Responses of Nitrifying Bacteria to Aquaculture Chemotherapeutic Agents

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ABSTRACT

As in any animal production industry, disease is inevitable; therefore, it is imperative that aquaculturists are able to effectively manage the disease and maintain their high production levels in an effort to bridge the gap between supply and demand in the seafood industry that has been caused in part by global over-fishing. This management responsibility lies not only in understanding the impact of the treatment on the cultured species, but also in understanding the impact of the treatment to the aquaculture system as an ecosystem. Currently, there is a narrow variety of chemicals approved by either the Food and Drug Administration (FDA) or the Environmental Protection Agency (EPA) for the treatment of disease outbreaks and water quality issues in aquaculture. Approved chemotherapeutants include oxytetracycline, Romet-30®, copper, and formalin. Additionally, a number of chemicals, such as Chloramine-T and potassium permanganate, are used off-label for the treatment of aquaculture systems. In this research, these six more commonly used chemotherapeutants were analyzed for their impacts to the nitrifying bacteria in aquaculture systems.

It was found that three of the chemotherapeutants: oxytetracycline, Romet-30®, and chelated copper caused inhibition to the nitrifying bacteria at the whole cell level as demonstrated in the results from water quality and specific oxygen uptake rate analyses. The nitrification process resumed once the chemotherapeutant was removed from the system, either by a mandatory water change or by natural degradation. The other three chemicals: formalin, Chloramine-T, and potassium permanganate did not result in any significant inhibition to the nitrification process. Experiments on laboratory-cultured nitrifying bacteria confirmed these findings. These experiments also resulted in the observation that the expression of *amoA* was upregulated by the copper exposure and inhibited by oxytetracycline and Romet-30®, but began to resume as the antibiotics degraded. Comprehensively, the findings of these analyses demonstrated that, although nitrifiers are well-known to be sensitive to their environment, the ability of nitrifying bacteria to continue their oxidative processes following exposure to chemical stress is inherent to the bacteria themselves rather than simply occurring under the protection of a biofilm community as has been suggested.

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Chapter 1: Literature Review

General Aquaculture

Recent census data gathered by the National Agricultural Statistics Service showed that U.S. sales of aquacultural products reached almost $1.1 billion dollars in 2005 – an increase of 11.7% since 1998. It is anticipated that this rapid growth will continue. Aquaculture is a large-scale production industry and, as such, it is under constant scrutiny by groups such as the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA). Effluent from aquaculture is biologically active with wastes being produced from direct excretion, uneaten feed and fecal matter (Black, 2001). The primary concerns from the effluent include solids and nutrients and EPA released a Final Rule regarding national effluent limitations guidelines and standards for concentrated aquatic animal facilities in 2004.

Aquaculture facilities may be defined as extensive (ponds) or intensive (indoor). One of the biggest advantages of indoor recirculating aquaculture systems is the level of control provided to the operator, especially those related to water quality and physical operation. Recirculating systems are very environmentally friendly because they reuse water, thus producing less waste than their flow-through counterparts. Typically, such systems discharge and replenish less than ten percent of the total system water volume on a daily basis ((Twarowska*, et al.*, 1996) and are highly engineered with respect to physical layout, hydrodynamic optimization, filtration and disinfection systems, and construction materials. Because of the limited resource requirements and controlled operations, recirculating aquaculture systems provide a means to produce and market a protein source that is safe from the contamination and bioterrorism threats to natural water supplies. Extensive outdoor systems are at the mercy of the natural environment.

The majority of the pollution in recirculating systems comes directly from the species being cultured and the feed that they receive (Wheaton, 2002). Because of the water reuse, waste products must be removed as quickly as possible to maintain the water quality in the system. For example, when waste solids are left in the system, they may generate additional oxygen demand and produce excess carbon dioxide and ammonia during decomposition (Twarowska*, et al.*, 1996). The primary concerns with respect to pollution from feed are associated with nutrient loadings of phosphorus and nitrogen. Nitrogen discharges can lead to increased oxygen demand and, in turn, dissolved oxygen sags in receiving waters. It is possible to reduce the release of nitrogen and phosphorus into the effluent level by lowering the levels of the nutrients in the diet.

Problems were observed with respect to growth rate and feed conversion efficiency when protein levels were reduced at a constant fat content (Lanari, *et al.*, 1995). Low levels of dietary phosphorus may be too low to ensure normal growth. When levels are increased, however, there is the risk that much of it will be undigestible and difficult to remove from the water (Flimlin, *et al.*, 2003). Excessive phosphorus loading may lead to the growth of algae and consumption of oxygen, which can be detrimental to the fish. Feed in which fish silage is used typically requires binders to improve the stability of the pellet. It also causes the fecal matter to remain intact rather than breaking down in the water (Fagbenro and Jauncey, 1995).

Although the majority of the nitrogen loading in an aquaculture system is rooted in that produced by the respiration and excretion of the fish, the feed is also a contributor. The nitrate and ammonia wastes are somewhat dependent on protein levels in the feed and the digestibility of that protein (Kolsater, 1995). As such, one would assume that an increased level of protein in the feed would have a negative impact on the water quality in an aquaculture system due to the increase in nitrogenous by-products. The use of extruded diets to improve growth rates and gross protein and energy retention seems to be a good way of reducing nitrogen loads into the water (Lanari, *et al.*, 1995). Between 15-25% of the feed will be excreted as solid waste and the food not consumed remains in the aquaculture systems (Bureau and Cho, 1999). One model shows that as the feed waste increases from 0-30%, the solid nitrogen waste quadruples, the total solid waste triples, and the solid phosphorus increases by about 60% (Bureau and Cho, 1999).

In addition to the nutrient threat to the environment, these nitrogen constituents, specifically unionized ammonia and nitrite, pose a toxicity threat to the fish themselves. In aquaculture, ammonia is a metabolic byproduct of the fish themselves with 60-90% of the total nitrogen being excreted from the gills and 9-27% of the soluble nitrogen coming from urea, thus comprising a very small portion of the total nitrogen (Hagopian and Riley, 1998). When combined with the nitrogenous loading presented by the feed, an already significant loading becomes more so. Unionized ammonia and nitrite are both toxic to fish. The fraction of unionized ammonia in the total ammonia present is dependent on pH and temperature and is described by an equation first derived in 1976 (Anthonisen, *et al.*, 1976):

$$NH_3 = \frac{[1.214\ TAN\ 10^{pH}]}{[e(6344/273+°C)+10^{pH}]} \qquad \text{Equation 1-1}$$

Toxic levels of ammonia cause irritation to the gills that, if exposure is prolonged, can cause the gills to swell and interfere with respiration and allow for opportunistic pathogens to grow. In extreme cases, ammonia poisoning can occur due to the inability to excrete the ammonia being produced. Acute toxicity from unionized ammonia in salmonids has been observed at 0.2mg/L NH_3, but 0.002mg/L NH_3 has been published as a maximum allowable concentration (Haywood, 1983; Hagopian and Riley, 1998). Nitrite, on the other hand, changes hemoglobin to methemoglobin, which is unable to transport oxygen. Nitrite has caused toxic effects in concentrations of around 1.8 mg/L over 24 hours in rainbow trout (Hagopian and Riley, 1998).

Biofilters are used in recirculating aquaculture systems to achieve biological reduction of pollutants, including ammonia. In some filters, such as bead filters, solids capture is also achieved. TAN concentrations have been shown to be reduced by an average of 20-40% in biofilters (Hargrove*, et al.*, 1996). Although the historically significant strains of bacteria found in biofilters have been shown to be *Nitrosomonas* and *Nitrobacter* species, more recent research has shown that the dominant strains in freshwater aquaculture systems included β-proteobacterial ammonia oxidizers (*Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus*) and *Nitrospira* nitrite oxidizers (Burrell*, et al.*, 1998; Hovanec*, et al.*, 1998; Burrell*, et al.*, 2001). In fact, in systems where *Nitrobacter* was added to aquaria to speed up the natural cycling process, nitrite levels initially decreased faster than those in the non-treated aquaria, but there was no evidence that these bacteria were actively growing within the systems, whereas *Nitrospira* species were easily observed (Hovanec*, et al.*, 1998). Although vital to the aquaculture systems, nitrifying bacteria are present in excessively low numbers. Samples analyzed using fluorescent in situ hybridization (FISH) resulted in less than about 3% of the total bacterial population (as determined by binding to a universal eubacterial probe) also binding to a probe that specifically targets nitrite-oxidizers (Burrell*, et al.*, 2001).

Because aquaculture is a high-volume production industry, disease is inevitable – caused primarily by high density grow-out conditions and less-than-optimal environmental conditions that cause stress to the fish and lead to disease outbreaks. Unfortunately, the treatment of these outbreaks is neither uniform nor sufficiently regulated (Benbrook, 2002). In addition to any physical signs of illness such as blisters and lesions, sick fish typically demonstrate reduced

feeding and general lethargy. The reduced feeding becomes an issue when one considers that antibiotics are most commonly administered in the form of medicated feed. Disease poses problems to aquaculturists primarily in financial losses due to fish mortality, treatment costs, and decreased production during treatment and mandatory holding periods; however, deterioration of the water quality due to the use of treatment may be equally problematic because of the impact to fish health and potential violation of discharge permits.

Nitrification and Nitrifying Bacteria Characteristics

Nitrification is the conversion of ammonia to nitrite and, further, to nitrate in a two-step oxidation process and a fundamental portion of the overall nitrogen cycle.

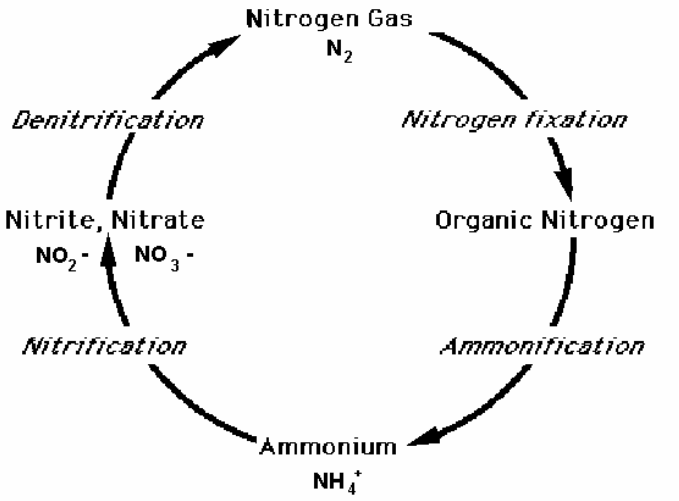

Figure 1-1. Simplified nitrogen cycle (http://ecosystems.mbl.edu/Research/Clue/nitrogen.html)

As wastewater discharge regulations continue to become increasingly stringent, especially with respect to the release of nutrients into the environment, understanding the nitrification process also becomes increasingly important to the field of environmental engineering. The first step of the nitrification process is ammonia oxidation. To complete this process, the ammonia is converted to hydroxylamine by the enzyme ammonia monooxygenase. This is followed by the conversion of hydroxylamine to nitrite by the enzyme hydroxylamine oxidoreductase. This first step is carried out by chemoautotrophic ammonia-oxidizing bacteria in the β and γ divisions of Proteobacteria (Woese, *et al.*, 1984; Woese, *et al.*, 1985; Prosser, 1989; Bano and Hollibaugh, 2000). Dominant genera of ammonia oxidizers include *Nitrosomonas*, *Nitrosospira*, and

Nitrosococcus and are primarily dictated by the ammonia concentration in the environment (Hovanec and DeLong, 1996; Mobarry, *et al.*, 1996; Wagner, *et al.*, 1996; Juretschko, *et al.*, 1998; Okabe, *et al.*, 1999; Kim and Ivanon, 2000; Biesterfield, *et al.*, 2001; Burrell, *et al.*, 2001; Rowan, *et al.*, 2003). The overall reaction by which ammonia is converted to nitrite releases 84 kcal/mol of energy (Hagopian and Riley, 1998). This is the combined equation of the two individual steps within the ammonia oxidation process in which hydroxylamine is produced and then consumed.

$$NH_3 + 1.5O_2 \leftrightarrow NO_2^- + H_2O + H^+ + 84 \text{ kcal/mol} \qquad \text{Equation 1-2}$$

The second step of the nitrification process is nitrite oxidation, which is carried out by the enzyme nitrite oxidase. Although some heterotrophic bacteria are capable of carrying out the nitrification process, autotrophic nitrifying bacteria rely on the process for cell maintenance and growth (Burrell, *et al.*, 1998). The primary genera of nitrite oxidizers include *Nitrobacter*, *Nitrospira*, and *Nitrococcus*. Like their ammonia oxidizing counterparts, most of the bacteria responsible for nitrite oxidizers are also aerobic Gram-negative chemoautotrophs that use both an inorganic electron source and inorganic carbon to produce biomass. The reaction by which nitrite is converted to nitrate releases 17.8 kcal/mol – only about ¼ of the energy released by the ammonia oxidation (Hagopian and Riley, 1998).

$$NO_2^- + 0.5O_2 \leftrightarrow NO_3^- + 17.8 \text{ kcal/mol} \qquad \text{Equation 1-3}$$

The typical metabolism of ammonia oxidizing bacteria allows the ammonia and oxygen to enter the periplasm from the environment through the cell wall. The ammonia monooxygenase, located in the cytoplasmic membrane, metabolizes these compounds to produce hydroxylamine, which is returned to the periplasm (Hollocher, *et al.*, 1981; Hagopian and Riley, 1998). Hydroxylamine oxidoreductase is located in the periplasm and it metabolizes the hydroxylamine to nitrite, which is released through the cell wall into the environment. The production of nitrite from hydroxylamine increases the acidity through the production of hydrogen ions and can quickly use up the alkalinity in a wastewater system, forcing supplementation of alkalinity to avoid pH-related inhibition (Hagopian and Riley, 1998). For nitrite oxidizing bacteria, the nitrite enters the periplasm from the environment through the cell wall. Nitrite oxidoreductase that is located in the cytoplasmic membrane, converts the nitrite to nitrate and releases the nitrate back

into the environment (Aleem and Sewell, 1981; Hagopian and Riley, 1998). Adding to their already intense energy requirements, nitrifiers require reverse electron transport in order to use electrons donated by from hydroxylamine and nitrite in the respiratory electron transport chain (Hagopian and Riley, 1998).

Nitrifying bacteria are Gram-negative and follow Monod growth kinetics. They prefer to clump into aggregates in both freshwater and marine environments and are very preferential to biofilms, where ammonia oxidizers and nitrite oxidizers are seen living in close proximity to one another. This preference for biofilm formation is so significant that it is estimated that between 70 and 95% of the nitrifying bacteria leave suspension and seek out a surface within thirty minutes of introduction into a system (Diab and Shilo, 1988; Hagopian and Riley, 1998). Although some nitrite oxidizing bacteria are able to use organic carbon sources, most nitrifying bacteria are autotrophic. This means that they require an inorganic carbon source, such as carbon dioxide, to survive and grow. Inorganic carbon is fixed and used to generate cell mass via the Calvin cycle. When coupled with the aforementioned need for a reverse electron transport mechanism, this cycle causes nitrifiers to be very inefficient. Autotrophic nitrifying bacteria to grow very slowly as a result of the intensity of their energy producing pathways and their doubling time is anticipated to be 7-8 hours under ideal conditions, which is an order of magnitude slower than for many heterotrophs (Hagopian and Riley, 1998). Other data for in situ conditions show the doubling times to be 26 hours for ammonia oxidizers and 60 hours for nitrite oxidizers (Belser, 1984; Hagopian and Riley, 1998).

Historically, it has been believed that *Nitrosomonas* and *Nitrobacter* were the two species of bacteria primarily responsible for ammonia oxidation and nitrite oxidation, respectively. More modern investigations have determined that while *Nitrosomonas* is a very common species of ammonia oxidizing bacteria, other species are more prevalent for nitrite oxidation while *Nitrobacter* is present to only a minimal degree (Mobarry, *et al.*, 1996; Wagner, *et al.*, 1996; Burrell, *et al.*, 1998). *Nitrospira*-like bacteria have been shown to be prevalent in wastewater treatment plants, ocean water, and laboratory reactors (Wagner, *et al.*, 1996; Juretschko, *et al.*, 1998; Schramm, *et al.*, 1998; Okabe, *et al.*, 1999; Daims, *et al.*, 2001). Although various *Nitrospira* species appear to be ubiquitous in nature, the lack of pure cultures limits widespread knowledge regarding the characteristics of the genus (Daims, *et al.*, 2001).

Even with the prevalence of *Nitrosomonas*, ammonia oxidizing bacteria have proven to be a very complex and diverse group (Juretschko*, et al.*, 1998; Schramm*, et al.*, 1998; McCaig*, et al.*, 1999; Whitby*, et al.*, 1999; Bano and Hollibaugh, 2000; Burrell*, et al.*, 2001). The historic reference to *Nitrosomonas* came about primarily as a result of the culturability of that species. That is, when cultures were started, that is the species that grew. Likewise, *Nitrobacter* was believed to be the dominate nitrite-oxidizer because, unlike its other nitrite-oxidizing counterparts that are obligately autotrophic, it has the capability to grow heterotrophically using pyruvate as the electron donor (Ehrich*, et al.*, 1995), thereby giving it some level of selective advantage in culturing methods (Burrell*, et al.*, 1998). Now that culture-independent methods have been developed, it is clear that additional ammonia oxidizers are present, and in some cases dominant, in a variety of environments (Purkhold*, et al.*, 2000; Burrell*, et al.*, 2001). It has also been shown that by varying the ammonia concentrations present, researchers are able to control which populations of nitrifiers are most abundant (Princic*, et al.*, 1998; Burrell*, et al.*, 2001; Biesterfield and Figueroa, 2002). From this, one can assume that similar manipulation is also occurring in natural systems making the strain of the dominant nitrifier heavily dependent on the environment and, thereby, correlating back to the significant sensitivity of the nitrifiers to their environmental conditions.

It has been difficult to study nitrifying bacteria due to their slow growth rate and the propensity of pure nitrifying cultures toward contamination with heterotrophs (Bano and Hollibaugh, 2000). Even though there have been significant advances made in the identification of nitrifying bacteria as a result of the culture-independent methods, including denaturing gel electrophoresis (DGGE) (Bano and Hollibaugh, 2000), they are not foolproof and are highly dependent on the environment being sampled (Hovanec and DeLong, 1996; Burrell*, et al.*, 2001). For example, it has been shown that *Nitrosospira* species are more abundant in natural environments, while *Nitrosomonas* species tend to be more prevalent in enriched cultures (Bano and Hollibaugh, 2000). Also, as ammonia concentrations vary, shifts in the dominant population of ammonia oxidizing bacteria in the sample occur. Low-ammonia environments demonstrate *Nitrosomonas marina*-like dominance. As the ammonia concentration increases, *Nitrosospira tenuis*-like and *Nitrosomonas europaea*-like bacteria become dominant until, at the highest concentrations, *Nitrosococcus mobilis*-like bacteria are the most prevalent (Prosser, 1989; Bano and Hollibaugh, 2000; Burrell*, et al.*, 2001).

Less work has been conducted to characterize the bacteria responsible for nitrite oxidation. Pure cultures of *Nitrospira* and other nitrite-oxidizing species have not been isolated from most environments, so knowledge of their physiology and genetics is very limited (Daims, *et al.*, 2001). *Nitrospira marina* and *Nitrospira moscoviensis* are both commonly seen in aquatic environments. They are both spiral-shaped, Gram-negative nitrite oxidizing species having wide and irregular periplasmic spaces (Ehrich, *et al.*, 1995). *Nitrospira* has shown an affinity for low nitrite and oxygen conditions and are referred to as "K strategists", while *Nitrobacter* species appreciate higher concentrations to thrive and are referred to as "r strategists" (Schramm, *et al.*, 1999; Nogueira, *et al.*, 2002; Kim and Kim, 2006). It is believed that the ratio of "K" to "r" may be responsible for the degree of inhibition of nitrite oxidizing bacteria by chemical stressors because these parameters are directly correlated to the oxidizing activity of the species of interest (Kim and Kim, 2006).

Stress Response of Nitrifying Bacteria

Nitrifiers have inherent survival mechanisms that allow them to exist in many environments (Hagopian and Riley, 1998). These mechanisms include the ability to create biomass through various pathways and maintain low-level metabolism even though periods of starvation by limiting their respiration and anabolic processes to levels that are nearly undetectable (Hagopian and Riley, 1998). The flipside to these mechanisms are the weaknesses displayed. Among these are sensitivity to light and temperature conditions. Research demonstrates that nitrification by starved *Nitrosomonas* cells is inhibited after approximately 10 minutes of exposure to direct light, but that this inihibition is overcome with 3-4 hours in the dark (Alleman, *et al.*, 1987). When grown in culture, cells in the stationary growth phase were not affected by light. On the other hand, starved cells that are in the exponential growth phase are extremely inhibited by light, while cells grown in the presence of ammonia were not as inhibited (Alleman, *et al.*, 1987; Abeliovich and Vonshak, 1993).

Growth and activity are both decreased significantly as a the pH falls below neutral as nitrifiers are unable to internally regulate for pH (Hagopian and Riley, 1998). Nitrifiers prefer to grow at a pH between 7.5 and 8.0, which can be difficult to maintain as ammonia oxidation consumes alkalinity and causes the pH to drop in aquatic systems if left

unbalanced. Temperatures above 42°C and below 5°C cause significant deterioration in nitrification (Painter, 1970; Hagopian and Riley, 1998), while the overall optimum temperature appears to be around 25°C (Hagopian and Riley, 1998).

In natural environments, microorganisms are constantly subjected to changes in environmental conditions and nutrient supply, requiring a bacterial response in order to survive and necessitating the ability to outcompete other bacterial populations once the stressor or starvation has been removed (Batchelor, *et al.*, 1997). It has been shown that the environment in which the bacteria grow or are cultured may also have an impact on their susceptibility to stressors such as antibiotics (Anwar, *et al.*, 1990). Bacteria in biofilms have demonstrated increased resistance to stress, but it is not known if this result is directly due to their exopolysaccharide (EPS) glycocalyx polymers or to the difference in specific growth rates among the species that make up the biofilm community (Batchelor, *et al.*, 1997). Growth and activity of nitrifying bacteria can be maintained in biofilms at significantly lower pH values that in suspended bacteria (Keen and Prosser, 1987; Allison and Prosser, 1993; Batchelor, *et al.*, 1997). Under starved conditions, lag periods in growth and activity were observed in suspended cultures of nitrifying bacteria; however, no lag period was observed in nitrifying biofilms (Batchelor, *et al.*, 1997), indicating that the biofilm community was either more resistant to the stress or able to recover more efficiently. Some believe that this ability to respond and recover is a direct result of an accumulation of quorum sensing molecules in concentrated biofilm cells and the ability of the EPS to absorb ammonia and gradually release it to the cells to maintain their operation at low levels (Batchelor, *et al.*, 1997; Benbrook, 2002; Wuertz, *et al.*, 2004).

In fluidized beds, oxygen limitation results in incomplete nitrification with the ammonia removal efficiency falling from 98% to 80% when the ammonia loading rate was increased from 2.5 kg NH_4-N/m^3-day to 3 kg NH_4-N/m^3-day (Botrous, *et al.*, 2004). In this same study, the nitrite concentration increased by approximately 20% when the dissolved oxygen was reduced from 1.0 mg/L to 0.4 mg/L. This is likely because nitrite oxidizing bacteria have a higher K_m (affinity constant) value for dissolved oxygen than ammonia oxidizers do (Aoi, *et al.*, 2004). In fact, in the same study by Aoi *et al.*, the nitrite oxidation was only 11% of the ammonia oxidation where the dissolved oxygen concentration was less than 1 mg/L. Concurrently, the nitrite oxidation rate increased almost ten-fold, while the ammonia

oxidation rate only increased by about 1.2 times, when that dissolved oxygen concentration was gradually raised to 3 to 6 mg/L. In low dissolved oxygen conditions, ammonia oxidizers are able to use nitrite as an election acceptor and generate nitrous oxide gas because both nitrification and denitrification are allowed to occur simultaneously (Hagopian and Riley, 1998). This activity allows the system to continuously remove ammonia.

Chemical exposure is another stressor to nitrifying bacteria. Antibacterial compounds and anti-parasitics can inhibit nitrification in freshwater systems (Collins, *et al.*, 1976) and it has been shown that recommended doses of certain drugs can be as toxic to the nitrifying bacteria as they are to the bacterial pathogens (Chun, *et al.*, 1978; Bower and Turner, 1982). When nitrification is inhibited by chemotherapeutics, the deterioration in water quality is thought to be more lethal to the aquatic animals than the pathogens themselves (Spotte, 1970; Bower and Turner, 1982). In addition, many chemicals are able to inhibit one step of the nitrification process without interfering with the other step (Hagopian and Riley, 1998). For example, the presences of many volatile fatty acids, especially formic acid, have been shown to cause inhibition of nitrite oxidation, but not ammonia oxidation. Other volatile fatty acids, such as isobutyric acid, inhibited both oxidation steps (Eilersen, *et al.*, 1994).

In addition to being growth limited because their energy requirements, nitrification is greatly impacted by the environment, especially when facing inhibition by organic compounds, metal concentrations, oxygen availability, and light. Nitrifiers can also be inhibited by the carbon/nitrogen ratio present in the environment. At high ratios, nitrifiers are routinely out-competed by heterotrophic bacteria for oxygen. The nitrification process can also be inhibited by its own oxidized products (Anthonisen, *et al.*, 1976). That is, nitrous acid inhibits ammonia-oxidizing bacteria, so nitrite oxidation and ammonia oxidation must be occurring simultaneously in order to avoid this type of inhibition. Accumulation of intermediate products, such as hydroxylamine and nitrite, can also be inhibitory and the build-up of these intermediate products along with the build-up of the ammonia can be toxic to aquatic animals (Kindaichi, *et al.*, 2004). There is no definite consensus on whether or not hydroxylamine inhibits ammonia oxidation. It has been shown that in hydroxylamine concentrations $\geq$ 0.5 mM, ammonia oxidation is inhibited in both dark and light conditions, but that the viability remained unaffected (Abeliovich and Vonshak, 1993). It has likewise been demonstrated that the addition of hydroxylamine actually stimulated ammonia oxidation

in low levels (< 2000 μM) and eliminated the presence of nitrite oxidizing bacteria (Kindaichi, *et al.*, 2004). It appears that the variation exists due to experimental conditions and physical parameters observed. Although the autotrophic nitrifiers consist of a very small portion of the microbial community in aquaculture systems – anywhere from 1-20% by some estimates – they are responsible for the majority of the nitrification taking place (Hovanec and DeLong, 1996; Burrell, *et al.*, 1998) and are, therefore, vital to the systems.

There are several ways to determine the stress on nitrifiers, including titrimetric biosensors that titrate the hydrogen ions that are produced during nitrification (Massone, *et al.*, 1998), assays that relate the disappearance of ammonia to nitrite and nitrate production (Daigger and Sadick, 1998), and monitoring of the dynamic response of nitrite production and oxygen uptake rates as they relate to the presence of a toxin (Eilersen, *et al.*, 1994).

Biofilms

Biofilms are clusters of microorganisms that bind together on a solid surface. They are heavily influenced by the exterior composition of the bacteria forming the biofilm heavily influences the formation of the biofilm. Development of biofilms follows a standard pattern of bacterial attachment, growth, sloughing, and reattachment that results in a complex tertiary structure (Stewart, *et al.*, 1995; vanLoosdrecht, *et al.*, 1995; deBeer, *et al.*, 1996; Thorn, *et al.*, 1996; Biesterfield and Figueroa, 2002). Because of their development pattern, biofilms in the environment are typically heterogeneous and rarely reach steady state (Flemming, 1995; vanLoosdrecht, *et al.*, 1995; Biesterfield and Figueroa, 2002). The spatial heterogeneity does not change with age. That is, when grown on coupons in natural systems, uncolonized areas will still be observed even in biofilms that are several hundred micrometers thick and/or many months old (e.g., 70-day biofilm) (Stewart, *et al.*, 1995; Thorn, *et al.*, 1996; Biesterfield and Figueroa, 2002). This indicates that as a biofilm grows, the new growth takes place over the old growth, thus growing "up" rather than "out" spatially. This would also account for the accumulation of inactive materials (e.g., dead cells) trapped within biofilms (Lazarova, *et al.*, 1998; Biesterfield and Figueroa, 2002)

In addition to being able to attach to each other, the bacteria must also be able to attach to surfaces (Anwar, *et al.*, 1989). It is this attachment to surfaces that give the biofilm many advantages over bacteria growing in suspension, include higher removal rates and good

performance stability (Botrous, *et al.*, 2004). Attachment is accomplished by production of exopolysaccharide (EPS) glycocalyx polymers that allows the bacteria to adhere to each other and the surface (Anwar, *et al.*, 1989; Anwar, *et al.*, 1990). The structure of a typical biofilm consists of 5 to 35% cells with the remainder occupied by the EPS matrix (Anwar, *et al.*, 1990). Much like the biofilm itself, the EPS is not homogeneous and is influenced by environmental conditions (Flemming, 1995; Schmitt, *et al.*, 1995). For example, in systems where the C/N ratio is reduced, the EPS produced by the heterotrophic bacteria serves as a space on which the nitrifying bacteria can grow and change their spatial distribution (Aoi, *et al.*, 2004). In Gram-negative bacteria, the EPS is comprised of various polysaccharides and proteins that are excreted and bound to the cell surface (Flemming, 1995). EPS tends to be highly hydrated, which gives EPS a slimy texture (Flemming, 1995). In addition to attachment, this polymer allows the biofilm to have a significantly greater viability and resistance to shocks than the same bacteria left in suspension, especially with respect to rejecting infection by pathogens, conditions of starvation, and antibiotic resistance, (Diab and Shilo, 1988; Anwar, *et al.*, 1989; Hagopian and Riley, 1998).

Biofilms are heavily influenced by their environment, most specifically the carbon/nitrogen (C/N) ratio, hydraulic conditions, pH, and dissolved oxygen (Gjaltema and Griebe, 1995; Aoi, *et al.*, 2004). The diffusion limitations of dissolved oxygen within the biofilms may, in turn, limit the nitrification rate (vanLoosdrecht, *et al.*, 1995; Jang, *et al.*, 2002). Nitrifying biofilms are greatly influenced by the C/N ratio. Previous studies demonstrated that ammonia oxidation was inhibited by a high C/N ratio due to an abundant growth of heterotrophs outcompeting the nitrifiers for growth requirements, such as dissolved oxygen (Aoi, *et al.*, 2004). Nitrification in a biofilm is not only directly dependent on the number of nitrifying bacteria present, but also indirectly dependent on the surface area available for the biofilm to form and not all available surface area on a specific medium is adequate for biofilm attachment (Bostock, *et al.*, 2002). Bacteria observed within a nitrifying biofilm through the use of cryosectioning showed that the bacterial distributions within the biofilm were widely variable based on the dissolved oxygen condition – with a primarily heterotrophic community at 2 mg/L dissolved oxygen and a significant increase in nitrifying bacteria at 10 mg/L dissolved oxygen (Jang, *et al.*, 2002).

Additionally, experiments conducted in a nitrifying trickling filter indicated that the biofilm growth was constant throughout the filter even if the growth of the nitrifying bacteria was not,

leading to the assumption that the non-nitrifying bacteria may play a larger role than originally thought (Biesterfield, *et al.*, 2001). Little is known about the adaptation of nitrifiers to competition with heterotrophs (Gieseke, *et al.*, 2001) One disadvantage of biofilm formation is that the sloughed biofilm or biofilm overgrowth may clog filters and lead to hydraulic problems in their respective systems (Botrous, *et al.*, 2004). An advantage of biofilm formation is that the slow-growing nitrifying bacteria experience reduced washout, even in fluidized beds, compared to bacteria that remain in suspension (Botrous, *et al.*, 2004).

In experiments performed in a chemostat, there is enough turbulence, due to the addition of fresh nutrients and oxygen, that the biofilm surface is continually sloughed and the thickness of the biofilm reaches a relatively steady state (Anwar, *et al.*, 1989). However, this thickness and steady state also appears to be dictated by the dissolved oxygen concentration in that the thicker and steadier biofilms were observed approximately ten days after the bulk dissolved oxygen concentration was increased from 2 mg/L to 10 mg/L (Jang, *et al.*, 2002). Experimentally, the maximum nitrification rate was obtained in biofilms between 15 and 25 μm as there is a balance between active and inactive biofilm materials (Liu and Capdeville, 1996).

As previously stated, biofilms in the natural environment rarely reach steady state and synthetic microbial communities are much less complex than their natural counterparts (Biesterfield and Figueroa, 2002). Although *in situ* sampling preserves the biofilm community, there are still problems associated with sampling both natural and synthetic systems. Many natural systems are very large and heterogeneous, which makes obtaining a representative sample very problematic (Biesterfield and Figueroa, 2002). For synthetic systems, problems primarily arise from the simplification and bias of the community and environmental characteristics (Biesterfield and Figueroa, 2002). Regardless of the system, biofilms are arranged in a specific way and the activities are based on the nature of the whole film rather than on the individual components (Amann and Kuhl, 1998).

Chemical stressors must interact with the surface of a bacterial cell in order to enter that cell and perform. Since the surface composition is altered and because biofilms are often comprised of many layers of bacteria, the interaction between the antibiotics and their targets is significantly more difficult (Anwar, *et al.*, 1990) and it has been shown, for example, that inhibition of nitrification in soil biofilm cultures is ten times less than the inhibition of nitrification in liquid cultures (Powell and Prosser, 1986; Hagopian and Riley, 1998). Further,

biofilms tend to form with the faster-growing bacteria on the outside of the film and the slower-growing bacteria protected on the inside, allowing the slower-growing cells to be spared during antibiotic treatments that target fast-growing species (Harrison, *et al.*, 2005).

Across the multitude of biological fields, few antibiotics have been discovered that can completely kill off biofilm bacteria (Anwar, *et al.*, 1989). When modeling biofilms, it is typically carried out by assuming that the mass transport and diffusion is perpendicular to the substrate to which the biofilm is attached (deBeer and Stoodley, 1995; deBeer, *et al.*, 1996). Real-time observation, however, indicates that this is not the case at all and, instead, biofilms demonstrate a complex community of clusters and voids. This structure not only makes it difficult for oxygen and nutrients to enter the biofilm, but also toxins such as antibiotics or other chemical stressors. Oxygen profiles through a biofilm are heavily dependent on the velocity of the bulk liquid and void areas do not enhance mass transport unless the velocity is high enough to force the mass transfer boundary layer to closely mimic the biofilm surface (deBeer, *et al.*, 1996). Because of these conditions, the planar model for mass transport in biofilms is only truly effective for biofilms under low velocity conditions.

Although the structure makes it difficult for toxins to reach the inner areas of the biofilm, EPS contains anionic groups as part of its composition. Some believe that this presents an opportunity for binding to metal cations, such as copper, cadmium, lead, and zinc (Flemming, 1995), while others cite this as protecting the community from the environment by restricting the entry of positively charged metal ions and some antibiotics (Harrison, *et al.*, 2005). Like other biofilm characteristics, this is also dependent on the environment. For example, biofilms under neutral pH conditions in freshwater lakes accumulated up to twelve times greater metal concentrations as those under lower pH conditions (Ferris, *et al.*, 1989).

Perhaps the most significant defense mechanism that biofilms display is quorum sensing. This allows the community to defend itself by means of expression or repression of certain genes. It may also defend the community against antibiotics through multidrug efflux pumps that reduce the accumulation of the antibiotics in the cells and, in some cases, allow the cells to continue growing in the presence of some concentration of the drugs (Harrison, *et al.*, 2005). In every biofilm, "persisters" (slow-growing variants of existing bacteria) have been found. These bacteria are genetically enabled to survive environmental stressors because of their ability to

generate a toxin (RelE) that causes the bacteria to enter a dormant state that is reversed once the stressor is gone (Harrison, *et al.*, 2005).

In nitrifying biofilms, *Nitrosospira* and *Nitrospira* were found in dense clusters with one another while *Nitrosomonas* was detected throughout that biofilm and *Nitrospira* was found only in the inner portions of the biofilm (Schramm, *et al.*, 1996; Schramm, *et al.*, 1998; Okabe, *et al.*, 1999; Burrell, *et al.*, 2001; Aoi, *et al.*, 2004). Nitrifying biofilms are documented as having a wet density of approximately 1.14 g/cm^3 with about 9.2% (w/w) nitrogen in the estimated 0.03 g/cm^3 of total solids per wet volume (Siegrist and Gujer, 1987; Hagopian and Riley, 1998). In biofilms that are comprised of both nitrifying bacteria and heterotrophic bacteria, the heterotrophic bacteria form a layer over the nitrifying bacteria due to their higher growth rate and ability to take advantage of the supply of dissolved oxygen and substrate (Aoi, *et al.*, 2004). As the C/N ratio was decreased, the thickness of this heterotrophic layer was reduced, the substrate reaches the nitrifying bacteria, and many of the nitrifying bacteria were able to extend their growth to the outer layers of the biofilm (Aoi, *et al.*, 2004). Likewise, the outgrowth of the nitrifying biofilm was increased in accordance with increased ammonia concentrations (vanLoosdrecht, *et al.*, 1995; Biesterfield and Figueroa, 2002). Nitrifying biofilms have been shown to sustain themselves quite well under starvation conditions. One study showed that in nitrifying communities with both ammonia oxidizers and nitrite oxidizers, established biofilms that had been starved up to seventeen days required only one hour to restart the nitrification process (Tappe, *et al.*, 1999; Biesterfield and Figueroa, 2002). A similar study was conducted using a pure culture to grow a biofilm and nitrification recovered with no lag time after a forty-three day starvation period (Batchelor, *et al.*, 1997; Biesterfield and Figueroa, 2002).

Even with the competition between heterotrophs and nitrifiers for space and sustenance, biofilms continue to be beneficial in many systems because of the dynamic and diverse communities. For example, a reactor has been proposed that will combine enhanced biological phosphate removal, nitrification, and denitrification into a single reactor (Gieseke, *et al.*, 2001). Analysis of this system by Gieseke *et al.* (2001) showed that during start-up of aeration, nitrifiers were oxygen deprived, but that over the course of the aeration period, the balance shifted from phosphate accumulation to nitrification, indicating a continuous trade-off in the function of the biofilm over the course of operation. As previously presented by other authors and discussed herein, the starvation period was not an issue with the performance of the nitrifying bacteria.

Common Aquaculture Chemotherapeutics

Controls regarding how chemotherapeutics may be used in the aquaculture industry vary greatly between countries. In the United States, regulatory control over the use of antimicrobial agents is very stringent (Alderman and Hastings, 1998) and it is estimated that of the total annual production of antibiotics, 50% are used for human consumption and the remaining 50% are used for agricultural and aquacultural purposes (Dietze, *et al.*, 2005). Antibiotics are usually provided to the fish as a coating adhered to the feed pellets at the time of manufacturing. The dose provided is dictated by the Food and Drug Administration (FDA) and has been approved following a rigorous review process. In the United States, oxytetracycline and Romet-30® are the two antibiotics approved for use in domestic aquaculture. In 2007, a third antibiotic (Aquaflor®) was approved by the FDA for the treatment of coldwater disease caused by *Flavobacterium psychrophilum*. This medication was the first antibiotic approved by the FDA for in-feed use in aquaculture in over twenty years, but has been used in other countries for as many as fifteen years (Feeks, 2007). Many believe that the use of chemotherapeutics in aquaculture is in great need of attention because of the limited number of approved chemicals causing the aquaculturists to use legal and illegal drugs and unapproved general-purpose chemicals to treat their systems for disease outbreaks (Benbrook, 2002).

Concerns regarding antibiotic resistance continue to increase, requiring additional focus to be placed on the volume and nature of the antibiotics entering the environment and the risks posed by the pathogens and bacteria that are developing resistance. Resistance comes about in many different forms depending on the bacteria that has become resistant: enzymatic inactivation that modify the drug itself upon entry into the cell, modification of the drug target site within the cell, and the adaptation of efflux pumps(Rajasekaran, *et al.*, 2007). In 1994, the American Society of Microbiology Task Force on Antibiotic Resistance reported that "the increasing problems associated with infectious diseases in fish, the limited number of drugs available for treatment and prevention of these diseases, and the rapid increase in resistance to these antibiotics represent major challenges for this source of food production worldwide." Some research programs subscribe to the belief that antibiotic-resistant fish pathogens are very unlikely to directly infect humans in temperate climates, but may be more likely to do so in warmer climates (Alderman and Hastings, 1998). Others operate under the belief that there is significant interaction between aquaculture and human disease (Rhodes, *et al.*, 2000). Some calculations

show that 70-80% of antibiotics used in the aquaculture industry enter the environment (Samuelsen, *et al.*, 1992; Hektoen, *et al.*, 1995) and that these antibiotics are creating "super-bugs" for which no antibiotic is currently available (Rajasekaran, *et al.*, 2007).

Whenever fish are treated with medicated feeds, some of the feed will go uneaten. In addition, much of the medication is eliminated by the fish through fecal matter (Agwuh and MacGowan, 2006). All of this solid matter accumulates on the bottom of the tank, pond, or cage and the medication leaches into the water column (Alderman and Hastings, 1998). Physical and chemical properties impact both the effectiveness of the antibiotics and their persistence in the environment (Alderman and Hastings, 1998). For example, oxytetracycline is complexed by calcium and magnesium ions and its availability to the diseased fish is thereby compromised (Jacobsen and Berglind, 1988; Alderman and Hastings, 1998). Over the years, aquaculture chemotherapeutics have been tested to determine their impact on nitrification in both freshwater (Collins, *et al.*, 1976) and in seawater (Bower and Turner, 1982) with varying results. In 1975 and 1976, Collins *et al.* tested many chemotherapeutic agents (antibacterials and antiparasitics) to determine the impact of these chemicals on nitrifying bacteria in recirculating systems. Through water quality analysis, this group showed that six of the antibacterial agents (including oxytetracycline and sulfamerazine) had no significant impact to nitrification. In 1975, the same primary investigator demonstrated that fish parasiticides also had no significant effect on nitrification and could be used with minimal concern of damaging the production system (Collins, *et al.*, 1975). Other investigators showed that oxytetracycline, chlorotetracycline, tiamulin, and streptomycin inhibited nitrification in activated sludge, but that sulfadiazine, oxolinic acid, olaquindox, and tylosin stimulated nitrification (Halling-Sorensen, 2001). This same investigation showed that growth inhibition occurred in *Nitrosomonas europaea* with antibiotics that had either a broad spectrum or were Gram-negative specific in the actions.

In addition to the antibiotics approved for use by the FDA, other treatment chemicals are approved for use by the FDA and the Environmental Protection Agency (EPA). These chemicals include formalin, potassium permanganate, and copper. Chloramine-T is currently being used off-label as it awaits FDA approval.

Oxytetracycline

Tetracyclines are part of a large group of broad-spectrum antibiotics that work against Gram-negative bacteria and became popular in clinical practice and development in the 1950's (Agwuh and MacGowan, 2006) after being discovered in the 1940's (Chopra and Roberts, 2001).

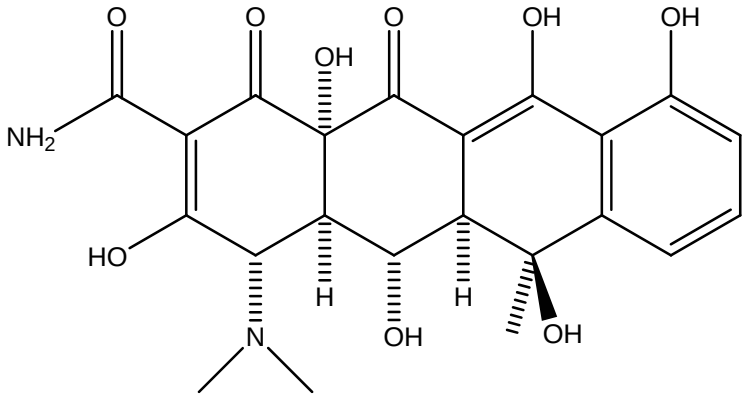

Figure 1-2. Oxytetracycline Structure (adapted from http://en.wikipedia.org/wiki/Oxytetracycline)

Oxytetracycline is one of the older oral antibiotics that has reduced absorption and is less lipophilic than some of the newer tetracyclines (Agwuh and MacGowan, 2006). In humans, oxytetracycline demonstrates approximately 58% absorption, mostly by the stomach, duodenum, and small intestine, and approximately 50% is eliminated by the body through the fecal matter (Agwuh and MacGowan, 2006). It is estimated that only 7-9% of the oxytetracycline is digested and absorbed by most fish, likely because the antibiotic binds to the calcium and magnesium in the feed and becomes unavailable (Bebak-Williams, *et al.*, 2002). Additionally, the partition coefficients of oxytetracycline make it unlikely that the chemical can be stored in lipid tissue (Pfizer, 1987). This absorption is estimated to be even less than in humans, and much of the residual antibiotic enters the environment in the form of uneaten feed in ranges of 15 – 40% depending on the feeding habits of the sick fish (Capone, *et al.*, 1996). In comparison, the digestibility of oxolinic acid was approximately 38% (Bjorklund, *et al.*, 1991).

The half-life of oxytetracycline in sediment has been reported as being anywhere from 9 days in a healthy, aerobic environment to as much as 419 days in an anoxic environment

(Jacobsen and Berglind, 1988; Bjorklund, *et al.*, 1990; Alderman and Hastings, 1998; Agwuh and MacGowan, 2006). This longer half-life was observed in thicker sediment located in a colder environment with less exposure to water currents, showing that these conditions may play a large part in the degradation and/or removal of the oxytetracycline. This half-life becomes very important when determining the fate of the antibiotic in the sediment and water as well as its persistence in the environment as the presence of the antibiotic in the environment may impact resistance not only of nitrifying bacteria, but also of other bacterial species in the microbial community (Samuelsen, *et al.*, 1994; Capone, *et al.*, 1996). The half-life in sediment is known to be dependent on the amount administered over the area, the amount of oxytetracycline that actually reaches the sediment versus being absorbed or washed away, the surface area of the sediment over which the oxytetracycline is administered, and the depth of the sediment in the area over which the oxytetracycline is administered (Coyne, *et al.*, 1994). In water columns, however, tetracyclines have been shown to degrade faster than in sediment because of photodegradation (Samuelsen, 1989; Bjorklund, *et al.*, 1991), with only 1% of the initial concentration present after 21 days of exposure to lighted conditions (Lunestad, *et al.*, 1995). 96% of the degradation occurred within the first week of light exposure. There was no apparent decrease in the inhibition zone when treatment occurred in the dark. Similarly, oxytetracycline-sediment that was covered with an additional layer of sediment demonstrated a longer half-life, likely also related to the lack of accessible light for photodegradation, and leading to the conclusion that the half-life of the oxytetracycline in sediment is directly related to the depth of the sediment (Hektoen, *et al.*, 1995). Oxytetracycline degrades faster in water than Romet-30 (Dietze, *et al.*, 2005) and it is anticipated that medications delivered in the form of medicated feed will not photodegrade as rapidly as those administered as baths due to the chemical being introduced into the lower regions of the aqueous environment (Lunestad, *et al.*, 1995).

Oxytetracycline chelates divalent cations and may accumulate in system water, biofilters, fecal matter, and uneaten feed (Bebak-Williams, *et al.*, 2002). In recirculating systems housing rainbow trout, Bebak-Williams *et al.* (2002) demonstrated that oxytetracycline reached peak concentrations on day ten of treatment and was at or below the detection limit (0.001 μg/g) within ten days after treatment ended. Likewise, the same group reported that the oxytetracycline concentration peaked on day ten of treatment and then consistently

decreased over the holding period. Unlike in the water column, the oxytetracycline residue was maintained longer in the sediments of the systems with an average concentration of 4.8 μg/g ten days after treatment ended (Bebak-Williams, *et al.*, 2002). In both the water column and the sediment, the residue concentration decreased sharply once dosing ended and did not increase again. That the oxytetracycline lingers in sediments is cause for concern with respect to the ingestion of these contaminated sediments by wild population fish that may linger around net pens.

Tetracyclines function by inhibiting protein synthesis and preventing the binding of aminoacyl-tRNA to the bacterial ribosome (Chopra and Howe, 1978; Chopra and Roberts, 2001). The tetracyclines have molecular weights of less than 500 daltons which allows them to diffuse through pores in the cell membrane of Gram-negative bacteria, especially in the case of oxytetracycline which is hydrophilic in nature (Chopra and Howe, 1978). Tetracyclines are acidic in nature and, as such, dissociate in aqueous solutions and cross the outer membrane of the Gram-negative bacteria by binding to a metal cation and passing through the OmpF and OmpC porins. The antibiotic accumulates in the periplasm before dissociating from the cation and diffusing through the lipid bilayer of the cytoplasmic membrane via proton motive force (Chopra and Howe, 1978; Chopra and Roberts, 2001). The minimum inhibitory concentration of oxytetracycline is estimated to be less than 1.56 mg/L for many strains of bacteria (Jacobsen and Berglind, 1988). A study evaluating oxytetracycline inhibition within a synthetic freshwater system resulted in the finding that oxytetracycline concentrations as low as 12.5 mg/L inhibit the nitrification process, but the authors recognize that the exposure to oxytetracycline in a bath treatment may have a substantially different effect on nitrification than exposure to oxytetracycline coated onto a feed (Klaver and Matthews, 1994).

Although controversial, tetracyclines are used in some countries at a level below that which is dosed for therapeutic purposes to promote growth and dissuade illness(Pfizer, 1987); however, this has potentially contributed to the emergence of bacterial resistance (Chopra and Roberts, 2001). Between 1917 and 1954, it was shown that of 433 different members of *Enterobacteriaceae* collected, only 2% were resistant to tetracyclines (Chopra and Roberts, 2001). That figure is suspected to be much higher now and, since this time, tetracyclines have been shown to induce antibiotic resistance in pathogenic bacteria

(Bjorklund, *et al.*, 1990). As of 2001, twenty-nine tetracycline resistant genes and three oxytetracycline resistant genes had been characterized and these genes were linked to various efflux pumps and ribosomal protection proteins (Chopra and Roberts, 2001). Other resistant genes continue to be identified and when expression of these genes occur, the bacteria is no longer susceptible to inhibition due to tetracycline exposure either because they are able to expel the tetracycline via efflux pump or because the tetracycline is unable to bind to the ribosome because protection proteins are bound to the ribosome instead. The binding of oxytetracycline to the ribosomes is reversible, however, and this explains how the antibiotic may inhibit the bacteria it affects without completely destroying them (Chopra and Roberts, 2001).

In aquaculture, oxytetracycline is used in finfish to treat ulcer disease, furunculosis, bacterial hemorrhagic septicemia, and pseudomonas disease (Benbrook, 2002). The dosage prescribed by the FDA is 2.5 – 3.75 g oxytetracycline per 100 lb fish per day for ten days. This dosage is pre-coated to the medicated feed at the manufacturing facility; therefore, the required feeding rate is typically provided by the manufacturer to ensure that the correct dose is obtained based on the feed itself rather than the medication. Oxytetracycline can be analyzed with the use of high performance liquid chromatography (Bjorklund, *et al.*, 1990; Du, *et al.*, 1995; Himmelsbach and Buchberger, 2005) with detection levels around 60 µg/L. Many variations of mobile phases have been tested and validated including mixtures of methanol/water or acetonitrile/water. HPLC separation of tetracycline and oxytetracycline peaks proved difficult, but was improved by the addition of 10mM oxalic acid (Himmelsbach and Buchberger, 2005).

Romet-30®

Romet-30 is a broad-spectrum antibiotic formulated from a 5 to 1 combination of sulfadimethoxine and ormetoprim, respectively.

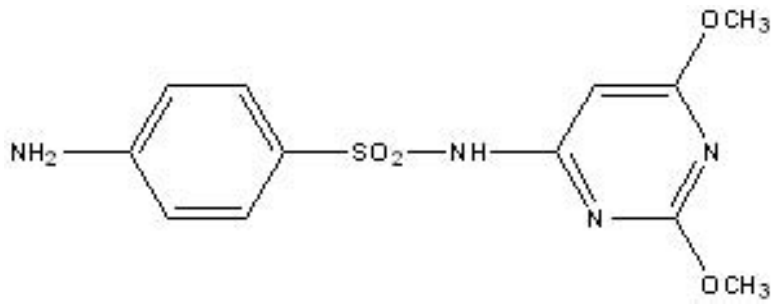

Figure 1-3. Structure of sulfadimethoxine (Hoffman-LaRoche, 1984)

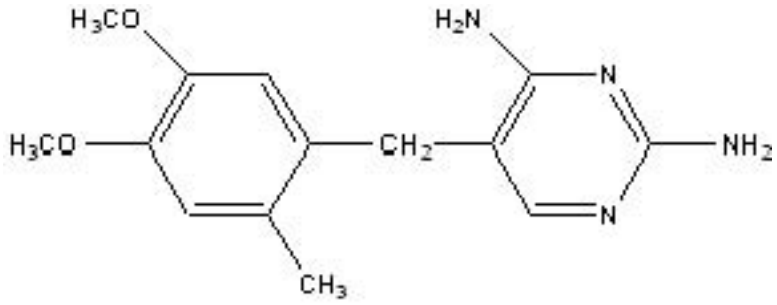

Figure 1-4. Structure of ormetoprim (Hoffman-LaRoche, 1984)

Romet-30® is a potentiated sulfonamide that blocks the synthesis of tetrahydrofolic acid, which is required for the synthesis of proteins and nucleic acid metabolism (FDA, 1984). This blocking takes place only in bacteria as opposed to animals because animals do not synthesize folic acid (Hoffman-LaRoche, 1984). This antibiotic is approved by the FDA for use in the control of furunculosis, ulcer disease, and bacterial hemorrhagic septicemia (FDA, 2003). Although each of these components has antibiotic characteristics on their own, the efficacy of the drugs in combination is greater at lower doses (Bakal, *et al.*, 2004). Unpotentiated sulfonamides are believed to have a low potency because of their means of operation in inhibiting the synthesis of folic acid (Halling-Sorensen, 2001). Romet-30® is applied to the feed in a dose of 2.2lb Romet/ton feed and the medicated feed is supplied to the treated fish at 1.5% body weight daily for a period of five days. There is a mandatory forty-two day holding period following this dosing to ensure that the fish is safe for consumption. By using the sulfadimethoxine in conjunction with ormetoprim, it is possible to reduce the amount of antibiotic required, thus reducing the potential for related antibiotic-

resistant bacteria, while actually enhancing the spectrum of bacteria over which the medication is effective (Hoffman-LaRoche, 1984).

Minimal work has been conducted regarding the fate of this antibiotic in the environment and the work that has been done has focused primarily on the fate in sediment (Samuelsen, *et al.*, 1994; Capone, *et al.*, 1996; Bakal and Stoskopf, 2001). These studies showed varied results with one study showing that the sulfadimethoxine concentrations in the soil degraded about 20% over a study period of 180 days (approximately 6 months) while the ormetoprim could not be detected after about one month (Samuelsen, *et al.*, 1994) and another showing that both portions of the Romet-30 were unstable and not detectable in soils anywhere from 21 to 62 days after treatment (Capone, *et al.*, 1996). These degradation rates, however, may prove to be very different from degradation rates observed in the water column as the chemical bound to substrate would be less available for microbial degradation (Bakal and Stoskopf, 2001). In comparison to oxytetracycline, this antibiotic is much more persistent in the water column (Dietze, *et al.*, 2005).

Unlike ormetoprim, the degradation of sulfadimethoxine appears to be highly temperature driven as Bakal *et al.* (2001) demonstrated that sulfadimethoxine declined by approximately half during refrigerated (4°C) conditions, but returned to initial concentrations at 25°C, likely due to the release of the drug into the water column at higher temperatures. Although this antibiotic is generated at a 5:1 ratio of the two components, it is not absorbed or excreted by the fish in a 5:1 ratio or any other constant ratio (Bakal, *et al.*, 2004) and it is, therefore, safe to assume that it also won't degrade at a constant ratio either. The bioavailability of Romet-30® is much greater than that of oxytetracycline, falling within 30 – 32% (Park, *et al.*, 1995).

Like oxytetracycline, Romet-30® is also photo-sensitive, but to a lesser degree. After 21 days, sulfadimethoxine degrades to 82% of the intial concentration and ormetoprim demonstrates no degradation (Lunestad, *et al.*, 1995). These chemicals showed no decrease in their inhibition zone when kept in the dark. Also like its oxytetracycline counterpart, it is anticipated that medications delivered in the form of medicated feed will not photodegrade as rapidly as those administered as baths due to the chemical being introduced into the lower regions of the aqueous environment (Lunestad, *et al.*, 1995). Romet-30 concentrations can

be analyzed in terms of its individual components (ormetoprim and sulfadimethoxine) using an HPLC method.

Copper

Although a required micronutrient for many species of bacteria, including nitrifying bacteria, higher concentrations of copper are toxic to nitrifying bacteria much like many other heavy metals. The toxic effect of copper, has been shown to increase with lower pH and/or higher free copper concentration. Metal inhibitions is a physicochemical process rather than a biological process because of the metal uptake due to sorption (Kim*, et al.*, 2006). In activated sludge systems, the inhibition of nitrification by copper was overcome with the addition of nitrilotriacetic acid (Braam and Klapwuk, 1981). The EC50 of free copper on the nitrification process (the concentration of free copper that causes a 50% decrease in nitrification activity) has been reported as 173 mg/L (Gernaey*, et al.*, 1997), but studies as early as 1961 showed that copper concentrations as low as 1 mg/L can inhibit the growth of *Nitrosomonas europaea* in pure culture. The difference in inhibition levels between operational systems such as activated sludge systems and pure culture systems has been attributed to the complexation of copper with organic matter (Bower and Turner, 1982). Following work with an activated sludge system exposed to free copper, a linear correlation ($r^2 = 0.89$) between nitrification rate and free copper concentrations was established for pH values between 6.5 and 8.0 when the exposure is for one day: nitrification rate = 8.35 + 1.22(pCu-11.0), where pCu is the negative logarithm of the copper concentration in mol/L (Braam and Klapwuk, 1981). Further experimentation by the same researchers showed that this correlation held true for at least three days of exposure, with some decrease in capacity on the third day.

Treatment of freshwater aquaria with 0.75 mg/L total copper (in the form of copper sulfate) resulted in no inhibition of nitrification (Collins*, et al.*, 1975); however, in this study, the copper concentration was added as three doses of 1 mg/L on alternate days until 3 mg/L (0.75 mg/L total copper) had been added. There was no subsequent monitoring of the copper concentration so any reduction in therapeutic concentration due to precipitation or complexation was not accounted for (Bower and Turner, 1982). Similarly, a study of nitrification inhibition by copper sulfate in seawater showed that slight ammonia oxidation

and moderate nitrite oxidation occurred following the 14-day application of 1.2 mg/L copper sulfate (0.3 mg/L total copper) followed by a total water change, but this inhibition was not replicated in duplicate aquaria (Bower and Turner, 1982). Further, this seawater study showed that following the required water change, the ammonia oxidation did not fully recover, but got no worse, while the nitrite oxidation process did appear to recover. Additionally this study showed that there was no statistical difference in the response of nitrification between new (rapid seeding with existing filtrant) filter beds and aged (approximately three months) filter beds.

Formalin

Formalin has historically been used as an embalming agent and now bath treatments are commonly used as an external antiparasitic treatment in aquaculture (Fajer-Avila*, et al.*, 2003; Buchmann*, et al.*, 2004). Formalin is a 37% solution of formaldehyde that typically has 10-15% methanol to stabilize the solution.

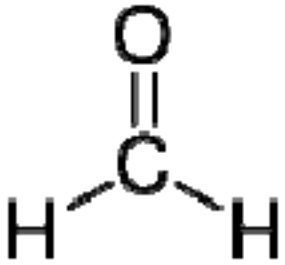

Figure 1-5. Formalin structure (adapted from http://en.wikipedia.org/wiki/Formaldehyde).

Formaldehyde is part of the chemical class of compounds called aldehydes. These compounds are generated by the oxidation of alcohols and formaldehyde is highly water soluble. For treatment purposes, formaldehyde works by dehydrating the target cells and coagulating in the place of the protoplasm, thereby altering the cellular composition and essentially destroying the cells (KoiFlorida). Formalin itself cannot differentiate between "good" cells and "bad" cells; therefore, any living cell is susceptible to formalin. Because of this, dosing must be strictly adhered to in order to avoid killing the fish. Formalin toxicity increases at high temperatures, low dissolved oxygen, and low hardness levels(Keck and Blanc, 2002)

As in other treatments, special care for the fish must be taken into consideration when using these chemicals even given that the FDA has approved many of these chemicals in terms of acute fish health and resistance. When the fish are over-exposed to formalin, gill damage can occur and the osmoregulation and respiration of the fish can be compromised (Keck and Blanc, 2002). Research has shown that rainbow trout can tolerate concentrations of formalin up to 50 ppm for twenty-four hours, 100 ppm for twelve hours, and concentrations of 200 and 300 ppm for one hour (Buchmann, *et al.*, 2004). Tolerance to chemicals is almost invariably dependent on the species and treatment conditions (Fajer-Avila, *et al.*, 2003). Even with the tolerance for these concentrations, differences were seen in the fish with respect to mucous density as a result to the exposure, which may indicate that even at low doses of formalin, fish can become more susceptible to opportunistic pathogens as a result of an open mucous layer or damaged mucous cells to which pathogens are able to attach (Buchmann, *et al.*, 2004). While the toxicity to various species in freshwater has been fairly well-documented, the toxicity in saltwater has been less so, but it was shown that formalin in saltwater systems was more effective in higher doses for shorter time periods than it was at lower doses for longer time period and the LC50 for saltwater tests using bullseye pufferfish corresponded with the efficacy (Fajer-Avila, *et al.*, 2003).

In saltwater systems, there was no observed effect of formalin to ammonia oxidizing bacteria in one-hour static exposures of concentrations greater than 40 mg/L and four-hour recirculating exposures of 60 mg/L; however, there was a significant effect to nitrite oxidizing bacteria under these same conditions (Keck and Blanc, 2002). To oppose this, other studies show no significant inhibition to nitrification during and following formalin treatments (Collins, *et al.*, 1975; Keck and Blanc, 2002), but these exposures were short-term and did not account for multiple exposures. One explanation for these results of no significant effect may be the requirement for system flushing following exposure. The nitrite impacts reported by Keck and Blanc (2002) were observed when the formalin was not flushed from the systems; thus concluding that impacts of formalin on nitrification is insubstantial.

Chloramine-T

Chloramine-T (n-sodium-n-chloro-ρ-toluenesulfonamide) is useful in aquaculture for the control of bacterial gill disease – a non-specific Gram-negative bacterial infection - and external fluke disease (From, 1980; Spangenberg, 1999; Dawson, *et al.*, 2003).

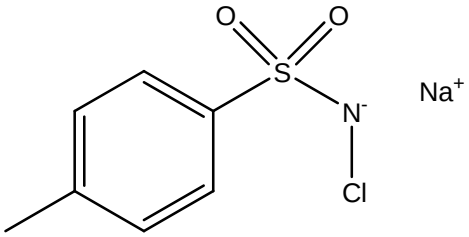

Figure 1-6. Chloramine-T structure

(adapted from http://en.wikipedia.org/wiki/Chloramine-T).

Although not currently approved by the FDA for use with food fish, it is currently being reviewed for approval and its off-label use is widespread as there is currently no other treatment approved to treat bacterial gill disease. Fish have demonstrated tolerance to this chemical as high as 43 mg/L for three hours (Dawson, *et al.*, 2003), but typical doses range from 10mg/L for one hour in hatcheries (Dawson, *et al.*, 2003) to 20 mg/L for four hours in cultured fish, depending on the pH and hardness of the water (Spangenberg, 1999). Even with this typical unapproved dose, the FDA has published that if approved, the therapeutic dose will be 10-20 mg/L for only one hour based on the hardness of the water. Because it contains an electrophilic chlorine in the chemical structure, it breaks down to hypochlorite – a potent disinfectant. Additionally, there is a sulfonamide component that inhibits bacteria in a fashion similar to the Romet-30 sulfonamide antibiotic. These characteristics allow Chloramine-T to treat the bacterial infection through a dual mode of action.

One of the major hurdles with the use of Chloramine-T is the instability in water causing the analysis of the concentration present to be difficult and time-sensitive. Historically, the method of choice has been analysis using high-performance liquid chromatography (HPLC), which is training and equipment intensive, especially in light of the instability of the chemical (Dawson and Davis, 1997; Dawson, *et al.*, 2003). In lieu of this procedure, it has been demonstrated that Chloramine-T can be detected in water through the use of DPD (N,N-diethyl-p-phenylenediamine) chlorine analysis as an indicator (Dawson, *et al.*, 2003). This

method correlates very well with the HPLC analysis and is colorometric in nature, making its use in a field environment very plausible while maintaining the sensitivity of the method if the samples are analyzed within two hours of collection. In order to obtain the Chloramine-T concentration, the bound chlorine concentration (the difference between the total and free chlorine) is multiplied by 3.21 - the ratio of anhydrous Chloramine-T to chlorine as Cl_2 (Dawson, *et al.*, 2003). Because of the successful correlation presented by Dawson *et al.*, the FDA has approved the DPD method for detection of Chloramine-T in aquaculture systems.

Potassium Permanganate

Potassium permanganate ($KMnO_4$) has long been used to remove iron and hydrogen sulfide smells.from drinking water. It can also be used as a disinfectant because of its oxidizing capabilities. Potassium permanganate is used in the U.S. aquaculture industry to control many bacterial, parasitic, and fungal exposures before they become systemic infections requiring more advanced and expensive treatments such as antibiotics (Francis-Floyd and Klinger, 1997; Lazur, 2002). This chemical is a strong oxidizer that works by eliminating organic matter. While this is beneficial because of the elimination of undesirable infectious agents, it can be detrimental due to the inability of the chemical to differentiate between undesirable matter and desirable matter (Francis-Floyd and Klinger, 2002). Because of the effect on organics, some sources advise removing the biological filtration from your system prior to treatment with potassium permanganate while others recommend leaving it in place due to the possibility of the infectious agents having taken up residence in the biofilters. Water changes are not required once the solution turns brown as this is indicative of the active chemical being spent. Water changes are sometimes carried out, however, for aesthetic purposes.

Potassium permanganate can be dosed in two different manners in aquaculture – a high-dose, short-term bath and a low-dose, long-term bath. The selection of treatment depends on factors such as the sensitivity of the fish in the system and the amount of organic matter present in the system that has to be overcome before the actual treatment occurs. The long-term bath consists of an exposure of no less than four hours at a minimum concentration of 2 mg/L. Because this chemical oxidizes organic matter in the systems, it is important to ensure that the purple color that is achieved by addition of the chemical remains for at least four

hours. As the chemical oxidizes, it will transition from purple to brown. If the brown color develops in less than four hours, another dose at 2 mg/L should be added. In the event that a concentration of 6 mg/L is reached and the chemical is oxidizing in less than the four hour period, the system should be cleaned and re-dosed (Francis-Floyd and Klinger, 1997). The second treatment option with potassium permanganate is a short-term bath at 10 mg/L for thirty minutes.

Oligonucleotide Probes and In Situ Hybridization

Monitoring the environment at the microscale is extremely difficult because for as much as the environment influences the bacteria, the bacteria also influence the environment. In addition, the techniques used must be respective of the colonies of interest with regards to minimal disturbance of properties such as the three-dimensional organization and the physicochemical gradient (Amann and Kuhl, 1998). Oligonucleotide probes have become increasingly common in the fields of microbial ecology and environmental biology, especially for organisms that do not culture well (Amann and Kuhl, 1998; Blackall, *et al.*, 1998; Biesterfield and Figueroa, 2002). However, there are still many problems with their widespread use, often as a result of their nomenclature and characterization (Alm, *et al.*, 1996). Alm *et al.* proposed a nomenclature standardization in 1996, but even that proposal has failed to become commonplace. Even with the issues related to correlating the use of specific probes between studies, these probes have proven to be invaluable to mixed culture analysis and observation. Oligonucelotide probes have shown phylogenetic relationships between microorganisms (Amann, *et al.*, 1990; Amann and Kuhl, 1998). While earlier studies required the extraction of nucleic acid, the probes have allowed investigators to assess communities using whole cells (Amann, *et al.*, 1990; Ivanov, *et al.*, 2003). Fluorescent *in situ* hybridization (FISH) was initially fairly slow to develop as cultivation of bacteria was required to produce species-specific probes. This led to the production of probes that were phylogenetic group-specific rather than species-specific. There are a small number of naturally occurring organisms that can be successfully cultured; therefore, probes which detected cells that were phylogenetically linked proved to be more useful in characterizing a natural population (Giovannoni, *et al.*, 1988). Because these probes bound to everything falling into that phylogenetic group, quantitation was difficult. Early on in the evolution of modern FISH techniques, oligonucleotide probes used radioactive labels to identify

individual cells, but these probes have more recently been labeled using fluorescent dye due to safety concerns as well as speed and sensitivity of detection (Jablonski, *et al.*, 1986; DeLong, *et al.*, 1989; Ivanov, *et al.*, 2003).

The 16S rRNA is the common target for developing the oligonucleotide probes (Giovannoni, *et al.*, 1988; Amann, *et al.*, 1995; Biesterfield, *et al.*, 2001; Biesterfield and Figueroa, 2002). RNA is a practical marker because it is present in all living organisms, has areas that are conserved within certain species, and is a dominant cellular marker (Muyzer and Ramsing, 1995). When these probes bind to their target DNA, a fluorescent signal is produced that can be observed under a fluorescent microscope. The intensity of the signal emitted is directly proportional to the physiological activity of the cells (DeLong, *et al.*, 1989). Probes that are targeting slow-growing organisms often provide a weak signal because there are fewer 16S rRNA sites available to bind to. This issue can often be overcome by using multiple probes to target the same molecules and thereby additively increase the signal (Amann, *et al.*, 1990). These probes are temperature specific in their function. This parameter, referred to as the temperature of dissociation (T_d) or melting temperature (T_m), is the temperature at which 50% of the probe and its complementary sequence bind. Probes do not bind well, or bind inappropriately at temperatures significantly lower or significantly higher than this temperature (Ivanov, *et al.*, 2003). This non-specific binding leads to false signals and background fluorescence.

In addition to characterizing the microbial community present in a specific environment, the use of probes have also been found to be useful in determining many of the parameters necessary to design nitrifying trickling filters (Biesterfield, *et al.*, 2001), to manage and characterize activated sludge (Blackall, *et al.*, 1998; Juretschko, *et al.*, 1998), and to develop biofilm models based on the ratio of nitrifiers to heterotrophs (Biesterfield, *et al.*, 2001). Investigations attempting to relate biofilm structure to biofilm function have been minimal (Biesterfield, *et al.*, 2001; Biesterfield and Figueroa, 2002). Some researchers have proposed that one of the largest possibilities for microbiological research lies in the combined use of FISH to obtain spatial distribution information with microsensors to obtain activity information (Amann and Kuhl, 1998), but two of the most common uses for FISH are to determine if decreased performance of components such as nitrifying biofilters is due to temporary inhibition or washout of bacteria (Biesterfield and Figueroa, 2002) and to determine the quantity of a specific organism within a

mixed community (Giovannoni, *et al.*, 1988). The FISH process involves annealing the RNA or DNA sequence of interest to its complementary genetic sequence within a morphologically intact cell. If there is no complementary sequence, no binding occurs and no fluorescent signal is produced.

Although proven to be a useful tool in microbiological investigations, there are several hurdles to *in situ* hybridization. One of the drawbacks to the use of fluorescent dye is the high background fluorescence of surrounding in situ material and autofluorescence of the bacteria themselves (Amann, *et al.*, 1992). To overcome some of the excess background fluorescence or cellular autofluorescence and increase the binding sensitivity, it has been shown that oligonucleotides can be bound to enzyme molecules, specifically horseradish peroxidase (HRP) prior to hybridization of the whole cells (Amann, *et al.*, 1992; Pernthaler, *et al.*, 2002). This modification to the traditional FISH protocol is referred to as catalyzed reporter deposition fluorescent in situ hybridization (CARD-FISH). The HRP assists in the deposition of fluorochrome-labeled tyramides within the target cells (Pernthaler, *et al.*, 2002; Ishii, *et al.*, 2004). Some tests have shown that the use of HRP-labeled oligonucleotides increased fluorescence by at least one order of magnitude (Amann and Kuhl, 1998; Pernthaler, *et al.*, 2002).

Cell population is also a hurdle as with only a minimal number of cells to target, the signal will be very difficult to detect. Typically, any population of less than 10^3 cells per cm^2 is extremely difficult to detect and these populations often have to be concentrated, usually by filtering the aquatic sample (Amann, *et al.*, 1995). As previously mentioned, another issue often encountered in hybridization is low signal intensity due to a low cell count or poor binding of the probe to the target organisms, often because of the inability of the probe to penetrate into the cell (Amann and Kuhl, 1998). Many methods to overcome some of these hurdles have been tested. Indirect assays attach a reporter molecule to the probe that is then detected by a labeled binding protein. There are issues with the method because the protein must penetrate the probe reporter molecules and these proteins are larger than fluorescently labeled oligonucleotides and, therefore harder to permeabilize (Amann, *et al.*, 1992; Amann and Kuhl, 1998). Multiple labeling, a method in which several oligonucleotides are used to attach to different target sites on the rRNA molecules of the cells, has also been tried. This method is restricted to the specificity and availability of target sites, which is often limited. (Amann, *et al.*, 1992).

Because it is estimated that only about 10% of microorganisms can be grown in culture, in situ hybridization helps to assess these microorganisms both independently and in biofilms (Amann and Kuhl, 1998). The percentage of the bacteria detected by in situ hybridization varies based on the environment, showing that in situ hybridization and cultures are actually complementary techniques in determining the characteristics of a microbial community (Amann and Kuhl, 1998). When the microorganisms leave suspension and form a biofilm, their properties often change drastically over small spaces (Amann and Kuhl, 1998). As such, it is very important to use techniques that disturb the biofilm as little as possible in order to capture these properties.

Successful correlation between in situ hybridization and bench-scale reactor rate data was reported by Biesterfield *et al.* (2002). In the same study, the researchers reported that while they were able to obtain a correlation for the nitrifier probe (Nso190), a similar correlation was not obtained using the bacterial probe (EUB338) that binds to active cells. More commonly used for nitrifier detection are Nso1225 for ammonia oxidizers (Daims, *et al.*, 2001) and Ntspa662 for nitrite oxidizers (Daims, *et al.*, 2001). Like other probes of interest, detailed probe information can be found on-line in probeBase (Loy, *et al.*, 2003).

Bioaugmentation

Traditionally, wastewater treatment plants have stabilized nitrification by increasing the solids retention time, but with this technique comes the requirement for an increased size in the reactors (Head and Oleszkiewicz, 2005). Bioaugmentation – the addition of a concentrated source of nitrifying bacteria – is an alternative that does not significantly change the concentration of biomass in the system because the mass of nitrifying bacteria required for nitrification is less than 5% of the total mass of solids (Metcalf and Eddy, 1991; Head and Oleszkiewicz, 2005). The theory behind bioaugmentation is to seed the systems with a culture of nitrifying bacteria from a dedicated external system so that the final sludge retention time calculates to be longer than the original sludge retention time of the treatment system (Rittmann, 1996; Wuertz, *et al.*, 2004).

Temperature – both that of the nitrifying bacteria culture and the treated system – has a significant impact on the bioaugmentation process. If the temperature of the receiving system is lower than that of the nitrifying culture, a decrease in nitrification rate is observed. For example,

it was shown that when a nitrifying culture maintained at 20°C was introduced to a bioreactor operating at 10°C, the nitrification rate was reduced by 58% (Head and Oleszkiewicz, 2005). The same study showed that when the nitrifying culture and bioreactor were both maintained at 10°C, there was no decrease in the nitrification rate and that the reactor continued to nitrify once the seeding ended as opposed to the reactor seeded with the 20°C culture, which failed.within a few days.

Although many aquaculturists use commercial products to seed their systems, it may be possible to use inocula from other operations. For example, it has been shown that bioaugmenting sequencing batch reactors with nitrifying bacteria from shrimp farms allowed the reactors to reduce more ammonia with fewer bacteria than those reactors that were supplemented with commercial bacteria (Paungfoo*, et al.*, 2003). The obstacle to pursuing this as an option lies in the concern of spreading pathogenic bacteria and disease from the shrimp systems to other production systems.

Justification for Work

While a large amount of work has been conducted in systems with nitrifiers in synthetic wastewater, little to no work has been conducted in stocked systems. Without the influence of the natural metabolism of the fish, the data collected remains guesswork. When working with a synthetic community, consideration must be given to the fact that the research is being conducted outside of its natural context (Biesterfield and Figueroa, 2002). For example, the yields, growth rates, and community structure of bacteria grown in a laboratory-controlled system vary greatly from those that are present in field conditions (Biesterfield*, et al.*, 2001; Biesterfield and Figueroa, 2002). By conducting experiments in the lab as well as in operational systems, we are beginning to bridge the gaps and make connections within the literature.

As stated in the "General Aquaculture" section, disease in a large-scale production facility is inevitable. If recirculating aquaculturists are going to continue to make a profit following a disease outbreak, they must know how their biofilters are going to respond to the additives used to combat the disease. Having an outbreak can be devastating enough, but not having the ability to return to normal operating conditions afterwards as a result of chemotherapeutic treatment, even those treatments that are approved by the FDA and/or EPA, begins to cut into the overhead and profit of the facility.

Literature Cited

Abeliovich, A. and A. Vonshak (1993). "Factors Inhibiting Nitrification of Ammonia in Deep Wastewater Reservoirs." Water Research **27**(10): 1585-1590.

Agwuh, K. N. and A. MacGowan (2006). "Pharmacokinetics and Pharmacodynamics of the Tetracyclines Including Glycyclines." Journal of Antimicrobial Chemotherapy **58**: 256-265.

Alderman, D. J. and T. S. Hastings (1998). "Antibiotic Use in Aquaculture: Development of Antibiotic Resistance - Potential for Consumer Health Risks." International Journal of Food Science and Technology **33**: 139-155.

Aleem, M. I. H. and D. L. Sewell (1981). "Mechanisms of Nitrite Oxidation and Oxidoreductase Systems in *Nitrobacter agilis*." Current Microbiology **5**: 267-272.

Alleman, J. E., V. Keramida and L. Pantea-Kiser (1987). "Light Induced *Nitrosomonas* Inhibition." Water Research **21**(4): 499-501.

Allison, S. M. and J. I. Prosser (1993). "Ammonia Oxidation at Low pH by Attached Populations of Nitrifying Bacteria." Soil Biology and Biochemistry **25**: 935-941.

Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl and L. Raskin (1996). "The Oligonucleotide Probe Database." Applied and Environmental Microbiology **62**(10): 3557-3559.

Amann, R. and M. Kuhl (1998). "*In Situ* Methods for Assessment of Microorganisms and Their Activities." Current Opinion in Microbiology **1**: 352-358.

Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux and D. A. Stahl (1990). "Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations." Applied and Environmental Microbiology **56**(6): 1919-1925.

Amann, R. I., L. Krumholz and D. A. Stahl (1990). "Fluorescent-Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental Studies in Microbiology." Journal of Bacteriology **172**(2): 762-770.

Amann, R. I., W. Ludwig and K.-H. Schleifer (1995). "Phylogenetic Identification and In Situ Detection of Individual Microbial Cells Without Cultivation." Microbiological Reviews **59**(1): 143-169.

Amann, R. I., B. Zarda, D. A. Stahl and K.-H. Schleifer (1992). "Identification of Individual Prokaryotic Cells by Using Enzyme-Labeled, rRNA-Targeted Oligonucleotide Probes." Applied and Environmental Microbiology **58**(9): 3007-3011.

Anthonisen, A. C., R. C. Loehr, T. B. S. Prakasam and E. G. Srinath (1976). "Inhibition of Nitrification by Ammonia and Nitrous Acid." Journal of Water Pollution Control Federation **48**(5): 835-852.

Anwar, H., M. K. Dasgupta and J. W. Costerton (1990). "Minireview: Testing the Susceptibility of Bacteria in Biofilms to Antibacterial Agents." Antimicrobial Agents and Chemotherapy **34**(11): 2043-2046.

Anwar, H., T. VanBiesen, M. Dasgupta, K. Lam and J. W. Costerton (1989). "Interaction of Biofilm Bacteria with Antibiotics in a Novel In Vitro Chemostat System." Antimicrobial Agents and Chemotherapy **33**(10): 1824-1826.

Aoi, Y., S. Tsuneda and A. Hirata (2004). "Transition of Bacterial Spatial Organization in a Biofilm Monitored by FISH and Subsequent Image Analysis." Water Science and Technology **49**(11-12): 365-370.

Bakal, R. S., S. A. Bai and M. K. Stoskopf (2004). "Pharmacokinetics of Sulfadimethoxine and Ormetoprim in a 5:1 Ratio Following Intraperitoneal and Oral Administration, in the Hybrid Striped Bass (*Morone chrysops x Morone saxitalis)*." Journal of Veterinary Pharmacology and Therapeutics **27**: 1-6.

Bakal, R. S. and M. K. Stoskopf (2001). "In Vitro Studies of the Fate of Sulfadimethoxine and Ormetoprim in the Aquatic Environment." Aquaculture **195**: 95-102.

Bano, N. and J. T. Hollibaugh (2000). "Diversity and Distribution of DNA Sequences with Affinity to Ammonia-Oxidizing Bacteria of the *B*-Subdivision of the Class *Proteobacteria* in the Arctic Ocean." Applied and Environmental Microbiology **66**(5): 1960-1969.

Batchelor, S. E., M. Cooper, S. R. Chhabra, L. A. Glover, G. S. A. B. Stewart, P. Williams and J. I. Prosser (1997). "Cell Density-Regulated Recovery of Starved Biofilm Populations of Ammonia-Oxidizing Bacteria." Applied and Environmental Microbiology **63**(6): 2281-2286.

Bebak-Williams, J., G. Bullock and M. C. Carson (2002). "Oxytetracycline Residues in a Freshwater Recirculating System." Aquaculture **205**: 221-230.

Belser, L. W. (1984). "Bicarbonate Uptake by Nitrifiers: Effects of Growth Rate, pH, Substrate Concentration, and Metabolic Inhibitors." Applied and Environmental Microbiology **48**(6): 1100-1104.

Benbrook, C. M. (2002). Antibiotic Drug Use in U.S. Aquaculture, The Northwest Science and Environmental Policy Center.

Biesterfield, S. and L. Figueroa (2002). "Nitrifying Biofilm Development with Time: Activitiy Versus Phylogenetic Composition." Water Environment Research **74**(5): 470 - 479.

Biesterfield, S., L. Figueroa, M. Hernandez and P. Russell (2001). "Quantification of Nitrifying Bacterial Populations in a Full-Scale Nitrifying Filter Using Fluorescent In Situ Hybridization." Water Environment Research **73**(3): 329-338.

Bjorklund, H., J. Bondestam and G. Bylund (1990). "Residues of Oxytetracycline in Wild Fish and Sediments from Fish Farms." Aquaculture **86**: 359-367.

Bjorklund, H. V., C. M. I. Rabergh and G. Bylund (1991). "Residues of Oxolinic Acid and Oxytetracycline in Fish and Sediments from Fish Farms." Aquaculture **97**: 85-96.

Black, K. D., Ed. (2001). Environmental Impacts of Aquaculture. Boca Raton, FL, SCRC Press LLC.

Blackall, L. L., P. C. Burrell, H. Gwilliam, D. Bradford, P. L. Bond and P. Hugenholtz (1998). "The Use of 16S rDNA Clone Libraries to Describe the Microbial Diversity of Activated Sludge Communities." Water Science and Technology **37**(4-5): 451-454.

Bostock, J. C., L. G. O. Montorio, J. F. Muir and A. E. D. McQueen (2002). Studies on Specific Surface Area and Surface Morphology of Commercial Granular Media Used as Biological Carriers of Fixed Biofilms. 4th International Conference on Recirculating Aquaculture, Roanoke, VA.

Botrous, A. E. F., M. F. Dahab and P. Mihaltz (2004). "Nitrification of a High-Strength Ammonium Wastewater by a Fluidized-Bed Reactor." Water Science and Technology **49**(5-6): 65-71.

Bower, C. E. and D. T. Turner (1982). "Effects of Seven Chemotherapeutic Agents on Nitrification in Closed Seawater Culture Systems." Aquaculture **29**: 331-345.

Braam, F. and A. Klapwuk (1981). "Effect of Copper on Nitrification in Activated Sludge." Water Research **15**: 1093-1098.

Buchmann, K., J. Bresciani and C. Jappe (2004). "Effects of Formalin Treatment on Epithelial Structure and Mucous Cell Densities in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), skin." Journal of Fish Diseases **27**: 99-104.

Bureau, D. P. and C. Y. Cho. (1999). "Nutritional Strategies for the Management of Aquaculture Wastes." Addressing Concerns for Water Quality Impacts from Large-Scale Great Lakes Aquaculture Retrieved June 27, 2007, from http://www.ijc.org/php/publications/html/aquaculture/app04.html.

Burrell, P. C., J. Keller and L. L. Blackall (1998). "Microbiology of a Nitrite-Oxidizing Bioreactor." Applied and Environmental Microbiology **64**(5): 1878-1883.

Burrell, P. C., C. M. Phalen and T. A. Hovanec (2001). "Identification of Bacteria Responsible for Ammonia Oxidation in Freshwater Aquaria." Applied and Environmental Microbiology **67**(12): 5791-5800.

Capone, D. G., D. P. Weston, V. Miller and C. Shoemaker (1996). "Antibacterial Residues in Marine Sediments and Invertebrates Following Chemotherapy in Aquaculture." Aquaculture **145**(55-75).

Chopra, I. and T. G. B. Howe (1978). "Bacterial Resistance to Tetracyclines." Microbiological Reviews **42**(4): 707-724.

Chopra, I. and M. Roberts (2001). "Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance." Microbiology and Molecular Biology Reviews **65**(2): 232-260.

Chun, S. K., I. B. Kim and D. S. Chang (1978). "Effects of Bacteriocides on the Nitrification in the Recirculating Aquarium." Bulletin of National Fisheries, University of Busan **18**: 69-81.

Collins, M. T., J. B. Gratzek, D. L. Dawe and T. G. Nemetz (1975). "Effects of Parasiticides on Nitrification." Journal of the Fisheries Research Board of Canada **32**: 2033-2037.

Collins, M. T., J. B. Gratzek, D. L. Dawe and T. G. Nemetz (1976). "Effects of Antibacterial Agents on Nitrification in an Aquatic Recirculating System." Journal of the Fisheries Research Board of Canada **33**(2): 215-218.

Coyne, R., M. Hiney, B. O'Connor, J. Kerry, D. Cazabon and P. Smith (1994). "Concentration and Persistence of Oxytetracycline in Sediments Under a Marine Salmon Farm." Aquaculture **123**: 31-42.

Daigger, G. T. and T. E. Sadick (1998). "Evaluation of Methods to Detect and Control Nitrification Inhibition with Specific Application to Incinerator Flue-Gas Scrubber Water." Water Environment Research **70**(7): 1248-1257.

Daims, H., J. L. Nielsen, P. H. Nielsen, K.-H. Schleifer and M. Wagner (2001). "In Situ Characterization of *Nitrospira*-Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants." Applied and Environmental Microbiology **67**(11): 5273-5284.

Dawson, V. K. and R. A. Davis (1997). "Liquid Chromatographic Determination of Chloramine-T and its Primary Decomposition Product p-Toluenesulfonamide in Water." Journal of the Association of Official Analytical Chemists **80**: 316-318.

Dawson, V. K., J. R. Meinertz, L. J. Schmidt and W. H. Gingerich (2003). "A Simple Analytical Procedure to Replace HPLC for Monitoring Treatment Concentrations of Chloramine-T on Fish Culture Facilities." Aquaculture **217**(1-4): 61-72.

deBeer, D. and P. Stoodley (1995). "Relation Between the Structure of an Aerobic Biofilm and Transport Phenomena." Water Science and Technology **32**(8): 11-18.

deBeer, D., P. Stoodley and Z. Lewandowski (1996). "Liquid Flow and Mass Transport in Heterogeneous Biofilms." Water Research **30**: 2761-2765.

DeLong, E. F., G. S. Wickham and N. R. Pace (1989). Phylogenetic Stains: Ribosomal RNA-Based Probes for the Identification of Single Cells. Science. **243:** 1360-1363.

Diab, S. and M. Shilo (1988). "Effect of Adhesion to Particles on the Survival and Activity of *Nitrosomonas* sp. and *Nitrobacter* sp." Archives of Microbiology **150**: 387-393.

Dietze, J. E., E. A. Scribner, M. T. Meyer and D. W. Kolpin (2005). "Occurrence of Antibiotics in Water From 13 Fish Hatcheries, 2001-2003." International Journal of Environmental Analytical Chemistry **85**(15): 1141-1152.

Du, W. X., M. R. Marshall, W. B. Wheeler, M. Mathews, D. Gatlin, S. D. Rawles, D.-H. Xu, W. A. Rodgers and C. I. Wei (1995). "Oxytetracycline, Sulfadimethoxine, and Ormetoprim Residues in Channel Catfish by HPLC." Journal of Food Science **60**(6): 1220-1224, 1227.

Ehrich, S., D. Behrens, E. Lebedeva, W. Ludwig and E. Bock (1995). "A New Obligately Chemolithoautotrophic, Nitrite-Oxidizing Bacterium, *Nitrospira moscoviensis* sp. nov. and its Phylogenetic Relationship." Archives of Microbiology **164**: 16-23.

Eilersen, A. M., M. Henze and L. Kloft (1994). "Effect of Volatile Fatty Acids and Trimethylamine on Nitrification in Activated Sludge." Water Research **28**(6): 1329-1336.

Fagbenro, O. and K. Jauncey (1995). "Water Stability, Nutrient Leaching and Nutritional Properties of Moist Fermented Fish Silage Diets." Aquacultural Engineering **14**: 143-153.

Fajer-Avila, E. J., I. Abdo-delaParra, G. Aguilar-Zarate, R. Contreras-Arce, J. Zaldivar-Ramirez and M. Betancourt-Lozano (2003). "Toxicity of Formalin to Bullseye Puffer Fish (*Sphoeroides annulatus*, Jenyns, 1843) and its Effectiveness to Control Ectoparasites." Aquaculture **223**: 41-50.

Feeks, J. (2007). AQUAFLOR(R) (florfenicol) Approved for Freshwater-reared Salmonids. P. Release. Kenilworth, New Jersey, Schering-Plough Corporation.

Ferris, F. G., S. Schultze, T. C. Witten, W. S. Fyfe and T. J. Beveridge (1989). "Metal Interactions with Microbial Biofilms in Acidic and Neutral pH Environments." Applied and Environmental Microbiology **55**: 1249-1257.

Flemming, H.-C. (1995). "Sorption Sites in Biofilms." Water Science and Technology **32**(8): 27-33.

Flimlin, G., S. Sugiura and R. P. Ferraris (2003). Examining Phosphorus in Effluents from Rainbow Trout (*Oncorhynchus mykiss*) Aquaculture, Rutgers Cooperative Extension - U.S. Department of Agriculture.

Francis-Floyd, R. and R. Klinger (1997). Fact Sheet FA37: Use of Potassium Permanganate to Control External Infections of Ornamental Fish, University of Florida, Institute of Food and Agricultural Sciences.

From, J. (1980). "Chloramine-T for Control of Bacterial Gill Disease." Progressive Fish-Culturist **42**: 85-86.

Gernaey, K., L. Verschuere, L. Luyten and W. Verstraete (1997). "Fast and Sensitive Acute Toxicity Detection with an Enrichment Nitrifying Culture." Water Environment Research **69**(6): 1163-1169.

Gieseke, A., U. Purkhold, M. Wagner, R. Amann and A. Schramm (2001). "Community Structure and Activity Dynamics of Nitrifying Bacteria in a Phosphate-Removing Biofilm." Applied and Environmental Microbiology **67**(3): 1351-1362.

Giovannoni, S. J., E. F. DeLong, G. J. Olson and N. R. Pace (1988). "Phylogenetic Group-Specific Oligonucleotide Probes for the Identification of Single Microbial Cells." Journal of Bacteriology **170**: 720-726.

Gjaltema, A. and T. Griebe (1995). "Laboratory Biofilm Reactors and On-line Monitoring: Report of the Discussion Session." Water Science and Technology **32**(8): 257-261.

Hagopian, D. S. and J. G. Riley (1998). "A Closer Look at the Bacteriology of Nitrification." Aquacultural Engineering **18**: 223-244.

Halling-Sorensen, B. (2001). "Inhibition of Aerobic Growth and Nitrification of Bacteria in Sewage Sludge by Antibacterial Agents." Archives of Environmental Contamination and Toxicology **40**: 451-460.

Hargrove, L. L., P. W. Westerman and T. M. Losordo (1996). "Nitrification in Three-stage and Single-stage Floating Bead Biofilters in a Laboratory-scale Recirculating Aquaculture System." Aquacultural Engineering **15**(1): 67-80.

Harrison, J. J., R. J. Turner, L. L. R. Marques and H. Ceri (2005). Biofilms. American Scientist. **November-December:** 508-515.

Haywood, G. P., Ed. (1983). Ammonia Toxicity in Teleost Fishes: A Review. Nanaimo, British Colombia, Canada, Department of Fisheries and Oceans.

Head, M. A. and J. A. Oleszkiewicz (2005). "Nitrifying Bacteria Addition into Reactors Operating Near Washout Conditions for Nitrification." Journal of Environmental Engineering **4**: 257-264.

Hektoen, H., J. A. Berge, V. Hormazabal and M. Yndestad (1995). "Persistence of Antibacterial Agents in Marine Sediments." Aquaculture **133**(175-184).

Himmelsbach, M. and W. Buchberger (2005). "Residue Analysis of Oxytetracycline in Water and Sediment Samples by High-Performance Liquid Chromatography and Immunochemical Techniques." Microchimica Acta **151**: 67-72.

Hoffman-LaRoche (1984). Romet-30 (R) Environmental Assessment.

Hollocher, T. C., M. E. Tate and D. J. D. Nicholas (1981). "Oxidation of Ammonia by *Nitrosomonas europaea*. Definitive 18O-tracer Evidence that Hydroxylamine Formation Involves a Monooxygenase." Journal of Biological Chemistry **256**: 10834-10836.

Hovanec, T. A. and E. F. DeLong (1996). "Comparative Analysis of Nitrifying Bacteria Associated with Freshwater and Marine Aquaria." Applied and Environmental Microbiology **62**(8): 2888-2896.

Hovanec, T. A., L. T. Taylor, A. Blakis and E. F. DeLong (1998). "*Nitrospira*-like Bacteria Associated with Nitrite Oxidation in Freshwater Aquaria." Applied and Environmental Microbiology **64**(1): 258-264.

Ishii, K., M. Mubmann, B. J. MacGregor and R. Amann (2004). "An Improved Fluorescence In Situ Hybridization Protocol for the Identification of Bacteria and Archaea in Marine Sediments." FEMS Microbiology Ecology **50**: 203-212.

Ivanov, V., S. T.-L. Tay and J.-H. Tay (2003). "Monitoring of Microbial Diversity of Fluorescence In Situ Hybridization and Fluorescence Spectrometry." Water Science and Technology **47**(5): 133-138.

Jablonski, E., E. W. Moomaw, R. H. Tullis and J. L. Ruth (1986). "Preparation of Oligodeoxynucleotide-Alkaline Phosphatase Conjugates and Their Use as Hybridization Probes." Nucleic Acids Research **14**(15): 6115-6128.

Jacobsen, P. and L. Berglind (1988). "Persistence of Oxytetracycline in Sediments from Fish Farms." Aquaculture **70**: 365-370.

Jang, A., P. L. Bishop, S. Okabe, S. G. Lee and I. S. Kim (2002). "Effect of Dissolved Oxygen Concentration on the Biofilm and In Situ Analysis by Fluorescence In Situ Hybridization (FISH) and Microelectrodes." Water Science and Technology **47**(1): 49-57.

Juretschko, S., G. Timmermann, M. Schmid, K.-H. Schleifer, A. Pommerening-Roser, H.-P. Koops and M. Wagner (1998). "Combined Molecular and Conventional Analyses of Nitrifying Bacterium Diversity in Activated Sludge: *Nitrosococcus mobilis* and *Nitrospira*-like Bacteria as Dominant Populations." Applied and Environmental Microbiology **64**(8): 3042-3051.

Keck, N. and G. Blanc (2002). "Effects of Formalin Chemotherapeutic Treatments on Biofilter Efficiency in a Marine Recirculating Fish Farming System." Aquatic Living Resources **15**: 361-370.

Keen, G. A. and J. I. Prosser (1987). "Interrelationship Between pH and Surface Growth of *Nitrobacter*." Soil Biology and Biochemistry **19**: 665-672.

Kim, D.-J. and S.-H. Kim (2006). "Effect of Nitrite Concentration on the Distribution and Competition of Nitrite-oxidizing Bacteria in Nitratation Reactor Systems and Their Kinetic Characteristics." Water Research **40**: 887-894.

Kim, I. S. and V. N. Ivanon (2000). "Detection of Nitrifying Bacteria in Activated Sludge by Fluorescent In Situ Hybridization and Fluorescent Spectrometry." World Journal of Microbiology and Biotechnology **16**: 425-430.

Kim, K. T., I. S. Kim, S. H. Hwang and S. D. Kim (2006). "Estimating the Combined Effects of Copper and Phenol to Nitrifying Bacteria in Wastewater Treatment Plants." Water Research **40**(561-568).

Kindaichi, T., S. Okabe, H. Satoh and Y. Watanabe (2004). "Effects of Hydroxyalamine on Microbial Community Structure and Function of Autotrophic Nitrifying Biofilms Determined by In Situ Hybridization and the Use of Microelectrodes." Water Science and Technology **49**(11-12): 61-68.

Klaver, A. L. and R. A. Matthews (1994). "Effects of Oxytetracycline on Nitrification in a Model Aquatic System." Aquaculture **123**: 237-247.

KoiFlorida. "Formalin." Retrieved June 27, 2007, from http://www.geocities.com/koifla/Formalin.htm.

Kolsater, L. (1995). "Feed Management and Reduction of Aquaculture Wastes." Water Science and Technology **31**(10): 213-218.

Lanari, D., E. D'Agaro and R. Ballestrazzi (1995). "Dietary N and P Levels, Effluent Water Characteristics and Performance in Rainbow Trout." Water Science and Technology **31**(10): 157-165.

Lazarova, V., D. Bellahcen, D. Rybacki, B. Rittman and J. Manem (1998). "Population Dynamics and Biofilm Composition in a New Three-phase Circulating Bed Reactor." Water Science and Technology **37**(4-5): 149-159.

Lazur, A. M. (2002). Fact Sheet FA23: The Use of Potassium Permanganate in Fish Ponds, University of Florida - Institute of Food and Agricultural Sciences.

Liu, Y. and B. Capdeville (1996). "Specific Activity of Nitrifying Biofilm in Water Nitrification Process." Water Research **30**(7): 1645-1650.

Loy, A., M. Horn and M. Wagner (2003). "ProbeBase- An Online Resource for rRNA-targeted Oligonucleotide Probes." Nucleic Acids Research **31**: 514-516.

Lunestad, B. T., O. B. Samuelsen, S. Fjelde and A. Ervik (1995). "Photostability of Eight Antibacterial Agents in Seawater." Aquaculture **134**: 217-225.

Massone, A., K. Gernaey, A. Rozzi and W. Verstaete (1998). "Measurement of Ammonium Concentration and Nitrification Rate by a New Titrimetric Biosensor." Water Environment Research **70**(3): 343-350.

McCaig, A. E., C. J. Phillips, J. R. Stephen, G. A. Kowalchuk, S. M. Harvey, R. A. Herbert, T. M. Embley and J. I. Prosser (1999). "Nitrogen Cycling and Community Structure of Proteobacterial Beta-Subgroup Ammonia-Oxidizing Bacteria Within Polluted Marine Fish Farm Sediments." Applied and Environmental Microbiology **65**: 213-220.

Metcalf and Eddy (1991). Advanced Wastewater Treatment. Wastewater Engineering: Treatment, Disposal and Reuse. McGraw-Hill. Boston, MA**:** 663-763.

Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann and D. A. Stahl (1996). "Phylogenetic Probes for Analyzing Abundance and Spatial Organization of Nitrifying Bacteria." Applied and Environmental Microbiology **62**(6): 2156-2162.

Muyzer, G. and N. B. Ramsing (1995). "Molecular Methods to Study the Organization of Microbial Communities." Water Science and Technology **32**(8): 1-9.

Nogueira, R., L. F. Melo, U. Purkhold, S. Wuertz and M. Wagner (2002). "Nitrifying and Heterotrophic Population Dynamics in Biofilm Reactors: Effects of Hydraulic Retention Time and the Presence of Organic Carbon." Water Research **36**: 469-481.

Okabe, S., H. Satoh and Y. Watanabe (1999). "In Situ Analysis of Nitrifying Biofilms as Determined by In Situ Hybridization and the Use of Microelectrodes." Applied and Environmental Microbiology **65**(7): 3182-3191.

Painter, H. A. (1970). "A Review of Literature on Inorganic Nitrogen Metabolism in Microorganisms." Water Research **4**: 393-450.

Park, E. D., D. V. Lightner, N. Milner, M. Mayersohn, D. L. Park, J. M. Gifford and T. A. Bell (1995). "Exploratory Bioavailability and Pharmacokinetic Studies of Sulphadimethoxine and Ormetoprim in the Penaeid Shrimp *Penaeus vannamei*." Aquaculture **130**: 113-128.

Paungfoo, C., P. Prasertsan, N. Intrasungkha, L. L. Blackall and R. Bhamidimarri (2003). "Enrichment of Nitrifying Microbial Communities from Shrimp Farms and Commercial Inocula." Water Science and Technology **48**(8): 143-150.

Pernthaler, A., J. Pernthaler and R. Amann (2002). "Fluorescence In Situ Hybridization and Catalyzed Reporter Deposition for the Identification of Marine Bacteria." Applied and Environmental Microbiology **68**(6): 3094.

Pfizer (1987). Terramycin (oxytetracycline) Environmental Assessment.

Powell, S. J. and J. I. Prosser (1986). "Inhibition of Ammonium Oxidation by Nitrapyrin in Soil and Liquid Culture." Applied and Environmental Microbiology **52**(4): 782-787.

Princic, A., I. Mahne, F. Megusar, E. A. Paul and H. Tiedje (1998). "Effects of pH and Oxygen and Ammonium Concentrations on the Community Structure of Nitrifying Bacteria from Wastewater." Applied and Environmental Microbiology **64**(10): 3584-3590.

Prosser, J. I. (1989). "Autotrophic Nitrification in Bacteria." Advanced Microbial Physiology **30**: 125-181.

Purkhold, U., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H.-P. Koops and M. Wagner (2000). "Phylogeny of All Recognized Species of Ammonia Oxidizers Based on Comparative 16S rRNA and *amoA* Sequence Analysis: Implications for Molecular Diversity Surveys." Applied and Environmental Microbiology **66**(12): 5368-5382.

Rajasekaran, P., N. Sriranganathan and S. M. Boyle (2007). Antibiotic Resistance - a Formidable Threat to the Human Race - Are We Losing the War Against Pathogens. 5th International Student/Young Pugwash Conference, Bari, Italy.

Rhodes, G., G. Huys, J. Swings, P. McGann, M. Hiney, P. Smith and R. W. Pickup (2000). "Distribution of Oxytetracycline Resistance Plasmids Between Aeromonads in Hospital and Aquaculture Environments: Implications of TN1 721 in Dissemination of the Tetracycline Resistance Determinant Tet A." Applied and Environmental Microbiology **66**(9): 3883-3890.

Rittmann, B. E. (1996). "How Input Active Biomass Affects Sludge Age and Process Stability." Journal of Environmental Engineering **122**: 4-8.

Rowan, A. K., G. Moser, N. Gray, J. R. Snape, D. Fearnside, T. P. Curtis, M. R. Barer and I. M. Head (2003). "A Comparative Study of Ammonia-Oxidizing Bacteria in Lab Scale Industrial Wastewater Treatment Reactors." Water Science and Technology **48**(3): 17-24.

Samuelsen, O. B. (1989). "Degradation of Oxytetracycline in Seawater at Two Different Temperatures and Light Intensities, and the Persistence of Oxytetracycline in the Sediment From a Fish Farm." Aquaculture **83**: 7-16.

Samuelsen, O. B., B. T. Lunestad, A. Ervik and S. Fjelde (1994). "Stability of Antibacterial Agents in an Artificial Marine Aquaculture Sediment Studied Under Laboratory Conditions." Aquaculture **126**: 283-290.

Samuelsen, O. B., V. Torsvik and A. Ervik (1992). "Long-range Changes in Oxytetracycline Concentration and Bacterial Resistance Towards Oxytetracycline in a Fish Farm Sediment After Medication." Science of the Total Environment **114**: 25-36.

Schmitt, J., D. Nivens, D. C. White and H.-C. Flemming (1995). "Changes of Biofilm Properties in Response to Sorbed Substances - an FITR-ATR Study." Water Science and Technology **32**(8): 149-155.

Schramm, A., D. DeBeer, J. C. VanDenHeuvel, S. Ottengraf and R. Amann (1999). "Microscale Distribution of Populations and Activities of *Nitrosospira* and *Nitrospira* spp. Along a Macroscrale Gradient in a Nitrifying Bioreactor: Quantification by In Situ Hybridization and the Use of Microsensors." Applied and Environmental Microbiology **65**(8): 3690-3696.

Schramm, A., D. DeBeer, M. Wagner and R. Amann (1998). "Identification and Activities In Situ of *Nitrosospira* and *Nitrospira* spp. as Dominant Populations in a Nitrifying Fluidized Bed Reactor." Applied and Environmental Microbiology **64**(9): 3480-3485.

Schramm, A., L. H. Larsen, N. P. Revsbech, N. B. Ramsing, R. Amann and K.-H. Schleifer (1996). "Structure and Function of a Nitrifying Biofilm as Determined by In Situ Hybridization and the Use of Microelectrodes." Applied and Environmental Microbiology **62**(12): 4641-4647.

Siegrist, H. and W. Gujer (1987). "Demonstrations of Mass Transfer and pH Effects in a Nitrifying Biofilm." Water Research **21**(12): 1481-1487.

Spangenberg, J. V. (1999). "Investigation of the Saftety and Efficacy of Chloramine-T in the Treatment of External Fluke Infestations."

Spotte, S. (1970). Fish and Invetebrate Culture: Water Management in Closed Systems. New York, NY, Wiley.

Stewart, P. S., R. Murga, R. Srinivasan and D. deBeer (1995). "Biofilm Structural Heterogeneity Visualized by Three Microscopic Methods." Water Research **29**: 2006-2009.

Tappe, W., A. Laverman, M. Bohland, M. Braster, S. Rittershaus, J. Groeneweg and H. W. VanVerseveld (1999). "Maintenance Energy Demand and Starvation Recovery Dynamics of *Nitrosomonas europaea* and *Nitrobacter winogradskyi* Cultivated in a Retentostat with Complete Biomass Retention." Applied and Environmental Microbiology **65**: 2471-2477.

Thorn, M., A. Mattsson and F. Sorensson (1996). "Biofilm Development in a Nitrifying Trickling Filter." Water Science and Technology **34**(1-2): 83-89.

Twarowska, J. G., P. W. Westerman and T. M. Losordo (1996). Water Treatment and Waste Characterization Evaluation of an Intensive Recirculating Fish Production System. 1st International Conference on Recirculating Aquaculture, Roanoke, VA.

vanLoosdrecht, M. C. M., D. Eikelboom, A. Gjaltema, A. Mulder, L. Tijhuis and J. J. Heignen (1995). "Biofilm Structures." Water Science and Technology **32**(8): 35-43.

Wagner, M., G. Rath, H.-P. Koops, J. Flood and R. Amann (1996). "In Situ Analysis of Nitrifying Bacteria in Sewage Treatment Plants." Water Science and Technology **34**(1-2): 237-244.

Wheaton, F. (2002). Recirculating Aquaculture Systems: An Overview of Waste Management. 4th International Conference on Recirculating Aquaculture, Roanoke, VA.

Whitby, C. B., J. R. Saunders, J. Rodriguez, R. W. Pickup and A. McCarthy (1999). "Phylogenetic Differentiation of Two Closely Related *Nitrosomonas* spp. That Inhabit Different Sediment Environments in an Oligotrophic Freshwater Lake." Applied and Environmental Microbiology **65**: 4855-4862.

Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig and E. Stackebrandt (1985). "The Phylogeny of Purple Bacteria: the Gamma Subdivision." Systematic and Applied Microbiology **6**: 25-33.

Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Kreig, H.-P. Koops, H. Harms and E. Stackebrandt (1984). "The Phylogeny of Purple Bacteria: the Beta Subdivision." Systematic and Applied Microbiology **5**: 327-336.

Wuertz, S., S. Okabe and M. Hausner (2004). "Microbial Communities and Their Interactions in Biofilm Systems: An Overview." Water Science and Technology **49**(11-12): 327-336.

Chapter 2: Impacts of Six Common Chemotherapeutic Agents on Nitrification in Operational Aquaculture Systems

Impacts of Six Common Chemotherapeutic Agents on Nitrification in Operational Aquaculture Systems

Abstract

In any mass production agricultural industry, disease is virtually inevitable and the aquaculture industry is no exception. Currently, there is a narrow variety of chemicals approved by either the Food and Drug Administration (FDA) or the U.S. Environmental Protection Agency (EPA) for the treatment of disease outbreaks and water quality issues in aquaculture. These chemicals include antiparasitics, antibiotics, and chemicals that area used off-label with therapeutic benefit. During these trials, the impacts of therapeutic concentrations of six chemotherapeutic agents on nitrification in operational aquaculture systems were evaluated. Aquaria (20-gallon) were set up with oxygen supplies and standard box filters. All controls and treatments were conducted in triplicate. The six chemotherapeutants analyzed were chelated copper, formalin (37% formaldehyde solution), oxytetracycline medicated feed, Romet-30® medicated feed, potassium permanganate, and Chloramine-T. All doses were based on approvals by either EPA or FDA or, in the case of off-label use, industry standards. One tilapia was housed in each aquarium to influence the filter system rather than conducting the experiments on synthetic systems. Treatments with the chelated copper, oxytetracycline, and Romet-30® all resulted in at least partial inhibition ($p < 0.05$) to the nitrification processes in the individual aquaria. In all of these, nitrification resumed upon completion of the treatment or following the required water change. Findings from specific oxygen uptake rate analysis (sOUR) supported the findings of the water quality analyses. Additionally, catalyzed reporter deposition – fluorescent in situ hybridization (CARD-FISH) analysis demonstrated that the decrease in nitrification was the result of cellular inhibition rather than from a reduction or shift in the nitrifier populations.

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Introduction

In any mass production agricultural industry, disease is virtually inevitable and the aquaculture industry is no exception. Currently, there is a narrow variety of chemicals approved by either the Food and Drug Administration (FDA) or the Environmental Protection Agency (EPA) for the treatment of disease outbreaks and water quality issues in aquaculture. Many believe that the use of chemotherapeutants in aquaculture is in great need of attention because of this limited number of approved chemicals available. This lack of availability leads aquaculturists to use legal and illegal drugs and unapproved general-purpose chemicals all in unapproved methods to treat their systems for disease outbreaks. Concerns over the impacts of these agents on the nitrifying bacteria began as early as the mid-1900's and, in 1976, experiments were conducted that demonstrated that the use of many antibiotics, including oxytetracycline, had no significant impact to the nitrification process (Collins*, et al.*, 1976). Chemical agents often have a significant impact on nitrification in seawater systems based on the investigations of seven common agents, including copper sulfate (Bower and Turner, 1982). The classification of aquaculture chemotherapeutants can be distributed into broad categories based on functionality, including antiparasitics, antibiotics, and off-label chemical treatments.

Two common antiparasitics in aquaculture are copper and formalin, both of which are approved for the treatment of external parasites. Copper is often dosed as copper sulfate as it is inexpensive and reasonably easy to use; however, chelated copper is favored at many facilities because it is less corrosive to equipment and more stable in solution than its copper sulfate counterpart. In small doses, copper is actually beneficial for the growth of nitrifying bacteria, serving as a micronutrient that is a component of many critical cellular enzymes. It has also been shown that copper concentrations <1 mg/L can cause inhibition of ammonia-oxidizing bacteria (AOB) grown in pure culture (Skinner and Walker, 1961); however, the approved dose is 0.25 mg/L free copper (AppliedBiochemists, 2002) – quite a bit less than this inhibitory threshold. Copper inhibits the bacteria either by the displacement of other essential metals from their metabolic sites by the copper or by interfering with the osmotic balance of the cell and alteration of the structures of nucleic acids and proteins (Hu*, et al.*, 2003; Borkow and Gabbay, 2005). The magnitude of the inhibition is dependent on the ratio of the copper dose to nitrifying biomass and the length of the exposure of the nitrifying biomass to the copper (Hu*, et al.*, 2003). Formalin

has been used in embalming practices and as a fixative for bacteria for many years. Formalin treatments on the biofilter in marine systems showed that while formalin had no apparent effect on the AOB, there was a significant effect on the nitrite-oxidizing bacteria (NOB) when the system was subjected to recirculating concentrations greater than 60 mg/L for four hours, causing the nitrite to build up in the systems (Keck and Blanc, 2002). Formalin is a 37% (by weight) formaldehyde solution that contains up to 15% methanol to stabilize the solution and prevent the formation of the highly toxic paraformaldehyde.

Of the few chemicals actually approved by FDA for use in food fish, antibiotics are among the fewest in number (USFDA, 2008). Oxytetracycline and Romet-30® have been approved for use by the FDA since 1970's and 1980's, respectively. Since then, only one additional antibiotic has been approved: a florfenicol marketed under the brand-name Aquaflor®, which was approved in 2007 (USFDA, 2008). With so few options, it is no surprise that the industry has some difficulty treating the disease outbreaks that come with the territory of working in a mass production industry. Additionally, with the emergence of antibiotic-resistant bacteria and "super bugs", FDA seems hesitant to continue approving additional antibiotics for use. When one considers that the antibiotic is typically coated to feed, poorly absorbed by the fish, and left to degrade in the environment, this hesitance is understandable.

Oxytetracycline (Figure 2-1) is a broad-spectrum antibiotic that works against Gram-negative bacteria. Tetracyclines as a group became popular in clinical practice and development for use in humans in the 1950's (Agwuh and MacGowan, 2006) after being discovered in the 1940's (Chopra and Roberts, 2001). Oxytetracycline is one of the older oral antibiotics that has reduced absorption and is less lipophilic than some of the newer tetracyclines (Agwuh and MacGowan, 2006). In humans, approximately 58% of oxytetracycline is absorbed, mostly by the stomach, duodenum, and small intestine, and approximately 50% is eliminated by the body through fecal matter (Agwuh and MacGowan, 2006). It is estimated that only 7-9% of the oxytetracycline is digested and absorbed by most fish, likely because the antibiotic binds to the calcium and magnesium in the feed and becomes unavailable (Bebak-Williams*, et al.*, 2002). Additionally, the partition coefficients of oxytetracycline make it unlikely that the chemical can be stored in lipid tissue (Pfizer, 1987). This absorption is estimated to be even less than in humans, and 15 - 40% of the residual antibiotic enters the environment in the form of uneaten feed depending on

the feeding habits of the sick fish (Capone, *et al.*, 1996). In comparison, the digestibility of oxolinic acid was approximately 38% (Bjorklund, *et al.*, 1991).

Oxytetracycline chelates divalent cations and may accumulate in system water, biofilters, fecal matter, and uneaten feed (Bebak-Williams, *et al.*, 2002). In recirculating systems housing rainbow trout, Bebak-Williams *et al.* (2002) demonstrated that oxytetracycline reached peak concentrations on day 10 of treatment and was at or below the detection limit (0.001 μg/g) within 10 days after treatment ended. Likewise, the same group reported that the oxytetracycline concentration peaked on day 10 of treatment and then consistently decreased over the holding period. Unlike in the water column, the oxtytetracycline residue was maintained longer in the sediments of the systems with an average concentration of 4.8 μg/g 10 days after treatment ended (Bebak-Williams, *et al.*, 2002). In both the water column and the sediment, the residue concentration decreased sharply once dosing ended and did not increase again. That the oxytetracycline lingers in sediments is cause for concern with respect to the ingestion of these contaminated sediments by wild population fish that may linger around net pens. In recirculating systems, the oxytetracycline is allowed to recirculate through the system as the antibiotic degrades. The fate of the oxytetracycline in the biofilters is highly dependent on the material in the filters, but deteriorates rapidly over the course of the treatment and holding periods (Bebak-Williams, *et al.*, 2002)

Tetracyclines function by inhibiting protein synthesis and preventing the binding of aminoacyl-tRNA to the bacterial ribosome (Chopra and Howe, 1978; Chopra and Roberts, 2001). The tetracyclines have molecular weights of less than 500 daltons which allows them to diffuse through pores in the cell membrane of Gram-negative bacteria, especially in the case of oxytetracycline which is hydrophilic in nature (Chopra and Howe, 1978). Tetracyclines are acidic in nature and, as such, dissociate in aqueous solutions and cross the outer membrane of the Gram-negative bacteria by binding to a metal cation and passing through the OmpF and OmpC porins. The antibiotic accumulates in the periplasm before dissociating from the cation and diffusing through the lipid bilayer of the cytoplasmic membrane via proton motive force (Chopra and Howe, 1978; Chopra and Roberts, 2001). The minimum inhibitory concentration of oxytetracycline is estimated to be less than 1.56 mg/L for many strains of bacteria (Jacobsen and Berglind, 1988). A study evaluating oxytetracycline inhibition within a synthetic freshwater system resulted in the finding that oxytetracycline concentrations as low as 12.5 mg/L inhibit the

nitrification process, but the authors recognize that the exposure to oxytetracycline in a bath treatment may have a substantially different effect on nitrification than exposure to oxytetracycline coated onto a feed (Klaver and Matthews, 1994).

Although controversial, tetracyclines are used in some countries at a level below that which is dosed for therapeutic purposes to promote growth and dissuade illness (Pfizer, 1987); however, this has potentially contributed to the emergence of bacterial resistance (Chopra and Roberts, 2001). Between 1917 and 1954, it was shown that of 433 different members of *Enterobacteriaceae* collected, only 2% were resistant to tetracyclines (Chopra and Roberts, 2001). That figure is suspected to be much higher now and, since this time, tetracyclines have been shown to induce antibiotic resistance in pathogenic bacteria (Bjorklund*, et al.*, 1990). As of 2001, twenty-nine tetracycline resistant genes and three oxytetracycline resistant genes had been characterized and these genes were linked to various efflux pumps and ribosomal protection proteins (Chopra and Roberts, 2001). Other resistant genes continue to be identified and, when expression of these genes occurs, the bacteria are no longer susceptible to inhibition due to tetracycline exposure. This is typically because the bacteria are able to expel the tetracycline via efflux pump or because the tetracycline is unable to bind to the ribosome because protection proteins are bound to the ribosome instead. The binding of oxytetracycline to the ribosomes is reversible, however, and this explains how the antibiotic may inhibit the bacteria it affects without completely destroying them (Chopra and Roberts, 2001).

Romet-30 ® is a broad-spectrum antibiotic formulated from a 5 to 1 combination of sulfadimethoxine and ormetoprim, respectively (Figures 2-2 and 2-3). Romet-30® is a potentiated sulfonamide that blocks the synthesis of tetrahydrofolic acid, which is required for the synthesis of proteins and nucleic acid metabolism (USFDA, 1984). This blocking takes place only in bacteria as opposed to animals because animals do not synthesize folic acid (Hoffman-LaRoche, 1984). Although each of these components has antibiotic characteristics on their own, the efficacy of the drugs in combination is greater at lower doses (Bakal*, et al.*, 2004). Unpotentiated sulfonamides are believed to have a low potency because of their means of operation in inhibiting the synthesis of folic acid (Halling-Sorensen, 2001). Romet-30® is applied to the feed in a dose of 2.2 lb Romet/ton feed and the medicated feed is supplied to the treated fish at 1.5% body weight daily for a period of five days. There is a mandatory 42-day holding period following this dosing to ensure that the fish is safe for consumption. By using the

sulfadimethoxine in conjunction with ormetoprim, it is possible to reduce the amount of antibiotic required, thus reducing the potential for related antibiotic-resistant bacteria, while actually enhancing the spectrum of bacteria over which the medication is effective (Hoffman-LaRoche, 1984).

Minimal work has been conducted regarding the fate of this antibiotic in the environment and the work that has been done has focused primarily on the fate in sediment (Samuelsen*, et al.*, 1994; Capone*, et al.*, 1996; Bakal and Stoskopf, 2001). These studies showed varied results with one study showing that the sulfadimethoxine concentrations in the soil degraded about 20% over a study period of 180 days (approximately 6 months), while the ormetoprim could not be detected after about one month (Samuelsen*, et al.*, 1994). Another study showed that both portions of the Romet-30 were unstable and not detectable in soils anywhere from 21 to 62 days after treatment (Capone*, et al.*, 1996). These degradation rates, however, may prove to be very different from degradation rates observed in the water column as the chemical bound to substrate would be less available for microbial degradation (Bakal and Stoskopf, 2001). In comparison to oxytetracycline, this antibiotic is much more persistent in the water column (Dietze*, et al.*, 2005).

Unlike ormetoprim, the degradation of sulfadimethoxine appears to be highly temperature driven as Bakal *et al.* (2001) demonstrated that sulfadimethoxine declined by approximately half during refrigerated (4°C) conditions, but returned to initial concentrations at 25°C, likely due to the release of the drug into the water column at higher temperatures. Although this antibiotic is generated at a 5:1 ratio of the two components, it is not absorbed or excreted by the fish in a 5:1 ratio or any other constant ratio (Bakal*, et al.*, 2004) and it is, therefore, safe to assume that it also will not degrade at a constant ratio either. The bioavailability of Romet-30® is much greater than that of oxytetracycline, normally between 30 – 32% (Park*, et al.*, 1995).

In aquaculture, oxytetracycline is used in finfish to treat ulcer disease, furunculosis, bacterial hemorrhagic septicemia, and pseudomonas disease (Benbrook, 2002). The dosage prescribed by the FDA is 2.5 – 3.75 g oxytetracycline per 100 lb fish per day for ten days. Romet is used in finfish to treat furunculosis, ulcer disease, and bacterial hemorrhagic septicemia (USFDA, 1984). The dosage prescribed by the FDA is 23 mg active ingredients per pound of live body weight of fish per day. The antibiotics are pre-coated to their respective medicated feeds at the manufacturing facility; therefore, the required feeding rate is typically provided by the

manufacturer to ensure that the correct dose is obtained based on the feed itself rather than the medication.

The literature is full of discrepancies regarding whether or not oxytetracycline has no effect on nitrifying bacteria, inhibits nitrifying bacteria, or kills them off completely. One of the reasons for this is the variations between the use of synthetic systems, the seemingly random selection of doses of each antibiotic, and the use of raw chemical versus pre-coated manufactured feed. The purpose of this study was to investigate the impacts of oxytetracycline and Romet-30® on nitrifying bacteria in an operational system under FDA-approved therapeutic conditions. Knowing that the spectra of both antibiotics encompasses Gram-negative bacteria and that the antibiotics degrade more quickly in an aqueous environment than in sediment, it is anticipated that the bacteria will be inhibited during the active dosing period when the antibiotic concentration is being sustained, but that the bacteria will start to recover once the treatment ends and the antibiotics begin to degrade. To theorize that the bacteria are destroyed by the antibiotics would force the use of the chemotherapeutants in production settings to be unrealistic as the biofilters would not survive the treatment period. It is further anticipated that no antibiotic will be detectable in the treated systems at the end of the holding period due to rapid degradation in the water column.

As a result of so few therapeutic agents being available, many production facilities use other chemicals off-label when there is proven or strongly perceived therapeutic value. Among these chemicals are Chloramine-T (n-sodium-n-chloro-ρ-toluenesulfonamide; Figure 2-4) and potassium permanganate, both of which are dosed as bath therapeutics for the treatment of bacterial gill disease and other bacterial, fungal, or parasitic exposures before they become systemic. Although not currently approved by the FDA for use with food fish, Chloramine-T is currently being reviewed for approval and its off-label use is widespread. Fish have demonstrated tolerance to this chemical as high as 43 mg/L for three hours (Dawson*, et al.*, 2003), but typical doses range from 10 mg/L for one hour in hatcheries (Dawson*, et al.*, 2003) to 20 mg/L for four hours in cultured fish, depending on the pH and hardness of the water (Spangenberg, 1999). Even with this typical unapproved dose, the FDA has published that if approved, the therapeutic dose will be 10-20 mg/L, based on the hardness of the water for only one hour. This treatment, however, may be repeated every other day as necessary for up to one week. Because it contains an electrophilic chlorine in the chemical structure, it breaks down to

hypochlorite – a potent disinfectant. Additionally, there is a sulfonamide component that inhibits bacteria in a fashion similar to the Romet-30 sulfonamide antibiotic. These characteristics allow Chloramine-T to treat the bacterial infection through a dual mode of action.

There are two potential treatments when using potassium permanganate ($KMnO_4$): a short-term, high-dose bath and a long-term, lower-dose bath. Potassium permanganate has long been used to remove hydrogen sulfide odors and iron from drinking water. It can also be used as a disinfectant because of its oxidizing capabilities. Potassium permanganate is used in the U.S. aquaculture industry to control many bacterial, parasitic, and fungal exposures before they become systemic infections, which require more advanced and expensive treatments, such as antibiotics (Francis-Floyd and Klinger, 1997; Lazur, 2002). This chemical is a strong oxidizer that works by eliminating organic matter. While this is beneficial because of the elimination of undesirable infectious agents, it can be detrimental due to the inability of the chemical to differentiate between undesirable matter and desirable matter. Because of the effect on organics, some sources advise removing the biological filtration from your system prior to treatment with potassium permanganate, while others recommend leaving it in place due to the possibility of the infectious agents having taken up residence in the biofilters. Water changes are not required once the solution turns brown, as this is indicative of the chemical being spent. Water changes are sometimes carried out, however, for aesthetic purposes.

Methods and Materials

Experimental Design and tank setup

The experiments on operational systems were conducted over the course of three separate trials: antiparasitics, antibiotics, and off-label chemicals. For each trial, 20-gallon aquaria were set up with triple-flow corner filters (Lee's Aquarium and Pet Products – San Marcos, CA). Three aquaria served as untreated control systems and three aquaria were established for each treatment. Oxygen was supplied via an oxygen line and air stone that was set into place using the connection on the filter. No material was used for filter fabric support to avoid the material (e.g., carbon or crushed coral) sorbing chemicals out of the water column prematurely. The tanks were spiked with BioSpira (Marineland Labs – Moorpark, CA) to initiate nitrification more rapidly than if the tanks were allowed to cycle

naturally. One ounce of BioSpira is adequate for aquarium volumes of up to 30 gallons per manufacturer's specifications. Each trial was repeated for validation of results.

Fish Care

One tilapia (Blue Ridge Aquaculture – Martinsville, VA) was placed in each aquarium to influence the biofilter rather than experimenting with synthetic solutions. The average starting weights for each trial are provided in Table 2-1. Fish were fed with a 40% protein, 10% fat feed (Finfish Silver, Zeigler Brothers – Gardners, PA) daily at 1.5% of their body weight. Although the 35% protein, 5% fat feed (Finfish Bronze, Zeigler Brothers – Gardners, PA) is recommended by the manufacturer for tilapia, the 40/10 feed most closely correlated with the composition of the feed required for the antibiotic trials (38% protein, 12% fat). No significant growth of the tilapia was observed during the trial periods.

Water quality sampling

Water quality was sampled pre-treatment to obtain baseline concentrations for the parameters of interest. Upon commencement of the dosing, the water quality was sampled daily during the active dosing periods and the subsequent holding periods. Total ammonia-nitrogen (TAN), nitrite-nitrogen (NO_2^--N), nitrate-nitrogen (NO_3^--N), free copper, formaldehyde, chlorine, and manganese were analyzed using Hach methods (Table 2-2). For the TAN analyses, the Nessler method was used in all of the trials except for the formalin trials. The salicylate method had to be used for the formalin trials due to the chemical interference between Nessler reagent and aldehydes. The amount of potassium permanganate in the system was monitored indirectly using Hach method 8034. This method measures periodate oxidation for high range manganese (0.1 – 20.0 mg/L). To obtain the amount of potassium permanganate, it was necessary to multiply the results by 2.88, the conversion value between potassium permanganate and manganese. Although this is not a highly sensitive method at values less than 1.5 mg/L $KMnO_4$, it was adequate for the purposes of this research. The analysis of Chloramine-T for this research was performed using the Hach DPD colorimetric method for the analysis of chlorine. In order to obtain the Chloramine-T concentration, the bound chlorine concentration is determined by subtracting the results of the free chlorine and total chlorine present in the sample. This bound chlorine concentration (in mg/L as Cl_2) is then multiplied by 3.21, which is the ratio of the molecular

weight of anhydrous Chloramine-T to chlorine as Cl_2. This method has been demonstrated to have a strong correlation with the more traditional HPLC analytical method when analyzed within 2 hours of sample collection (Dawson*, et al.*, 2003). Dissolved oxygen and temperature were measured using a YSI 55 portable meter and pH was measured with a Fisher Scientific Accumet Model 10 benchtop meter with an Accumet probe.

Analysis of antibiotic concentrations with high performance liquid chromatography (HPLC)

Oxytetracycline, sulfadimethoxine, and ormetoprim were all analyzed by HPLC using a Pinnacle DB C18 5um, 250x4.6 mm (length x inner diameter) column and a diode array detector (DAD). The mobile phase consisted of a 72.7:22.7:4.6 mixture of running buffer, acetonitrile, and methanol. The running buffer was comprised of 0.1M potassium phosphate, 0.05M triethanolamine, and 1% acetic acid. A standard curve for both the oxytetracycline and sulfadimethoxine was generated using raw antibiotics obtained from Fisher Scientific. It was not possible to obtain raw ormetoprim; therefore, the data reported represent the area under the HPLC peak rather than an actual concentration within the solution. It was observed that the compounds eluted later as the eluent aged past three days; therefore, fresh eluent was used for each analysis.

Specific oxygen uptake rates

Oxygen uptake rate samples were collected from the biofilters twice per week and immediately analyzed in accordance with *Standard Methods* (Clesceri*, et al.*, 1998) using a dual dissolved oxygen probe system connected to a computer on which LabView software (National Instruments, Austin, TX) was installed to automatically collect time-step data. Sampling more frequently caused the filters to become inoperable due to depletion of the bacterial population. Samples were analyzed in duplicate with one replicate being altered with 2-chloro-6 (trichloromethyl) pyridine (TCMP) at 3mg TCMP/300mL in order to inhibit the nitrogenous oxygen demand. This addition allowed us to observe the difference between the oxygen uptake rate of the total bacterial population and that of the carbonaceous bacterial population. The difference between the two rates was representative of the oxygen uptake rate for the nitrifying bacteria alone. The samples were analyzed for volatile solids concentration in accordance with *Standard Methods* (Clesceri*, et al.*, 1998). The oxygen

uptake rates were then normalized to the concentration of volatile solids to obtain the specific oxygen uptake rates (sOUR) for each sample.

Catalyzed reporter deposition-fluorescent *in-situ* hybridization (CARD-FISH)

Teflon-coated microscope slides, each with eight wells, were suspended into each tank using nylon line which spanned the tank and was held in place using binder clips. By placing the slides directly into the tanks, biofilm was collected as it naturally formed *in-situ* and the bias that is introduced by manipulating the bacterial community was reduced. Two slides were harvested from the tanks every three days and immediately fixed. The slides were then analyzed using CARD-FISH to observe variations in population density with regards to the ratio of AOB (primarily *Nitrosomonas* species) to total bacterial population and the ratio of NOB (*Nitrospira* species) to total bacterial population.

The use of CARD-FISH as opposed to traditional FISH has shown results with increased and more accurate binding and increased signal intensity – often to a degree of at least one order of magnitude – in samples such as these in which the target organisms are slow-growing and few in number (Amann and Kuhl, 1998; Pernthaler, *et al.*, 2002). Nso1225, Ntspa662, EUB338, EUB338-II, and EUB338-III conjugated with horseradish peroxidase (HRP) on the 5' end were used to hybridize the slides (Thermo Scientific GmbH – Ulm, Germany). Details regarding the probe characteristics are provided in Table 2-2. One slide was hybridized for AOB (Nso1225) and total bacteria (EUB series), while the other was hybridized for NOB (Ntspa662) and total bacteria (EUB series). Six wells on each slide were hybridized – three with a hybridization solution containing active probe and three with a hybridization buffer containing no probe to serve as controls for non-specific binding and auto-fluorescence. Two wells were not utilized for the hybridization protocol due to the risk of the biofilm in these wells having been disrupted while suspended in the aquaria. The protocol used for the analysis was adapted from published protocols (Pernthaler, *et al.*, 2002) and the slides were hybridized sequentially for the multiple probes based on increasing stringency. The EUB probe used is a composite probe generated from equimolar concentrations of EUB338, EUB338-II, and EUB338-III.

The fixed slides were incubated in lysozyme for 30 minutes at room temperature, followed by triple washing for one minute (per wash) in Nanopure water. The fixed slides

were then incubated in Proteinase K for 5 minutes at room temperature, followed by triple washing for one minute (per wash) in Nanopure water. The slides were incubated in a quenching solution for 30 minutes in a 37°C hybridization oven and then triple washed in Nanopure water for one minute per wash. The slides were dehydrated in an ethanol series: 5 minutes in 50% ethanol, 1 minute in 80% ethanol, and 1 minute in 96% ethanol. The slides were dried in the hybridization oven at 37°C oven for 2 minutes.

Hybridization buffer was prepared based on the formamide concentration for the probes used (Table 2-3). Hybridization solution was prepared such that the probe was present at a 0.5 ng/μL concentration in the hybridization buffer. Two strips of filter paper moistened with hybridization buffer were placed in a 50 mL conical centrifuge tube and placed in the hybridization oven at 35°C to generate a humidified chamber for the hybridization process. 10 μL of the hybridization buffer (no probe) was evenly applied to the top three wells on the microscope slide to serve as negative controls for autofluorescence of the bacteria. 10 μL of the hybridization solution (probe added) was evenly applied to the bottom three wells on the microscope slide to hybridize with the species of interest. The slides were inserted into the humidified centrifuge tubes and incubated in a dark hybridization oven at 35°C for at least 2 hours. The tube was tightly sealed to ensure that the tubes remained humidified.

Following hybridization, the slides were incubated in pre-heated washing buffer in a dark hybridization oven at 37°C for 10 – 15 minutes. The slides were next incubated in 1X PBS (pH 7.6) for 30 minutes at room temperature. 10 μL of fluorescent tyramide working solution (NEN Life Sciences/Perkin Elmer, Waltham, MA) was added to each well while still wet in accordance with manufacturer recommendations and incubated in the dark in humidified tubes for 30 minutes in a 37°C hybridization oven. Cy3-Tyramide for was used for the nitrifiers and Cy5-Tyramide was used for total bacteria. The slides were removed and washed in 96% ethanol in the dark at room temperature for 1 minute and double-washed in 1X PBS for 1 minute (per wash) at room temperature.

Microscopic analysis was conducted using an epifluorescence microscope (Axioskop 2; Carl Zeiss, Thornwood, NY) and a Zeiss MRm camera. The images were captured using Axiovision 4.0 software. Three photos from each well were captured to ensure a comprehensive cross-section of the processed biofilm.

Chelated copper trials

A concentration of 0.25 ppm free copper was maintained in the chelated copper treated systems for a period of ten days. Water changes of at least 75% were conducted after the treatment period to remove the excess remaining copper. After this treatment, the systems were held for seven days to observe system response and then re-treated for an additional ten days to mimic the repetition of treatments that are often necessary in production facilities. Water quality was monitored in accordance with the "water quality sampling" section above and sOUR analyses were conducted in accordance with the "specific oxygen uptake rates" section above. Microscope slides were harvested every three days, fixed, and frozen for future CARD-FISH analysis in accordance with the "catalyzed reporter deposition – fluorescent *in-situ* hybridization" section above. The trial was then replicated to validate results observed during the first trial.

Formalin trials

A concentration of 150 ppm formalin (37% formaldehyde) was maintained in the formalin treated systems for a period of one hour. Although concentrations of up to 250 ppm are allowed, the dosing is highly temperature dependent as the toxicity of formalin increases at temperatures above 70°F (21°C). Given the operating temperature of 28-29°C, the lower concentration was required. Water changes were conducted after the treatment period and the treatment was repeated every other day for seven days to mimic the repetition of treatments that often occurs in production facilities. After this treatment, the systems were held untreated and monitored for seven days before repeating the treatment regimen. Water quality was monitored in accordance with the "water quality sampling" section above and sOUR analyses were conducted in accordance with the "specific oxygen uptake rates" section above. Microscope slides were harvested every three days, fixed, and frozen for future CARD-FISH analysis in accordance with the "catalyzed reporter deposition – fluorescent *in-situ* hybridization" section above. The trial was replicated to validate results obtained during the first trial.

Oxytetracycline trials

The oxytetracycline feed (Finfish Booster – Zeigler Brothers, Gardners, PA) is a 38% protein, 12% fat feed coated with 2.5 g oxytetracycline per pound of feed. This feed was

provided to the fish per the manufacturer specifications at 1.5% body weight per day for ten days. The mandatory 21-day post-treatment holding period was then observed, during which time the fish were returned to a feeding regimen with the unmedicated feed. Water quality was monitored in accordance with the "water quality sampling" section above and sOUR analyses were conducted in accordance with the "specific oxygen uptake rates" section above. The oxytetracycline concentration in the systems was analyzed in accordance with the "analysis of antibiotic concentrations with high performance liquid chromatography (HPLC)" section above. Microscope slides were harvested every three days, fixed, and frozen for future CARD-FISH analysis in accordance with the "catalyzed reporter deposition – fluorescent *in-situ* hybridization" section above. The trial was then replicated to validate results observed during the first trial.

Romet-30® trials

The Romet-30® feed (Finfish Xtra Booster – Zeigler Brothers, Gardners, PA) is also a 38% protein, 12% fat feed coated with 22.2 lbs Romet-30® per ton of feed. This feed was provided to the fish per the manufacturer's specifications at 1.5% body weight per day for five days. The mandatory 42-day post-treatment holding period was then observed, during which time the fish were returned to a feeding regimen with the unmedicated feed. Water quality was monitored in accordance with the "water quality sampling" section above and sOUR analyses were conducted in accordance with the "specific oxygen uptake rates" section above. The amounts of sulfadimethoxine and ormetoprim in the systems were analyzed in accordance with the "analysis of antibiotic concentrations with high performance liquid chromatography (HPLC)" section above. Microscope slides were harvested every three days, fixed, and frozen for future CARD-FISH analysis in accordance with the "catalyzed reporter deposition – fluorescent *in-situ* hybridization" section above. The trial was replicated to validate results obtained during the first trial.

Potassium permanganate trials

As previously discussed, two treatments with potassium permanganate are available – a high-dose, short-term trial and a lower-dose, long-term trial. The high-dose, short-term trial consisted of a 10 mg/L treatment with potassium permanganate for thirty minutes. Following this exposure, a water change is conducted at the end of the exposure period. Over-exposure

to the chemical can burn the gill tissues and mucous of treated fish. The low-dose, long-term trial consisted of a total treatment of 4 mg/L of potassium permanganate for four hours, dosed in 2 mg/L increments until the purple color was retained. Following the four-hour retention period, a water change was conducted. The tanks were exposed to this dose only once per week for two weeks to minimize over-exposure. Water quality was monitored in accordance with the "water quality sampling" section above and sOUR analyses were conducted in accordance with the "specific oxygen uptake rates" section above. Microscope slides were harvested every three days, fixed, and frozen for future CARD-FISH analysis in accordance with the "catalyzed reporter deposition – fluorescent *in-situ* hybridization" section above. The trial was replicated to validate results obtained during the first trial.

Chloramine-T trials

Chloramine-T was dosed to the three Chloramine-T treatment aquaria at 20 mg/L for one hour. Other researchers dosed the chemical for a period of four hours (Spangenberg, 1999); however, documentation published by the FDA during its on-going approval process have indicated that only a one-hour exposure period will be authorized when and if the chemical is approved (Gingerich*, et al.*, 2001). The data provided to the FDA by Gingerich *et al.* (2001) for freshwater salmonids exposed to treatments of 12 – 20 mg/L for one-hour, up to three times a week has been accepted for use as an external microbiocide in static or flow-through systems. Their guidance is that if the fish is to be treated more than once, treatment should occur on alternate days. This trial was conducted in accordance with these guidelines. Water quality was monitored in accordance with the "water quality sampling" section above and sOUR analyses were conducted in accordance with the "specific oxygen uptake rates" section above. Microscope slides were harvested every three days, fixed, and frozen for future CARD-FISH analysis in accordance with the "catalyzed reporter deposition – fluorescent *in-situ* hybridization" section above. The trial was replicated to validate results obtained during the first trial.

Results

Copper Trials

As shown in Figures 2-5 through 2-8, the results of the copper trials indicated that the inhibition of nitrification occurred almost immediately following initial exposure with respect to both TAN and nitrite. In the first trial, the pre-treatment TAN concentration averaged 0.07 mg/L and the average TAN concentration during treatment peaked on day 7 of each of the two dosing periods at 1.37 mg/L and 1.39 mg/L, respectively. Prior to the second treatment, the pre-treatment TAN had returned to pre-dose conditions with an average concentration of 0.08 mg/L. The replicate trial demonstrated a similar response, reaching a peak concentration of 1.25 mg/L and 1.35 mg/L on day 7 of each dosing period. In comparison, the untreated control systems maintained TAN concentrations below 0.11 mg/L in both the initial and replicate trials. The nitrification process began to recover between days 7 and 10 of the treatment and the TAN concentrations returned to levels comparable to those observed during the pre-treatment period, once the treatment ended and water changes of approximately 50% were made. In both trials, the peak TAN concentration was slightly higher in the second dose than in the initial dose. Statistically significant differences ($p<0.05$) in TAN concentrations were observed between the control systems and the systems treated with chelated copper in both replicate trials. The water change resulted in a TAN concentration reduction of approximately 24% when analyzed approximately 24 hours after the change was conducted. Forty-eight hours after the water change, the TAN concentration had been reduced by approximately 90% from the concentration observed immediately before the water change.

Like the TAN, the nitrite concentration also increased in the treated systems as a result of the chelated copper treatment regimen and was slower to recover from the exposure (Figures 2-3 and 2-4). In the first trial, the pre-treatment nitrite concentration averaged 0.01 mg/L. The average nitrite concentration peaked at 1.29 mg/L on day 7 of treatment before returning to an average concentration of 0.01 mg/L, following the end of the treatment and the subsequent water change. During the second dose of the trial, the water was slightly more compromised with a peak concentration of 1.485 mg/L on day 8 of treatment before returning to an average of 0.01 mg/L post-treatment. The replicate trial mimicked the first

with pre-treatment concentrations averaging 0.01 mg/L, a peak concentration of 1.25 mg/L observed on day 7 of treatment, and a post-treatment concentration of 0.01 mg/L after the post-treatment water changes. Statistically significant differences ($p<0.05$) in nitrite concentrations were observed between the control systems and the systems treated with chelated copper. During the second dose of the trial, the water was once again slightly more compromised than in the initial dose with a peak concentration of 1.57 mg/L on day 8 of treatment before returning to an average of 0.01 mg/L post-treatment. Statistically significant differences ($p<0.05$) in the nitrite concentrations were observed between the control tanks and the tanks treated with chelated copper in both replicate trials. The water change resulted in a nitrite concentration reduction of approximately 15% when analyzed approximately 24 hours after the change was conducted. The nitrite concentration had been reduced by approximately 60% and 89% of the concentration observed immediately before the water change at 48 and 72 hours, respectively.

Nitrate accumulation continued throughout the course of the treatments; however, it did so at a slower pace once the treatments began (Figures 2-9 and 2-10). This further supports the hypothesis that nitrification was inhibited, but did not stop completely. There were no statistically significant differences ($p>0.05$) in nitrite between the treated and untreated systems. In the treated systems, both the TAN and nitrite concentrations were elevated during the treatment with the copper. Coupled with the continued increase in nitrate concentration, this indicates that the inhibition of each step was only partial.

The sOUR results support the findings of the water quality trials; however, there was negligible difference between the sample treated with TCMP and the sample that was not. This indicates that, as expected, the nitrifying bacteria population comprised only a very small portion of the total bacteria population. As shown in Figures 2-11 and 2-12, the oxygen uptake rate decreased as the treatment progressed. Only sOUR analysis during the first copper trial indicated a statistically significant difference from the control, but a very distinct response trend was observed in direct correlation to the chelated copper dosing during both the initial and the replicate trials. Although the data indicate that the oxygen uptake ability of the bacteria recovered once treatment ended, complete recovery was not observed over the experimental period. That is, the uptake rates never returned to their pre-treatment levels and continuously decreased following treatment. The rate observed after

completion of the first treatment was less than the pre-treatment rate, and the rate after completion of the second treatment was, in turn, less than the rate observed after the first treatment. This also mimics the peak TAN concentration shift that was observed in the water quality analysis in which the peak TAN concentration during the second dose was consistently a bit higher than the peak TAN concentration during the initial dose. The sOUR results for the untreated control remained reasonably constant over the course of the treatments. CARD-FISH analysis showed that there was no significant change in the population density of the nitrifying bacteria as a proportion of the total bacteria population (Table 2-4).

Formalin Trials

As shown in Figures 2-13 through 2-16, there was negligible impact to the water quality as a result of the formalin trials with regards to TAN or nitrite. Although the data look highly variable at first glance, the TAN concentration remained within a boundary of 0.04 and 0.11 mg/L over the course of the formalin treatments. The TAN concentrations for the control remained within a similar boundary. Additionally, in the first dosing of the first trial, there appeared to be a trend indicating an increase in the TAN; however, this trend occurred at very low TAN concentrations (<0.10 mg/L) and was not observed in the second dosing period, thus making it inconsequential. A similar observation was made in the replicate trial; but, in the replicate trial, there was no trend observed during the first dosing period and a low-dose upward trend (<0.08 mg/L TAN) in the second dosing period. Even with these minor trends, there were no statistically significant differences ($p>0.05$) between the TAN concentrations observed in the control systems versus the systems treated with formalin.

The nitrite concentrations demonstrated results similar to those observed with the TAN concentrations. There were statistically significant differences ($p<0.05$) between the nitrite concentrations for the control systems and those for the systems treated with formalin in the first trial; however, there was no discernible trend to the concentration data as a response to the treatment. When comparing the nitrite concentrations during the treatment periods with those during the holding and recovery periods within the same trial, no statistical differences observed ($p>0.05$). This statistical difference was not observed during the replicate trial, nor was there any trend in the data. As in the copper trials, nitrate continued to accumulate in the

systems over the course of the formalin treatments (Figures 2-17 and 2-18), further indicating that nitrification continued throughout the treatments. The sOUR results validated the lack of an observed impact (Figures 2-19 and 2-20) to nitrification. Because no impact to nitrification was observed in the water quality or sOUR experiments, CARD-FISH analysis was not conducted.

Oxytetracycline trials

The results of the water quality analyses indicated that oxytetracycline inhibited nitrification within the treated systems in regards to ammonia oxidation (Figure 2-21). In the first trial, the peak TAN concentration of 0.82 mg/L occurred on day 6 of the ten day required dose. After reaching this peak, the concentration began to decrease. Interestingly, this peak concentration did not correspond to the peak concentration of oxytetracycline, which reached 1.58 mg/L on day 9 of the 10-day required dose. Statistical analysis revealed that there were significant differences ($p<0.05$) between the treated systems and the untreated controls. Prior to treatment, the average TAN concentration was 0.06 mg/L. TAN concentrations in the treated tanks returned to their pre-treatment levels approximately five days after treatment ended. At this point in time, the oxytetracycline had degraded to levels that were below the detection limit of the HPLC.

Like its TAN counterpart, the nitrite concentrations in the first trial also increased over the course of active treatment (Figure 2-23), peaking on day 5 of the ten day dosing period at a concentration of 0.43 mg/L. Prior to treatment, the average nitrite concentration was 0.01 mg/L. The nitrite concentrations in the treated tanks returned to their pre-treatment levels approximately eight days after the treatment ended. At this point in time, the oxytetracycline had degraded to levels that were below the detection limit of the HPLC. Statistical analysis showed that there were significant differences ($p<0.05$) between the treated systems and the untreated control systems. Nitrate levels continued to increase over the course of the treatment with no significant differences between the rate of increase in the control and the rate of increase in the treatments (Figures 2-25).

Similar results were observed in the replicate trial (Figure 2-22). The peak TAN concentration of 1.05 mg/L occurred on day 5 of the ten day required dose. After reaching this peak, the concentration began to decrease. Interestingly, this peak concentration did not

correspond to the peak concentration of oxytetracycline, which reached 0.87 mg/L on day 6 of the ten day required dose. Statistical analysis revealed that there were significant differences ($p<0.05$) between the treated systems and the untreated controls. Prior to treatment, the average TAN concentration was 0.05 mg/L. TAN concentrations in the treated tanks returned to their pre-treatment levels approximately 12 days after treatment ended. At this point in time, the oxytetracycline had degraded by approximately 75%.

The nitrite concentrations in the replicate trial (Figure 2-24) also increased over the course of active treatment, peaking on day 9 of the 10-day dosing period at a concentration of 0.52 mg/L. Prior to treatment, the average nitrite concentration was 0.01 mg/L. The nitrite concentrations in the treated tanks did not return to their pre-treatment concentrations until the last day of the 21-day holding period. At this point in time, the oxytetracycline had degraded by somewhere between 75 and 90%. Statistical analysis showed that there were significant differences ($p<0.05$) between the treated systems and the untreated control systems. Nitrate levels continued to increase over the course of the treatment with no significant differences between the rate of increase in the control and the rate of increase in the treatments (Figure 2-26).

The sOUR results support the findings of the water quality trials; however, there was negligible difference between the sample treated with TCMP and the sample that was not. This indicates that, as expected and as observed in the other trials, the nitrifying bacteria population comprised only a very small portion of the total bacteria population. In the first trial (Figures 2-27 and 2-28), the uptake rate was most reduced on day 9 of the 10-day dosing period. At this point, the uptake rate was 1.205 mg DO/g VS-hr, a reduction of approximately 77% from the pre-dose uptake rate of 5.173 mg DO/g VS-hr. In the replicate trial (Figure 3-9), the uptake rate was most reduced two days after the end of the 10-day dosing period. At this point, the uptake rate was 1.101 mg DO/g VS-hr, a reduction of approximately 78% from the pre-dose uptake rate of 4.892 mg DO/g VS-hr. Statistical analysis showed that there was a significant difference ($p<0.05$) between the sOUR data for the treated systems and the untreated controls. After reaching this low point in the treated systems, the sOUR began to recover; however, it never returned to its pre-treatment levels, having only returned to a rate of 4.112 mg DO/g VS-hr at the end of the mandatory 21-day holding period in the first trial and a rate of 3.568 mg DO/g VS-hr in the replicate. Analysis

with CARD-FISH indicated that there were no significant changes to the population density of either the AOB or the NOB (Table 2-5).

Romet-30® trials

As with oxytetracycline, the results of the water quality analyses indicated that Romet-30® inhibited nitrification within the treated operational systems, and it did so at a much higher degree than the oxytetracycline. In the first trial (Figure 2-29), the peak TAN concentration of 1.79 mg/L occurred two days after the end of the five day required dose. After reaching this peak, the concentration began to decrease. This peak concentration did not correspond to the peak concentration of sulfadimethoxine, which reached 1.04 mg/L two days after the end of the five day required dose. Likewise, the ormetoprim concentration reached its peak on this same day, with an area under the curve of 30.19. Statistical analysis revealed that there were significant differences ($p<0.05$) between the treated systems and the untreated controls. Prior to treatment, the average TAN concentration was 0.06 mg/L. TAN concentrations in the treated tanks returned to their pre-treatment levels approximately 17 days after treatment ended. At this point in time, the sulfadimethoxine had degraded by approximately 88% and the ormetoprim had degraded by approximately 91%.

The nitrite concentrations in the first trial (Figure 2-31) also increased over the course of active treatment, peaking four days after the end of the five day dosing period at a concentration of 1.62 mg/L. Prior to treatment, the average nitrite concentration was 0.01 mg/L. The nitrite concentrations in the treated tanks did not return to their pre-treatment levels until the very end of the mandatory 42-day holding period. At this point in time, both the sulfadimethoxine and ormetoprim had degraded by approximately 90%, but had begun to reappear in the water column and were being detected at levels of about 50% of the peak concentration. This re-emergence was likely due to a release of antibiotic that had been bound to organic matter in the system. Statistical analysis showed that there were significant differences ($p<0.05$) between the treated systems and the untreated control systems. Nitrate levels continued to increase over the course of the treatment with no significant differences between the rate of increase in the control and the rate of increase in the treatments (Figure 2-33).

In the replicate trial (Figure 2-30), the peak TAN concentration of 1.53 mg/L occurred two days after the end of the five day required dose. After reaching this peak, the concentration began to decrease. This peak concentration did not correspond to the peak concentration of sulfadimethoxine, which peaked at 1.42 mg/L, four days after the end of the five day required dose and two days after the peak TAN concentration was observed. Likewise, the peak concentration did not correspond to the ormetoprim, with the area under the ormetoprim curve peaking at 41.42 on the same day as the peak sulfadimethoxine concentration. Statistical analysis revealed that there were significant differences ($p<0.05$) between the treated systems and the untreated controls. Prior to treatment, the average TAN concentration was 0.04 mg/L. TAN concentrations in the treated tanks returned to their pre-treatment levels approximately seventeen days after treatment ended. At this point in time, the oxytetracycline had degraded by approximately 50%.

The nitrite concentrations in the replicate trial (Figure 2-32) also increased over the course of active treatment, peaking three days after the end of the five day dosing period at a concentration of 1.05 mg/L. Prior to treatment, the average nitrite concentration was 0.01 mg/L. The nitrite concentrations in the treated tanks did not return to their pre-treatment concentrations until 34 days after the five day dosing period. At this point in time, both the sulfadimethoxine and ormetoprim had degraded by approximately 90%, but had begun to re-emerge and were being detected at levels of about 50% of the peak concentration. This reappearance of antibiotic was likely due to the release of antibiotic that had previously been bound to organic matter present within the system. Statistical analysis showed that there were significant differences ($p<0.05$) between the treated systems and the untreated control systems. Nitrate levels continued to increase over the course of the treatment with no significant differences between the rate of increase in the control and the rate of increase in the treatments (Figure 2-34)

The sOUR results support the findings of the water quality trials; however, there was negligible difference between the sample treated with TCMP and the sample that was not. This indicates that, as expected and as observed in the other trials, the nitrifying bacteria population comprised only a very small portion of the total bacteria population. In the first trial (Figure 2-35), the uptake rate was most reduced four days after the end of the five day dosing period. At this point, the uptake rate was 1.149 mg DO/g VS-hr, a reduction of

approximately 77% from the pre-dose uptake rate of 5.045 mg DO/g VS-hr. In the replicate trial (Figure 2-36), the uptake rate was most reduced two days after the end of the ten day dosing period. At this point, the uptake rate was 1.411 mg DO/g VS-hr, a reduction of approximately 73% from the pre-dose uptake rate of 5.157 mg DO/g VS-hr. Statistical analysis showed that there was a significant difference ($p<0.05$) between the sOUR data for the treated systems and the untreated controls. After reaching this low point in the treated systems, the sOUR began to recover; however, it never returned to its pre-treatment levels, having only returned to a rate of 4.602 mg DO/g VS-hr at the end of the mandatory 21-day holding period in the first trial and a rate of 4.761 mg DO/g VS-hr in the replicate. Analysis with CARD-FISH indicated that there were no significant changes to the population density of either the AOB or the NOB (Table 2-5).

Long-term Potassium permanganate trials

The results of the low-dose, long-term potassium permanganate trials (4 mg/L for four hours) indicated a significant difference ($p < 0.05$) in the data between the control systems and the systems treated with the long-term exposure to potassium permanganate. This significant difference was not, however, observed in the replicate trial. Although the TAN concentrations in the systems were higher during and after dosing than they were prior to the exposure, this was true in the control systems, as well, and the maximum TAN concentration reached in the systems was 0.10 mg/L in the first trial and 0.11 mg/L in the replicate trial – levels that pose no threat to the fish or threat to the environment as an effluent. Additionally, although statistically significant differences in the data were observed in the first trial, there was no indication that the water quality would be impaired as a result of the exposure. The average TAN concentrations for each trial are provided in Figures 2-37 and 2-38.

Similar to the results of the TAN analyses, there was also no evidence of inhibition to the nitrite-oxidizing step of the nitrification process. Unlike the TAN data, however, statistical analysis of the nitrite data showed no statistically significant differences between the control systems and the long-term treated systems in either the initial trial or the replicated trial. The nitrite concentrations also never reached a level that would prove to be hazardous to either the fish or the natural environment, with maximum concentrations reaching only 0.01 mg/L and 0.01 mg/L in the initial and replicate trials, respectively. The average nitrite

concentrations observed over the testing period are provided in Figures 2-39 and 2-40. Nitrate accumulated in the systems; however, the rate at which nitrate was produced decreased during the exposure (Figures 2-41 and 2-42). This further supports the hypothesis that nitrification was inhibited, but did not stop completely. There were no statistically significant differences ($p>0.05$) between the treated and untreated systems.

The sOUR analyses (data provided in Figures 2-43 and 2-44) forces us to re-evaluate the findings of "no effect", but like the TAN, analysis of the data sets indicated that only one of the trials resulted in sOUR data for which the treated systems were significantly different ($p<0.05$) than the untreated control systems. The trend for the sOUR data in both trials decreased slightly over the course of each trial, with the downward trend of the replicate being more pronounced. This downward trend was not observed in the control systems. There was minimal difference between the sOUR sample analyzed in the presence of TCMP and the sample analyzed without it, indicating that the population of nitrifiers in our overall trial was quite low. This indicates that, as expected and as observed in the other trials, the nitrifying bacteria population comprised only a very small portion of the total bacteria population.

Short-term potassium permanganate trials

Unlike the long-term, low-dose potassium permanganate exposure, statistical data analysis indicated that there was a consistent statistically significant difference ($p<0.05$) between the TAN concentrations in the untreated controls and the systems treated with a short-term, high-dose exposure (10 mg/L for thirty minutes) to potassium permanganate. Even with this statistical difference, there was no distinct dose response. That is, the TAN concentrations increased from the levels observed pre-dose; however, the same was true in the control systems. Additionally, although an increase was observed, the maximum TAN concentrations observed were 0.09 mg/L and 0.11 mg/L in the initial and replicate trials, respectively. These concentrations were no threat to the fish housed in the systems, nor do they pose an environmental threat as an effluent parameter. Because of this, the results of the trials were deemed to be insignificant. These data are provided in Figures 2-45 and 2-46.

With regards to the nitrite concentrations, only one of the trials resulted in data that were shown to be statistically different ($p<0.05$) from the untreated controls. No distinct dose-

response trend was observed in the initial trial, but in the replicate trial, the nitrite continued to increase until the water change was conducted following the second exposure. Like the TAN, however, the concentrations observed were not deemed to be of aquacultural or environmental significance, with maximum concentrations reaching 0.01 mg/L and 0.01 mg/L in the initial and replicate trials, respectively. These data are presented in Figures 2-47 and 2-48. Nitrate accumulation continued throughout the course of the treatments; however, it did so at a slower rate once the treatments began (Figures 2-49 and 2-50). This further supports the hypothesis that nitrification was inhibited, but did not stop completely. There were no statistically significant differences ($p>0.05$) between the treated and untreated systems.

Both of the sOUR trials resulted in data that were shown to be statistically different ($p<0.05$) from the untreated controls. However, only the initial trial indicated a distinct trend. The data for this trial demonstrated a downward trend over the course of the trial, but it was a very minor deterioration. The pre-treatment rate was 5.196 mg DO/g VS-hr and the final rate was 5.084 mg DO/g VS-hr. These data are provided in Figures 2-51 and 2-52. There was minimal difference in uptakes observed between the samples analyzed in the presence of TCMP and the samples analyzed without this addition. This indicates that, as expected, the nitrifying population was quite low. As in the long-term, low-dose exposure of potassium permanganate, it is possible that the potassium permanganate impacted the heterotrophic community of bacteria more than the nitrifying bacteria, causing the sOUR to be affected while the nitrifying process was seemingly unaffected.

Chloramine-T trials

Following Chloramine-T exposure (20 mg/L for one hour) the TAN concentrations in the initial trial showed a significant difference ($p<0.05$) from the untreated controls; however, the concentrations observed in the replicate trial did not show a comparably significant difference ($p>0.05$). Even with these significant data differences, the maximum TAN concentrations observed in the treated tanks only reached a maximum concentration of 0.11 mg/L in both the initial and replicate trials – a concentration that is not of aquatic or environmental significance. Further, while there was a downward trend in the TAN

concentration after the first dose of the initial trial, this trend was not consistently observed in response to the various dosing periods. These data are provided in Figures 2-53 and 2-54.

The nitrite concentration data showed a significant difference ($p<0.05$) between the treated systems and untreated controls in only one of the trials. Like the TAN concentrations, even with this data significance, there was no distinct dose-response trend in either of the trials and, in the replicate trial, the increase in nitrite concentration observed in the treated systems was also seen in the control systems. The maximum nitrite concentrations observed were only 0.01 mg/L and 0.02 mg/L in the initial and replicate trials, respectively. Again, these concentration levels are not of aquatic or environmental significance. The nitrite data are presented in Figures 2-55 and 2-56. Nitrate accumulation continued throughout the course of the treatments, however, it did so at a slower rate once the treatments began (Figures 2-57 and 2-58). This further supports the hypothesis that nitrification was inhibited, but did not stop completely. There were no statistically significant differences in nitrate concentrations ($p>0.05$) between the treated and untreated systems.

Like the nitrite concentrations, only the replicate sOUR trial resulted in data for the treated systems that were of statistically significant difference ($p<0.05$) from the control data. Regardless of this lack of significant difference across both trials, both trials did show a decrease in sOUR over the duration of the trial. The maximum difference observed between the pre-treatment sOUR and the most reduced sOUR for the treated systems was 0.524 mg DO/g VS-hr in the initial trial and 0.239 mg DO/g VS-hr in the replicate trial. After reaching this point, the rates began to increase. Additionally, the difference between the pre-treatment sOUR and the sOUR at the end of the trial was only 0.064 mg DO/g VS-hr in the initial trial and 0.086 mg DO/g VS-hr in the replicate trial, demonstrating almost total recovery from the metabolic inhibition that may have occurred. These data are provided in Figures 2-59 and 2-60. As observed in the potassium permanganate trials, there was minimal difference observed between the samples analyzed with TCMP and those analyzed without, indicating that the nitrifying bacteria population was low in comparison to the heterotrophic population. It is possible that the heterotrophic bacteria were also more impacted by the chemical exposure than the nitrifying bacteria given that there was no substantial increase in nitrification parameters, but there was a distinct response in the sOUR of the samples.

Discussion and Conclusions

Antiparasitic Trials

Copper sulfate is a compound that is widely used in freshwater farm ponds and aquaculture as an algicide and antiparasitic (Watson and Yanong, 2002). Copper sulfate is approved by the EPA for the treatment of algae, but it also sometimes used to control external parasites of fish. In small amounts, copper is a required micronutrient for metabolic activity in nitrifying bacteria, but in higher concentrations – greater than 0.50 mg/L in water and greater than 150 mg/L in activated sludge – this heavy metal may become inhibitory (Braam and Klapwijk, 1981; Kim*, et al.*, 2006). Over the years, chelated copper has replaced copper sulfate as the copper treatment of choice among aquaculturists due to its increased stability and less corrosive nature. Unlike copper sulfate, chelated copper stays in solution so it was unnecessary to add significant amounts of the solution on a daily basis in order to maintain the therapeutic concentration of 0.25 ppm free copper. Copper exposure to nitrifiers growing in pure culture has been shown to have more of an impact than that to nitrifiers in complex media, such as activated sludge (Tomlinson*, et al.*, 1966). The reason for this is believed to be the complexation of copper with readily available organic matter. The biofilter slurry sampled in this study had a volatile solids (organic matter) content as high as 4,900 mg/L, which was equivalent to 30-40% of the total solids present. No carbon or crushed coral was used in the filters in order to prevent absorption of the chemicals of interest.

As in this study, others have found inconsistencies in their results for systems treated with copper (Bower and Turner, 1982). As demonstrated in our findings, they also found that ammonia oxidation was inhibited, but recovered following a water change to eliminate or greatly reduce the copper presence; however, their work showed that nitrite oxidation was uninhibited. Even with the slight variations in the data and significance between replicates in this study, the hypothesis that the bacteria would be inhibited by the copper exposure was supported. Likewise, our hypothesis that there would be a stronger impact to the NOB was upheld. When comparing the pre-dose concentration to the peak concentration, the peak concentration was 160-fold higher for nitrite and only 19-fold higher for TAN.

We observed an initial decrease in the concentration of copper present in our systems after the first addition. This concentration was supplemented with additional chelated copper (as Cutrine-Plus, Applied Biochemists, Alpharetta, GA) to maintain the 0.25 ppm therapeutic concentration. No further additions were necessary over the course of treatment. It is likely that the copper that was initially added complexed with the organic matter present in the filters. Copper also complexes with unionized ammonia to produce a copper hydroxide precipitate and the ammonium ion. Cutrine Plus is manufactured from copper-ethanolamine complexes. These complexes tend to resist absorption by the hardness in the water and remain fully charged. It was shown that the amine complexes destroy parasites more rapidly and at lower concentrations, resulting in less toxicity risk to the fish and more rapid recovery than their chloride complex counterparts, including copper sulfate (Morin, 2004). This is believed to be because the amine complex is larger and positively charged, thereby making the passage through cellular membranes more difficult.

In the aquatic environment, copper is primarily found in its divalent state. Free copper is very reactive and, in a water column, it binds to organic matter or forms complexes with other ions to form precipitates. Ionized copper is internalized by *Nitrosomonas europaea* and reaches intracellular equilibrium after only 4 hours of exposure. This is very rapid in comparison to other metals, including zinc, cadmium, and nickel, the intracellular concentrations of which continued to increase beyond 12 hours of exposure. This indicates that the mechanism of inhibition by copper is likely different than that of other metals and may primarily involve cell membrane disruption and rupture rather than the disruption of nucleic acids and protein structures (Hu*, et al.*, 2003). Traditionally, copper sulfate has been used in aquaculture. Commercial copper sulfate is a complex with five waters of hydration in its chemical structure. When copper sulfate is added to an aquatic sample with a dilute ammonia concentration, some copper hydroxide forms, but ammonia is also removed from the water column to form a copper-ammonia complex ion. As the ammonia levels increase, only a portion of this ammonia is complexed by the copper sulfate. The complexation by copper – not just with ammonia, but also with other organics, hydroxyl ions, and so forth – makes treatment with copper very difficult and unpredictable. Chelated copper behaves very differently in the water column because it is very stable and already bound; therefore, traditional copper chemistry does not apply to the behavior of the copper that was observed

and explains why the chelated copper remains in solution over the duration of the treatment period.

The therapeutic copper concentrations is documented as being sufficient to kill parasites as copper is known to be highly toxic to parasites and invertebrates at very low concentrations; however, it was insufficient to kill the bacteria or to cause complete inhibition of the nitrification process. The primary action of copper against parasites is osmotic shock. Like the fish being treated, bacterial cells have mechanisms for osmoregulation. In fish, this regulation is primarily achieved by the gills. It is important to note, however, that one of the impacts of ammonia to fish is the disruption of osmoregulation by damaging the gill, thereby making the fish more susceptible to the copper if the ammonia concentration becomes too elevated. In bacteria, there are proteins that bind to copper and control its uptake, transport, and efflux (Andreini*, et al.*, 2008). In *Nitrosomonas europaea*, three copper resistance proteins are expressed (CopA, CopB, and CopD), along with a copper binding protein (CopC), and an inner membrane copper tolerance protein (Chain*, et al.*, 2003). These proteins belong to the cation transport ATPase family and prevent copper toxicity while promoting copper utilization. These transport systems do have thresholds above which the copper cannot be removed as quickly as it is crossing the membrane. These thresholds vary between systems and bacteria. Once this threshold is exceeded, the copper accumulates in the cell and begins to cause inhibition, most likely by damaging the cellular membrane. Once the copper is removed from the aquatic system, the bacteria that remain viable following the exposure are able to begin effluxing the excess copper from the cell.

Formalin is an antiparasitic and algicide comprised of 37% formaldehyde in solution with 10 – 15% methanol to prevent the formation of paraformaldehyde, which is toxic to fish (Francis-Floyd, 1996). This chemical is approved for use as an antiparasitic by FDA (USFDA, 2008). Prolonged exposure of fish to formalin treatments has been known to cause gill damage, mucous secretion, and toxicity to epidermal cells, leaving the fish less resistant to pathogens (Keck and Blanc, 2002; Buchmann*, et al.*, 2004). There is no mandatory holding period following treatment with formalin. The data collected refuted the initial hypothesis that the formalin would inhibit the nitrifying bacteria. No statistically significant impact ($p>0.05$) was observed on the TAN concentrations and, while there were statistically significant differences between the control systems and the systems treated with formalin,

there were no statistically significant differences observed within the formalin systems themselves during the dosing and holding periods.

Given the historical uses of formalin as an agent for fixing bacteria and embalming practices, it was surprising that the formalin did not inhibit or kill off the nitrifying bacteria. However, aquaculturists have reported for years that formalin is only effective as a parasiticide when used in conjunction with other treatments. In agreement with the findings of this study, other authors have refuted the common belief that formalin will kill off the nitrifying bacteria in aquaculture systems. One such study showed that concentrations ranging from 167 ppm to 300 ppm did not have a significant impact to the nitrification in a recirculating fluidized-sand bed (Schwartz*, et al.*, 2000). In another study, the investigators showed that neither one-hour static exposures up to 90 mg/L nor recirculating exposures of 60 mg/L for up to six hours had any significant impact to ammonia oxidation, but significant nitrite oxidation inhibition occurred in the recirculating systems (Keck and Blanc, 2002). Although it may not explain the results of our colleagues, it is likely that the lack of inhibition in our trials was primarily due to the short-term exposure immediately followed by a water change of at least 75%. In the studies where formalin did inactivate bacteria, concentrations of 25 to 75 ppm were typically maintained and allowed to naturally degrade over at least twenty-four hours. If there is an impact of formalin on nitrification, it is minor.

One of the largest sources of nitrogen in an aquaculture system is from the feed. Proteins in the feed are composed of approximately 16% nitrogen. The feed used in the antiparasitic trials was 40% protein, resulting in 64 mg nitrogen added per gram of feed. Given the experimental parameters of the antiparasitic trials, approximately 442 mg nitrogen was added to each 20-gallon aquarium each day. This is equivalent to approximately 5.8 mg/L nitrogen added in the form of feed per day. Estimates show that approximately 50% of the feed is utilized by the fish for growth and metabolism while the remaining 50% is put into the water column in the form of uneaten feed and waste (Craig and Helfrich, 2002). Based on these estimates, approximately 2.9 mg/L nitrogen was released into the aquarium each day during the antiparasitic experiments. Water quality data for these experiments demonstrated that the total nitrogen in the aquarium water sampled – determined by adding the concentrations of TAN, nitrite, and nitrate – increased by only an average of 0.5 mg/L per day over the course of the treatments. Of the 50% of the nitrogen released into the systems, it is estimated that

10% is uneaten feed, 10% is fecal matter, and 30% is liquid waste (Craig and Helfrich, 2002). Using these estimates for the treated systems would lead to an estimate of 0.87 mg/L nitrogen being added directly to the water column each day. This is very close approximation to the calculated 0.5 mg/L average daily increase in this experiment. The difference in the numbers can be accounted for by the estimates and assumptions made as these figures vary with factors such as species and system conditions. There were no statistically significant differences ($p > 0.05$) between the total nitrogen in the treated systems versus the untreated controls. The primary contributor to the total nitrogen in the system is in the form of nitrate. This indicates that nitrification continued over the course of the treatment; however, the increased levels of both TAN and nitrite in the copper-treated systems indicate that the conversion was slow in comparison to the untreated controls.

Antibiotic Trials

As expected due to its potency, the water quality deteriorated to a larger degree as a result of Romet-30® exposure than as a result of the oxytetracycline exposure, even though the dosing period was only half as long. Potentiated sulfonamides have replaced unpotentiated sulfonamides over the years because they are much more potent and effective. This is because the potentiator (in the case of Romet-30®, ormetoprim) exerts its antibacterial measures in a step immediately following that of the sulfonamide. Specifically, when these antibiotics are combined, the sulfadimethoxine blocks the synthesis of folic acid by replacing the para-aminobenzoic acid (PABA) molecule in dihydrofolic acid. Subsequently, the ormetoprim stops the production of tetrahydrofolic acid and folate from dihydrofolic acid (U.S.Pharmacopeia, 2007). Additionally, potentiated sulfonamides have been shown to have some antibiotic effect on bacteria that are sulfonamide resistant.

Within each nitrification parameter, we observed a reversal of the inhibition that occurred during and immediately following exposure to the antibiotic medicated feeds. The rates of recovery, however, were inconsistent even between replicate treatments. The oxytetracycline in the replicate trial was much slower to degrade in the replicate trial than it was in the first trial, which may explain the difference in the amount of time taken before the water quality returned pre-treatment conditions. The degradation of oxytetracycline is highly dependent on

environmental factors, including photo-exposure and the amount of organic matter to which the oxytetracycline can bind and persist.

Other authors have noted that oxytetracycline degraded in the water column faster than the components of Romet-30® (Samuelsen, 1989; Samuelsen, *et al.*, 1994; Park, *et al.*, 1995; Capone, *et al.*, 1996; Bakal and Stoskopf, 2001; Bakal, *et al.*, 2004; Dietze, *et al.*, 2005). It was believed that the components of the antibiotic, which are combined at a 5:1 ratio during production, would degrade at different rates once they began to break down in the water column. Our findings were somewhat different. Instead, the degradation of the two chemicals was very similar with the area under the sulfadimethoxine peak consistently being 10-fold higher than the area under the ormetoprim peak. Although we were unable to determine exact ormetoprim concentrations due to the lack of a calibration curve, this still represents a correlation between the degradation of the two chemicals in the water column.

Antibiotics can act as an inducer or a regulator of bacterial efflux pumps. In Gram-negative bacteria, these efflux pumps are comprised of membrane transporter proteins in the cellular membrane (Lee, *et al.*, 2000). In addition to these pumps, the concerns regarding antibiotic resistance continue to increase, requiring additional focus to be placed on the volume and nature of the antibiotics entering the environment and the risks posed by the pathogens that are developing resistance. Resistance is manifested in many ways, depending on the bacteria that have become resistant: enzymatic inactivation that modify the drug itself upon entry into the cell, modification of the drug target site within the cell, and the adaptation of efflux pumps (Nikaido, 1998; Rajasekaran, *et al.*, 2007). Bacteria can have either single-component efflux pumps or multi-component efflux pumps, which are found exclusively in Gram-negative bacteria. Gram-negative bacteria have a much more sophisticated efflux system than their Gram-positive counterparts, due in part to the presence of an outer membrane that serves as a barrier and in part to the need to pump the toxins out of both the periplasmic space and the cytoplasmic space (Nikaido, 1996). Because of this most Gram-negative bacteria have pumps that span between both membranes through the use of three membrane proteins: a transporter protein in the cytoplasmic membrane, an outer membrane channel, and a linker protein that connects the two (Nikaido, 1996). For example, in *E. coli*, tetracyclines are pumped out of the system by the AcrAB complex.(Ma, *et al.*, 1993; Okusu, *et al.*, 1996). *Nitrosomonas* possesses a similar system involving the TolC protein in the

outer membrane as well as tetracycline-repressor proteins that assist in the efflux of the tetracycline from the cell (Nikaido, 1996).

In more intensive production environments, it may be discovered that the nitrifying bacteria are unable to efflux the amounts of antibiotic introduced into the systems as rapidly as it was effluxed in these experimental systems. For example, our average fish weight was just shy of 500 grams in 20-gallons of water (25 grams fish/gallon), resulting in an average of 0.375 grams/gallon of medicated feed being introduced into the system daily. In intensive tilapia culture, it is not uncommon for production densities to reach 6 pounds per cubic foot (364 grams fish/gallon) (Rakocy, 2005), resulting in the addition of at least 5.46 grams/gallon of medicated feed being introduced daily and increasing the antibiotic loading accordingly.

Feed is the primary source of nitrogen addition to the systems. Proteins in the feed are composed of approximately 16% nitrogen. The feed used as a control in the antibiotic trials was 40% protein, resulting in 64 mg nitrogen added per gram of feed. The medicated feed was 38% protein, resulting in 61 mg nitrogen added per gram of feed. Given the experimental parameters of the antibiotic trials, an average of 465 mg nitrogen was added to each 20-gallon aquarium each day. This is equivalent to approximately 6.1 mg/L nitrogen added in the form of feed per day. Based on these estimates provided by Craig and Helfrich (2002) already described, approximately 3.1 mg/L nitrogen was released into the aquarium each day during the antibiotic experiments. Water quality data for these experiments demonstrated that the total nitrogen in the aquarium water sampled – determined by adding the concentrations of TAN, nitrite, and nitrate – increased by only an average of 0.14 mg/L per day over the course of the treatments. Further, an estimate of 0.92 mg/L nitrogen was calculated as being added directly to the water column each day. This is much higher than the observed daily increase in total nitrogen in the system. A portion of the difference in the numbers can be accounted for by the estimates and assumptions made as these figures vary with factors such as species and system conditions. There were no statistically significant differences ($p > 0.05$) between the total nitrogen in the treated systems versus the untreated controls.

The primary contributor to the total nitrogen in the system is in the form of nitrate. The rate at which the nitrate accumulated in the treated systems decreased over the course of the trial, potentially indicating that the cells were indeed damaged during the antibiotic

treatments. This tapering off in accumulation rate was not observed in the untreated control systems. Because the nitrate comprised such a large portion of the total nitrogen, had the rate of accumulation continued at the rate observed in the early stages of the trials, there would have been much less variation between the calculated daily nitrogen input to the water column and the average observed daily nitrogen concentration. This continued accumulation indicates that nitrification continued over the course of the treatments; however, the increased levels of both TAN and nitrite in the copper-treated systems indicate that the conversion in both steps of the nitrification process was slow in comparison to the untreated controls.

Off-label Chemical Trials

Although there was no discernible impact to the nitrifying bacteria, sOUR data indicate that there was some impact to the overall bacterial community. Because of the low density of nitrifiers, it is interpreted as an impact to the heterotrophic community. Potassium permanganate is unstable in water and dissociates into a potassium ion (K+) and a permanganate ion (MnO_4^-). The permanganate ion then releases an oxygen molecule (O_2) that interacts with organic material to oxidize the cell material and leaves manganese oxide (MnO_2). Manganese oxide is relatively inert and insoluble in water. Because of the lack of selectivity between organic matter, potassium permanganate attacks heterotrophic bacteria in the same manner that it attacks other bacteria, such as nitrifiers. Heterotrophic bacteria are more densely populated in aquacultural systems than nitrifying bacteria. This is primarily due to their ability to reproduce much more efficiently and rapidly than their nitrifying counterparts. Given this low population, it may be that the impact of the potassium permanganate was more pronounced in the heterotrophic community, yielding results that showed the sOUR of the total community declined, but in which the nitrification parameters showed little to no difference from the controls

In biofilters, the attached growth process allows more time for the slow-growing nitrifiers to reproduce than suspended growth systems, where washout is a factor. As the bacteria attach, the rapid growth of the heterotrophs causes them to form the bulk of the biofilm, and overlay and surround the nitrifying bacteria that become embedded in the film. This embedment is a blessing and a curse for these bacteria as it protects them from toxins, but

also puts them at a disadvantage with regards to competing for nutrients and oxygen. The toxicity of potassium permanganate to the nitrifying bacteria is highly dependent on the amount of organic material in the system, which includes heterotrophic bacteria. When there is more organic material in the system, there is less likelihood that the nitrifying bacteria that are buried in a biofilter will be exposed to the potassium permanganate. By the same token, however, if there is too much organic matter in the system, the dose will be consumed by this matter and the treatment will be ineffective against the target organisms. As the potassium permanganate oxidizes and kills off the organic matter, some oxygen is given off in the process, slightly increasing the dissolved oxygen concentration in the water. Simultaneously, the redox potential is increased. This slight increase in oxygen coupled with the heterotrophic layer of the biofilm offering up a more readily available source of organic matter allows the nitrifiers to take advantage of the reduced competition for oxygen and also protects them from being oxidized by the potassium permanganate over the short exposure times.

Chloramine-T works through a dual-mode of action:. There are differing opinions on the mode of action of Chloramine-T: (1) as a hypochlorite disinfectant and (2) as a member of the sulfonamide antibiotic family. If one subscribes to the mechanism, that it works as a hypochlorite disinfectant, chlorine chemistry shows that at approximately pH 7.5, hypochlorous acid and the hypochlorite ion exist in almost equal forms in the water column. Below this pH, hypochlorous acid is more dominant, while at pH values above this, the hypochlorite ion is more dominant. The systems used in these trials were maintained at around pH 7.1 with minimal variation. Hypochlorous acid is a stronger oxidant than its hypochlorite counterpart and because of the neutral electrical charge, it is more likely to penetrate bacterial membranes. Additionally, hypochlorous acid will react with the ammonia to form chloramines, pulling the ammonia out of solution. Although the experimental conditions were not ideal for the creation of chloramines, the requirements vary in accordance with other environmental conditions (such as temperature), so this could explain there was no clear inhibition to the nitrification process based on analysis of the water quality. Thus, the increase in TAN may have been mitigated by the presence of Chloramine-T in the system that degraded and reacted with the “excess” ammonia. Additionally, as discussed in relation to potassium permanganate, the nitrifying bacteria are embedded within

the biofilm. Chloramine-T degrades rapidly and will not have sufficient time to diffuse into the inner regions of the biofilm in order to reach the nitrifying bacteria (Schmidt, *et al.*, 2002).

The second mechanism would suggest that Chloramine-T remains stable in solution and is metabolized to p-toluenesulfonamide (p-TSA). Although this is a sulfonamide with antibacterial properties, microorganisms do not develop a resistance as they often do with typical sulfonamide antibiotics (Haneke, 2002). Although there is some concern that p-TSA may posses some carcinogenic traits because of its relationship to saccharin, it is known that Chloramine-T is poorly absorbed from the water and there is no current evidence that the compound bioaccumulates. Instead, it biodegrades fairly rapidly in aerobic environments (Haneke, 2002). The first mechanism must hold some validity or the DPD method of analysis that is based on the difference between the free and total chlorine present in the aquatic system would not be applicable for determination of the Chloramine-T concentration in the water. This is because the presumably stable Chloramine-T would not be releasing any chlorine. It is possible that both mechanisms occur simultaneously and that the Chloramine-T remaining in the water column follows the chlorine chemistry, but that the small amount of Chloramine-T that is absorbed by the fish is actually metabolized into p-TSA, providing some antibacterial benefit. Although there was no discernible inhibition to the nitrification process, the sOUR analysis indicated that there was an impact to the total bacterial community. Because the nitrifier population was low, the data indicate that the impact of the Chloramine-T may have been to the heterotrophic population.

As previously acknowledged, the feed was the primary source of nitrogen addition to the systems. Proteins in the feed are composed of approximately 16% nitrogen. The feed used in the odd-label chemical trials was 40% protein, resulting in 64 mg nitrogen added per gram of feed. Given the experimental parameters of the off-label chemical trials, approximately 478 mg nitrogen was added to each 20-gallon aquarium each day. This is equivalent to approximately 6.3 mg/L nitrogen added in the form of feed per day. Based on the estimates provided by Craig and Helfrich (2002) already described, approximately 3.1 mg/L nitrogen was released into the aquarium each day during the off-label chemical experiments. Water quality data for these experiments demonstrated that the total nitrogen in the aquarium water sampled – determined by adding the concentrations of TAN, nitrite, and nitrate – increased

by only an average of 0.3 mg/L per day over the course of the treatments. Additional calculations result in an estimate of 0.93 mg/L nitrogen being added directly to the water column each day. This is substantially higher than the 0.3 mg/L average daily increase observed in this experiment. A portion of the difference in the numbers can be accounted for by the estimates and assumptions made as these figures vary with factors such as species and system conditions, thus further research to delineate these contributions would be required. There were no statistically significant differences ($p > 0.05$) between the total nitrogen in the treated systems versus the untreated controls.

The primary contributor to the total nitrogen in the system is in the form of nitrate. The rate at which the nitrate accumulated in the treated systems remained fairly steady over the course of the treatments. This steady and continued accumulation indicates that nitrification continued over the course of the treatments coupled with the lack of significant increase in either the ammonia or nitrite concentrations supports the finding that nitrification was not inhibited by the treatment with these off-label chemicals at levels that are considered therapeutic.

Acknowledgements

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References

Agwuh, K. N. and A. MacGowan (2006). "Pharmacokinetics and Pharmacodynamics of the Tetracyclines Including Glycyclines." Journal of Antimicrobial Chemotherapy **58**: 256-265.

Amann, R. and M. Kuhl (1998). "*In Situ* Methods for Assessment of Microorganisms and Their Activities." Current Opinion in Microbiology **1**: 352-358.

Andreini, C., L. Banci, I. Bertini and A. Rosato (2008). "Occurrence of Copper Proteins Through the Three Domains of Life: A Bioinformatic Approach." Journal of Proteome Research **7**: 209-216.

AppliedBiochemists. (2002). "Cutrine-Plus." from http://www.dunnsfishfarm.com/cutrineliquidlabel.pdf.

Bakal, R. S., S. A. Bai and M. K. Stoskopf (2004). "Pharmacokinetics of Sulfadimethoxine and Ormetoprim in a 5:1 Ratio Following Intraperitoneal and Oral Administration, in the Hybrid Striped Bass (*Morone chrysops x Morone saxitalis)*." Journal of Veterinary Pharmacology and Therapeutics **27**: 1-6.

Bakal, R. S. and M. K. Stoskopf (2001). "In Vitro Studies of the Fate of Sulfadimethoxine and Ormetoprim in the Aquatic Environment." Aquaculture **195**: 95-102.

Bebak-Williams, J., G. Bullock and M. C. Carson (2002). "Oxytetracycline Residues in a Freshwater Recirculating System." Aquaculture **205**: 221-230.

Benbrook, C. M. (2002). Antibiotic Drug Use in U.S. Aquaculture, The Northwest Science and Environmental Policy Center.

Bjorklund, H., J. Bondestam and G. Bylund (1990). "Residues of Oxytetracycline in Wild Fish and Sediments from Fish Farms." Aquaculture **86**: 359-367.

Bjorklund, H. V., C. M. I. Rabergh and G. Bylund (1991). "Residues of Oxolinic Acid and Oxytetracycline in Fish and Sediments from Fish Farms." Aquaculture **97**: 85-96.

Borkow, G. and J. Gabbay (2005). "Copper as a Biocidal Tool." Current Medicinal Chemistry **12**(18): 2163-2175.

Bower, C. E. and D. T. Turner (1982). "Effects of Seven Chemotherapeutic Agents on Nitrification in Closed Seawater Culture Systems." Aquaculture **29**: 331-345.

Braam, F. and A. Klapwijk (1981). "Effects of Copper on Nitrification in Activated Sludge." Water Research **15**(9): 1093-1098.

Buchmann, K., J. Bresciani and C. Jappe (2004). "Effects of Formalin Treatment on Epithelial Structure and Mucous Cell Densities in Rainbow Trout, *Oncorhynchus mykiss* (Walbaum), skin." Journal of Fish Diseases **27**: 99-104.

Capone, D. G., D. P. Weston, V. Miller and C. Shoemaker (1996). "Antibacterial Residues in Marine Sediments and Invertebrates Following Chemotherapy in Aquaculture." Aquaculture **145**(55-75).

Chain, P., J. Lamerdin, F. Larimer, W. Regala, V. Lao, M. Land, L. Hauser, A. Hooper, M. Klotz, J. Norton, L. Sayavedra-Soto, D. Arciero, N. Hommes, M. Whittaker and D. Arp (2003). "Complete Genome Sequence of the Ammonia-Oxidizing Bacterium and Obligate Chemolithoautotroph *Nitrosomonas europaea*." Journal of Bacteriology **185**(9): 2759-2773.

Chopra, I. and T. G. B. Howe (1978). "Bacterial Resistance to Tetracyclines." Microbiological Reviews **42**(4): 707-724.

Chopra, I. and M. Roberts (2001). "Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance." Microbiology and Molecular Biology Reviews **65**(2): 232-260.

Clesceri, L., A. Greenberg and A. Eaton, Eds. (1998). Standard Methods for the Examination of Water and Wastewater. Washington, DC.

Collins, M. T., J. B. Gratzek, D. L. Dawe and T. G. Nemetz (1976). "Effects of Antibacterial Agents on Nitrification in an Aquatic Recirculating System." Journal of the Fisheries Research Board of Canada **33**(2): 215-218.

Craig, S. and L. A. Helfrich (2002). Understanding Fish Nutrition, Feeds, and Feeding, Virginia Cooperative Extension, Publication Number 420-256.

Dawson, V. K., J. R. Meinertz, L. J. Schmidt and W. H. Gingerich (2003). "A SImple Analytical Procedure to Replace HPLC for Monitoring Treatment Concentrations of Chloramine-T on Fish Culture Facilities." Aquaculture **217**(1-4): 61-72.

Dietze, J. E., E. A. Scribner, M. T. Meyer and D. W. Kolpin (2005). "Occurrence of Antibiotics in Water From 13 Fish Hatcheries, 2001-2003." International Journal of Environmental Analytical Chemistry **85**(15): 1141-1152.

Francis-Floyd, R. (1996). Fact Sheet VM-77: Use of Formalin to Control Fish Parasites, University of Florida, Institute of Food and Agricultural Sciences.

Francis-Floyd, R. and R. Klinger (1997). Fact Sheet FA37: Use of Potassium Permanganate to Control External Infections of Ornamental Fish, University of Florida, Institute of Food and Agricultural Sciences.

Gingerich, W. H., G. R. Stehly, J. J. Rach, J. R. Meinertz, J. A. Bernardy, J. E. Rivera, C. Vue, M. P. Gaikowski, L. J. Schmidt, R. A. Schnick, B. R. Griffin, D. Erdahl, J. Bowker and

D. Carty (2001). Seventh Midyear Report of Progress - Approval of Drugs for Public Fish Production.

Halling-Sorensen, B. (2001). "Inhibition of Aerobic Growth and Nitrification of Bacteria in Sewage Sludge by Antibacterial Agents." Archives of Environmental Contamination and Toxicology **40**: 451-460.

Haneke, K. E. (2002). Chloramine-T and Metabolite p-Toluenesulfonamide: A Review of Toxicological Literature, Integrated Laboratory Systems.

Hoffman-LaRoche (1984). Romet-30 (R) Environmental Assessment.

Hu, Z., K. Chandran, D. Grasso and B. F. Smets (2003). "Impact of Metal Sorption and Internalization on Nitrification Inhibition." Environmental Science and Technology **37**(4): 728-734.

Jacobsen, P. and L. Berglind (1988). "Persistence of Oxytetracycline in Sediments from Fish Farms." Aquaculture **70**: 365-370.

Keck, N. and G. Blanc (2002). "Effects of Formalin Chemotherapeutic Treatments on Biofilter Efficiency in a Marine Recirculating Fish Farming System." Aquatic Living Resources **15**: 361-370.

Kim, K. T., I. S. Kim, S. H. Hwang and S. D. Kim (2006). "Estimating the Combined Effects of Copper and Phenol to Nitrifying Bacteria in Wastewater Treatment Plants." Water Research **40**(561-568).

Klaver, A. L. and R. A. Matthews (1994). "Effects of Oxytetracycline on Nitrification in a Model Aquatic System." Aquaculture **123**: 237-247.

Lazur, A. M. (2002). Fact Sheet FA23: The Use of Potassium Permanganate in Fish Ponds, University of Florida - Institute of Food and Agricultural Sciences.

Lee, A., W. Mao, M. S. Warren, A. Mistry, K. Hoshino, R. Okumura, H. Ishida and O. Lomovskaya (2000). "Interplay Between Efflux Pumps May Provide Either Additive or Multiplicative Effects on Drug Resistance." Journal of Bacteriology **182**(11): 3142-3150.

Ma, D., D. N. Cook, M. Alberti, G. Pon, H. Nikaido and J. E. Hearst (1993). "Molecular Cloning and Characterization of *acrA* and *acrE* genes of *Escherichia coli*." Journal of Bacteriology **175**: 6299-6313.

Morin, L. G. (2004). "Disease Prevention Control." 2008, from http://www.seachem.com/support/Articles/DiseasePrvntnCntrl02.html.

Nikaido, H. (1996). "Multidrug Efflux Pumps of Gram-Negative Bacteria." Journal of Bacteriology **178**(10): 5853-5859.

Nikaido, H. (1998). "Multiple Antibiotic Resistance and Efflux." Current Opinion in Microbiology **1**: 516-523.

Okusu, H., D. Ma and H. Nikaido (1996). "AcrAB Efflux Pump Plays a Major Role in the Antibiotic Resistance Phenotype of *Escherichia coli* Mulitple-Antibiotic-Resistance (Mar) Mutants." Journal of Bacteriology **178**: 306-308.

Park, E. D., D. V. Lightner, N. Milner, M. Mayersohn, D. L. Park, J. M. Gifford and T. A. Bell (1995). "Exploratory Bioavailability and Pharmacokinetic Studies of Sulphadimethoxine and Ormetoprim in the Penaeid Shrimp *Penaeus vannamei*." Aquaculture **130**: 113-128.

Pernthaler, A., J. Pernthaler and R. Amann (2002). "Fluorescence In Situ Hybridization and Catalyzed Reporter Deposition for the Identification of Marine Bacteria." Applied and Environmental Microbiology **68**(6): 3094.

Pfizer (1987). Terramycin (oxytetracycline) Environmental Assessment.

Rajasekaran, P., N. Sriranganathan and S. M. Boyle (2007). Antibiotic Resistance - a Formidable Threat to the Human Race - Are We Losing the War Against Pathogens. 5th International Student/Young Pugwash Conference, Bari, Italy.

Rakocy, J. E. (2005). Tank Culture of Tilapia, Southern Regional Agricultural Center and Texas Aquaculture Extension Service.

Samuelsen, O. B. (1989). "Degradation of Oxytetracycline in Seawater at Two Different Temperatures and Light Intensities, and the Persistence of Oxytetracycline in the Sediment From a Fish Farm." Aquaculture **83**: 7-16.

Samuelsen, O. B., B. T. Lunestad, A. Ervik and S. Fjelde (1994). "Stability of Antibacterial Agents in an Artificial Marine Aquaculture Sediment Studied Under Laboratory Conditions." Aquaculture **126**: 283-290.

Schmidt, L. J., M. P. Gaikowski, W. H. Gingerich, G. R. Stehly and W. J. Larson (2002). Use of Chloramine-T in Intensive Freshwater Aquaculture: An Evaluation of Potential Environmental Fate and Effects, U.S. Food and Drug Administration Center for Veterinary Medicine.

Schwartz, M. F., G. L. Bullock, J. A. Hankins, S. T. Summerfelt and J. A. Mathias (2000). "Effects of Selected Chemotherapeutants on Nitrification in Fluidized-Sand Biofilters for Coldwater Fish Production." International Journal of Recirculating Aquaculture **1**.

Skinner, F. A. and N. Walker (1961). "Growth of *Nitrosomonas europaea* in batch and continuous culture." Archives of Microbiology **38**: 339-347.

Spangenberg, J. V. (1999). "Investigation of the Saftety and Efficacy of Chloramine-T in the Treatment of External Fluke Infestations."

Tomlinson, T. G., A. G. Boon and C. N. A. Trotman (1966). "Inhibition of NItrification in the Activated Sludge Process of Sewage Disposal." Journal of Applied Bacteriology **29**: 266-291.

U.S.Pharmacopeia. (2007). "Potentiated Sulfonamides." from http://www.usp.org/pdf/EN/veterinary/potentiatedSulfonamides.pdf.

USFDA (1984). Environmental Impact Analysis Report: Romet-30 (Sulfadimethoxine and Ormetoprim) Medicated Premix for Control of Certain Salmonid Diseases (NADA 125-933_EA).

USFDA. (2008). "FDA/CVM Approved Drugs for Use in Aquaculture." from http://www.fda.gov/cvm/drugsuseaqua.htm.

Watson, C. and R. P. E. Yanong (2002). Fact Sheet FA-13: Use of Copper in Freshwater Aquaculture and Farm Ponds, University of Florida Institute of Food and Agricultural Sciences.

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Tables

Table 2-1. Initial fish weights for each set of trials

System	First Trial (g)	Replicate Trial (g)
Antiparasitic trials	484	463
Antibiotic trials	498	493
Off-label chemical use trials	483	513

Table 2-2. Hach methods used for water quality analysis

Hach Method Number	Description
8038	Total ammonia-nitrogen (TAN) – Nessler
10023	Total ammonia-nitrogen (TAN) - Salicylate
8507	Nitrite-nitrogen
8039	Nitrate-nitrogen
8506	Free copper
8110	Formaldehyde
8210	Free and Total Chlorine
8034	Manganese

Table 2-3. Oligonucleotides used in CARD-FISH application

Oligonucleotide Name	**Probe Sequence**	**Formamide Concentration**	**Target organisms**
Nso1225	CGC CAT TGT ATT TGT GA	35%	β-proteobacterial ammonia-oxidizers
Ntspa662	GGA ATT CCG CGC TCC TCT	35%	Genus *Nitrospira*
Eub338	GCT GCC TCC CGT AGG AGT	0-50%	Most bacteria
Eub338-II	GCA GCC ACC CGT AGG TGT	0-50%	Planctomycetales
Eub338-III	GCT GCC ACC CGT AGG TGT	0-50%	Verrucomicrobiales

Table 2-4. Results of CARD-FISH analyses in the chelated copper trials

Sample Day	Average AOB/EUB Ratio (%)		Average NOB/EUB Ratio (%)	
	Control	Treated	Control	Treated
-3	3.5	3.7	2.9	3.4
6	3.2	3.2	3.1	3.8
9	3.8	3.1	3.0	3.4
12	3.5	3.7	3.3	2.7
15	3.1	2.6	3.6	3.1
18	3.9	2.7	3.2	2.9
21	3.3	2.4	3.5	3.1
24	3.6	3.0	3.1	2.4
27	3.5	2.8	3.0	3.7
30	3.8	2.4	3.6	2.8
33	3.4	2.7	4.0	3.5

Table 2-5. Results of CARD-FISH analyses for the antibiotic trials

Sample Day	Average AOB/EUB Ratio (%)			Average NOB/EUB Ratio (%)		
	Control	OTC-treated	Romet-treated	Control	OTC-treated	Romet-treated
-3	4.5	4.7	5.2	5.2	4.7	4.3
3	4.1	4.1	4.6	4.7	4.5	3.7
6	3.7	4.2	4.1	5.3	4.5	3.9
9	4.4	4.5	4.6	5.1	4.4	4.1
12	4.9	4.8	5.2	4.7	5.1	4.5
15	5.3	5.1	4.8	4.8	4.3	5.0
18	4.5	5.0	5.3	5.2	4.8	4.9
22	4.7	4.5	4.2	5.4	4.1	4.2
25	4.4	3.7	4.0	5.5	4.2	4.6
29	4.6	4.8	4.4	5.1	5.7	5.3
32	4.9	5.0	3.9	5.3	5.1	4.8
36	4.1	4.5	4.7	4.8	4.9	4.1
39	4.7	4.9	4.2	4.7	5.3	4.6
43	5.2	5.2	4.1	4.1	5.7	4.9
46	4.9	4.6	4.8	4.3	4.8	5.2

Figures

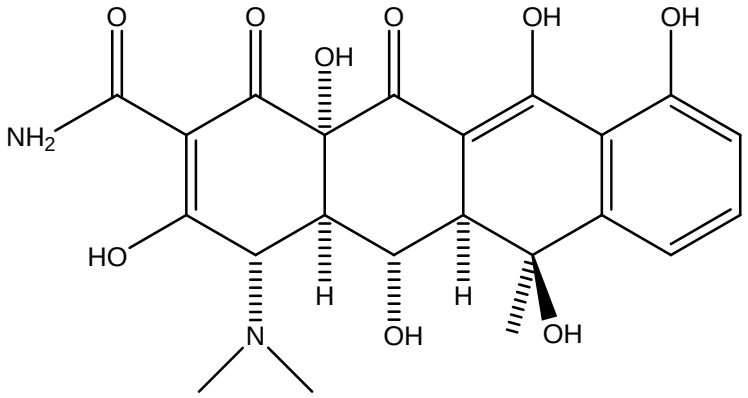

Figure 2-1. Oxytetracycline structure (adapted from http://en.wikipedia.org/wiki/Oxytetracycline)

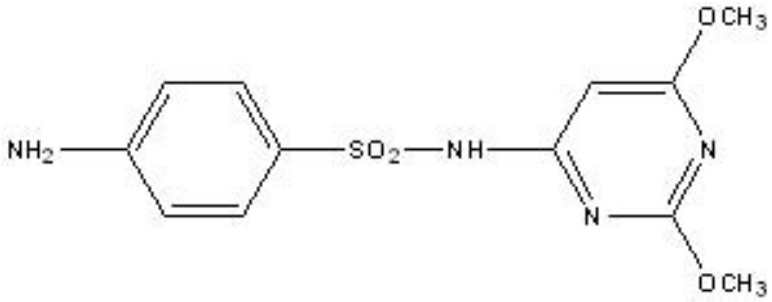

Figure 2-2. Sulfadimethoxine structure adapted from (Hoffman-LaRoche, 1984)

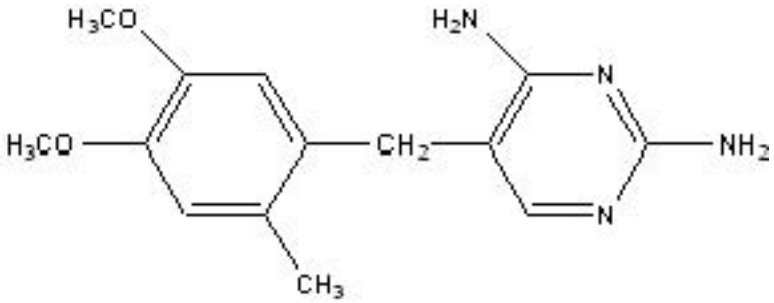

Figure 2-3. Ormetoprim structure adapted from (Hoffman-LaRoche, 1984)

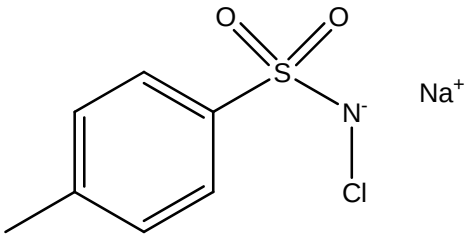

Figure 2-4. Chloramine-T structure (from http://en.wikipedia.org/wiki/Chloramine-T)

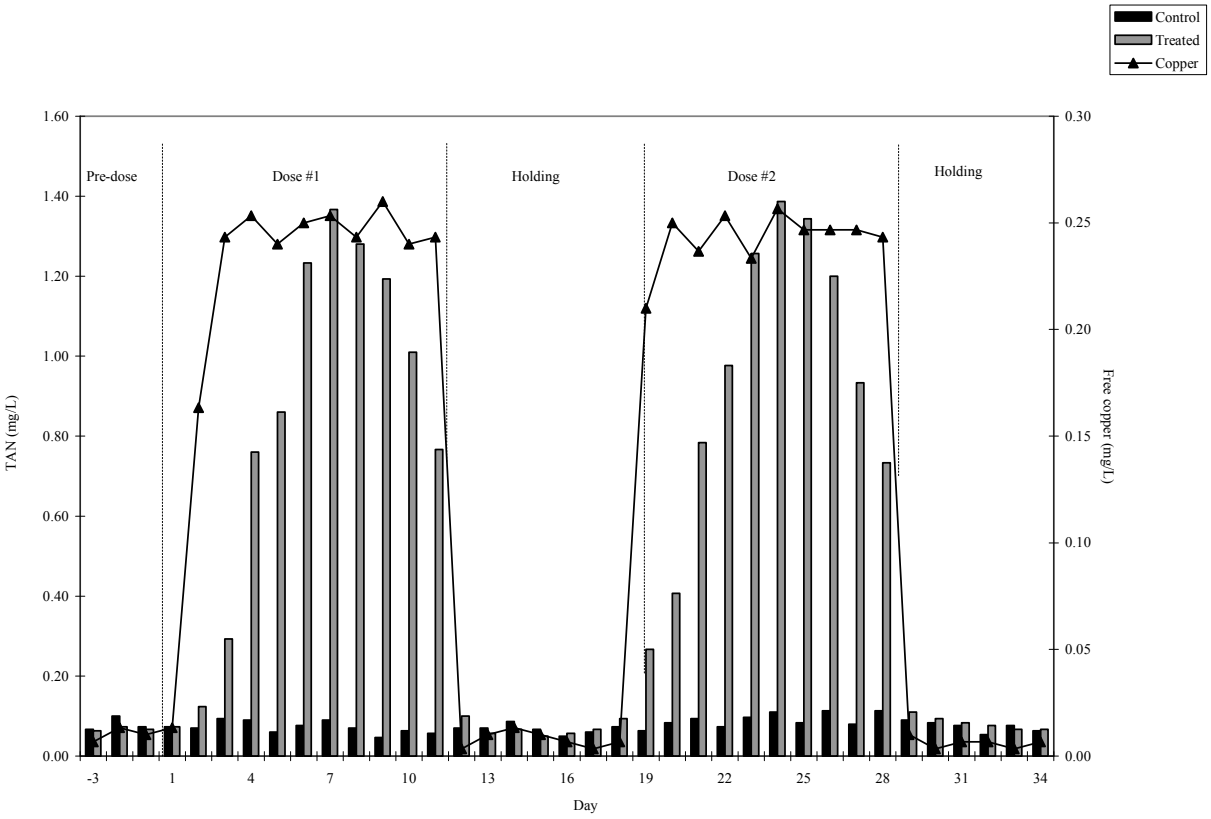

Figure 2-5. Total ammonia-nitrogen (TAN) response to treatment with chelated copper (0.25 ppm free copper) during the first trial

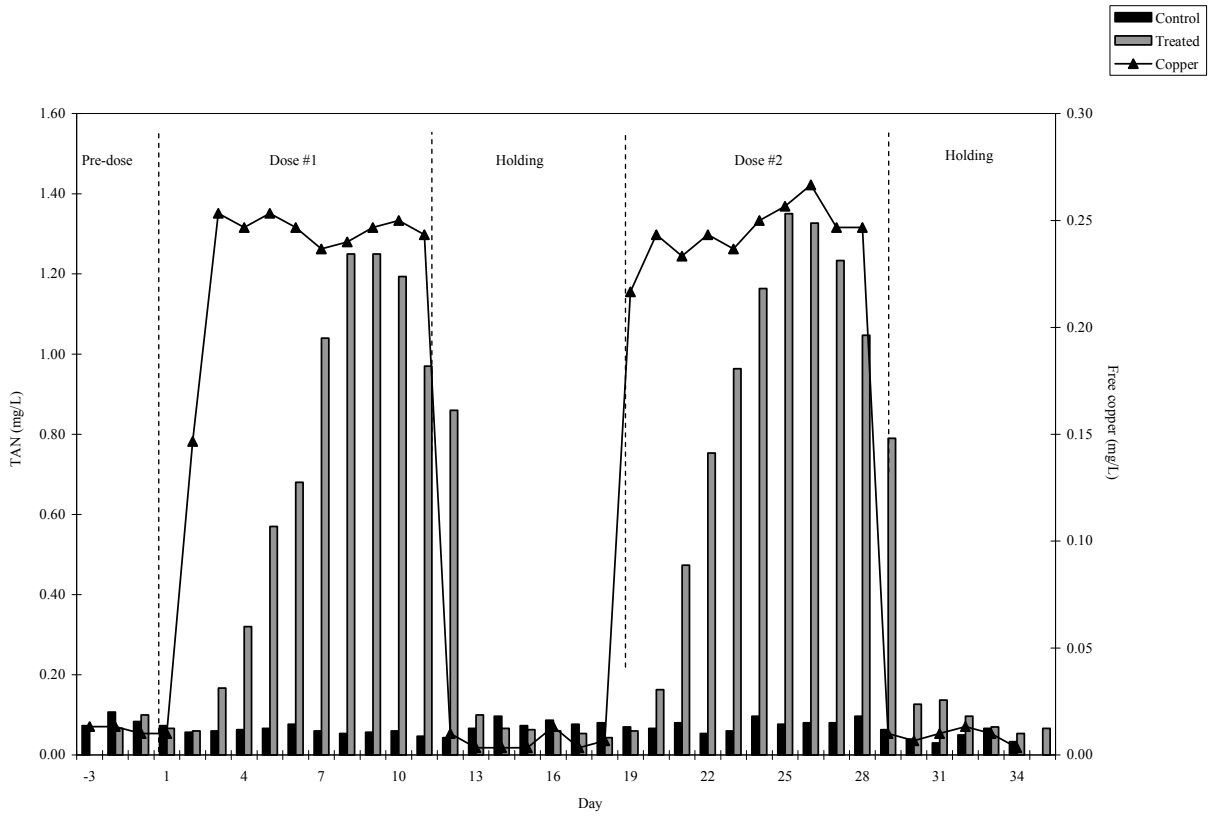

Figure 2-6. Total ammonia-nitrogen (TAN) response to treatment with chelated copper (0.25 ppm free copper) during the replicate trial

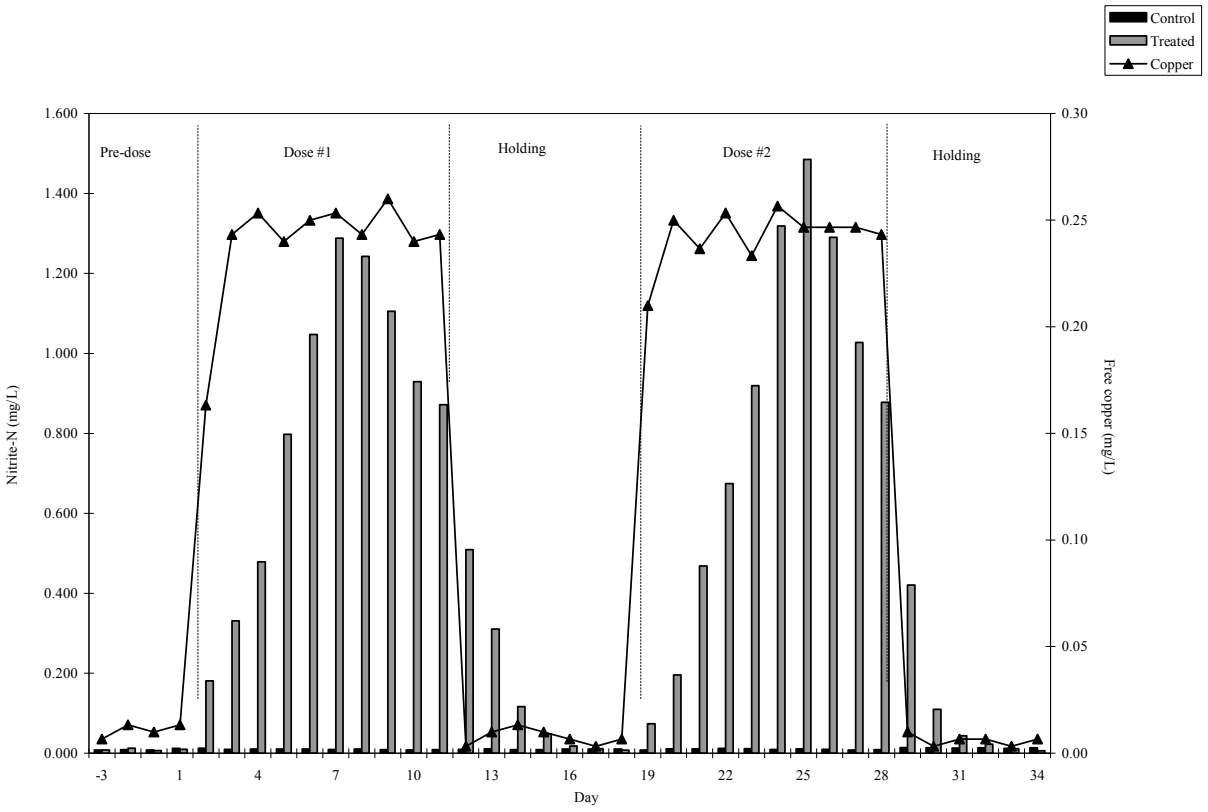

Figure 2-7. Nitrite-nitrogen response to treatment with chelated copper (0.25 ppm free copper) during the first trial

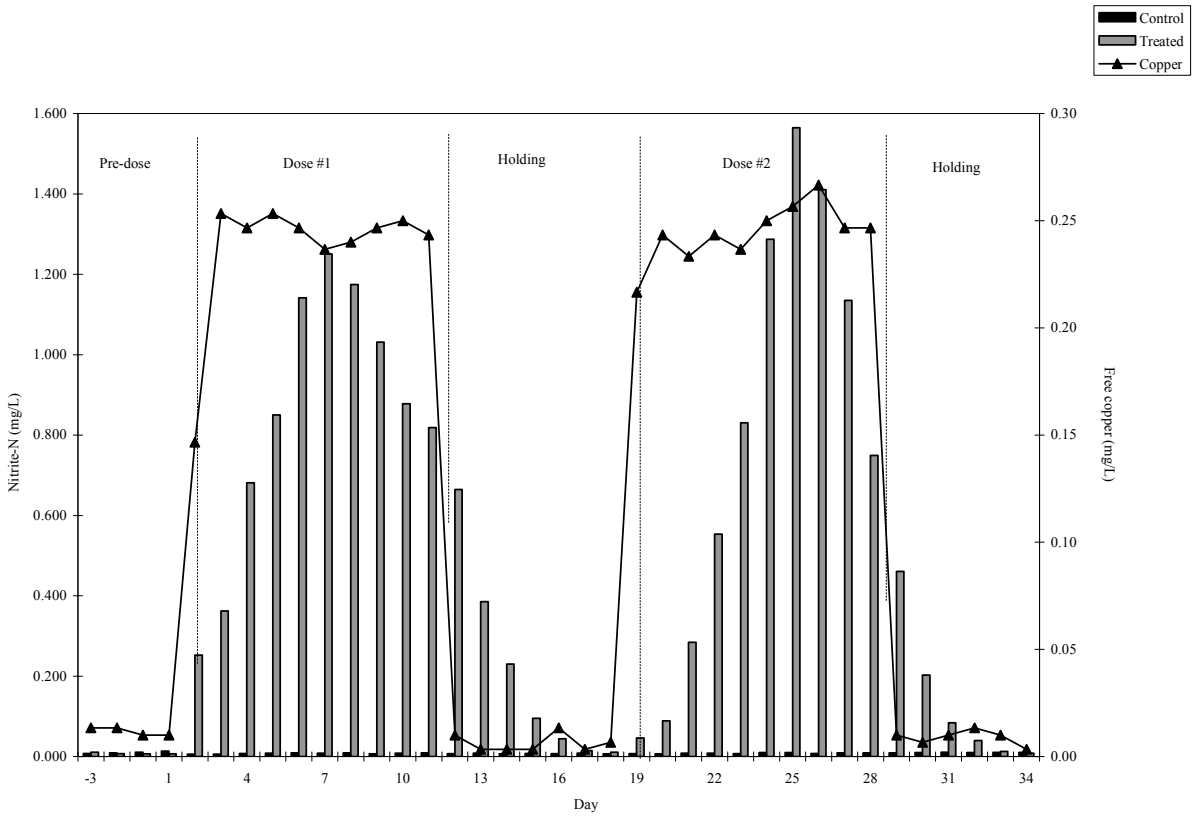

Figure 2-8. Nitrite-nitrogen response to treatment with chelated copper (0.25 ppm free copper) during the replicate trial

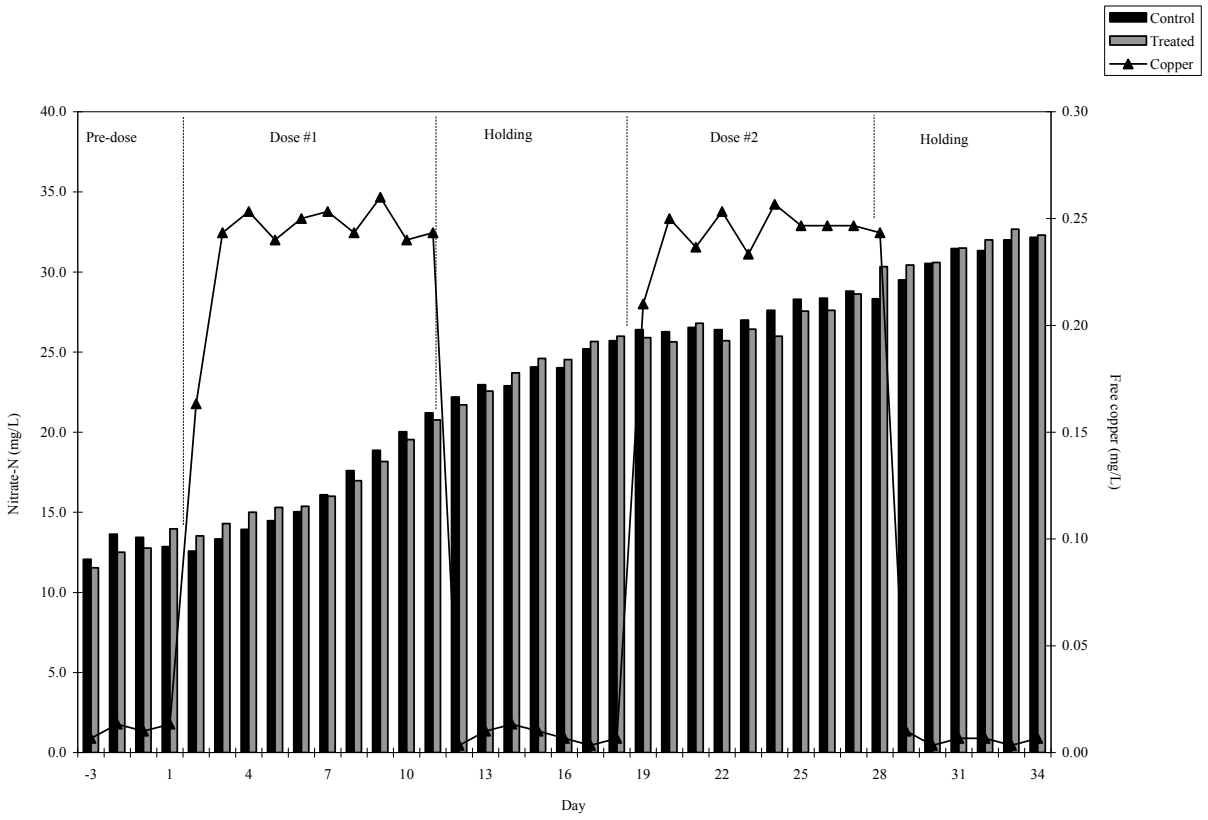

Figure 2-9. Nitrate-nitrogen response to treatment with chelated copper (0.25 ppm free copper) during the first trial

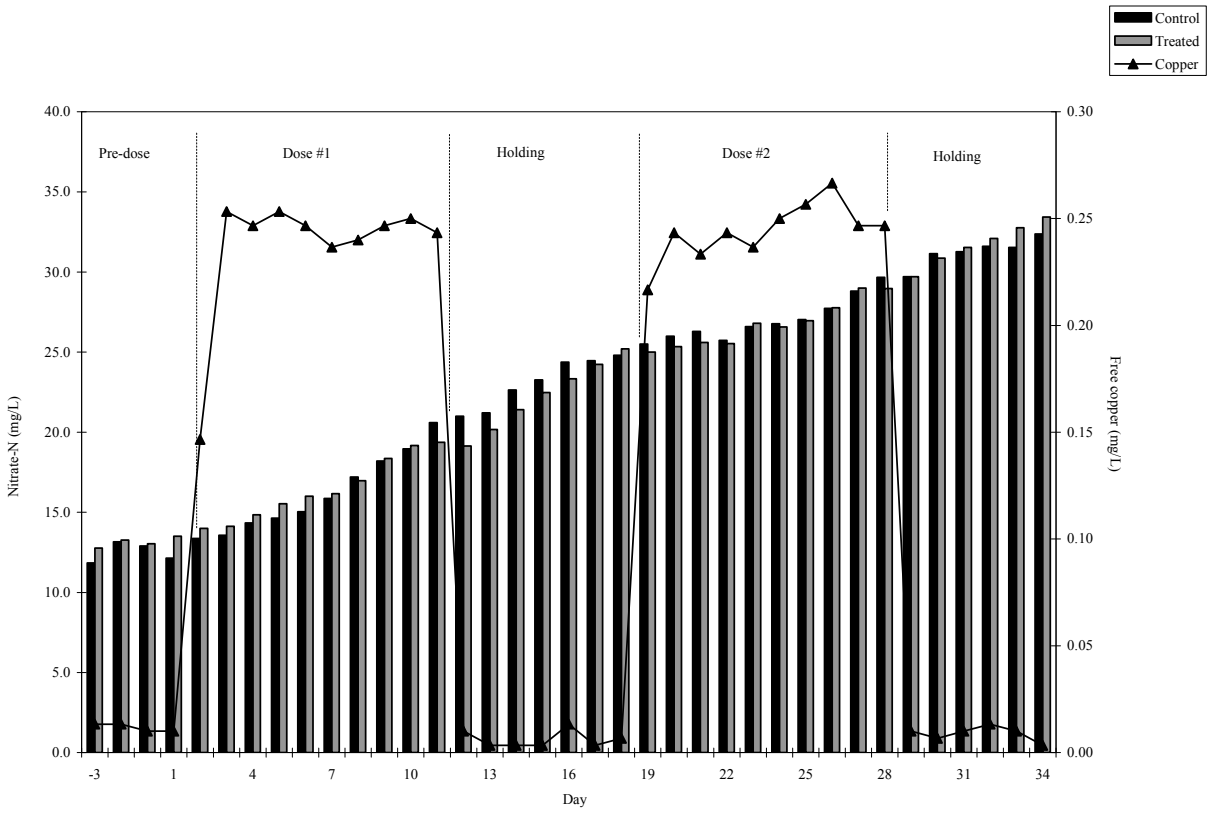

Figure 2-10. Nitrate-nitrogen response to treatment with chelated copper (0.25 ppm free copper) during the replicate trial

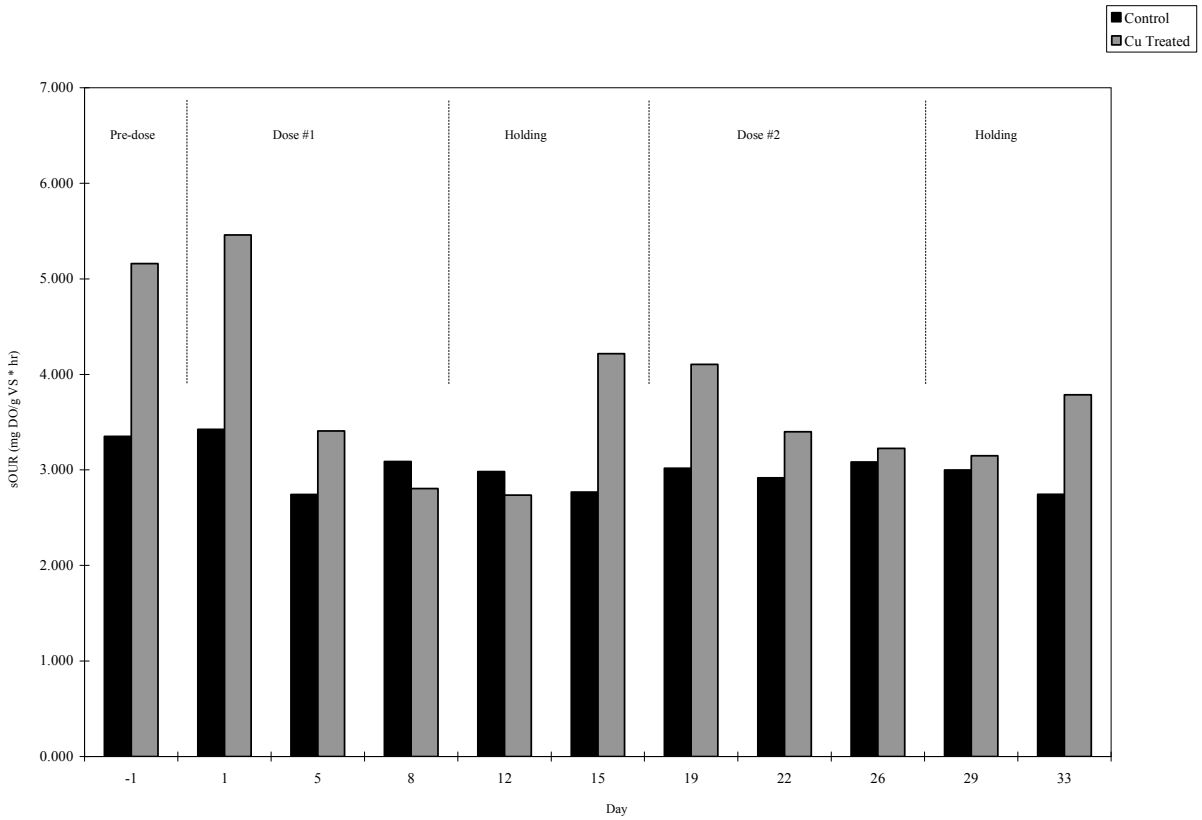

Figure 2-11. Specific oxygen uptake rate (sOUR) response to treatment with chelated copper (0.25 ppm free copper) during the first trial

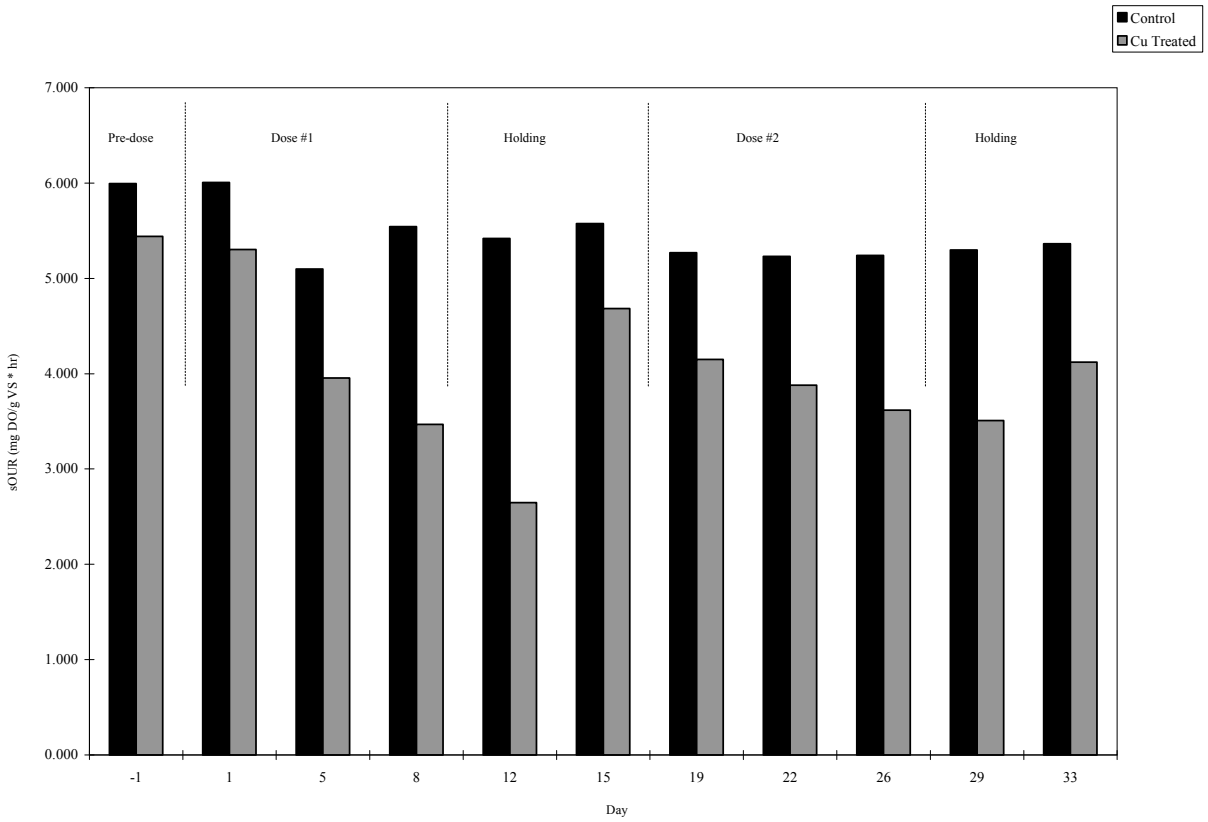

Figure 2-12. Specific oxygen uptake rate (sOUR) response to treatment with chelated copper (0.25 ppm free copper) during the replicate trial

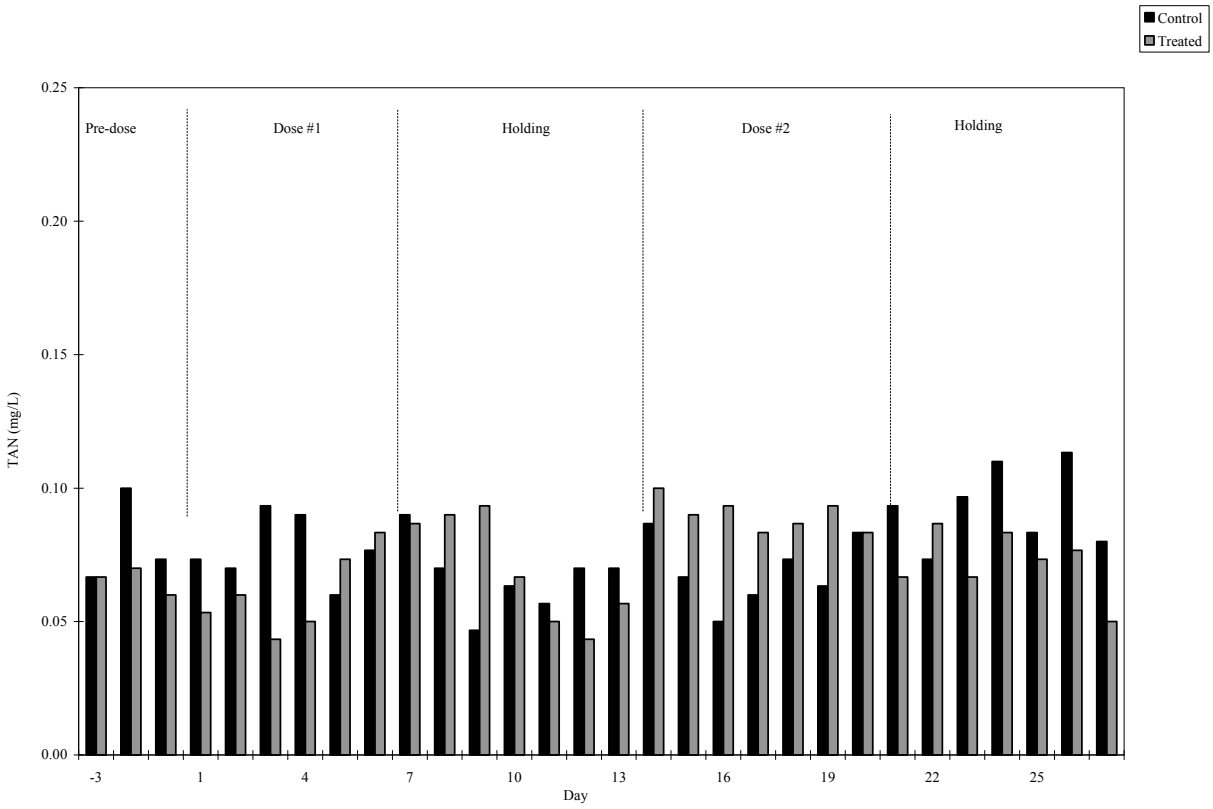

Figure 2-13. Total ammonia-nitrogen (TAN) response to treatment with formalin (150 ppm) during the first trial

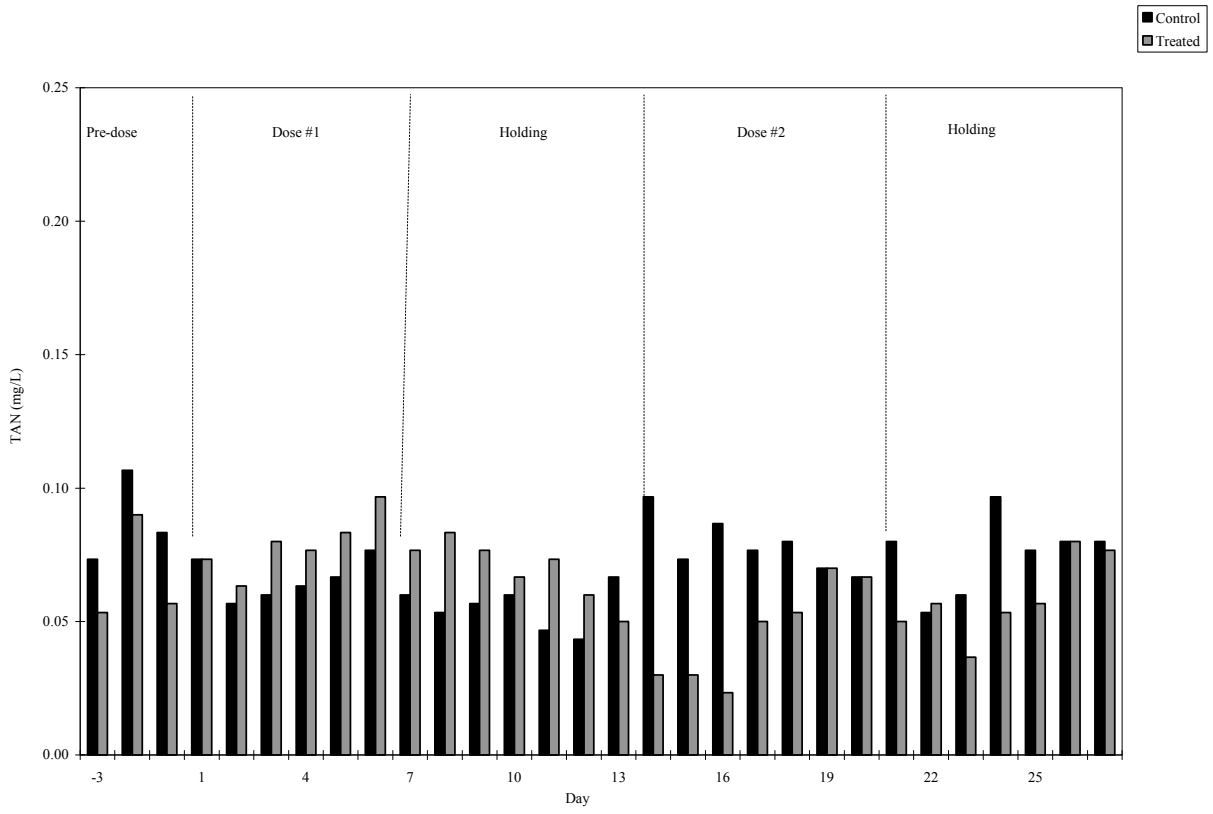

Figure 2-14. Total ammonia-nitrogen (TAN) response to treatment with formalin (150 ppm) during the replicate trial

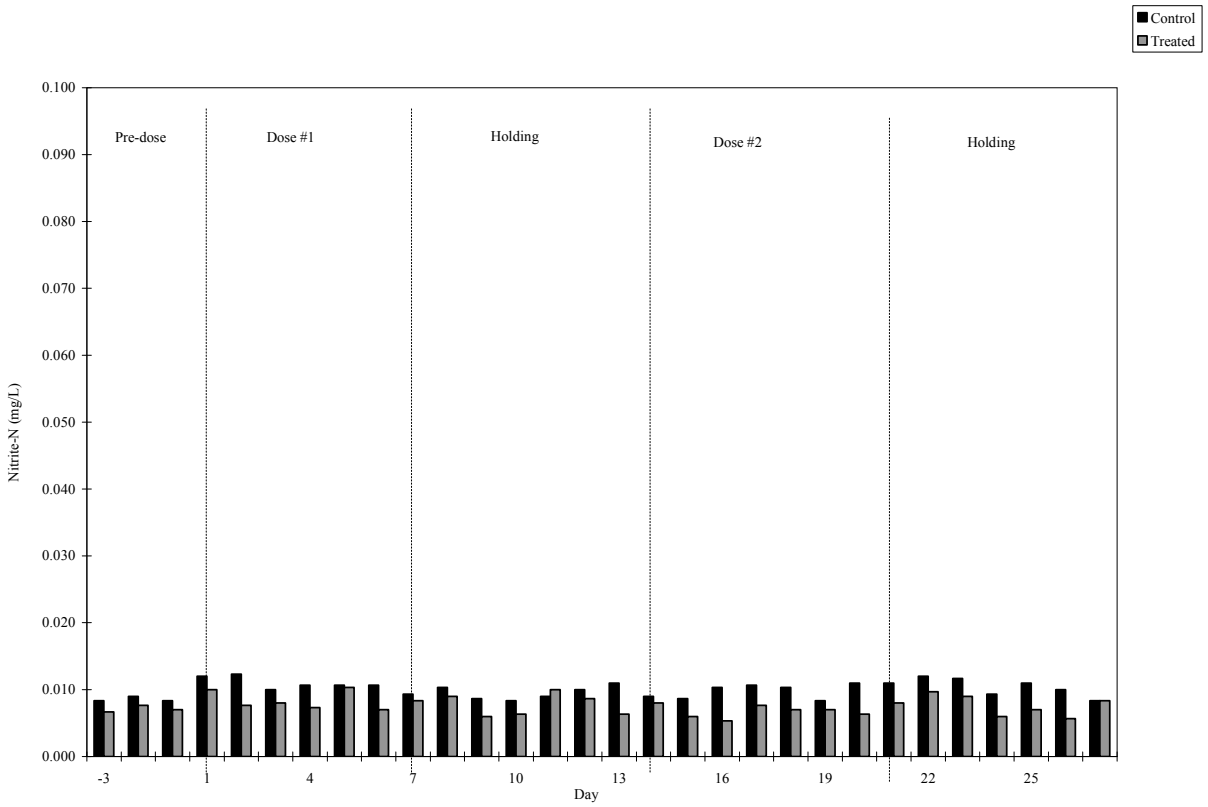

Figure 2-15. Nitrite-nitrogen response to treatment with formalin (150 ppm) during the first trial

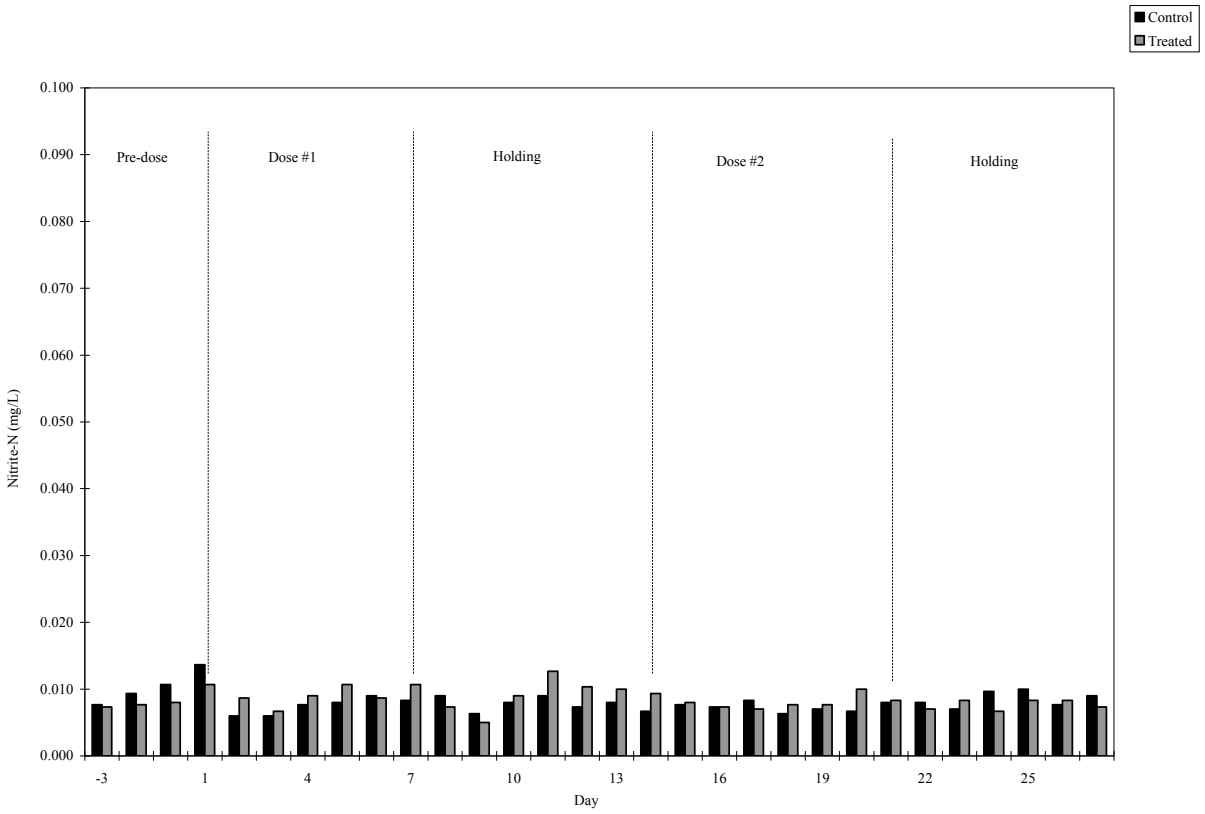

Figure 2-16. Nitrite-nitrogen response to treatment with formalin (150 ppm) during the replicate trial

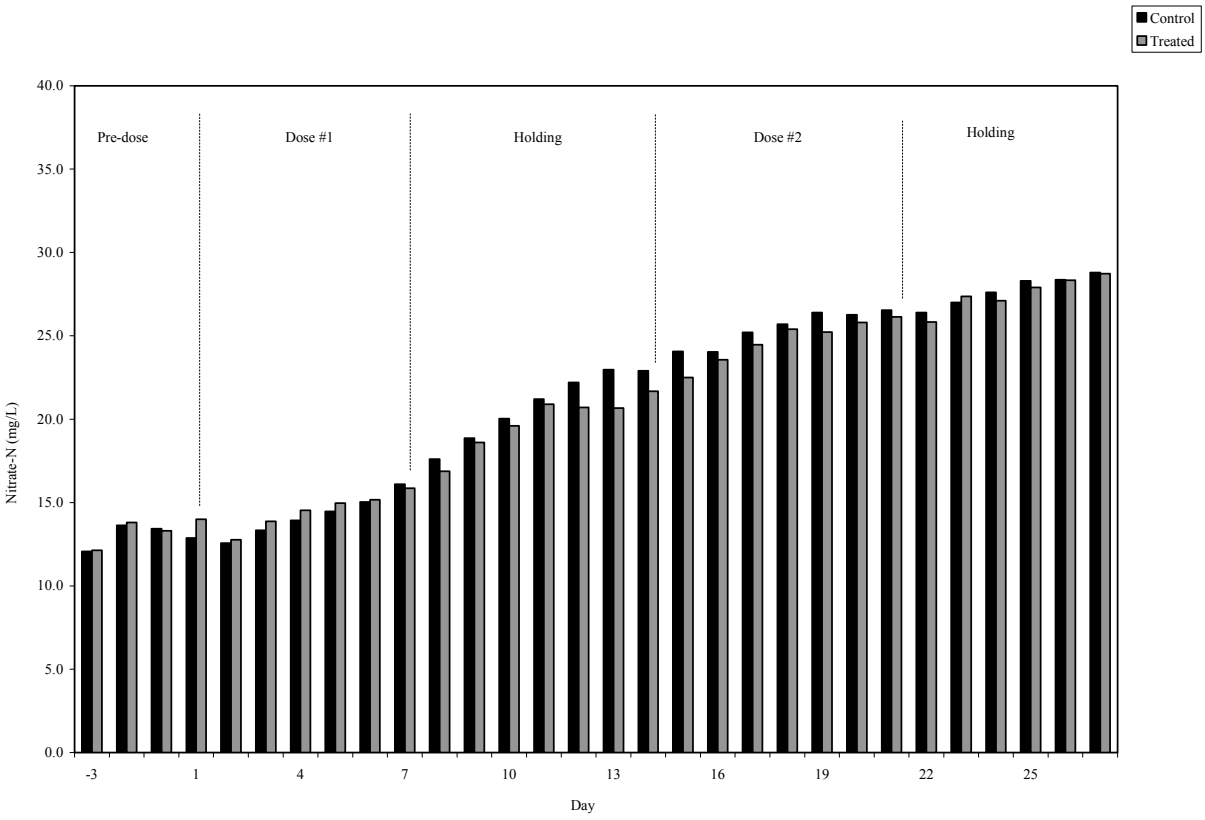

Figure 2-17. Nitrate-nitrogen response to treatment with formalin (150 ppm) during the first trial

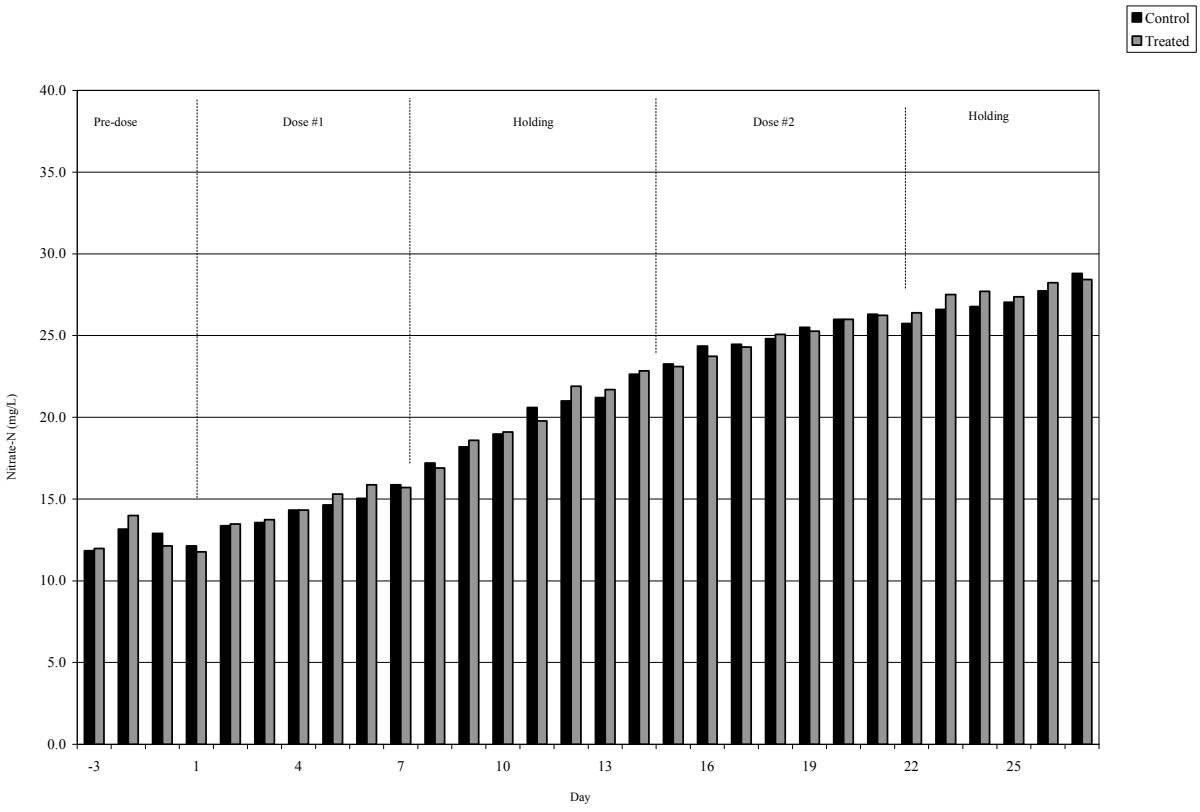

Figure 2-18. Nitrate-nitrogen response to treatment with formalin (150ppm) during the replicate trial

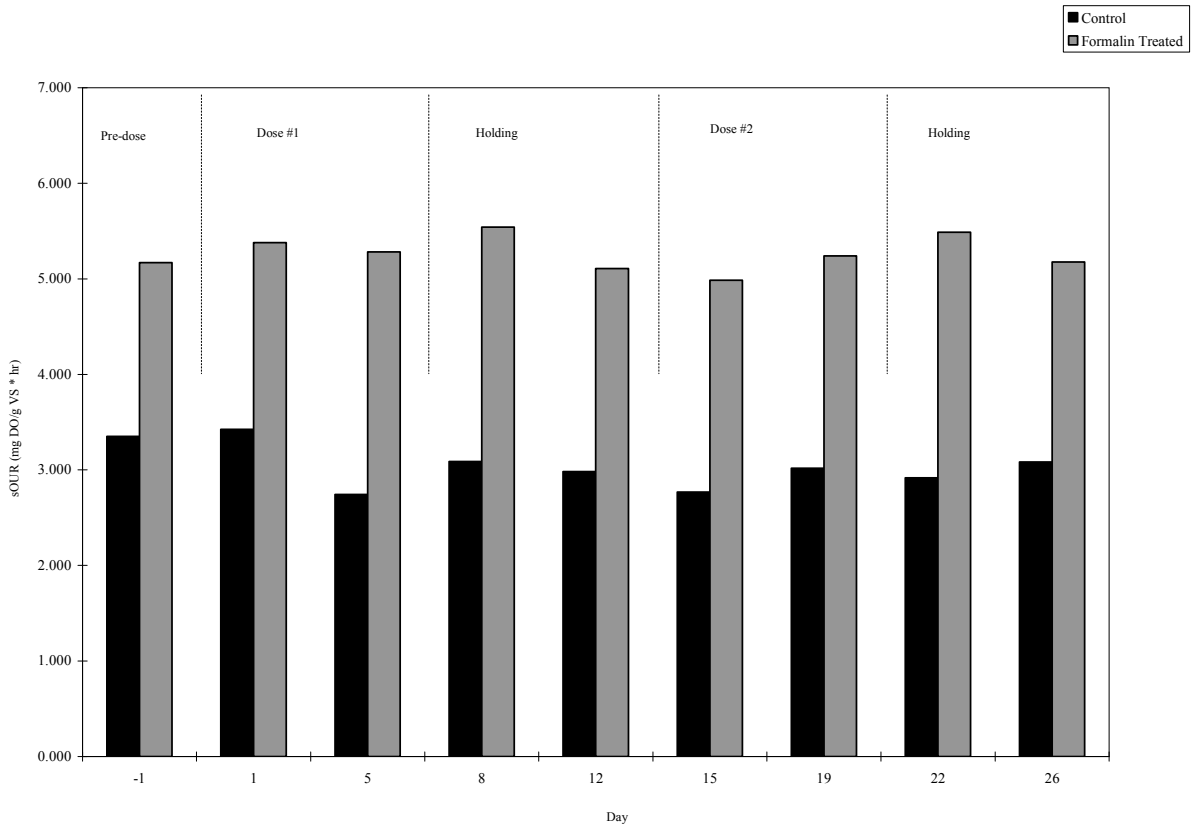

Figure 2-19. Specific oxygen uptake rate (sOUR) response to treatment with formalin (150ppm) during the first trial

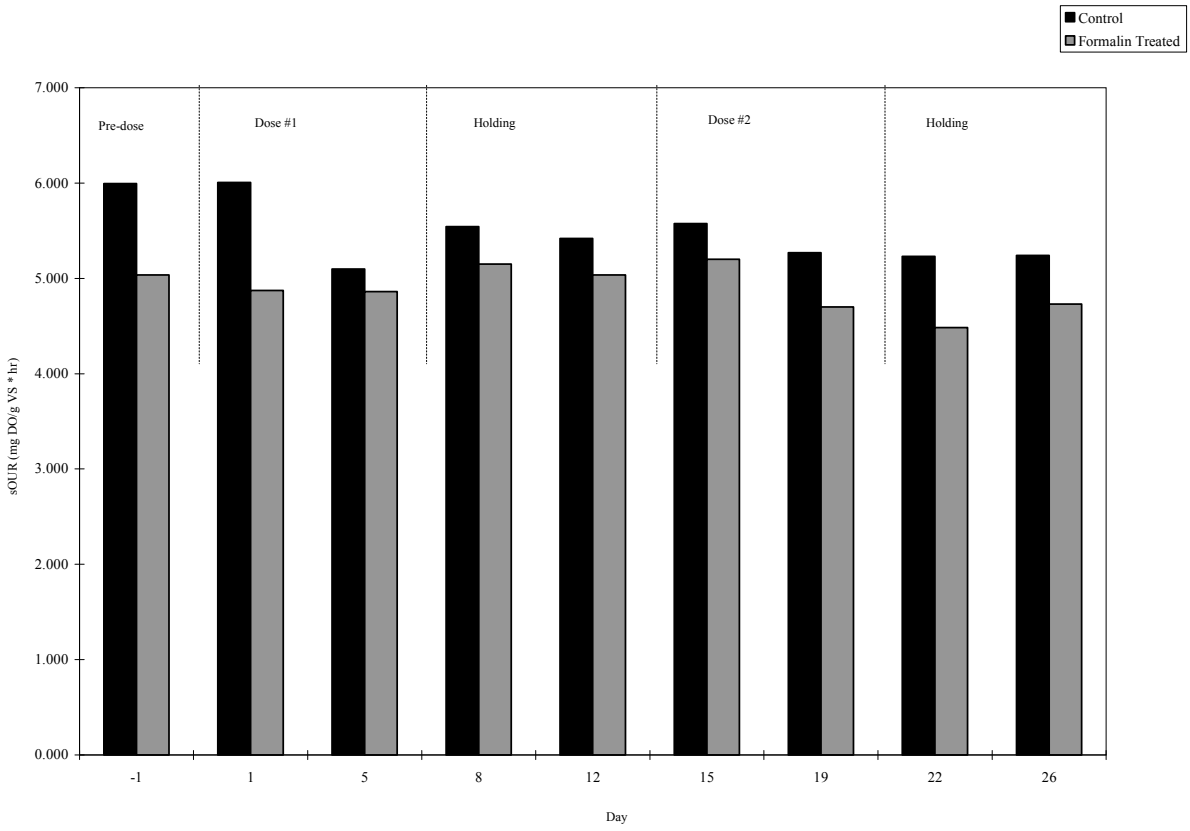

Figure 2-20. Specific oxygen uptake rate (sOUR) response to treatment with formalin (150 ppm) during the replicate trial

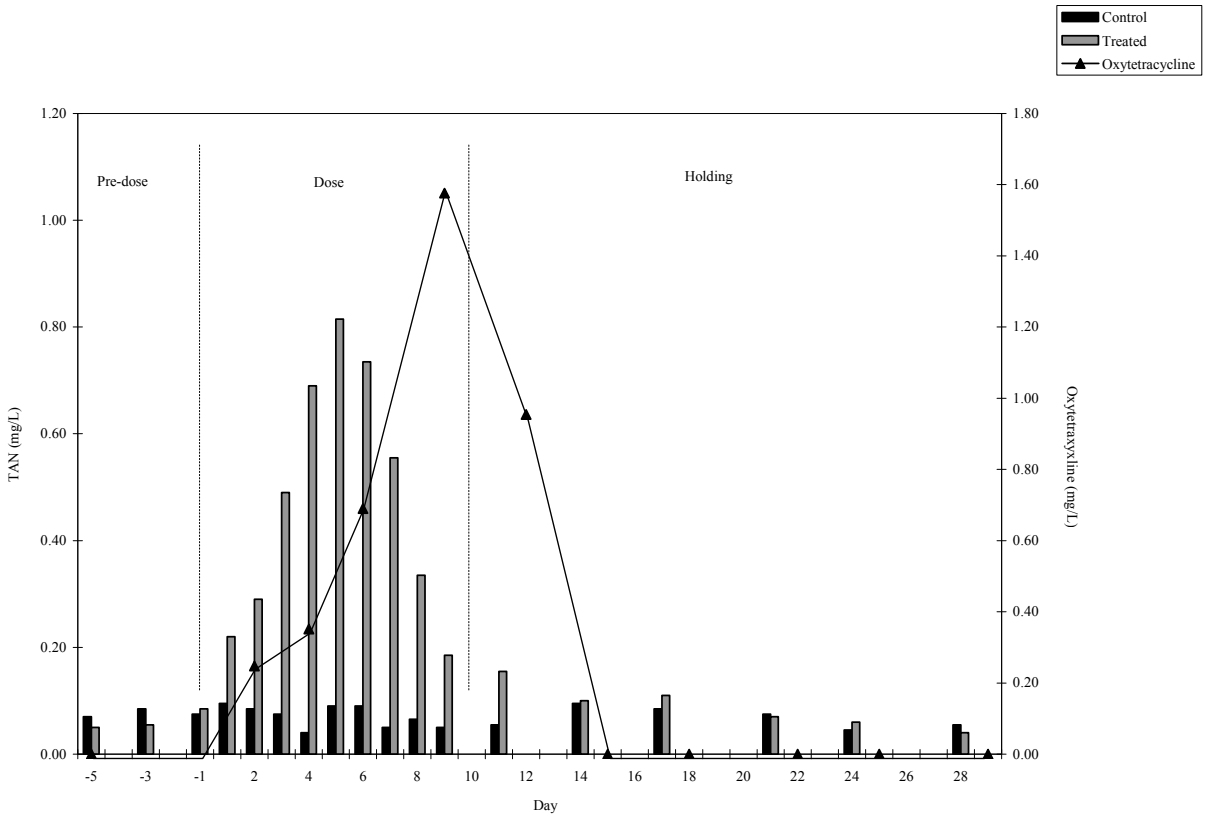

Figure 2-21. Total ammonia-nitrogen (TAN) response to treatment with oxytetracycline medicated feed during the first trial

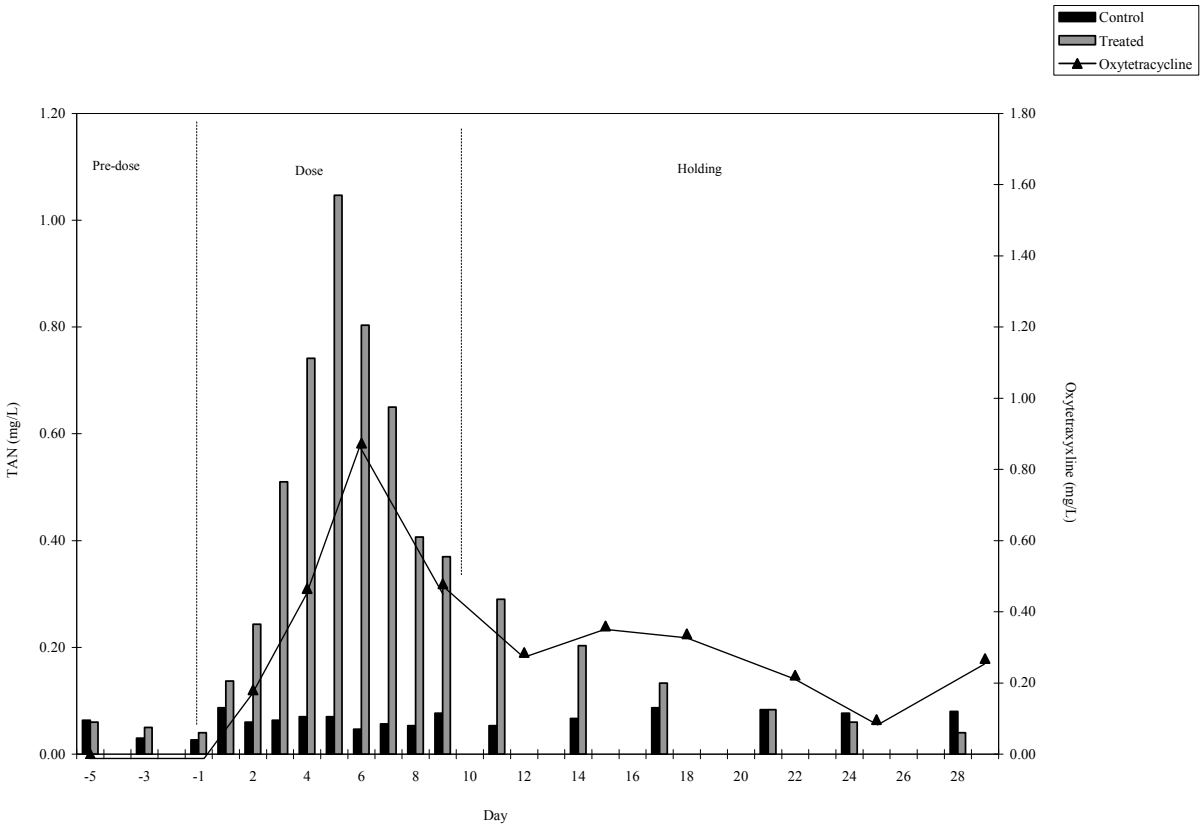

Figure 2-22. Total ammonia-nitrogen (TAN) response to treatment with oxytetracycline medicated feed during the replicate trial

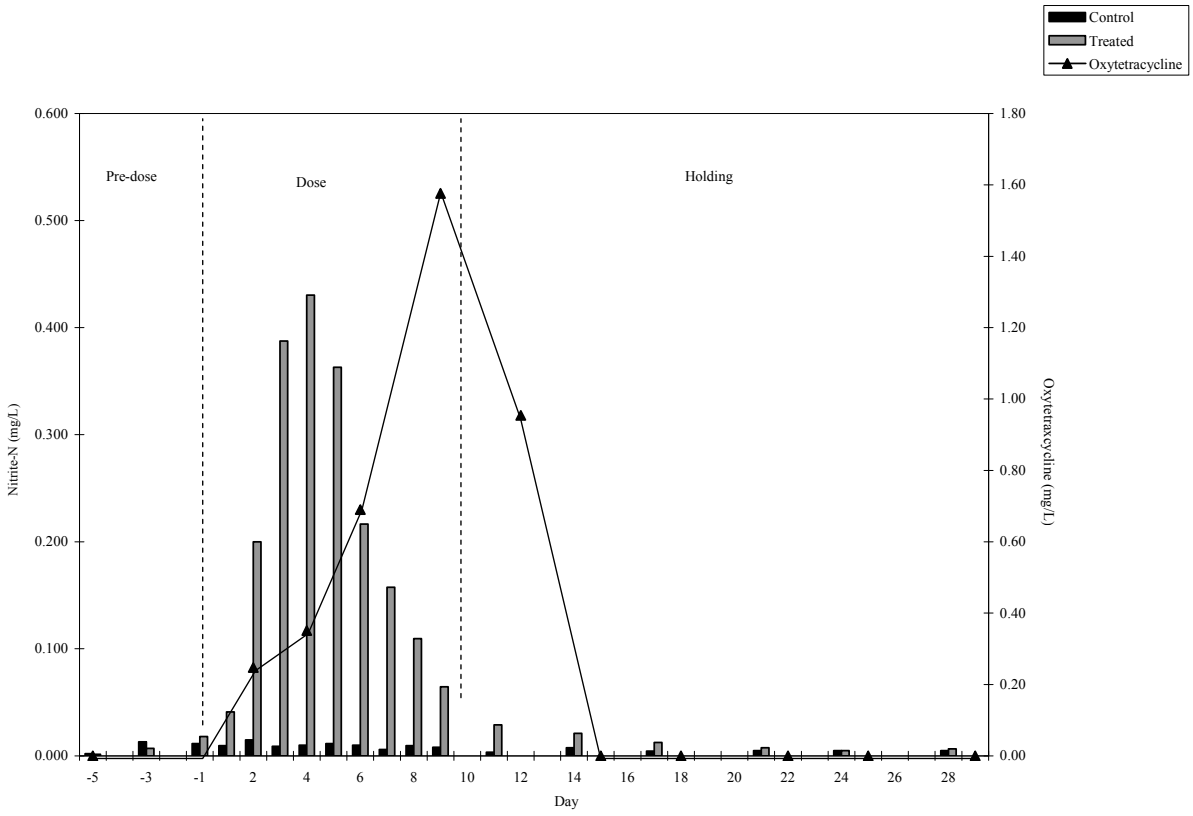

Figure 2-23. Nitrite-nitrogen response to treatment with oxytetracycline medicated feed during the first trial

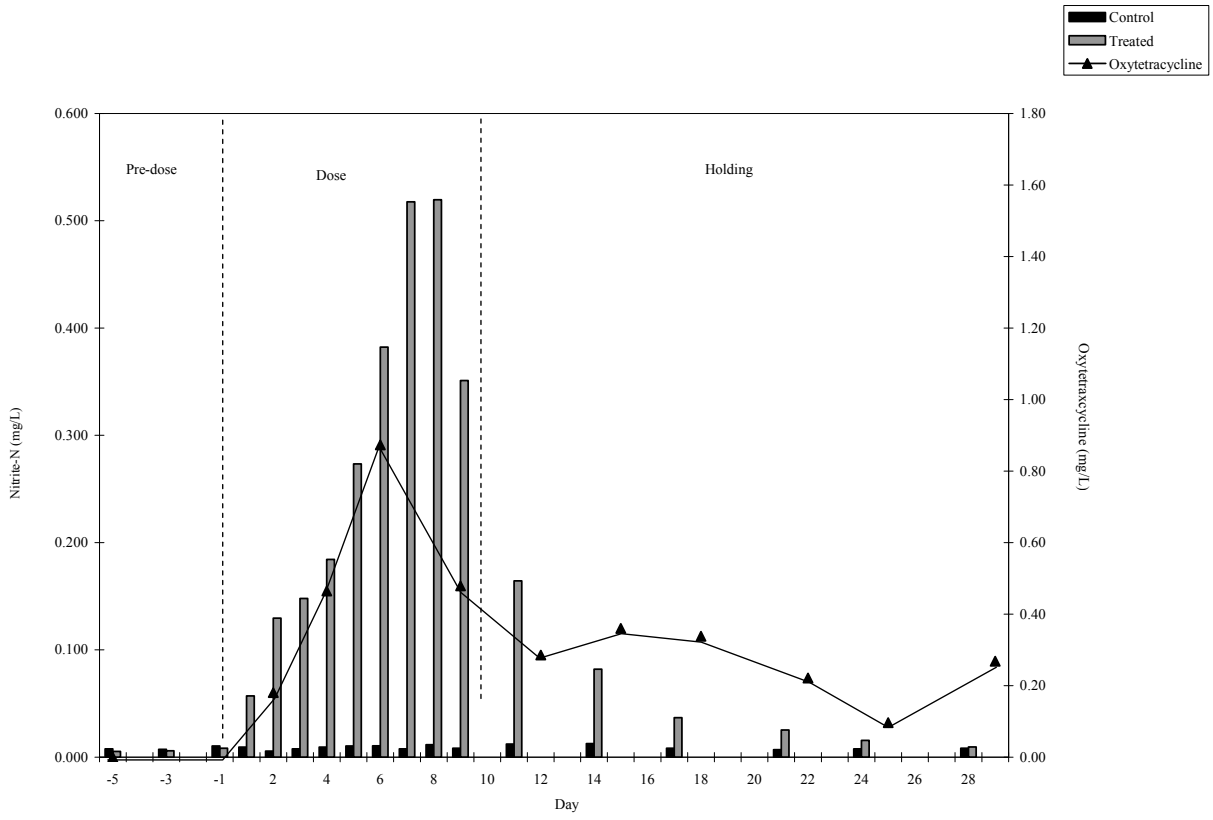

Figure 2-24. Nitrite-nitrogen response to treatment with oxytetracycline medicated feed during the replicate trial

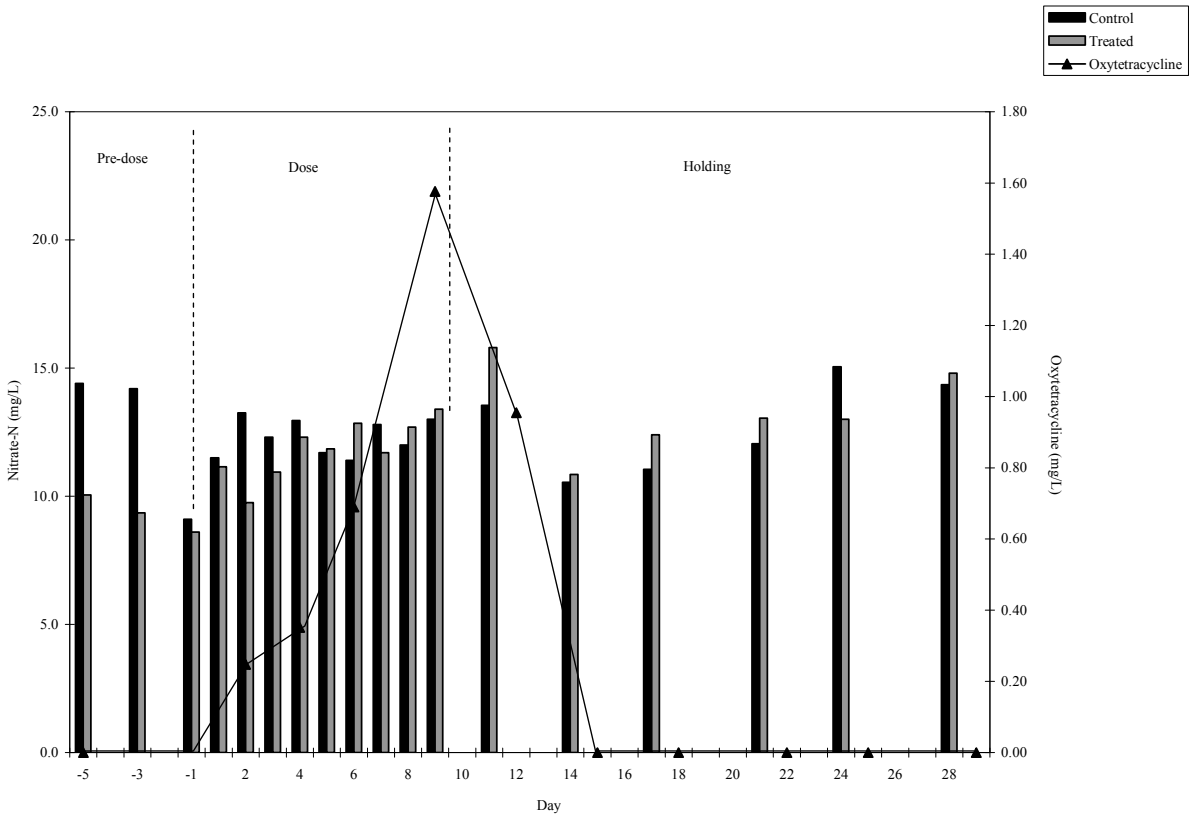

Figure 2-25. Nitrate-nitrogen response to treatment with oxytetracycline medicated feed during the first trial

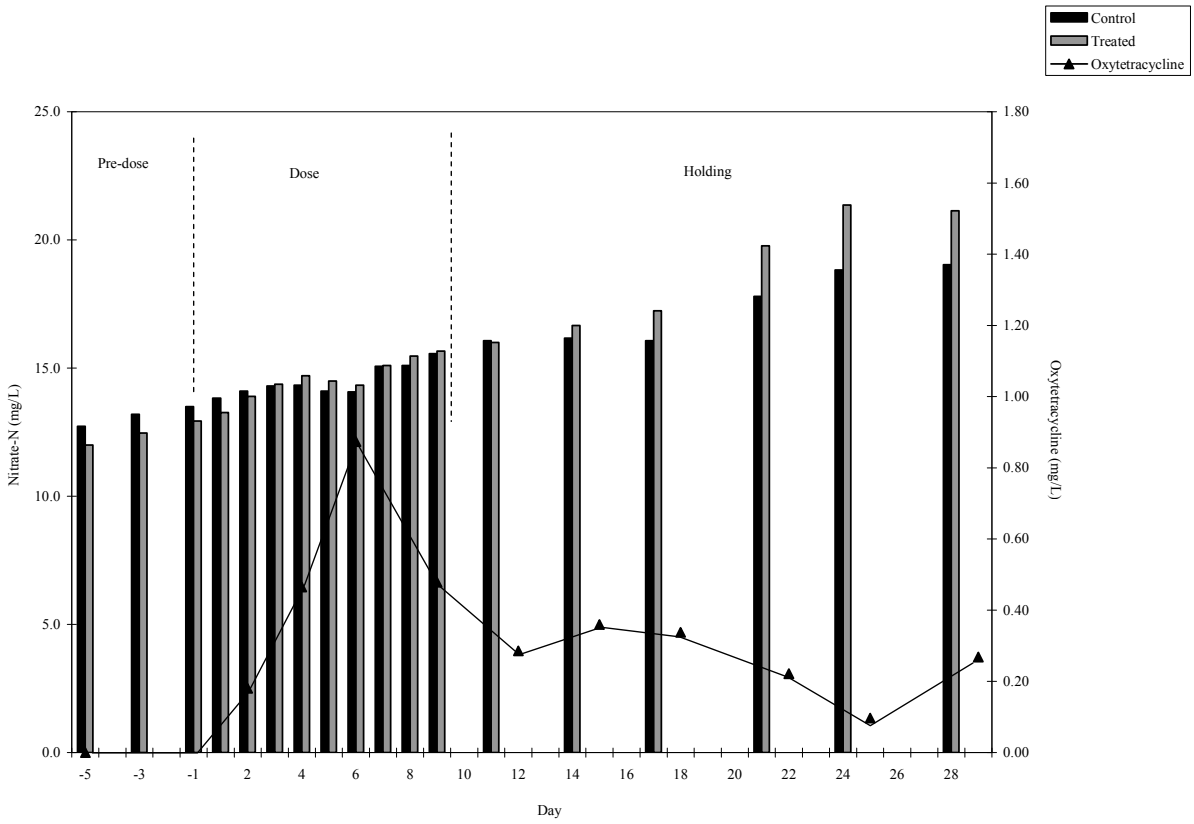

Figure 2-26. Nitrate-nitrogen response to treatment with oxytetracycline medicated feed during the replicate trial

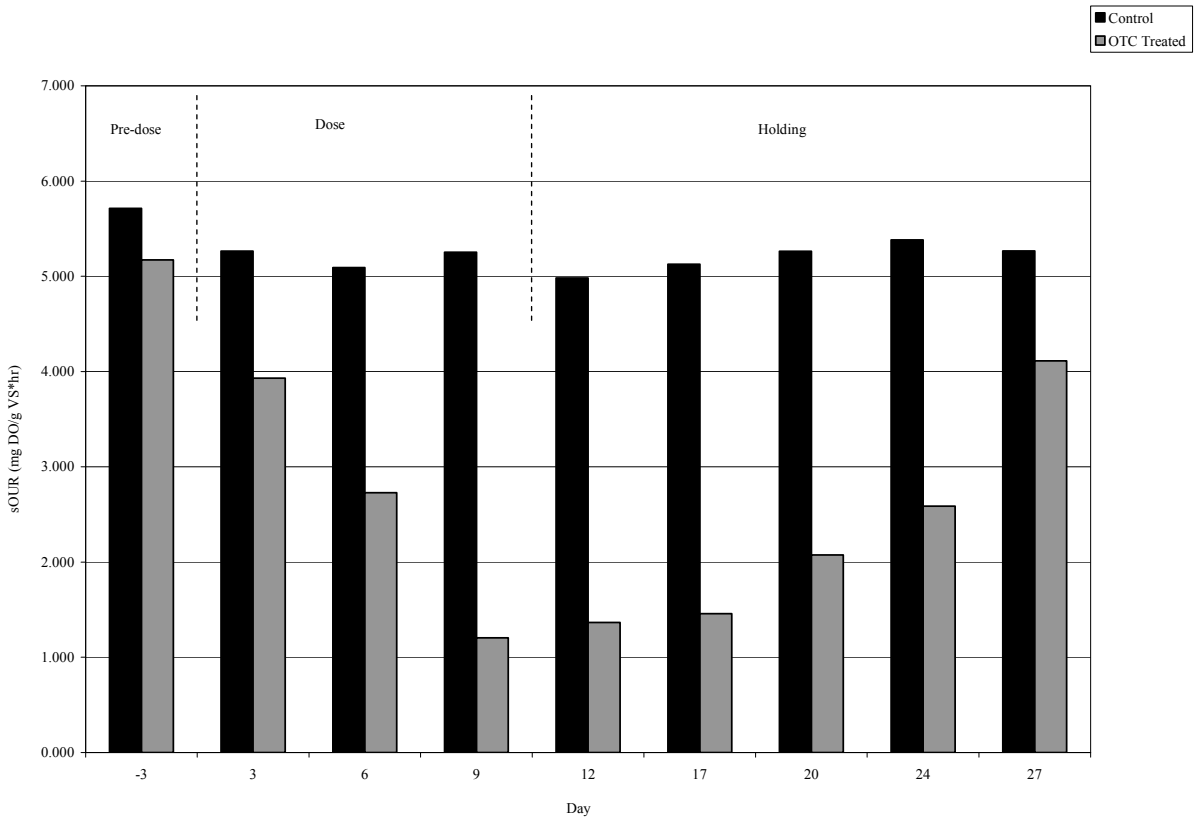

Figure 2-27. Specific oxygen uptake rate (sOUR) response to treatment with oxytetracycline medicated feed during the first trial

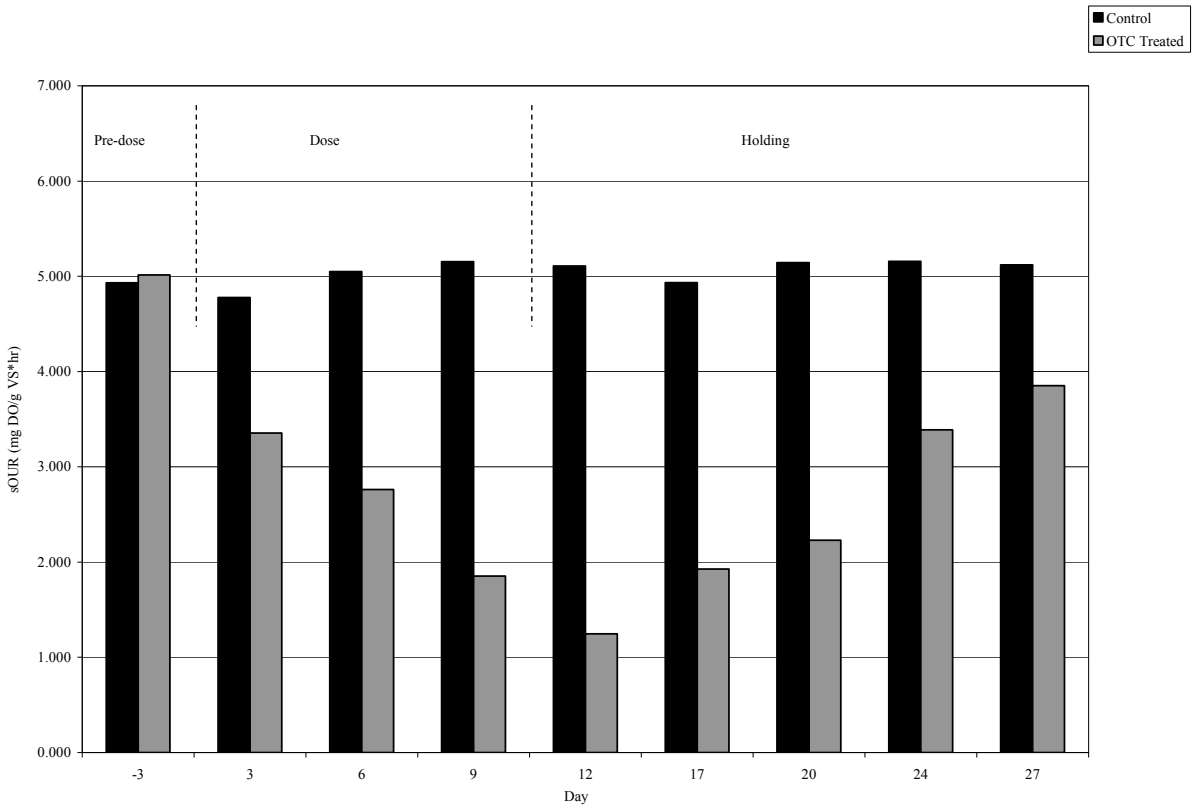

Figure 2-28. Specific oxygen uptake rate (sOUR) response to treatment with oxytetracycline medicated feed during the replicate trial

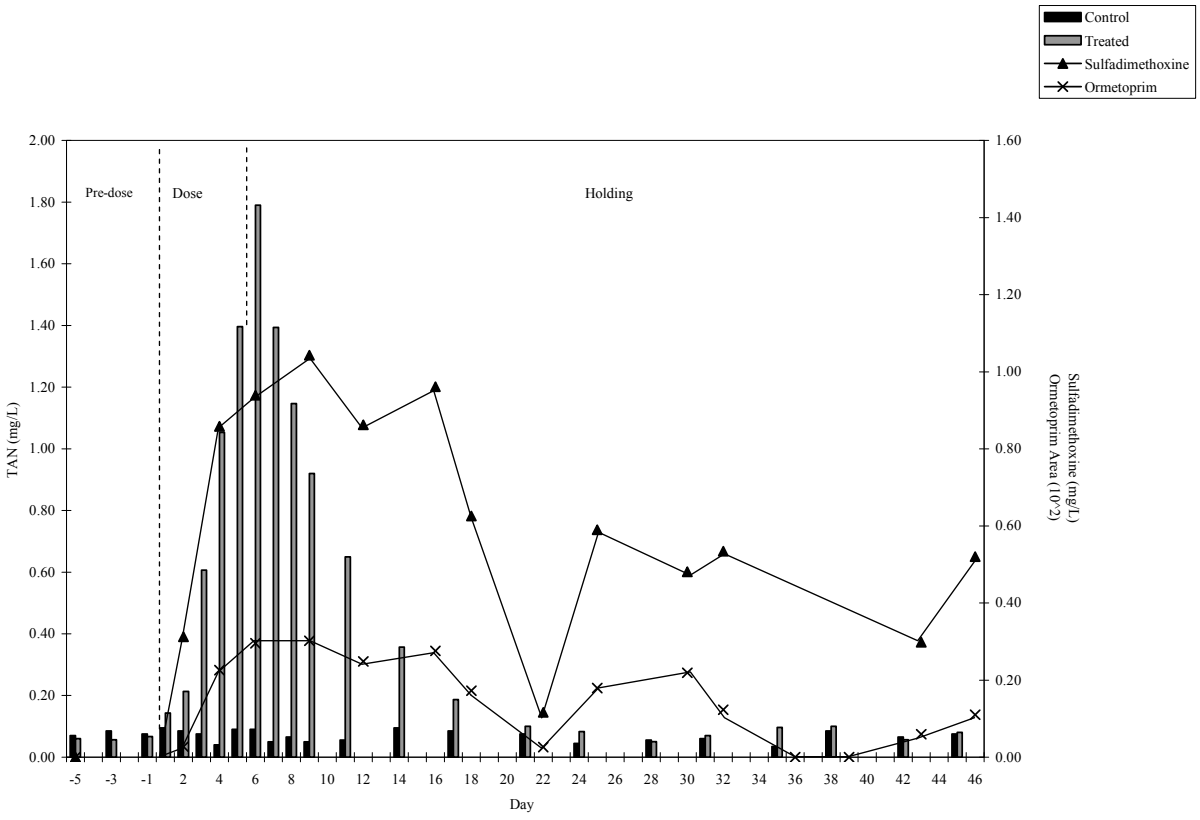

Figure 2-29. Total ammonia-nitrogen (TAN) response to treatment with Romet-30® medicated feed during the first trial

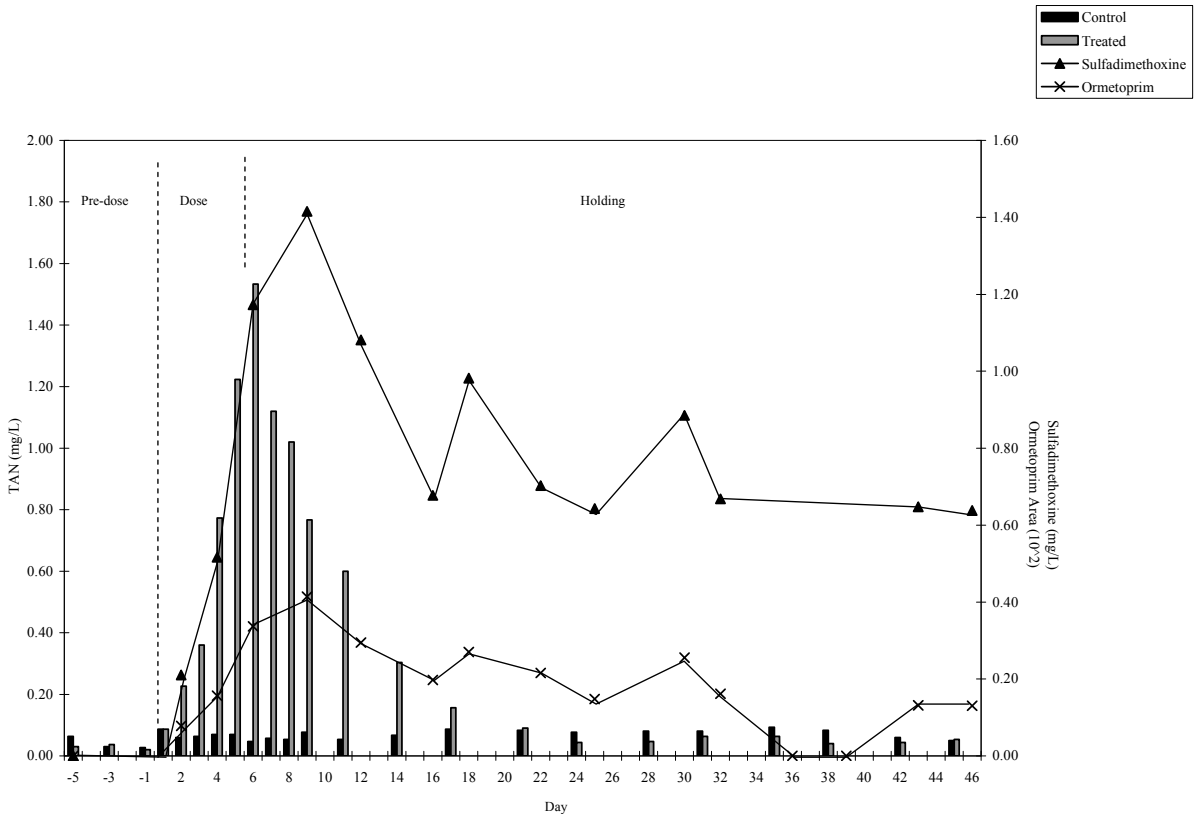

Figure 2-30. Total ammonia-nitrogen (TAN) response to treatment with Romet-30® medicated feed during the replicate trial

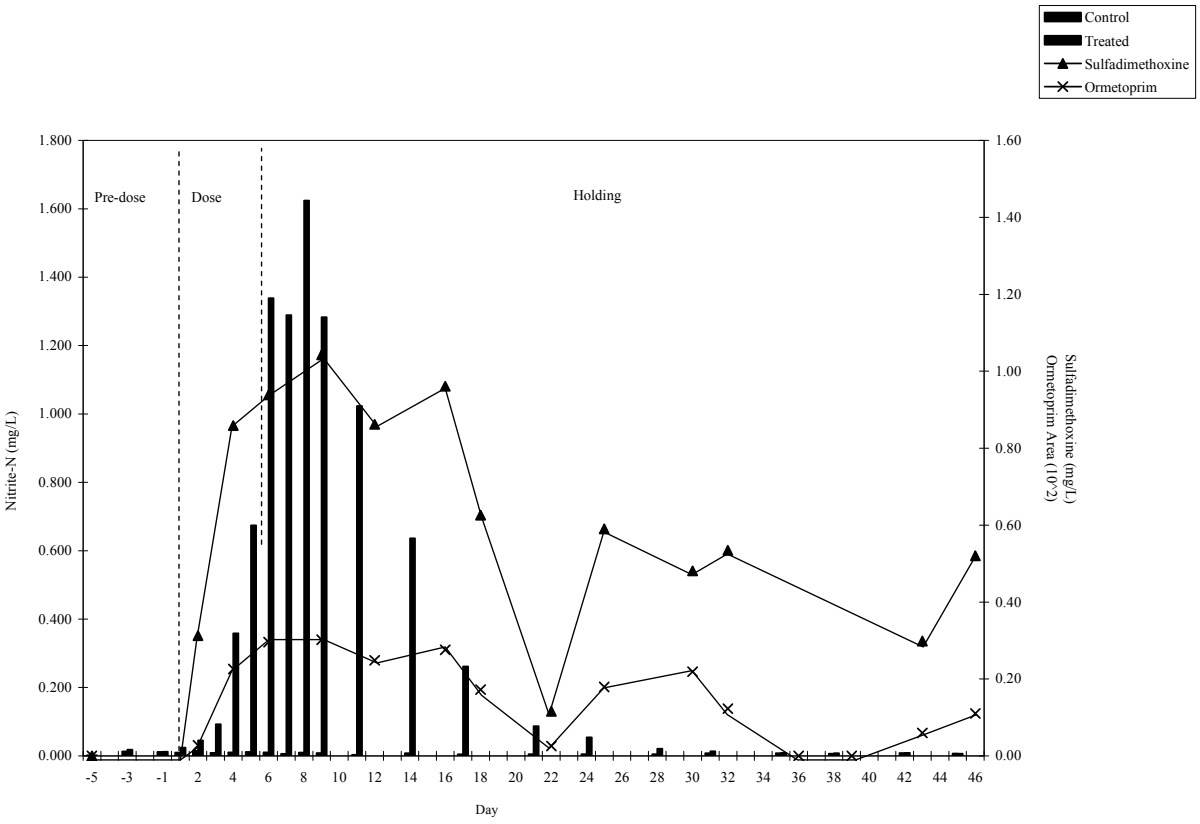

Figure 2-31. Nitrite-nitrogen response to treatment with Romet-30® medicated feed during the first trial

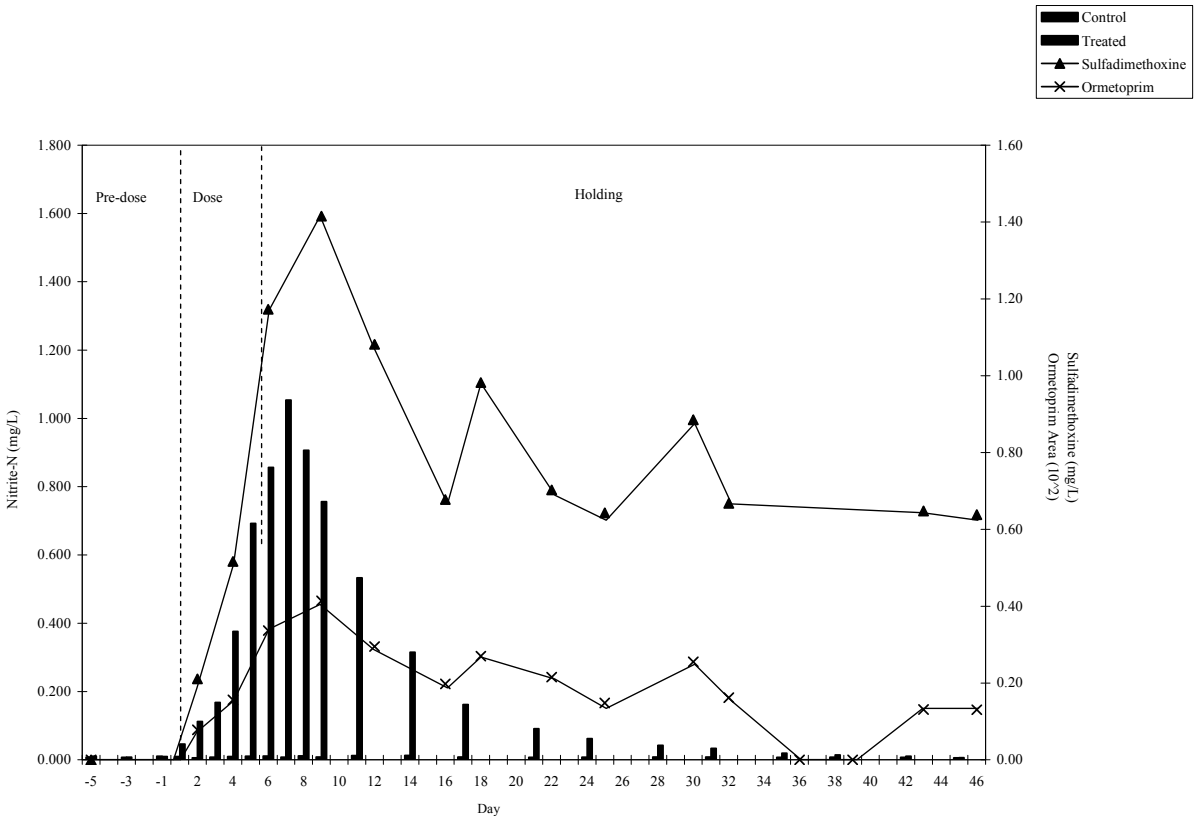

Figure 2-32. Nitrite-nitrogen response to treatment with Romet-30® medicated feed during the replicate trial

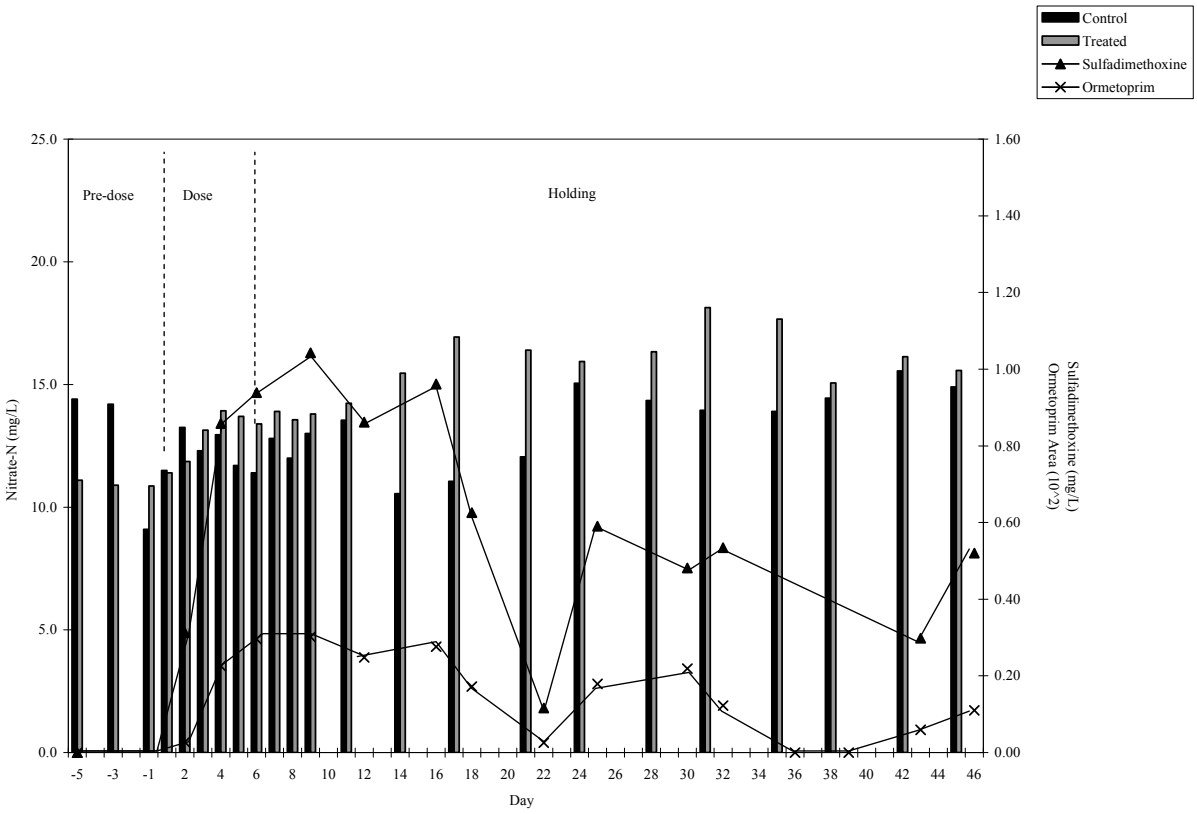

Figure 2-33. Nitrate-nitrogen response to treatment with Romet-30® medicated feed during the first trial

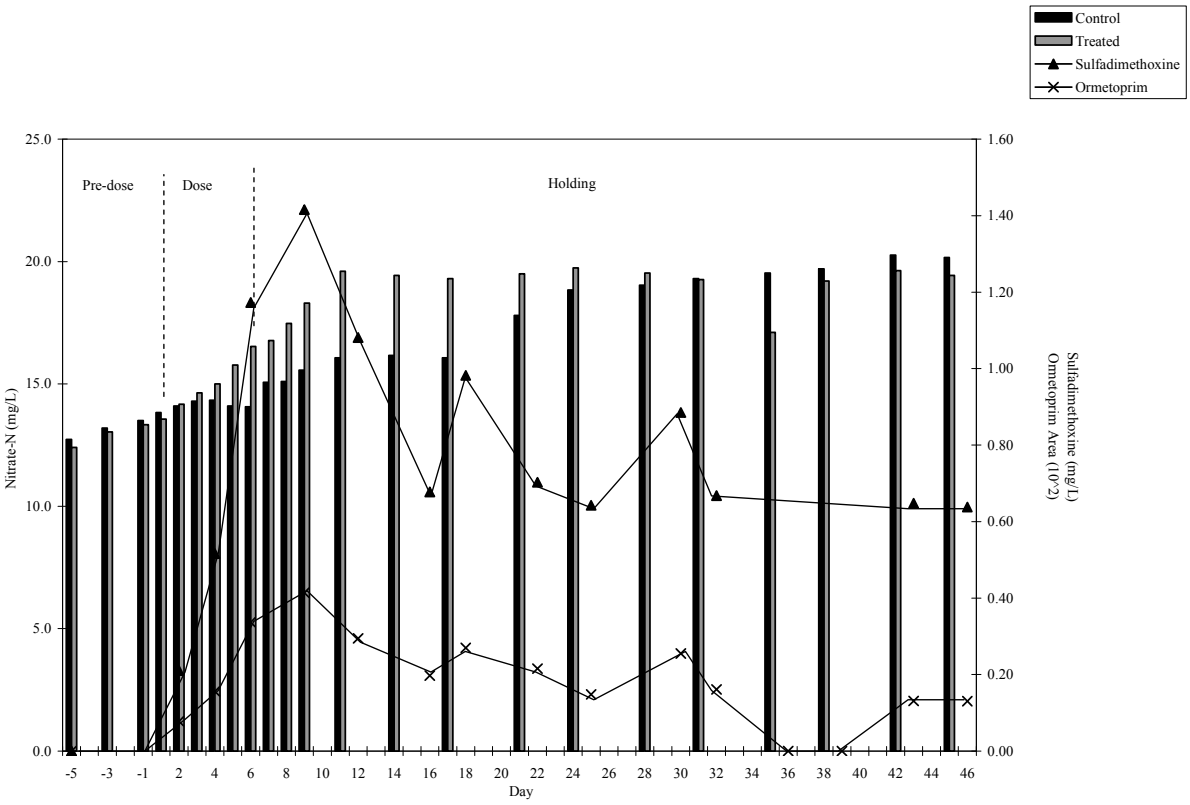

Figure 2-34. Nitrate-nitrogen response to treatment with Romet-30® medicated feed during the replicate trial

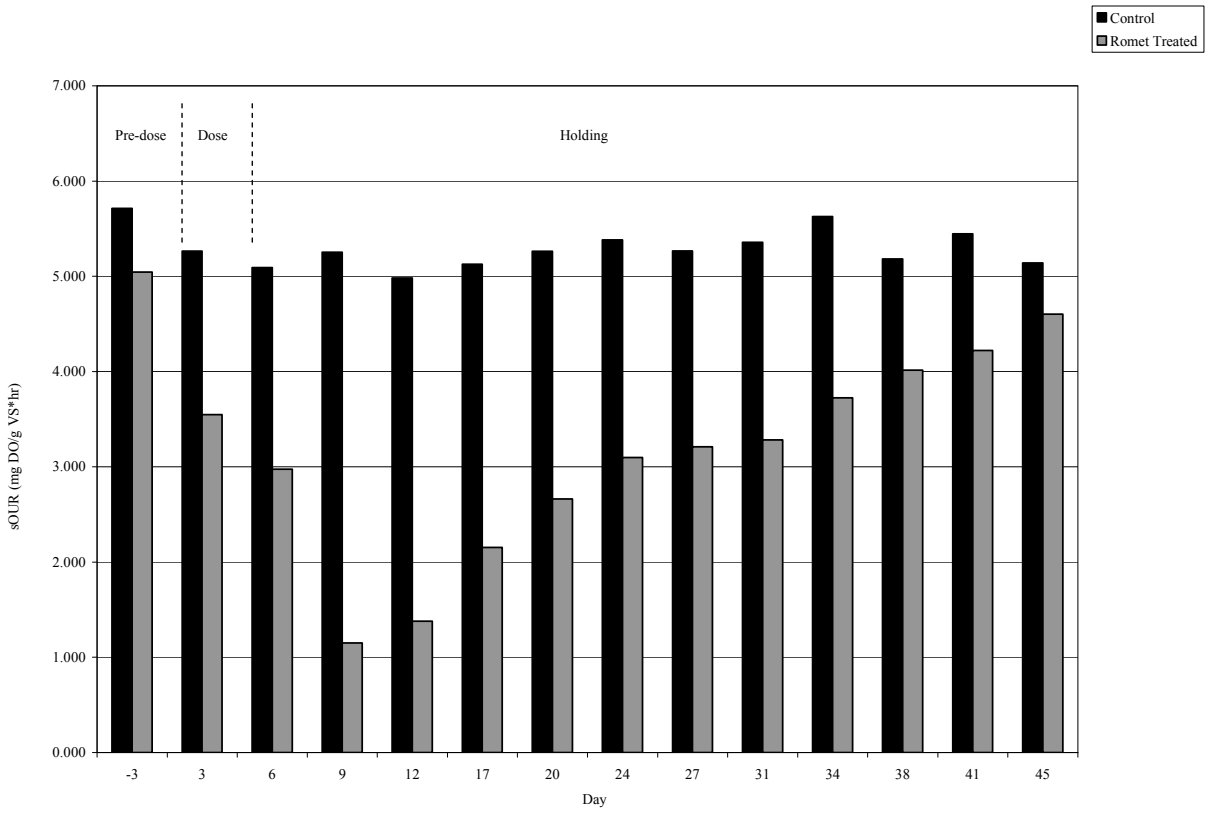

Figure 2-35. Specific oxygen uptake rate (sOUR) response to treatment with Romet-30® medicated feed during the first trial

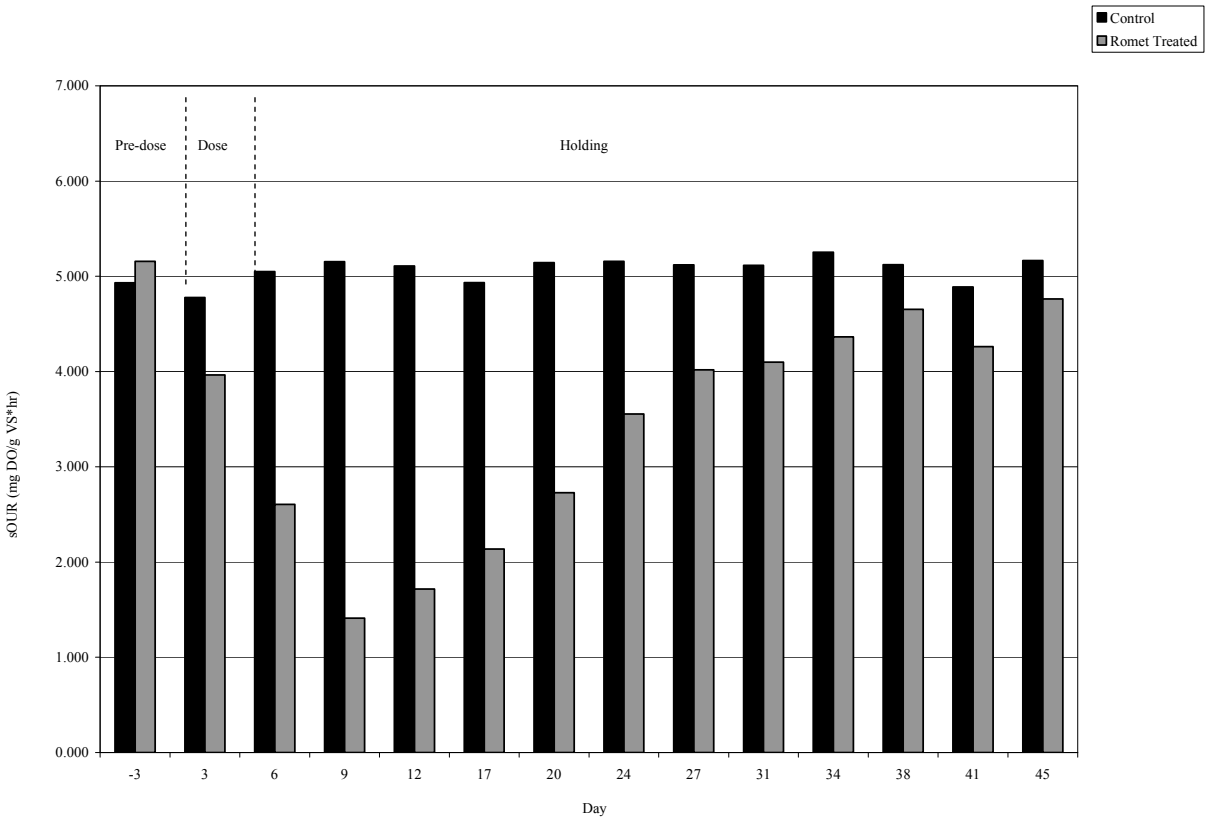

Figure 2-36. Specific oxygen uptake rate (sOUR) response to treatment with Romet-30® medicated feed during the replicate trial

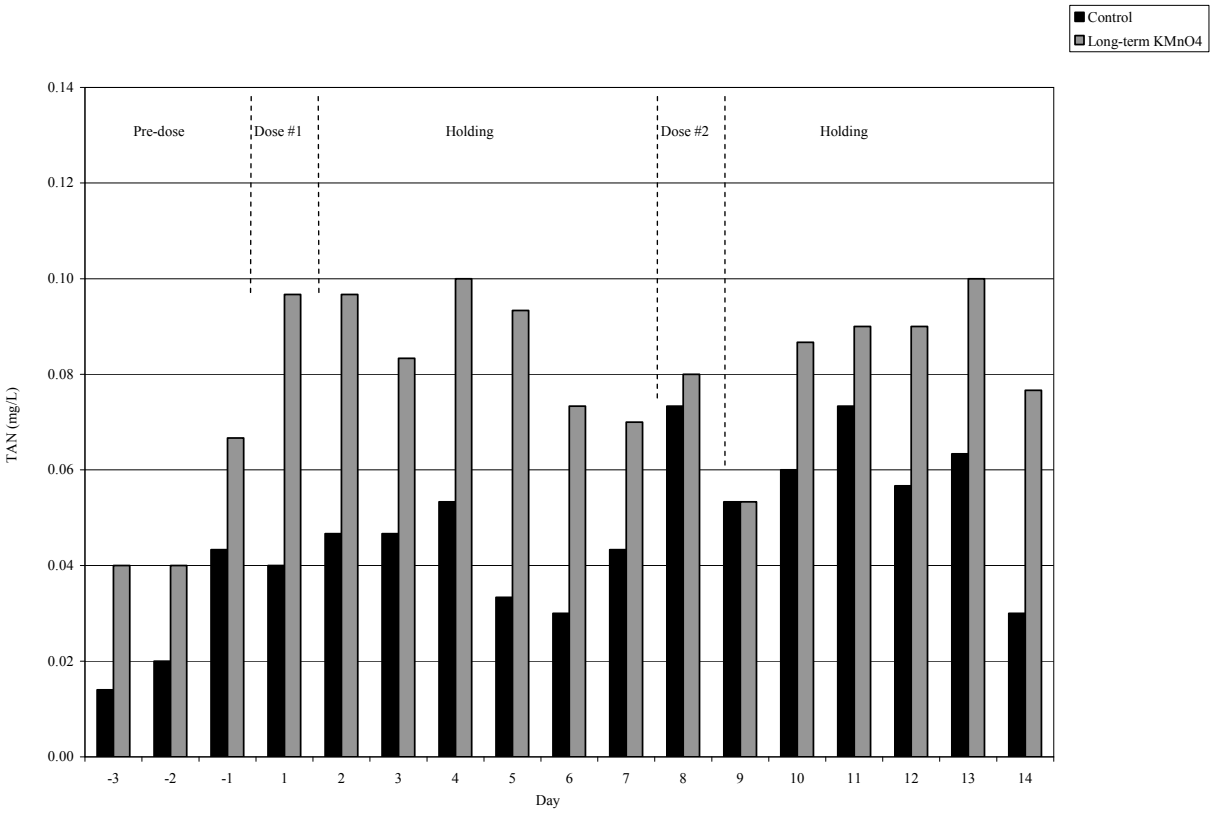

Figure 2-37. Total ammonia-nitrogen (TAN) response to treatment with long-term potassium permanganate during the first trial

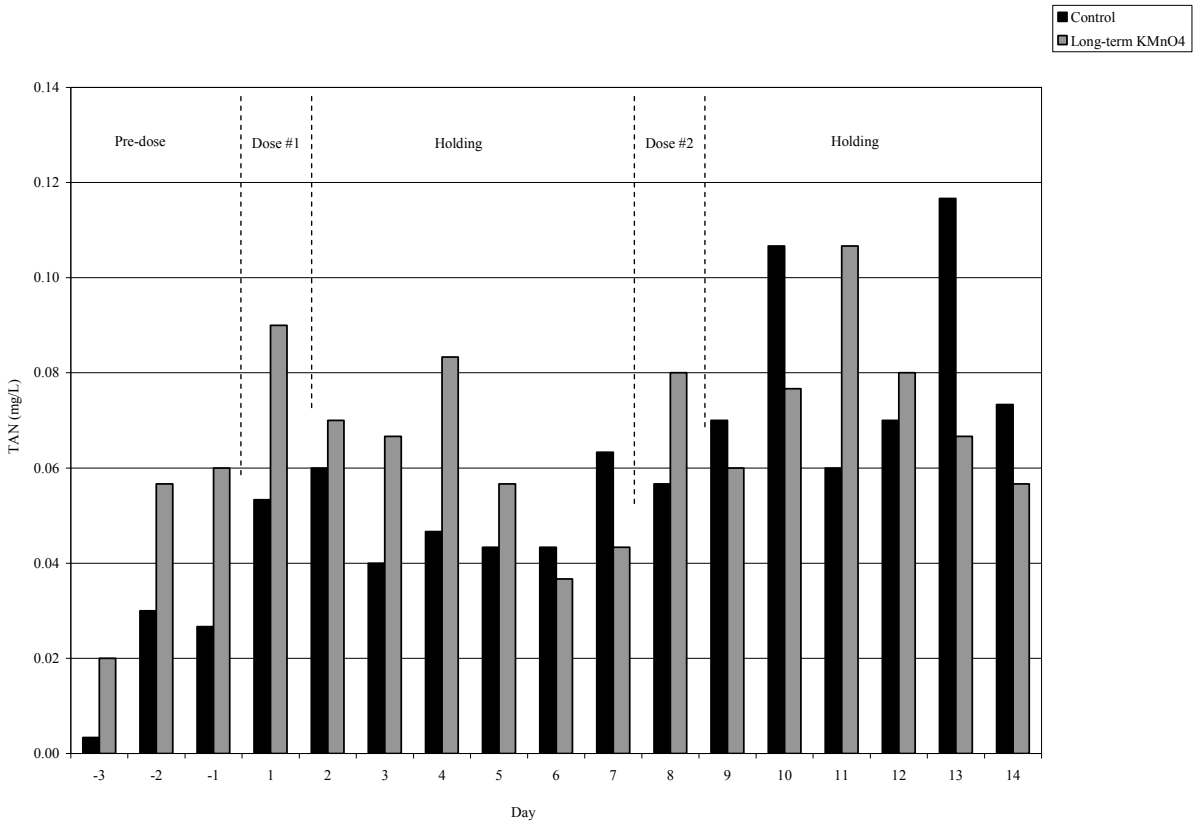

Figure 2-38. Total ammonia-nitrogen (TAN) response to treatment with long-term potassium permanganate during the replicate trial

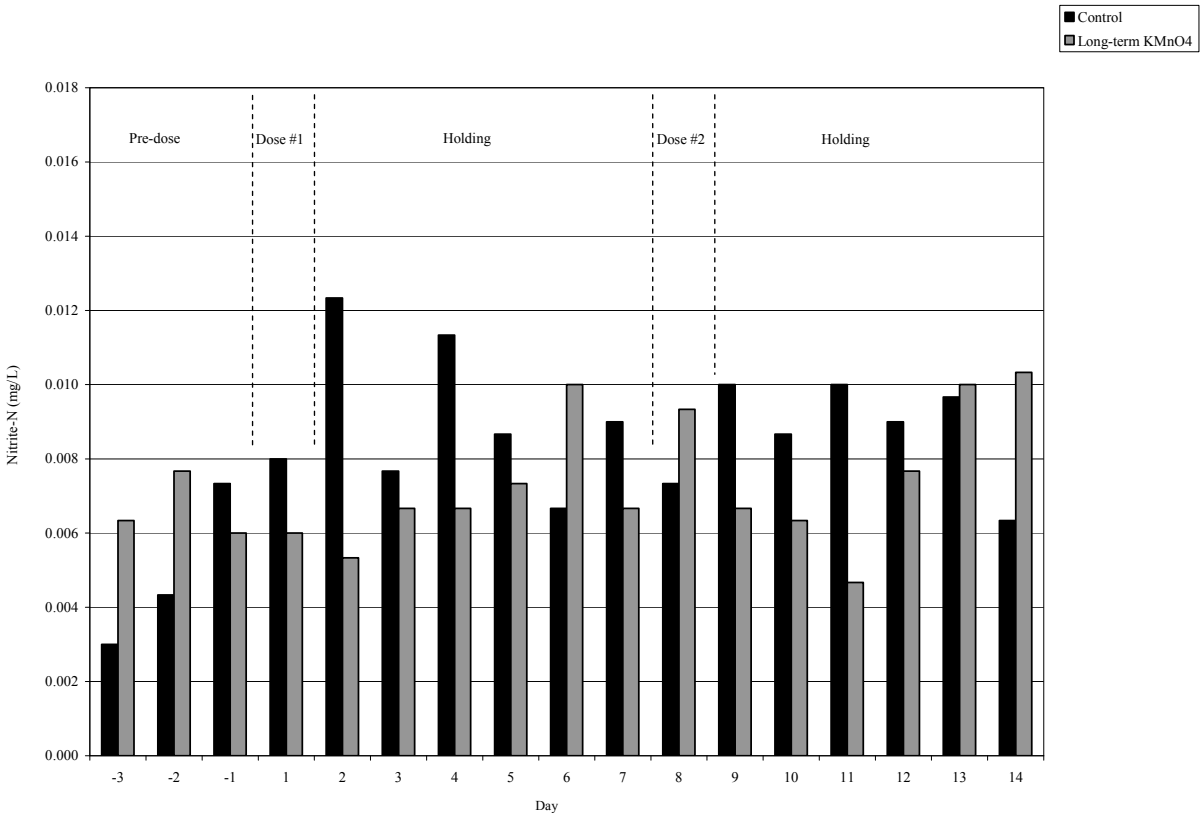

Figure 2-39. Nitrite-nitrogen response to treatment with long-term potassium permanganate during the first trial

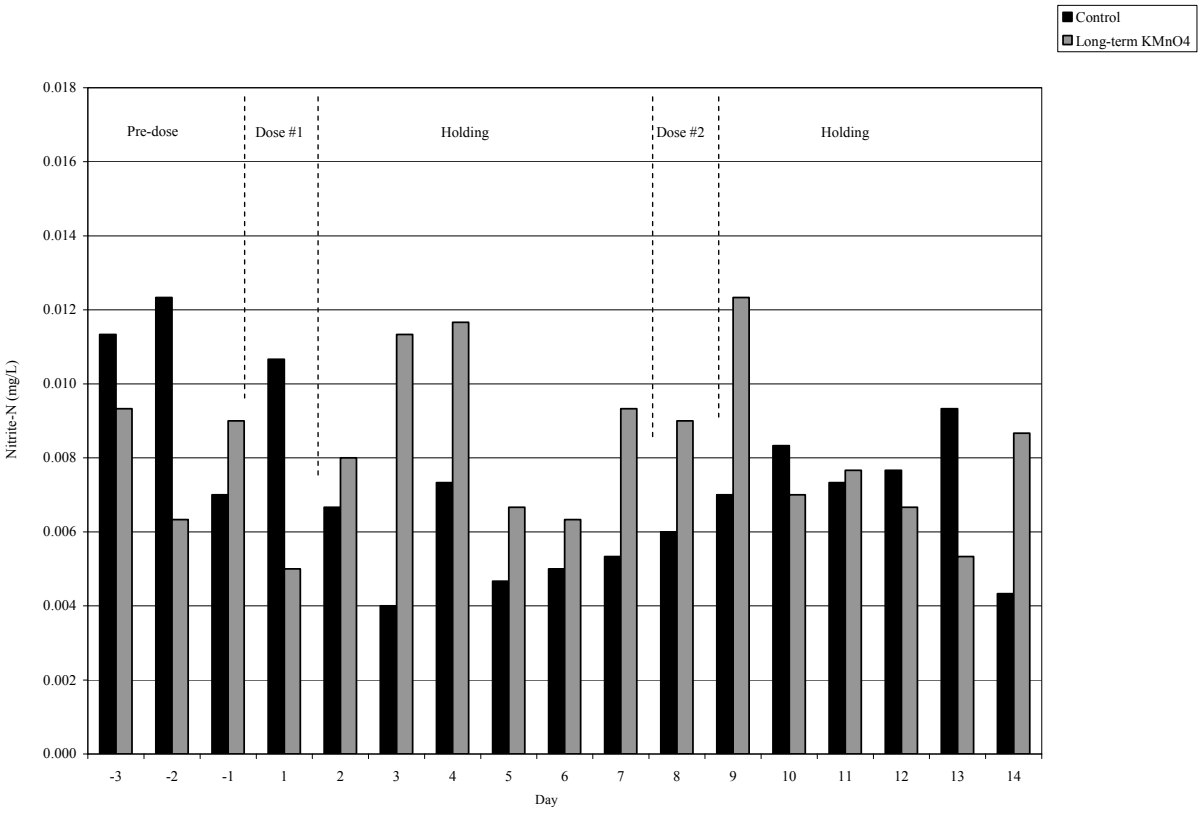

Figure 2-40. Nitrite-nitrogen response to treatment with long-term potassium permanganate during the replicate trial.

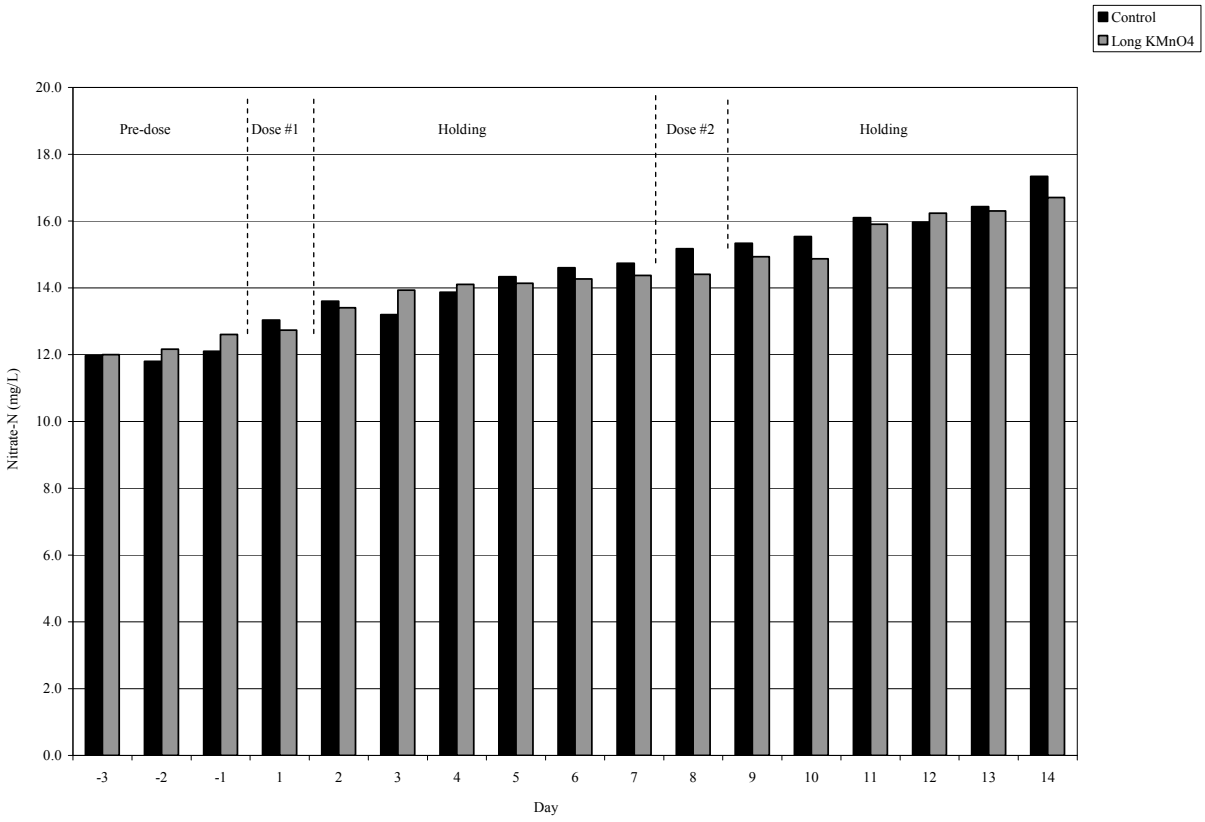

Figure 2-41. Nitrate-nitrogen response to treatment with long-term potassium permanganate during the first trial.

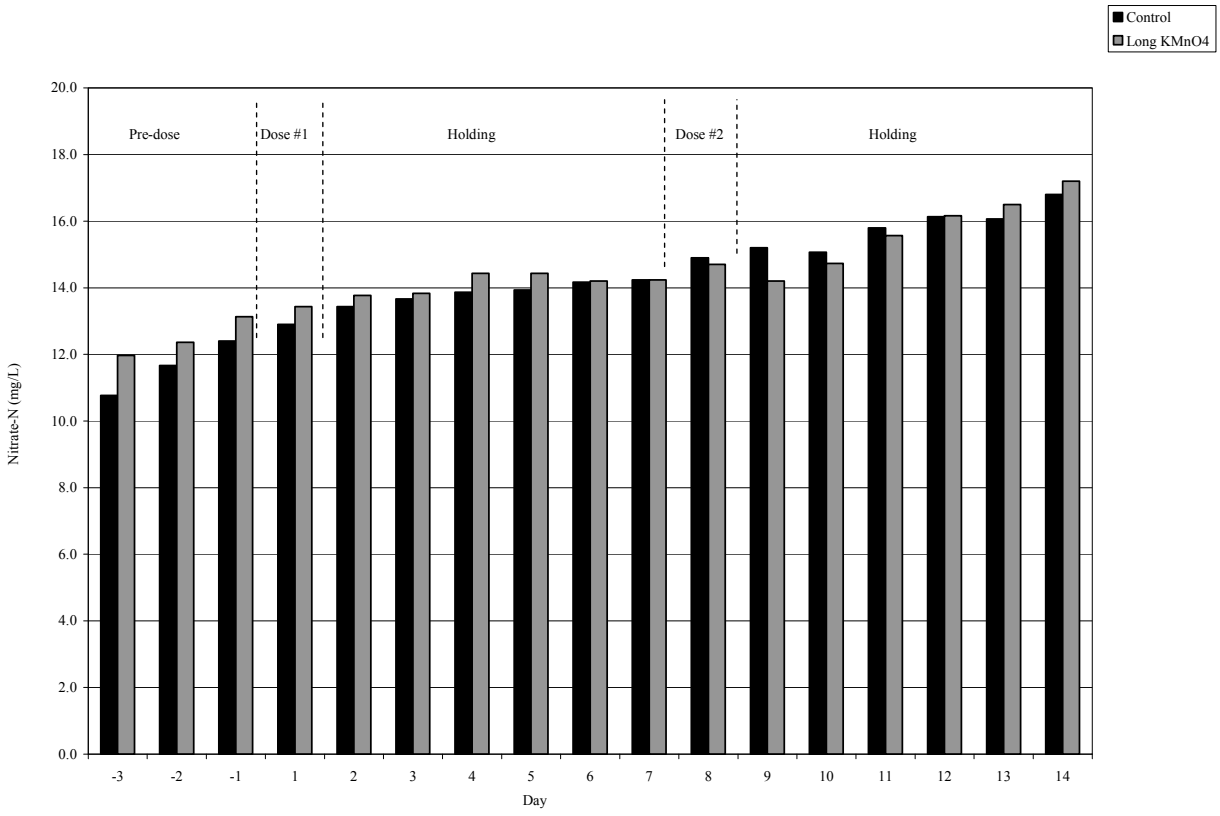

Figure 2-42. Nitrate-nitrogen response to treatment with long-term potassium permanganate during the replicate trials

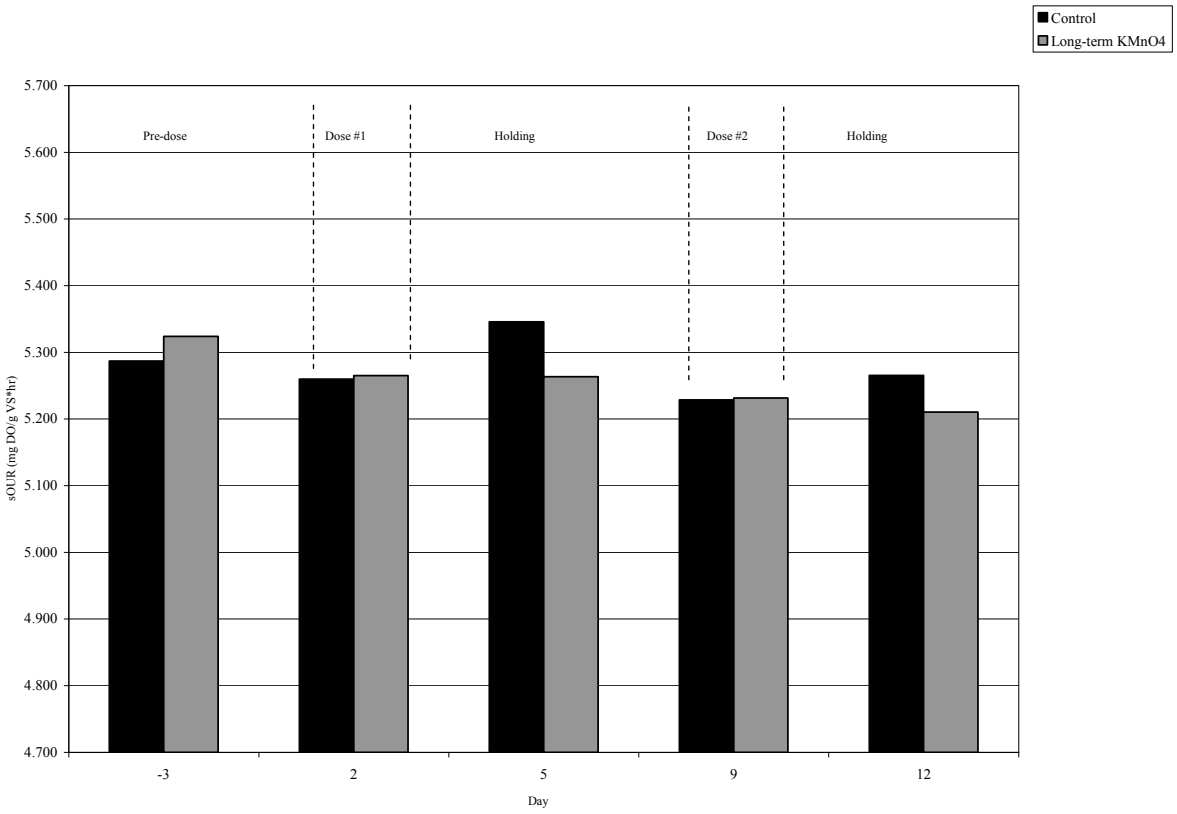

Figure 2-43. Specific oxygen uptake rate (sOUR) response to treatment with long-term potassium permanganate during the first trial

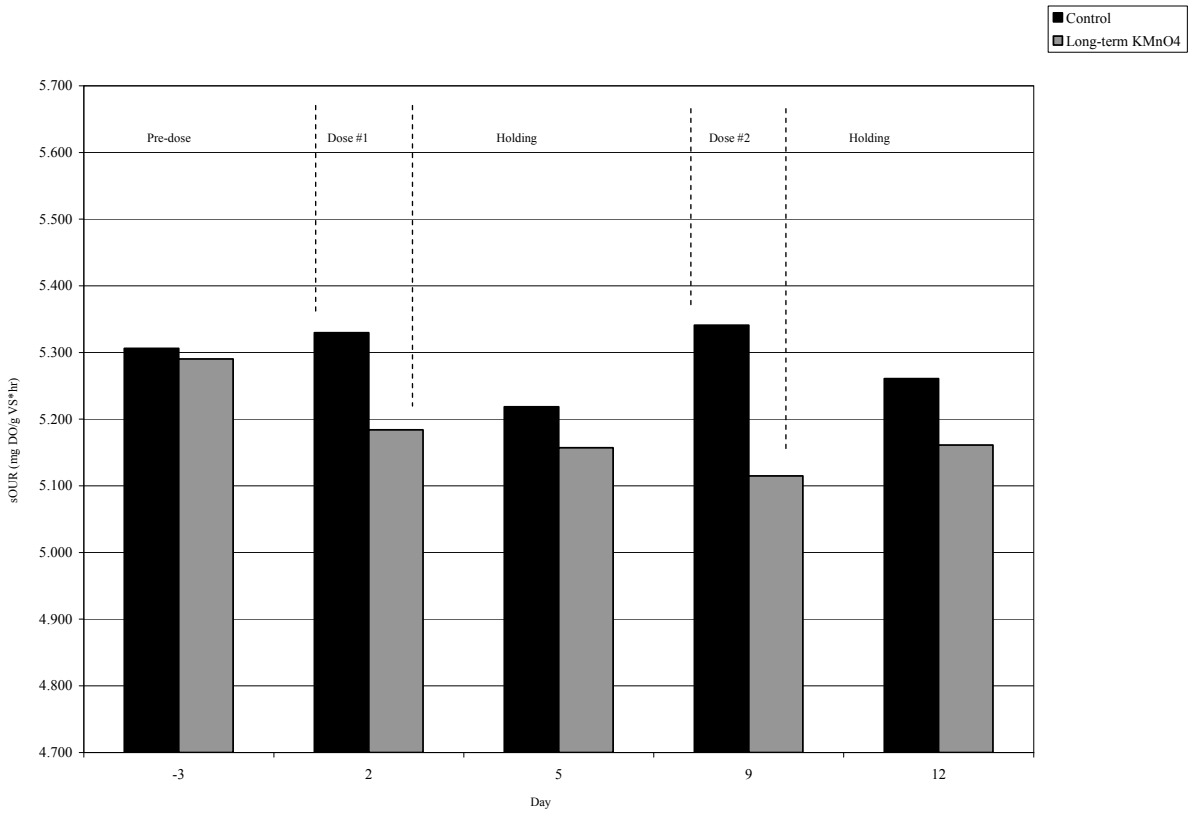

Figure 2-44. Specific oxygen uptake rate (sOUR) response to treatment with long-term potassium permanganate during the replicate trial

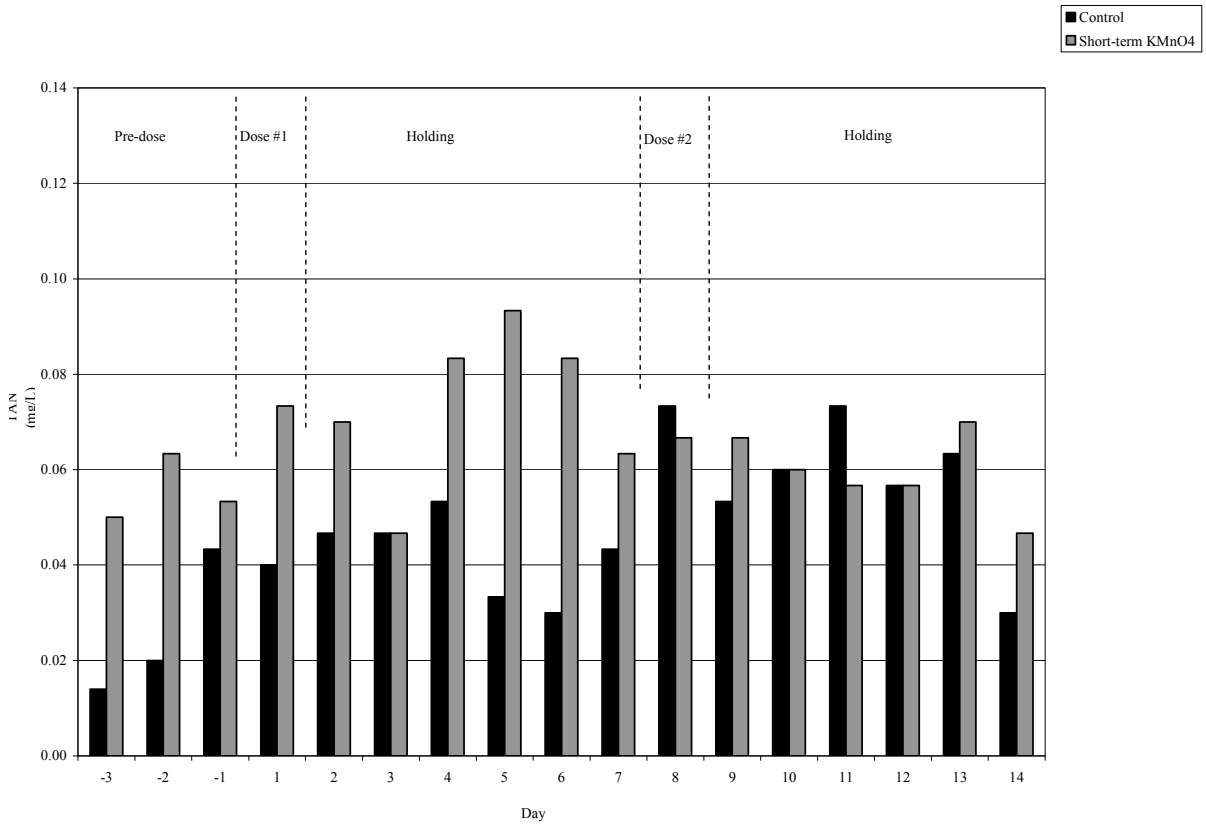

Figure 2-45. Total ammonia-nitrogen (TAN) response to treatment with short-term potassium permanganate during the first trial

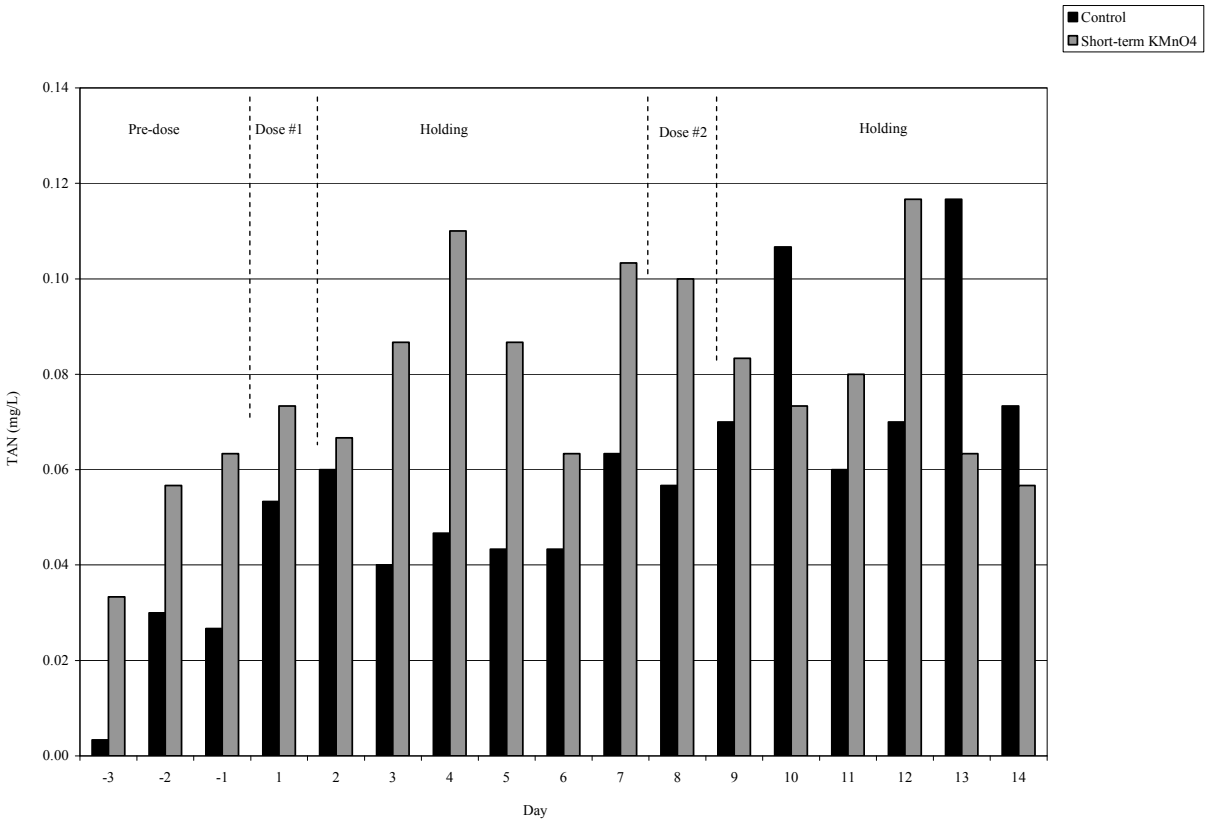

Figure 2-46. Total ammonia-nitrogen (TAN) response to treatment with short-term potassium permanganate during the replicate trial

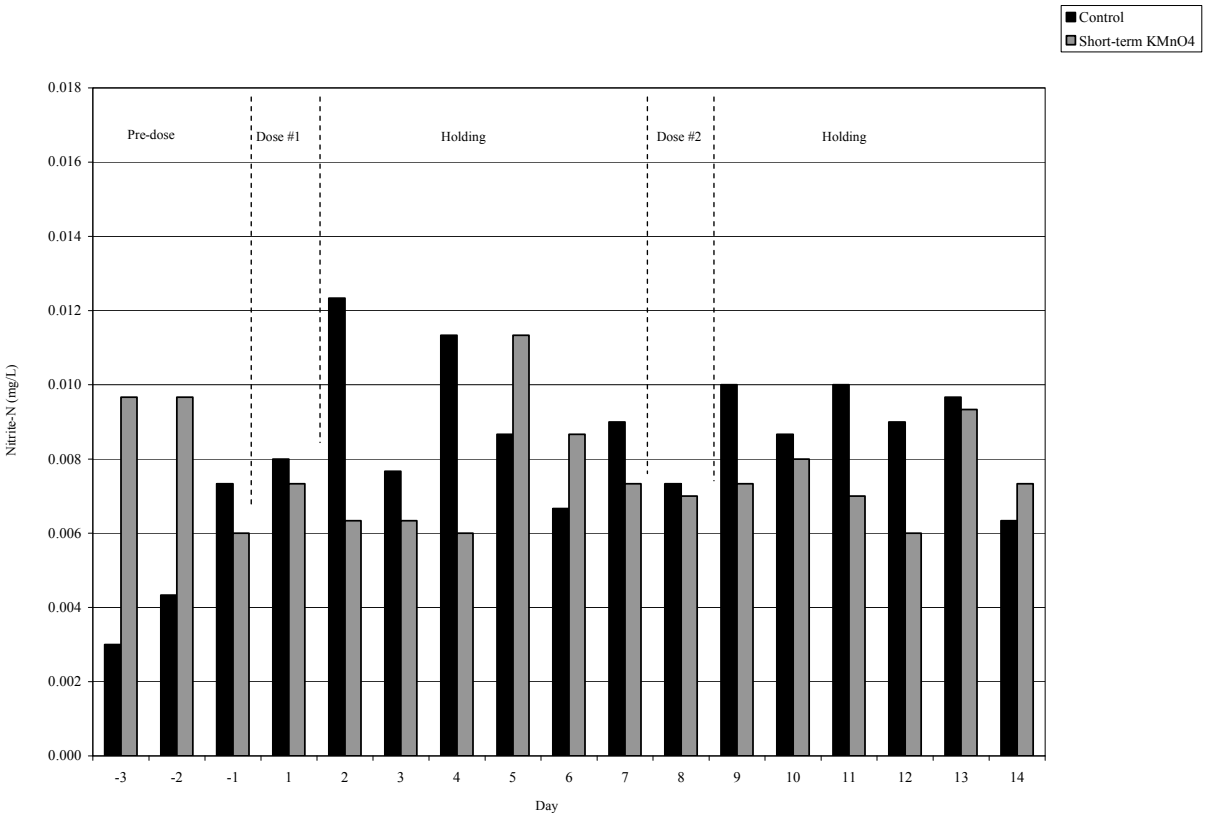

Figure 2-47. Nitrite-nitrogen response to treatment with short-term potassium permanganate during the first trial

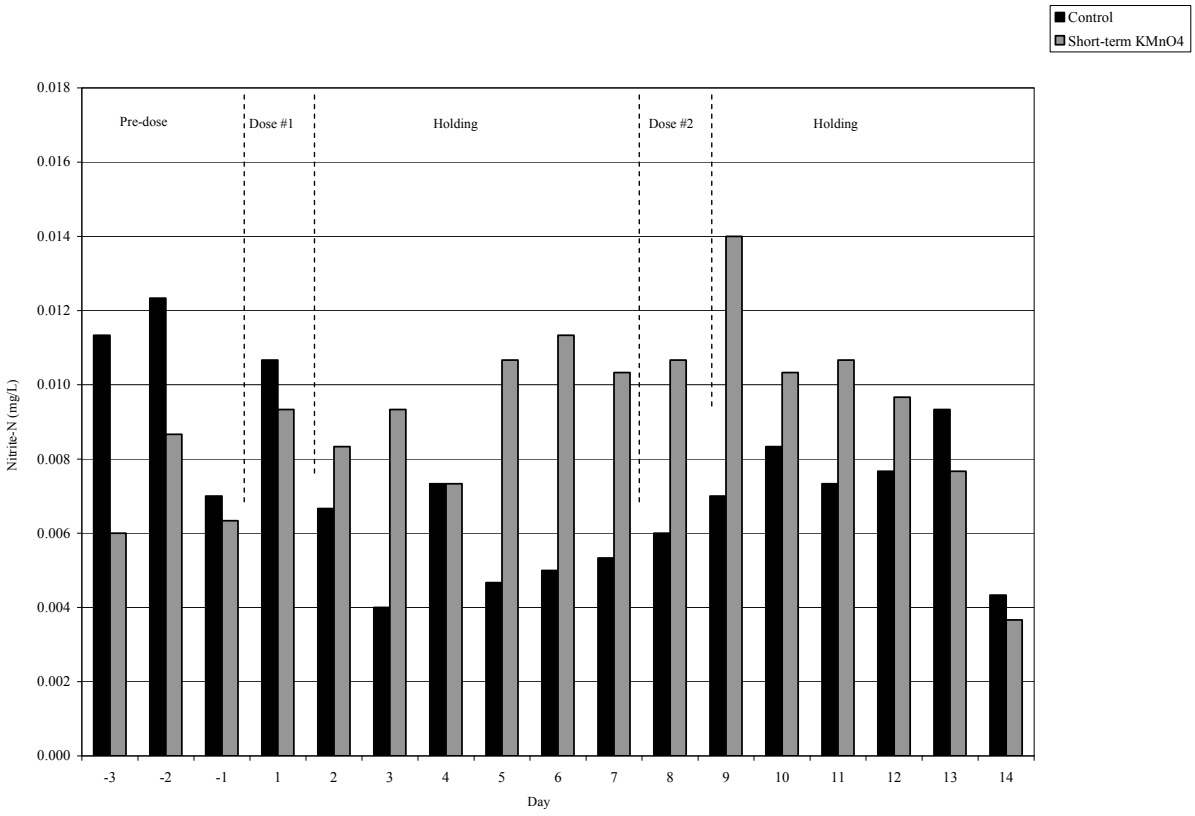

Figure 2-48. Nitrite-nitrogen response to treatment with short-term potassium permanganate during the replicate trial

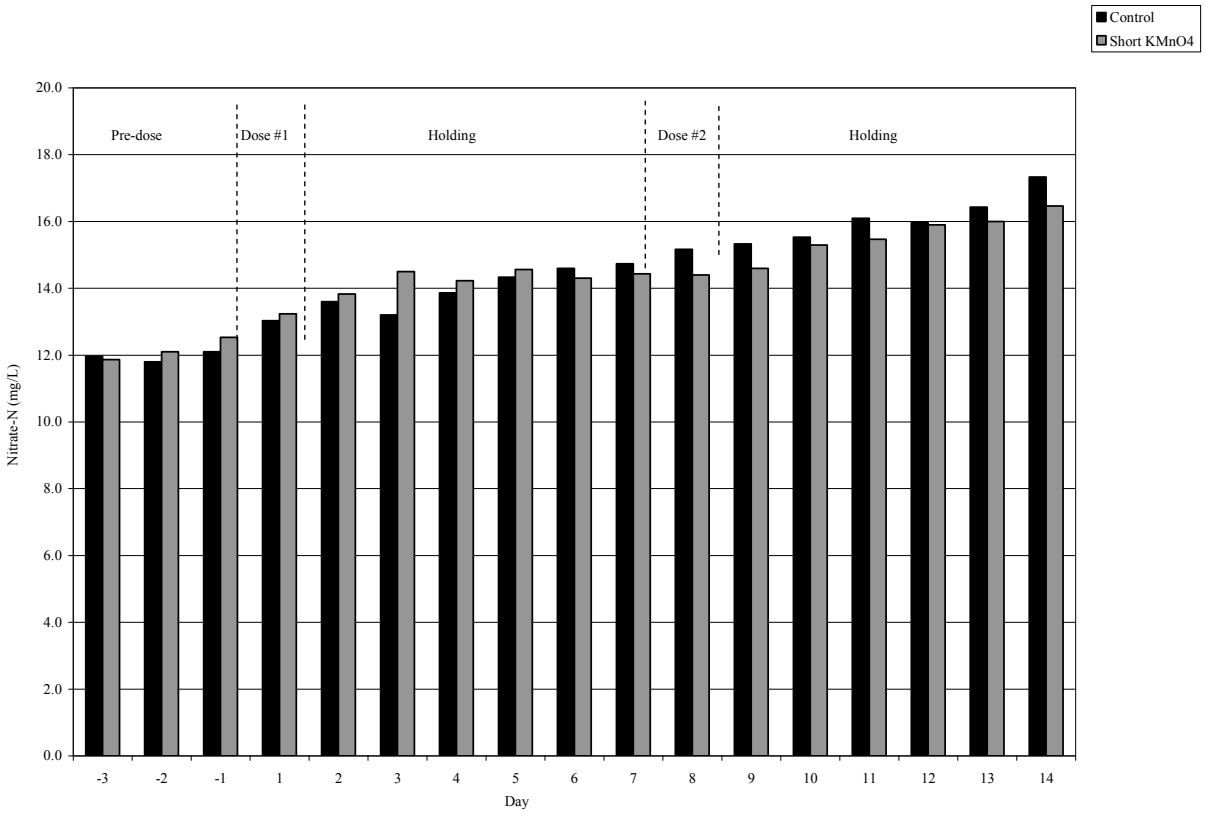

Figure 2-49. Nitrate-nitrogen response to treatment with short-term potassium permanganate during the first trial

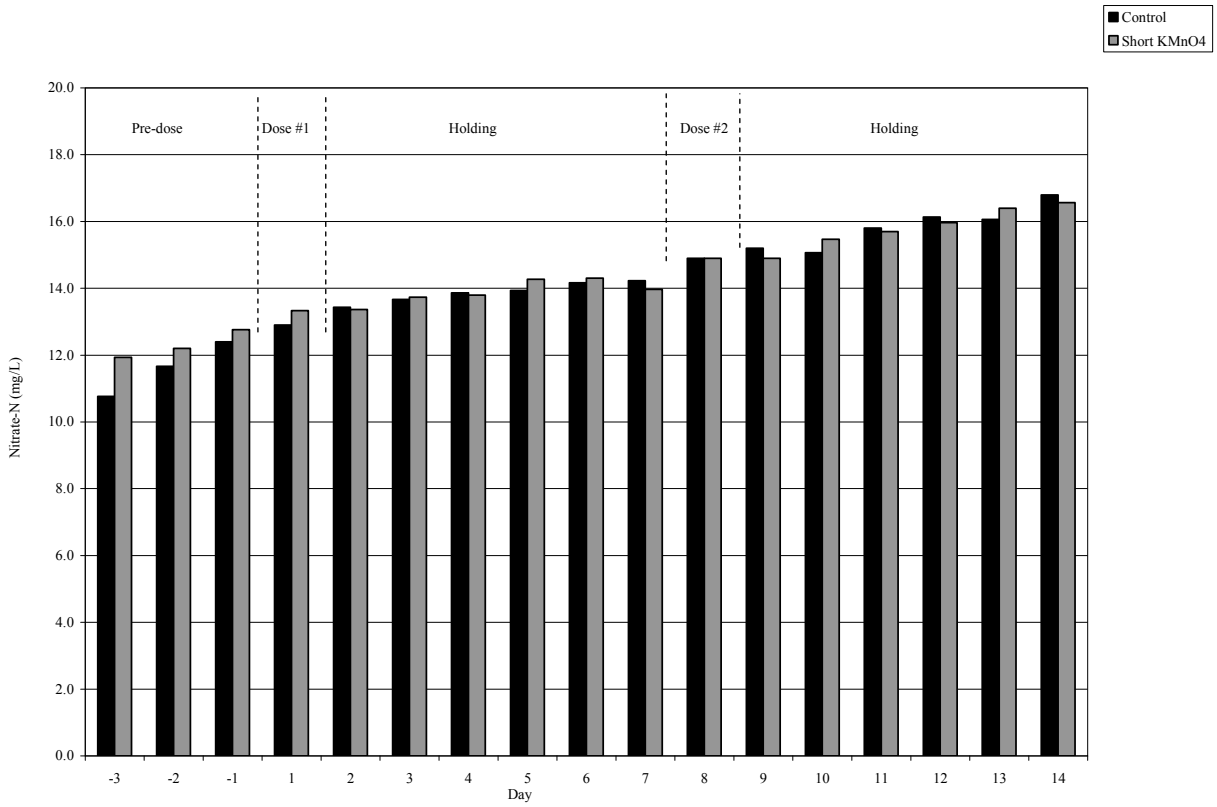

Figure 2-50. Nitrate-nitrogen response to treatment with short-term potassium permanganate during the replicate trial

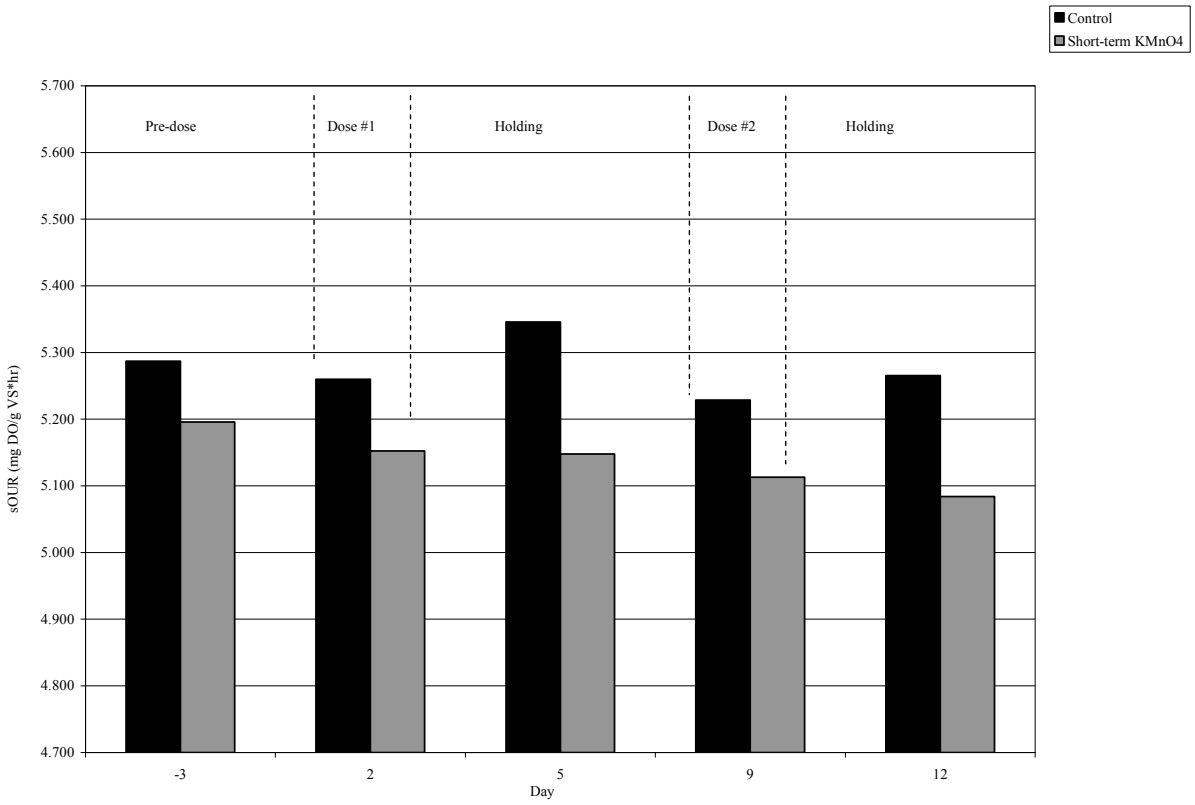

Figure 2-51. Specific oxygen uptake rate (sOUR) response to treatment with short-term potassium permanganate during the first trial

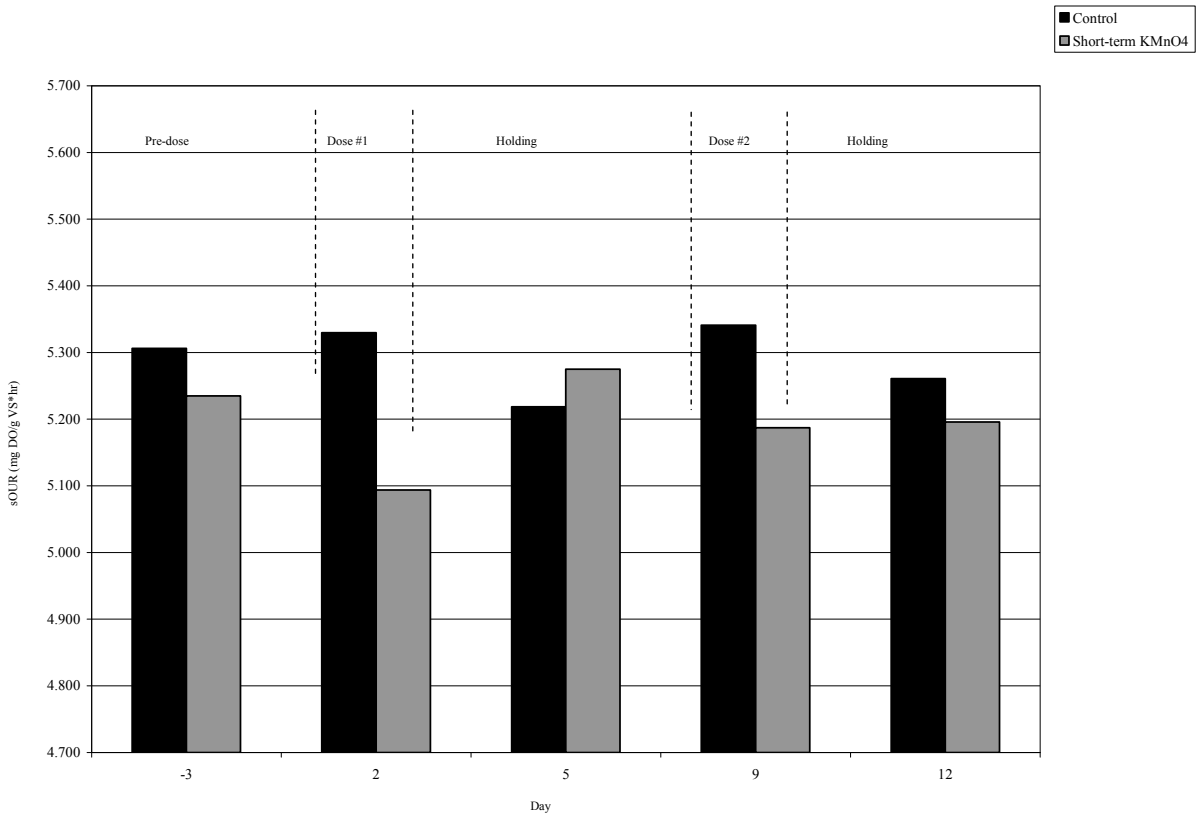

Figure 2-52. Specific oxygen uptake rate (sOUR) response to treatment with short-term potassium permanganate during the replicate trial

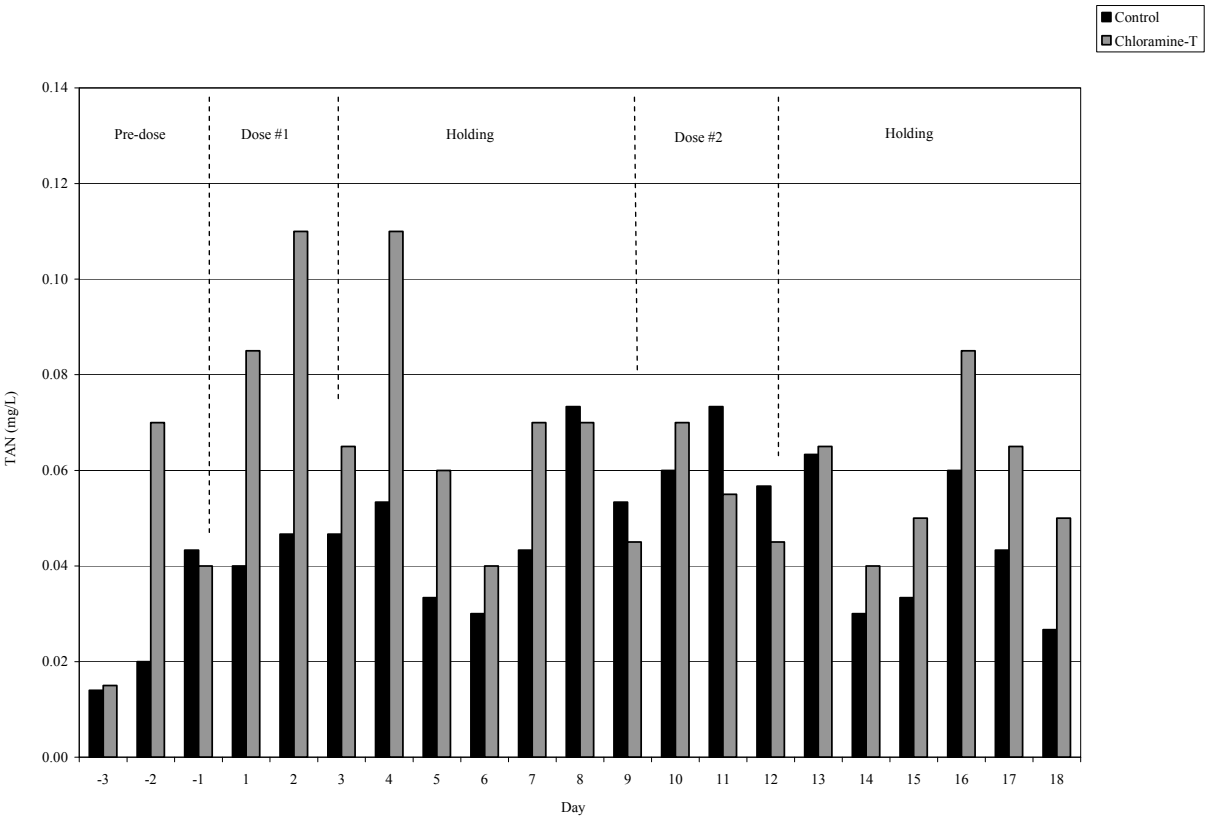

Figure 2-53. Total ammonia-nitrogen (TAN) response to treatment with Chloramine-T during the first trial

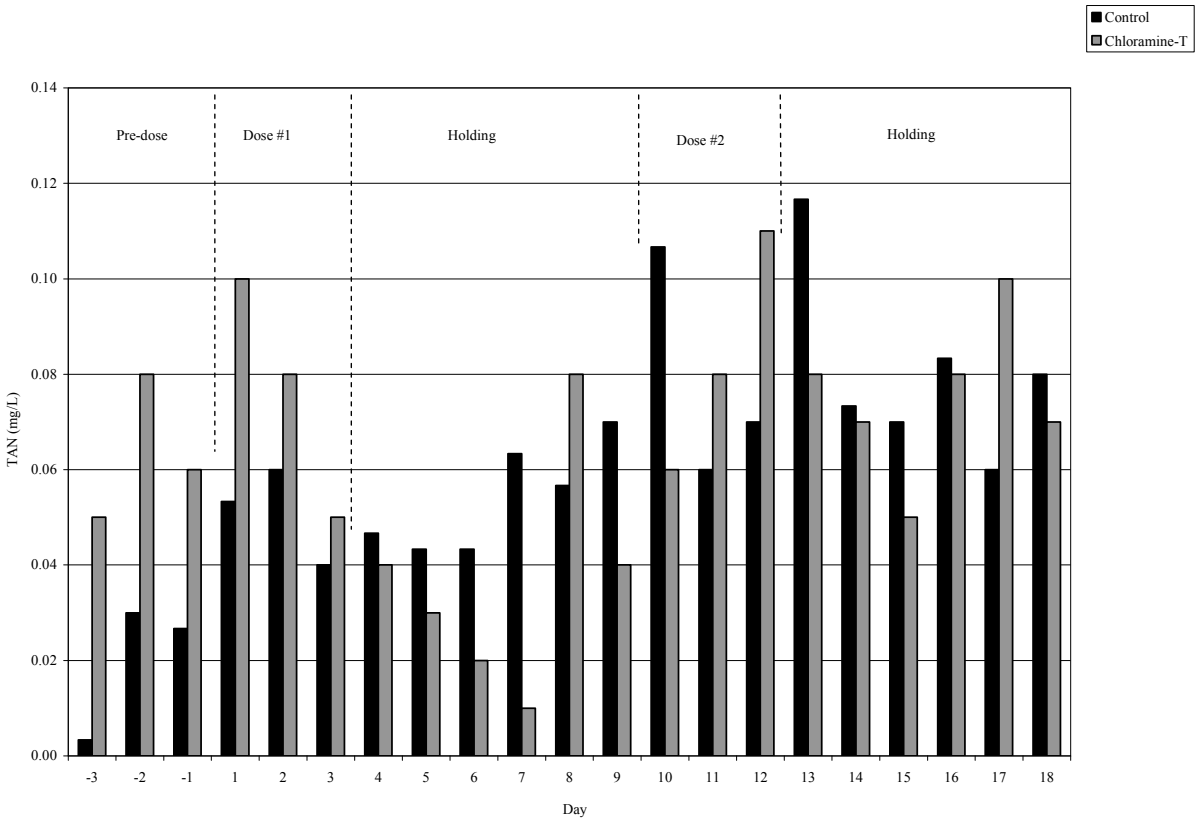

Figure 2-54. Total ammonia-nitrogen (TAN) response to treatment with Chloramine-T during the replicate trial

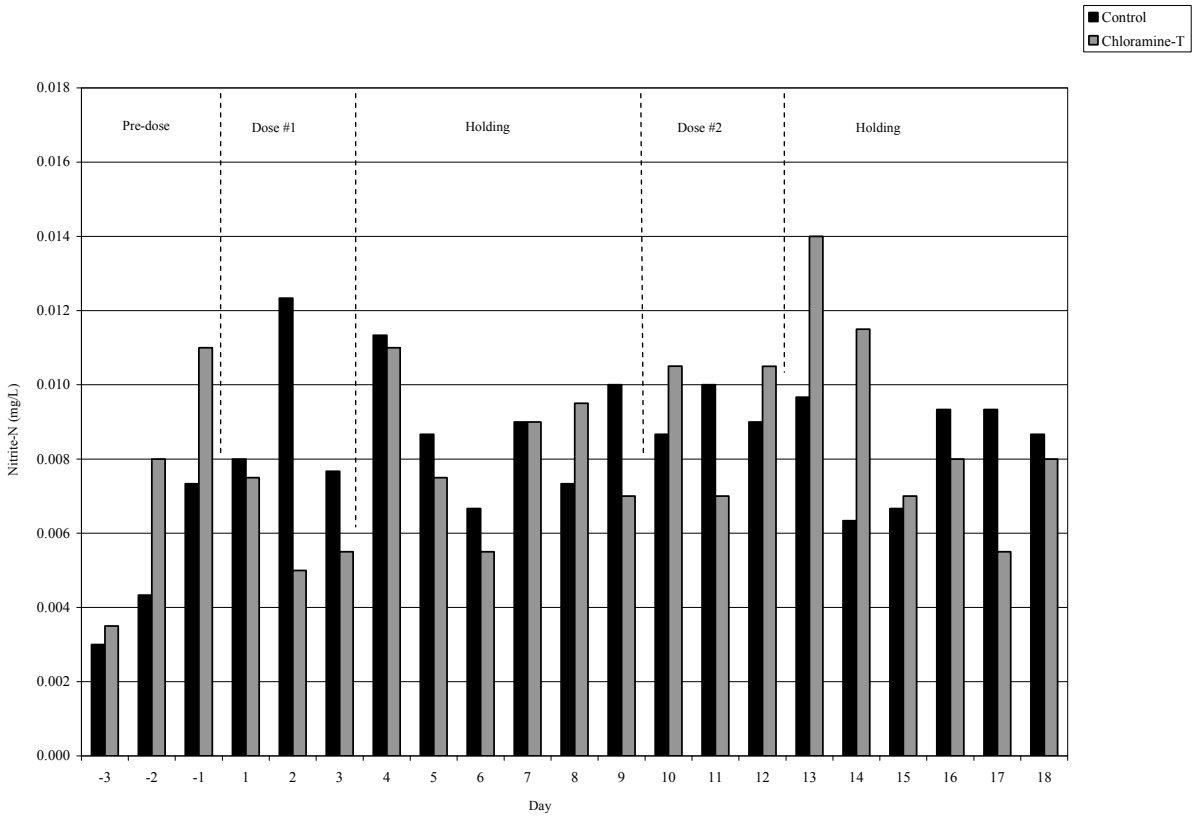

Figure 2-55. Nitrite-nitrogen response to treatment with Chloramine-T during the first trial

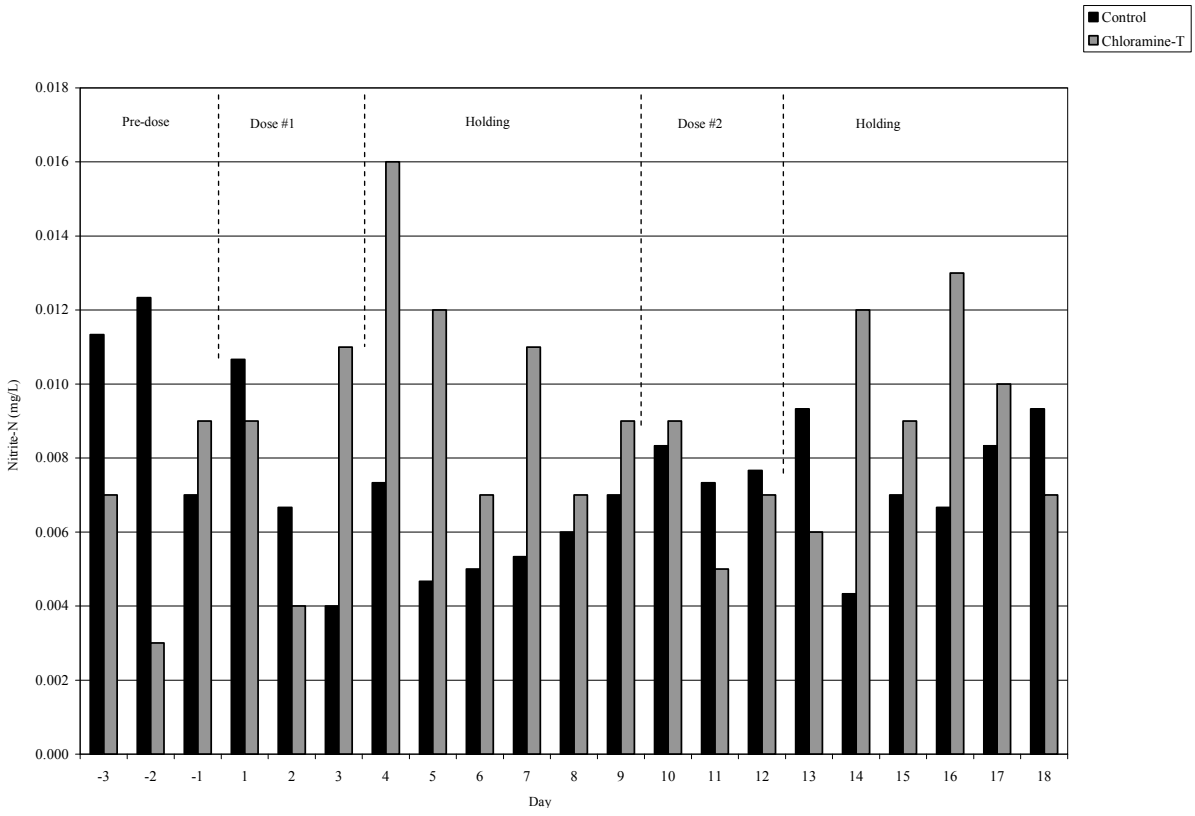

Figure 2-56. Nitrite-nitrogen response to treatment with Chloramine-T during the replicate trial

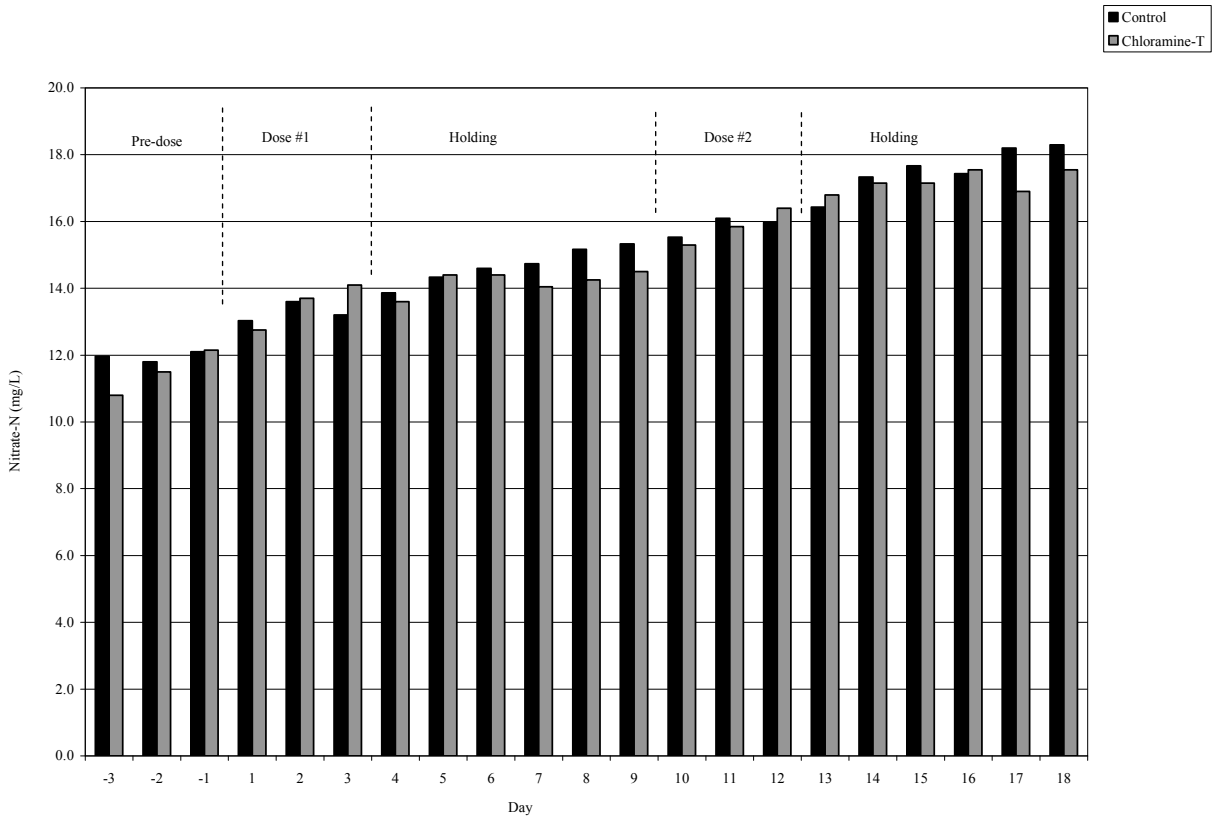

Figure 2-57. Nitrate-nitrogen response to treatment with Chloramine-T during the first trial

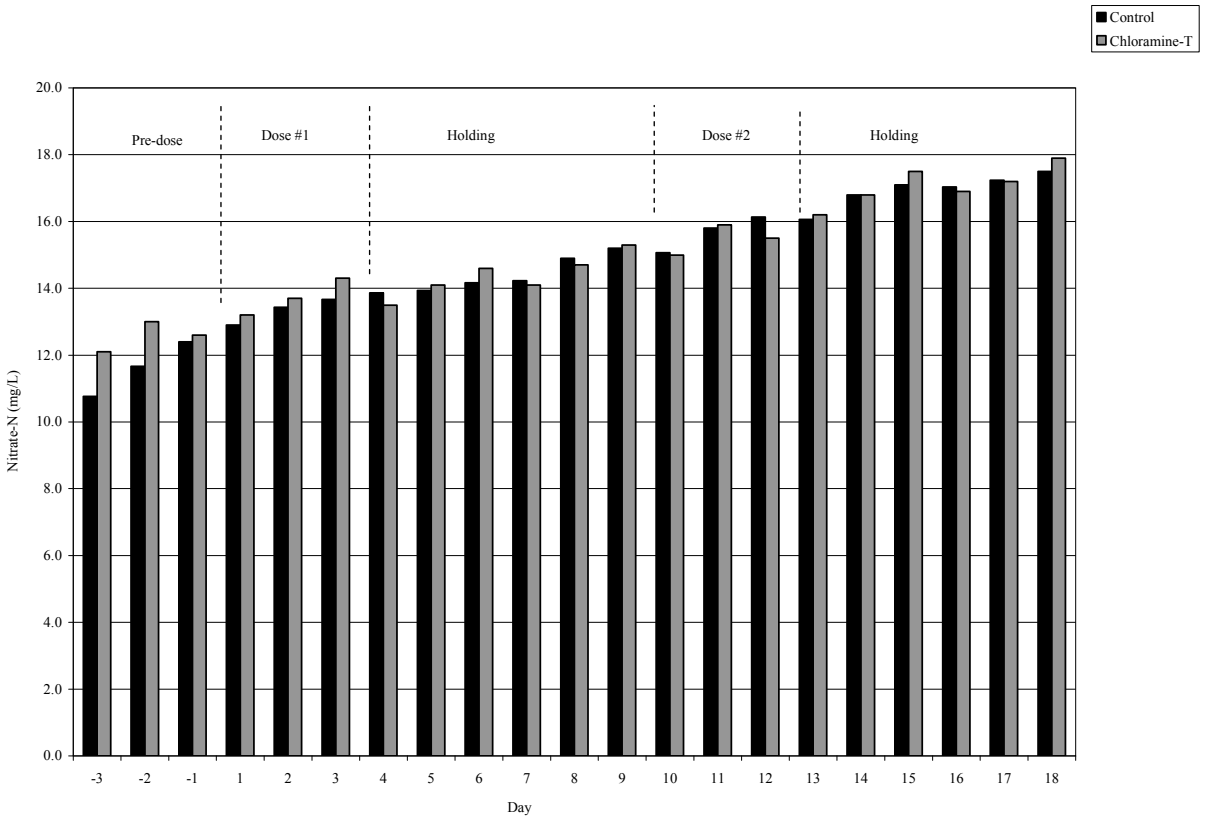

Figure 2-58. Nitrate-nitrogen response to treatment with Chloramine-T during the replicate trial

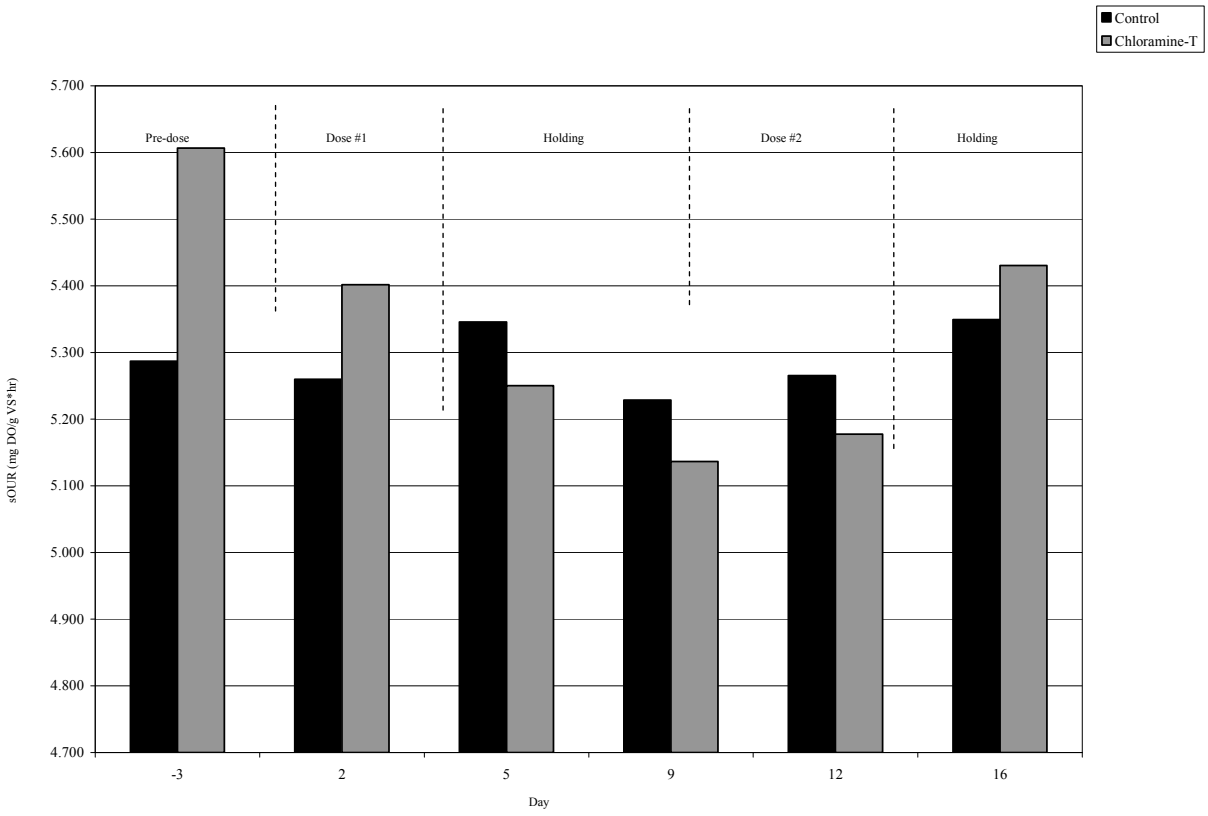

Figure 2-59. Specific oxygen uptake rate (sOUR) response to treatment with Chloramine-T during the first trial

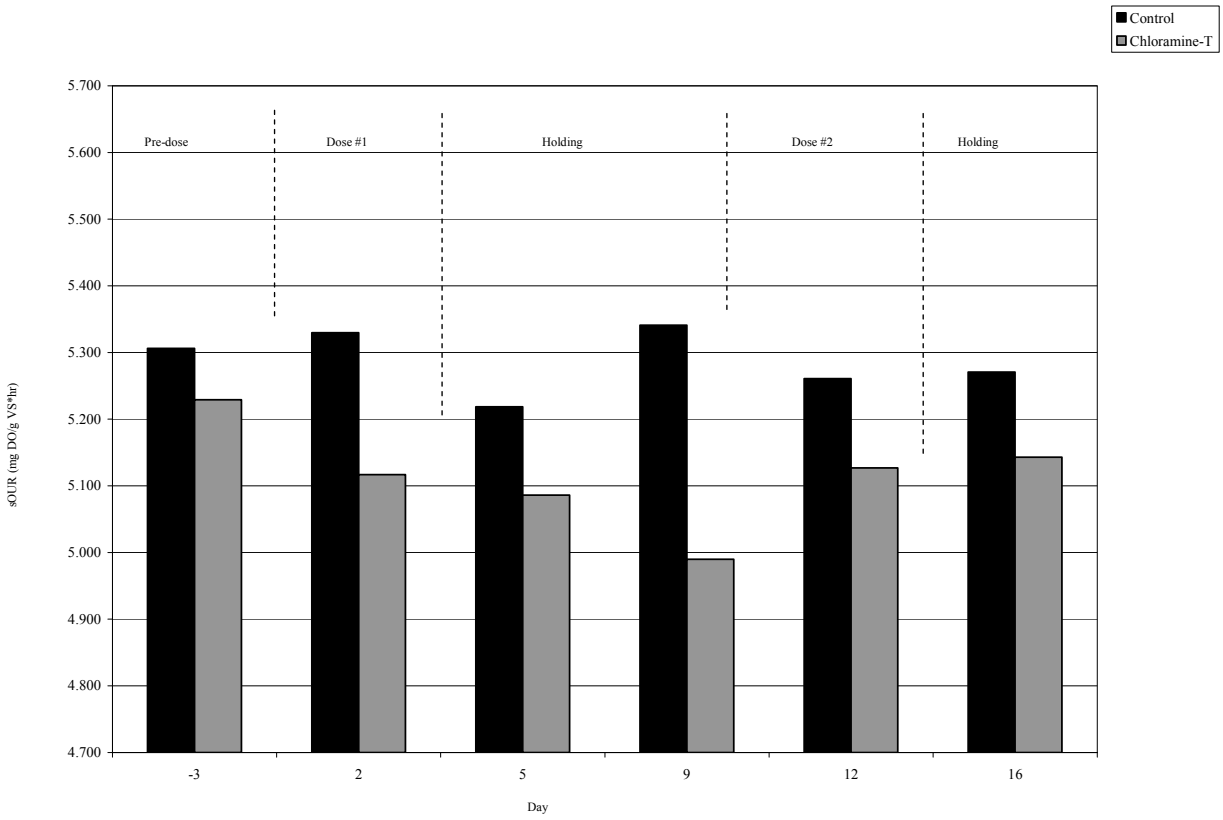

Figure 2-60. Specific oxygen uptake rate (sOUR) response to treatment with Chloramine-T during the replicate trial

Chapter 3: Impacts of Aquaculture Chemotherapeutants on Laboratory-cultured Nitrifying Bacteria

Impacts of Aquaculture Chemotherapeutants on Laboratory-cultured Nitrifying Bacteria

Abstract

Currently, there are only a limited number of chemicals approved by the Food and Drug Administration (FDA) for use in aquaculture. Previous investigations in operational aquaculture systems stocked with tilapia indicated that nitrifying bacteria were inhibited in the presence of therapeutic doses of copper as well as in the presence of the antibiotics oxytetracycline and Romet-30®, a commercially available sulfadimethoxine-ormetoprim combination antibiotic. Because the population of nitrifying bacteria in operational aquaculture systems has been shown to average between 3% and 5% of the total bacterial present and although there are distinct differences in the characteristics of laboratory-cultured bacteria and bacteria that grow naturally in an environment, laboratory trials were conducted to better evaluate the potential impacts of the chemotherapeutants to the nitrifying bacteria.

Three 1 L reactors were set-up for each of the chemotherapeutic treatments: oxytetracycline, Romet-30®, low-dose copper (0.125 mg/L free copper), therapeutic-dose copper (0.25 mg/L free copper), and high-dose copper (0.5 mg/L free copper). These reactors were treated in accordance with FDA guidelines for each chemical and analyzed for 24-hour total ammonia-nitrogen (TAN) uptake, specific oxygen uptake rates (sOUR), ammonia monooxygenase subunit A (*amoA*) expression, and for population density using catalyzed reporter deposition-fluorescent in situ hybridization (CARD-FISH). Results showed that the TAN uptake was reduced by 98% during the oxytetracycline treatments, 98% during the Romet-30® treatments, 94% during the low-dose copper treatments, 94% during the therapeutic-dose copper treatments, and 92% during the high-dose copper treatments. These reductions were observed both in comparisons to the pre-dose uptakes for each reactor as well as in comparison to the untreated control cultures.

The sOUR analyses confirmed that the bacteria were inhibited by the exposure to the therapeutic chemicals as shown through a significant reduction, but not elimination, in the uptake of the oxygen by the aerobic nitrifiers. CARD-FISH analyses showed that the population density continued to increase over the course of all treatments, but that this increase was not as rapid in those systems being treated with chemotherapeutants. This indicates that the bacteria were able

to sustain themselves and achieve limited growth rather than being eliminated. There were no significant fluctuations in the ratio of ammonia-oxidizing bacteria (AOB) or nitrite-oxidizing bacteria (NOB) to total DNA as represented by a DAPI counterstain. The *amoA* expression analyses demonstrated that the antibiotics inhibited the bacteria at the genetic expression level and that the expression of *amoA* began to resume once the active dosing of the antibiotics ended and the antibiotic started to degrade in the water column. These analyses also showed that the expression of *amoA* was up-regulated by the addition of copper, even though this copper was inhibitory to whole cells. Comprehensively, the findings validated the previous work in operational systems that showed that the bacteria were significantly inhibited, but likely not destroyed as a result of the exposure to any of the chemicals at therapeutic concentrations.

Keywords: Nitrifying bacteria; copper; oxytetracycline; Romet-30®

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Introduction

Inherently, the responses of laboratory-cultured bacteria following exposure to stressors, including chemotherapeutants, are markedly different than the responses of the same bacteria in the natural environment. For the aquaculture industry, the challenge lies in determining the nature and magnitude of these differences in order to manage them and sustain mass production through times of sickness or less than ideal conditions. In studies previously conducted on operational aquaculture systems, six common chemotherapeutants were tested to determine their impact to the nitrification process: chelated copper, formalin, potassium permanganate, Chloramine-T, oxytetracycline medicated feed, and Romet-30® medicated feed. Of these, only chelated copper, oxytetracycline (Figure 3-1), and Romet-30® were observed as causing significant inhibition to the nitrification process. For this research, these three chemotherapeutants were provided to laboratory-cultured nitrifying bacteria to observe the impact to the bacteria in a more densely populated culture and to compare the observed impacts between the natural and synthetic systems.

Copper is used in freshwater aquaculture as an algicide and antiparasitic treatment. In small amounts, copper is beneficial to cellular metabolism, but quickly becomes inhibitory at increasing levels. It has been shown that concentrations of free copper less than 1 mg/L can cause inhibition of ammonia oxidizing bacteria (AOB) grown in pure culture (Skinner and Walker, 1961; Lee*, et al.*, 2008). The Environmental Protection Agency (EPA) has approved copper sulfate for use in aquaculture and provides guidelines for its use at a sustained dose of 0.25 ppm free copper (USJSA, 2007); however, in recent years, chelated copper has become the formulation of choice among aquaculturists as it is more stable in solution and less corrosive to equipment . Copper has been shown to have more of an impact to nitrifiers growing in pure culture than to nitrifiers in complex media, such as activated sludge (Tomlinson*, et al.*, 1966). The reason for this is believed to be the complexation of copper with readily available organic matter. For these reasons, it is hypothesized that the bacteria in the pure cultures will be inhibited by the copper exposure because the dosing has been established at levels to effectively eliminate parasites in natural systems, but there is a lack of organic matter to complex with the excess amounts of copper residing in the reactors.

Few antibiotics are approved by the U.S. Food and Drug Administration (FDA) for use in aquaculture (USFDA, 2008). Two of these are oxytetracycline and Romet-30®, which are most commonly provided to the fish in the form of medicated feed. Tetracyclines function by inhibiting protein synthesis and preventing the binding of aminoacyl-tRNA to the bacterial ribosome (Chopra and Howe, 1978; Chopra and Roberts, 2001). The tetracyclines have molecular weights of less than 500 daltons which allows them to diffuse through pores in the cell membrane of Gram-negative bacteria, especially in the case of oxytetracycline which is hydrophilic in nature (Chopra and Howe, 1978). Tetracyclines are acidic in nature, dissociate in aqueous solutions, and cross the outer membrane of the Gram-negative bacteria by binding to a metal cation and passing through the OmpF and OmpC porins (Chopra and Roberts, 2001). The antibiotic accumulates in the periplasm before dissociating from the cation and diffusing through the lipid bilayer of the cytoplasmic membrane via proton motive force (Chopra and Howe, 1978; Chopra and Roberts, 2001). The binding of oxytetracycline to the ribosome is reversible and this explains how the antibiotic may inhibit bacteria without completely destroying them under the appropriate dosing (Chopra and Roberts, 2001). As such, it is hypothesized that the oxytetracycline will inhibit the bacteria and that some reduction in population will be observed. Additionally, it is hypothesized that some minimal recovery will be observed as the therapeutant degrades.

Romet-30® is a potentiated sulfonamide developed from a 5 to 1 ratio of sulfadimethoxine and ormetoprim, respectively (Figures 3-2 and 3-3). This antibiotic functions by blocking the synthesis of tetrahydrofolic acid, which is required for the synthesis of proteins and nucleic acid metabolism.(USFDA, 1984). This blocking takes place only in bacteria as opposed to animals because animals do not synthesize folic acid (Hoffman-LaRoche, 1984). Originally, each of the components was administered to animals as independent antibiotics, but the efficacy of the drugs is greater at lower doses (Bakal*, et al.*, 2004) when used in combination. By using the antibiotics in unison as a single antibiotic, it is possible to reduce the dose required, thus reducing the potential for antibiotic-resistant bacteria, while actually enhancing the spectrum of bacteria over which the medication is effective (Hoffman-LaRoche, 1984). Because of the extremely broad spectrum of this antibiotic coupled with the potency of the combination of antibiotics, it is hypothesized that this antibiotic will inhibit the bacteria and that some decrease in population will be observed. Likewise, Romet-30® functions differently than oxytetracycline, so it is not

anticipated that the effects of this antibiotic will be shown to be reversible, resulting in a lack of recovery of the bacteria in suspended culture.

Materials and Methods

Reactor Setup

One-liter (1 L) glass batch reactors were set-up in triplicate for each of the treatment conditions and untreated controls. These treatment conditions included oxytetracycline exposure, Romet-30® exposure, 0.125 mg/L free copper (low-dose), 0.25 mg/L free copper (therapeutic-dose), and 0.5 mg/L free copper (high-dose). These reactors were equipped with an oxygen supply and were wrapped in aluminum foil to prevent photoinhibition of the bacteria. Twenty-five milliliters (25 mL) of batch-grown nitrifying bacteria cultured from a commercially-available concentrated culture (Pond Protect – Novozymes, Salem, VA) was added to 1 L fresh nitrifying bacteria medium in each reactor. The recipe for this medium was provided by Novozymes (Salem, VA) and all bacteria were cultured at room temperature.

Antibiotic Exposures

Three reactors were exposed to therapeutic doses of oxytetracycline medicated feed (Finfish Booster – Zeigler Brothers, Gardners, PA). This antibiotic is coated to the feed at 2.5g oxytetracycline per pound of feed and provided to fish at a rate of 1.5% body weight for 10 days. One hundred milligrams (100 mg) of oxytetracycline medicated feed was added to the oxytetracycline-treated reactors each day for 10 days. This dose was based on data obtained using stocked aquaculture systems in earlier experiments. Three reactors were exposed to therapeutic doses of Romet-30® medicated feed (Finfish Xtra Booster – Zeigler Brothers, Gardners, PA). This antibiotic is coated to the feed at 22.2 lb Romet-30® per ton of feed and provided to fish at a rate of 1.5% body weight for 5 days. One hundred milligrams (100 mg) of Romet-30® medicated feed was added to the Romet-30®-treated reactors each day for 5 days. This dose was based on data obtained using stocked aquaculture systems in earlier experiments. Three reactors were also maintained as control reactors to which no antibiotics were added.

Antibiotic concentrations in the samples were analyzed on a high performance liquid chromatography (HPLC) unit using a Pinnacle DB C18 5um, 250x4.6 mm (length x inner diameter) column and a diode array detector (DAD). The mobile phase consisted of a 72.7:22.7:4.6 mixture of running buffer, acetonitrile, and methanol. The running buffer was comprised of 0.1M potassium phosphate, 0.05M triethanolamine, and 1% acetic acid. A standard curve for both the oxytetracycline and sulfadimethoxine was generated using raw antibiotics obtained from Fisher Scientific. It was not possible to obtain raw ormetoprim; therefore, the data reported represent the area under the HPLC peak rather than an actual concentration within the solution. Oxytetracycline eluted at 4.3 minutes, while ormetoprim eluted at 4.5 minutes and sulfadimethoxine eluted at 14.3 minutes. Fresh eluent was used for each analysis as it was observed that eluent that was more than three days old caused the compounds to elute much later.

Chelated Copper Exposures

Nine reactors were exposed to chelated copper in varying concentrations of free copper. Three reactors were exposed to a low dose (0.125 mg/L) free copper; three reactors were exposed to a therapeutic dose (0.25 mg/L) free copper; and three reactors were exposed to a high dose (0.5 mg/L) free copper. The FDA-approved dose for copper treatments in aquacultures in 0.25 mg/L free copper. By providing doses 50% above and 50% below the approved dose, the impacts to nitrifying bacteria as a result of moderate under- and over-dosing could be assessed. Three reactors were also maintained as control reactors to which no copper was added.

Total Ammonia-Nitrogen (TAN) Uptake

TAN uptake was monitored in each reactor on every fifth day during exposure to the chemotherapeutants to observe potential metabolic inhibition. Ammonium chloride equivalent to 10 mg/L TAN was added to the reactors and allowed to be metabolized by the bacteria over a 24-hour period. The reactors were sampled for TAN immediately after dosing and then at times 1, 4, 8, 12, 18, and 24-hours post-dose to generate an uptake curve. TAN was analyzed using the Hach Nesslerization Method (Hach Method 8038) with a Hach DR2800 spectrophotometer.

Specific Oxygen Uptake Rate (sOUR)

Samples for specific oxygen uptake rate (sOUR) were collected every fifth day and immediately analyzed in accordance with *Standard Methods* (Clesceri*, et al.*, 1998) using a dual dissolved oxygen probe system connected to a computer on which LabView software (National Instruments, Austin, TX) was installed to automatically collect time-step data. Samples were analyzed in duplicate with one replicate being altered with 2-chloro-6 (trichloromethyl) pyridine (TCMP) at 3 mg TCMP/300 mL in order to inhibit the nitrogenous oxygen demand. This addition allowed for the observation of the difference between the oxygen uptake rate of the total bacterial population and that of the carbonaceous bacterial population. The difference between the two rates was representative of the oxygen uptake rate for the nitrifying bacteria alone.

Catalyzed Reporter Deposition-Fluorescent in-situ hybridization (CARD-FISH)

Samples of bacteria culture to be used for CARD-FISH analysis were collected every fifth day. The bacteria were immediately fixed and applied to Teflon-coated slides with eight wells. The slides were then analyzed using CARD-FISH protocols (Pernthaler*, et al.*, 2002) to observe variations in population density with regards to the ratio of AOB (primarily *Nitrosomonas* species) and NOB (*Nitrobacter* species) to the total DNA present (as represented by DAPI counterstain). These bacteria were known to be present because of the seed culture analysis provided by Novozymes, Inc. (Salem, VA).

The use of CARD-FISH as opposed to traditional FISH has shown results with increased and more accurate binding and increased signal intensity – often to a degree of at least one order of magnitude – in samples such as these in which the target organisms are slow-growing and few in numbers (Amann and Kuhl, 1998; Pernthaler*, et al.*, 2002). Nso1225 and NIT3 conjugated with HRP on the 5' end were used to hybridize the slides (Thermo Scientific GmbH – Ulm, Germany). Details regarding the probe characteristics are provided in Table 3-1. Six wells on each slide were hybridized – three with a hybridization solution containing active probe and three with a hybridization buffer containing no probe to serve as controls for non-specific binding and auto-fluorescence. The protocol used for the analysis was adapted from published protocols (Pernthaler*, et al.*, 2002) and the slides were hybridized sequentially for the multiple probes based on increasing stringency.

The fixed slides were incubated in Lysozyme for 30 minutes at room temperature, followed by triple washing for one minute (per wash) in Nanopure water. The fixed slides were then incubated in Proteinase K for 5 minutes at room temperature, followed by triple washing for one minute (per wash) in Nanopure water. The slides were incubated in a quenching solution for 30 minutes in a 37°C hybridization oven and then triple washed in Nanopure water for one minute per wash. The slides were dehydrated in an ethanol series: 5 minutes in 50% ethanol, 1 minute in 80% ethanol, and 1 minute in 96% ethanol. The slides were dried in the hybridization oven at 37°C oven for 2 minutes.

Hybridization buffer was prepared based on the formamide concentration for the probes used (Table 2-3). Hybridization solution was prepared such that the probe was present at a 0.5 ng/μL concentration in the hybridization buffer. Two strips of filter paper moistened with hybridization buffer were placed in a 50 mL conical centrifuge tube and placed in the hybridization oven at 35°C to generate a humidified chamber for the hybridization process. 10 μL of the hybridization buffer (no probe) was evenly applied to the top three wells on the microscope slide to serve as negative controls for autofluorescence of the bacteria. 10 μL of the hybridization solution (probe added) was evenly applied to the bottom three wells on the microscope slide to hybridize with the species of interest. The slides were inserted into the humidified centrifuge tubes and incubated in a dark hybridization oven at 35°C for at least 2 hours. The tube was tightly sealed to ensure that the tubes remained humidified.

Following hybridization, the slides were incubated in pre-heated washing buffer in a dark hybridization oven at 37°C for 10 – 15 minutes. The slides were next incubated in 1X PBS (pH 7.6) for 30 minutes at room temperature. 10 μL of fluorescent tyramide working solution (NEN Life Sciences/Perkin Elmer, Waltham, MA) was added to each well while still wet in accordance with manufacturer recommendations and incubated in the dark in humidified tubes for 30 minutes in a 37°C hybridization oven. Cy3-Tyramide for was used for the AOB and Cy5-Tyramide was used for the NOB. The slides were removed and washed in 96% ethanol in the dark at room temperature for 1 minute and double-washed in 1X PBS for 1 minute (per wash) at room temperature. The slides were then counterstained using DAPI per manufacturer recommendations to observe the total population present in the sample.

Microscopic analysis was conducted using an epifluorescence microscope (Axioskop 2; Carl Zeiss, Thornwood, NY) and a Zeiss MRm camera. The images were captured using Axiovision 4.0 software. Three photos from each well were captured to ensure a comprehensive cross-section of the processed biofilm.

amoA Expression

Samples (25 mL) to be analyzed for amoA expression were collected from reactors over the course of the TAN uptake procedure immediately after dosing the reactors with ammonium chloride solution and again 4, 12, and 24 hours after the dosing. The samples were immediately frozen at -80°C until the time of analysis. Biomass for RNA analysis were centrifuged for 30 minutes at 5000 rpm and 4°C for harvesting bacteria cells and immediately stabilized with RNAprotect bacteria reagent (Qiagen - Valencia, CA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen – Valencia, CA) according to manufacturer recommendations and cDNA reverse transcription was carried out using Quantitect Reverse Transcription Kit (Qiagen – Valencia, CA). qPCR with SYBR Green assay was then conducted by the target primer sets and the cDNA that was generated during the reverse transcription process. Primers for AOB 16S rRNA and *amoA* were used for RT-qPCR amplification (Table 3-2).

Standard plasmid DNAs were generated with *Nitrosomonas europaea* 19718 pure culture genomic DNA. PCR was conducted in accordance with published protocols (Hermansson and Lindgren, 2001; Nicolaisen and Ramsing, 2002). PCR products were purified using the QIAEX Purification Kit (Qiagen – Valencia, CA) according to manufacturer specifications. Purified PCR products were then inserted into TOPO vector using the TOPO TA® cloning kit for sequencing (Invitrogen – Carlsbad, CA) according to manufacturer specifications. Plasmid DNA was purified using the PureLink^{TM} Quick Plasmid Miniprep Kit (Invitrogen – Carlsbad, CA). Serial 10-fold dilutions of plasmid DNA inserted for each target gene were used to generate standard curves. All data were collected and analyzed with iCycler (Bio-Rad Laboratories – Hercules, CA) and triplicates of the samples with the SYBR Green assay was conducted for all qPCR quantification. Collected *amoA* expression data were normalized with the reference gene, 16S rRNA of AOB.

Results and Discussion

Antibiotic Trials

Over the course of the 15-day trial, the ability of the bacteria culture to take up the TAN was reduced by 98% in both the oxytetracycline and Romet-30® trials. This was a significant reduction ($p<0.05$) from both the untreated control systems as well as from the pre-dose uptake capabilities. The ability of the control bacteria to uptake the TAN improved over the course of the trial, likely due to the maturation and growth of the bacteria population. For the oxytetracycline trial, statistical evaluation of the data for individual sample dates show that the difference was significant ($p<0.05$) on days 10 and 15. For the Romet-30® trial, statistical evaluation of the data for individual sample dates shows that the difference was statistically significant by day 5 ($p<0.05$) and remained significant ($p<0.05$) over the 15-day period. TAN uptake data over the 15-day trial are shown in Figures 3-4 through 3-7.

The sOUR data confirmed these findings, showing a dramatic increase (12-fold) in the oxygen uptake rates of the control bacteria over the course of the trial and a decrease in the oxygen uptake rates of the bacteria cultures exposed to oxytetracycline and Romet-30®. The differences in the sOUR data for the antibiotic-treated reactors and the untreated control reactors were significant ($p<0.05$) for oxytetracycline and Romet-30®. The response of the oxygen uptake rates to the antibiotic exposure is provided in Figure 3-8.

HPLC analysis revealed oxytetracycline concentrations of 0 mg/L on day 0, 2.05 mg/L on day 5, 3.84 mg/L on day 10, and 0.50 mg/L on day 15. These concentrations demonstrated an increase in concentration over the 10-day active dosing period and a rapid degradation in the water column between days 10 and 15 when the daily treatments had ceased. HPLC analysis revealed sulfadimethoxine concentrations of 0 mg/L on day 0, 1.11 mg/L on day 5, 2.55 mg/L on day 10, and 0.40 mg/L on day 15. Likewise, the area under the ormetoprim HPLC peak was 0 on day 0, 32.71 on day 5, 83.51 on day 10, and 1.17 on day 15. These concentrations demonstrated the expected increase in concentration over the 5-day active dosing period. The concentration of antibiotic continued to increase for at least five more days once dosing ended, indicative of a slower leaching of the antibiotic from the medicated feed into the water column than was observed with the oxytetracycline-medicated

feed. Once the degradation began and the daily dosing ended, the antibiotic had rapidly degraded by day 15.

amoA expression analyses showed that the antibiotics inhibited the nitrifying bacteria through day 10 of the treatments; however, the expression had started to recover by day 15. This demonstrated a distinct pattern of inhibition and recovery by the nitrifying bacteria. The control bacteria increased expression of *amoA* at increasing magnitude over the course of the experimental period (Figure 3-9).

CARD-FISH showed an increase in the population density of the nitrifying bacteria in the untreated controls over the duration of the 15-day trial. Likewise, the population density of the bacteria in the antibiotic-treated reactors increased over the duration of the trial, but did so at a slower rate than observed in the controls. This indicates that the nitrifying bacteria were still able to grow over the course of the trial, but that the growth rate was inhibited by the exposure to the antibiotics. There were no significant fluctuations in the ratio of AOB or NOB to total DNA. Supporting data are provided in Table 3-3.

Oxytetracycline (a tetracycline antibiotic) and Romet-30® (a potentiated sulfonamide antibiotic) both encompass a broad spectrum of activity and are proven to treat Gram-negative bacteria, making nitrifying bacteria part of the target organism field (Halling-Sorensen, 2001). The results of this research indicate that at the therapeutic doses established by FDA, the bacteria are inhibited, but not eliminated from the systems at a level of significance ($p>0.05$). In other experiments conducted on operational systems, the nitrifying bacteria were in a strongly heterogeneous community and recovery was observed during the treatment cycle. In regards to whole cell impact (N-uptake and sOUR analyses), the antibiotics inhibited the bacteria and recovery was not observed over the course of the treatment. With regards to oxytetracycline, this refutes the original hypothesis that some recovery would occur. That said, the hypothesis that Romet-30® would inhibit the bacteria to a larger degree than the oxytetracycline was upheld.

Nitrosomonas europaea are equipped with tetracycline-specific repressor proteins that efflux the tetracycline from the cells (Nikaido, 1996); however, when the concentration of oxytetracycline exceeds the capability of these efflux pumps, the oxytetracycline accumulates in the cell and inhibition or destruction occurs. It is believed that similar pumps may be present for sulfonamides, such as Romet-30® (Recchia and Hall, 1997; Skold, 2001).

Whole cell experiments resulted in data showing almost complete inhibition of the AOB with no recovery over the course of the experimental period. The genetic expression of *amoA* was inhibited during exposure to each of the antibiotics; however, small levels of expression were detected once the antibiotic was no longer being added to the reactor and the antibiotic began to degrade. Even though the N-uptake and sOUR results showed no recovery, the eventual upregulation of *amoA* expression as the concentration of the antibiotics degraded may indicate that the nitrification process would resume if provided ample recovery time.

Copper Trials

Over the course of the 10-day trial, the ability of the bacteria culture to take up the TAN was reduced by 94% in the low-dose systems, 94% in the therapeutic-dose systems, and 92% in the high-dose systems. There was negligible change in the uptake capabilities of the control systems. The reductions observed in the copper-treated reactors were significant ($p<0.05$) in relation to both the untreated control systems as well as the pre-dose uptake capabilities. The difference between the uptake of TAN by the copper-treated systems and the untreated controls over the course of the 10-day trial was statistically significant ($p<0.05$) at all three levels of copper treatment. Statistical evaluations of the data collected on the individual sample dates indicate that the difference was observed for all sampling events after the dosing began. TAN uptake data over the 10-day trial are shown in Figures 3-10 through 3-12.

The sOUR data confirmed these findings, showing an increase (approximately 6-fold) in the oxygen uptake rates of the control bacteria over the course of the trial. The oxygen uptake rates for all of the copper-treated reactors increased between days 0 and 5 followed by a tapering off in the rate of increase in the uptake rates between days 5 and 10. This demonstrates that although the bacteria were able to metabolize the oxygen over the course of the exposure, this metabolism was stifled as the duration of the exposure progressed. This impact was not seen in the copper treatments to the operational systems because the FDA requires a water change to remove the excess copper following the 10-day exposure. Although there were distinct trends to the data indicating impact by the copper, these trends were not shown to be significant ($p>0.05$). The response of the oxygen uptake rates to the copper exposure is provided in Figure 3-13.

amoA expression analyses showed that the copper upregulated the expression of the *amoA* gene at free copper concentrations of 0.125 ppm and 0.25 ppm. This upregulation is in keeping with other reports of the upregulation of *amoA* expression by copper (Hu, *et al.*, 2003; Hu, *et al.*, 2004; Park and Ely, 2008). At 0.5 ppm free copper, the genetic expression remained relatively stable, but was inhibited in comparison to the expression observed in the untreated controls (Figure 3-14).

CARD-FISH showed an increase in the population density of the nitrifying bacteria in the untreated controls over the duration of the 10-day trial. Likewise, the population density of the bacteria in the copper-treated reactors increased over the duration of the trial, with the highest level of growth occurring in the high-dose (0.5 mg/L) treatment. This went against expectations for this study. The other two copper treatments did not result in any significant difference in growth rates. This data demonstrate that the bacteria were able to continue to grow during exposure to the therapeutic concentrations of copper, even while their ability to metabolize ammonia-nitrogen and oxygen was somewhat reduced. There were no significant fluctuations in the ratio of AOB or NOB to total DNA. Supporting data are provided in Table 3-3.

Copper inhibited the nitrification process at the whole cell level (N-uptake and sOUR analyses); however, there were no distinct trends that could be related to the dose provided. That is, there was minimal difference in the N-uptake patterns and magnitudes between the three treatments. With regards to the oxygen uptake, all three treatments showed an increase in oxygen uptake capability over time; however, the capability of the high-level dose (0.5 ppm) increased at a slower rate than the other two treatments and the control. There was negligible differentiation between the low (0.125 ppm) and therapeutic (0.25 ppm) dose. Copper upregulates the expression of *amoA* (Hu, *et al.*, 2003; Hu, *et al.*, 2004; Park and Ely, 2008); however, copper is shown to be inhibitory at the whole cell level as observed in the N-uptake and sOUR analyses. Additionally, copper has been shown to induce cytotoxicity and to cause the loss of intracellular potassium, indicative of a loss of membrane integrity (Hu, *et al.*, 2004). Unlike other heavy metals, copper was observed to cause lesions in the cellular membrane that allowed approximately 70% of the intracellular potassium to leave the cell (Borkow and Gabbay, 2005). Intracellular potassium is a major cation that is important for maintaining the membrane potential of the cell and impacts to the potassium balance directly

impact the viability of the cell. These findings further support that theory of inhibition at the whole cell level in our experiments. In addition to damaging the cellular membrane, copper acts like other heavy metals and binds to and denatures DNA helix structures (Borkow and Gabbay, 2005). This binding to the DNA receptor sites is reversible; however, higher concentrations and/or longer exposure times may result in the destruction of the cell rather than significant inhibition.

Copper exposure to nitrifiers growing in pure culture has been shown to have more of an impact than that to nitrifiers in complex media, such as activated sludge (Tomlinson, *et al.*, 1966). The reason for this is believed to be the complexation of copper with readily available organic matter. In laboratory cultures, there is negligible organic matter aside from the biomass present in the reactors, thus the copper remains in solution and reacts only with the bacteria and media. Ionized copper is internalized by *Nitrosomonas europaea* and reaches intracellular equilibrium after only 4 hours of exposure. This is very rapid in comparison to other metals, including zinc, cadmium, and nickel, the intracellular concentrations of which continued to increase beyond 12 hours of exposure (Hu, *et al.*, 2003). When an excess of copper or another heavy metal is present, it bombards the cells and may be taken up through non-specific transport pathways. These pathways cannot be closed and contribute to the heightened toxicity of heavy metals (Nies, 1999).

sOUR has been shown to be a sensitive indicator of inhibition (Chandran and Love, 2008). With regards to these experiments, the sOUR correlated well to the hypothesized responses of the bacteria to the copper exposure in terms of the physiological condition of the bacteria. In each of the treatments, the ability of the untreated control cultures to utilize the oxygen in the reactors increased as the cultures matured and the healthy population increased. Accordingly, the sOUR results for the treated systems indicated that the physiological state of the nitrifying cultures was impaired during exposure to the antibiotics and the copper treatments. For slow-growing bacteria, sOUR continues to be a fast and easy method for determining the overall health and metabolic activity of a bacterial community.

The *amoA* expression in the AOB community was upregulated by approximately 3-fold when exposed to the therapeutic concentration of 0.25 ppm free copper (as chelated copper). This upregulation confirmed findings by other authors (Park and Ely, 2008). Even though

the expression of the *amoA* was upregulated, it was not adequate to allow the cell to overcome the impacts of the copper at the cellular level.

All treated systems (in the copper trials and in the antibiotic trials) showed an increase in population density over the treatment periods. The difference, however, was distinct in the rate of the increases over time. There was no significant difference between the control systems for the copper-treated systems and the control systems for the antibiotic-treated systems. Generally speaking, the growth rate relationship can be expressed as:

Control Systems > Low Copper > Medium Copper ~ High Copper > Oxytetracycline > Romet-30®

Based purely on the reported potency of the treatments, this sequence was to be expected with the exception of the therapeutic (0.25 ppm) and high (0.5 ppm) doses of copper being roughly the same. The findings of this research are very promising for aquaculturists in that it demonstrates that the ability of nitrifiers to recover following intense treatments with copper or antibiotics is not simply a function of bacterial protection by biofilms, but rather a mechanism that is inherent to the nitrifiers themselves that is observed, albeit to a lesser degree, in suspended laboratory cultures.

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References

Amann, R. and M. Kuhl (1998). "*In Situ* Methods for Assessment of Microorganisms and Their Activities." Current Opinion in Microbiology **1**: 352-358.

Bakal, R. S., S. A. Bai and M. K. Stoskopf (2004). "Pharmacokinetics of Sulfadimethoxine and Ormetoprim in a 5:1 Ratio Following Intraperitoneal and Oral Administration, in the Hybrid Striped Bass (*Morone chrysops x Morone saxitalis)*." Journal of Veterinary Pharmacology and Therapeutics **27**: 1-6.

Borkow, G. and J. Gabbay (2005). "Copper as a Biocidal Tool." Current Medicinal Chemistry **12**(18): 2163-2175.

Chandran, K. and N. G. Love (2008). "Physiological State, Growth Mode, and Oxidative Stress Play a Role in Cd(II)-Mediated Inhibition of *Nitrosomona europaea* 19718." Applied and Environmental Microbiology **74**(8): 2447 - 2453.

Chopra, I. and T. G. B. Howe (1978). "Bacterial Resistance to Tetracyclines." Microbiological Reviews **42**(4): 707-724.

Chopra, I. and M. Roberts (2001). "Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance." Microbiology and Molecular Biology Reviews **65**(2): 232-260.

Clesceri, L., A. Greenberg and A. Eaton, Eds. (1998). Standard Methods for the Examination of Water and Wastewater. Washington, DC.

Halling-Sorensen, B. (2001). "Inhibition of Aerobic Growth and Nitrification of Bacteria in Sewage Sludge by Antibacterial Agents." Archives of Environmental Contamination and Toxicology **40**: 451-460.

Hermansson, A. and P.-E. Lindgren (2001). "Quantification of Ammonia-Oxidizing Bacteria in Arable Soil by Real-Time PCR." Applied and Environmental Microbiology **67**(2): 972-976.

Hoffman-LaRoche (1984). Romet-30 (R) Environmental Assessment.

Hu, Z., K. Chandran, D. Grasso and B. F. Smets (2003). "Impact of Metal Sorption and Internalization on Nitrification Inhibition." Environmental Science and Technology **37**(4): 728-734.

Hu, Z., K. Chandran, D. Grasso and B. F. Smets (2004). "Comparison of Nitrification Inhibition by Metals in Batch and Continuous Flow Reactors." Water Research **38**: 3949-3959.

Lee, L. Y., S. L. Ong, H. Y. Ng, J. Y. Hu and Y. N. Koh (2008). "Simultaneous Ammonium-nitrogen and Copper Removal, and Copper Recovery Using Nitrifying Biofilm From the Ultra-Compact Biofilm Reactor." Bioresource Technology **99**(14): 6614-6620.

Nicolaisen, M. H. and N. B. Ramsing (2002). "Denaturing Gradient Gel Electrophoresis (DGGE) Approaches to Study the Diversity of Ammonia-Oxidizing Bacteria." Journal of Microbiological Methods **50**(2): 189-203.

Nies, D. H. (1999). "Mini-review: Microbial Heavy-metal Resistance." Applied Microbiology and Biotechnology **51**: 730-750.

Nikaido, H. (1996). "Multidrug Efflux Pumps of Gram-Negative Bacteria." Journal of Bacteriology **178**(10): 5853-5859.

Park, S. and R. L. Ely (2008). "Candidate Stress Genes of *Nitrosomonas europaea* for Monitoring Inhibition of Nitrification by Heavy Metals." Applied and Environmental Microbiology **74**(17): 5475-5482.

Pernthaler, A., J. Pernthaler and R. Amann (2002). "Fluorescence In Situ Hybridization and Catalyzed Reporter Deposition for the Identification of Marine Bacteria." Applied and Environmental Microbiology **68**(6): 3094.

Recchia, G.D. and R.M. Hall (1997). "Origins of the mobile gene cassettes found in integrons." Trends in Microbiology **5**: 389-394.

Skinner, F. A. and N. Walker (1961). "Growth of *Nitrosomonas europaea* in batch and continuous culture." Archives of Microbiology **38**: 339-347.

Skold, O. (2001). "Resistance to trimethoprim and sulfonamides." Veterinary Research **32**: 261-273.

Tomlinson, T. G., A. G. Boon and C. N. A. Trotman (1966). "Inhibition of Nitrification in the Activated Sludge Process of Sewage Disposal." Journal of Applied Bacteriology **29**: 266-291.

USFDA (1984). Environmental Impact Analysis Report: Romet-30 (Sulfadimethoxine and Ormetoprim) Medicated Premix for Control of Certain Salmonid Diseases (NADA 125-933_EA).

USFDA. (2008). "FDA/CVM Approved Drugs for Use in Aquaculture." from http://www.fda.gov/cvm/drugsuseaqua.htm.

USJSA. (2007). "Guide to Drug, Vaccine, and Pesticide Use in Aquaculture." from http://aquanic.org/jsa/wgqaap/drugguide/drugguide.htm.

Tables

Table 3-1. Oligonucleotides used in CARD-FISH application

Oligonucleotide Name	Sequence (5' – 3')	Formamide Concentration	Target organisms
Nso1225	CGC CAT TGT ATT TGT GA	35%	β-proteobacterial ammonia-oxidizers
Nit3	CCT GTG CTC CAT GCT CCG	40%	*Nitrobacter* spp.

Table 3-2. RT-qPCR primer sets for *amoA* mRNA gene expression

Primer	Sequence(5'-3')	Target	Reference
CTO 189fA/B	GGAGRAAAGCAGGGGATCG	AOB 16s rRNA	(Hermansson and Lindgren, 2001)
CTO 189fC	GGAGGAAAGTAGGGGATCG		
RT1r	CGTCCTCTCAGACCARCTACTG		
amoA-1F	GGGGTTTCTACTGGTGGT	*amoA* mRNA	(Nicolaisen and Ramsing, 2002)
amoA-2R-TC	CCCCTCTGCAAAGCCTTCTTC		

Table 3-3. Results of CARD-FISH analysis

Sample Day	Average AOB/DAPI (%)						
	Antibiotic Control	**OTC-treated**	**Romet-treated**	**Copper Control**	**Low-dose**	**Therapeutic dose**	**High dose**
0	41 ± 20	42 ±15	45 ± 22	43 ± 17	47 ± 14	45 ± 22	44 ± 19
5	45 ± 32	39 ± 25	42 ± 28	42 ± 21	44 ± 25	41 ± 31	43 ± 34
10	47 ± 28	41 ± 37	39 ± 24	45 ± 30	42 ± 22	47 ± 38	48 ± 29
15	45 ± 17	43 ± 21	40 ± 32	--	--	--	--

Sample Day	Average NOB/DAPI (%)						
	Antibiotic Control	**OTC-treated**	**Romet-treated**	**Copper Control**	**Low-dose**	**Therapeutic dose**	**High dose**
0	33 ± 25	35 ± 18	31 ± 29	41 ± 23	42 ± 21	45 ± 15	39 ± 24
5	32 ± 22	37 ± 28	38 ± 33	47 ± 20	44 ± 26	41 ± 30	42 ± 18
10	29 ± 17	33 ± 22	32 ± 26	45 ± 27	39 ± 24	42 ± 21	37 ± 30
15	31 ± 30	34 ± 32	31 ± 24	--	--	--	--

Figures

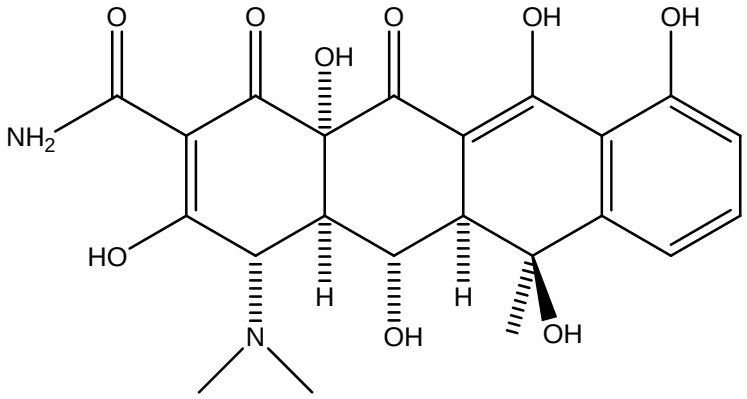

Figure 3-1. Oxytetracycline structure (adapted from http://en.wikipedia.org/wiki/Oxytetracycline)

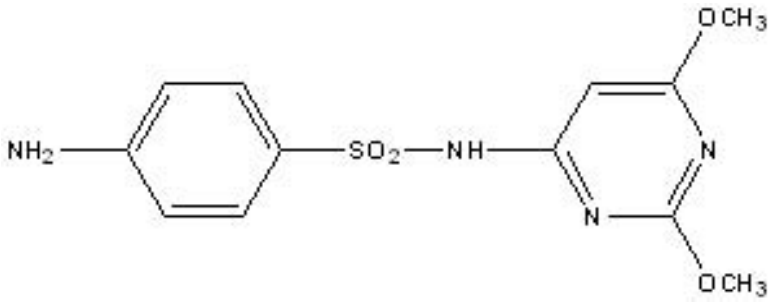

Figure 3-2. Sulfadimethoxine structure adapted from (Hoffman-LaRoche, 1984)

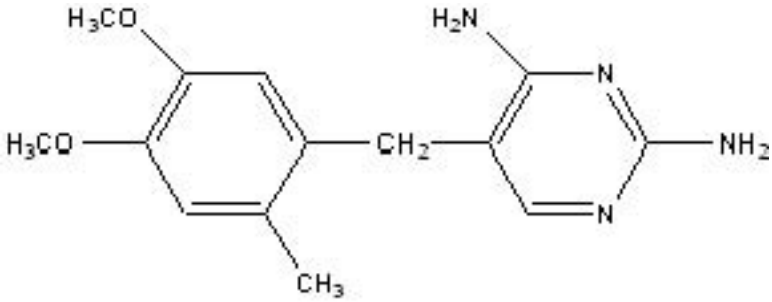

Figure 3-3. Ormetoprim structure adapted from (Hoffman-LaRoche, 1984)

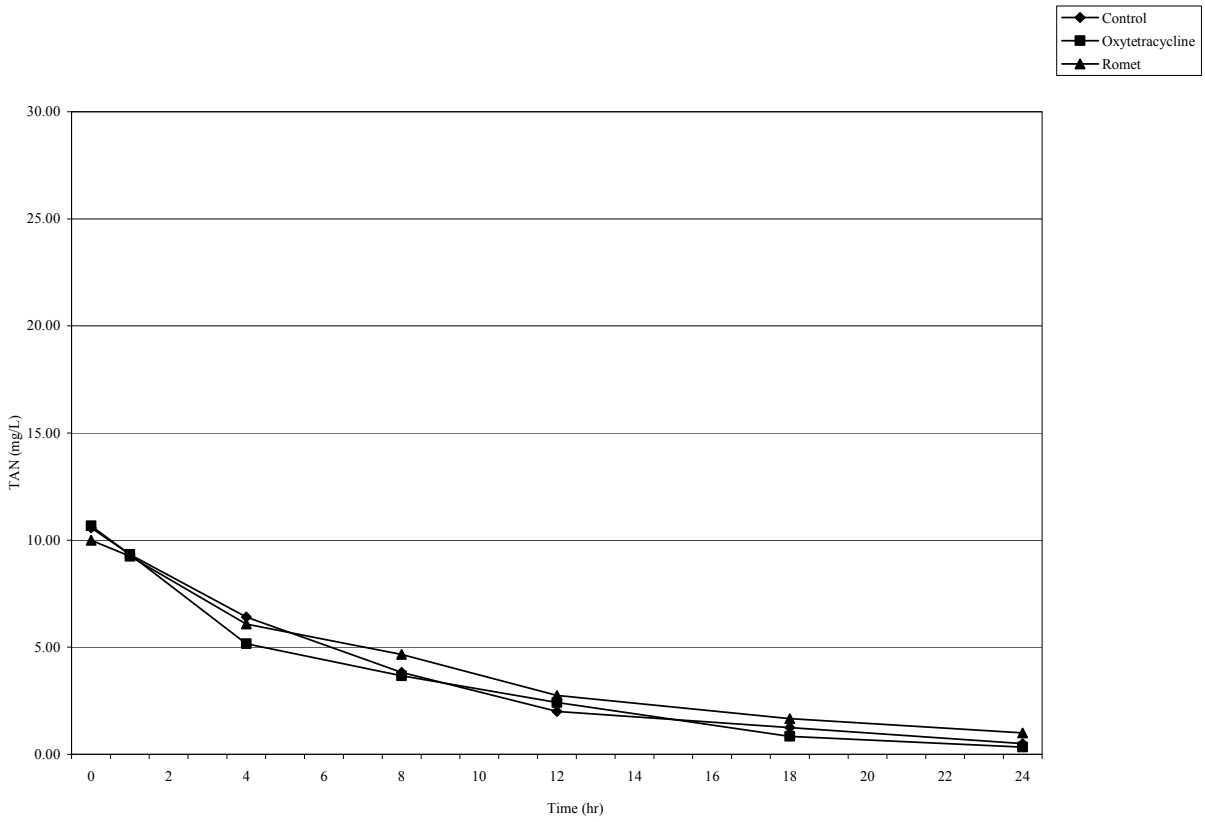

Figure 3-4. TAN response to antibiotics; Day 0; Before antibiotics were added to the reactors

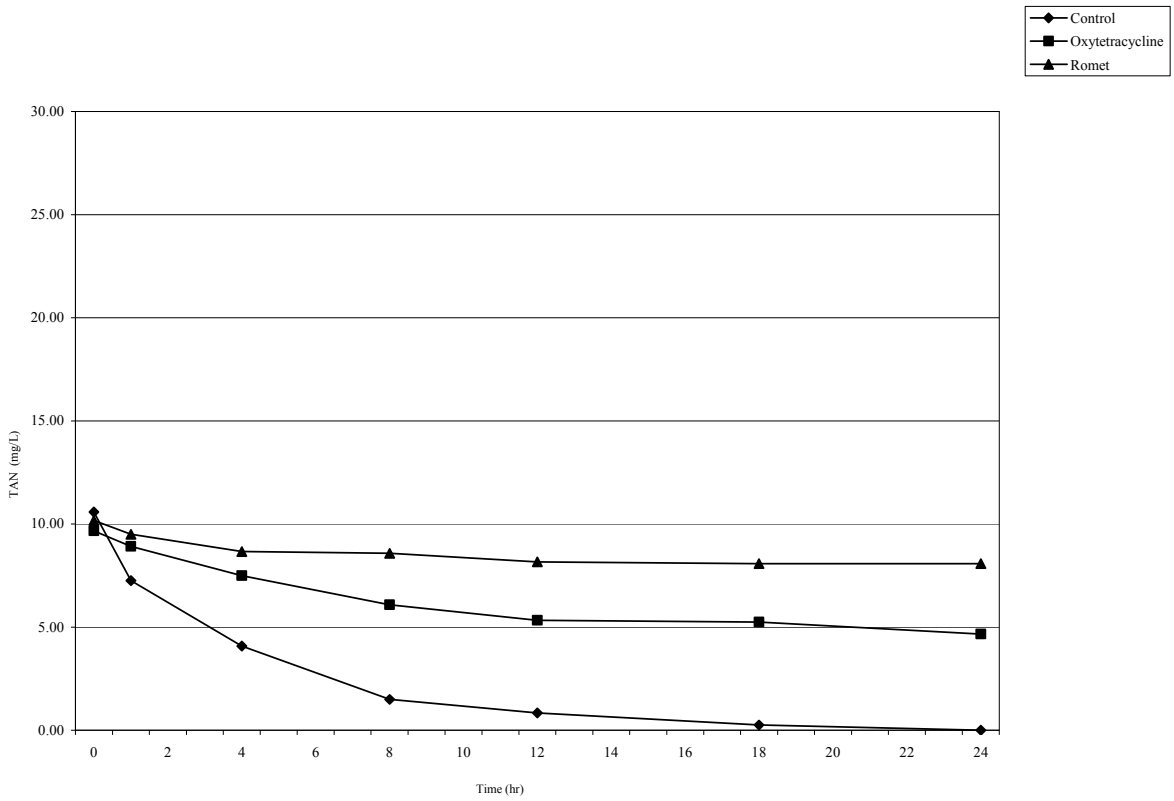

Figure 3-5. TAN response to antibiotics; Day 5; Oxytetracycline concentration = 2.05 mg/L, Sulfadimethoxine concentration = 1.11 mg/L, Ormetoprim area under peak = 0.33(10^2)

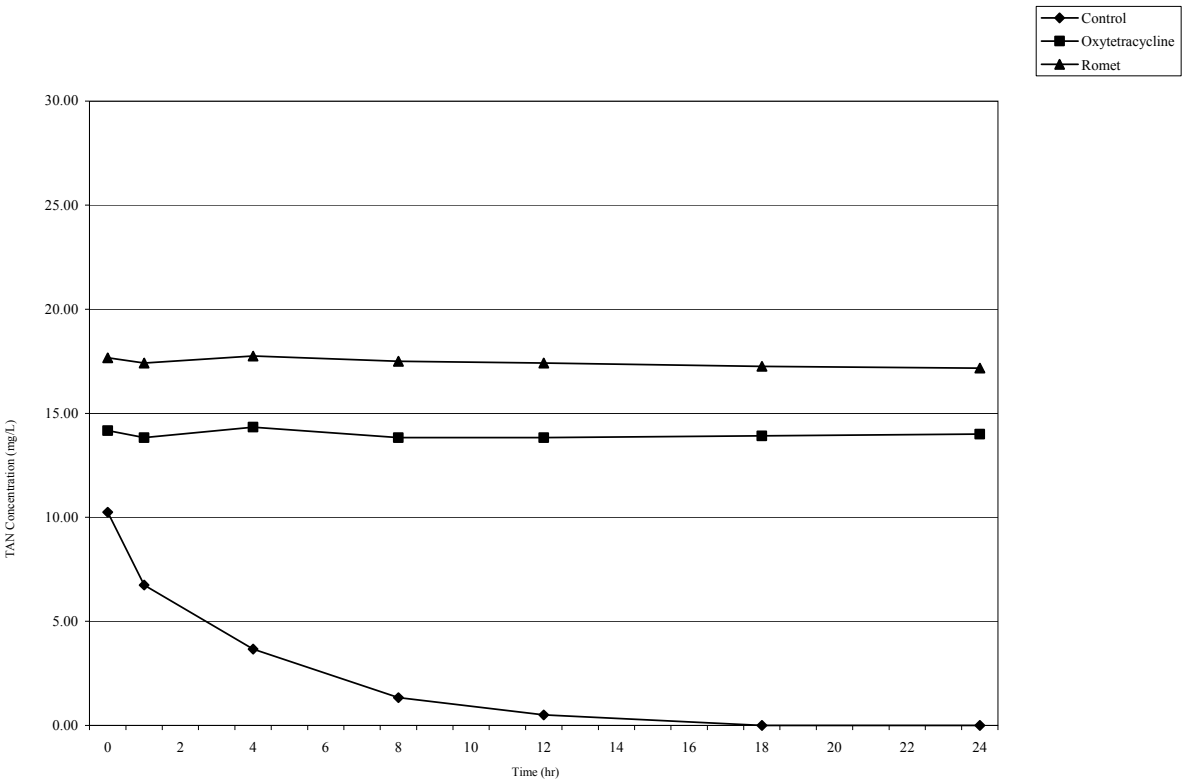

Figure 3-6. TAN response to antibiotics; Day 10; Oxytetracycline concentration = 3.84 mg/L,Sulfadimethoxine concentration = 2.55 mg/L, Ormetoprim area under peak = 0.84(10^2)

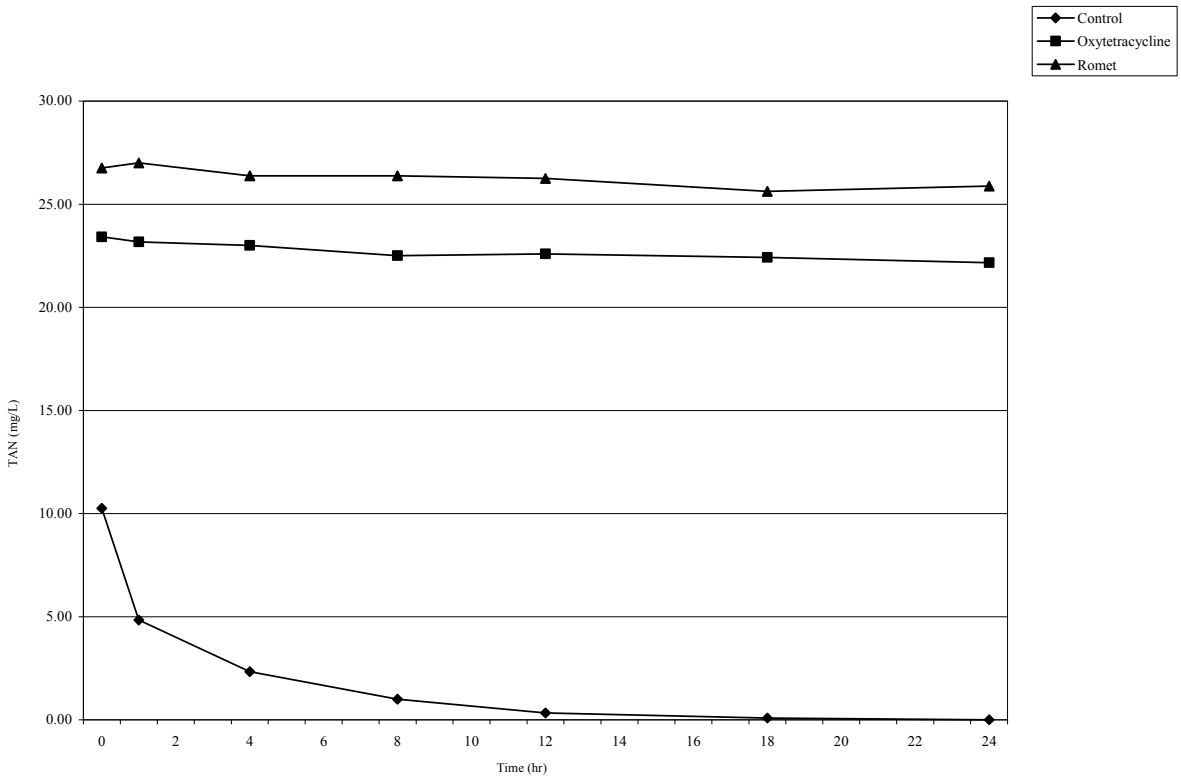

Figure 3-7. TAN response to antibiotics; Day 15; Oxytetracycline concentration = 0.50 mg/L, Sulfadimethoxine concentration = 0.40 mg/L, Ormetoprim area under peak = 0.01(10^2)

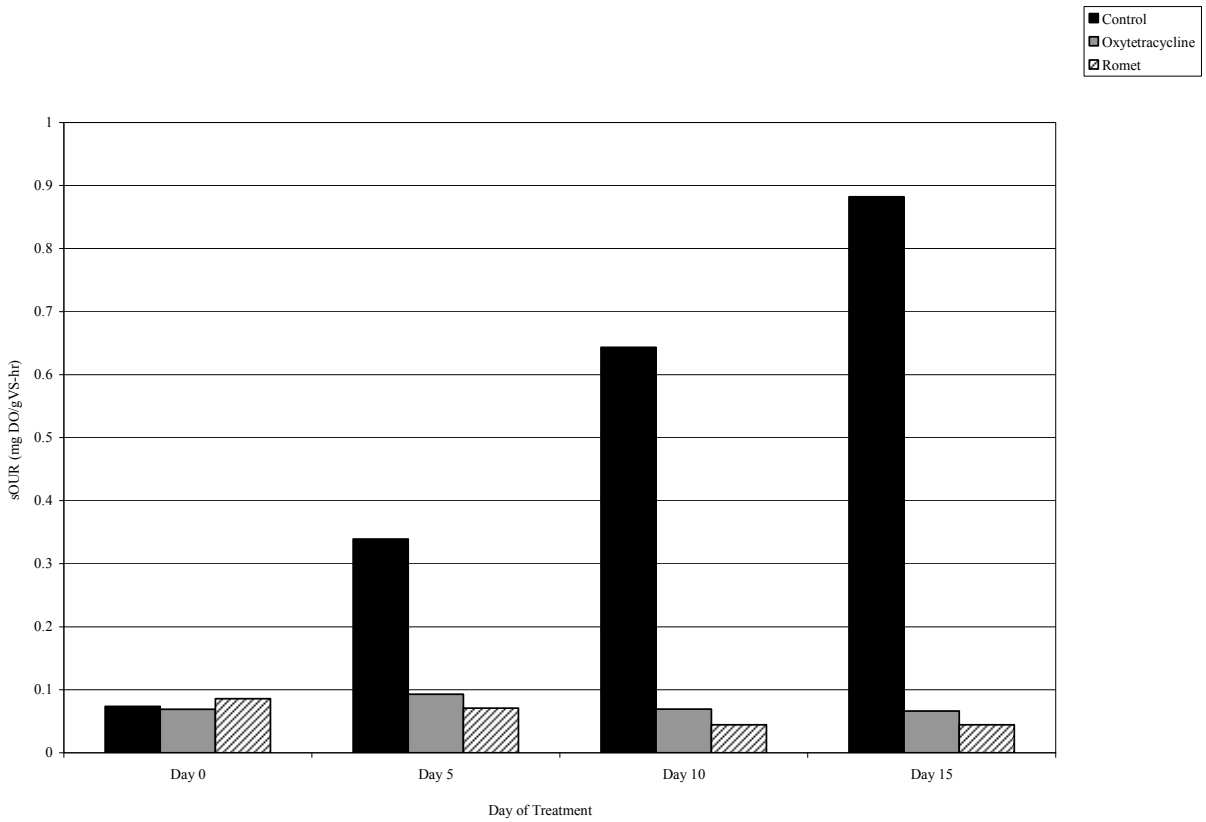

Figure 3-8. sOUR response to antibiotics

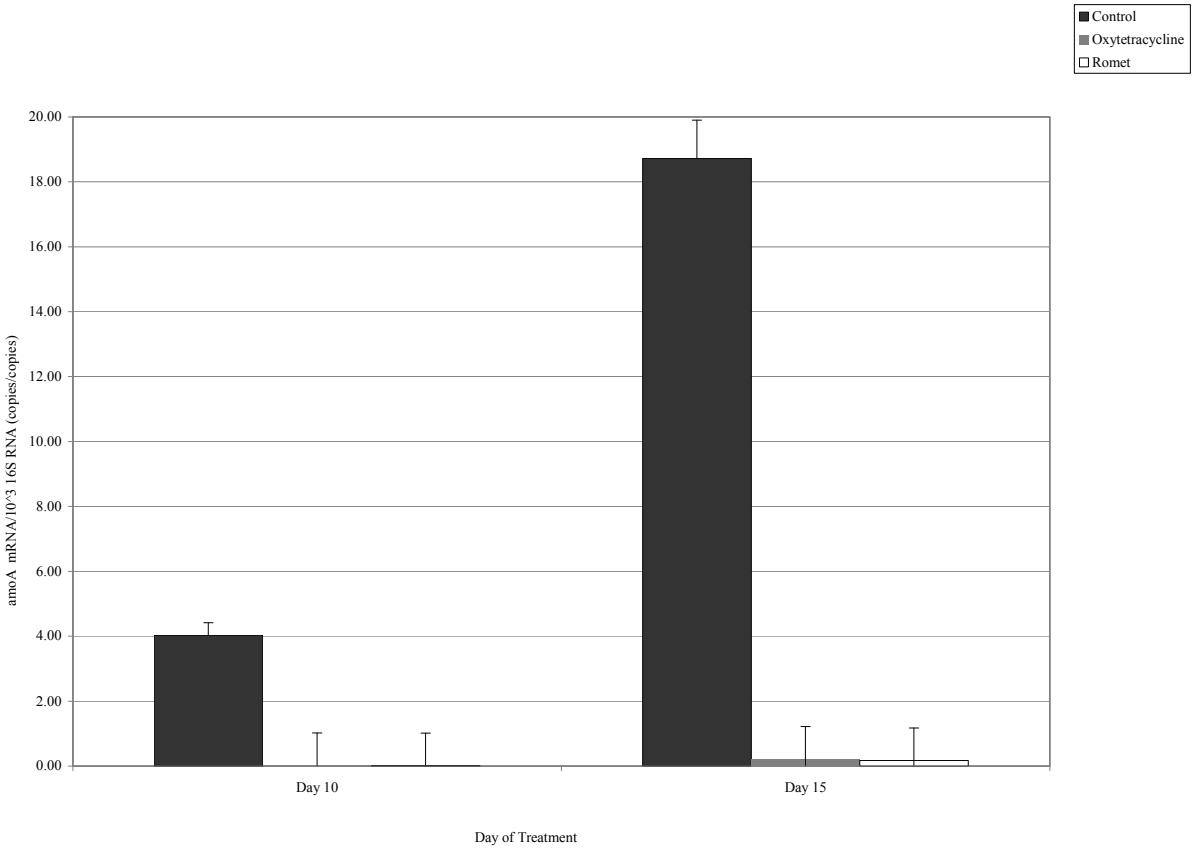

Figure 3-9. *amoA* expression following exposure to antibiotics

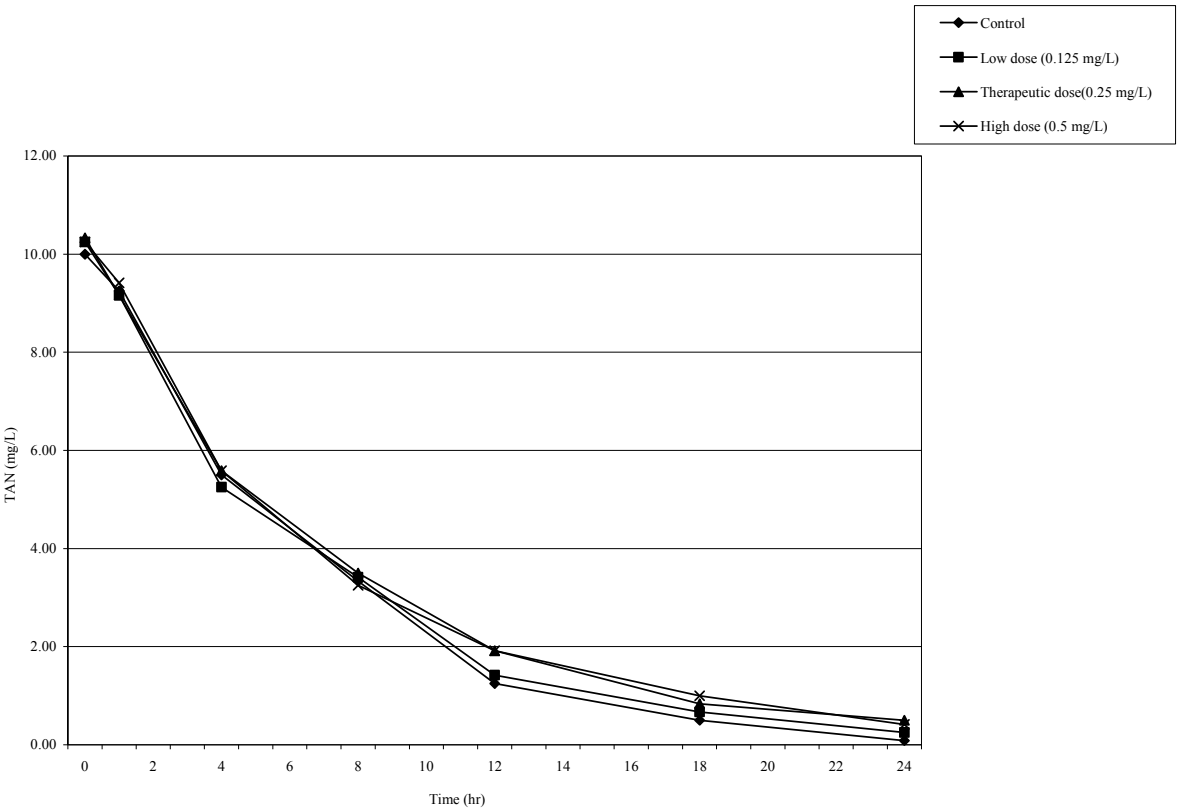

Figure 3-10. TAN response to copper; Day 0

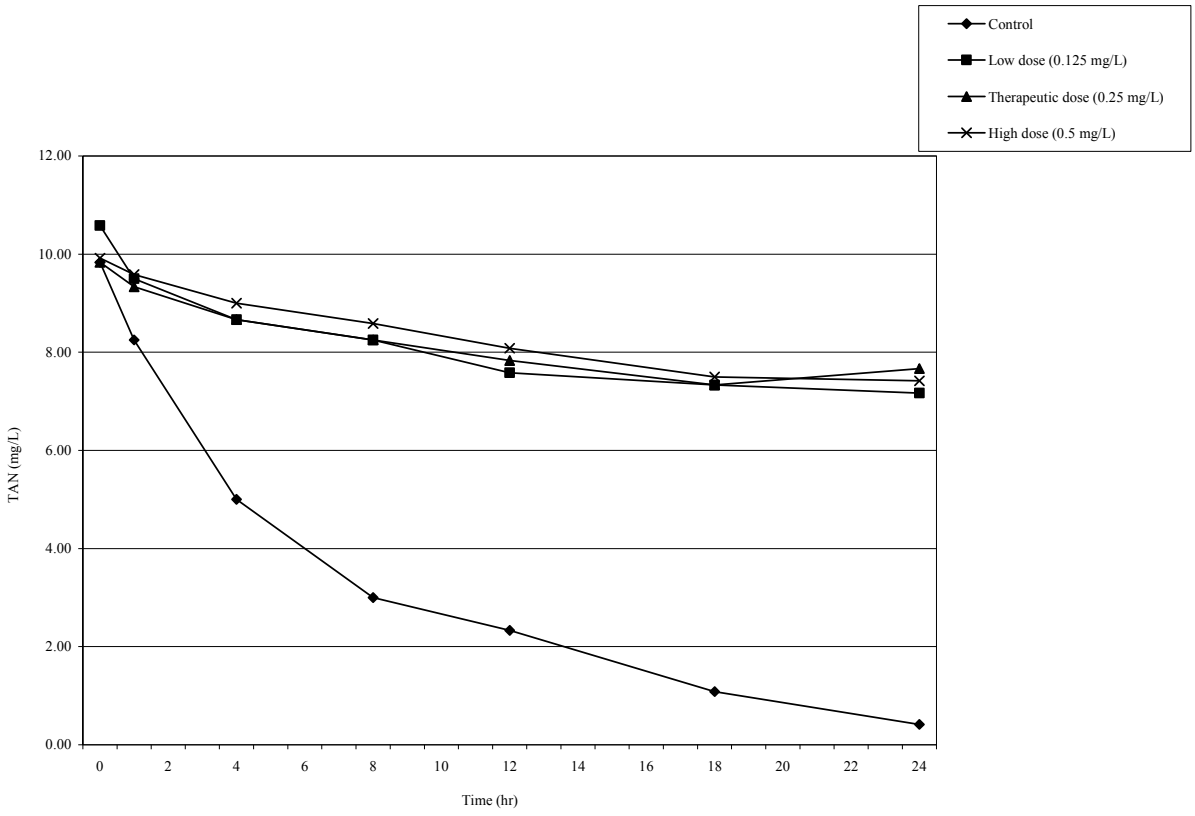

Figure 3-11. TAN response to copper; Day 5

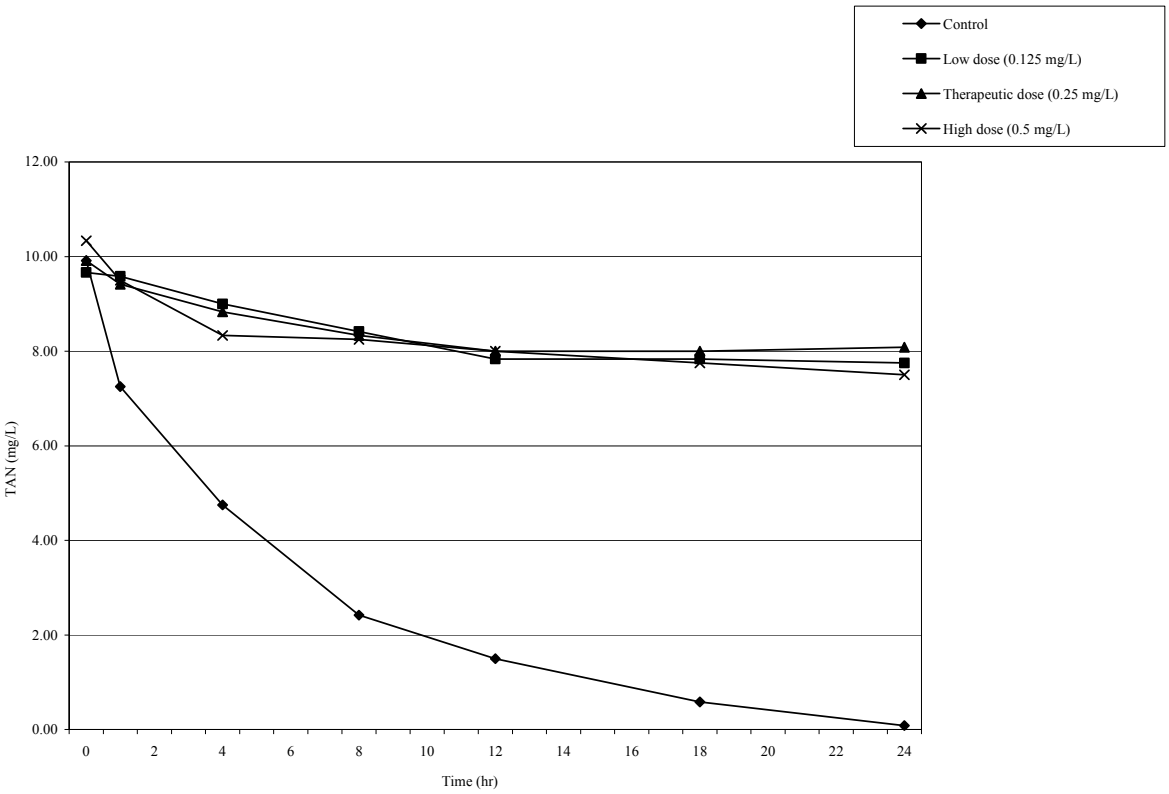

Figure 3-12. TAN response copper; Day 10

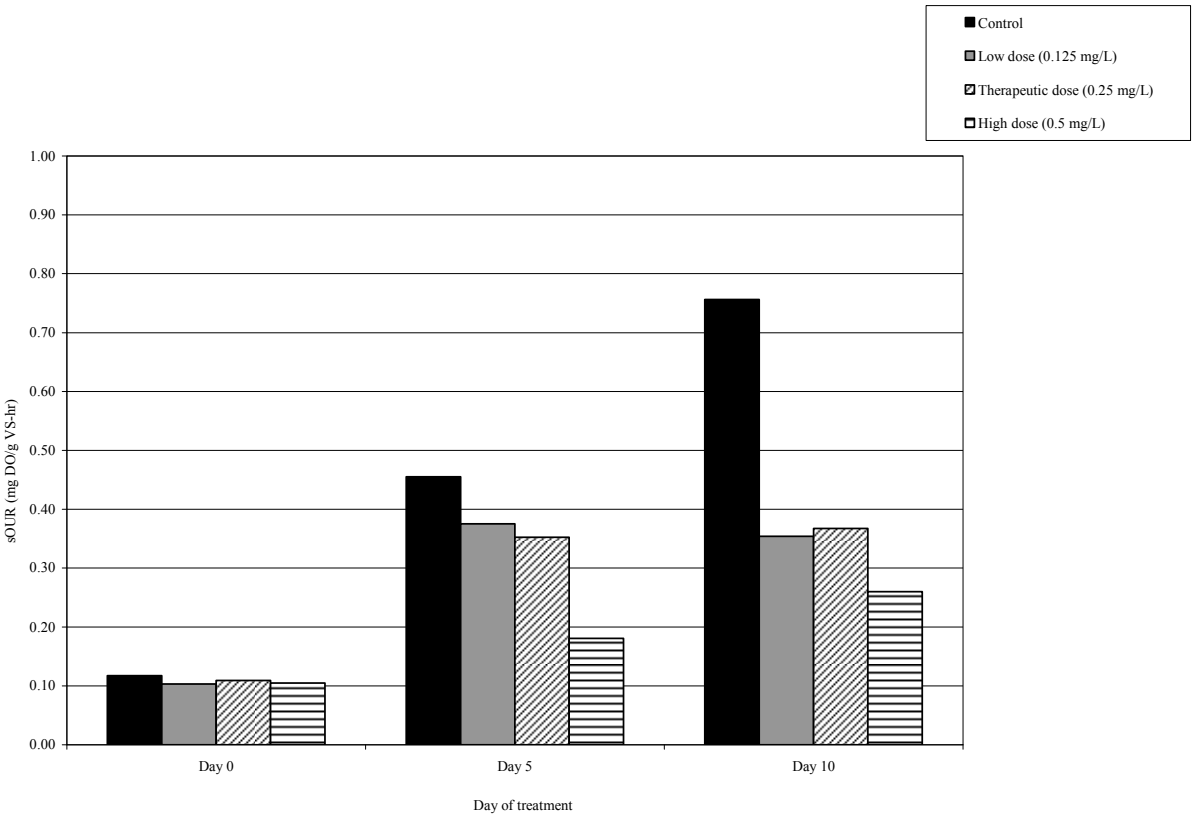

Figure 3-13. sOUR response to copper

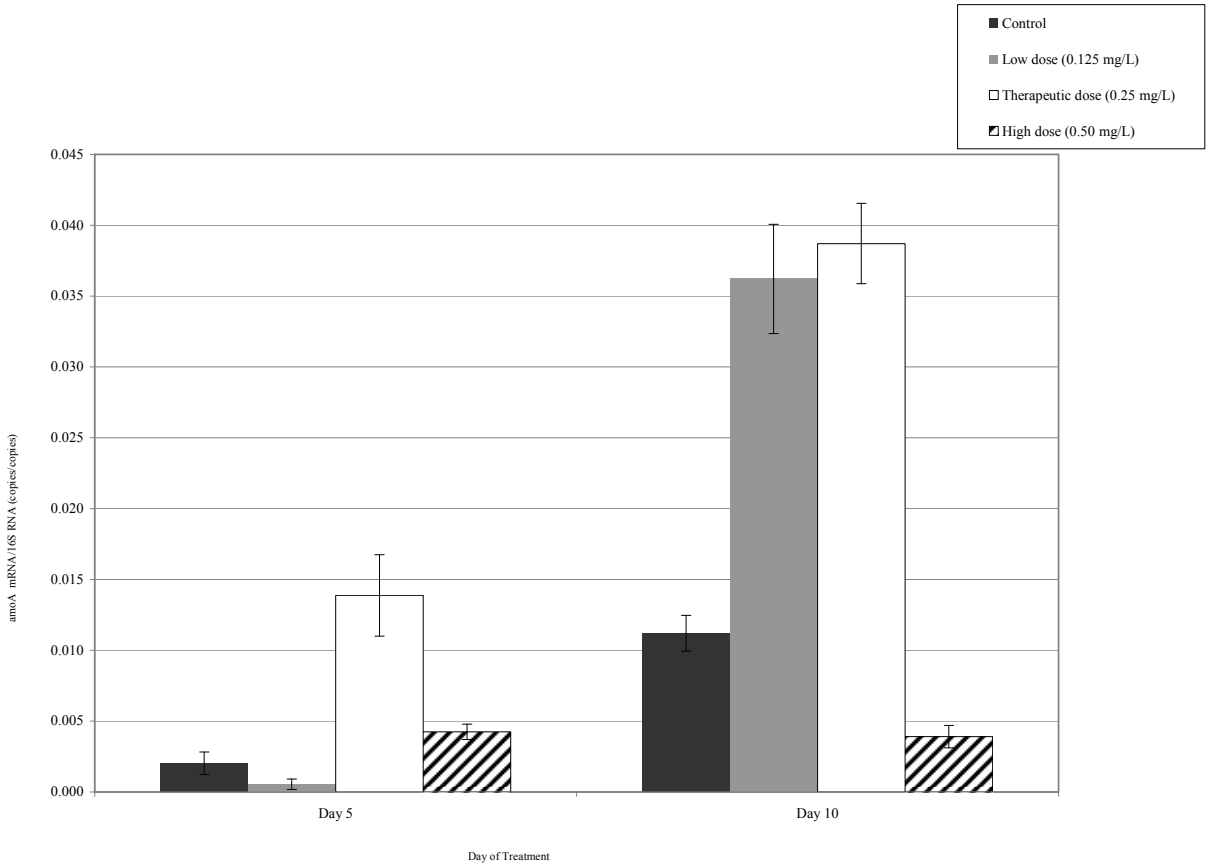

Figure 3-14. *amoA* expression following exposure to free copper

Chapter 4: Summary of Significant Findings and Their Effect on the Aquaculture Industry

Summary of Significant Findings and Their Effect on the Aquaculture Industry

Conflicts in literature

Throughout the course of this research, the primary focus has been to more effectively determine the impacts of chemotherapeutants to the nitrifying bacteria in aquaculture systems. The basis for this investigation has been the presence of extremely conflicting information in the literature. These conflicts were found to be rooted in several baseline conditions of the experiments: the use of synthetic water versus natural or operational (stocked) systems, the dose of the chemotherapeutant at levels significantly higher or lower than the approved therapeutic doses, the use of suspended laboratory-cultured nitrifying bacteria versus heterogeneous communities and biofilms, and the use of outdated or inappropriate analytical methods. The following sections outline the findings in regards to these conditions.

Synthetic water versus natural systems

Many of the previously published experiments utilized waters that had been generated under laboratory conditions to mimic the conditions of a natural or operational aquaculture system rather than actually using waters that had been stocked with fish and managed accordingly. Without the influence of the fish in the system, it is not possible to adequately mimic the fluctuations in nitrogen resulting from factors such as the natural fish metabolism and the addition of feed over a given period of time. Additionally, the parameters of the water (hardness, alkalinity, micronutrients, etc.) are entirely subjective to the desires of the researcher, allowing for ideal conditions to be maintained rather than allowing for realistic ones. Although these environmental factors and fluctuations are what often present a challenge when using operational systems, they remain more reflective of natural conditions and obstacles that would arise in a production facility.

Dose of the chemotherapeutants

The allowable dose of specific chemotherapeutants for use in aquaculture is established by the Food and Drug Administration (FDA) or by the Environmental Protection Agency (EPA)

based on many different factors. Among these are the levels and longevity of bioaccumulation in the fish and residues in the environment. As such, it is very important that operators of production facilities adhere to these dosing requirements and, likewise, it is very important that a researcher follow these dosing guidelines. Although "pushing" the systems often provides for dynamic research results, it does not provide for realistic results and may actually result in creating a panic among aquaculturists regarding the safe use of approved chemotherapeutants where negligible concern is actually warranted.

Laboratory-cultured bacteria versus heterogeneous communities

Nitrifying bacteria are highly preferential to attached growth (i.e., biofilms) and prior research has demonstrated that bacteria that exist in biofilms are as much as 1000 times more able to withstand stressed conditions than their suspended-growth counterparts. It is likely that this played a role in the ability of the nitrification process to recover following exposure to various chemotherapeutants when dosed to the operational systems used in this research. In suspended culture, the nitrifying bacteria did not demonstrate significant recovery at the whole cell level under the same dosing plan. Coupled with this protection from biofilms, it appeared that the nitrifiers had an inherent ability to withstand the exposure to the chemotherapeutants and to re-initiate the nitrification process once the stressors were removed. This was further supported by evidence in the laboratory-cultures that expression of *amoA* restarted as the chemotherapeutants degraded. Finally, laboratory-cultured bacteria required a specific set of parameters be met in order for the culture to be successful. These parameters are very often not in keeping with the values for the same parameters observed in operational systems. Because nitrifying bacteria are extremely sensitive to their environment, shifts in these factors ultimately change the nature of the culture that is being observed.

Outdated or inappropriate analytical methods

Historically, the analysis of the impacts of chemotherapeutants to aquaculture systems was limited to the observation of water quality parameters, especially with regards to the nitrogenous components: total ammonia-nitrogen (TAN), nitrite, and nitrate. Although these are good raw indicators of the systematic ability to metabolize nitrogen and may provide compelling evidence for a determination of "no significant effect" to the nitrifying bacteria, water quality alone does

not paint a comprehensive picture of inhibition, especially in the presence of a heterogeneous bacterial community where nitrifying bacteria may only represent about 3% of the total bacterial population. It has been shown that other bacteria are able to metabolize nitrogen as part of their function, which adds to the ineffectiveness of solely using nitrogen concentrations as an indicator for nitrifier inhibition.

More recent methods have allowed for the use of molecular techniques to analyze the population constituency and metabolic activity of nitrifying bacteria. These methods include traditional fluorescent in situ hybridization (FISH) and genetic expression analyses, such as for *amoA* – a subunit of the gene encoding ammonia monooxygenase , the enzyme responsible for the conversion of ammonia to nitrite. These techniques have become increasingly popular with bacteria that are considered non-culturable or difficult to culture; however, as investigation into the functionality of these methods continues, deficiencies are being identified. One of the deficiencies most significant to this research is the finding that FISH is not a good indicator of metabolic inhibition under stressed conditions for slow-growing bacteria, such as nitrifiers, that do not express their rRNA quickly enough to be captured by this method. Additionally, traditional FISH is not sufficiently sensitive to populations in which the species of interest are small in proportion, as is the case with nitrifiers in aquaculture. When one combines these deficiencies, traditional FISH becomes a decidedly poor choice for trying to observe metabolic impacts in stressed systems.

One method that is being used with increasing frequency for aquaculture samples is CARD-FISH. Although this method does not overcome the deficiency related to slow growth, it is notably more sensitive and can be used for determining shifts in population density and composition. This method can be correlated with modern genetic expression techniques to establish inhibition within the nitrifying community. The level of *amoA* expression is directly related to the ability of the ammonia-oxidizing bacteria to function. By combining these methods, researchers can determine (1) if there are increases or decreases or shifts in the population of nitrifiers or other bacteria within the total population and (2) if ammonia-oxidation has been inhibited by exposure to the chemotherapeutants.

Molecular analysis is not always feasible, especially for small private companies or field laboratories, so specific oxygen uptake rate (sOUR) analysis is a traditional, yet effective tool for determining the overall metabolic capacity of the aerobic bacteria. An advantage to this method

is that a user can differentiate between nitrogenous oxygen uptake and carbonaceous oxygen uptake through the use of a nitrification inhibitor, such as TCMP. When TCMP is added to the samples, the nitrogenous oxygen uptake is eliminated, resulting in only the carbonaceous oxygen uptake being represented. The difference between the total oxygen uptake and the carbonaceous oxygen uptake provides the nitrogenous oxygen uptake. This research showed, however, that sOUR analysis may be fairly ineffective for sample populations in which the nitrifying community is small. In these cases, there is little differentiation between the sOUR results for the carbonaceous and nitrogenous oxygen uptakes. Without a marked differentiation, one cannot definitively determine if the nitrogenous uptake experiences any significant inhibition as a result of exposure to the chemotherapeutant. Instead, the researcher is only able to adequately report inhibition to the total bacteria population in relation to untreated controls. To overcome this hurdle, nitrite/nitrate generation rates (NGR) are sometimes determined. The NGR allows for the evaluation of the nitrification process independent of heterotrophic activity by correlating the consumption and production of nitrification parameters. This rate can then be converted to specific nitrification rate by normalizing the results to the biomass concentration in the sample.

Summary of findings and value to aquaculture production facilities

Over the course of these experiments, nitrification in the operational systems was inhibited during exposure to both antibiotics (oxytetracycline and Romet-30®) and chelated copper. There were no significant impacts to nitrification observed under therapeutic doses of formalin, potassium permanganate, or Chloramine-T. As expected, the findings of this research further indicated that if the chemotherapeutants are dosed to the operational systems in accordance with FDA- and EPA-approved methods, including required or recommended water changes and holding periods, the nitrification process will recover without the need for taking the biofilters off-line during the treatment process. Recovery was also observed in the laboratory-cultured nitrifying bacteria, showing that this response and recovery is not only a result of protection by the heterotrophic bacteria present in biofilms, but also as a result of inherent properties of the nitrifiers themselves.

Additionally, this research validates the methodology of combining traditional methods, such as water quality analysis and sOUR analysis, with more modern techniques, such as genetic expression and CARD-FISH, to characterize complex bacterial systems. Through this type of

combined analysis, one is able to appropriately determine the impacts to a bacterial community without the bias that is introduced by culturability. For example, if one was to simply use nitrogen uptake assays to observe nitrification impacts following copper exposure, inhibition would be demonstrated because the impact of the copper to the bacteria initially occurs at the whole cell level, but the fact that the *amoA* expression is actually upregulated by the copper would be unaccounted for and it would be impossible to determine the extent of the inhibition to the cell.

For the average aquaculturist, the true value of this research lies in the response and recovery that was documented in both the operational and the laboratory-controlled experiments. This documentation enables them to operate and treat their systems in a more predictable manner. Additionally, the findings of no impact for formalin, potassium permanganate, and Chloramine-T refute much of the literature that warns users of the destruction of their biofilters when left on-line during treatment.

Appendix A: Data Associated with Chapter 2

Table A1. Average water quality results from untreated control systems during the first antiparasitic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	37	6.93	7.83	7.416	0.254	7.331	7.500
Temperature (°C)	37	28.6	28.7	28.65	0.05	28.63	28.67
Dissolved Oxygen	37	6.46	7.19	6.837	0.138	6.791	6.883
Total Ammonia-N	37	0.05	0.11	0.077	0.017	0.072	0.083
Nitrite-N	37	0.008	0.014	0.0105	0.0017	0.0100	0.0111
Nitrate-N	37	12.1	32.2	22.79	6.56	20.60	24.98
Orthophosphate	7	0.92	4.47	2.616	1.392	1.329	3.904
Carbon Dioxide	7	9.5	10.8	10.15	0.50	9.68	10.61
Total Organic Carbon	7	1.060	4.015	2.5700	1.0505	1.5984	3.5415
Hardness	7	118	124	120.9	2.4	118.6	123.2
Alkalinity	7	102	113	107.4	3.9	103.8	111.1

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Table A2. Average water quality results from copper-treated systems during the first antiparasitic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	37	7.02	8.00	7.401	0.266	7.312	7.489
Temperature (°C)	37	28.5	28.7	28.62	0.05	28.60	28.63
Dissolved Oxygen	37	6.45	6.95	6.798	0.108	6.762	6.834
Total Ammonia-N	37	0.05	1.39	0.525	0.509	0.356	0.695
Nitrite-N	37	0.006	1.485	0.4940	0.4913	0.3302	0.6578
Nitrate-N	37	11.5	32.7	22.83	6.57	20.64	25.02
Orthophosphate	7	1.06	4.45	2.754	1.294	1.558	3.951
Carbon Dioxide	7	10.3	11.5	11.05	0.46	10.63	11.48
Total Organic Carbon	7	0.993	4.653	2.7758	1.3436	1.5332	4.0184
Hardness	7	116	124	120.2	2.7	117.7	122.8
Alkalinity	7	104	111	108.6	2.4	106.4	110.8

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Table A3. One-way ANOVA for total ammonia-N from copper-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	37	0.077	0.000	0.0028	3.713	1	3.713	28.64	<0.0001
Copper	37	0.525	0.259	0.0837					

Table A4. One-way ANOVA for nitrite-N from copper-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	37	0.0105	0.0000	0.00028	4.3238	1	4.3238	35.83	<0.0001
Copper	37	0.4940	0.2414	0.08077					

Table A5. One-way ANOVA for nitrate-N from copper-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	37	22.79	43.03	1.078	0.03	1	0.03	0.00	0.9808
Copper	37	22.83	43.18	1.080					

Table A6. Average water quality results from formalin-treated systems during the first antiparasitic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	30	7.43	8.03	7.695	0.195	7.622	7.768
Temperature (°C)	30	28.3	28.6	28.52	0.07	28.49	28.54
Dissolved Oxygen	30	6.47	7.10	6.838	0.100	6.800	6.875
Total Ammonia-N	30	0.04	0.10	0.073	0.017	0.067	0.079
Nitrite-N	30	0.005	0.010	0.076	0.0014	0.0011	0.0018
Nitrate-N	30	12.1	28.7	20.59	5.53	18.53	22.66
Orthophosphate	6	0.93	4.88	2.998	1.441	1.486	4.511
Carbon Dioxide	6	10.6	12.6	11.42	0.92	10.45	12.38
Total Organic Carbon	6	0.990	3.844	2.3106	1.0864	1.1706	3.4507
Hardness	6	116	122	119.0	2.9	116.0	122.0
Alkalinity	6	106	112	109.6	2.2	107.3	111.8

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Table A7. One-way ANOVA for total ammonia-N from formalin-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	30	0.077	0.000	0.0030	0.000	1	0.000	0.84	0.3639
Formalin	30	0.073	0.000	0.0030					

Table A8. One-way ANOVA for nitrite-N from formalin-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	30	0.0100	0.0000	0.00022	0.0001	1	0.0001	52.75	<0.0001
Formalin	30	0.0076	0.0000	0.00025					

Table A9. One-way ANOVA for nitrate-N from formalin-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	30	20.93	34.08	1.066	1.70	1	1.70	0.05	0.8194
Formalin	30	20.59	30.56	1.009					

Table A10. Average water quality results from untreated control systems during the replicate antiparasitic trial (mg/L)

	Descriptive Statistics						
	N	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	37	6.72	7.81	7.412	0.315	7.307	7.517
Temperature (°C)	37	28.5	28.8	28.68	0.06	28.66	28.70
Dissolved Oxygen	37	6.58	7.01	6.856	0.071	6.833	6.880
Total Ammonia-N	37	0.03	0.11	0.068	0.018	0.062	0.074
Nitrite-N	37	0.006	0.014	0.0085	0.0016	0.0013	0.0021
Nitrate N	37	11.8	32.4	22.47	6.58	20.27	24.66
Orthophosphate	7	0.77	5.22	2.987	1.664	1.448	4.526
Carbon Dioxide	7	8.6	12.0	10.89	1.12	9.85	11.93
Total Organic Carbon	7	1.072	4.564	2.8751	1.2501	1.7190	4.0313
Hardness	7	118	124	121.2	2.2	119.1	123.2
Alkalinity	7	106	114	110.9	2.7	108.4	113.4

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Table A11. Average water quality results from copper-treated systems during the replicate antiparasitic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	37	7.05	8.05	7.580	0.255	7.494	7.665
Temperature (°C)	37	28.5	28.8	28.65	0.07	28.63	28.67
Dissolved Oxygen	37	6.57	7.04	6.833	0.083	6.806	6.861
Total Ammonia-N	37	0.04	1.35	0.510	0.490	0.346	0.673
Nitrite-N	37	0.007	1.565	0.5047	0.4904	0.3988	0.6370
Nitrate-N	37	12.8	33.4	22.46	6.46	20.31	24.61
Orthophosphate	7	1.01	5.73	3.200	1.730	1.600	4.801
Carbon Dioxide	7	7.7	11.9	10.89	1.46	9.54	12.24
Total Organic Carbon	7	1.030	4.306	2.7400	1.1841	1.6450	3.8351
Hardness	7	117	123	120.4	1.9	118.6	122.1
Alkalinity	7	98	109	104.1	4.0	100.4	107.8

Table A12. One-way ANOVA for total ammonia-N from copper-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	37	0.068	0.000	0.0029	3.612	1	3.612	30.02	<0.0001
Copper	37	0.063	0.000	0.0806					

Table A13. One-way ANOVA for nitrite-N from copper-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	37	0.0085	0.0000	0.00027	4.5564	1	4.5564	37.90	<0.0001
Copper	37	0.5047	0.2405	0.08062					

Table A14. One-way ANOVA for nitrate-N from copper-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	37	22.47	43.25	1.081	0.00	1	0.00	0.00	0.9957
Copper	37	22.46	41.68	1.061					

Table A15. Average water quality results from formalin-treated systems during the replicate antiparasitic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	30	7.37	8.01	7.651	0.208	7.574	7.729
Temperature (°C)	30	28.3	28.7	28.57	0.09	28.54	28.61
Dissolved Oxygen	30	6.72	6.98	6.848	0.069	6.822	6.874
Total Ammonia-N	30	0.02	0.10	0.063	0.018	0.056	0.070
Nitrite-N	30	0.005	0.013	0.0085	0.0016	0.0013	0.0021
Nitrate-N	30	11.8	28.4	20.61	5.69	18.49	22.74
Orthophosphate	6	0.83	4.67	2.718	1.495	1.150	4.287
Carbon Dioxide	6	8.4	12.1	10.72	1.27	9.39	12.05
Total Organic Carbon	6	1.089	3.651	2.3994	0.9500	1.4024	3.3964
Hardness	6	119	124	120.9	2.2	118.6	123.3
Alkalinity	6	100	108	103.5	2.7	100.6	106.4

Table A16. One-way ANOVA for total ammonia-N from formalin-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	30	0.068	0.000	0.0027	0.001	1	0.001	3.07	0.0851
Formalin	30	0.063	0.000	0.0034					

Table A17. One-way ANOVA for nitrite-N from formalin-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	30	0.0081	0.0000	0.00029	0.0000	1	0.0000	0.72	0.4010
Formalin	30	0.0085	0.0000	0.00029					

Table A18. One-way ANOVA for nitrate-N from formalin-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	30	20.47	31.61	1.026	0.32	1	0.32	0.01	0.9203
Formalin	30	20.61	32.33	1.038					

Table A19. Specific Oxygen Uptake Rate results during the first antiparasitic trial (mg DO/g VS*hr)

	Descriptive Statistics						
	N	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated Control	11	2.742	3.424	3.0104	0.2260	2.8585	3.1622
Copper-treated	11	2.736	5.359	3.7679	0.8971	3.1652	4.3705
Formalin-treated	9	4.986	5.542	5.2629	0.1800	5.1245	5.4013

Table A20. One-way ANOVA for sOUR from copper-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	11	3.0104	0.0511	0.06815	3.1561	1	3.1561	7.38	0.0133
Copper	11	3.7679	0.8047	0.27048					

Table A21. One-way ANOVA for sOUR from formalin-treated systems during the first antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	11	3.0104	0.0511	0.06815	22.2089	1	22.2089	514.72	<0.0001
Formalin	11	5.2629	0.0324	0.06000					

Table A22. Specific Oxygen Uptake Rate (sOUR) results during the replicate antiparasitic trial (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated control	11	5.098	6.007	5.4583	0.3016	5.2557	5.6610
Copper-treated	11	2.648	5.440	5.3668	0.1658	5.2554	5.4782
Formalin-treated	9	4.482	5.202	5.4307	0.2489	5.2394	5.6220

Table A23. One-way ANOVA for sOUR from copper-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	11	5.4583	0.0910	0.09095	0.0461	1	0.0461	0.78	0.3883
Copper	11	5.3668	0.0275	0.05000					

Table A24. One-way ANOVA for sOUR from formalin-treated systems during the replicate antiparasitic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	11	5.4583	0.0910	0.09095	0.0141	1	0.0141	0.17	0.6898
Formalin	11	5.4037	0.0619	0.08296					

Table A25. One-way ANOVA for CARD-FISH of AOB from copper-treated systems during the antiparasitic trials

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	11	3.51	0.06	0.077	1.80	1	1.80	13.19	0.0017
Copper	11	2.94	0.21	0.138					

Table A26. One-way ANOVA for CARD-FISH of NOB from copper-treated systems during the antiparasitic trials

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	11	3.30	0.11	0.102	0.10	1	0.10	0.67	0.4236
Copper	11	3.16	0.19	0.132					

Table A27. Average water quality results from untreated control systems during the first antibiotic trial (mg/L)

	Descriptive Statistics						
	N	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	23	6.40	7.03	6.677	0.201	6.590	6.764
Temperature (°C)	23	26.7	28.0	27.32	0.33	27.18	27.47
Dissolved Oxygen	23	6.58	7.47	6.942	0.205	6.853	7.030
Total Ammonia-N	23	0.04	0.10	0.070	0.018	0.062	0.077
Nitrite-N	23	0.002	0.015	0.0079	0.0031	0.0066	0.0093
Nitrate-N	23	9.1	15.6	12.95	1.62	12.25	13.65
Orthophosphate	8	1.34	4.11	2.919	1.163	1.947	3.892
Carbon Dioxide	8	8.9	12.4	11.31	1.18	10.32	12.30
Total Organic Carbon	8	1.472	2.730	2.0486	0.4846	1.6435	2.4537
Hardness	8	119	127	121.3	2.5	119.2	123.4
Alkalinity	8	100	111	107.0	4.1	103.6	110.4

Table A28. Average water quality results from oxytetracycline-treated systems during the first antibiotic trial (mg/L)

	Descriptive Statistics						
	N	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	18	6.83	7.39	7.044	0.186	6.952	7.137
Temperature (°C)	18	26.8	28.2	27.45	0.40	27.25	27.64
Dissolved Oxygen	18	6.64	7.44	6.976	0.231	6.861	7.091
Total Ammonia-N	18	0.04	0.82	0.280	0.262	0.150	0.410
Nitrite-N	18	0.002	0.431	0.1154	0.1453	0.0432	0.1877
Nitrate-N	18	8.6	15.8	11.92	1.85	11.00	12.84
Orthophosphate	6	1.11	3.00	2.214	0.637	1.546	2.883
Carbon Dioxide	6	11.4	13.3	12.05	0.67	11.35	12.75
Total Organic Carbon	5	1.051	3.024	1.9567	0.8755	0.8695	3.0438
Hardness	7	116	128	121.4	4.3	117.4	125.4
Alkalinity	7	102	113	107.9	4.1	104.1	111.6

Table A29. One-way ANOVA for total ammonia-N from oxytetracycline-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	18	0.071	0.000	0.0043	0.393	1	0.393	11.40	0.0018
OTC	18	0.280	0.069	0.0617					

Table A30. One-way ANOVA for nitrite-N from oxytetracycline-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	18	0.0081	0.0000	0.00083	0.1037	1	0.1037	9.82	0.0035
OTC	18	0.1154	0.0211	0.03424					

Table A31. One-way ANOVA for nitrate-N from oxytetracycline-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	18	12.51	2.32	0.359	3.15	1	2.15	1.09	0.3028
OTC	18	11.92	3.44	0.437					

Table A32. Average water quality results from Romet-30®-treated systems during the first antibiotic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	23	7.04	7.38	7.229	0.075	7.197	7.262
Temperature (°C)	23	26.6	27.8	27.15	0.28	27.03	27.27
Dissolved Oxygen	23	6.96	8.05	7.253	0.328	7.111	7.395
Total Ammonia-N	23	0.05	1.79	0.464	0.543	0.229	0.699
Nitrite-N	23	0.002	1.624	0.3866	0.5405	0.1529	0.6204
Nitrate-N	23	10.9	18.1	14.32	2.16	13.39	15.26
Orthophosphate	8	0.86	3.43	2.557	0.885	1.817	3.297
Carbon Dioxide	8	10.9	12.1	11.69	0.38	11.37	12.01
Total Organic Carbon	8	1.192	3.728	2.3111	0.9058	1.5538	3.0684
Hardness	8	117	123	120.3	2.1	118.5	122.0
Alkalinity	8	103	112	109.1	2.8	106.8	111.4

Table A33. One-way ANOVA for total ammonia-N from Romet-30®-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	23	0.070	0.000	0.0038	1.791	1	1.791	12.12	0.0011
Romet	23	0.464	0.295	0.1133					

Table A34. One-way ANOVA for nitrite-N from Romet-30®-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	23	0.0079	0.0000	0.00065	1.6492	1	1.6492	11.29	0.0016
Romet	23	0.3866	0.2922	0.11271					

Table A35. One-way ANOVA for nitrate-N from Romet-30®-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	23	12.95	2.62	0.337	21.55	1	21.55	5.92	0.0191
Romet	23	14.32	4.66	0.450					

Table A36. Average water quality results from untreated control systems during the replicate antibiotic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	23	7.28	7.58	7.437	0.060	7.411	7.463
Temperature (°C)	23	26.5	28.0	27.29	0.48	27.08	27.50
Dissolved Oxygen	23	7.39	8.81	7.994	0.298	7.865	8.123
Total Ammonia-N	23	0.03	0.09	0.066	0.018	0.058	0.074
Nitrite-N	23	0.006	0.013	0.0086	0.0019	0.0078	0.0095
Nitrate N	23	12.7	20.3	16.21	2.52	15.12	17.30
Orthophosphate	8	0.84	3.98	2.902	1.122	1.964	3.840
Carbon Dioxide	8	8.2	13.3	12.40	1.71	10.97	13.82
Total Organic Carbon	8	1.344	3.636	2.4429	0.7683	1.8005	3.0852
Hardness	8	116	128	121.3	3.6	118.4	124.3
Alkalinity	8	106	114	109.5	2.9	107.1	112.0

Table A37. Average water quality results from oxytetracycline-treated systems during the replicate antibiotic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	18	7.32	7.71	7.495	0.106	7.443	7.548
Temperature (°C)	18	26.7	28.0	27.18	0.42	26.97	27.39
Dissolved Oxygen	18	7.42	8.78	8.076	0.309	7.923	8.230
Total Ammonia-N	18	0.04	1.05	0.326	0.307	0.173	0.479
Nitrite-N	18	0.005	0.520	0.1620	0.1752	0.0749	0.2492
Nitrate-N	18	12.0	21.4	15.60	2.75	14.23	16.97
Orthophosphate	6	0.63	5.18	3.379	1.832	1.457	5.302
Carbon Dioxide	6	7.8	13.3	12.24	2.18	9.95	14.53
Total Organic Carbon	5	1.301	2.720	2.0722	0.5438	1.3969	2.7475
Hardness	7	114	129	121.8	4.7	117.5	126.2
Alkalinity	7	105	114	109.7	3.1	106.8	112.5

Table A38. One-way ANOVA for total ammonia-N from oxytetracycline-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	18	0.064	0.000	0.0041	0.618	1	0.618	13.07	0.0010
OTC	18	0.326	0.094	0.0724					

Table A39. One-way ANOVA for nitrite-N from oxytetracycline-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	18	0.0090	0.0000	0.00046	0.2107	1	0.2107	13.73	0.0007
OTC	18	0.1620	0.0307	0.04129					

Table A40. One-way ANOVA for nitrate-N from oxytetracycline-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	18	15.21	3.37	0.433	1.36	1	1.36	0.25	0.6213
OTC	18	15.60	7.58	0.649					

Table A41. Average water quality results from Romet-30®-treated systems during the replicate antibiotic trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	23	7.32	7.66	7.501	0.071	7.471	7.532
Temperature (°C)	23	26.5	28.0	27.48	0.39	27.31	27.65
Dissolved Oxygen	23	7.45	8.63	8.068	0.261	7.955	8.181
Total Ammonia-N	23	0.02	1.53	0.378	0.464	0.178	0.579
Nitrite-N	23	0.006	1.054	0.2732	0.3445	0.1242	0.4221
Nitrate-N	23	12.4	19.7	17.07	2.56	15.97	18.18
Orthophosphate	8	1.39	4.95	3.887	1.312	2.790	4.984
Carbon Dioxide	8	7.1	13.5	12.28	2.11	10.52	14.04
Total Organic Carbon	8	1.687	3.792	2.5985	0.5937	2.1022	3.0948
Hardness	8	116	130	122.3	4.0	118.9	125.6
Alkalinity	8	104	114	110.1	3.1	107.5	112.8

Table A42. One-way ANOVA for total ammonia-N from Romet-30®-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	23	0.066	0.000	0.0037	1.122	1	1.122	10.42	0.0024
Romet	23	0.378	0.215	0.0967					

Table A43. One-way ANOVA for nitrite-N from Romet-30®-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	23	0.0086	0.0000	0.00040	0.8048	1	0.8048	13.56	0.0006
Romet	23	0.2732	0.1187	0.07183					

Table A44. One-way ANOVA for nitrate-N from Romet-30®-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	23	16.21	6.37	0.526	8.59	1	8.59	1.33	0.2554
Romet	23	17.07	6.56	0.534					

Table A45. Specific Oxygen Uptake Rate results during the first antibiotic trial (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated Control	14	4.981	5.713	5.2924	0.2017	5.1759	5.4088
Oxytetracycline-treated	9	1.205	5.173	2.7371	1.3951	1.6647	3.8095
Romet-30®-treated	14	1.149	5.045	3.2189	1.1244	2.5697	3.8681

Table A46. One-way ANOVA for sOUR from oxytetracycline-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	14	5.2924	0.0407	0.05390	28.6411	1	28.6411	28.80	<.0001
OTC	9	2.7371	1.9463	0.46504					

Table A47. One-way ANOVA for total sOUR from Romet-30®-treated systems during the first antibiotic trial

Group	Count	Average	Variance	SE	SS	Df	MS	F	P-value
Control	14	5.2924	0.0407	0.05390	30.0937	1	30.0937	46.12	<0.0001
Romet	14	3.2189	1.2643						

Table A48. Specific Oxygen Uptake Rate (sOUR) results during the replicate antibiotic trial (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated Control	14	4.778	5.253	5.0658	0.1324	4.9893	5.1422
Oxytetracycline-treated	9	1.246	5.014	2.8470	1.1653	1.9513	3.7427
Romet-30®-treated	14	1.411	5.157	3.5306	1.1967	2.8397	4.2216

Table A49. One-way ANOVA for sOUR from oxytetracycline-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	14	5.0658	0.0175	0.03540	21.6753	1	21.6753	31.52	<0.0001
OTC	9	2.8470	1.3580	0.38844					

Table A50. One-way ANOVA for total sOUR from Romet-30®-treated systems during the replicate antibiotic trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	14	5.0658	0.0175	0.03540	16.4961	1	16.4961	22.76	<0.0001
Romet	14	3.5306	1.4322	0.31984					

Table A51. One-way ANOVA for CARD-FISH of AOB from oxytetracycline-treated systems during the antibiotic trials

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	15	4.59	0.18	0.110	0.02	1	0.02	0.09	0.7619
OTC	15	4.64	0.17	0.105					

Table A52. One-way ANOVA for CARD-FISH of NOB from oxytetracycline-treated systems during the antibiotic trials

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	15	4.95	0.16	0.105	0.56	1	0.56	2.63	0.1161
OTC	15	4.67	0.26	0.132					

Table A53. One-way ANOVA for CARD-FISH of AOB from Romet-treated systems during the antibiotic trials

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	15	4.59	0.18	0.110	0.02	1	0.02	0.11	0.7443
Romet	15	4.54	0.21	0.119					

Table A54. One-way ANOVA for CARD-FISH of NOB from Romet-treated systems during the antibiotic trials

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	15	4.95	0.16	0.105	1.24	1	1.24	6.28	0.0183
Romet	15	4.54	0.23	0.124					

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Table A55. Average water quality results from untreated control systems during the first off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	21	6.89	7.08	6.999	0.050	6.976	7.022
Temperature (°C)	21	27.7	28.0	27.83	0.09	27.78	27.87
Dissolved Oxygen	21	6.75	7.08	6.907	0.088	6.867	6.947
Total Ammonia-N	21	0.01	0.07	0.045	0.016	0.038	0.052
Nitrite-N	21	0.003	0.012	0.0083	0.0021	0.0073	0.0092
Nitrate-N	21	11.8	18.3	15.08	2.04	14.15	16.01
Orthophosphate	3	0.97	1.89	1.424	0.460	0.281	2.567
Carbon Dioxide	3	10.4	11.0	10.70	0.27	10.04	11.36
Total Organic Carbon	3	1.490	2.087	1.8356	0.3094	1.0669	2.6043
Hardness	3	118	119	118.3	0.6	116.9	119.8
Alkalinity	3	103	105	103.9	1.0	101.4	106.4

Table A56. Average water quality results from high-dose, short-term $KMnO_4$-treated systems during the first off-label chemical trial (mg/L)

	Descriptive Statistics						
	N	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	17	6.98	7.14	7.053	0.045	7.030	7.076
Temperature (°C)	17	27.7	28.1	27.84	0.10	27.79	27.89
Dissolved Oxygen	17	6.71	7.08	6.881	0.095	6.833	6.930
Total Ammonia-N	17	0.05	0.09	0.065	0.013	0.058	0.072
Nitrite-N	17	0.006	0.011	0.0077	0.0016	0.0069	0.0085
Nitrate-N	17	11.9	16.5	14.34	1.32	13.66	15.02
Orthophosphate	3	1.06	1.93	1.434	0.451	0.315	2.554
Carbon Dioxide	3	11.0	11.4	11.18	0.17	10.75	11.60
Total Organic Carbon	3	1.187	2.549	1.6939	0.7449	-0.1565	3.5444
Hardness	3	118	120	119.1	0.8	117.0	121.2
Alkalinity	3	101	108	104.2	3.7	95.1	113.3

Table A57. One-way ANOVA for total ammonia-N from high-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.046	0.000	0.0041	0.003	1	0.003	13.17	0.0010
$KMnO_4$	17	0.065	0.000	0.0032					

Table A58. One-way ANOVA for nitrite-N from high-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.0082	0.0000	0.00057	0.0000	1	0.0000	0.56	0.4583
$KMnO_4$	17	0.0077	0.0000	0.00038					

Table A59. One-way ANOVA for nitrate-N from high-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	14.42	2.70	0.398	0.05	1	0.05	0.02	0.8761
$KMnO_4$	17	14.34	1.75	0.321					

Table A60. Average water quality results from low-dose, long-term $KMnO_4$-treated systems during the first off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	17	6.94	7.25	7.132	0.095	7.083	7.181
Temperature (°C)	17	27.6	28.0	27.85	0.14	27.77	27.92
Dissolved Oxygen	17	6.55	7.00	6.828	0.097	6.778	6.878
Total Ammonia-N	17	0.04	0.10	0.079	0.019	0.069	0.089
Nitrite-N	17	0.005	0.010	0.0073	0.0017	00064	0.0081
Nitrate-N	17	12.0	16.7	14.30	1.44	13.56	15.03
Orthophosphate	3	1.17	1.95	1.498	0.408	0.485	2.511
Carbon Dioxide	3	11.2	13.2	12.34	1.02	9.81	14.88
Total Organic Carbon	3	1.149	2.106	1.4814	0.5409	0.1378	2.8250
Hardness	3	119	121	120.0	0.9	117.8	122.2
Alkalinity	3	109	114	111.1	2.9	103.9	118.3

Table A61. One-way ANOVA for total ammonia-N from low-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.046	0.000	0.0041	0.009	1	0.009	27.35	0.0001
$KMnO_4$	17	0.079	0.000	0.0047					

Table A62. One-way ANOVA for nitrite-N from low-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.0082	0.0000	0.00057	0.0000	1	0.0000	1.74	0.1965
$KMnO_4$	17	0.0073	0.0000	0.00041					

Table A63. One-way ANOVA for nitrate-N from low-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	14.42	2.70	0.398	0.13	1	0.13	0.05	0.8198
$KMnO_4$	17	14.30	2.06	0.348					

Table A64. Average water quality results from Chloramine-T-treated systems during the first off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	21	7.01	7.26	7.137	0.064	7.108	7.167
Temperature (°C)	21	27.5	28.0	27.72	0.15	27.65	27.78
Dissolved Oxygen	21	6.70	6.98	6.839	0.082	6.802	6.876
Total Ammonia-N	21	0.02	0.11	0.062	0.023	0.052	0.073
Nitrite-N	21	0.004	0.014	0.0082	0.0026	0.0070	0.0094
Nitrate-N	21	10.8	17.6	14.80	2.01	13.89	15.72
Orthophosphate	3	1.05	1.80	1.400	0.375	0.470	2.330
Carbon Dioxide	3	10.8	13.9	12.28	1.58	8.37	16.20
Total Organic Carbon	3	1.230	2.000	1.5316	0.4109	0.5108	2.5524
Hardness	3	118	121	119.7	1.4	116.1	123.3
Alkalinity	3	114	121	118.5	3.9	108.8	128.2

Table A65. One-way ANOVA for total ammonia-N from Chloramine-T treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	21	0.045	0.000	0.0036	0.003	1	0.003	7.82	0.0079
Cl-T	21	0.062	0.001	0.0050					

Table A66. One-way ANOVA for nitrite-N from Chloramine-T treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	21	0.0083	0.0000	0.00047	0.0000	1	0.0000	0.01	0.9313
Cl-T	21	0.0082	0.0000	0.00056					

Table A67. One-way ANOVA for nitrate-N from Chloramine-T treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	21	15.08	4.15	0.444	0.81	1	0.81	0.20	0.6581
Cl-T	21	14.80	4.05	0.439					

Table A68. Average water quality results from untreated control systems during the replicate off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	21	6.95	7.32	7.115	0.115	7.063	7.168
Temperature (°C)	21	27.7	28.0	27.85	0.09	27.81	27.89
Dissolved Oxygen	21	6.63	6.97	6.815	0.080	6.778	6.851
Total Ammonia-N	21	0.00	0.12	0.060	0.026	0.048	0.072
Nitrite-N	21	0.004	0.012	0.0074	0.0023	0.0064	0.0084
Nitrate-N	21	10.8	17.5	14.76	1.91	13.89	15.63
Orthophosphate	3	1.15	2.14	1.607	0.496	0.374	2.839
Carbon Dioxide	3	9.8	13.8	11.84	1.98	6.92	16.77
Total Organic Carbon	3	1.632	2.379	1.9847	0.3751	1.0529	2.9164
Hardness	3	117	117	117.2	0.2	116.7	117.7
Alkalinity	3	107	109	108.1	1.3	105.0	111.2

Table A69. Average water quality results from high-dose, short-term $KMnO_4$-treated systems during the replicate off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	17	6.93	7.20	7.087	0.082	7.045	7.129
Temperature (°C)	17	27.7	28.0	27.82	0.09	27.77	27.87
Dissolved Oxygen	17	6.68	6.96	6.822	0.069	6.786	6.857
Total Ammonia-N	17	0.03	0.12	0.077	0.022	0.066	0.089
Nitrite-N	17	0.004	0.014	0.0091	0.0024	0.0078	0.0103
Nitrate-N	17	11.9	16.6	14.33	1.40	13.61	15.05
Orthophosphate	3	1.07	1.94	1.449	0.449	0.334	2.564
Carbon Dioxide	3	10.6	12.8	11.81	1.08	9.12	14.50
Total Organic Carbon	3	1.543	2.153	1.8632	0.3063	1.1023	2.6242
Hardness	3	120	122	121.1	0.8	119.0	123.2
Alkalinity	3	101	108	104.0	3.4	95.6	112.4

Table A70. One-way ANOVA for total ammonia-N from high-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.057	0.001	0.0066	0.004	1	0.004	6.01	0.0199
$KMnO_4$	17	0.077	0.000	0.0053					

Table A71. One-way ANOVA for nitrite-N from high-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.0073	0.0000	0.00060	0.0000	1	0.0000	4.48	0.0421
$KMnO_4$	17	0.0092	0.0000	0.00058					

Table A72. One-way ANOVA for nitrate-N from high-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	14.18	2.69	0.397	0.19	1	0.19	0.008	0.7743
$KMnO_4$	17	14.33	1.95	0.339					

Table A73. Average water quality results from low-dose, long-term $KMnO_4$-treated systems during the replicate off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	17	7.02	7.26	7.130	0.068	7.095	7.165
Temperature (°C)	17	27.5	27.9	27.67	0.08	27.63	27.71
Dissolved Oxygen	17	6.67	6.92	6.820	0.070	6.784	6.856
Total Ammonia-N	17	0.02	0.11	0.065	0.021	0.055	0.076
Nitrite-N	17	0.005	0.012	0.0082	0.0022	0.0071	0.0093
Nitrate-N	17	12.0	17.2	14.40	1.38	13.70	15.11
Orthophosphate	3	1.07	1.85	1.446	0.393	0.470	2.421
Carbon Dioxide	3	10.8	12.6	11.72	0.87	9.57	13.88
Total Organic Carbon	3	1.877	2.477	2.1233	0.3143	1.3427	2.9040
Hardness	3	120	122	121.2	1.2	118.3	124.1
Alkalinity	3	104	106	105.3	0.9	103.1	107.5

Table A74. One-way ANOVA for total ammonia-N from low-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.057	0.001	0.0066	0.001	1	0.001	1.07	0.3080
$KMnO_4$	17	0.065	0.000	0.0050					

Table A75. One-way ANOVA for nitrite-N from low-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	0.0073	0.0000	0.00060	0.0000	1	0.0000	1.29	0.2640
$KMnO_4$	17	0.0082	0.0000	0.00056					

Table A76. One-way ANOVA for nitrate-N from low-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	17	14.18	2.69	0.397	0.44	1	0.44	0.19	0.6643
$KMnO_4$	17	14.40	1.90	0.334					

Table A77. Average water quality results from Chloramine-T-treated systems during the replicate off-label chemical trial (mg/L)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
pH	21	6.98	7.25	7.110	0.077	7.075	7.146
Temperature (°C)	21	27.5	27.8	27.62	0.10	27.57	27.66
Dissolved Oxygen	21	6.63	7.02	6.809	0.092	6.767	6.851
Total Ammonia-N	21	0.01	0.11	0.064	0.027	0.052	0.076
Nitrite-N	21	0.003	0.016	0.0087	0.0031	0.0073	0.0101
Nitrate-N	21	12.1	17.9	14.96	1.68	14.19	15.72
Orthophosphate	3	1.18	2.14	1.560	0.510	0.292	2.828
Carbon Dioxide	3	11.7	13.7	12.63	1.01	10.13	15.13
Total Organic Carbon	3	1.740	2.421	2.1157	0.3461	1.2560	2.9753
Hardness	3	117	128	121.3	5.9	106.8	135.9
Alkalinity	3	103	121	113.0	9.2	90.2	135.8

Table A78. One-way ANOVA for total ammonia-N from Chloramine-T treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	21	0.060	0.001	0.0056	0.000	1	0.000	0.24	0.6254
Cl-T	21	0.064	0.001	0.0058					

Table A79. One-way ANOVA for nitrite-N from Chloramine-T treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	21	0.0074	0.0000	0.00049	0.0000	1	0.0000	2.39	0.1302
Cl-T	21	0.0087	0.0000	0.00068					

Table A80. One-way ANOVA for nitrate-N from Chloramine-T treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	21	14.76	3.65	0.417	0.43	1	0.43	0.13	0.7186
Cl-T	21	14.96	2.83	0.367					

Table A81. Specific Oxygen Uptake Rate results during the first off-label chemical trial (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated Control	6	5.229	5.349	5.2893	0.0488	5.2381	5.3405
High-dose, short-term $KMnO_4$	5	5.084	5.196	5.1386	0.0424	5.0859	5.1913
Low-dose, long-term $KMnO_4$	5	5.210	5.324	5.2588	0.0429	5.2056	5.3120
Chloramine T-treated	6	5.137	5.607	5.3338	0.1781	5.1469	5.5207

Table A82. One-way ANOVA for sOUR from high dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	5	5.2773	0.0024	0.01946	0.0481	1	0.0481	26.05	0.0009
$KMnO_4$	5	5.1386	0.0018	0.01898					

Table A83. One-way ANOVA for total sOUR from low-dose $KMnO_4$-treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	5	5.2773	0.0024	0.01946	0.0009	1	0.0009	0.46	0.5167
$KMnO_4$	5	5.2588	0.0018	0.01917					

Table A84. One-way ANOVA for total sOUR from Chloramine-T treated systems during the first off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	6	5.2893	0.0024	0.05331	0.0059	1	0.0059	0.35	0.5681
Cl-T	6	5.3338	0.0317	0.05331					

Table A85. Specific Oxygen Uptake Rate (sOUR) results during the replicate off-label chemical trial (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated control	6	5.219	5.341	6.2878	0.0463	5.2393	5.3364
High-dose, short-term $KMnO_4$	5	5.121	5.273	5.1813	0.0626	5.1035	5.2591
Low-dose, long-term $KMnO_4$	5	5.115	5.290	5.1815	0.0657	5.0999	5.2631
Chloramine T-treated	6	4.990	5.229	5.1153	0.0780	5.0335	5.1972

Table A86. One-way ANOVA for sOUR from high dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	5	5.2913	0.0021	0.02275	0.0302	1	0.0302	9.28	0.0159
$KMnO_4$	5	5.1813	0.0039	0.02802					

Table A87. One-way ANOVA for total sOUR from low-dose $KMnO_4$-treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	5	5.2913	0.0021	0.02275	0.0301	1	0.0301	8.72	0.0184
$KMnO_4$	5	5.1815	0.0043	0.02939					

Table A88. One-way ANOVA for total sOUR from Chloramine-T treated systems during the replicate off-label chemical trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	6	5.2878	0.0021	0.01889	0.0893	1	0.0893	21.72	0.0009
Cl-T	6	5.1153	0.0061	0.03183					

Appendix B: Data Associated with Chapter 3

Table B1. 24-hour total ammonia-nitrogen (TAN) – antibiotic control average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	0.50	10.58	4.845	4.010	1.137	8.554
Day 5 (mg/L)	7	0.00	10.58	3.500	4.052	-0.247	7.247
Day 10 (mg/L)	7	0.00	10.25	3.214	3.953	-0.442	6.871
Day 15 (mg/L)	7	0.00	10.25	2.690	3.749	-0.776	6.157

Table B2. 24-hour total ammonia-nitrogen (TAN) – oxytetracycline-treated average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	0.33	10.67	4.631	4.032	0.902	8.360
Day 5 (mg/L)	7	4.67	9.67	3.500	4.052	-0.247	7.247
Day 10 (mg/L)	7	13.83	14.33	3.214	3.953	-0.442	6.871
Day 15 (mg/L)	7	22.17	23.42	2.690	3.749	-0.776	6.157

Table B3. 24-hour total ammonia-nitrogen (TAN) – Romet-30®-treated average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	1.00	10.00	4.631	4.032	0.902	8.360
Day 5 (mg/L)	7	8.08	10.17	8.750	0.799	8.011	9.489
Day 10 (mg/L)	7	17.17	17.75	17.452	0.209	17.259	17.646
Day 15 (mg/L)	7	25.63	27.00	26.321	0.472	25.884	26.758

Table B4. 24-hour total ammonia-nitrogen (TAN) – copper control average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	0.08	10.00	4.2738	4.1005	0.4815	8.0661
Day 5 (mg/L)	7	0.42	9.83	4.2738	3.5984	0.9459	7.6018
Day 10 (mg/L)	7	0.08	9.92	3.7857	3.6874	0.3754	7.1960

Table B5. 24-hour total ammonia-nitrogen (TAN) – low-dose copper average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	0.25	10.25	4.3452	4.0553	0.5947	8.0958
Day 5 (mg/L)	7	7.17	10.58	8.4405	1.2507	7.2838	9.5971
Day 10 (mg/L)	7	7.75	9.67	8.5833	0.8361	7.8101	9.3566

Table B6. 24-hour total ammonia-nitrogen (TAN) – medium-dose copper average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	0.50	10.33	4.5476	3.9598	0.8854	8.2098
Day 5 (mg/L)	7	7.33	9.83	8.4167	0.9141	7.5712	9.2621
Day 10 (mg/L)	7	8.00	9.92	8.6548	0.7629	7.9492	9.3603

Table B7. 24-hour total ammonia-nitrogen (TAN) – high-dose copper average

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Day 0 (mg/L)	7	0.42	10.25	4.5476	3.9907	0.5947	8.0958
Day 5 (mg/L)	7	7.42	9.92	8.5833	0.9778	7.6791	9.4876
Day 10 (mg/L)	7	7.50	10.33	8.5238	1.0214	7.5791	9.4685

Table B8. Specific oxygen uptake rate (sOUR) results during antibiotic trials (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated Control	4	0.073	0.882	0.5108	0.3720	-0.0811	1.1027
Oxytetracycline-treated	4	0.066	0.093	0.0744	0.0125	0.0545	0.0943
Romet-30®-treated	4	0.044	0.086	0.0614	0.0205	0.0288	0.0939

Table B9. Specific oxygen uptake rate (sOUR) results during copper trials (mg DO/g VS*hr)

	Descriptive Statistics						
	n	Min	Max	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Untreated Control	3	0.118	0.756	0.4429	0.3195	-0.3507	1.2365
Low-dose (0.125 ppm)	3	0.103	0.375	0.2775	0.1511	-0.0979	0.6528
Medium-dose (0.25 ppm)	3	0.109	0.368	0.2764	0.1450	-0.0840	0.6367
High-dose (0.5 ppm)	3	0.105	0.260	0.1817	0.0777	-0.0113	0.3746

Table B10. One-way ANOVA of CARD-FISH analysis of AOB population during oxytetracycline trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	4	44.5	6.3	1.26	21.1	1	21.1	4.57	0.0764
OTC	4	41.3	2.9	0.85					

Table B11. One-way ANOVA of CARD-FISH analysis of NOB population during oxytetracycline trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	4	31.3	2.9	0.85	24.5	1	24.5	8.40	0.0274
OTC	4	34.8	2.9	0.85					

Table B12. One-way ANOVA of CARD-FISH analysis of AOB population during Romet trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	4	44.5	6.3	1.26	18.0	1	18.0	2.70	0.1515
Romet	4	41.5	7.0	1.32					

Table B13. One-way ANOVA of CARD-FISH analysis of NOB population during Romet trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	4	31.3	2.9	0.85	6.1	1	6.1	0.86	0.3896
Romet	4	33.0	11.3	1.68					

Table B14. One-way ANOVA of CARD-FISH analysis of AOB population during low-dose copper trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	3	43.3	2.3	0.88	1.5	1	1.5	0.35	0.5879
Copper	3	44.3	6.3	1.45					

Table B15. One-way ANOVA of CARD-FISH analysis of NOB population during low-dose copper trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	3	44.3	9.3	1.76	10.7	1	10.7	1.36	0.3081
Copper	3	41.7	6.3	1.45					

Table B16. One-way ANOVA of CARD-FISH analysis of AOB population during therapeutic-dose copper trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	3	43.3	2.3	0.88	0.7	1	0.7	0.20	0.6779
Copper	3	42.7	4.3	1.20					

Table B17. One-way ANOVA of CARD-FISH analysis of NOB population during therapeutic-dose copper trial

Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	3	44.3	9.3	1.76	4.2	1	4.2	0.61	0.4785
Copper	3	42.7	4.3	1.20					

Table B18. One-way ANOVA of CARD-FISH analysis of AOB population during high-dose copper trial

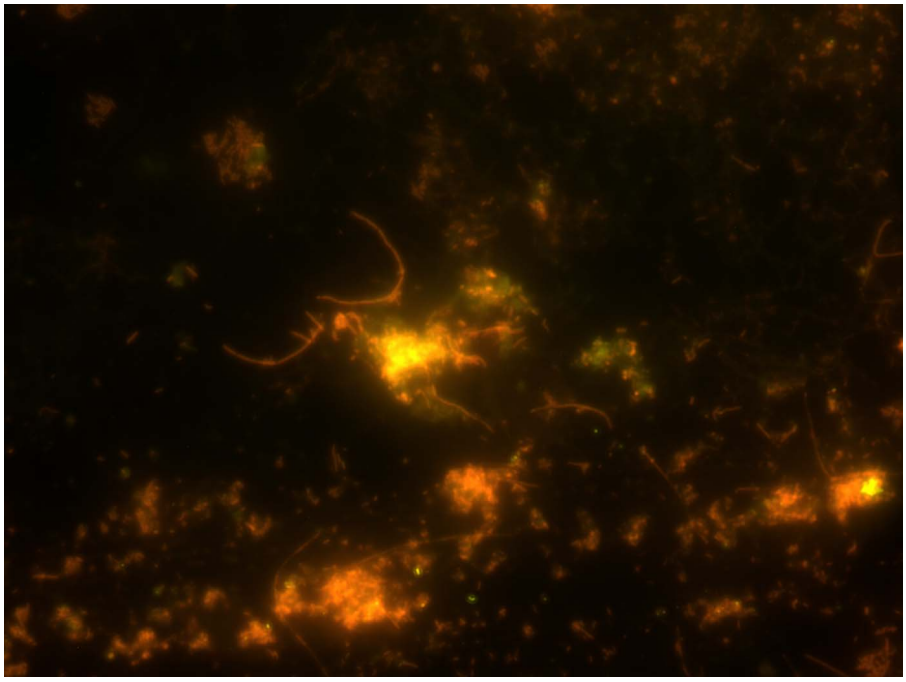
Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	3	43.3	2.3	0.88	4.2	1	4.2	0.89	0.3982
Copper	3	45.0	7.0	1.53					

Table B19. One-way ANOVA of CARD-FISH analysis of NOB population during high-dose copper trial

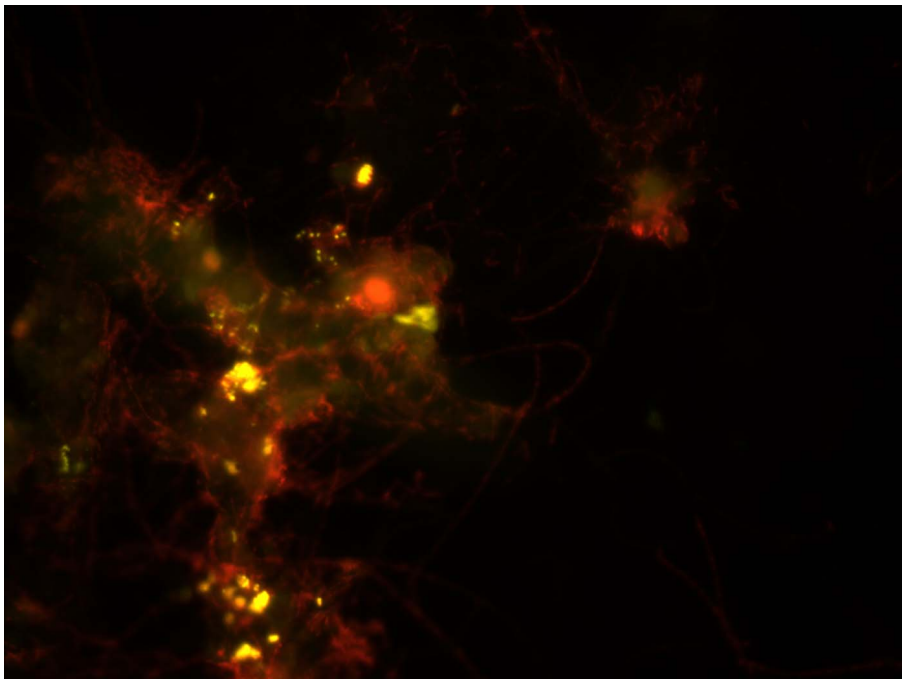
Group	Count	Average	Variance	SE	SS	df	MS	F	P-value
Control	3	44.3	9.3	1.76	37.5	1	37.5	4.79	0.0939
Copper	3	39.3	6.3	1.45					

Appendix C: Sample CARD-FISH Images

(a)



(b)



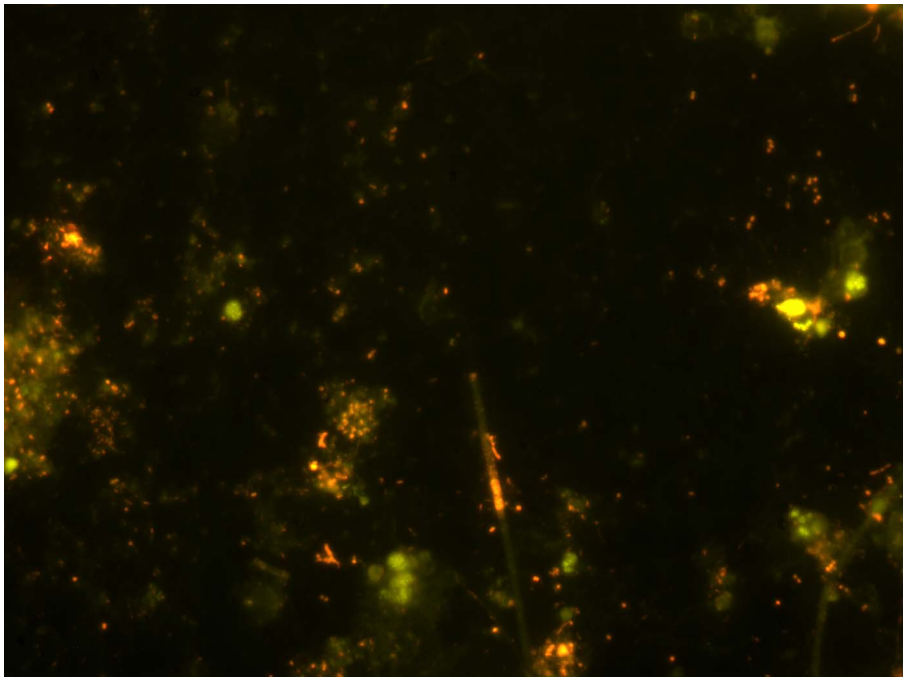
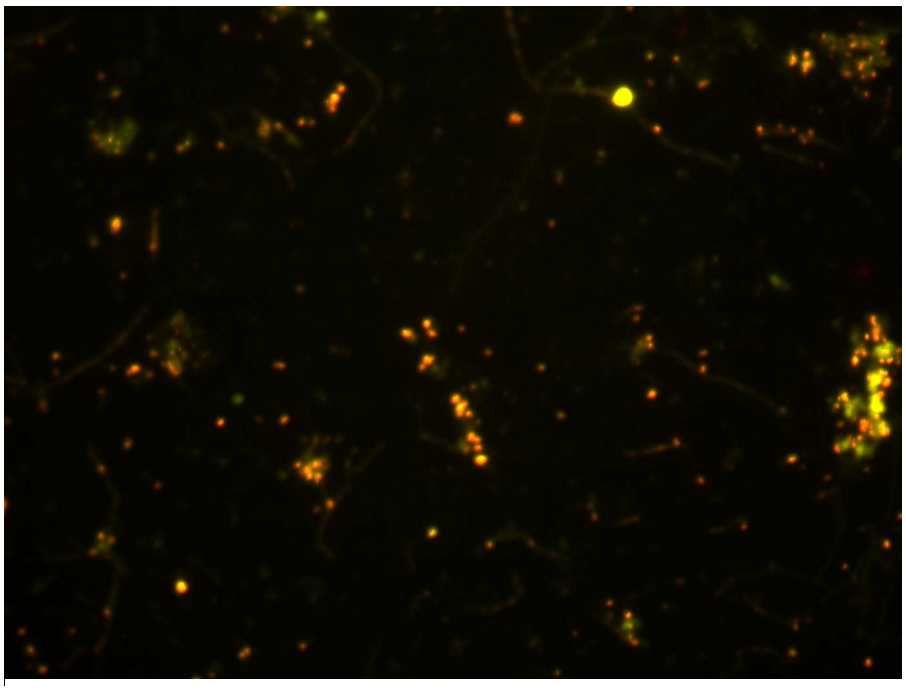
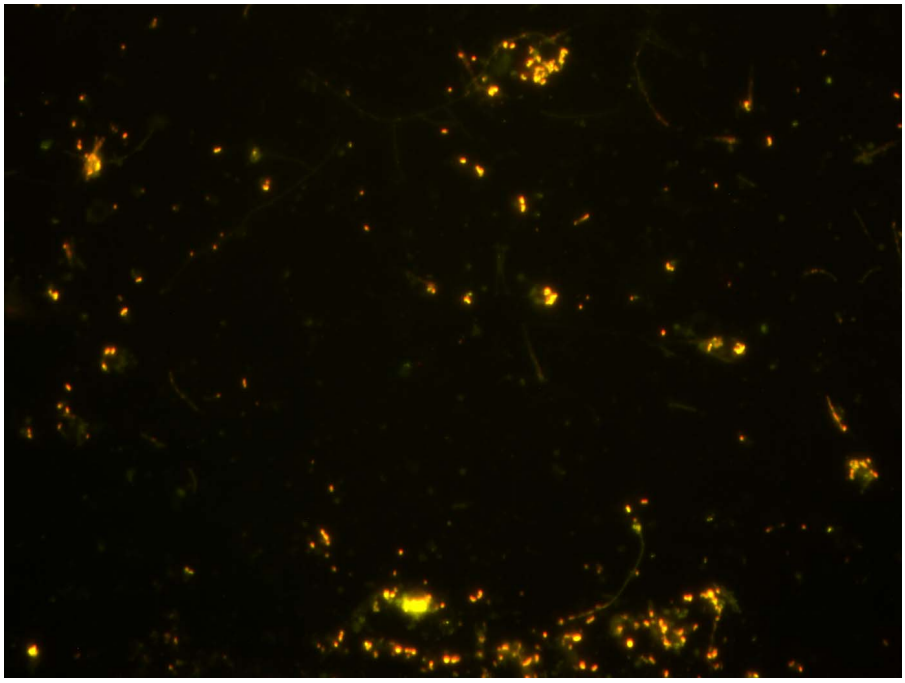
(c) 

Figure C1. Sample images from (a) untreated control, (b) oxytetracycline-treated, and (c) Romet-treated aquaria during antibiotic trials; Green – AOB, Red – EUB

(a)



(b)



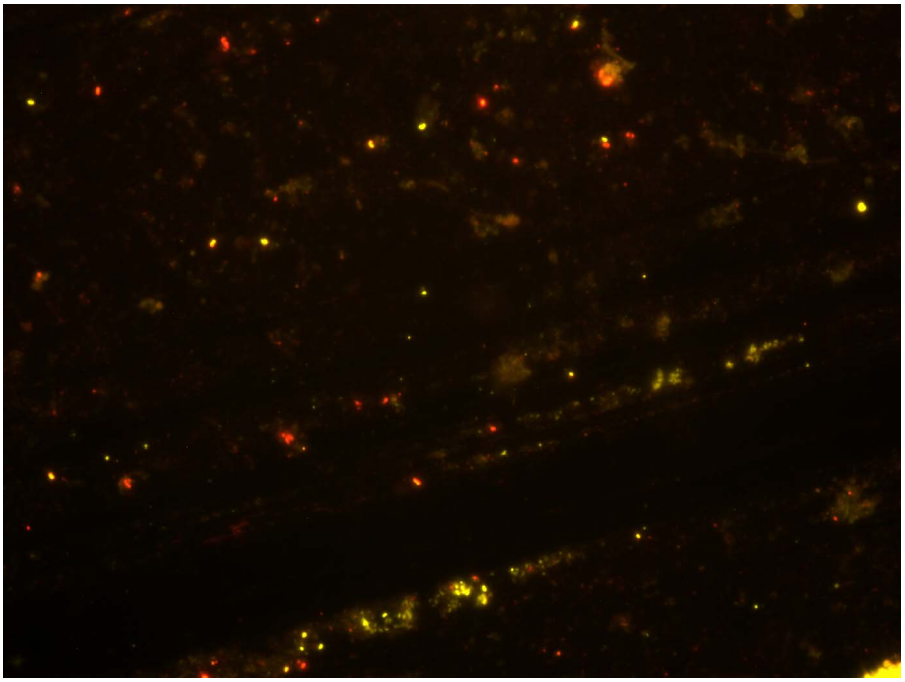
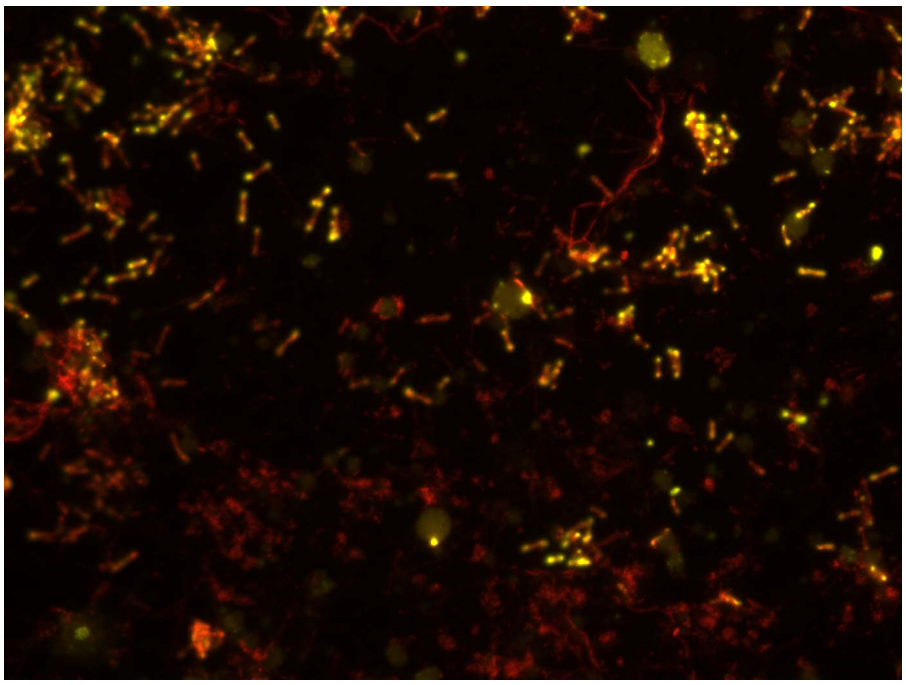
(c) 

Figure C2. Sample images from (a) untreated control, (b) oxytetracycline-treated, and (c) Romet-treated aquaria during antibiotic trials; Green – NOB, Red – EUB

(a)



(b)

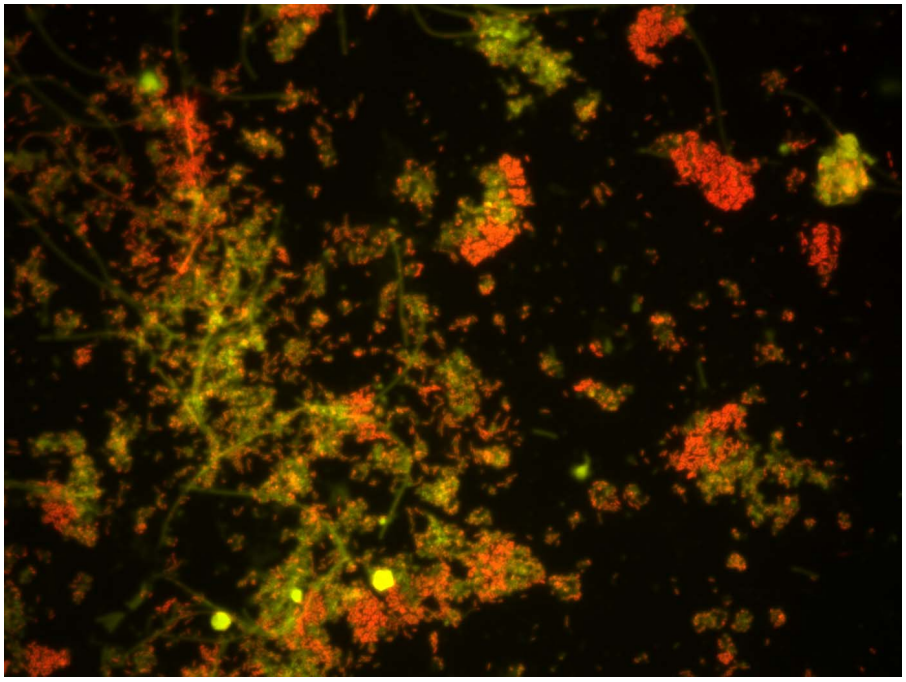
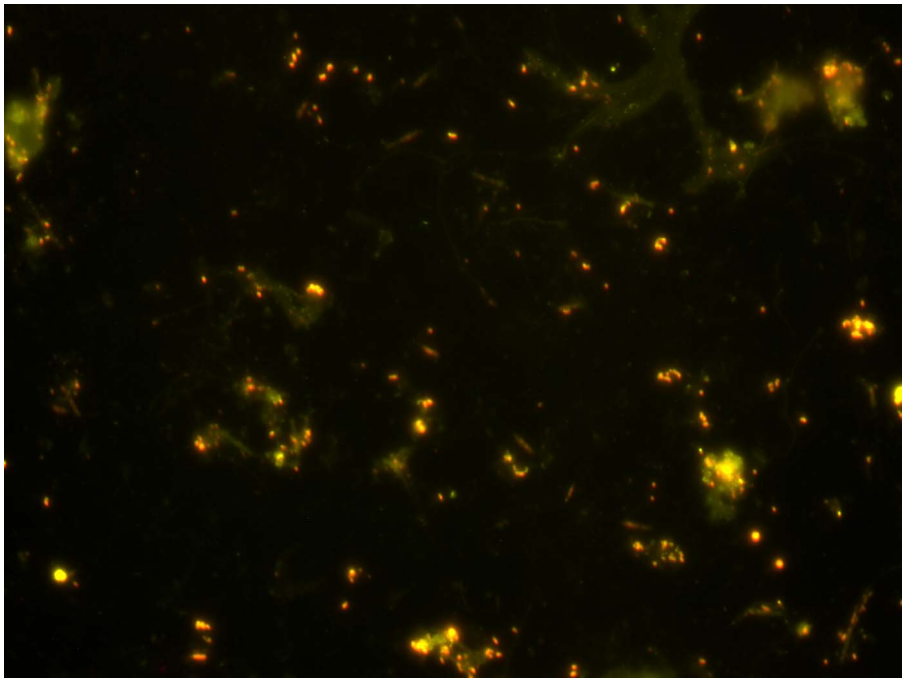


Figure C3. Sample images from (a) untreated control and (b) copper-treated aquaria during copper trials; Green – AOB, Red – EUB

(a)



(b)

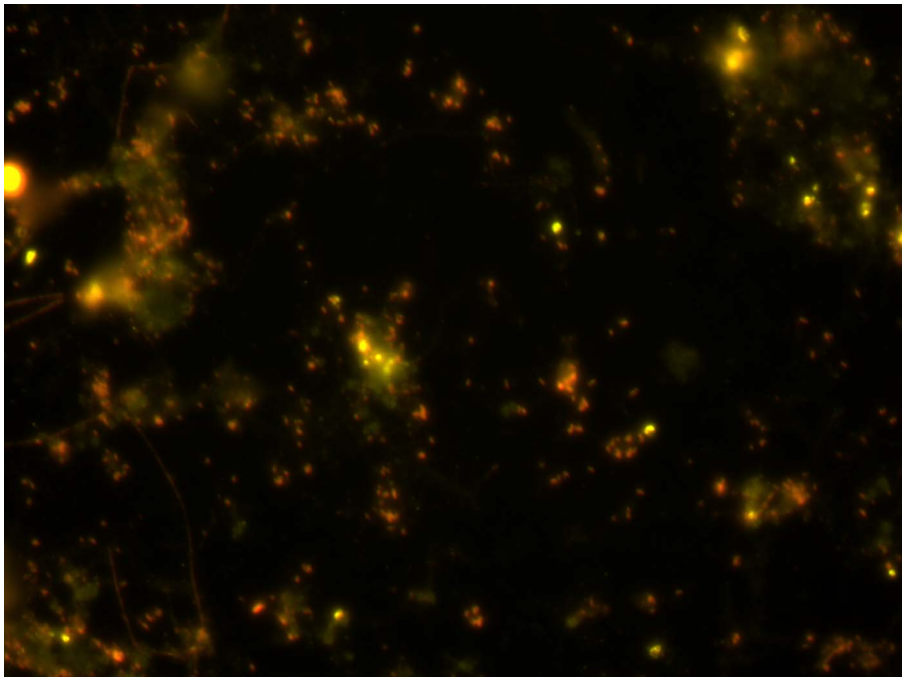


Figure C4. Sample images from (a) untreated control and (b) copper-treated aquaria during copper trials; Green – NOB, Red – EUB