasmid was extracted using GeneJET Plasmid Midiprep Kit (Thermo, Lithuania) following the manufacturer's procedure. Gene expression analysis was normalized with 16s rRNA concentration using primers also indicated in Table 1.

Table 1 Primers used for both Endpoint PCR and qPCR

Primer	Sequence for PCR	Target	Ref.
amoA-1F	5'-GGG GTT TCT ACT GGT GGT-3'	amoA	Rotthauwe *et al.* 1997
amoA-2R	5'-CCC CTC KGS AAA GCC TTC TTC-3'		
KNO50F	5'-TNA NAC ATG CAA GTC GAI CG-3'	16s rDNA	Moyer *et al.* 1994
KNO51R	5'-GGY TAC CTT GTT ACG ACT T-3'		
Primer	**Sequence for qPCR**	**Target**	**Ref.**
amoAFq	5'-GGA CTT CAC GCT GTA TCT G-3'	amoA	Chandran and Love 2008
amoARq	5'-GTG CCT TCT ACA ACG ATT GG-3'		
16sRDNA 341F	5'-CCT ACG GGA GGC AGC AG-3'	16s rDNA	Muyzer *et al.* 1993
534R	5'-ATT ACC GCG GCT GCT GG-3'		

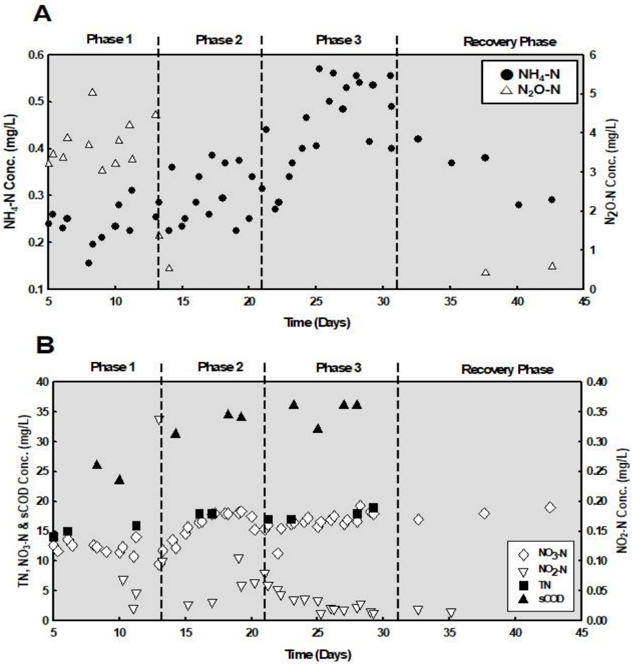

Fig. 1 (a) Ammonia and nitrous oxide and (b) other water quality observation in the recirculating aqua culture system. Phase 1 is the control phase, Phase 2 and Phase 3 were dosed with oxytetracycline to maintain 1 and 2 mg/L concentration, respectively. The system was not dosed during the recovery phase

3. Results

Fig. 1 exhibits a direct influence of oxytetracycline on nitrogenous compounds that are critical to the aquaculture operation. The oxytetracycline concentrations in water were regularly checked (2 times a week) and they had been maintained within 0.3-0.8 mg L^{-1}, 0.7-1.5 mg L^{-1} in phase 1 and phase 2, respectively. Julie Bebak-Williams (2002) researched that these oxytetracycline residue concentrations are similar range 0.39-0.72 mg L^{-1}.

Time profile of NH_4-N concentration in each phase was presented in Fig. 1(a). Unlike phase 1, the NH_4-N concentration continuously increased in phases 2 and 3(Fig.1A). On the contrary, the emission of N_2O dropped significantly almost zero when oxytetracycline dosing started in phase 2 and 3(Fig. 1(b)). NO_2-N concentration profile was also presented in Fig 1B. In Phase 1, it's concentration was below 0.06 mg L^{-1}. After dosing oxytetracycline (phase 2,3), NO_2-N concentration was instantaneously increased around 0.12 mg L^{-1} but decreased to 0.02 mg L^{-1} after 15 hour later (Fig. 1(b)) After that, nitrite level was pretty stable below 0.02 mg L^{-1} during phase 2 and 3. Meanwhile, Nitrate levels significantly increased during phase 2 (Fig. 1(b)) with an average of 12.61 mg L^{-1}, 15.71 mg L^{-1} ,16.42 mg L^{-1} in phase 1, 2, and 3, respectively. In terms of N_2O, the emission of N_2O significantly reduced when oxytetracycline dosing started and the N_2O gas emission was not detectable after 14 days (Fig. 1(b)). In recovery phase (Fig. 1), the most visual change of nitrogenous species is the decrease of the average NH_4-N concentration from 0.42 mg L^{-1} to 0.28 mg L^{-1} (Fig. 1(a)). During the whole experiment time, organic nitrogen was almost not detected because total nitrogen concentration was similar to the summation of NH_4-N, NO_2-N and NO_3-N concentrations.

4. Discussions

The purpose of our study is to investigate the fate of nitrogen species under the oxytetracycline presence. As shown in Fig. 1, NH_4-N and NO_3-N concentration were

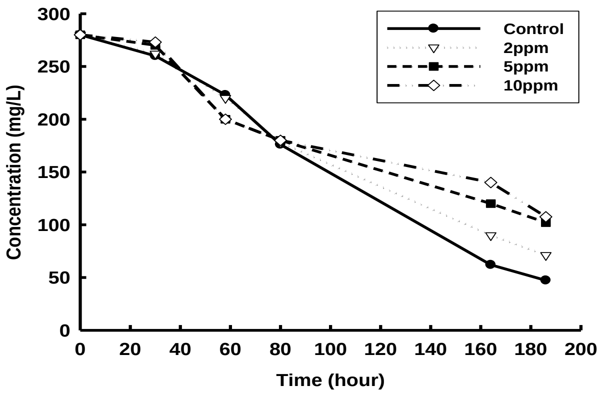

Fig. 2 Ammonia oxidation inhibition by oxytetracycline (0,2,5,10 mgL^{-1}) in Nitrosomonas europaea pure culture

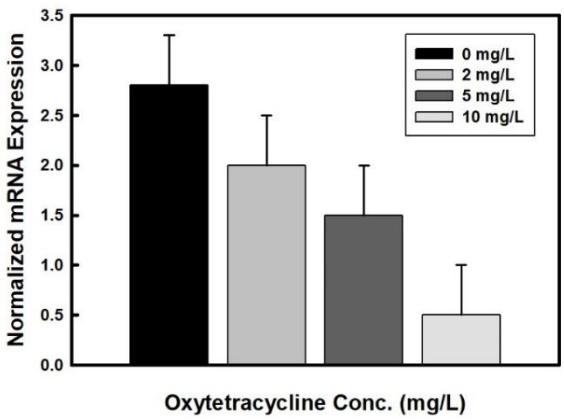

Fig. 3 Normalized mRNA expression of amoA gene with different oxytetracycline concentration at 90 hr exposure with 280 mg L^{-1} NH_4-N. Performed in triplicates, error bars correspond to 95% confidence intervals

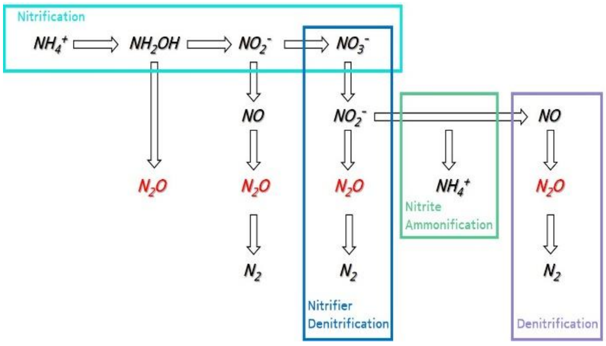

Fig. 3 Normalized mRNA expression of amoA gene with different oxytetracycline concentration at 90 hr exposure with 280 mg L^{-1} NH_4-N. Performed in triplicates, error bars correspond to 95% confidence intervals

accumulated under the presence of oxytetracycline (Phase 2 and 3) but NO_2-N concentration was fluctuated. In case of N_2O, its emission was rapidly decreased in phase 2 and there no N_2O concentrations were detected in phase 3. In recovery phase, accumulated NH_3-N level started to be decreased but nitrate concentration was still increased. N_2O emission was observed again in recovery phase. In this study, one of the most striking observations was that there was evident inhibition to N_2O consumption in the presence of oxytetracycline. In Phase 1, without oxytetracycline dose, relatively stable ammonia and nitrate, sCOD concentrations were observed (Fig. 1). However, when oxytetracycline was dosed (phase 2 and 3), ammonia, nitrate and sCOD concentrations were increased and accumulated indicating that there was nitrification and denitrification inhibition by oxytetracycline.

For more carefully characterizing N_2O emission process in nitrification, authors examined that N_2O emission from nitrification process using pure culture, *Nitrosomonas europeaea*, which is a model nitrifier. It was cultivated in a batch reactor and its NH_4-N oxidation profile was monitored with different oxytetracycline concentrations (Fig. 2).

NH_4-N oxidation rate was decreased as oxytetracycline concentration was increased from 0 to 10 mg L^{-1}. The HRT of RAS system was 20day. In the pure culture experiments, the decay constants for each oxytetracycline concentration were 0.089, 0.068, 0.050, 0.048 hr^{-1} in control (0 mg L^{-1}) for 2 mg L^{-1}, 5 mg L^{-1} and 10 mg L^{-1}, respectively. As shown in Fig. 2, 2 ppm of oxytetracycline inhibited the nitrification about 8.5%. Accordingly, these results confirmed that our oxytetracycline dose can inhibit some nitrification process but not fully.

Fig. 3 shows that Normalized mRNA expression of *amoA* gene with different oxytetracycline concentration at 90 hr exposure in *Nitrosomonas europaea* pure culture. *amoA* gene plays an important role for nitrification since it codes for the production of the enzyme that converts ammonia to an intermediate, hydroxylamine (NH_2OH), which is the first step in the organism's energy production (Fig. 4).

In our pure culture study, oxytetracycline-dosed cultures showed the expression of *amoA* gene were proportionally related to the oxytetracycline concentration (Fig. 3).

For example, 2 mg L^{-1} dose of oxytetracycline in RAS could be considered as enough dose for some accumulation of NH_4-N because of depressed *amoA* gene. Also, we can assume that N_2O production process from hydroxylamine (nitrification process) is more vulnerable rather than nitrite production by oxytetracycline since N_2O is significantly reduced and completely disappeared but nitrate and ammonia are accumulated in Phase 2 and 3 (Fig. 4).

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The other interesting aspect is that continuous NO_3-N and soluble COD accumulation but no N_2O emission in Phase 2 and 3. This indicated that N_2O emission from RAS is not solely from nitrification process but also denitrification process in Phase 1 since NO_3-N and soluble COD accumulation should be related to the denitrification inhibition.

Kox and Jetten (2015) studied that nitrogenous compound species distributions in aquatic environment are mainly facilitated by three types of organisms, ammonia oxidizers, nitrite oxidizers, and denitrifiers. As shown in Fig. 5, N_2O can be emitted in both nitrification and denitrification processes. In nitrification process, Bock (1995) showed nitrifier denitrification step is responsible for N_2O emission. Anderson, Poth, Homstead and Burdige (1993) explained. N_2O emission from heterotrophic denitrifiers are well known fact.

Fig. 1(b) showed that increased NO_3-N concentration was about 7.98 mg L^{-1} and soluble COD was 26 mg L^{-1}. Metcalf and Eddy (1979) explained the consumption rate of substrate defined to use carbon source for denitrification, ratio of sCOD to NO_3-N is 2.86. Based on this calculation, accumulated sCOD for inhibited denitrification of 7.98 mg L^{-1} was about 22.82 mgL^{-1}, which are very similar value with 26 mg L^{-1}.

Accordingly, it is apparent that oxytetracycline also had an impact not just on nitrification, but also on the denitrification process. In Phase 2 and 3, the concentration of sCOD increased indicating that oxytetracycline may have inhibited denitrification, thus reducing substrate consumption by denitrifying bacteria. During the recovery phase, confirmatory tests show that concentrations of compounds returned to levels similar to phase 1. In reverse phase, NH_4-N concentration decreased during the first week

(0.31 mg L^{-1}) while some emission of N_2O (1.33 mg L^{-1}) during this phase. Although sCOD data was not documented in this period, average higher NO_3-N concentrations were observed (Fig. 2). This might indicate that some N_2O production in this period might be more likely from nitrification but not denitrification process.

The influence of oxytetracycline in this lab-scale experiment gives an insight on how xenobiotics affect natural processes. As for this study, intensive aquaculture systems with antibiotic, setting aside other effects like antibiotic resistance and the transfer of resistance genes, seemed to have some disadvantageous. Oxytetracycline counters nitrification and denitrification process and it might deteriorate the water quality such as NH_4-N, NO_3-N and sCOD accumulation, which have potential negative effect to fish. Melissa J. Eichner (1989) said one of the potential advantage could be the reduction of N_2O emission, which is well known greenhouse gas, but it is not clear benefit from this practice.

5. Conclusions

The results imply that oxytetracycline, even at low concentrations, is a potential factor that affects NH_4-N, NO_3-N, and N_2O which are all significant in terms of operational and environmental impacts of aquaculture systems. The residual concentration of oxytetracycline that is highly found in intensive aquaculture ponds may contribute to the increased toxicity to the culture and deteriorate water quality. The findings in this study especially the reduced production of N_2O is observed because of oxytetracycline to the both nitrification and denitrification process but denitrification process by oxytetracycline.

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