METAL ACCUMULATION IN GILL EPITHELIUM AND LIVER TISSUE IN STEELHEAD (*Oncorhynchus mykiss*) REARED IN RECLAIMED WASTEWATER

By

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ABSTRACT

Metal Accumulation in Gill Epithelium and Liver Tissue in Steelhead (*Oncorhynchus mykiss*) Reared in Reclaimed Wastewater

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The purpose of this study was to assess the bioaccumulation of copper, cadmium and zinc in steelhead trout (*Oncorhynchus mykiss)* gill epithelium and liver tissue subsequent to rearing in reclaimed wastewater for 12 months. Copper, cadmium and zinc are metals commonly found in wastewater and at certain concentrations can be toxic to fish. Previous studies found copper, cadmium and zinc had the highest accumulation in gill epithelium and liver tissue of trout species. Copper found in gill and liver tissue of trout in Summer Ponds 1 and 2 at the end of twelve months was lower than the reference site, conversely, mean aqueous copper concentrations in Summer Ponds were higher then the reference site. The bioaccumulation of copper in the liver tissue of trout reared in wastewater was considered not to be at toxic levels. Cadmium in gill epithelium and liver tissue and copper in gill epithelium was below levels of detection before and after twelve months exposure. Zinc found in gill and liver tissue of trout in Summer Ponds 1 and 2 at the end of twelve months was higher than the reference site. Mean aqueous zinc concentrations at Summer Pond 1 and 2 were higher than the reference site. Zinc was not considered to be at toxic levels. In summary, concentrations of copper, cadmium and zinc in the Summer Ponds were below toxic concentrations and the wastewater Summer Ponds met the necessary metabolic requirements for rearing trout.

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INTRODUCTION

Accumulation of metals in tissues of salmonids raised in reclaimed wastewater is virtually unexplored due to the novelty of rearing salmonids in wastewater (Allen 1987). One serious bottleneck for wastewater aquaculture expansion is the subsequent risks heavy metals present to public health due to heavy metal concentrations in wastewater (Phillips and Russo 1978, Sorensen 1991, Deb and Santra 1997, Saiki et al. 2005, Spencer and Sickle 2006, Ciardullo et al. 2008). In this study, I evaluated the accumulation of copper, cadmium and zinc in gill epithelium and liver tissues of steelhead (*Oncorhynchus mykiss*) reared for twelve months in reclaimed wastewater.

Aquaculture faces mounting pressure to provide an abundant, inexpensive and renewable source of dietary protein (Castle 2002). One reason is local and regional populations of salmonids have severely declined (California Fish and Game 2004, 2007). Loss and alteration of habitat are believed to have been the major factors (Bohn and Kershner 2002). However, in the United States, efforts have been made to restore salmonid populations through aquaculture.

Reclaimed wastewater has been widely used in most of the world to rear non-salmonid species (Allen 1998). Health guidelines for the use of recycled wastewater aquaculture have been compiled by the World Health Organization (1989, 2001). In the last several decades, there has been increasing concern and environmental controversy revolving around industrialized aquaculture or “fish ranching”. These issues include the impact of escapees on wild populations of fish, waste from net pens as a source of water pollution, toxins and pressure on local freshwater sources (Castle 2002). The possibility of using reclaimed wastewater can help to conserve freshwater resources by providing an alternative water source for aquaculture.

Wastewater reclamation processes and effluent quality is influenced by technology, social, and environmental factors. Industrial and non-industrial municipal wastewater contents vary and may require further levels of treatment to meet the specific requirements necessary for aquaculture. Humans, through recreational fishing, could eventually consume trout reared in wastewater. Therefore, in order to reduce the risks to fish health and public safety metal concentration in wastewater should be considered before reuse. In ideal situations, public health risk from fish reared in reclaimed wastewater may be no greater than risk from uncontrolled harvesting of fish from waters that receive discharged effluent from sewage plants (Hejkal et al. 1983). However, studies evaluating metal toxcicity in humans and the correlation with fish consumption cannot be ignored (Barber et al. 2000, Zhang et al. 2001, EPA 816-F-02-013 2002, Safe 2003, Spencer and Sickle 2006, Ciardullo et al. 2008).

Copper, cadmium, and zinc are metals commonly found in wastewater and are toxic to trout at certain levels. External factors involved in metal uptake in fish include source, exposure level, distance from contamination, presence of other ions, and speciation of the metal. A major pathway of metals incorporation from water into fish is by absorption across gill surfaces and intestinal mucosa. In heavily contaminated environments, heavy metals in invertebrates and sediment are incorporated into fish through the uptake of food (Sorensen 1991).

In general, the toxic action of metals in fishes cause osmoregulatory stress and precipitation of mucus on gills with death being attributed to suffocation as well necrotic damage of gill epithelium depending on length and levels of exposure (Cusimano, et al. 1986). A number of studies have examined how toxicity of metals relates to various water quality parameters such as pH, water hardness, and complexation of ions. Howarth and Sprague (1978) found that with a change in pH, there is a change in metal speciation, which can lead to a change in biological availability and that changes in oxygen levels may influence oxidation state of metals. Sorenson (1991) found acute cadmium toxicity and salinity are inversely related. Other studies have shown that with an increase in water hardness there is an increase in fish resistance to metal toxicity (Pagenkopf 1983, Cusimano et al. 1986, Sorenson 1991).

Davies and Woodling (1980) found concentrations of metals that induced acute mortality in unacclimated rainbow trout were not acutely toxic to resident salmonid populations. Other studies have also found previous that exposure to metals increases trout tolerance to heavy metals (Seim et al. 1984, Sorenson 1991). Cusimano et al. (1986) measured the toxicity of copper, cadmium and zinc on unacclimated steelhead trout under laboratory conditions designed to mimic natural environments with soft waters and low dissolved solids. The study determined the LC50 after 96 hours for steelhead without previous exposure to copper was 0.003 mg/L, cadmium <0.001 mg/L and zinc 0.066 mg/L.

Numerous studies have shown that both gill and liver tissues in fish accumulate copper, cadmium and zinc (Mount and Stephan 1967, Matthiessen and Brafield 1977, Phillips and Russo 1978, Pagenkopf 1983, Sorensen 1991, Deb and Santra 1997, McGreer et al. 2000). In a study by McGreer et al. (2000), fish exposed to either cadmium or copper ions experienced significant accumulation in the gills as well as the liver and kidney. Buckley et al. (1982) found copper homeostasis to be regulated by the liver and may be concentration dependent while gill tissue resulted in slight accumulation of copper at sublethal concentrations. Mount and Stephan (1967) suggested gill tissues are an accurate measure of acute cadmium poisoning while the liver may indicate long term exposure. Cadmium can displace zinc from high molecular weight proteins that are believed to shuttle cadmium and zinc across membranes since zinc and cadmium are similar to each other chemically and metabolically (Sorenson 1991). A study by Matthienssen and Brafield (1977) supported zinc accumulation from water in euryhaline fish species readily occurs in both gill and liver tissues. Watson and Beamish (1980 as cited in Sorenson 1991) found that zinc accumulation could be regulated in anadromous species by active transport of zinc at the gills. Matthissen and Brafield (1977) determined that metabolic rate was correlated with metal accumulation and at higher temperatures metabolism increases.

In addition, other studies took into consideration the toxicity of copper, cadmium and zinc with age class, previous exposure and length of exposure of fish (Mount and Stephan 1967, Ogino and Yang 1978, Phillips and Russo 1978, Chapman 1978, Chapman and Stevens 1978, Siem, et al. 1984, Deb and Santra 1997, McGreer et al. 2000). Bioassays of alevin fry with heavy metals are approximately equivalent to those obtained from year-long studies on all life stages (Sorenson 1991). Safe levels for heavy metals for some species in the alevin-fry life stage under certain water quality conditions may be as low as 0.04 ppb (Sorenson 1991).

In summary, the aquaculture industry can help conserve freshwater resources by utilizing reclaimed wastewater. The risk to fish and subsequent human health from heavy metals in wastewater is a concern that may deter the use of reclaimed wastewater in aquaculture. Studies on the effects of metals in salmonids reared specifically in wastewater have not been conducted due to the novelty of rearing salmonids in wastewater. The purpose of this study is to examine the accumulation of copper, cadmium, and zinc in salmonids at the Arcata Wastewater Aquaculture Facility.

STUDY SITE

This study was performed *in situ* with an established reclaimed wastewater pond system at the Arcata Wastewater Aquaculture Project facility. The Arcata Wastewater Aquaculture Project facility is part of the Arcata Marsh and Wildlife Sanctuary located adjacent to Humboldt Bay in Arcata, California, and is located just south of the Arcata Wastewater Treatment Plant (Figure 1). Collection sites consisted of two replicate pond sites, Summer Pond 1 and Summer Pond 2, and the Humboldt State University Fish Hatchery reference site.

Multiple fish species have been reared by the Arcata Wastewater Aquaculture Project including Chinook salmon (*Oncorhynchus tshawytscha*), Coho salmon (*Oncorhynchus kisutch*), steelhead trout (*Oncorhynchus mykiss*), cutthroat trout (*Oncorhynchus clarki*), and sturgeon (*Acipenser medirostris*). At the Arcata Wastewater Treatment Plant, sewage undergoes secondary treatment within three 16 ha oxidation ponds followed by three 3 ha treatment marshes, chlorination and three 12 ha polishing marshes before entering Humboldt Bay. A sump pump located at the treated wastewater effluent discharge point is diverted to the Arcata Wastewater Aquaculture Facility yearly ponds and Summer Ponds. Effluent is mixed with bay water and is gravity fed through the rearing ponds. The Summer Ponds also receive water from the Bay which diffuses through a dike that separates the Summer Ponds from the bay. The Summer Pond study sites cover an area of approximately 0.012 hectares and depths average 2.7m.

Figure 1. Satellite photo of the Arcata Marsh and Wetland Sanctuary on Humboldt Bay, Humboldt County, California including the Arcata Wastewater Aquaculture Project located south of the Arcata Wastewater Treatment Plant (photo adapted from website www.appropedia.org/Arcata_marsh, accessed August 25, 2008).

Trout in the Summer Ponds are supplementary fed approximately 85 percent less than the conventional ration of fish feed while the rest of their diet included various macroinvertebrates already established in the pond system.

A study in 2005 found copper, cadmium and zinc were heavy metals highest in concentration in the Arcata Wastewater Treatment Plant effluent (Brenneman and Lasko 2005). According to the Arcata Wastewater Treatment Plant discharge permit (National Pollutant Elimination Discharge System Permit No. CA0022713) the average monthly level permitted for copper is 0.003 mg/L and zinc 0.047 mg/L. Cadmium was not identified as a pollutant of concern in the Arcata Wastewater Treatment Plant discharge permit.

The reference site, Humboldt State University Fish Hatchery, is part of the Fisheries Aquaculture Program located on the campus of Humboldt State University, Arcata, CA. The hatchery grounds consist of a laboratory, egg incubators, rearing and research tanks, 60 meter long raceways, six 3 meter circular tanks, an aeration tower and an 85 cubic meter redwood tank reservoir. The hatchery is a closed system and obtains its water supply from Fern Lake reservoir which is fed by Jolly Giant Creek. Municipal city water only is used as a backup supply during summer. The water is stored in the redwood tank and used for back flushing and other operational needs.

MATERIALS AND METHODS

Before the study steelhead trout were brought in as eyed eggs from Trout Lodge Hatchery (Sumner, Washington) and hatched in vertical tray fish egg incubators at the Arcata Wastewater Aquaculture Project hatchery (AWWAP) and the reference site Humboldt State University Fish Hatchery. Humboldt State University Fish Hatchery and the Arcata Wastewater Aquaculture Project hatcheries prohibit transferring live fish from hatchery to hatchery; thus, eggs from the same batch were reared at each hatchery.

Most metal toxicity studies in a controlled setting use 5 to 30 fish per treatment (Ogino and Yang 1978, Hejkal et al. 1983, Guerrin et al. 1990, Liang et al. 1999, Chen et al. 2000, Barber et al. 2003). Based on such studies, a sample size of 50 fish was collected for each sampling period (Figure 2). At time zero, 50 four- month old fingerlings were collected at each hatchery. At the Arcata Wastewater Aquaculture Facility, fingerlings were randomly selected and sacrificed for tissue samples. The remaining fingerlings were separated into two equal groups and introduced into the wastewater rearing ponds, Summer Pond 1 and Summer Pond 2. Trout placed in the Summer Ponds were marked by removing the adipose fin. To prevent predation by larger trout in the ponds, fingerlings were kept in net-pens for two months and then released into the general population. Concurrently, 50 fingerlings from the HSU reference site were sacrificed for analysis (Figure 2). Remaining reference steelhead trout were reared in an indoor rearing tank for five months and then a 3 meter circular for seven months.

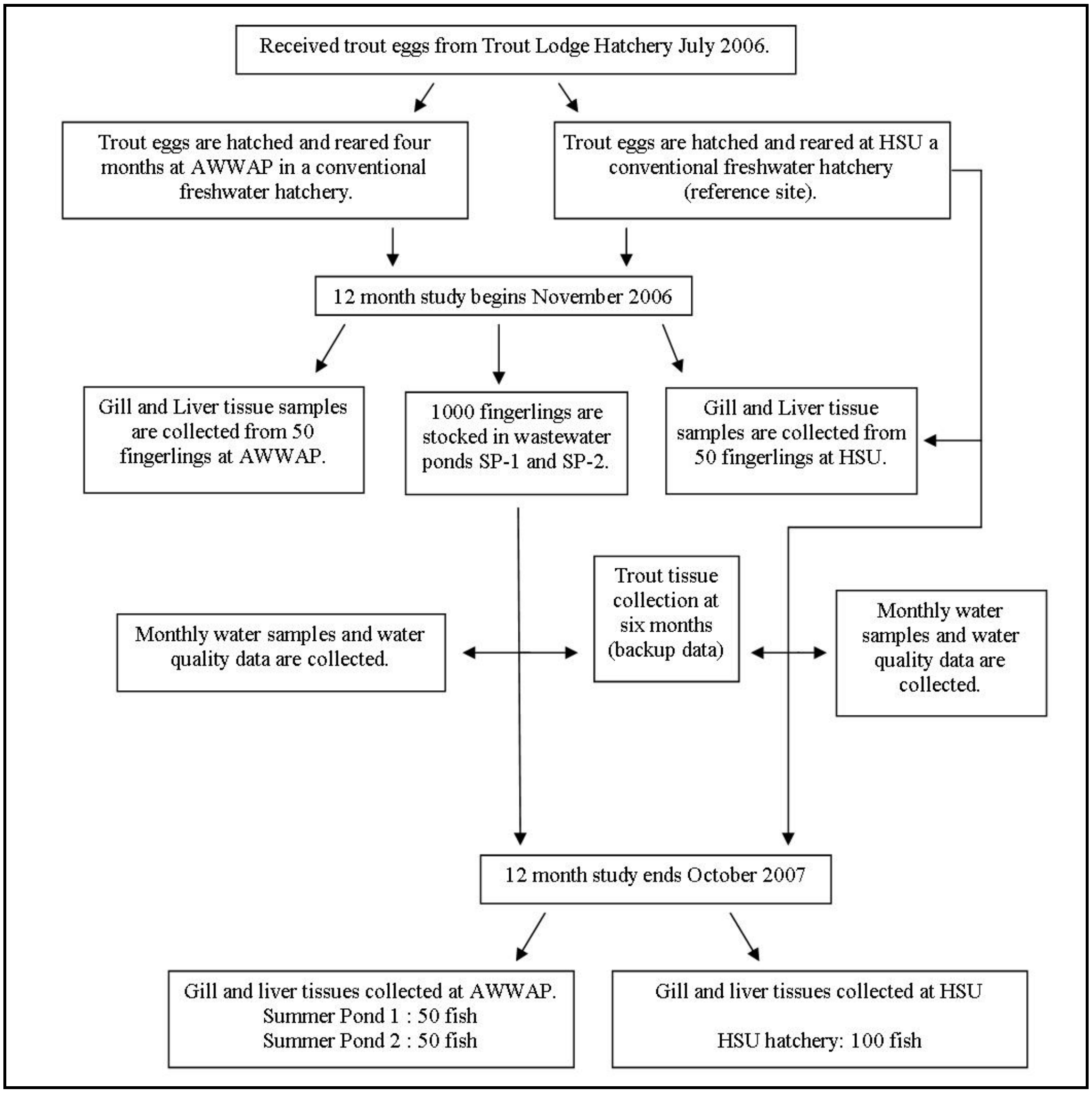

Figure 2. A time line flow chart for the study of metal accumulation in gill epithelium and liver tissue in steelhead (*Oncorhynchus mykiss*) reared at Arcata Wastewater Aquaculture Project (AWWAP) and Humboldt State University (HSU), Arcata, California.

As a precaution, tissue samples were collected at six months as back up in the event of sudden heavy mortalities or other unforeseen events (Figure 2). At month 12 in October 2007, 50 fish were collected from Summer Pond 1 and Summer Pond 2 and 100 fish from the Humboldt State University Fish Hatchery for tissue analysis (Figure 2). Collection of gill and liver tissue samples before and after 12 months exposure to wastewater was executed in compliance with the city of Arcata, Environmental Protection Agency standard methods and the Humboldt State University Institutional Animal Care and Use Committee permit (No.05/06F.21-A).

Analysis of Metals

Concentrations of cadmium, copper and zinc in gill and liver tissue of trout reared in reclaimed wastewater are unknown. Several studies on metal bioaccumulation in freshwater *Tilapia* reared in wastewater were considered in developing the methods for this study and estimating possible metal concentrations in trout tissue (Guerrin et al. 1990, Deb and Santra 1997, Chen et al. 2000).

Fingerlings and fish at the study and reference sites were removed using a handheld fish net. Adults reared in Summer Ponds were captured by seining. Fish to be sacrificed were placed in a five gallon bucket containing 10mg/L MS-222 solution (tricaine methanesulfonate) an anesthetic for aquatic animals which has been used for sacrificing trout in similar metal detection experiments (Seim et al. 1984). Each fish was wiped dry of excessive water, weighed to the nearest milligram with a Mettler Toledo

B2002-S analytical balance, and fork length measured to the nearest millimeter with a ruler. Adult fish (1+ year) were sexed by observing the gonads.

Gill and liver tissues were removed from each fish with dissection scissors for analysis. Once removed, tissues were given an identification number, stored in Ziploc® plastic bags and stored at 2˚C until further processing. All laboratory ware was cleaned with Alconox® liquid soap and either submerged or filled with a 1:5 dilution of concentrated nitric acid and metal free nano-filtered MilliQ water (Millipore Corporation, Billerica, MA.) wash for three days. Between samples all laboratory ware was washed, rinsed and soaked in the nitric acid solution for 12 hours (M. Hurst, personal communication). Acid washed laboratory ware was rinsed three times with MilliQ water and air dried. At the time of analysis, used nitric acid solution was labeled and discarded according to Humboldt State University waste management protocol. Labeled ceramic crucibles were set in tin dishes and tongs were used to transport the crucibles during the weighing procedure. Each batch of ten tissue samples included one blank and one control containing standard reference material 1577b bovine liver (Gaithersburg, MD) with known metal concentrations (National Institute of Standards and Technology, 1991).

Frozen tissue samples were thawed and wet weight measured to the nearest 0.01g using a Mettler Toledo B2002-S analytical balance. Each tissue sample was assigned a laboratory identification number. Tissue samples were placed in ceramic crucibles and dehydrated at 110º C (± 5º C) in a Grieve Laboratory Oven model LO-201C for 12 hours or until a constant weight was obtained. The length of drying time was determined with 4-5 initial weights of several samples every few hours until a standard drying time was established. After drying time was established, samples were weighed twice at separate times to verify dried condition before the digestion procedure began. The crucibles were handled with tongs and brought to room temperature in a desiccator before being weighed.

Each dehydrated tissue sample was ground in the crucible using a pestle, weighed on a Sartorius micro balance to the nearest 0.001g and stored in a digestion tube. Ten milliliter glass chemical oxygen demand tubes with screw caps were used for digestion. According to Standard Methods for the Examination of Water and Wastewater 3030 D, digestion of tissues reduces interference from organic matter and will convert metals associated with particulates to a form that can be determined by atomic absorption spectroscopy (Standard Methods for the Examination of Water and Wastewater 1998). For the digestion of tissue samples, a ratio of 10ml 36.5 – 38.0 percent metal grade hydrochloric acid per one gram of dry tissue was used according to standard method 3050 B (Standard Methods for the Examination of Water and Wastewater 1998). The digestion procedure occurred in two parts. Tubes containing a ground tissue sample and digestion acid were capped and left to digest for 12 hours at ambient temperatures. Tubes were then placed under a fume hood in a Hach chemical oxygen demand reactor (Hach, Loveland CO) at 95 ºC ± 5 ºC and refluxed for 15 minutes according to standard method 3050 B (Standard Methods for the Examination of Water and Wastewater 1998). After cooling to room temperature, the digested solution was diluted 200 times with MilliQ water to a pH=0. All digested samples were vortexed between dilutions with a Fisher Scientific mini vortexer to insure homogeneous mixture. Of the final diluted solution, a sample of 2.0 mls was analyzed for each metal using a flame atomic absorption spectroscopy with a Perkin Elmer Aanalyst 400 (Perkin Elmer, Waltham Mass) (Figure 9). Metals concentration was determined per dry weight of tissue sample (Mount and Stephan 1967, Deb and Santra 1997). The concentrations of copper, cadmium and zinc are reported for tissue dry-weight in μg/g (Equation 2).

$$\text{Tissue Metal Concentration: } \mu g / g = A \times B \times C \qquad (2)$$

Where

A = concentration of metal (mg/L)

B = dilution factor of digestion acid

C= dilution factor of nano-filtrated water

To assess metal concentrations in the water throughout the study period duplicate water samples were collected at the study and reference sites for 12 months (n=72). Water sample collection began November 2006 and ended October 2007 (Figure 2). Concurrently, monthly water quality data were collected for temperature, dissolved oxygen, pH and salinity between the months of November 2006 until October 2007. HOBO temperature data loggers were installed at both study and reference sites.

Sample containers were 1000 ml polypropylene or polyethylene according to water collection Standard Method 3010 B. (Standard Methods for the Examination of

Water and Wastewater 1998). Bottles were cleaned for three days with an acid wash of nitric acid and metal free nano-filtered water at a 1:5 dilution (~3.0 M). Bottles were conditioned for three days with 2.0 mls of hydrochloric acid per 1000 mls MilliQ water (M. Hurst, personal communication). At the collection site, bottles were empted of the conditioning solution and rinsed three times in sample water away from the immediate collection area. A grab sample was collected 15 cm below the surface on the north side of each pond. Concurrently, water quality data for temperature, dissolved oxygen, pH, conductivity and salinity was measured using Yellow Springs Instrument (YSI) Model 55 Dissolved Oxygen Meter (Yellow Springs, OH) Hanna pH meter (Woonsocket, RI) and YSI 30 Salinity/Conductivity Meter (Yellow Springs, OH), respectively. Bottles were stored in a Ziploc gallon bag and labeled. Throughout the study HOBO (Bourne, MA) water temperature data loggers were installed at 1.5 meters depth at the study and reference site to collect continuous water temperature data.

In the laboratory, water samples were acidified to pH=2 with 2.0 ml of metal grade nitric acid approximately one month before analysis. Duplicate water samples were divided and analyzed randomly in two separate sets. Each set consisted of five batches of six to seven water samples and one blank. To recover and concentrate the soluble trace metals, water samples were buffered with 5 M ammonium acetate to a pH of 5.3 then pumped through a column containing Chelex 100 ion exchange resin. Chelex 100 is a chelating ion exchange mechanism that preferentially binds copper, iron and other heavy metals over such cations as sodium, potassium and calcium. The selectivity of the Chelex 100 for divalent over monovalent ions is approximately 5,000 and has a very strong attraction for transition metals in highly concentrated salt solutions (Bio-Rad Laboratories, 1996). The quantity of cations chelated by the Chelex resin is a function of pH with maximum absorption values above pH 4. Any metal removed from solution is replaced by an equivalent amount of sodium ions originally on the resin.

The procedural steps for the column method to recover copper, cadmium and zinc from water samples using Chelex 100 ion exchange resin was adopted from the Bio-Rad Laboratories Chelex Instruction Manual (Bio-Rad Laboratories, 1996) In each column the bed volume of Chelex resin was 1.8 -2.0 mls. Resin was rinsed with four bed volumes of 0.5 M sodium acetate and five bed volumes of metal free water to buffer the resin to approximately pH 5. A water sample of 400-500 mls was run through the column with a Rainin RP-1 Peristaltic pump (Oakland, CA) at a flow rate of 2.0-2.5 mls/min/cm^2 for ions exchange. Elution of metals was performed by using 1 M nitric acid and collected in an acid washed polypropylene tube until further analysis. The resin was then rinsed with five bed volumes of nano-filtrated water and regenerated with four bed volumes of 0.5 M ammonium acetate and five bed volumes water rinse. The analysis of metals collected in the nitric acid was performed with flame atomic absorption spectroscopy on a Perkin Elmer Analyst 400. The concentration of heavy metals in the water samples was calculated with equation 3.

$$\text{Concentration of heavy metals in water samples (mg/L)} = \frac{A}{B} - C \qquad (3)$$

Where

A = Concentration of heavy metals (mg/L)

B = Concentration factor (volume of sample/ volume of eluded nitric acid)

C = Contamination in blank (mg/L)

The final metal concentration for each month at each site was calculated from the mean of the duplicate samples.

Each batch of ten tissue samples included a method blank and a quality control sample of Standard Reference Material 1577b bovine liver with known metal concentrations. The digestion tube blank was used to detect contamination of copper, cadmium or zinc throughout the procedure. A quality control sample of Standard Reference Material 1577b bovine liver with known metal concentrations was used to verify effectiveness of the tissue digestion and calibration of the spectrometer Analyst 400 (National Institute of Standards and Technology, 1991).

To determine metal concentrations in water samples, a pilot study measured the recovery of wastewater samples spiked with a known concentration of heavy metals. The results showed 80 percent-120 percent recovery of metals. Based on this, I concluded that constituents such as ethylenediamine tetraacetic acid (EDTA), typically found in natural waters, that could complex with heavy metals and thereby compete with the Chelex 100 ion exchange resin would not be a concern. Each batch of six to seven water samples included a blank that was used to identify contamination. Initial calibration of the Analyst 400 instrument used metal standards and a blank. Standards had dilution differences no greater then one order of magnitude and the calibration-instrument response correlation coefficient was ≥ 0.995. The instrument was calibrated daily.

Calibration was verified after every ten samples with standard reference material or a check standard. Standard reference material and check standards with metal determinations outside of 90- 110 percent of the established range was considered unacceptable.

The optimum concentration range for direct aspiration atomic absorption according to wastewater standard method 3111 A. for cadmium is 2.0-0.05 mg/L, copper 10.0-0.2 mg/L and zinc 2.0-0.05 mg/L. The instrument detection limit for direct aspiration atomic absorption for cadmium is 0.002 mg/L, copper 0.01 mg/L and zinc 0.005 mg/L. The instrument detection limit for copper, cadmium and zinc is based on both sensitivity and signal stability (Standard Methods for the Examination of Water and Wastewater 1998).

RESULTS

Throughout the study, mean weekly water temperature in the Summer Ponds was 15.67ºC and 15.93ºC, respectively and the reference site was 14.32ºC (Table 1). A Tukey-Kramer Multiple Comparison test showed no significant difference in mean temperature among all sites (Table 1); however, water temperature at the reference site was slightly cooler then the Summer Ponds March through September 2007 (Figure 3). The statistical significant level was set at $\alpha < 0.05$ for all tests.

The hydrogen ion (pH) was similar ($p = 0.212$) in both Summer Ponds and the reference site throughout the study (Table 1). The reference site pH shifted a month earlier then the study site (Figure 4). Analog mean pH in the Summer Ponds 1 and 2 was 7.9 and 8.1, respectively, and the reference site 7.6. Although pH differences were not significantly different ($p = 0.212$) the reference site maintained the lowest throughout the study and Summer Pond 2 had the highest pH from April to August. A cursory sampling of water from all sites found zero water hardness in the form of calcium carbonate.

Summer Pond 1 had the lowest percentage oxygen saturation while Summer Pond 2 and the reference site were consistently ≥ 100% saturated throughout the study (Figure 5). Mean dissolved oxygen in Summer Pond 1 and 2 was 8.4 mg/L and 9.6 mg/L respectively, the reference site was slightly higher at 10.5 mg/L and was less variable in concentration compared to the Summer Ponds throughout the study (Figure 6).

Table 1. Results of ANOVA used to determine differences in mean weekly temperature, and mean monthly pH, salinity, conductivity corrected (25˚C), dissolved oxygen and percentage oxygen saturation in Summer Ponds 1 and 2 at the Arcata Wastewater Aquaculture Project and the reference site Humboldt State University Fish Hatchery collected from November 2006 to October 2007 Arcata, California.

	Summer Pond 1		Summer Pond 2		Reference			
Variable	Mean	SE	Mean	SE	Mean	SE	n	P
Temperature (°C)	15.67†	0.69	15.93†	0.69	14.32†	0.69	44	0.212
pH	7.9†	0.26	8.1†	0.26	7.6†	0.26	12	0.107
Salinity (ppt)	7.81	0.36	2.33	0.36	0.1	0.36	12	<0.001
Conductivity (μmhos)	11.38	0.57	3.64	0.57	nd	0.57	12	<0.001
Dissolved Oxygen (mg/L)	8.4	0.29	9.6†	0.29	10.5†	0.29	12	<0.001
Percentage Oxygen Saturation	83.51	1.63	96.14†	1.63	100.74†	1.63	12	<0.001

† Means did not differ ($p<0.05$) based on a Tukey-Kramer Multiple-Comparison Test

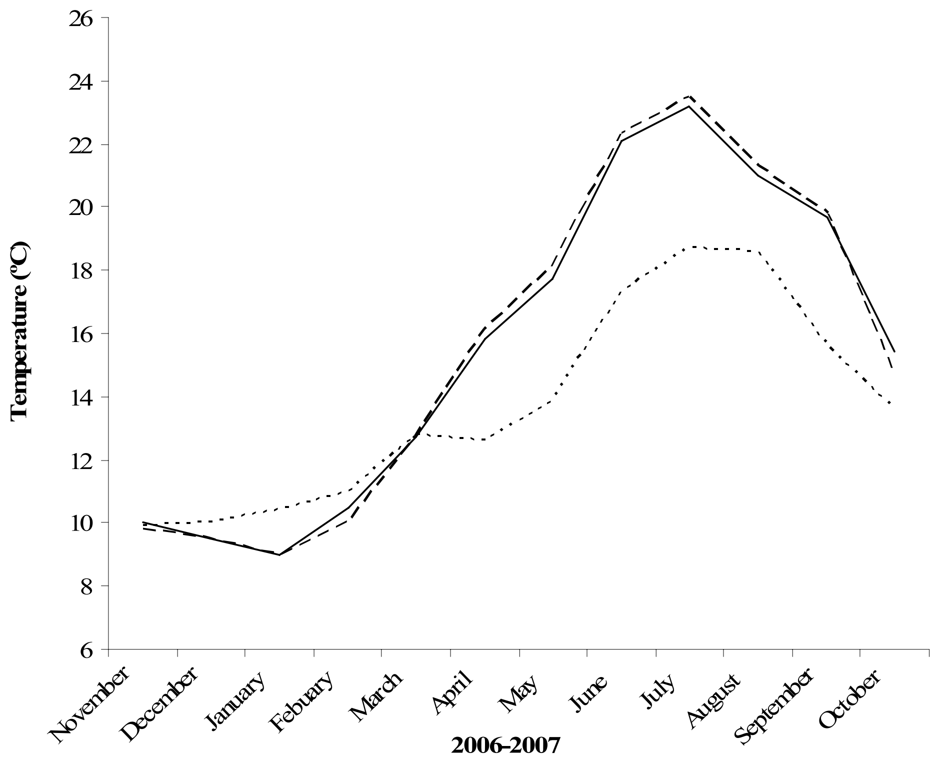

Figure 3. Mean weekly water temperature (˚C) from HOBO temperature meters in Summer Ponds 1 and 2 at Arcata Wastewater Aquaculture Facility and reference site Humboldt State University Fish Hatchery, Arcata, California from November 2006 to October 2007 (—— Summer Pond 1, ------ Summer pond 2, ⋯⋯ reference site).

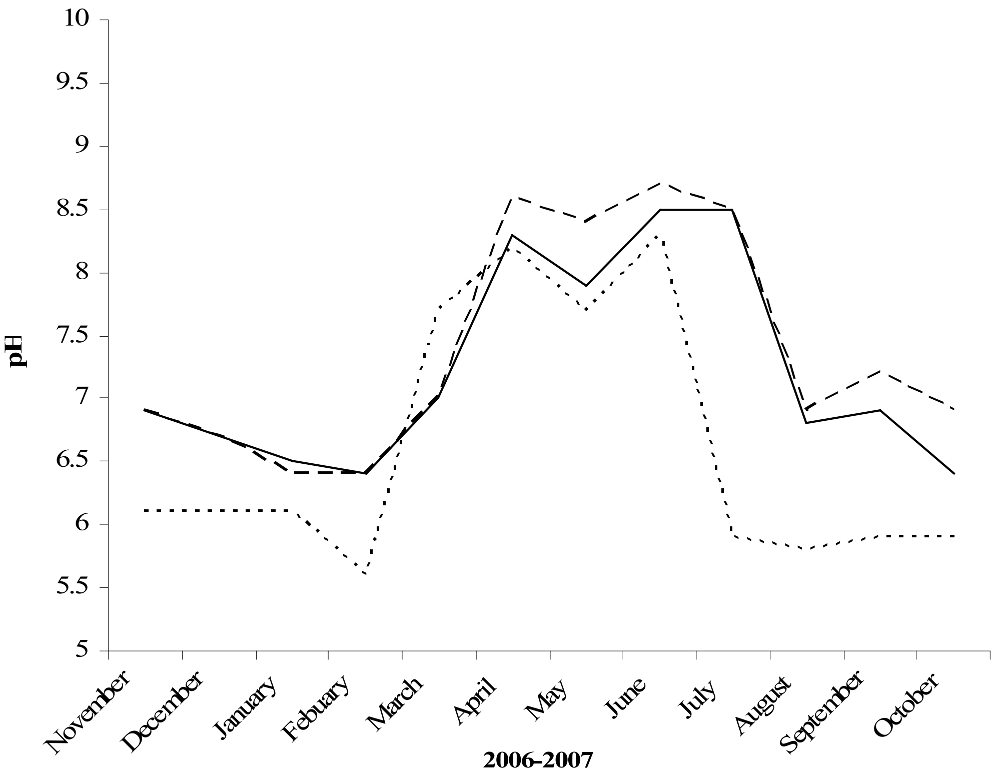

Figure 4. The pH in Summer Ponds 1 and 2 at Arcata Wastewater Aquaculture Facility and Humboldt State University Fish Hatchery, Arcata, California, from November 2006 to October 2007 (—— Summer Pond 1, ------ Summer pond 2, …… reference site).

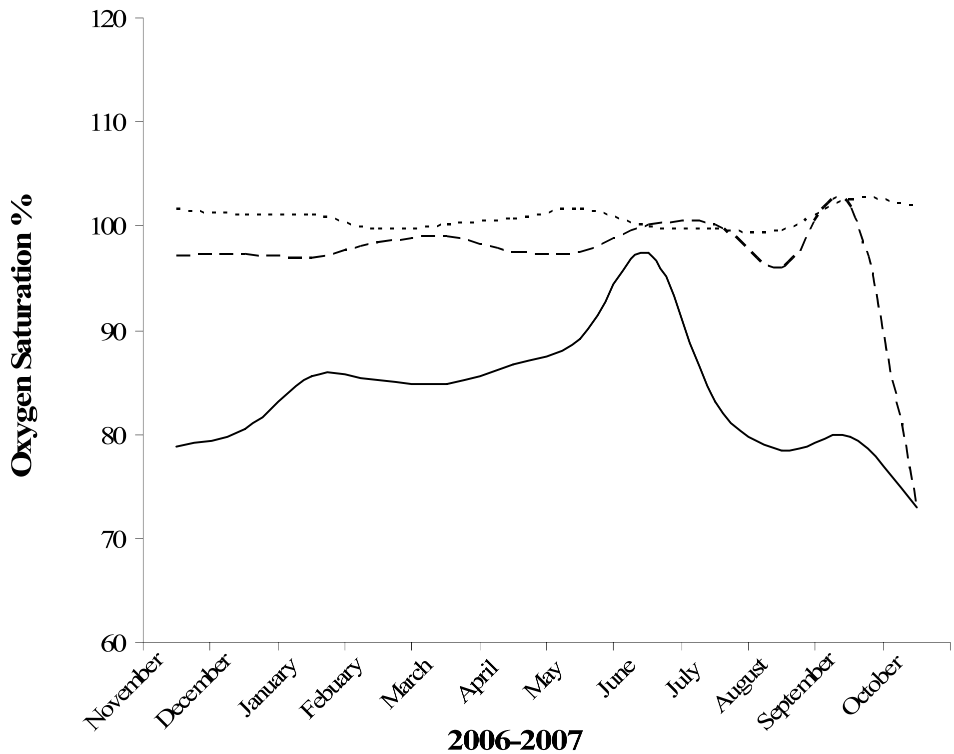

Figure 5. Oxygen saturation percentage in Summer Ponds 1 and 2 at Arcata Wastewater Aquaculture Facility and reference site Humboldt State University Fish Hatchery, Arcata, California (—— Summer Pond 1, ------ Summer pond 2, ⋯⋯ reference site).

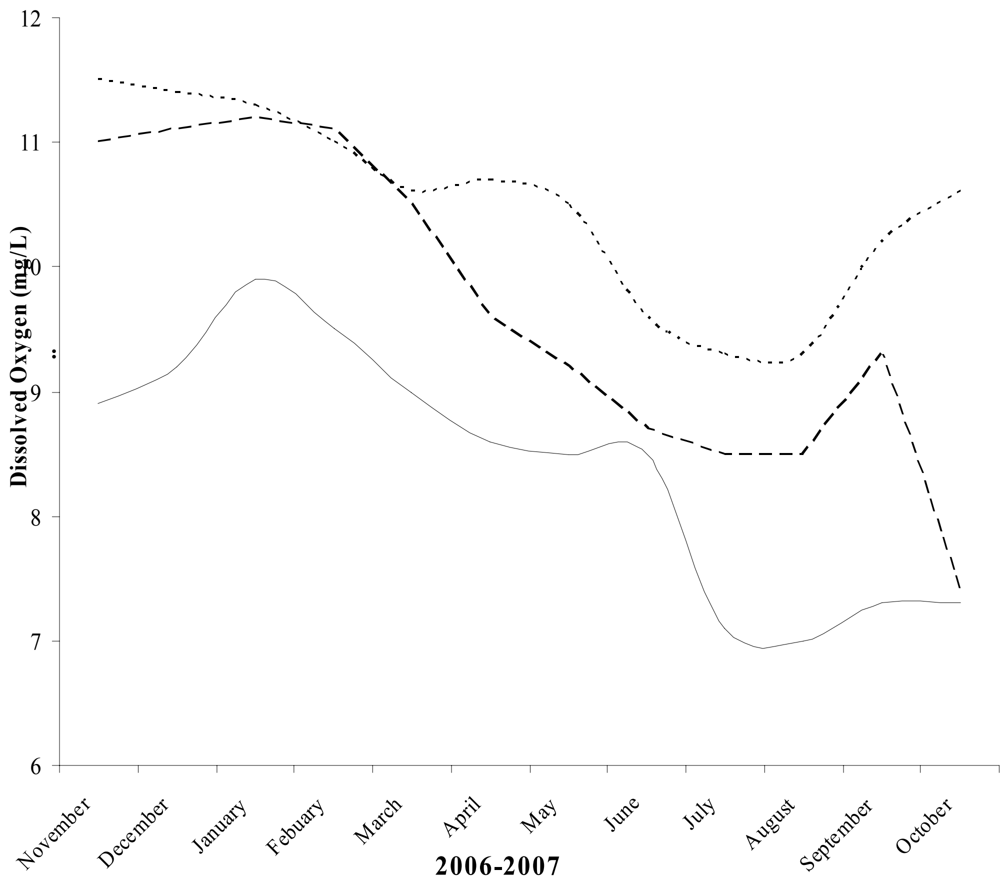

Figure 6. Dissolved oxygen in Summer Ponds 1 and 2 at Arcata Wastewater Aquaculture Facility and reference site Humboldt State University Fish Hatchery, Arcata, California (—— Summer Pond 1, ------ Summer pond 2, ⋯⋯ reference site).

Dissolved oxygen and percentage oxygen saturation in Summer Pond 1 was significantly different ($p < 0.001$) than Summer Pond 2 and the reference site based on a Tukey-Kramer Multiple Comparison test (Table 1).

Mean salinity in Summer Ponds 1 and 2 was 7.8 ppt and 2.3 ppt, respectively and the reference site was 0.1 ppt (Table 1). Mean conductivity (corrected to 25ºC) in Summer Ponds 1 and 2 was 11.38 μmhos/cm and 3.64 μmhos/cm, respectively and the reference site was < 1.0 μmhos/cm (Table 1). Salinity and conductivity in Summer Pond 1 was higher and more variable when compared to Summer Pond 2 and the reference site (Figures 7 and 8). Salinity and conductivity were found to be different ($p < 0.001$) among all sites as determined by a Tukey-Kramer Multiple Comparison test (Table 1).

The mean body weight and fork length of steelhead trout before introduction to the Summer Ponds was greater than the reference site, Humboldt State University Fish Hatchery. At the end of the study the mean body weight of trout and the mean fork length of trout from Summer Pond 1 and 2 varied little from the reference site (Tables 2 and 3). A two tail Student's t-test showed a significant increase ($p < 0.001$) in trout weight (g) and fork length (cm) at all sites at the end of the twelve month study. A Tukey-Kramer multiple comparison test showed no significant difference ($p = 0.648$) in fork length among all sites at month 12. A Tukey-Kramer multiple comparison test found body weight in Summer Pond 2 was significantly greater ($p < 0.001$) then Summer Pond 1 and the reference site at month 12. No mortalities were observed at the study and reference sites throughout the study.

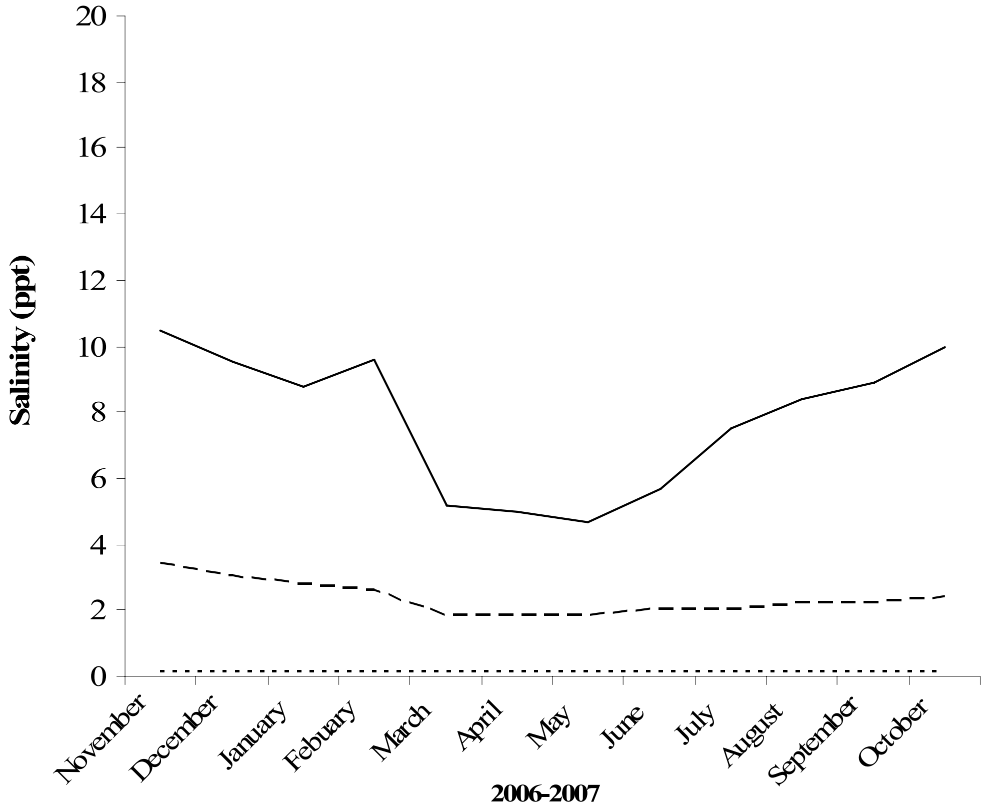

Figure 7. Salinity (ppt) in Summer Ponds 1 and 2 at Arcata Wastewater Aquaculture Facility and reference site Humboldt State University Fish Hatchery, Arcata, California from November 2006 to October 2007 (—— Summer Pond 1, ------ Summer pond 2, ⋯⋯ reference site).

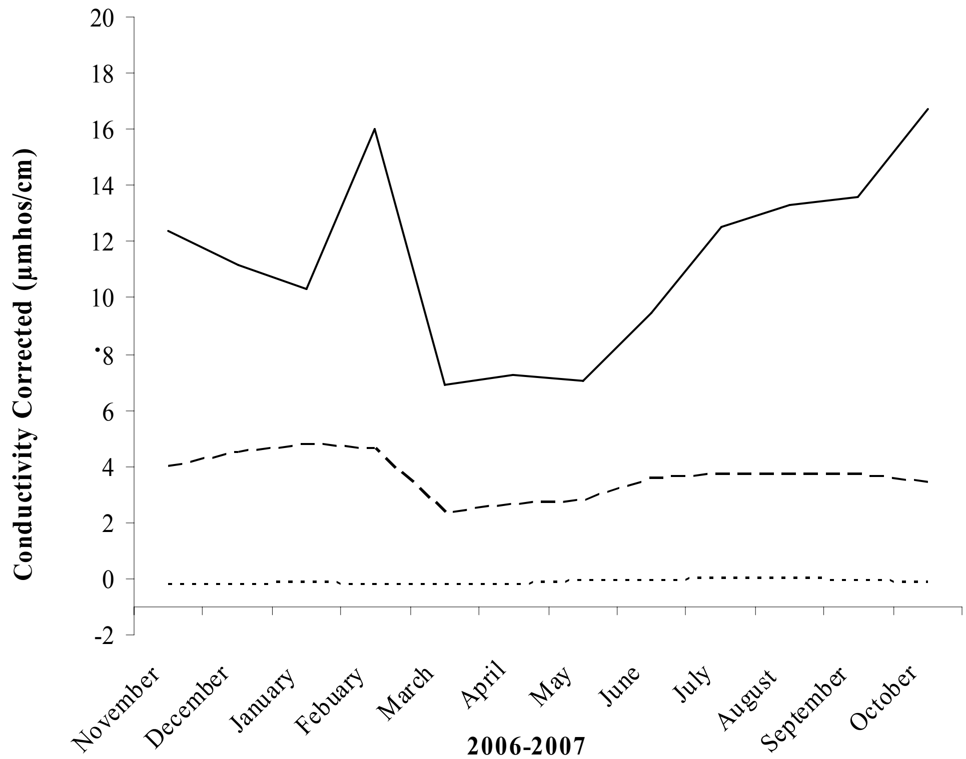

Figure 8. Conductivity (µmhos/cm) in Summer Ponds 1 and 2 at Arcata Wastewater Aquaculture Facility and reference site Humboldt State University Fish Hatchery, Arcata, California from November 2006 to October 2007 (conductivity is corrected to 25˚ C) (—— Summer Pond 1, ------ Summer pond 2, ⋯⋯ reference site).

Table 2. Mean body weight (g) of steelhead trout at Arcata Wastewater Aquaculture Facility (AWWAP) before exposure to wastewater (November 2006) and Summer Ponds 1 and 2 after 12 months exposure to wastewater (October 2007) and reference site, Humboldt State University Fish Hatchery in Arcata, California.

Trout Body Weight								
	Before				After			
Site	Mean (g)	SE (±)	n	Site	Mean (g)	SE (±)	n	P
AWWAP	19.51	0.68	37	Summer Pond 1	116.50†	3.51	50	<0.001
				Summer Pond 2	136.67	5.55	50	<0.001
Reference	12.43	0.33	50	Reference	120.14†	5.55	100	<0.001

† Means among sites did not differ (p<0.05) based on a Tukey-Kramer Multiple-Comparison Test.

Table 3. Mean fork length (cm) of steelhead trout at Arcata Wastewater Aquaculture Facility (AWWAP) before exposure to wastewater (November 2006) and Summer Ponds 1 and 2 after 12 months exposure to wastewater (October 2007) and reference site Humboldt State University Fish Hatchery in Arcata, California.

Trout Fork Length								
	Before				After			
Site	Mean (cm)	SE (±)	n	Site	Mean (cm)	SE (±)	n	P
AWWAP	11.09†	0.75	37	Summer Pond 1	21.29†	1.74	50	<0.001
				Summer Pond 2	22.36†	2.13	50	<0.001
Reference	9.47†	0.65	50	Reference	23.66†	3.22	100	<0.001

† Means among sites did not differ (p<0.05) based on a Tukey-Kramer Multiple-Comparison Test.

Metal Analysis

Duplicate water samples collected monthly were analyzed for copper, cadmium and zinc. No significant difference ($p < 0.001$) in metal concentration was found between duplicate water samples.

Throughout the study mean copper concentrations in monthly water samples in the Summer Ponds were both 0.003mg/L and the reference site was 0.001mg/L (Table 4). a Tukey-Kramer Multiple Comparison test showed the mean copper concentration in the Summer Ponds were significantly different from the reference site
(Table 4). There were no apparent seasonal trends in aqueous copper at all sites.

Copper was below levels of detection in gill epithelium from the study the reference sites throughout the study. Copper in liver tissue at the beginning of the study had a mean of 113.86 ± 8.56 μg/g and was significantly different ($p <0.001$) from the reference site with a mean of 39.48 ± 4.86 μg/g (Figure 9). There was no increase in mean copper concentration in liver tissue of trout from the study site, however, there was an increase in copper in liver tissue at the reference site at the end of the study (Figure 9). A two-tail Student's t-test ($p<0.05$) showed a significant increase in copper accumulation in liver tissue at the reference site, but no significant difference in copper accumulation in liver tissue of trout from the Summer Ponds at the end of the study (Table 5). A Tukey-Kramer Multiple Comparison test showed copper concentration at the reference site was significantly less ($p < 0.001$) than the study site at the start of the study; however, copper accumulation at the reference site was significantly greater ($p < 0.001$) than Summer Pond 2 at the end of the study (Table 4).

Table 4. Mean copper concentration (mg/L) from duplicate samples collected monthly at Arcata Wastewater Aquaculture Facility Summer Ponds 1 and 2 and Humboldt State University Fish Hatchery from November 2006 to October 2007 Arcata, California.

	November	December	January	February	March	April (mg/L)	May	June	July	August	September	October	n	Mean	SE
Summer pond 1	0.002	0.003	0.004	0.005	0.006	0.001	0.005	0.005	0.003	0.005	0.000	0.003	24	0.003†	0.001
Summer pond 2	0.003	0.003	0.004	0.005	0.002	0.005	0.003	0.005	0.003	0.005	0.001	0.003	24	0.003†	0.000
Reference Site	0.001	0.000	0.001	0.002	0.001	0.003	0.000	0.000	0.001	0.000	0.001	0.000	24	0.001	0.000

† Means among sites did not differ ($p<0.05$) based on a Tukey-Kramer Multiple-Comparison Test.

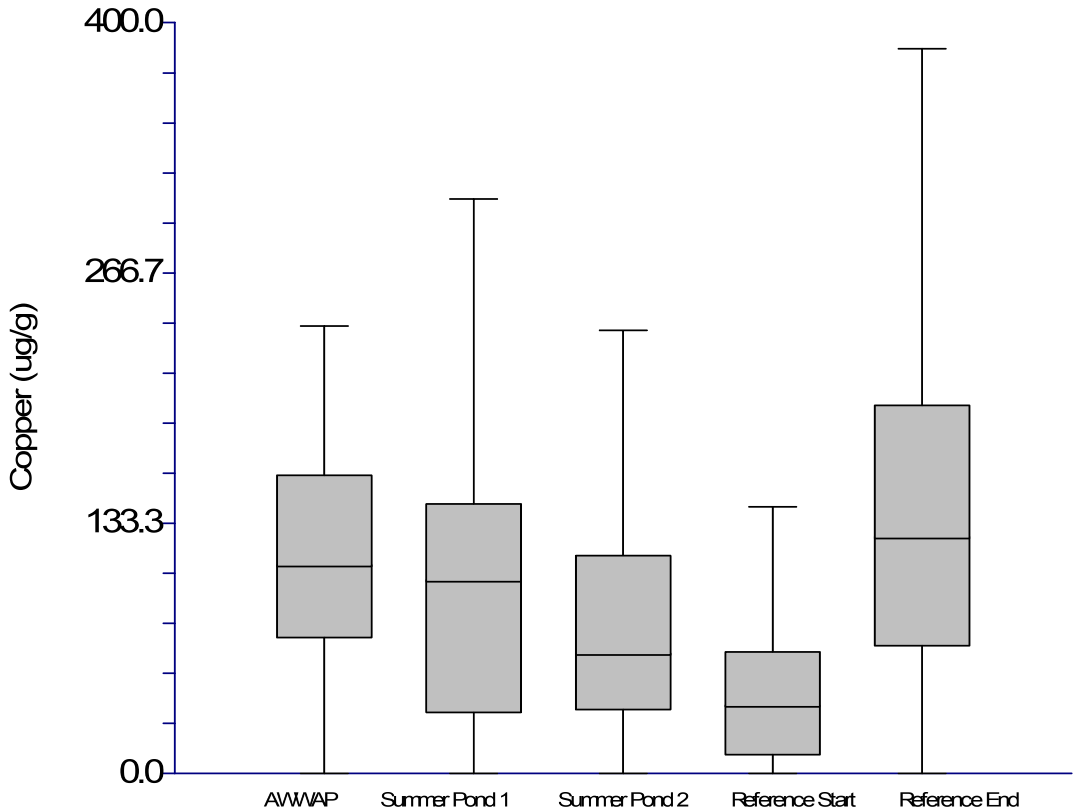

Figure 9. Box plot analysis of copper (μg/g) in liver tissue of steelhead trout at the start of the study at Arcata Wastewater Aquaculture Facility (AWWAP) and after twelve months in Summer Pond 1 and 2 and the reference site at the start and end of the study in Arcata, California from November 2006 to October 2007.

Table 5. Results of a Two Sample T-test used to determine a difference in mean copper values (µg/g) dry weight in liver tissue of steelhead trout at Arcata Wastewater Aquaculture Facility (AWWAP) before exposure to wastewater (November 2006) and Summer Ponds 1 and 2 after 12 months exposure to wastewater (October 2007) and reference site Humboldt State University Fish Hatchery in Arcata, California.

Copper in Trout Liver

Before				After				
Site	Mean (µg/g)	SE (±)	n	Site	Mean (µg/g)	SE (±)	n	P
AWWAP	113.86	8.56	49	Summer Pond 1	120.08 †‡	14.82	50	0.71
				Summer Pond 2	109.59 †	16.20	49	0.81
Reference	39.48	4.86	51	Reference	150.38‡	9.91	89	<0.001

† Means and ‡ means among sites did not differ ($p<0.05$) based on a Tukey-Kramer Multiple-Comparison Test.

Cadmium was found to be below the detection limit in tissue samples throughout the study. The gill epithelium and liver tissue of 30 trout from the Summer Ponds and the reference site at the beginning and end of the 12 month study were analyzed for cadmium. Analysis for cadmium was not performed on water samples since cadmium accumulated in trout tissue samples was below levels of detection and concurrent studies in Humboldt Bay, associated with the study site, found cadmium concentrations below the detection limit of this method (Martin and Hurst 2008).

Throughout the study mean zinc concentrations in monthly water samples at Summer Pond 1 and 2 were 0.010 mg/L and 0.027 mg/L, respectively; whereas the reference site was 0.007mg/L. A Tukey-Kramer Multiple Comparison test showed zinc concentrations in Summer Pond 2 were significantly different ($p < 0.001$) from Summer Pond 1 and the reference site (Table 6). There was no apparent seasonal trend in aqueous zinc in Summer Pond 1; however, Summer Pond 2 had a slight decrease in zinc from April to July 2007. The reference site showed a spike in aqueous zinc in April 2007 and September 2007.

Zinc in gill epithelium at the beginning of the study had a mean of 141.00±6.27 µg/g and at the reference site a mean of 112.54 ± 6.42 µg/g (Figure 10). Final mean zinc concentration in gill epithelium from the Summer Ponds 1 and 2 was 168.93 ± 6.20 µg/gand 164.89 ± 6.33 µg/g, respectively, and the reference site was 130.30 ± 4.56 µg /g (Figure 10). A two-tail Student's t-test showed a significant increase ($p < 0.001$) in mean zinc concentrations in gill epithelium of trout at the end of the study in the Summer

Table 6. Mean zinc concentration (mg/L) from duplicate samples collected monthly at Arcata Wastewater Aquaculture Facility Summer Ponds 1 and 2 and Humboldt State University Fish Hatchery from November 2006 to October 2007 Arcata, California.

	November	December	January	February	March	April (mg/L)	May	June	July	August	September	October	n	Mean	SE
Summer Pond 1	0.001	0.011	0.008	0.021	0.017	0.005	0.011	0.008	0.006	0.006	0.001	0.012	24	0.010†	0.001
Summer Pond 2	0.032	0.034	0.040	0.044	0.027	0.014	0.015	0.021	0.012	0.042	0.010	0.003	24	0.027	0.003
Reference Site	0.005	0.003	0.001	0.005	0.004	0.022	0.004	0.005	0.002	0.000	0.027	0.003	24	0.007†	0.002

† Means among sites did not differ ($p<0.05$) based on a Tukey-Kramer Multiple-Comparison Test.

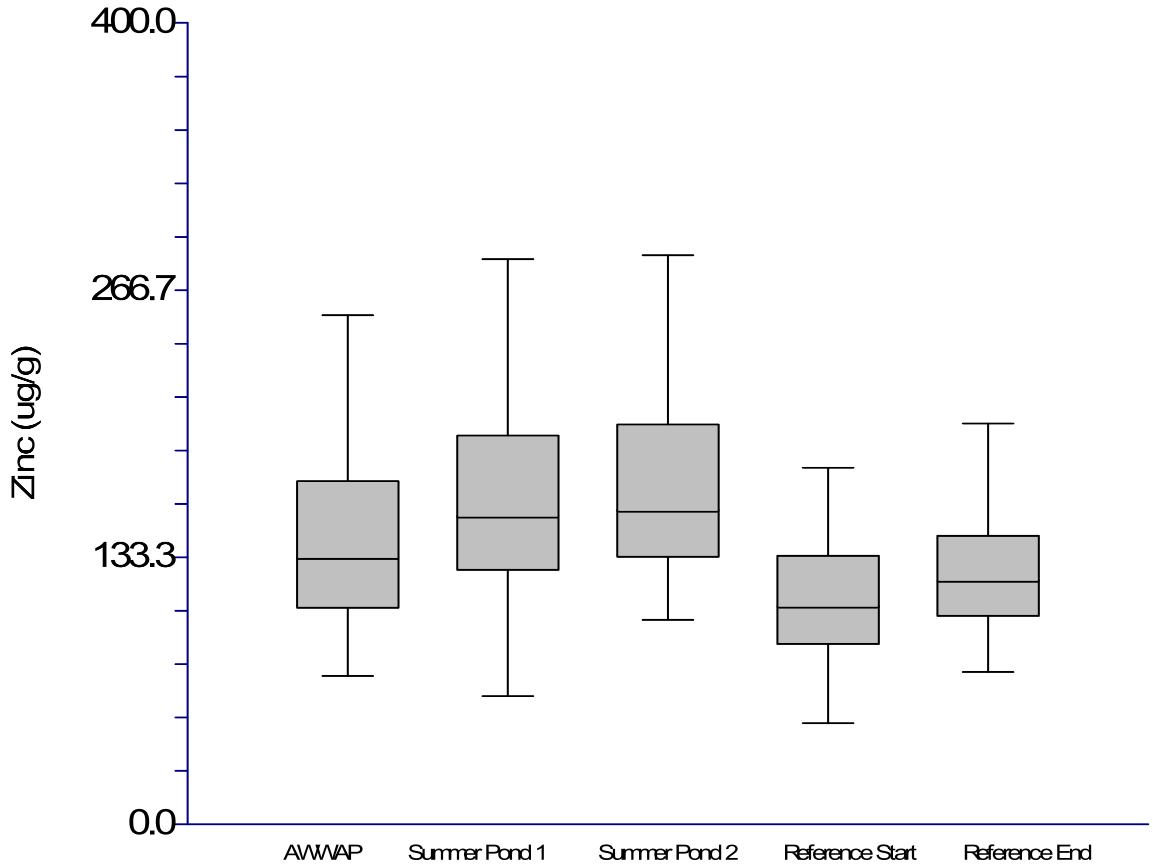

Figure 10. Box plot analysis of zinc (μg/g) in gill epithelium of steelhead trout at the start of the study at Arcata Wastewater Aquaculture Facility (AWWAP) and after twelve months in Summer Pond 1 and 2 and the reference site at the start and end of the study in Arcata, California from November 2006 to October 2007.

Ponds and the reference site (Table 7). A Tukey-Kramer Multiple Comparison test showed a significant difference in mean zinc concentration in gill epithelium among sites at the beginning of the study and end of the study (Table 7).

Zinc in liver tissue at the beginning of the study had a mean of 90.30 ± 6.17 µg/g and at the reference site 97.40 ± 6.05 µg/g (Figure 11). At the end of the study mean zinc concentration in liver tissue of trout from the Summer Ponds 1 and 2 had increased with similar concentrations (Figure 11). A two-tail Student's t-test showed a significant increase ($p < 0.001$) in mean zinc concentrations in liver tissue of trout in the Summer Ponds and the reference site at the end of the study (Table 8). A Tukey-Kramer Multiple Comparison test showed a significant difference ($p = 0.032$) in mean zinc concentration in liver tissue among sites at the beginning of the study, however the Summer Pond fish had accumulated significantly ($p < 0.001$) more zinc in the liver tissue than the reference site fish after one year (Table 8).

Table 7. Results of a Two Sample T-test used to determine a difference in mean zinc values (µg/g) dry weight in gill epithelium of steelhead trout at Arcata Wastewater Aquaculture Facility before exposure to wastewater (November 2006) and Summer Ponds 1 and 2 after 12 months exposure to wastewater (October 2007) and reference site Humboldt State University Fish Hatchery in Arcata, California.

Zinc in Trout Gill Epithelium								
	Before				After			
Site	Mean (µg/g)	SE (±)	n	Site	Mean (µg/g)	SE (±)	n	P
AWWAP	141.00	6.27	48	Summer Pond 1	168.93†	6.20	49	<0.001
				Summer Pond 2	164.89†	6.33	48	<0.001
Reference	112.54	6.42	50	Reference	130.30	4.56	99	<0.001

† Means among sites did not differ (p<0.05) based on a Tukey-Kramer Multiple-Comparison Test.

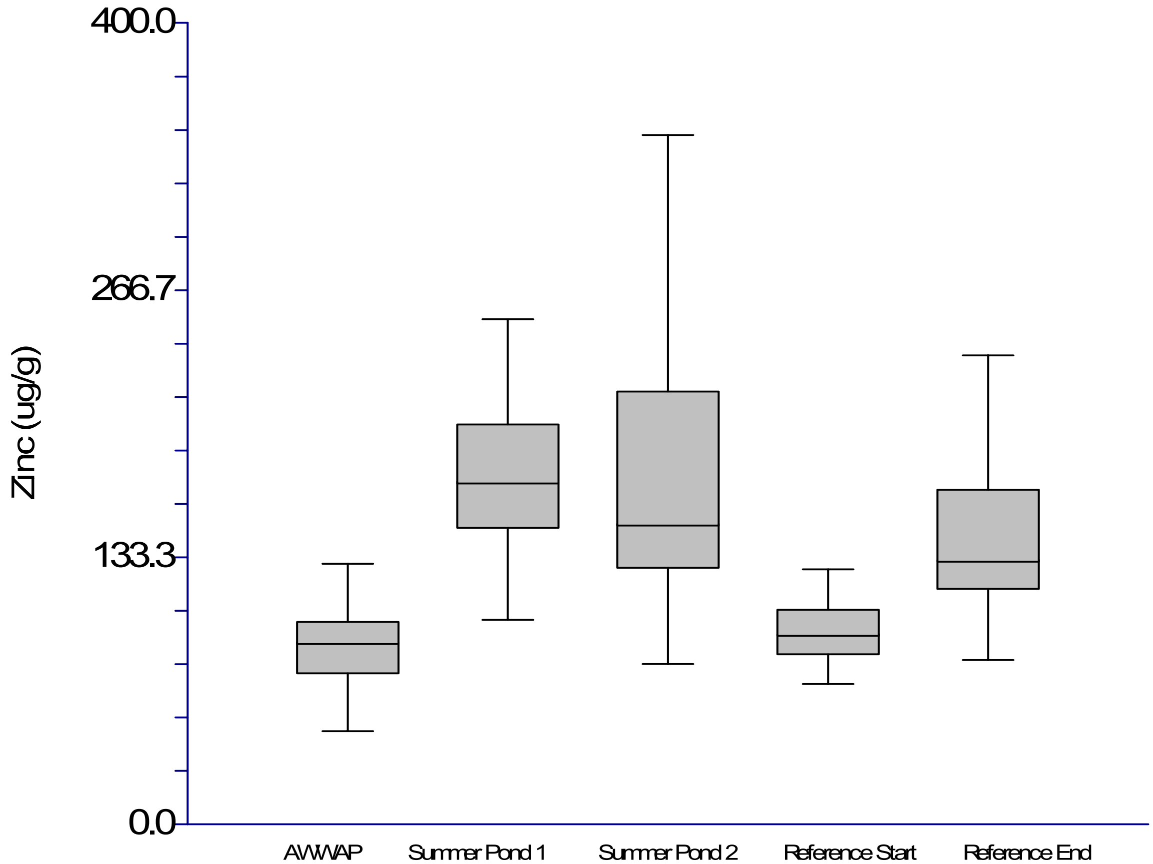

Figure 10. Box plot analysis of zinc (µg/g) in liver tissue of steelhead trout at the start of the study at Arcata Wastewater Aquaculture Facility (AWWAP) and after twelve months in Summer Pond 1 and 2 and at the reference site at the start and end of the study in Arcata, California from November 2006 to October 2007.

Table 8. Results of a Two Sample T-test used to determine a difference in mean zinc values (µg/g) dry weight in liver tissue of steelhead trout at Arcata Wastewater Aquaculture Facility (AWWAP) before exposure to wastewater (November 2006) and Summer Ponds 1 and 2 after 12 months exposure to wastewater (October 2007) and reference site Humboldt State University Fish Hatchery in Arcata, California.

Zinc in Trout Liver								
	Before				After			
Site	Mean (µg/g)	SE (±)	n	Site	Mean (µg/g)	SE (±)	n	P
AWWAP	90.30†	6.17	48	Summer Pond 1	176.65†	6.17	49	<0.001
				Summer Pond 2	185.79†	7.10	48	<0.001
Reference	97.40†	6.05	51	Reference	151.95	4.34	98	<0.001

† Means among sites did not differ (p<0.05) based on a Tukey-Kramer Multiple-Comparison Test.

DISCUSSION

This study found no significant evidence of copper, cadmium and zinc toxicity in steelhead trout (*Oncorhynchus mykiss)* after being reared for 12 months in ponds containing wastewater. In general, the necessary environmental conditions in the ponds were met for rearing trout. The temperature in the ponds ranged from 6˚ C to 22˚ C (Figure 3) and was often below 20˚ C, the preferred maximum mean daily water temperature for trout growth (Southern California Edison Company 2007). Maximum water temperature at the study site in the summer months was 22˚ C. A water temperature of 24˚ C is expected to be stressful for trout; however, there were no observed mortalities in the Summer Ponds during the study. The Summer Ponds are steep sided and deep (2.5-3 meters) allowing cooler water to sink providing a thermal refuge during warmer seasons. Temperature changes in the Summer Ponds were gradual and mostly controlled by the seasons (Figure 3). High and rapid changes of temperature are more hazardous to fish exposed to metals such as zinc (Sorenson 1991).

The pH in the ponds ranged from 6.5 – 8.5 (Figure 4) and dissolved oxygen levels in the ponds maintained above 7 mg/L (Figure 6) both of which are ideal environmental conditions for rearing trout (Helfman et al. 1999). Low levels of dissolved oxygen can increase stress in fish decreasing their resistance to disease (Helfman et al. 1999). Results from this study found that Summer Pond 2 had a higher ratio of wastewater than Summer Pond 1 (Figure 7). Based on the higher salinity in Summer Pond 1 it was determined that the less saline Summer Pond 2 received more wastewater. Due to lower salinity, Summer Pond 2 could have higher phytoplankton production which could also increase dissolved oxygen levels thus supporting increased numbers of invertebrates and providing more food for trout.

When considering body weight and fork length, trout from the study and reference sites were similar over all indicating that growth of trout in the Summer Ponds was similar to a conventional hatchery. Interestingly, mean body weight for trout from Summer Pond 2 was significantly different from Summer Pond 1 and the reference site, while fork length for trout from the Summer Ponds and reference site were not different (Tables 2 and 3). Trout from Summer Pond 2 had a higher body weight due to a higher ratio of nutrient rich wastewater which resulted in higher primary production and more food for trout. Arcata Wastewater Aquaculture Project trout were supplementary fed approximately 85 percent less than the conventional ration. Availability of food likely fluctuated with environmental conditions, but appeared substantial enough for trout survival.

This study found no evidence for copper toxicity in trout from the Summer Ponds throughout the study. Despite a higher ratio of wastewater to bay water in Summer Pond 2 the concentration of aqueous copper was the same in both Summer Ponds, therefore wastewater may not be a significant source of copper. The concentration of copper at the study site could reflect current concentrations in bay waters. The concentration of total aqueous copper in the Summer Ponds averaged 0.003 mg/L throughout the study (Table 4). Davis and Woodling (1980) determined the LC_{50} of copper for unacclimated trout was

0.023 mg/L in a riverine system. Cusimano et al. (1986) found the LC50 of copper for non-acclimated trout in a laboratory setting was 0.003 mg/L.

Copper concentrations were below detection limits in gill epithelium of trout from the study and reference sites. Buckley, et al. (1982) stated copper accumulates in gill tissue when trout are exposed to sublethal concentrations of copper. In this study, because copper was below levels of detection in gill epithelial the concentration of copper biologically available in the Summer Ponds was likely below sublethal concentrations.

Copper in the liver tissue of trout at time zero was significantly higher than trout from the reference site (Table 5). The difference in copper concentration in the liver tissue at the beginning of the study is likely due to the difference in exposure to aqueous copper during hatching and rearing. Studies have shown copper homeostasis to be regulated by the liver and may be concentration dependent (Buckley et al.1982, McGreer et al. 2000). Trout before exposure to wastewater were hatched and reared in city water, which reported copper levels below regulatory action level of 1.3 mg/L (City of Arcata Drinking Water 2006 Water Quality Report). Trout from the reference site were exposed to aqueous copper at concentrations of 0.001 mg/L prior and during the 12 month study.

At month 12, concentration of copper in liver tissue from fish reared in Summer Ponds 1 and 2 had not significantly increased ($p = 0.71$ and $p = 0.81$, respectively). During the same period, liver tissue from fish reared at the reference site exhibited a significant increase in copper (Figure 9). Wastewater in the Summer Ponds contained higher organic and inorganic material than the reference site hatchery water that likely influenced the availability of free copper.

Previous studies found that free copper was reduced by organic complexing agents and adsorption to inorganic particles (Wilson 1972, Brown et al. 1974, Sylva 1976 as cited in Sorenson 1991). For example, Brown et al. (1974 as cited in Sorenson 1991) found copper toxicity in brown trout decreased as a function of organic matter. Additionally, pH in Summer Pond 2 ranged from 6.5-8.5 (Figure 4) where the toxic form of copper, Cu (II), changes speciation to non toxic forms (Sorensen 1991).

A physiological mechanism that can influence the toxicity of copper in trout is developed through previous exposure. Copper levels are regulated in the liver to protect against toxic and acute concentrations of copper; however, studies have not found a correlation with copper toxicity and the accumulation of copper in liver tissue (Sorenson 1991). Dixon and Sprague (1981 as cited in Sorensen 1991) found increased tolerance of copper appeared to be correlated to elevated levels of metal binding hepatoproteins which sequesters the metal, increasing accumulation of copper in the tissues before it reaches toxic levels. Roch et al. (1982 as cited in Sorensen 1991) stated that trout from a polluted river system have increased hepatic levels of copper and cadmium as well as increased levels of hepatoproteins.

Considering the environmental and physiological mechanisms stated previously, the LC_{50} of 0.003mg/L does not appear to be applicable probably due to the reduction of biologically available free copper in the presence of organic and inorganic material. Concentrations of copper in the Summer Ponds were below lethal levels for trout.

This study found cadmium to be below detection limits (0.002 mg/L) in trout tissue and water samples from the study and reference sites. Previous studies have found cadmium typically had the lowest concentration in fish tissue and is often below detection limits (Chen et al. 2000, Guerrin et al. 1990, Deb and Santra 1997); however, many fish are extremely sensitive to cadmium and lethality can occur at whole body concentrations of less than 1ppm (McGreer et al. 2000). Toxic concentrations of aqueous cadmium for trout is <0.0005 mg/L (Davis and Woodling 1980, Cusimano et al. 1986). Concurrent studies found cadmium in Humboldt Bay, associated with the study site, to be in parts-per-trillion and below levels of detection for this method (Martin and Hurst 2008). With respect to cadmium levels, there was no apparent threat to trout health throughout the study.

Mean aqueous concentrations of zinc in Summer Pond 1 was 0.010 mg/L and was not significantly different from the reference site (0.007 mg/L; $p = 0.18$) (Table 6). Aqueous zinc concentrations at the reference site were consistent throughout the study except for a marked increase in both April and September. The increase in zinc at these times may be due to the use of city water to backwash the filters in the hatchery system (Table 6). Summer Pond 2 had a higher ratio of wastewater and a significantly higher mean of aqueous zinc (0.027 mg/L). Aqueous zinc concentrations in Summer Pond 2 decreased slightly from April to August 2007 suggesting that zinc may have been removed at increased rates by phytoplankton production. Previous studies found zinc LC_{50} was 0.035 mg/L in a riverine system and 0.066 mg/L under laboratory conditions (Davis and Woodling 1980, Cusimano et al. 1986). In context of these analyses, concentrations of zinc in the Summer Ponds were below lethal levels for trout.

Upon initiation of the study, concentrations of zinc in gill epithelium from trout reared at the study site were significantly higher than the reference site; however, there was no difference in liver concentrations. At month 12, concentration of zinc in gill epithelium and liver tissue had increased significantly in fish reared at all sites (Tables 8 and 9) and the study site was significantly higher than the reference site. The highest mean accumulation of zinc in trout was 168.93 µg/g in gill tissue from Summer Pond 1. Bioaccumulation of zinc in *Tilapia* after prolonged exposure to wastewater can range from 1.0 µg/g to 140.0 µg/g (Deb and Santra 1997, Barber et al. 2000, McGreer et al. 2000).

Zinc accumulation in gill epithelial and liver tissues between the Summer Ponds was not significantly different despite the different concentrations of zinc in the water (Tables 7 and 8). This suggested that the study trout were able to regulate levels of zinc in their tissues despite being reared in different concentrations of aqueous zinc. Watson and Beamish (1980 as cited in Sorenson 1991) found anadromous fish respond differently to elevated levels of zinc than freshwater fish because of a homeostatic response mechanism. This response mechanism is required to differentially transport salts and other ions such as zinc across the gill surface in chemically different environments.

Likewise, Roch et al. (1982) found hepatic levels of zinc in rainbow trout living in a polluted river did not increase despite the high concentrations of aqueous zinc further supporting homeostatic control of zinc in trout. The innate ability of trout to regulate zinc accumulation and therefore further reduce the risk of toxicity can be of special interest to the aquaculture industry. Anadromous fish such as trout would have an advantage over freshwater fish because they can be reared in water with nominally elevated levels of zinc without notable toxicity. The trout aquaculture industry could take advantage of reclaimed wastewater as a water source with less risk of metal toxicity.

From March to September 2007, temperatures at the study site were higher than the reference site (Figure 3). The significantly higher accumulation of zinc in trout from Summer Ponds compared to the reference site could have been influenced by temperature (Table 6). Matthissen and Brafield (1977) determined that metabolic rate was correlated with metal accumulation and at higher temperatures metabolism increases. The few months that the Summer Ponds had higher temperatures may account for the difference in zinc accumulation in trout tissues between the study and reference sites.

When considering the toxic forms of zinc, Cairns et al. (1971 as cited in Sorenson 1991) stated zinc (II) (toxic form) does not cause mortalities above pH 8 when most of the available zinc has converted into hydroxides. Summer Ponds reached a pH above 8 in the summer months thereby reducing toxic forms of zinc. In general, environmental conditions including temperature, pH and dissolved oxygen in the Summer Ponds were favorable for rearing trout.

In conclusion, the successful growth of trout in this established aquaculture pond system indicates significant benefits can be gained from using wastewater in trout aquaculture. In general, environmental conditions including temperature, pH and dissolved oxygen in the Summer Ponds were favorable for rearing trout. Toxic levels of heavy metals in wastewater are a concern for public health and for fish health. This study found the Summer Ponds contained only a nominal concentration of copper when compared with the reference site. Concentrations were below toxic levels for trout, when compared with other toxicity studies. Increased wastewater constituents including organic and inorganic material in Summer Pond 2 further reduced the accumulation of copper in fish tissues when compared with the reference site. Cadmium was below levels of detection in trout tissue and in the Summer Ponds. While other studies have found that cadmium toxicity in fishes can occur at very low concentrations, cadmium was below detection limits and thus likely below toxic levels. Concentrations of zinc were below toxic levels when compared with other toxicity studies. Wastewater provided an increased load of zinc in Summer Pond 2; however, the lack of difference in accumulation in trout tissues between ponds suggested that trout have homeostatic control mechanisms which allows trout to avoid toxic accumulation levels. Additionally, wastewater is not always a significant source of toxic levels of heavy metals. Still, wastewater constituents vary greatly among municipalities and heavy metal concentrations should be considered before establishing an aquaculture facility utilizing wastewater. This study utilized a mixture of bay water with reclaimed wastewater which provided ideal conditions for rearing trout and would be ideal for trout aquaculture in coastal regions. Further studies should be considered if establishing a wastewater aquaculture facility that is exclusively freshwater. The mechanisms that reduce metal toxicity in trout in saline water, such as the ability to actively transport zinc out of the gills, may be inhibited or reversed in freshwater. In addition, other constituents in wastewater, such as toxic nitrogenous forms, combined with freshwater may not provide the necessary conditions for rearing trout.

Future studies should consider other parameters of concern in wastewater such as trace levels of endocrine disrupting chemicals, hormone-disrupting compounds and pharmaceuticals before establishing an aquaculture industry that produces trout for human consumption or restoration purposes. Overall, increased demands on local water sources as well as an increased demand for trout production needs efficient and sustainable solutions such as the use of recycled, already established water sources. Wastewater can be a valuable renewable resource solution for the aquaculture industry.

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