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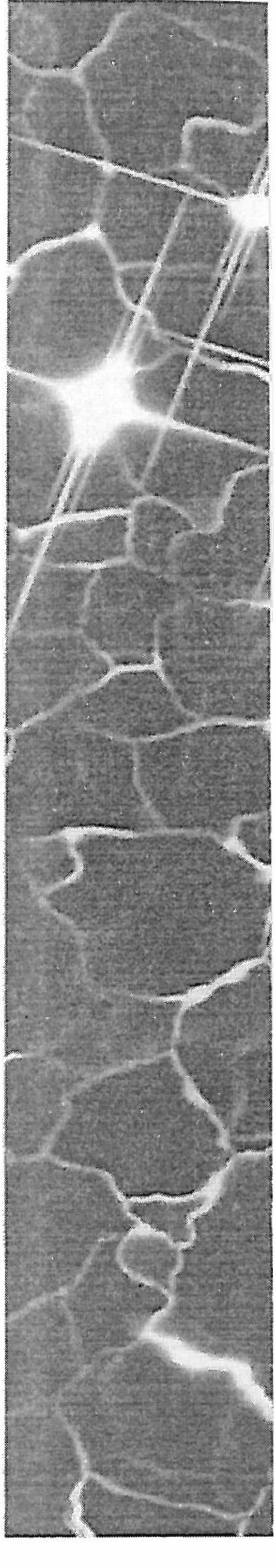

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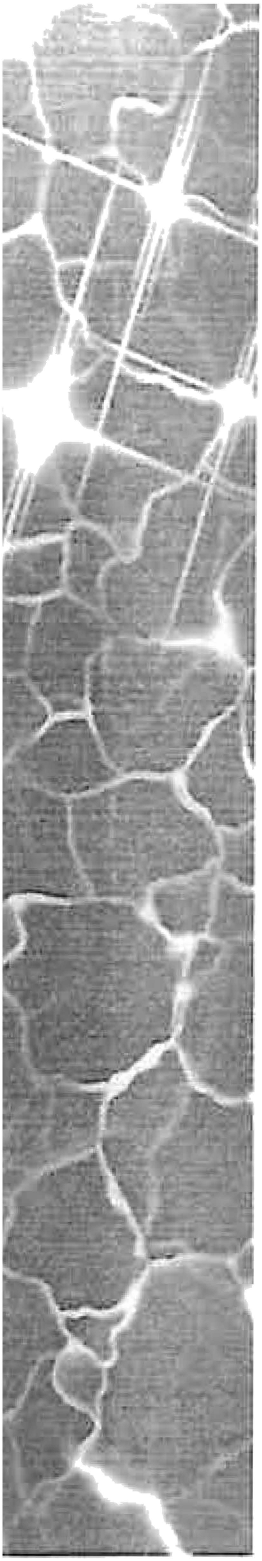

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Dear Readers:

We are greatly pleased to present to you the **International Journal of Recirculating Aquaculture**. This refereed publication represents the next logical step in the consolidation of research and applications expertise in the area of recirculation systems, and is intended to be a forum for the open exchange of reliable information on the subject. The journal will be published biannually, or more frequently as submissions warrant.

As everyone involved in the fisheries industry is already aware, the future of our wild fisheries is at best unpredictable. Commercial scale wild harvesting of fish and shellfish puts a heavy burden on marine and freshwater ecosystems, and is subject to ever-changing regulations, tariffs, and limitations. At the same time, the demand for high-quality, low fat protein sources is increasing. Aquaculture fits neatly into this market niche, but must be responsive to those who are critical of its impact on the environment. Aquaculture has the potential to reduce pressures on specific species and habitats, but it will not do so unless aquaculturists keep ecological concerns a high priority. In the near future, aquaculture operations will face new regulations on effluents, and the industry must be prepared to face these challenges.

Recirculating aquaculture, both as a concept and as an industry, requires that its workforce be well-versed in a variety of fields, from animal husbandry, to economics, to water chemistry, to systems engineering. Aquaculturists must select a species that will be valuable in the marketplace, and provide products to their purchasers without diminishing food quality or safety. It is this need for varied and reliable information that led to the idea of a journal where research in areas germane to the recirculating aquaculturist could be compiled and presented. The papers presented will cover a variety of topics, with *recirculation* as their common element.

We invite you to look the journal over, and to give us your comments. Consider what the journal has the potential to contribute to the emerging recirculating aquaculture industry over time. Consider it as a place to add your ideas and your work to the field.

Recirculating systems hold the promise of being the best examples of responsible and resource-efficient aquaculture operations. We hope that you will join us as we chronicle the progress and the problems that face our industry.

A.I. Correa, and the IJRA Executive Board

Note: *Subscription information and guidelines for manuscript submission can be found in the back of the journal.*

Hematology and Plasma Chemistry Values for Production Tilapia (*Oreochromis* hybrid) Raised in a Recirculation System

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ABSTRACT

Tilapia are a frequently aquacultured fish, yet little is known about their normal physiology and response to disease. To assess specific diseases in mammals, blood constituents are routinely analyzed and compared to previously determined standardized values. Research to establish hematologic values for normal healthy fish is required before blood analysis can be used for diagnostic purposes in fishes. This study determined hematology and plasma chemistry values for production tilapia (*Oreochromis* hybrids) raised in a recirculation system. Using standard clinical techniques, the following hematologic parameters were determined: PCV (packed cell volume), plasma protein and MCV (mean cell volume) values; and erythrocyte, leukocyte, lymphocyte (small and large), neutrophil, monocyte, eosinophil, thrombocyte-like-cell, and thrombocyte numbers. Additionally, the following plasma chemistry values were determined: total protein, albumin, globulin, creatinine, total bilirubin, ALP (alkaline phosphatase), AST (aspartate aminotransferase), sodium, potassium, chloride, calcium, phosphorus, magnesium, glucose, cholesterol, ammonia and osmolality. Analysis of blood parameters can enhance production of hybrid tilapia by providing a means for the early detection of infectious diseases, and by assisting in the identification of sub-clinical conditions affecting production performance.

INTRODUCTION

Tilapia are the second most commonly cultured fish in the world, and are a food staple in many parts of Africa, Asia, and South America (CEAH 1995; Anonymous 1996). In the USA, tilapia consumption has increased and these fish are the fourth most commonly cultured fish. Intensive aquaculture of tilapia, as with other species of finfish, is adversely affected by production-related disorders and infectious diseases (CEAH 1995). This is particularly true of fish raised in recirculating systems, where high stocking densities promote the spread of infectious diseases. As the aquaculture industry expands, there is an increasing need for improved diagnostic methods to detect disease and to diagnose causes of poor production performance. Hematology and clinical chemistry analysis are not regularly used in fishes, as baseline values are not available for most species; however, blood analysis can provide significant diagnostic information once baseline values are established. Successful culture and maintenance of tilapia can be enhanced by developing such a clinical tool to monitor the health of production fish. Previously reported blood values for tilapia are from non-production or low intensity production tilapia (Terao and Ogawa 1984; Haniffa and Vijayarani 1989; Hussein et al. 1996). This present study determined a complete profile of hematologic and biochemical blood values for production hybrid tilapia raised in a commercial recirculating system. This profile can serve as a guide for comparisons in the analyses of blood values from other production hybrid tilapia.

MATERIALS AND METHODS

Hybrid tilapia were stocked into a 231,900 L recirculation system as fingerlings. At the time of sampling, the fish had an average weight of 551 g, a length of 26.7 cm and were stocked at a density of 70 g/L. The fish were fed a commercial tilapia feed (Tilapia Grower - floating, Southern States Cooperative, Richmond, VA, USA) at 2% of body weight per day. Water quality was monitored at the times of sampling for standard parameters and the values are shown in Table 1. Fish with any gross abnormalities were not included in the study.

The fish were rapidly netted, anesthetized in aerated buffered tricaine methanesulfonate (MS-222, Sigma Chemical Co. St. Louis, MO, USA)

Table 1. Mean water quality values for hybrid tilapia maintained in high density (70 g/L) recirculating systems.

PARAMETER	VALUE
Temperature (°C)	29
pH	7.2
NH_3 un-ionized (mg/L)	0.017
NO_2-N (mg/L)	0.39
NO_3-N (mg/L)	54
Alkalinity (mg/L)	188
Hardness (mg/L)	154
DO (mg/L)	8.0

and bled with a 23 gauge needle and a 3 mL syringe from the caudal vessels. The collected blood was placed in blood tubes containing either ethylenediamine tetra-acetic acid (EDTA) for hematological analysis, or lithium heparin for chemistry analysis. Any hemolyzed or clotted samples were discarded before analysis.

Blood from the EDTA tube was drawn into microhematocrit tubes (Fisher Scientific, Norcross, GA, USA) and the packed cell volume (PCV) determined after centrifugation at 10,000 x g for 5 min. Plasma protein was determined with a clinical refractometer using plasma from the microhematocrit tube. The total red cell count and total white cell counts were determined manually as previously described (Hrubec et al. 1996a) with a Neubauer hemacytometer using Natt-Herrick's solution (Natt and Herrick 1952) as a diluent stain. Briefly, as thrombocytes can not be reliably distinguished from leukocytes on the hemacytometer, both were counted to give a combined leukocyte/thrombocyte count. Thrombocytes were then enumerated and subtracted from the combined count during the differential. Manual determination of total red and white cell counts is recommended for use with fish blood, as the nucleated red cells prevent accurate enumeration using automated analysis (Huffman et al. 1997). Blood smears, using the EDTA treated blood, were stained with Wright's Geimsa stain and used for the differential count as described previously (Hrubec et al. 1996a).

Blood in the heparinized tubes was centrifuged (14,000 x g) for 5 min and the plasma collected and frozen at -10°C until analyzed (approximately 3 weeks later). Plasma samples were analyzed using an automated dry chemistry system (Kodak Ektachem 700, Rochester, NY, USA) for total protein, albumin, creatinine, total bilirubin, alkaline phosphatase (ALP), aspartate aminotransferase (AST), sodium, chloride, potassium, calcium, magnesium, phosphorus, glucose, cholesterol, and ammonia. Dry chemistry analysis systems are standardized and automated to reduce variation in results. They are used routinely in human and veterinary medicine, and have been documented to be accurate for fish samples (Warner et al. 1978; Warner et al. 1979; Smith and Ramos 1980). Globulin was calculated from the difference between the total protein and albumin. Osmolality was determined using an osmometer (Multiosmette Micro-Osmometer 2430, Precision Systems Inc., Natick, MA, USA).

Table 2. Hematologic values for production tilapia reared in recirculating systems.

Analyte	**n**[1]	**Range**	**Mean**	**SEM**[2]
PCV[3] (%)	35	23-35	28	0.5
Plasma Protein (mg/dl)	35	4.9-8.4	6.3	0.2
Erythrocytes (x 10^6/µL)	35	1.96-2.91	2.43	0.046
MCV[4] (fl)	35	90-143	114	2
Leukocytes (#/µL)	31	13,200-110,300	55,700	4111
Lymphocytes (#/µL)				
Small	31	9,300-78,900	44,200	3170
Large	31	700-11,700	4,000	469
Neutrophils (#/µL)	30	1000-9,300	3,200	348
Monocytes (#/µL)	30	0-3,300	900	148
Eosinophils (#/µL)	31	0-1,600	500	83
TLC[5] (#/µL)	31	500-5,700	1,800	235
Thrombocytes (#/µL)	31	18,800-80,300	39,500	2787

[1] *Number of fish,*
[2] *Standard error of the mean,*
[3] *Packed cell volume,*
[4] *Mean cell volume,*
[5] *Thrombocyte-like-cell*

RESULTS and DISCUSSION

Although tilapia are one of the most frequently cultured fish in the world, there are surprisingly few papers that present normal blood values (Terao and Ogawa 1984; Haniffa and Vijayarani 1989; Hussein et al. 1996). In these previous studies, only a few fish were used, or only a small number of analytes were determined. Additionally, these studies were conducted on pure species of non-production tilapia and not the more commonly cultured hybrids. The blood values of the pure strains may be quite different from the values for a production hybrid. The data presented in the present study should be relevant for production hybrids raised in recirculating systems under similar culture conditions.

The values for the hematologic parameters are given in Table 2. The plasma protein, white cell count and differential white cell count can provide valuable information about the immune and inflammatory responses of an individual. The packed cell volume, red cell count and MCV can provide information about anemias and red cell production and hydration status. The hematologic values determined in this study are comparable to those published previously with the exception of Haniffa and Vijayarani (1989) who reported lower PCV (10%), and erythrocyte counts (0.9 x10^6/µL) in *Oreochromis mossambicus*. Conversely, Terao and Ogawa (1984) reported higher PCV values (35%) in *Tilapia nilotica*. The leukocyte types observed in the fish from the present study: small lymphocytes, large lymphocytes, neutrophils, monocytes, and eosinophils, are similar to those described for *Oreochromis mossambicus* (Doggett et al. 1987) and other species of fish (Ellis 1977). The thrombocyte-like-cell (TLC) is a cell type that has been identified in different species, and superficially resembles a thrombocyte and probably represents a maturational stage of one of the leukocytes (Hrubec et al. 1996a).

The values for the plasma chemistry parameters are given in Table 3. Total protein, albumin and globulin can provide information about the immune status, liver function, hydration and osmoregulation. The blood chemistries: AST, ALP, total bilirubin, creatinine, and cholesterol can indicate changes in organ function; while the electrolytes and plasma osmolality are indicators of osmoregulatory ability. Electrolytes and glucose can also provide information about stress levels. Comparison of previously reported blood values with those determined in this study

Table 3. Serum biochemical values for production tilapia reared in recirculating systems.

Parameters	n[1]	Range	Mean	SEM[2]
Total Protein (g/dL)	40	3.9-8.6	5.8	0.3
Albumin (g/dL)	40	1.8-3.0	2.4	0.1
Globulin (g/dL)	40	2.1-5.6	3.4	0.2
Creatinine (mg/dL)	40	0.2-0.6	0.4	0.02
Total bilirubin (mg/dL)	40	0.1-0.5	0.2	0.02
ALP[3] (U/L)	37	12-48	28	1
AST[4] (U/L)	28	21-770	238	44
Sodium (mEq/L)	40	141-161	151	1
Potassium (mEq/L)	40	3.57-6.16	4.84	0.1
Chloride (mEq/L)	39	110-129	121	1
Calcium (mg/dL)	40	16.5-165.0	60.1	6.58
Phosphorus (mg/dL)	38	9.8-60.1	21.5	1.7
Magnesium (mg/dL)	38	2.8-5.0	3.7	0.1
Glucose (mg/dL)	39	49-120	78	3
Cholesterol (mg/dL)	40	126-313	208	8
Ammonia (µmol/L)	39	157-500	324	17
Osmolality (mOsm)	39	310-359	328	2

[1] *Number of fish,*
[2] *Standard error of the mean,*
[3] *Alkaline phosphatase*
[4] *Aspartate aminotransferase (SGOT)*

revealed similar values for most analytes. Terao and Ogawa (1984) did report higher levels of creatinine (4.3 mg/dL), chloride (192 mEq/dL), cholesterol (567 mg/dL), and glucose (408 mg/dL) and lower levels of calcium (11.8 mg/dL) in *Tilapia nilotica*. Hussein et al. (1996) reported slightly lower levels of total protein, albumin and globulin (3.4, 0.7, and 2.7 g/dL respectively). The blood values reported in this present study are generally consistent with those of other species of finfish (McDonald and Milligan 1992). The calcium and phosphorus levels are higher than usually reported for other species, but the reason for this is unknown.

Differences in hematologic and biochemical blood values may be due to a wide variety of factors. Blood values in fishes are affected by capture and sample collection technique, environmental factors, culture conditions, diet, and age and sex of the fish (McDonald and Milligan 1992; Lane 1979; Ram-Bhaskar and Srinivasa-Rao 1989; Hrubec et al. 1996a,b; Hrubec et al. 1997a,b). Additionally, as reported previously, different hybrids of striped bass have different normal blood values (Hrubec et al. 1996a). In the current study, the tilapia were hybrids of the parental species used in the previously published tilapia hematology studies. The water quality in the production tanks, although typical for intensively reared hybrid tilapia in recirculating systems, is different from the water quality of non-production tanks. These differences may account for some of the variance observed between studies.

In order to develop hematology and clinical chemistry as diagnostic tools for use with fishes, one must determine normal values and then determine whether environmental factors influence these values. Finally, one determines how the values change under pathologic conditions. In the early stages of this process, the range of normal blood values may appear unduly broad until the effects of external factors that influence the blood values (water quality, diet, age, culture conditions, etc.) are identified and mitigated. This is the most likely cause for the range of values observed in this study. Similar ranges of normal values were observed in hybrid striped bass from high density recirculating systems (Hrubec et al. 1996a,b).

Taken together, the analyses of hematologic and biochemical parameters provide direct information on the overall immune status and health of an individual, contributing information relevant to disease diagnosis. As the aquaculture industry expands, tools to monitor the health status of fishes using standardized non-lethal and inexpensive methods will be needed. This study provides baseline blood values for production hybrid tilapia in a recirculation system. Generating these values is the first step in developing hematology as a diagnostic tool for use in these fish.

ACKNOWLEDGEMENTS

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REFERENCES

Anonymous. Status of the World Aquaculture 1995. pp. 6-27, In: *Aquaculture Magazine Buyer's Guide '96*, **1996**.

CEAH, Centers for Epidemiology and Animal Health. Overview of Aquaculture in the United States. Centers for Epidemiology and Animal Health, USDA:APHIS:VS, 555 South Howes, Fort Collins, CO **1995**.

Doggett, T.A.,Wrathmell, A.B., Harris, J.E. A Cytochemical and Light Microscopical Study of the Peripheral Leukocytes of *Oreochromis mossambicus* Cichlidae. *Journal of Fish Biology* **1987**, 31,147-154.

Ellis, A.E. The Leukocytes of Fish: A Review. *Journal of Fish Biology* **1997**,11, 453-491.

Haniffa, M.A., Vijayarani, S.M. Hematological Effects of Textile Mill Effluent on Freshwater Fish *Oreochromis mossambicus* (Trewaves). *Indian Journal of Experimental Biology* **1989**, 27, 476-478.

Hrubec, T.C., Smith, S.A., Robertson, J.L., Feldman, B., Veit, H.P., Libey, G.S., Tinker, M.K. Comparison of Hematologic Reference Intervals Between Culture System and Type of Hybrid Striped Bass. *American Journal of Veterinary Research* **1996**a, 57, 618-623.

Hrubec, T.C., Smith, S.A., Robertson, J.L., Feldman, B., Veit, H.P., Libey, G.S., Tinker, M.K. Blood Biochemical Reference Intervals for Sunshine Bass (*Morone chrysops* x *Morone saxatilis*) in Three Culture Systems. *American Journal of Veterinary Research* **1996**b, 57, 618-623.

Hrubec, T.C., Robertson J.L., Smith, S.A. Effects of Temperature on Hematologic and Serum Biochemical Profiles of Hybrid Striped Bass (*Morone chrysops* x *Morone saxatilis*). *American Journal of Veterinary Research* **1997**a, 58, 126-130.

Hrubec, T.C., Robertson, J.L., Smith, S.A. Effects of Ammonia and Nitrate Concentration on Hematologic and Serum Biochemical Profiles of Hybrid Striped Bass (*Morone chrysops* X *Morone saxatilis*). *American Journal of Veterinary Research* **1997**b, 58, 131-136.

Huffman, P.A., Arkoosh, M.R., Casillas, E. Characteristics of Peripheral Blood Cells from Rainbow Trout Evaluated by Particle Counter Image Analysis and Hemocytometric Techniques. *Journal of Aquatic Animal Health* **1997**, 9, 239-248.

Hussein, S.Y., El-Nasser, M.A., Ahmed, S.M. Comparative Studies on the Effect of the Herbicide Atazine on Freshwater Fish *Oreochromis niloticus* and *Chrysichthyes auratus* at Assiut, Egypt. *Bulletin of Environmental Contamination and Toxicology* **1996**, 57,503-510.

Lane, H.C. Progressive Changes in Hematology and Tissue Water of Sexually Mature Trout, *Salmo gairdneri* Richardson During the Autumn and Winter. *Journal of Fish Biology* **1979**, 15, 425-436.

McDonald, D.G., Milligan, C.L. Chemical Properties of Blood. pp. 56-135, In: Fish Physiology Vol. 12 Part B. The Cardiovascular System. Hoar, W.S., Randall, D.J., Farrell, A.P. (eds.), Academic Press: New York, NY, **1992**.

Natt, M.P., Herrick, C.A. A New Blood Diluent for Counting Erythrocytes and Leukocytes of the Chicken. *Poultry Science* **1952**, 31, 735-738.

Ram-Bhaskar, B.R., Srinivasa-Rao, K.S. Influence of Environmental Variables on Hematology, and Compendium of Normal Hematological Ranges of Milkfish, *Chanos chanos* (Forskal) in Brackish Culture. *Aquaculture* **1989**, 83, 123-136.

Smith, A.C., Ramos, F. Automated Chemical Analysis in Fish Health Assessment. *Journal of Fish Biology* **1980**, 17, 445-450.

Terao, T., Ogawa, T. On the Biochemical Components in the Blood of the Cultured Cichlid Fish *Tilapia nilotica. Scientific Reports of the Hokkaido Fish Hatchery* **1984**, 39, 83-88.

Warner, M.C., Diehl, S.A., Tomb, A.M. Effects of Dilution and Temperature of Analysis on Blood Serum Values in Rainbow Trout *Salmo gairdneri. Journal of Fish Biology* **1978**,13, 257-263.

Warner, M.C., Tomb, A.M., Diehl, S.A. Variability and Stability of Selected Components in Rainbow Trout *Salmo gairdneri* Serum and the Precision of Automated Analysis in Measuring These Components. *Journal of Fish Biology* **1979**,15, 141-151.

Economic Analysis of an Aquaponic System for the Integrated Production of Rainbow Trout and Plants

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ABSTRACT

Conventional treatment alternatives for phosphorus in wastewater, whether they employ chemical precipitation, physical removal, or land application technologies, represent a significant additional cost to the owner of an aquaculture operation. Plant-based removal of nutrients has the potential to generate additional revenues, which can offset treatment costs. The objective of this analysis was to describe the economic relationship between a 22,680 kg per year recirculating rainbow trout (*Oncorhynchus mykiss* Walbaum) production system and a hydroponic treatment unit, growing 'Ostinata' lettuce (*Lactuca sativa* L.) and sweet basil (*Ocimum basilicum* L.), capable of reducing phosphorus concentration levels in the fish farm effluent to less than 0.1 mg/L. The integration of the fish and plant production system (aquaponics) produces economic cost savings over either system alone. Shared cost savings come from spreading out operating costs (e.g., management, water, nutrients, and overhead charges) and capital costs (e.g., backup generator, used truck, and office equipment) over the two systems. The investment analysis demonstrates the profitability of this combined system over its 20-year expected life. Net present values are positive for a wide range of discount rates. Internal rate of return analysis shows that for a total investment of $244,720 this system

can potentially provide a return of 12.5%. The hydroponic system drives the potential profitability of the combined system with 67% of annual returns derived from plant production.

INTRODUCTION

Consumer demand for fish has been increasing, but ocean fish catches continue to decline. Aquaculture, the cultivation of freshwater and marine plants and animals, is one of the fastest growing segments of U.S. agriculture. The increase in farm-raised fish is leading to increased concerns regarding discharges from those facilities. Therefore, treatment of fishery effluents needs to be considered when planning aquacultural production systems. Aquacultural effluents are difficult to treat because of large volume flows carrying relatively dilute nutrients (< 1 mg/L P) (Adler et al. 1996e; Heinen et al. 1996a). However, it may be important to treat the nutrients in aquaculture effluents because, depending on the quality of receiving water, the total nutrient mass loading can contribute significantly to environmental degradation. Land-based recycle aquaculture facilities release dissolved nitrogen and phosphorus to the water environment, which contributes to the undesirable growth of macro and micro algae in receiving waters. All states in the Northeastern U.S. have regulations regarding the discharge of aquacultural effluents (Ewart et al. 1995). Technologies are available to reduce the concentration of nutrient discharge from these facilities to regulated levels based on United States EPA (Environmental Protection Agency) water quality drinking standards. Some common solutions to reducing nutrient discharge include reducing excess phosphorus concentrations in the feed (Heinen et al. 1996a; Jacobsen and Borresen 1995; Ketola and Harland 1993), reducing the amount of uneaten feed entering the rearing system (Asgard et al. 1991; Summerfelt et al. 1995), aggressive separation of uneaten feed and feces from the waste stream (Summerfelt 1996), biological, chemical, and physical nutrient removal systems (Adler et al. 2000; Metcalf and Eddy Inc., 1991), and plant-based nutrient removal systems (Adler et al. 1996d,e; Adler 1998; Rakocy and Hargreaves 1993). Of these solutions, plant-based removal of nutrients has the potential to offset treatment costs with additional revenues (Adler et al. 2000). Byproduct utilization is an important strategy to enhance both the economic and environmental sustainability of aquaculture (Adler et al. 1996c).

Integrated hydroponic and fish production systems are an example of nutrient recycling which can reduce nutrient discharge to the environment and generate additional revenues. Economic analysis of warmwater fish species (eg., tilapia) of small research-scale (Jenkins et al. 1996; Jenkins and Wade 1997) and commercial-scale (Bailey et al. 1997) systems have been published. In these types of systems, fish-rearing water is applied to plants that absorb dissolved nitrogen and phosphorus from the water. The water is then returned to the fish-rearing unit for reuse. This technology is impractical, however, in coldwater recycle systems (e.g., rainbow trout, arctic charr) due to temperature elevation in the plant treatment phase. For this reason, plant-based nutrient removal from coldwater fish rearing systems must take place after final discharge from the fish rearing system.

Hydroponic production of lettuce and basil using thin-film technology, also known as NFT - Nutrient Film Technique, was investigated as a method to remove P to low levels from an aquaculture effluent. Thin-film technology is a hydroponic crop production system in which plants grow in water that flows continuously as a thin film over their roots. Water flow across the roots decreases the stagnant boundary layer surrounding each root which, in turn, enhances the mass transfer of nutrients to the root surface and permits crops to maintain high productivity at steady-state P levels above 0.3 mg/L (Chen et al. 1997). The rainbow trout effluent in this study contained between 0.5 and 0.7 mg P/L. So, conventional hydroponic technology (where all plants in the trough are the same age) could only remove about 50% of the P while producing a marketable product. Although lettuce produced using NFT can remove P to <0.3 mg/L, a reduction in growth will coincide with a further reduction in solution P concentrations. A conveyor production system made it possible for plants to remove >95% of the P (to < 0.01 ppm P) in the rainbow trout effluent while producing a marketable product.

The objective of this analysis was to describe the economic relationship between a 22,680-kg per year recirculating trout production system and a hydroponic treatment unit capable of reducing phosphorus concentration levels in the fish farm effluent to less than 0.1 mg/L. Adler et al. (2000) conducted a study which compared the cost of alternative nutrient discharge treatment options including chemical and filtration methods and hydroponics. However it did not describe the economic relationship between the fish production system and the greenhouse treatment system as a combined business enterprise. The economics of this integrated relationship must be quantified to properly assess the viability of this technology.

MATERIALS AND METHODS

The economic feasibility of a small-scale trout production system with an associated hydroponic treatment system was evaluated using data from studies conducted at the Conservation Fund's Freshwater Institute during 1994 and 1995. Inputs for the fish production system are based on United States Department of Agriculture (USDA) sponsored research evaluating water reuse technologies for cold water trout culture (Hankins et al. 1995; Hankins et al. 1996; Heinen et al. 1996b). Inputs for the hydroponic treatment system are based on USDA-ARS (Agricultural Research Service) research designed to economically reduce nutrient discharge from a cold water trout production system (Adler et al. 1996b).

Rainbow Trout Production System

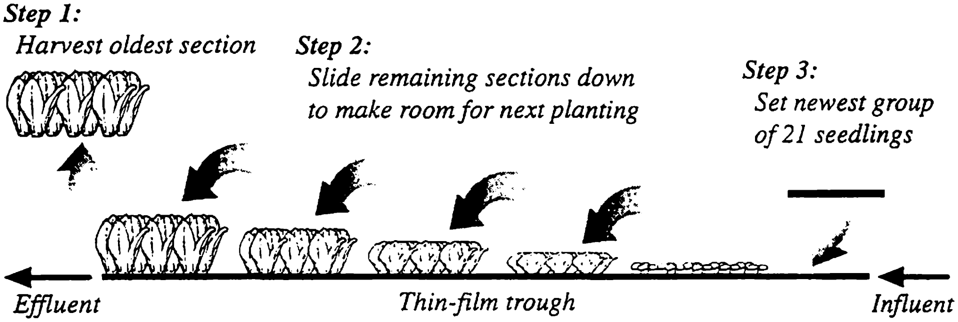
The Freshwater Institute maintains a high-density recirculating rainbow trout production facility near Shepherdstown, WV, USA. The facility evaluated in this analysis utilizes intensive water reuse production technology. Approximately 109 m^3 of trout effluent are produced daily. All fish production takes place inside an insulated metal building (239 m^2). The production system consists of 2 independent fish rearing systems composed of a single fish tank and filtration loop. The fish tanks are cross flow raceways (19,000 L). The filtration loops include drum filters, fluidized sand filters, carbon dioxide strippers, and low head oxygenators (Figure 2). The production schedule utilizes a continuous stocking strategy where 10.2-cm fingerlings are stocked every two months and size graded harvests of the largest fish are made on a weekly basis. Approximately 10% of the system biomass (431 kg) of market size fish are harvested weekly. The mean full production cycle per stocked cohort is ten months. The average production per year at steady state is 22,680 kg of market size fish.

Rainbow Trout Effluent Characteristics

The bulk effluent from the recirculating system for rainbow trout production at The Conservation Fund's Freshwater Institute typically has a pH of 7.2 and contains about 6 mg/L total suspended solids (TSS) and the following macronutrients (mg/L): NO_3-N (25), P (0.7), K (5), Ca (55), Mg (20), and S (9). In contrast, the spring water that supplied the fish culture system typically contained (mg/L): NO_3 (3), P (<0.001), and K (3). In this effluent, nutrients most limiting to plants (in decreasing order) are Fe, Mn, Mo, and K. A plant's productivity is determined by the nutrient present in lowest supply relative to its requirements. When other nutrients limit plant growth, P removal can be increased by adding those nutrients that are most limiting. To maximize P removal, the following nutrients were added to make P the most limiting nutrient: 0.1 mg/L Fe-EDDHA (LibFer SP, Allied Colloids Inc., Suffolk, VA, USA), 0.1 mg/L Mn-EDTA (Librel Mn, Allied Colloids Inc., Suffolk, VA, USA), 0.004 mg/L Mo (as $(NH_4)_6Mo_7O_{24}$), and 15 mg/L K (as K_2SO_4).

Conveyor Production System

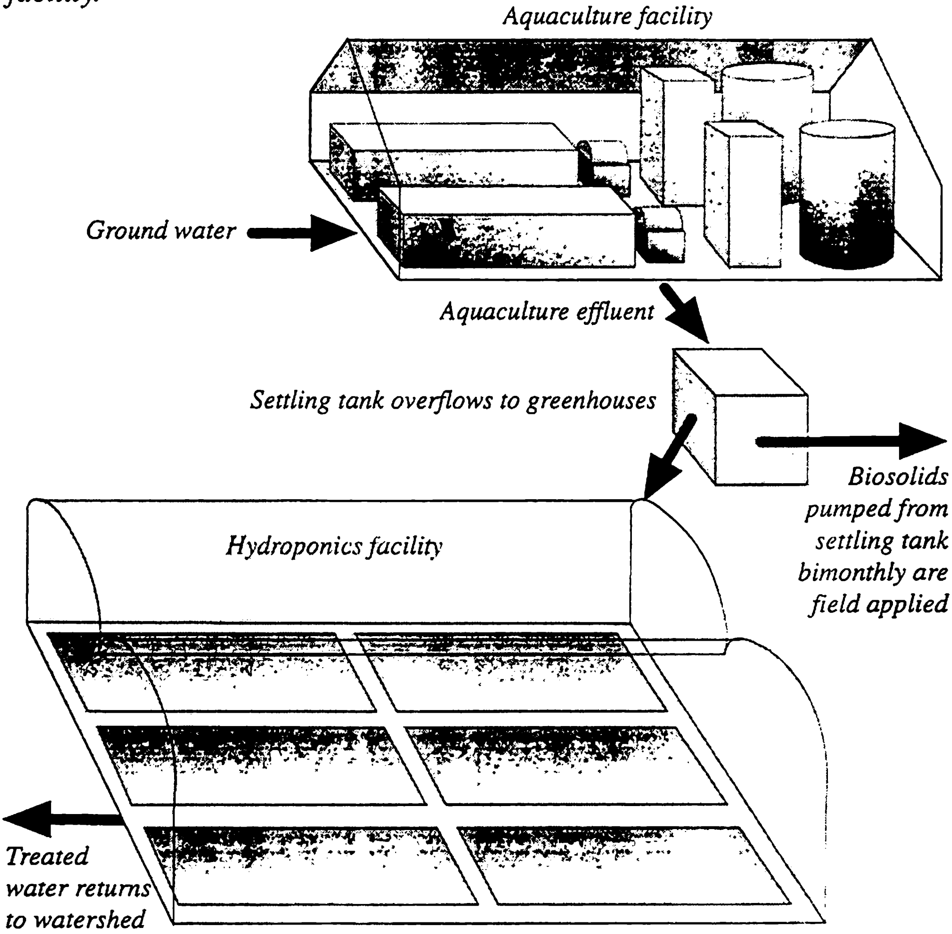
Figure 1. Conveyor crop production schematic for hydroponic lettuce and basil.



'Ostinata' lettuce (*Lactuca sativa* L.) and sweet basil (*Ocimum basilicum* L.) were seeded into Oasis® cubes (Smithers-Oasis, Kent, OH, USA). The lettuce and basil seedlings were placed into thin-film troughs and watered for the first 20 days with a recirculated complete nutrient solution (Adler et al. 1996e). After about 3 weeks lettuce and basil were moved to a nonrecirculating thin-film system configured with the conveyor production sequence. Adler et al. (1996e) describe the system in more detail. With the conveyor production strategy, seedlings are set at time intervals (e.g., every 4 days) near the inlet of a thin-film system and progressively moved in sequence as they matured towards the outlet (Figure 1). This cycle is repeated 6 times to move a given set of plants completely through the system to harvest. The number of sections can be greater or less than 6. Increasing the number of sections decreases the percentage of biomass removed with any one harvest and results in a more stable outlet concentration.

Plants have the capacity to absorb and store nutrients in excess of their immediate needs, a process called luxury consumption (Marschner 1995). The conveyor crop production strategy enables plants to store P early in their growth cycle (when they are younger and closer to the inlet where the P concentration is higher). This stored reservoir of P can be remobilized to meet current plant needs and supplement the lower P

Figure 2. Schematic of the integrated fish and hydroponic plant production facility.



influx rate, which occurs as P drops below about 0.3 mg/L in the effluent. Phosphorus remobilization will maintain growth as long as the tissue P concentration remains above the critical deficiency level (about 0.35-0.4% P on a dry weight basis in lettuce). At the front end of the thin-film troughs, where nutrient concentrations were highest, young plants absorbed and stored nutrients in excess of their immediate needs. Luxury consumption of nutrients during this early growth phase sustained the plants later when they were moved towards the trough outlet where nutrient concentrations in solution were too low for absorption kinetics to meet their growth needs. Cellular nutrient concentrations were sufficient to sustain growth even after nutrients within the flow were limiting.

This conveyor crop production system permitted the removal of P to very low levels (ppb), without an apparent reduction in plant productivity (Adler 1998). This is in contrast to a conventional production system where a gradient in growth and a reduction in plant quality would accompany the reduction in nutrient levels. Using the conveyor production strategy, lettuce and basil were able to remove dissolved P levels to less than 0.01 mg/L and developed to a marketable product with no apparent reduction in productivity.

Because plants remove nutrients continuously, effluent storage facilities are not necessary to temporarily hold effluents that are generated 24 h a day. Previous research found that N absorption varied with the day/night cycle while P absorption has very little diurnal variation (Adler et al. 1996a).

Sizing Criteria for Greenhouse Hydroponic System

Greenhouse sizing assumptions for this analysis are based on the plant mass required to reduce the concentration of the phosphorus in the fish system effluent to a level of 0.1 mg/L (Adler et al. 2000). After solids collection, the recycle fish system discharges 22.6 kg of phosphorus a year (Heinen et al. 1996a). Research has shown that using a hydroponic system, phosphorus concentrations can be reduced from 0.6 mg/L to 0.1 mg/L in the greenhouse. Nitrate concentrations can be reduced from 15 mg/L to 6 mg/L, well below the 10 NO_3-N mg/L allowable limit for nitrate in drinking water. Optimal placement for a greenhouse treatment system would be downhill from the fish facility to take advantage of gravity to move the water from one to the other (Figure 2).

The hydroponic treatment system consists of a complete greenhouse facility capable of year round plant production. A system capable of treating the daily effluent from production of 22,680 kg of trout would require 3, 9.1 m x 40.2 m arch-style greenhouses. In addition to a hydroponic trough rearing system for the plants, greenhouses were assumed to include cooling and heating systems and supplemental lighting (Adler et al. 2000).

Table 1. Component fixed costs of the fish production system.

Fish system components	**Estimated fixed cost ($U.S.)**	**Projected life (years)**
Building	43,300	20
Plumbing general	13,000	5
Oxygen equipment	10,700	5
Tanks	10,000	20
Drum filters	9,200	5
Fluidized sand filter	7,800	20
Furnace and heating	5,200	10
Feeders/nets, etc.	4,600	5
Well pump	4,300	5
Computers/phones	4,000	3
Monitor and controls	3,800	5
CO_2 stripper	2,400	20
Hatchery equipment	2,300	10
Recirculating pumps	2,200	5
Total fish system	**$122,800**	

Table 2. Component fixed costs for the greenhouse structure and hydroponic system.

Greenhouse components	**Estimated fixed cost ($U.S.)**	**Projected life (years)**
Frames & sidewalls	13,430	20
Gable ends	2,400	20
Wood baseboard	560	10
Double polyethylene film (0.15mm)	2,350	3
Exhaust fans & vents	9,000	10
Heater	5,400	10
Crushed stone base	600	20
Light fixtures	18,480	10
Electrical installation	2,970	20
Lamps	6,470	8
Evaporative cooler	3,000	10
Construction costs	14,110	20
Total greenhouse system	**$78,770**	

Hydroponic components	**Estimated fixed cost ($U.S.)**	**Projected life (years)**
Tray supports	3,780	20
Hydroponic trays & covers	7,990	10
Supply line	260	5
Feeder tubes & fittings	1,410	5
Injector pumps	1,650	5
Solenoid valves	280	5
Nutrient tanks	90	5
Submersible pumps	1,690	5
Total hydroponic system	**$17,150**	
Total greenhouse and hydroponics system	**$95,920**	

Table 3. Combined facility fixed costs.

Facility components	Fixed costs ($U.S.)
Fish system	122,800
Greenhouse system	95,920
Backup generator (50-kilowatt)	21,000
Office equipment	2,000
Land	3,000
Total combined system	**$244,720**

ECONOMIC ANALYSIS

Evaluation of the economic viability of the combined fish production and greenhouse hydroponic system requires the consideration of initial and replacement capital costs, annual operating costs, and annual revenues. The flow of costs and returns over the projected 20-year life of the system were evaluated using investment choice criteria including net present value (NPV), internal rate of return (IRR), and payback period.

Fixed Costs

The initial fixed costs for the fish production system total approximately $122,800 (Table 1). The total initial fixed costs for the greenhouse and hydroponic system is about $95,920 (Table 2). As indicated by the expected life of the individual components, many items will need to be replaced over the course of the 20-year investment. A component with a 3-year expected life will need to be replaced 6 times; with a 5-year life, 3 times; with an 8-year life, 2 times; and with a 10-year life, 1 time. Combined system costs, including capital items shared by both systems (office equipment, backup generator, and land) are summarized in Table 3.

Annual Variable Costs

Annual variable costs for the combined system are presented in Table 4. Approximately 60% of the combined system costs represent employee

Table 4. Annual variable costs of the greenhouse and fish systems.

Fish system	**Variable costs ($U.S.)**
Labor	25,000
Feed	23,300
Energy	17,440
Transportation	2,000
Maintenance	1,500
Fish eggs	880
Overhead (at ~2%)	1,400
Total fish system	**$71,520**
Greenhouse system	**Variable costs ($U.S.)**
Harvest materials	12,290
Energy	26,510
Labor	113,130
Seeding materials	10,890
Transportation and marketing	16,290
Fertilizer and pesticides	540
Overhead (at ~2%)	3,590
Total greenhouse system	**$183,240**
Total system variable costs	**Variable costs ($U.S.)**
Fish system	71,520
Greenhouse system	183,240
Manager	35,000
Total	**$289,760**

Table 5. Estimated annual revenues, expenses, and cash flows for a 20-year fish and hydroponic production system investment.

Year	Annual fixed cost ($U.S.)	Annual revenues ($U.S.)	Tax-deductible expenses[1] ($U.S.)	After-tax income[2] ($U.S.)	Annual cash flow[3] ($U.S.)
1	244,720	236,422	313,554	-53,338	-298,058
2	0	351,392	351,392	61,632	61,632
3	0	351,392	351,392	61,632	61,632
4	6,350	351,392	315,010	48,390	42,040
5	0	351,392	313,554	54,333	54,333
6	53,180	351,392	313,554	1,153	-52,027
7	6,350	351,392	313,554	47,983	41,633
8	0	351,392	313,554	54,333	54,333
9	6,470	351,392	313,554	47,863	41,393
10	6,350	351,392	313,554	47,983	41,633
11	53,180	351,392	313,554	1,153	-52,027
12	0	351,392	313,554	54,333	54,333
13	6,350	351,392	313,554	47,983	41,633
14	0	351,392	313,554	54,333	54,333
15	0	351,392	313,554	54,333	54,333
16	59,530	351,392	313,554	-5,197	-64,727
17	6,470	351,392	313,554	47,863	41,393
18	0	351,392	313,554	54,333	54,333
19	6,350	351,392	313,554	47,983	41,633
20	0	351,392	313,554	54,333	54,333

[1] *Includes cash costs, depreciation, and allocation of net operating loss from year 1 in years 2-4.*

[2] *Revenues minus cash operating expenses and taxes (15% on taxable income up to $25,350 and 28% on amounts above $25,350, but less than $61,400).*

[3] *After-tax income minus annual investment.*

Table 6. Net present value (NPV), internal rate of return (IRR), and payback period for the integrated trout and basil/lettuce production system.

Discount rate	NPV
4%	$266,139
6%	$176,797
8%	$107,580
10%	$53,197
IRR:	12.5%
Payback period:	7.5 years

payroll expenses (for hourly laborers and the system manager). Energy costs (for fuel and electricity) account for 15% of the combined system costs. Costs relating solely to the greenhouse system constitute 63% of the total system cost, reflecting the large amount of labor required by this system.

Annual Revenues

Annual revenues for the combined system are based on the yearly sale of 22,680 kg of trout and 398,600 plants of lettuce and basil (assuming 95% packout). Annual revenues for the hydroponic system are based on 50% production of lettuce and 50% production of basil. Annual revenues are estimated to be $236,422 in year 1 and $351,392 in years 2-20 (Table 5). Production of trout in year 1 is assumed to be only 25% of production in years 2-20 due to time required to get the fish system up to a steady state. Production of plants in the first year is reduced by 12% due to the time required to get the hydroponics systems up to a steady state. In years 2-20, revenues from basil and lettuce account for 67% of the annual total for the system. Prices of $14/box of lettuce (24 heads per box), $0.60/plant of basil, and $5.09/kg of trout were used to calculate annual revenues. The vegetable prices are conservative and reflect typical marketing efforts. The assumed price for trout is below the weighted average stocker price of $5.53/kg and is above the weighted average food fish price of $3.64/kg for the Northeast U.S. over the years from 1991 to 1995 (USDA-ERS 1996). Breakeven price analysis indicates that at steady state yield levels, prices could fall to $12.60/box for lettuce, $0.54/plant for basil, and $4.98/kg for trout and still cover the annual variable costs of production and depreciation expenses.

Investment Analysis

The purpose of an investment analysis is to demonstrate the profitability over the expected life of the investment. The economic viability of this combined fish and plant production system was evaluated using net present value analysis (NPV), which takes into account the time value of money and the variability of annual cash flows over time. The NPV method is used to reduce the 20-year stream of revenues and expenses from the combined system (Table 5) to a single number in which these future annual cash flows are discounted. A description of this method of analysis as it applies to fish farming projects is available in O'Rourke (1991).

NPVs of the combined system assuming various discount rates are found in Table 6. The NPV indicates the value of the investment in the combined system over its 20-year life in terms of today's dollars. For example, at a discount rate of 8%, the NPV of the combined system is $107,580. Lower discount rates increase the NPV because the value of future cash flows are reduced less than they would be for higher discount rates. Choice of an appropriate discount rate is up to the individual decisionmaker and depends on their preference for current versus future consumption, the cost of the investment capital (personal or borrowed funds), and the availability and riskiness of other investment opportunities. In selecting between competing investment opportunities, the decisionmaker would select the one that maximizes expected NPV.

The IRR is the discount rate that equates a project's initial cost with the sum of its discounted future cash flows. In other words, the IRR is the discount rate which would reduce the NPV of a project to zero. The results of the IRR analysis shows that for the initial investment of $244,720 this system can potentially provide a return of 12.5% over the life of the facility.

Another widely used investment criterion is the payback period. This is the length of time required to recover the initial fixed costs of an investment. According to this criterion, it will take approximately 7.5

years to recover the initial fixed costs of $244,720. Although widely used as an evaluation tool, the payback period approach has two major deficiencies: 1) it fails to consider the time value of money and 2) it ignores the length and magnitude of cash flows after the payback period. The payback period should not be used to compare investment alternatives unless they are of the same magnitude and expected life.

Cost and revenue estimates made in this analysis are conservative in order to offset production variability caused by potential production and marketing inefficiencies and occasional disease setbacks. Experienced commercial growers could potentially build and operate the system at higher levels of profit. The addition of the hydroponic production system results in significantly higher profitability than the fish system alone, especially when considering the cost of wastewater treatment, which could potentially be charged to the fish system. With only 39% of the total fixed cost and 63% of the total annual variable costs, the hydroponic system generates 67% of the annual revenue for the combined system. The revenue from the greenhouse system also helps offset first year operating losses from the fish system. The economics of the fish system could be improved by increasing the scale of production (Wade et al. 1996) and by the utilization of higher valued fish species such as arctic charr, which has similar production requirements and a higher market price than rainbow trout.

In addition to the economic benefits, there are non-monetary societal benefits associated with this type of system integration. There are the benefits of using recycle technology to reduce water consumption by the fish system; it uses only 3% of the water of traditional raceway technology. Also, the integration of the two systems reduces the combined consumption of water by reusing water discharged from the fish production system for plant production. Water reuse increased and the majority of this water is returned to the environment in excellent condition. This makes the combined systems largely non-consumptive and non-polluting users of the water resource.

This analysis demonstrates that the integration of recycle aquaculture systems with hydroponic vegetable production can generate a profit and still be environmentally friendly. These types of systems, when designed properly can reduce water use and greatly reduce the discharge of unwanted nutrients to the environment. Conventional treatment alternatives for phosphorus in wastewater, whether they employ chemical precipitation, physical removal, or land application technologies, represent a significant additional cost to the owner of an aquaculture operation. Treatment costs vary from a low of $0.18/m^3 for land application using alfalfa as the recipient crop to a high of $1.26/m^3 for reverse osmosis and electrodialysis (Adler et al. 2000). They also involve moderate to large investments in capital items that have no alternative uses. In contrast, the conveyor production system generates income while nutrients are removed to a very low level (Adler et al. 1996c,f). Treatment of fishery effluent using hydroponic crop production represents a potentially profitable additional enterprise for the aquaculture producer. Regardless of the crop chosen (lettuce or basil), expected crop prices appear to be more than sufficient to cover the costs of production at expected yields. The primary drawbacks of hydroponic production as a treatment alternative would be the added technical sophistication, labor, and marketing expertise required. Various production and market risks could significantly reduce the projected returns. These include fish and plant disease, mechanical failure, regulatory changes, and market variation. Development of marketing plans for both fish and produce is crucial. Sufficient attention must be paid to the day-to-day operation and the development and servicing of markets or the profitability advantage of combining the two production systems could rapidly disappear.

REFERENCES

Adler, P.R., Harper, J.K., Takeda, F., Wade, E.M., Summerfelt, S.T. Economic Evaluation of Hydroponics and Other Treatment Options for Phosphorus Removal in Aquaculture Effluent. *Hort Science* **2000**. (in press).

Adler, P.R. Phytoremediation of Aquaculture Effluents. *Aquaponics Journal* **1998**. 4(4):10-15.

Adler, P.R., Summerfelt, S.T. , Glenn, D.M., Takeda, F. Evaluation of a Wetland System Designed to Meet Stringent Phosphorus Discharge Requirements. *Water Environmental Research* **1996**a. 68(5):836-840.

Adler, P.R., Summerfelt, S.T., Glenn, D.M., Takeda, F. Evaluation of the Effect of a Conveyor Production Strategy on Lettuce and Basil Productivity and Phosphorus Removal from Aquaculture Wastewater. In: Staudenmann, J., Schönborn, A., and Etnier. C. (eds.), Recycling the Resource - Ecological Engineering for Wastewater Treatment. Proceedings of the Second International Conference, Waedenswil - Zurich, Switzerland; 18-22 September 1995. Trans Tech Publications, Switzerland. Environmental Research Forum **1996**b. 5-6:131-136.

Adler, P.R., Takeda, F., Glenn, D.M., Summerfelt, S.T. Utilizing Byproducts to Enhance Aquaculture Sustainability. *World Aquaculture* **1996**c. 27(2):24-26.

Adler, P.R., Takeda, F., Glenn, D.M., Wade, E.M.,Summerfelt, S.T., Harper, J.K. Phytoremediation of Wastewater Using Lettuce and Basil. *Proceedings of the American Society for Plasticulture* **1996**d. 26:253-258.

Adler, P.R., Takeda, F., Glenn, D.M., Wade, E.M.,Summerfelt, S.T., Harper, J.K. Conveyor Production Strategy Enhances Nutrient Byproduct Recovery from Aquaculture Wastewater, pp. 431-440, In: Libey, G.S., and Timmons, M.B. (eds.) Successes and Failures in Commercial Recirculating Aquaculture Conference Proceedings **1996**e. NRAES-98. Northeast Regional Agricultural Engineering Service: Ithaca, NY. July 1996.

Adler, P.R., Takeda, F., Glenn, D.M.,Wade, E.M., Summerfelt, S.T., Harper, J.K. Nutrient Removal: Ecological Process Sows a Cost-saving Idea for Enhancing Water Quality. *Water Environment & Technology* **1996**f. 8:23-24.

Asgard, T., Langaker, R.M., Shearer, K.D., Austreng, E., Kittelsen, A. Ration Optimalization to Reduce Potential Pollutants - Preliminary Results. *American Fisheries Society Symposium* **1991**. 10:410-416.

Bailey, D.S., Rakocy, J.E., Cole, W.M., Shultz K.A. Economic Analysis of a Commercial-scale Aquaponic System for the Production of Tilapia and Lettuce. pp. 603-612, In: Fitzsimmons, K. (ed.) Tilapia Aquaculture: Proceedings from the Fourth International Symposium on Tilapia in Aquaculture **1997**. NRAES, Ithaca, NY.

Chen, X.G., Gastaldi, C., Siddiqi, M.Y., Glass, A.D.M. Growth of a Lettuce Crop at Low Ambient Nutrient Concentrations: A Strategy Designed to Limit the Potential for Eutrophication. *Journal of Plant Nutrition* **1997**. 20:1403-1417.

Ewart, J.W., Hankins, J., Bullock, D. State Policies for Aquaculture Effluents and Solid Wastes in the Northeast Region. *NRAC Bulletin* No. 300 **1995**. Northeastern Regional Aquaculture Center, University of Massachusetts-Dartmouth.

Hankins, J.A., Summerfelt, S.T., Durant, M.D. Impacts of Feeding and Stock Management Strategies Upon Fish Production Within Water Recycle Systems. pp. 70-86, In: Timmons, M.B. (ed.), Aquacultural Engineering and Waste Management **1995**. Northeast Regional Agricultural Engineering Service, Ithaca, New York.

Hankins, J. A., Summerfelt, S.T., Durant, M.D., Weber, A.L., Summerfelt, S.R. Effect of Cohort Size Distribution on Production and Growth Within a Sequentially Stocked and Harvested Rearing System. p 41, In: Aquaculture America 1996. Arlington Texas, Book of Abstracts **1996**. World Aquaculture Society, Baton Rouge, Louisiana.

Heinen, J.M., Hankins, J.A., Adler, P.R. Water Quality and Waste Production in a Recirculating Trout-culture System With Feeding of a Higher-energy or a Lower-energy Diet. *Aquaculture Research* **1996**a. 27:699-710.

Heinen, J.M., Hankins, J.A., Weber, A.L., Watten, B.J. A Semiclosed Recirculating-water System for High-density Culture of Rainbow Trout. *The Progressive Fish-Culturist* **1996**b. 58:11-22.

Jacobsen, C., Borresen, T. Formulation of Fish Diets with Reduced Phosphorous Content. *Water Science Technology* **1995**. 31(10): 167-173.

Jenkins, M.R., Wade, E.M., Yohn, C.W. The Opportunity for Small-scale Aquaculture as an Alternative Farm Enterprise: An Economic Analysis of a Small-scale Partial Reuse Trout Production System in West Virginia. *Journal of the ASFMRA* **1996**. p. 81-89.

Jenkins, M.R., Wade, E.M. A Preliminary Economic Analysis of a Low-cost, Small-scale, Natural Gas Powered, Integrated Recycle Aquaculture-hydroponic System for Tilapia. In: Fitzsimmons, K. (ed.), Tilapia Aquaculture: Proceedings from the Fourth International Symposium on Tilapia in Aquaculture **1997**. NRAES, Ithaca, NY.

Ketola, H.G., Harland B.F. Influence of Phosphorus in Rainbow Trout Diets on Phosphorus Discharges in Effluent Water. *Transactions of the American Fisheries Society* **1993**. 122, 1120-1126.

Marschner, H. Mineral Nutrition of Higher Plants. 2nd ed. Academic Press. Harcourt Brace & Co. **1995**. London, England. p. 466.

Metcalf and Eddy, Inc. Wastewater Engineering: Treatment, Disposal, and Reuse, Third Edition, revised by Tchobanoglous, G. and Burton, F.L. **1991**. McGraw-Hill, Inc., New York, NY.

O'Rourke, P. D. Current Status on Profits in Recirculating Systems. pp. 65-99, In: Regional Workshop on Commercial Fish Culture Using Water Recirculating Systems **1991**. IL-IN-SG-E-91-8. Illinois/ Indiana Sea Grant, Illinois State University, Normal, Illinois.

Rakocy, J.E., Hargreaves, J.A. Integration of Vegetable Hydroponics with Fish Culture: a review. pp. 112-136, In: Wang, J.K. (ed.), Techniques for Modern Aquaculture **1993**. ASAE, St. Joseph, MI.

Summerfelt, S., Holland, K.H., Hankins, J.A., Durant, M.D. A Hydroacoustic Waste-feed Controller for Tank Systems. *Water Science and Technology* **1995**. 31(10):123-129.

Summerfelt, S.T. Engineering Design of a Water Reuse System. pp. 277-309, In: Walleye Culture Manual, Summerfelt, R.C. (ed.), NRAC Culture Series 101 **1996**. Central Regional Aquaculture Center Publication Center, Iowa State University, Ames, IA.

United States Department of Agriculture, Economic Research Service. Aquaculture Situation and Outlook Report. USDA-ERS, Commodity Economics Division **1995**. Washington, D.C.

Wade, E.M., Summerfelt, S.T., Hankins, J.A. Economies of Scale in Recycle Systems, pp. 575-588, In: Libey, G.S., and Timmons, M.B. (eds.), Successes and Failures in Commercial Recirculating Aquaculture Conference Proceedings **1996**. NRAES-98. Northeast Regional Agricultural Engineering Service: Ithaca, NY. July 1996.

Effects of High-density Stocking in a Recirculating Aquaculture System on Gill Morphology of Hybrid Striped Bass (*Morone saxatilis* x *M. chrysops*)

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ABSTRACT

The types and distribution of gill lesions observed in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) reared in a commercial-scale recirculating aquaculture system are described. When placed in the system as fingerlings and reared there for eight months at typical stocking density, the gills of all examined fish presented a variety of extensive, non-specific lesions typically resulting from poor water quality. Lesions included epithelial cell hyperplasia, infiltration of the interfilamental region by mixed inflammatory cells, hyperplasia of mucous and lamellar epithelium, lamellar fusion and occasional filamental fusion. Up to 76% of the gill sample surface of individual fish was affected, with lesions being most severe in the distal filamental regions. Fish transferred to and maintained at low stocking densities in water of superior quality demonstrated that all lesions were fully reversible by five weeks post-transfer. This study demonstrates that culture of hybrid striped bass under intensive aquaculture management induced pathological changes in the gills, and suggests that maintenance of fish under improved water quality conditions will reduce gill lesions, which could potentially increase the fishes' performance.

INTRODUCTION

Recirculation aquaculture systems have become increasingly significant in the culture of numerous species of economically important fish. Benefits offered by recirculation systems include conservation of water and energy, decreased environmental pollution, and increased flexibility in the choice of aquaculture site location (Lucchetti and Gray 1988; Liao and Mayo 1974). Hybrid striped bass (*Morone saxatilis* x *M. chrysops*) are among an increasing number of species being successfully raised in intensive recirculation systems. In the interest of maximizing economic return, fish are commonly stocked at the highest population density possible without overtly affecting the fishes' health or growth. Unfortunately, high-density stocking practices exacerbate the inherent tendency of water in recirculation systems to carry high levels of contaminants such as particulates, metabolic wastes, parasites, and bacteria (Allen and Kinney 1981; Spotte 1979). The gills, being continuously and unavoidably in direct contact with these substances, respond with various structural and functional changes that can compromise respiratory, excretory, and osmoregulatory efficiency. (Roberts 1989; Ferguson 1989). By affecting gill function, even moderate changes in gill structure can affect the fishes' behavior, appetite, and homeostasis, and thus also have the potential to adversely affect growth and development.

The effects on gill morphology of rearing fish in recirculating systems under present management practices have not been closely scrutinized. However, conditions documented as significant stress factors, including poor water quality, overcrowding, and excessive handling, have long been known to occur in commercial recirculation systems (Amend 1970). Water quality parameters experienced in such systems may induce types and degrees of pathological change in the gills that have the potential to decrease fish performance and thereby decrease economic return. This study was undertaken to examine the hypothesis that high-density stocking of hybrid striped bass in recirculation systems as is commonly practiced in commercial aquaculture operations would induce significant gill pathology. Further, this study also aimed to characterize the nature of any gill lesions that developed, as well as to determine whether the resulting pathological changes were reversible.

METHODS

Fingerling hybrid striped bass (7-10 cm in length) were obtained from a commercial source where they had been reared in ponds. Six fish were arbitrarily selected as an incoming group to examine the microscopic anatomy of the gills at the outset of the study and determine whether significant underlying gill pathology was present at the outset of the study. These six fish were immediately anesthetized with tricane methanesulfonate (MS-222, Sigma Chemical Co., St. Louis, MO, USA) and killed by cervical dislocation. Tissue samples consisting of the gill arch and associated holobranchs were obtained from the second gill arch of each fish and placed in fixative (5% glutaraldehyde, 4% formaldehyde, and 2.75% picric acid in 0.05% cacodylate buffer, pH = 7.4).

The remaining fish were divided into two groups and placed in recirculating systems where they were reared for eight months. One group (control; n = 21) was maintained at low population density (≤ 2.5 kg/m^3) in 1,893 liter (500 gallon) circular tanks with a sand filter for mechanical filtration and a trickle filter with Bio-Pac 150 (NSW Corp., Roanoke, VA, USA) as the biological filter. Water quality in this tank was main-tained within parameters defined as superior (i.e., they were better than those currently considered acceptable by the production industry: temperature 26 ± 1°C; pH 7.2-7.4; TAN < 0.1 mg/L; $NO_2 <$ 0.01 mg/L; $NO_3 <$ 50 mg/mL; DO 8-10 mg/L; alkalinity > 150 mg/L; hardness > 200 mg/L) throughout the eight-month period. The second group (experimental) (5000 fingerlings with fish graded and removed at appropriate intervals) was reared for the eight-month cycle in a 11,356 liter (3000 gallon) recirculation system with a sump discharge for mechanical filtration and a rotating biological contactor for biological filtration. Fish in both groups were fed a commercial diet (Floating Fish Nuggets, 40% protein, Zeigler Bros., Garners, PA, USA) twice a day at 5-6% body weight. At the end of the production cycle, fish density in the control group neared 2.5 kg/m^3, while that in the experimental group had reached 130 kg/m^3 of water. Water quality parameters in both systems were determined weekly. When water quality deteriorated beyond acceptable industry limits in the experimental group, appropriate water changes were performed.

At the end of the 8-month period, representatives from both the control and the experimental groups (six and nine fish, respectively) were arbitrarily removed and gill samples obtained as previously described. The remaining 15 control fish were maintained in their original system, in which the stocking density remained low. From the experimental group, 15 fish were arbitrarily selected to investigate the reversibility of any lesions that had developed. These fish were placed in recirculation systems like that in which the control fish were held, and in which the population density was kept low (less than ≤ 2.5 kg/m^3) and the water was maintained at superior quality. Gill samples were obtained weekly as previously described from three arbitrarily-selected fish in each of the groups for a total of five weeks. Gill tissues from the experimental group were compared to those of control fish as well as to published normal anatomy for striped bass and their hybrids (Groman 1982; Pfeiffer et al. 2000).

Tissues were prepared for light microscopy (LM) by routine histological technique (Luna 1968; Hinton 1990), embedded in EM 400 embedding medium (Surgipath Medical Industries, Richmond, IL, USA), and sectioned at 6 µm. Separate slides from each sample were stained with hematoxylin/eosin (H&E) and the periodic acid-Schiff (PAS) reaction. For LM evaluation, samples were standardized to include a length of gill arch that included 10 to 20 gill filaments presenting

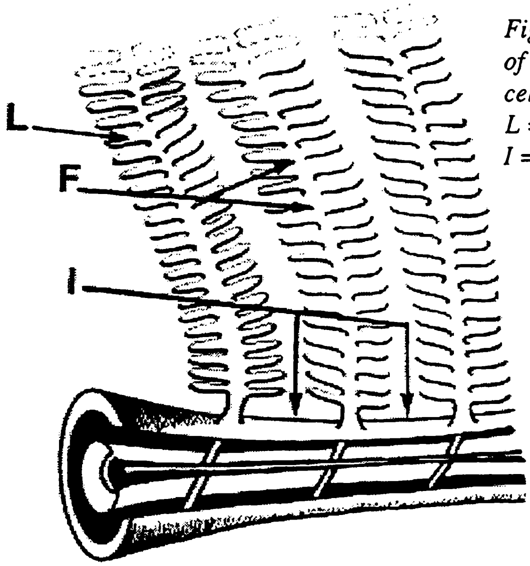

Figure 1. Schematic representation of sites on the gill at which mucous cells were enumerated. L = lamellar, F = filamental, and I = interfilamental sites.

lamellae along both sides of the length of the filament. For each sample, ten filaments were evaluated for morphological structure and pathologic change. Pathologic changes were evaluated using H&E-stained slides, characterized descriptively, and expressed as a percentage of filament length involved. To calculate the percentage of affected gill surface, the length of filament showing structural anomalies was divided by the total length of the filament. Slides stained with PAS were evaluated to detail alterations in number and location of mucous cells. Mucous cells were enumerated in the interfilamental regions at the base of 10 filaments, in the interlamellar spaces between the bases of the lamellae, and on the surfaces of the lamellae (Figure 1).

For transmission electron microscopy (TEM), tissue was fixed immediately at 4°C, washed in buffer, postfixed in 1% osmium tetroxide in 0.1 M cacodylate, washed in buffer again, dehydrated through a graded series (15% to 100%) of ethanol, transferred to propylene oxide, and embedded in Polybed 812 (Polysciences Inc., Warrington, PA, USA). Thin sections were doubly stained in lead citrate and uranyl acetate, and viewed in a JEOL/00 CX-11 transmission electron microscope (JEOL, Peabody, MA, USA) operating at 80 kv.

For statistical evaluation, the percentage abnormal gill surface and counts of the number of mucous cells in the interfilamental regions at the bases of the lamellae, in the interlamellar spaces, and on the surfaces of the lamellae were measured on 10 lamellae for each fish. For each of the four response variables, one measurement per fish was calculated by averaging the values from the 10 lamellae. Separate one-way ANOVA models were used to test whether sampling time had an effect on the percent abnormal gill surface and mucous cell counts from the three areas evaluated. ANOVA assumptions were evaluated by looking at normal probability plots of the residuals and plots of residuals against the predicted values. The normality of the residuals and equality of variances were substantially improved by a natural log transformation of the data. Sheffe's multiple comparisons procedure was used to make pairwise comparisons between sampling times. For all tests, a p-value of <0.05 was considered significant.

RESULTS

Incoming Fish

The histologic structure of the gills in the incoming group of fish was considered normal as compared with published reports of normal anatomy (Groman 1982; Pfeiffer et al. 2000).

Control Fish

Water Quality -- Water quality parameters remained within superior limits during the entire rearing period (data not shown).

Clinical Appearance of Control Fish -- Control fish appeared clinically normal on daily visual inspection throughout the study. The fish displayed normal swimming, feeding, social, and resting behavior, and grew at a rate typical of hybrid striped bass in commercial production.

Gill Histology -- The histologic structure of at least 94% of the gill surface in control samples was normal (Table 1, Figure 2) as was comparable to normal anatomy (Groman 1982; Pfeiffer et al. 2000). Filaments (Figure 3) were regularly arranged along the gill arch, with lamellae projecting freely and uniformly along the entire length of the filament. Interfilamental cells at the base of the filaments were present in appropriate types and numbers, consisting of a thin layer of mainly

Table 1. Percent Abnormal Gill Area in Control, High-Density, and Recovering Fish

	Percent Abnormal Gill Area									
	Number of Fish									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Overall Mean**
Control	5.9	5.1	5.4	5.1	5.7	5.6	--	--	--	5.5
High-density	61.5	71.5	64.0	67.5	76.1	73.7	72.8	69.9	71.7	69.9
Week 1 *Recovery*	45.9	48.9	47.0	--	--	--	--	--	--	47.3
Week 2 *Recovery*	25.9	27.4	25.6	--	--	--	--	--	--	26.3
Week 3 *Recovery*	23.3	23.8	23.3	--	--	--	--	--	--	23.5
Week 4 *Recovery*	11.0	10.9	10.2	--	--	--	--	--	--	10.5
Week 5 *Recovery*	5.7	4.8	5.3	--	--	--	--	--	--	5.3

For individual fish identified by number, each percentage reported is the mean for 10 filaments counted per fish. For the overall mean, the number reported is the arithmetic mean of all values within that group. For the control group, n = 6; for the high-density group, n = 9; for the recovering fish, n = 3. Dashes indicate a zero value in that category.

Table 2. Mucous Cell Numbers in Examined Regions of the Gill of Control, High-Density, and Recovering Fish

A. **Interfilamental Space**

	Number of Fish									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Overall Mean**
Control	3.9	3.7	2.8	3.0	3.3	3.2	--	--	--	3.3
High-density	9.0	11.5	8.3	11.0	9.3	10.1	8.2	9.6	10.5	9.7
Week 1 *Recovery*	5.4	5.7	4.9	–	–	–	–	–	–	5.3
Week 2 *Recovery*	4.5	4.8	5.0	–	–	–	–	–	–	4.8
Week 3 *Recovery*	3.5	3.7	3.1	–	–	–	–	–	–	3.4
Week 4 *Recovery*	3.2	3.1	3.2	–	–	–	–	–	–	3.2
Week 5 *Recovery*	2.8	2.9	3.2	–	–	–	–	–	–	2.9

For individual fish identified by number, the number reported is the mean of values for 10 interfilamental spaces in each fish. For the overall mean, the number reported is the arithmetic mean of all fish within that group. Dashes indicate a zero value in that category.

B. **On Filament**

	Number of Fish									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Overall Mean**
Control	26.1	28.8	28.6	27.4	28.5	28.2	–	–	–	27.9
High-density	220.6	203.2	227.2	220.6	226.6	240.8	214.5	218.8	219.1	221.3
Week 1 *Recovery*	163.6	162.5	159.0	–	–	–	–	–	–	161.7
Week 2 *Recovery*	106.6	103.7	104.0	–	–	–	–	–	–	104.8
Week 3 *Recovery*	52.4	46.8	55.8	–	–	–	–	–	–	51.7
Week 4 *Recovery*	31.8	29.1	28.8	–	–	–	–	–	–	29.6
Week 5 *Recovery*	27.4	29.0	27.9	–	–	–	–	–	–	28.1

For individual fish identified by number, the number reported is the mean of values for 10 filaments in each fish. For the overall mean, the number reported is the arithmetic mean of all fish within that group. Dashes indicate a zero value in that category.

C. **On Lamellae**

	Number of Fish									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Overall Mean**
Control	0.7	1.0	0.5	1.0	0.4	0.8	–	–	–	0.7
High-density	41.5	44.1	44.7	35.4	42.3	40.8	44.4	44.8	46.5	42.7
Week 1 *Recovery*	1.4	1.2	1.3	–	–	–	–	–	–	1.3
Week 2 *Recovery*	1.2	1.0	0.9	–	–	–	–	–	–	1.03
Week 3 *Recovery*	1.1	0.9	1.1	–	–	–	–	–	–	1.03
Week 4 *Recovery*	1.2	1.0	1.2	–	–	–	–	–	–	1.1
Week 5 *Recovery*	0.7	0.5	0.8	–	–	–	–	–	–	0.4

For individual fish identified by number, the number reported is the mean of values for 10 lamellae in each fish. For the overall mean, the number reported is the arithmetic mean of all fish within that group. Dashes indicate a zero value in that category.

undifferentiated cells, together with small numbers of chloride cells and isolated migrating leukocytes and eosinophilic granulocytes. Normal lamellar structure was typified by pillar cells separating capillary spaces, with the external lamellar surface covered by squamous epithelial cells. Occasional mucous cells were present in the interfilamental spaces. Greater numbers of mucous cells were found along the filaments, but were rare or absent along the lamellae (Table 2). Ultrastructurally, control gills (Figure 4) were typified by normal cellular structure of the principal cell types, including the surface epithelial cells of the secondary lamellae, pillar cells, and vascular endothelial cells.

Histological abnormalities were rarely observed in the control gills, and were restricted to small, localized sites of minor proliferation of interlamellar epithelial cells at the base of the lamellae, or, more rarely, in the interlamellar space.

Experimental (High-Density Stocked)Fish

Water Quality -- Experimental group water quality parameters generally remained within limits considered acceptable by production industry standards, though departures from acceptable limits occasionally occurred (Table 3). Near the end of the production cycle, despite the water quality parameters being generally acceptable, a higher concentration of suspended feed particles and organic debris often caused the water to become visibly turbid and brownish.

Clinical Appearance -- Throughout the study, the experimental fish appeared clinically normal on daily visual inspection. As with controls, the fish displayed normal swimming, feeding, social, and resting behavior, and appeared by visual inspection to grow at a rate typical of healthy hybrid striped bass.

Gill Histology of Experimental (High-Density Stocked) Fish -- Lesions of various types involving the majority of the gill surface (Table 1, Figure 2) were manifested in all market-aged fish at all levels along the length of the filament, though the full length of individual filaments was never involved. Twisting of filaments near lesions were generally most severe at the bases of and along the distal half of the filaments (Figure 5). The mid-regions of the filaments were affected to a lesser degree or not at all. Areas of severely affected tissue were often flanked by areas with less pathologic change.

Table 3. Water Quality Parameters from Tanks Holding Hybrid Striped Bass at High Density

Week	Temp. (22-28°C)	pH (7.2-8.6)	TAN (<2.0 mg/L)	NO_2 (<1.0 mg/L)	NO_3 (<150 mg/L)	DO (>5.0 mg/L)	ALK (>125 mg/L $CaCO_3$)	Hardness (>200 mg/L $CaCO_3$)
1	25.9	8.3	0.25	0.39	5	10.3	160	325
2	25.5	7.8	0.31	0.23	11	12.0	128	180
3	26.0	7.7	0.47	0.28	15	10.4	149	312
4	27.5	7.5	0.61	0.27	22	12.5	172	368
5	25.5	7.7	0.70	0.36	30	12.8	NA	NA
6	26.7	7.8	0.83	0.21	33	11.2	236	456
7	27.4	7.9	0.72	0.15	26	11.0	274	500
8	27.8	7.8	0.85	0.10	31	11.3	142	450
9	27.5	7.5	1.04	0.19	77	11.0	115	400
10	26.5	7.8	1.12	0.28	83	9.4	105	492
11	28.0	7.6	1.21	0.54	40	8.7	110	NA
12	28.2	7.6	1.28	0.19	83	11.5	115	NA
13	27.6	7.6	1.30	0.52	91	12.8	124	658
14	26.9	7.3	1.26	0.12	115	11.9	117	578
15	27.4	7.3	1.07	0.34	169	12.2	109	604
16	27.0	7.9	0.75	0.38	141	14.1	117	630
17	27.0	7.9	0.95	0.49	258	15.5	216	471
18	25.9	7.4	0.87	0.79	227	14.4	161	545
19	26.9	7.3	0.75	0.15	119	13.0	150	489
20	25.7	7.4	0.65	0.16	152	14.3	215	524
21	24.6	7.7	0.80	0.35	115	15.4	179	382
22	24.3	7.6	0.88	0.36	116	13.3	230	371
23	24.1	7.8	0.83	0.45	43	14.7	252	329
24	25.6	7.7	1.18	0.24	111	13.2	143	280
25	23.7	8.0	1.13	0.25	30	13.5	201	386
26	22.7	8.0	1.65	0.34	98	14.3	203	428
27	24.4	7.6	1.56	0.56	116	13.0	225	379
28	24.4	7.6	1.04	0.61	81	13.4	171	450
29	24.4	7.6	1.18	0.16	18	14.7	169	338
30	24.3	7.4	1.22	0.54	109	14.4	139	401
31	24.6	7.3	1.51	0.36	64	15.3	130	340
32	24.1	7.3	1.47	1.53	175	12.6	94	419
33	24.1	7.3	1.47	1.47	97	14.8	125	350

Numbers in parentheses under each parameter indicate desired limits for intensively-raised hybrid striped bass (modified for recirculation systems from Harrell et al. 1990). TAN indicates total ammonia nitrogen, *NA indicates* not available.

Lesions of the basal interfilamental region were typified by a thickening of the interfilamental cellular layers, caused by proliferation of epithelial cells and marked infiltration of the interfilamental region by mixed inflammatory cells. The thickening often extended to a level that engulfed some of the most proximal lamellae (Figure 6). The deepest layer of this thickened region contained primarily lymphocytes and macrophages, which extended from the basal region into and throughout the superficial layers as well. In the intermediate layers, these cells were joined by inflammatory cells possessing large eosinophilic granules. The superficial-most layer demonstrated increased numbers of mucous cells.

Lesions of the distal filaments were typified by proliferation of both epithelial and mucous cells on the lamellae (Figures 7-9). The number of mucous cells was significantly increased along both the filament and on the lamellae (Table 2; Figures 8,9). Mucous and other epithelial cellular proliferation and accumulation were sufficient to produce prominent, widespread lamellar fusion (Figures 8,9), which involved as much as a mean of 40% of the surface of affected filaments. In some areas, the fusion was so severe as to require careful examination of lamellar capillaries to distinguish adjacent lamellae (Figure 8).

Ultrastructurally, the gills of high-density stocked fish demonstrated a variety of pathologic changes. The lamellae demonstrated disruption of the pillar cells which resulted in the separation from their flanges supporting the central blood spaces (Figures 10,11). Further, marked enlargement of the subepithelial lymphatic spaces was evident, and the presence of granulocytes within these spaces was greatly increased (Figures 10,11). Membranous inclusions of obscure origin were common, both within the central blood spaces and the subepithelial lymphatic spaces. Small, electron-dense cytoplasmic granules became numerous within apices of the outer epithelial cells lining the secondary lamellae (Figure 10), though they were not prevalent in the control epithelia (Figure 4). Small cytoplasmic vacuoles were also noted in the outer epithelial cells covering the secondary lamellae.

Recovering Fish Transferred to Water of Superior Quality

Figure 2 and Table 1 show the percentage of gill surface with histopathological abnormalities in recovering fish. Evaluation of mucous cell numbers in these same fish is presented in Table 2.

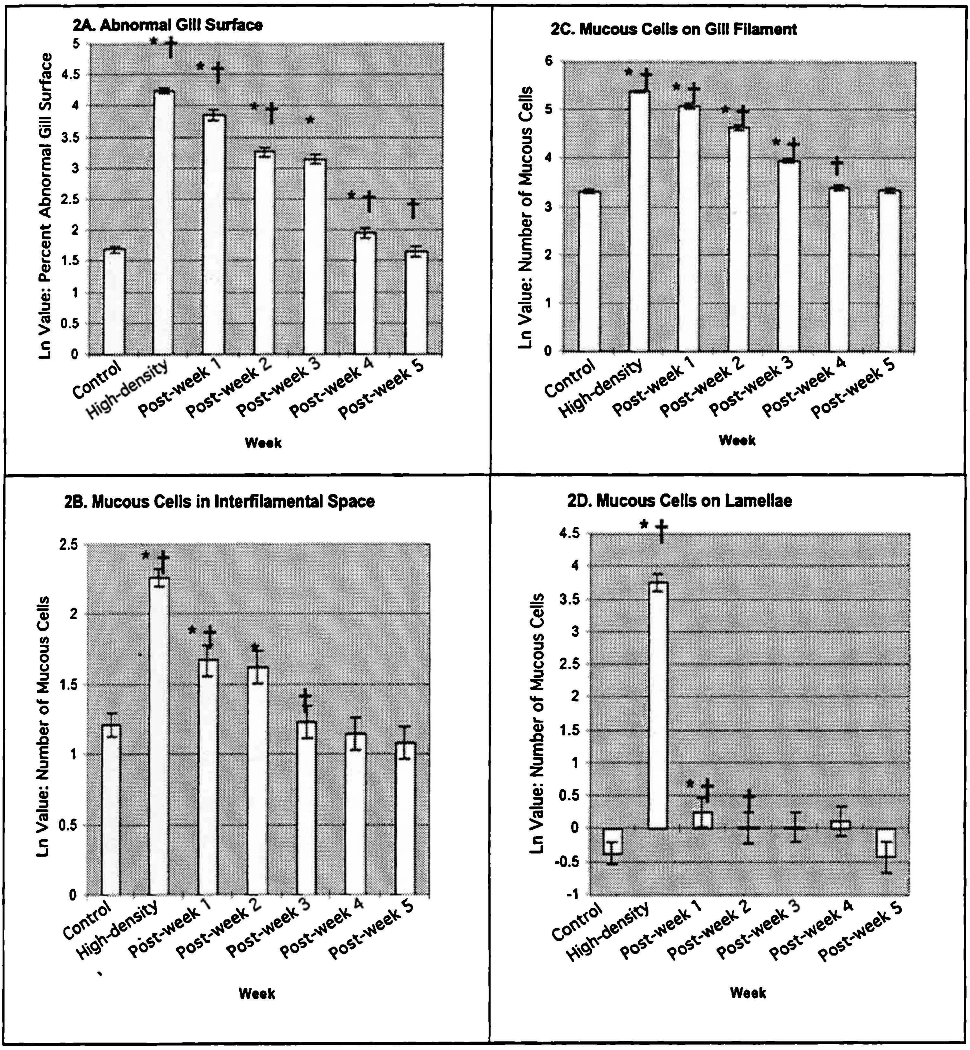

Figure 2. Percent abnormal gill surface (2A), and numbers of mucous cells in the interfilamental space (2B), on the filament (2C), and on the lamellae (2D) in control (n = 6), high-density (n = 9), and recovering (n = 3 in each group) fish.

Values shown are the group means of the natural log (Ln) transformed data. Error bars are 2x the SE of the mean.

** Indicates values significantly different from control,*

† Indicates significantly different from the immediately preceding measurement, $p < 0.05$.

One week post-transfer, gill tissue occasionally demonstrated resolving mild lesions of terminal vessel dilation (telangiectasis). Proliferative lesions and lamellar fusion in the distal region of the filaments were reduced to a mean of 45.9% of the gill surface (Table 1, Figure 2). The severity of these lesions was also greatly reduced as reflected in decreases in depth of proliferated cells in the interfilamental spaces and in the length of the filaments exhibiting lamellar fusion, as well as in absence of fusion of adjacent gill filaments. Mucous cells, though still present in elevated numbers (Table 3, Figure 2), appeared to be less active than in samples from high-density stocked fish, being smaller in size and presumably containing less mucus.

After two weeks of maintenance in superior quality water, areas of proliferation were further significantly decreased to a mean of approximately 25.9% of the gill surface (Table 1, Figure 2). The number of mucous cells on the lamellae were reduced to a level similar to controls, and remained so for the duration of the study (Table 3, Figure 2). Proliferation of mucous and epithelial cells as well as infiltration of inflammatory cells persisted in the regions between the basal interfilamental region, but were reduced from the one week post-transfer fish. The number of mucous cells in the interlamellar regions and on the filaments was decreased (Table 3, Figure 2), and the thickness of proliferated cells in the interlamellar spaces as well as along the lamellae was also reduced.

The types of lesions observed in the three week post-transfer fish were similar in distribution to the two week fish, but lesion severity continued to decrease. Numbers of mucous cells in the interfilamental regions and on the filaments approached control levels (Table 3, Figure 2). The thickness of proliferated cells in the interlamellar and interfilamental regions was further reduced, as was the proximal-to-distal length of involved lamellar regions. Lamellar fusion was no longer evident in any region of the gill.

By five weeks post-transfer, nearly all gill filament samples were similar in appearance to control tissues. Departures from normal histological structure were reduced to a mean of less than 10% of the gill surface (Table 1, Figure 2), and were characterized by only occasional areas of minor proliferation of epithelial cells in the interlamellar areas. Numbers of mucous cells were comparable to control levels in all locations.

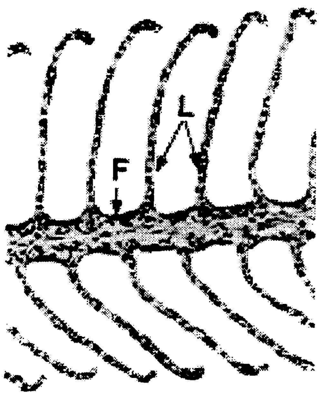

Fig.3

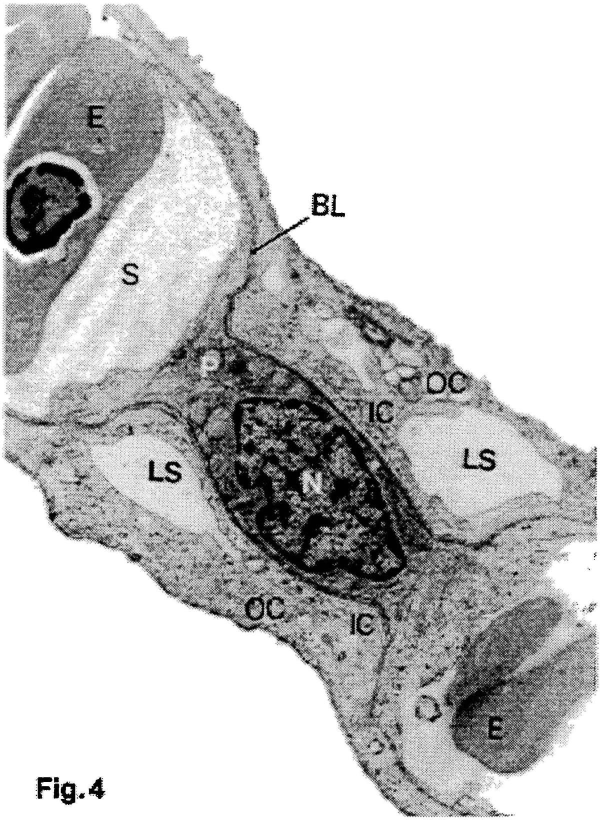

Fig.4

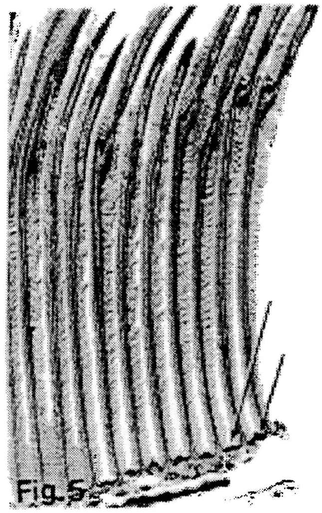

Fig.5

Figures 3-5

Figure 3. Light micrograph of normal gill filament. Lamellae (L) project freely from each side of the filament (F) with abundant water space between them. Squamous epithelial cells cover the surface of the lamellae, and the blood-water barrier is appropriately thin. Hematoxylin and eosin, x = 300.

Figure 4. Transmission electron micrograph of normal morphology of a secondary gill lamella showing pillar cell (P) with large, centrally-located nucleus (N) and a distinct basal lamina (BL) surrounding both the pillar cells and the central blood spaces (S). Portions of erythrocytes (E) are present within the respiratory channels. Outer (OC) and inner (IC) epithelial cells can be observed covering the lamellae, as well as subepithelial pymphatic spaces (LS). x = 19,440.

Figure 5. Light micrograph showing distribution of lesions along the length of gill filaments of high-density stocked fish. The basal interfilamental regions demonstrate some thickening of epithelial cell layers (arrows). Lamellar lesions are most prominent alone the distal region of the filaments, with lamellae along the more proximal part of the filament far less affected. Hematoxylin and eosin, x = 15.

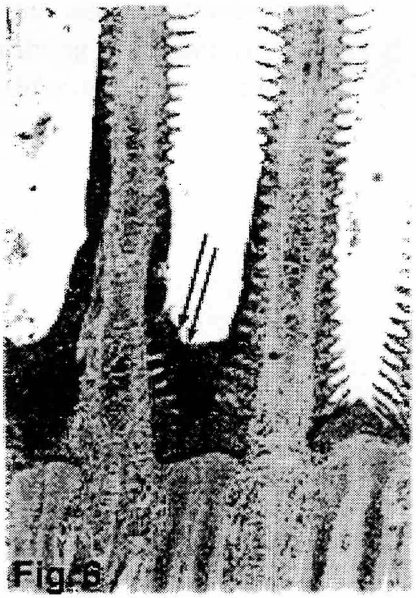

Fig.6

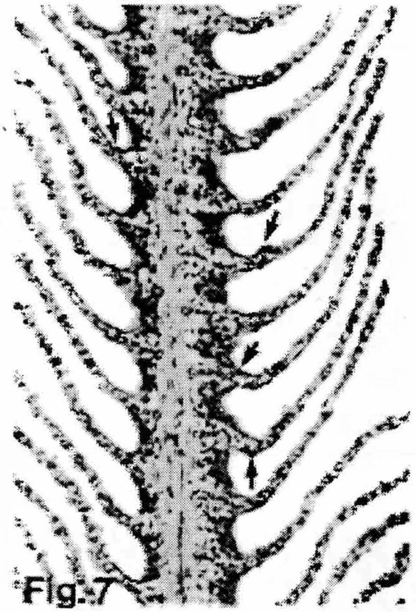

Fig.7

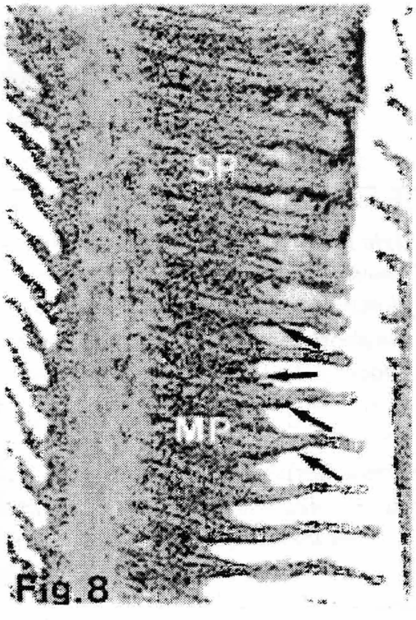

Fig.8

Figures 6-8.

Figure 6. Light micrograph demonstrating thickening of epithelial cells in the basal interfilamental region in a high-density stocked fish. The layers of proliferated epithelial cells are infiltrated by mixed inflammatory cells, and mucous cells are numerous in the most superficial layer (arrows). Hematoxylin and eosin, x = 75.

Figure 7. Light micrograph demonstrating mild epithelial cell proliferation in high-density stocked fish. Proliferated layers of epithelial cells in the interlamellar spaces have extended only slightly distally along the length of the lamellae. Though the proliferation of epithelial cells is only mild, mucous cells (arrows) are greatly increased. Periodic acid-Schiff, x = 240.

Figure 8. Light micrograph of a segment of a filament from a high-density stocked fish showing moderate (MP) and severe (SP) epithelial cell proliferation. In the severe region, proliferation has proceeded to the point of lamellar fusion. Elevated numbers of mucous cells (arrows) are clearly demonstrated. Periodic acid-Schiff, x = 150.

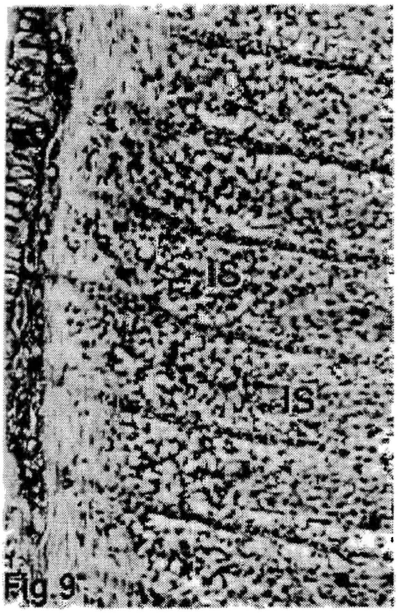

Figure 9. Light micrograph of a region of severe lamellar fusion. The entire interlamellar space (IS) is filled with proliferated and/or sloughed epithelial cells, effectively removing this area of the gill from respiratory function. Periodic acid-Schiff, x = 300.

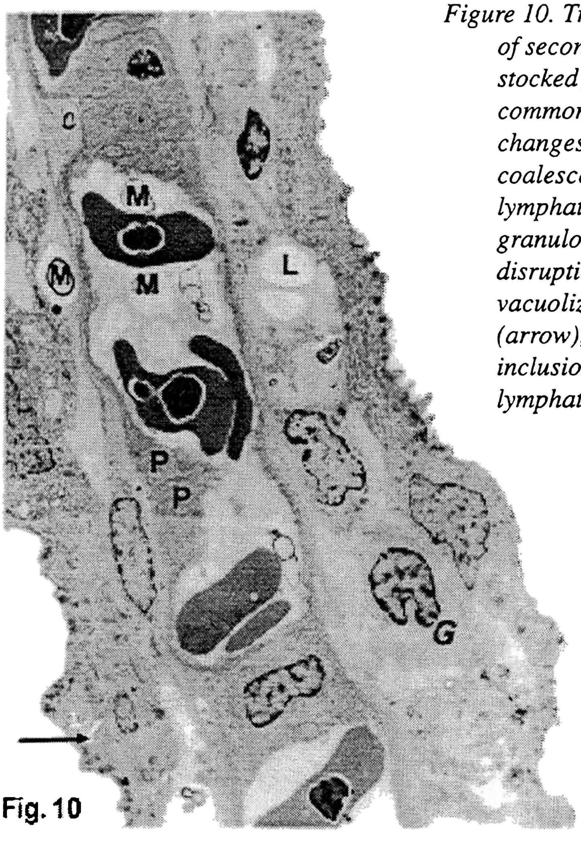

Fig. 10

Figure 10. Transmission electron micrograph of secondary gill lamella of high-density stocked fish showing numerous, commonly-observed cytopathologic changes including enlargement and coalescence of the subepithelial lymphatic spaces (L), accumulation of granulocytes (G) within those spaces, disruption of pillar cells (P), vacuolization of epithelial cell cytoplasm (arrow), and presence of membranous inclusions (M) in the vascular and lymphatic spaces. x = 7540.

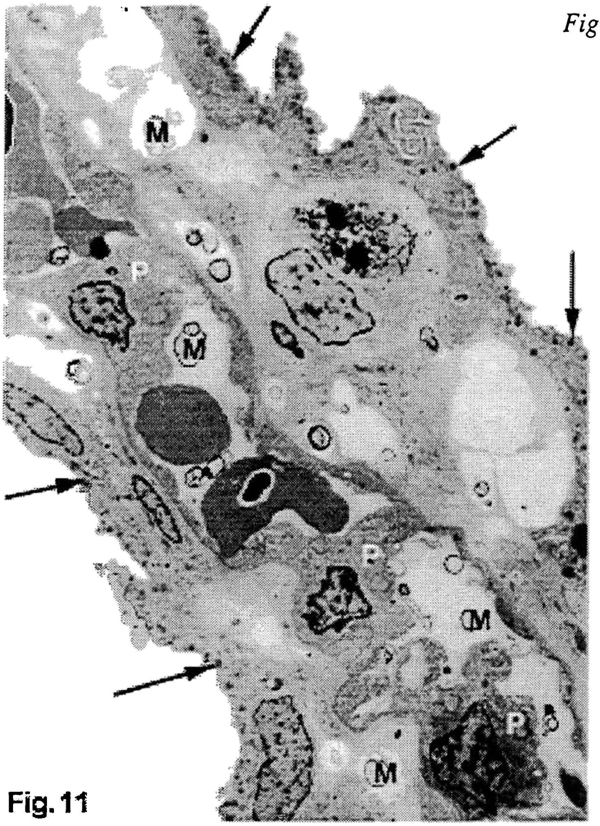

Fig. 11

Figure 11. Transmission electron micrograph of secondary gill lamella of high-density stocked fish showing alterations similar to those demonstrated in Figure 10, but at a more advanced stage of pathology. Greater thickening of gill lamella, more pronounced disruption of pillar cells (P), more numerous membranous inclusions (M) in both the vascular and lymphatic spaces, and enhanced accumulation of small, dense granules in the cytoplasm of the outer epithelial cells (arrow) are observed. x = 7540.

DISCUSSION

The minor areas of proliferative tissue present in the control fish were considered unremarkable. Such variation is typical of many species of wild and cultured fish inhabiting clean water (Ferguson 1989; Solangi and Overstreet 1982; Haensly et al. 1982; Ribelin and Migaki 1975), and hence is representative of the range of variation typically present in normal healthy fish. Though such foci do represent departures from normal, when small and few they are not considered significantly pathological to the gill as a functioning organ (Haensly et al. 1982). The similar foci observed in the five week post-transfer fish were also considered to be within normal limits.

The ultrastructural data clearly illustrate not only some specific cytopathological changes in the secondary gill lamellae resulting from over-crowding of the fish, but also the structural basis for impairment of respiratory gaseous transport as well as excretory and osmoregulatory functions. In normal fish, the water-blood barrier consists of pillar cells flanges, a basal lamina, and thin layers of epithelial cell cytoplasm. In fish showing pathological changes, this transport barrier was significantly increased by either fusion of some of the secondary gill lamellae, or by a several-fold increase in the portion of the barrier consisting of the two-cell thick epithelial cell layer, as well as by mucus accumulation and enlargement/extension of the subepithelial lymphatic spaces. The origin of the membranous inclusions noted in the vascular and subepithelial lymphatic spaces remains obscure, but probably represent fragments of cellular membrane products (Hinton, personal communication). Nonetheless, their common occurrence in the crowded fish and absence in the uncrowded fish suggests that these bodies develop in response to some factor(s) experienced by the fish under high-density stocking. Such factors could involve stress on the fish, water quality, some other unidentified factor, or a combination of multiple factors. The ultrastructural changes observed in this study of hybrid striped bass resembled the acute inflammatory effects in gills reported in rainbow trout exposed to a specific water contaminant, zinc sulfate (Skidmore and Tovell 1972), which resulted in respiratory collapse and death.

Development of gill lesions was anticipated in fish reared at high population density in the intensive recirculating system. Though water quality parameters generally remained within limits considered acceptable, levels of potentially irritating substances such as suspended particulates or metabolic wastes were nonetheless elevated as compared to what would be encountered in open, unpolluted waters. Continuous exposure to these physical and chemical irritants likely contributed to the development of the changes observed.

The lesions that developed in these gills were non-specific in nature (Roberts 1989; Mallat 1985; Ribelin and Migaki 1975), and were similar to what has been described in studies involving exposure to diverse substances such as crude oil, ammonia, molluscicides, herbicides, and pesticides (Cruz et al. 1988; Soderberg 1985; Haensly et al. 1982; Solangi and Overstreet 1982; Eller 1969; Eller 1971). Further, similar effects have been reported in salmonids reared using re-used water or in recirculation systems (MacConnell 1989; Morrison and Piper 1988). The relatively few cell types present in the gill tissue together with the simple structural arrangement of those components result in only a limited range of possible histopathological responses to any of a wide variety of insults (Roberts 1989). In particular, epithelial hyperplasia with or without fusion of adjacent lamellae, mucous cell hyperplasia, and inflammatory cell infiltration are characteristic of a chronic stress response (Roberts 1989; Ferguson 1989; Mallat 1985). This contrasts with certain conditions that induce specific changes, such as hyperplastic gill response in fish exposed to ammonia (Smart 1976; Larmoyeux and Piper 1973; Burrows 1964), increased chloride cells following exposure to acid water (Karlsson-Norrgren et al.1986; Leino and McCormick 1984) or nitrite (Gaino et al. 1984), and chloride cell degeneration and necrosis in nitrite and cadmium toxicity (Ferguson 1989). Proliferation of epithelial cells and particularly of mucous cells can contribute to the lamellar fusion that was prominent in the market-aged fish (Ferguson 1989; Roberts,1989).

Gill lesions from a number of etiologies may be distributed diffusely along the gill arch (Ferguson 1989; Mallat 1985), while other etiologies such as exposure to crude oil and other factors (Ferguson 1989; Solangi and Overstreet 1982) are characterized by a distal-filamental localization of lesions. Alterations observed in this study were more similar to the latter, being distributed mainly along the distal third to half of the arch. Severe histopathological alteration such as lamellar fusion effectively reduces the surface area of gills available for respiratory and other functions. Decreased oxygen intake caused by severe proliferative lesions and resulting impairment of respiratory exchange can be the primary cause of death in certain conditions such as zinc toxicity (Hughes 1972; Skidmore 1972,1970; Burton et al. 1972). However, when such lesions are only moderate and/or when not diffusely spread among the filaments, exchange across less-affected regions of the gills may adequately compensate for the damaged regions (Roberts 1989). Modifications in cardiovascular function have also been postulated to contribute to such compensation (Goldes et al. 1988). This is particularly true when fish are not achieving or sustaining high levels of physical activity (similar to the fish in this study and other intensive aquaculture systems), since in such instances the distal regions of the filament are not fully perfused (Ferguson 1989). Pantothenic acid deficiency (classical "nutritional gill disease") of rainbow trout presents a similar situation in which fish having severe lamellar fusion along the distal filamental region but little involvement of the more proximal filamental region show little clinical evidence of distress when maintained under inactive conditions (Karges and Woodward 1984; Poston and Page 1982).

Dilation of small or terminal gill vessels (telangiectasis) has been described in association with chemical exposure, parasitic infestation, or metabolic wastes (Roberts 1989; Haensly et al. 1982; Redner and Stickney 1979; Smart 1976). This lesion also commonly develops in farmed fish following their physical handling associated with size grading and/or pond transfer (Roberts 1989). In the present work, resolving telangiectasis was observed only in the group sampled shortly after transference between tanks, rather than in any of the fish in groups maintained in the recirculation system for extended periods of time before sampling. This suggests that the telangiectasis developed related to the handling of the fish during transfer, rather than to water conditions in the recirculation system.

The alacrity with which mucous cell number on the gill lamellae is striking. The numbers of mucous cells decreased precipitously during the first week, decreasing from an overall mean of 42.7 cells in the high-density fish to 1.3 cells at 1 week post-transfer (Table 2, Figure 2). Lamellar mucous cell numbers then reached normal levels during the second week. In contrast, the number of mucous cells in the interfilamental spaces and on the gill filament did not reach normal levels until the third and fourth weeks, respectively. Further, the decrease in mucous cells in these other areas was more gradual, rather than being abrupt as on the lamellar surface. That the lamellar surface should clear of abnormally high numbers of mucous cells both rapidly and before other areas, may present an adaptive response in that the swift return of this area, which is the main respiratory surface, to normal structure would promote an expedient return to a normally-functioning state of this strategic portion of the gill.

The rapid resolution of the proliferative lesions was likely related to the extensive epithelial tissue component of the gills, the nature of the lesions, and the placement of the fish in clean water (i.e., removal from inciting conditions caused by less than superior water quality in the recirculation system). Epithelial tissues inherently possess rapid regenerative capacity, and organs with large amounts of epithelium are often capable of swift recovery from minor to moderate damage once the insult is removed. In keeping with such characteristics, gills are well-characterized as able to recover rapidly from sub-lethal injury (Ferguson 1989; Goldes 1988; Fukuda 1983). Degenerative lesions resolve more quickly than necrotic lesions, since generation of new cells is not necessary. In this regard, the preservation of the basement membrane observed in this study likely facilitated rapid recovery. Placing the fish in superior quality water eliminated stimuli for continued excessive epithelial and mucous cell proliferation, as well as for excessive mucus production by proliferated cells. The flushing action of clean water is also documented as contributing to the removal of accumulated mucus and cells (Roberts 1989).

Though development of gill pathology in the experimental group was expected, the extent of the gill surface involvement was not. With significant structural alterations distributed over more than half of the gill surface, the absence of overt effects on the behavior and growth of the fish is noteworthy. Such apparent well-being of the fish could be misleading. Despite acceptable growth, the deteriorated condition of the gills could contribute to increased susceptibility of the fish to stressors like bacterial, viral, fungal, or parasitic infections or other forms of challenge. Further, resulting morbidity could be more severe than in fish where the gills were in better condition. For instance, increased water temperature and resulting decreased oxygen saturation could induce mortality in fish with damaged gills that otherwise might survive (Roberts 1989). Similarly, overcrowding of fish is known to be an important predisposing factor in numerous diseases (Roberts 1989; Ferguson 1989; Ribelin and Migaki 1975). Therefore, impairment of normal respiratory, excretory, and osmoregulatory function resulting from structural and functional compromise of the gill caused by such conditions may be a major contributing factor in the susceptibility of fish to other disease conditions, despite seemingly healthy condition and adequate growth.

Despite the fact that fish raised at high population densities in recirculation systems with less than superior quality water can survive and grow at an economically-profitable rate, the results of this study have important implications for the aquaculture industry. First, this study demonstrates that fish raised under present aquaculture practices in these systems develop extensive structural alterations along the majority of the exchange area of the gill surface, the character of which could potentially alter respiratory, excretory, and osmoregulatory functions. By affecting such functions, growth performance and feed efficiency of the fish could be negatively impacted, as has been previously shown in channel catfish (Robinette, 1976; Soderberg et al. 1984). Therefore, production performance of fish as commonly reared in intensive recirculation systems, though considered acceptable, may not necessarily reach full potential. The reversibility of the lesions suggest that fish in production facilities could respond quickly to significant improvements in water quality. Practical and economic considerations of most production facilities preclude the maintenance of water conditions similar to those used in the recovery phase of this study. Nonetheless, moderate improvement of water conditions would likely decrease gill lesion severity. Such improvement could improve respiratory, osmoregulatory, and excretory functions of the gill, thereby potentially improving both the performance of the fish and ultimately increase economic return. Further, improving gill health prior to and after inducing significant stress on the fish by stressors such as handling procedures could assist in maintaining physical and physiological condition of the fish. Whether increased return generated by such practices will exceed the cost and logistics of implementation is yet to be determined, but invites investigation.

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REFERENCES

Allen, L.J., Kinney, E.C., (eds.), In: Proceedings of the Bio-engineering Symposium for Fish Culture, American Fisheries Society. Fish Culture Section **1981**, Bethesda, Maryland.

Amend, D.F. Myxobacterial Infections of Salmonids: Prevention and Treatment. Diseases of Fishes and Shellfishes. *American Fisheries Society Spec* **1970**, Publ 5, 258-265.

Burrows, R.E. Effects of Accumulated Excretory Products on Hatchery-reared Salmonids. *Research Reports of the U. S. Fish and Wildlife Service* **1964**, 66, 1-12.

Burton, D.J., Jones, A.H., Cairns, J. Acute Zinc Toxicity to Rainbow Trout (*Salmo gairdneri*): Confirmation of Hypothesis that Death is Related to Tissue Hypoxia. *Journal of the Fisheries Research Board of Canada*, **1972**, 29, 1463-1466.

Cruz, E.R., de la Cruz, M.C., Sunaz, N.A. Hematological and Histopathological Changes in *Oreochromis mossambicus* After Exposure to the Molluscicides Aquatin and Brestan. pp. 99-110, In: The Second International Symposium on Tilapia in Aquaculture. ICLARM Conference Proceedings **1988**, 15. Pullin, R.S.V., Bhukaswan. T., Tonguthai, K., and Maclean, J.L., (eds.)

Eller, L.L. Pathology of Redear Sunfish Exposed to Hydrothol 191. *Transactions of the American Fisheries Society* **1969**, 98, 52-29.

Eller, L.L. Histopathologic Lesions in Cutthroat Trout (*Salmo clarki*) Exposed Chronically to the Insecticide Endrin. *American Journal of Pathology* **1971**, 164, 321-336.

Ferguson, H.W. **1989**, Systemic Pathology of Fish. Iowa State University Press, Ames, Iowa.

Fukuda, Y. Specific Reaction of Goldfish Gills Exposed to Linear Alkylbenzene Sulphonate. *Japanese Journal of Ichthyology* **1983**, 30, 268-274.

Gaino. E., Arillo, A., Mensi, P. Involvement of the Gill Chloride Cells of Trout under Acute Nitrite Intoxication. *Comparative Biochemistry and Physiology* **1984**, 77A, 611-617.

Goldes, S.A., Ferguson, H.W., Moccia, R.D., Daoust, P.Y. Histological Effects of the Inert Suspended Clay Kaolin on the Gills of Juvenile Rainbow Trout, *Salmo gairdneri* Richardson. *Journal of Fish Diseases* **1988**, 11, 23-34.

Groman, D.B. Histology of the Striped Bass. American Fisheries Society Monograph Number 3 **1982**. Bethesda, MD. 116 pp.

Haensly, W.E., Nef, J.M., Sharp, J.R., Morris, A.C., Bedgood, M.F., Boem, P.D. Histopathology of *Pleuronectes platessa* L. from Aber Wrac'h and Aber Benoit, Brittany, France: Long-term Effects of the Amoco Cadiz crude oil spill. *Journal of Fish Diseases* **1982**, 5, 365-391.

Harrel, R.M., Kerby, J.H., Minton, R., (eds.) **1990**. Culture and Propagation of Striped Bass and its Hybrids. Striped Bass Committee, Southern Division, American Fisheries Society, Bethesda, MD.

Hinton, D.E. Histological techniques. pp. 191-209, In: Methods for Fish Biology. **1990**; Schreck, C.B., Moyle, P.B., (eds.), American Fisheries Society, Bethesda, MD.

Hughes, G.M. Morphometrics of Fish Gills. *Respiratory Physiology* **1972**, 14, 1-26.

Karges, R.G., Woodward, B. Development of Lamellar Epithelial Hyperplasia in Gills of Pantothenic Acid-deficient Rainbow Trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **1984**, 25, 57-62.

Karlsson-Norrgren, L., Bjorklund, I., Ljungberg, O., Runn, P. Acid Water and Aluminum Exposure: Experimentally Induced Gill Lesions in Brown Trout, *Salmo trutta*. L. *Journal of Fish Diseases* **1986**, 9, 11-25.

Larmoyeux, J.D., Piper, R.G. Effects of Water Re-use on Rainbow Trout in Hatcheries. *Progressive Fish Culturist* **1973**, 35, 2-8.

Leino, R.L., McCormick, J.H. Morphological and Morphometrical Changes in Chloride Cells of the Gills of *Pimephales promelas* After Chronic Exposure to Acid Water. *Cell and Tissue Research* **1984**, 236, 121-128.

Liao, P.B., Mayo, R.D. Intensified Fish Culture Combining Water Reconditioning with Pollution Abatement. *Aquaculture* **1974**, 3, 61-85.

Luna, L.G. (ed.) **1968**. Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology, third edition. McGraw-Hill, New York. 258 pp.

Luchetti, G.L., Gray, G.A. Water Reuse Systems: A Review of Principal Components. *Progressive Fish Culturist* **1988**, 1, 50, 1-6.

MacConnell. E. Effects of Water Reuse on Lake Trout. *Progressive Fish Culturist* **1989**, 51, 33-37.

Mallat, J. Fish Gill Structural Changes Induced by Toxicants and Other Irritants: A Statistical Review. *Canadian Journal of Fisheries and Aquatic Science* **1985**, 42, 630-648.

Morrison, K.J., Piper, R.G. Effects of Reused Water on Atlantic Salmon. *Progressive Fish Culturist* **1988**, 50, 110-112.

Pfeiffer, C.J., Smith, B.J., Smith, S.A. Ultrastructural Morphology of the Gill of the Hybrid Striped Bass (*Morone saxatilis* x. *M. chrysops*). *The Journal of Veterinary Medicine*, Series C. Anatomy Histology and Embryology 28:337-44.

Poston, H.A., Page, J.W. Gross and Histological Signs of Dietary Deficiencies of Biotin and Pantothenic Acid in Lake Trout, *Salvelinus namaycush. Cornell Veterinarian* **1982**, 72, 242-261.

Redner, B.D., Stickney, R.R. Acclimation to Ammonia by *Tilapia aurea. Transactions of the American Fisheries Society* **1979**, 108, 383-388.

Ribelin, W.E., Migaki, G. (eds.), **1975**. The Pathology of Fishes. University of Wisconsin Press, Madison, WI.

Roberts, R.J. (ed.), **1989**. Fish Pathology, second edition. Balliere Tindall, Philadelphia, PA. 467 pp.

Robinette, H.R. Effect of Selected Sublethal Levels of Ammonia on the Growth of Channel Catfish (*Ictalurus punctatus*). *Progressive Fish Culturist* **1976**, 38, 26-29.

Skidmore, J.F. Respiration and Osmoregulation in Rainbow Trout with Gills Damaged by Zinc Sulfate. *Journal of Experimental Biology* **1970**, 52, 484-494.

Skidmore, J.F., Tovell, P.W.A. Effects of Zinc Sulfate on the Gills of Rainbow Trout. *Water Research* **1972**, 6, 217-230.

Smart, G. The Effect of Ammonia Exposure on Gill Structure of the Rainbow Trout (*Salmo gairdneri*). *Journal of Fish Biology* **1976**, 8, 471-475.

Soderberg, R.W. Histopathology of Rainbow Trout, *Salmo gairdneri* Richardson, Exposed to Diurnally Fluctuating Un-ionized Ammonia Levels in Static-water Ponds. *Journal of Fish Diseases* **1985**, 8, 57-64.

Soderberg, R.W., McGee, M.V., Grizzle, J.M., Boyd, C.E. Comparative Histology of Rainbow Trout and Channel Catfish Grown in Intensive Static Water Aquaculture. *Progressive Fish Culturist*, **1984**, 46, 195-199.

Solangi, M.A., Overstreet. R.M. Histopathological Changes in Two Estuarine Fishes, *Menidia beryllina* (Cope) and *Trinectes maculatus* (Bloch and Schneider), Exposed to Crude Oil and its Water-soluble Fractions. *Journal of Fish Diseases* **1982**, 5, 13-35.

Spotte, S. **1979**. Fish and Invertebrate Culture: Water Management in Closed Systems, second edition. Wiley-Interscience, New York.

Effects of Selected Chemotherapeutants on Nitrification in Fluidized-Sand Biofilters for Coldwater Fish Production

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ABSTRACT

Four fish chemotherapeutants, formalin, benzalkonium chloride, chloramine-T, and hydrogen peroxide were evaluated for their effect on the nitrification efficiency of fluidized-sand biofilters. The chemotherapeutants were added at conventional concentrations to two small-scale (2,200 L) coldwater recirculating rainbow trout (*Oncorhynchus mykiss*) culture systems each containing six fluidized-sand biofilters operating in parallel. Nitrification efficiency of biofilters was calculated before and after chemotherapeutant treatments by determining ammonia removal efficiency at ambient conditions, and also when challenged with a sudden increase of ammonium chloride at a concentration four times that of the ambient total ammonia-nitrogen (TAN). Two formalin treatments in recycle bath mode at 167 and 300 ppm were conducted with only the 300 ppm treatment having a significant negative effect on biofilter nitrification efficiency. Four single benzalkonium chloride treatments of one and 2 ppm were conducted; two static bath treatments and two recycle bath treatments. Of these four tests, only the recycle bath treatments caused biofilter nitrification efficiency to be significantly impaired. Two multiple treatments with benzalkonium chloride were conducted: one static bath treatment and one recycle bath treatment. These treatments caused ammonia removal efficiency to decrease by 18% in the static bath treatment and by 63% in the recycle bath treatment. Of these two tests, only the recycle bath treatment caused a significant impairment of nitrification. Single static bath and recycle bath treatments with 9 ppm of chloramine-T both resulted in significant impairment of nitrification, as did a 12 ppm multiple static bath treatment. A single static bath treatment with 100

ppm of hydrogen peroxide caused almost total failure of nitrification within 24 h of treatment but biofilters were able to remove 23% of TAN within 48 h of treatment.

INTRODUCTION

As land and water resources become increasingly limited, interest in recirculating aquaculture systems as a sustainable form of food production is growing. In order to be economical, recirculating systems must maintain high densities of fish, a condition that provides favorable conditions for the outbreak and spread of disease (Noble and Summerfelt 1996). Typical disease treatment protocols often require the availability of large volumes of water (Noga 1996). Given that one of the primary reasons for operating recirculating systems is to conserve water, conducting disease treatments without flushing the system with fresh water after the treatment would be preferable. In addition, in some cases there is not enough water available for the complete water exchange necessary to flush the chemotherapeutant from the system.

A typical recirculating system generally possesses two separate flows: the system flow, and the make-up flow. The system flow is the internal flow rate of the water passing through the tanks and other system components, while the make-up flow is the flow rate of fresh water entering and leaving the system. In coldwater aquaculture the make-up flow rate typically ranges from 1-20% of the system flow rate and is used for the control of temperature and water quality.

The typical methods of disease treatment within recirculating systems are either a static bath treatment or a flow-through treatment (Noga 1996). Static bath treatments are conducted by treating the culture organism in a static volume of water followed by flushing. Flow-through treatments are conducted by allowing water to flow one way through the system in a single pass and constantly adding the chemotherapeutant to maintain the desired concentration. Another option, unique to recirculating systems, is a recycle bath treatment where the chemotherapeutant is added to the culture system under normal operating conditions. From a disease management perspective, disease treatment using a recycle bath treatment might be desirable in order to decrease the possibility that the biofilter could become a reservoir for pathogens

(Noble and Summerfelt 1996). Therefore, recycle bath treatments would be preferred from the standpoint of efficacy and system management if the chemotherapeutant did not impair nitrification.

Most tank based aquaculture systems rely on flow and fresh water inputs to remove toxic metabolites and add oxygen. Treatments that require static volumes are difficult or impossible to sustain unless special design considerations, such as in-tank oxygenation and plumbing are made. Disease treatment using the static bath method will result in a lower concentration of chemotherapeutant exposure to the biofilters, because the chemotherapeutant that reaches the biofilters is diluted by the water volume residing in other compartments of the system once normal flows are resumed. If static bath treatments are not an option, then the recirculating aquaculturist must use a recycle bath treatment. A recycle bath treatment can maintain flow within the culture tank but also results in a continual exposure of the biofilter to the chemotherapeutant, which could result in impairment or failure of nitrification.

An important component of recirculating systems, biofilters support living populations of nitrifying bacteria that transform ammonia and nitrite, which are toxic to fish, into nitrate, which is relatively non-toxic. It is important that biofilters operate at peak efficiency during disease outbreaks because any impairment of biofiltration will serve to increase the stress on the fish through the reduction of water quality (Klontz 1993). Because of the biological nature of biofilters, they are often presumed to be sensitive to the biocidal agents added to recirculating systems for the control of pathogens. For these reasons it is important that aquaculturists know the effects of commonly used chemotherapeutants on biofilters, and how extensive these effects may be. In a previous study using fluidized-sand biofilters it was determined that formalin treatments at levels commonly used in fish culture caused no apparent effect on biofilter performance when tested under ambient conditions (Heinen et al. 1995). Given that most commonly used chemotherapeutants in aquaculture are biocides, it was assumed that they must have some effect on the microbial community associated with biofilters.

Fluidized-sand biofilters are typically designed with excess nitrification capacity (Summerfelt 1996; Summerfelt and Cleasby 1996) in the form of surface area available for microbial colonization. This excess capacity allows fluidized-sand biofilters to nitrify more ammonia and nitrite than they are exposed to under normal operating concentrations. Because of this property it was hypothesized that a change in the microbial community caused by a chemotherapeutant treatment that was not evident when a biofilter was tested under ambient conditions would become evident when the biofilter was "challenged" with a spike of higher than normal ammonia concentration. Challenging the biofilters under normal conditions should allow for the determination of their maximum instantaneous capacity, which could then be used as a benchmark to compare biofilter performance after exposure to a chemotherapeutant. If a chemotherapeutant treatment caused an impairment of maximum biofilter nitrification capacity that was not apparent under ambient conditions, it should become apparent when the biofilters are challenged. Hence, it was thought that the effect of chemotherapeutants on biofilter nitrification capability might be ascertained through the determination of diminished maximum capacity.

With this in mind, an investigation into the effect of formalin, benzalkonium chloride, chloramine-T, and hydrogen peroxide on biofilter efficiency was undertaken. The goal of this study was to determine what effect the method of treatment might have on biofilter efficiency, and to prescribe modifications of these methods to minimize the effect of a given therapeutant on biofiltration. The four chemicals tested were chosen because of their widespread historical use in coldwater aquaculture (Noble and Summerfelt 1996). Formaldehyde, benzalkonium chloride and chloramine-T are in use in the countries of the European Union and Iceland (Schlotfeldt 1990). However, within the United States, only formalin is approved by the U.S. Food and Drug Administration (FDA) for use on food fish. Benzalkonium chloride is approved for use only as a disinfectant in aquaculture. Hydrogen peroxide is not approved, but is considered of low regulatory priority and the FDA is unlikely to object to its use. Attempts to register chloramine-T for treatment of bacterial gill disease are presently underway (J. Bowker, personal communication).

MATERIALS AND METHODS

Recirculating System

All tests were conducted using two identical recirculating systems (Figure 1). Each system contained one 1,500-L culture tank; one drum filter; one pump sump; two degassers with sumps; and six identical biofilters operating in parallel. Each 15 cm inside diameter fluidized-sand biofilter contained 4,700 cm^3 of silica sand and treated a flow of nine liters/min (L/min) for a total system flow rate of 54 L/min. The average diameter of the sand used in the biofilters was 0.17 mm and the static height of each sand bed was 30.5 cm. The system was stocked with rainbow trout (*Oncorhynchus mykiss*) maintained at a density that ranged from 23-38 kg/m^3. Fish were fed continually using mechanical feeders at a rate of approximately 2% of their body weight per day with Southern States 3/32" 40% Protein Trout Grower Feed[1] (Southern States Cooperative, Richmond, VA, USA). The make-up flow, a hard spring water (300 ppm as $CaCO_3$) at 11.5°C, was added at a rate of 5% of the system flow to provide approximately two system volume turnovers per day. Temperatures within the culture system ranged from 14-16°C. Biofilter influent samples were collected from sampling ports in the common influent line for each set of three biofilters while biofilter effluent samples were collected from sampling ports at the top of each individual biofilter.

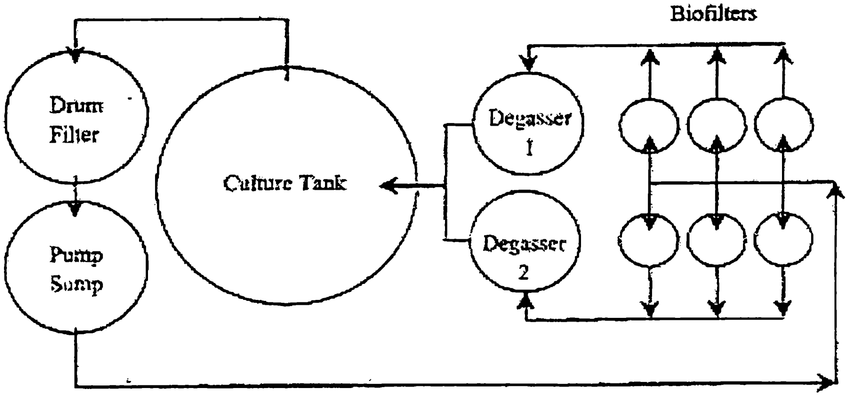

Figure 1. Diagram of recirculating systems used for chemotherapeutant experiments

Chemotherapeutant Treatments

Formalin is typically used for the treatment of external parasites at a concentration of 167 ppm for one hour followed by flushing (Noga 1996). Formalin treatments (37% solution of Formalin-F®[1] (formaldehyde), Natchez Animal Supply, Natchez, MS, USA) were conducted at 167 and 300 ppm in recycle bath mode with single treatments.

A historical treatment for bacterial gill disease with benzalkonium chloride is to treat with 1-2 ppm for one hour followed by flushing (Bullock 1990; Noga 1996). Noble and Summerfelt (1996) reported that an effective treatment technique for benzalkonium chloride was three 2 ppm treatments 48 hours apart. Single treatments of benzalkonium chloride (50% solution of benzalkonium chloride (dimethyl benzyl ammonium chloride), Argent Chemical Laboratories[1], Redmond, WA, USA) were conducted using both static bath and recycle bath treatments at 1 and 2 ppm. Multiple treatments, consisting of three treatments 48 h apart, with benzalkonium chloride at 2 ppm were also conducted in both static bath and recycle bath mode.

Chloramine-T is used as a bactericide at concentrations ranging from 9-12 ppm for one-hour static bath treatments either singly, or in a series of three treatments given on alternate days (Bills et al. 1988; Bullock et al. 1991). Single static bath and recycle bath treatments with 9 ppm of chloramine-T (N-chloro-p-toluene sulfonamide sodium salt, Sigma Chemical Co.[1], St. Louis, MO, USA) were conducted first, and then a multiple static bath treatment consisting of three treatments at 12 ppm was performed on alternate days.

Hydrogen peroxide is used as a bactericide and fungicide with a one hour static bath treatment at concentrations ranging from 100-500 ppm (Arndt and Wagner 1997; Rach et al. 1997). The hydrogen peroxide treatment (35% solution of Peroxyclear®[1] (hydrogen peroxide), EKA Chemicals, Marietta, GA, USA) consisted of one static bath treatment at 100 ppm. This concentration was chosen because previous unpublished work indicated that the peroxide treatment would significantly impair biofilter performance. Hence, a concentration at the lower end of the reported range was chosen.

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[1] Use of trade names or specific manufacturers or suppliers does not indicate endorsement.

Before a given chemotherapeutant test the ambient biofilter water chemistry was analyzed, then the biofilters were challenged. The chemotherapeutant was added to the system immediately after the challenge. Chemotherapeutant concentration was determined every twenty minutes in the culture tank and in the biofilter influent and effluent lines. Formalin concentrations were measured directly using the Purpald colorimetric method (Chemetrics[1], Calverton, VA, USA). The concentration of benzalkonium chloride was determined by analyzing the quaternary ammonium compounds (QAC) present in the water using the Direct Binary Complex colorimetric method (Hach Chemical Co.[1], Loveland, CO, USA) and establishing the relationship between benzalkonium chloride concentration and measured QAC. The concentration of chloramine-T was determined by analyzing the concentration of total chlorine present in the water using the DPD (N, N-diethyl-p-phenylenediamine) method (Hach Chemical Co., Loveland, CO, USA) and establishing the relationship between calculated chloramine-T concentration and measured free chlorine. Hydrogen peroxide concentrations were measured directly using the thiocyanate method (Chemetrics, Calverton, VA, USA).

At least 4 weeks were allowed to elapse between tests with a given chemotherapeutant to allow the microbial flora of the biofilters time to stabilize from any perturbations caused by previous treatments. The maximum time that elapsed between the conclusion of one set of chemotherapeutant tests and the onset of tests with the next chemotherapeutant was two months.

Static Bath Treatments

Static bath treatments were conducted by turning off the make-up flow to prevent dilution of the chemotherapeutant, and isolating the biofilters in a separate recirculating loop to maintain fluidization. The chemotherapeutant was then added to the static culture tank and the above conditions were maintained for an hour after which normal operating conditions were resumed. In this type of treatment, biofilters were exposed to the chemotherapeutant only after normal operations were resumed, at which time the chemotherapeutant would have been diluted by water volume residing in other compartments of the system. In the case of this experiment, chemotherapeutants in the culture tank were diluted by 40% once normal operations were resumed.

Recycle Bath Treatments

Recycle bath treatments were conducted by leaving all flow processes in their normal mode with the only difference being that the make-up flow was turned off to prevent dilution of the chemotherapeutant. The chemotherapeutant was then added in aliquots throughout the system. Normal make-up flow operating conditions were resumed after one hour. During recycle bath treatments the biofilters were left connected to the main flow and as such were continually exposed to the chemotherapeutant during treatment.

Ammonia Challenge Tests

As a preliminary step the biofilters were challenged at various total ammonia nitrogen (TAN) concentrations up to five times higher than ambient in order to determine the maximum TAN concentration that could be assimilated without a significant drop in biofilter efficiency. A TAN concentration five times higher than ambient resulted in a significant reduction in ammonia removal (30%), whereas at concentrations approximately four times higher than ambient, there was very little difference in biofilter efficiency under ambient and challenge conditions.

The fact that a recirculating system was used required two issues to be addressed by the experimental design in order for the challenge test to be successful: time of sample collection, and prevention of contamination by the recirculating spike. The time when the peak concentration of injected TAN occurred was determined by proportionally metering a concentrated solution of ammonium chloride into the system pump intake. Samples were then collected at the site of biofilter influence every 30 sec and analyzed for TAN. The time required for injected material to recirculate back to the point of injection was determined by injecting a 10 mL aliquot of dye(red food coloring) into the pump intake and collecting samples at regular intervals. The absorbance of these samples was recorded at 500 nm with a Hach DR2000 spectrophotometer. It typically took 9-10 min for the dye to return to the point of injection at the pump intake. The hydraulic retention time of the biofilters was approximately 20 sec. The TAN concentration reached a peak at 5 min after injection. To achieve a consistent TAN spike concentration across tests, the biofilter influent and effluent samples were collected at precisely six min after the onset of the ammonium chloride solution injection. Collecting the samples at six min was long enough to ensure that the ammonia concentration was at its peak level but was still short enough to prevent the ammonia spike from recirculating through the system.

Before and 24 h after each chemotherapeutant treatment, ambient biofilter performance was measured and then the biofilters were challenged with a spike of ammonium chloride solution approximately four times that of the ambient influent TAN concentration. The ammonium chloride solution (8.93 g NH_4Cl/L) was metered directly into the pump intake for six minutes with samples collected from the biofilter influent and effluent at the end of this time period. Parameters measured were: temperature, pH, dissolved oxygen, TAN, and nitrite-nitrogen. Water quality parameters were all analyzed according to standard methods (APHA 1989).

Biofilter nitrification efficiency was calculated by subtracting the outlet concentration from the inlet concentration and dividing the difference by the inlet concentration. The statistical significance of differences between removal efficiencies was determined using a one-tailed Wilcoxon paired-sample test (Zar 1974) on the mean of six biofilters. A non-parametric test was chosen because the data was not distributed normally.

The experimental protocol and methods described were in compliance with Animal Welfare Act (9CFR) requirements and were approved by the Freshwater Institute Institutional Animal Care and Use Committee.

RESULTS

Under ambient conditions, influent concentrations for TAN and nitrite ranged from 0.18-0.52 mg/L and 0.009-0.086 mg/L, respectively (after treatment with hydrogen peroxide the ambient TAN peaked at 1.8 mg/L and dropped back to normal levels after three days). During the biofilter challenges influent concentrations of TAN and nitrite ranged from 1.07 - 1.52 mg/L and 0.014-0.105 mg/L, respectively. Total suspended solids measurements during the testing period averaged 2.9 mg/L with little difference between biofilter influent and effluent being observed.

Table 1. Summary of effects of formalin recycle bath treatments on biofilter nitrification efficiency. Values are means of six biofilters with the standard error. Double asterisks indicate a highly significant difference ($p<0.01$ *between before and after values for one particular test.*

		Removal Efficiency	
Treatment		**Ambient TAN**	**Challenged TAN**
167 ppm Formalin			
	Before	0.91 ± 0.02	0.69 ± 0.04
	After (24 h)	0.90 ± 0.04	0.81 ± 0.06
300 ppm Formalin			
	Before	0.79 ± 0.02	0.68 ± 0.05**
	After (24 h)	0.76 ± 0.03	0.55 ± 0.06

Table 2. Summary of single benzalkonium chloride treatments at different concentrations and treatment modes and their effect on biofilter nitrification efficiency. Values are means of six biofilters with the standard error. Single asterisks indicate a significant difference ($p<0.05$*) between before and after values for one particular test, while double asterisks indicate a highly significant difference (*$p<0.01$*).*

		Removal Efficiency	
Treatment		**Ambient TAN**	**Challenged TAN**
1 ppm Benzalkonium Chloride *Static Bath*			
	Before	0.76 ± 0.02	0.79 ± 0.04
	After (24 h)	0.95 ± 0.03	0.82 ± 0.05
1 ppm Benzalkonium Chloride *Recycle Bath*			
	Before	0.79 ± 0.02	0.78 ± 0.05**
	After (24 h)	0.82 ± 0.02	0.68 ± 0.06
2 ppm Benzalkonium Chloride *Static Bath*			
	Before	0.35 ± 0.03	0.61 ± 0.03
	After (24 h)	0.85 ± 0.04	0.68 ± 0.05
2 ppm Benzalkonium Chloride *Recycle Bath*			
	Before	0.96 ± 0.03*	0.83 ± 0.06**
	After (24 h)	0.74 ± 0.10	0.56 ± 0.11

The concentration of formalin remained relatively constant during the tests while the concentration of benzalkonium chloride decreased by about 50% over the space of an hour. During static bath treatments the concentration of chloramine-T decreased on average by 18%. The decrease in chloramine-T concentrations during recycle bath treatments averaged 43%. The concentration of hydrogen peroxide declined by 25% during the last 30 min of treatment after staying constant for the first 30 min.

The 167 ppm formalin test produced a slight reduction in ambient TAN removal (AAR) and a 12% increase in challenged TAN removal (CAR) (Table 1). After the 300 ppm formalin treatment there was a slight decrease of both AAR and CAR.

A 1 ppm benzalkonium chloride single static bath treatment resulted in a slight increase of both AAR and CAR (Table 2). The single 1 ppm benzalkonium chloride recycle bath treatment resulted in a slight increase of AAR and a 10% decrease in CAR. The single 2 ppm benzalkonium chloride static bath treatment caused an increase in both AAR and CAR. Of the single benzalkonium chloride treatments, the most dramatic effect was observed in the 2 ppm recycle bath treatment. Both AAR and CAR were reduced by over 20%. There was an 18% reduction of both AAR and CAR after the end of the multiple benzalkonium chloride static bath treatment. At the end of the multiple recycle bath treatment AAR had decreased by 63% and CAR by 46% (Table 3). The comparative effects of the multiple benzalkonium chloride treatments are displayed in Figure 2.

After the 9 ppm single chloramine-T static bath treatment AAR increased 20% and CAR decreased 5% (Table 4). The AAR decreased 10% and the CAR decreased 9% after the 9 ppm single chloramine-T recycle bath treatment. After the set of multiple 12 ppm chloramine-T static bath treatments there was only a slight decrease in AAR while CAR decreased by 8%. The longer term effects of the chloramine-T treatments are displayed in Figure 3.

The 100 ppm single hydrogen peroxide static bath treatment caused almost total impairment of nitrification (Table 5). Twenty-four hours after treatment the AAR was reduced by 84% and the CAR by 57%. From Figure 4, it can be seen that the biofilters had significantly recovered 11 days after treatment.

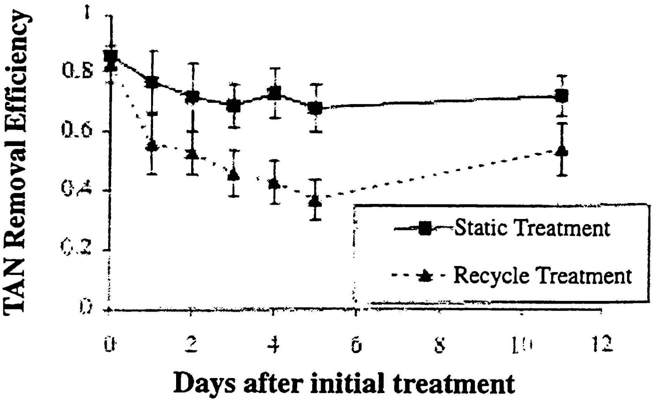

Figure 2. Effect of multiple benzalkonium chloride treatments (static bath and recycle bath) on challenged biofilter TAN removal efficiency. Values are means ± standard errors of six biofilters.

Table 3. Summary of effects of three consecutive benzalkonium chloride treatments on biofilter efficiency under two different treatment modes. Values are means of six biofilters with the standard error. Double asterisks indicate a highly significant difference (p<0.01) between initial and final values for one particular test.

	Removal Efficiency	
Treatment	**Ambient TAN**	**Challenged TAN**
2 ppm Benzalkonium Chloride		
(Three Static Bath Treatments/Alternate Days)		
Before	0.86 ± 0.04	0.86 ± 0.04
After (24 h)	0.87 ± 0.05	0.77 ± 0.11
Before	0.64 ± 0.04	0.72 ± 0.12
After (24 h)	0.78 ± 0.06	0.69 ± 0.08
Before	0.59 ± 0.06	0.73 ± 0.08
After (24 h)	0.68 ± 0.08	0.68 ± 0.08
2 ppm Benzalkonium Chloride		
(Three Recycle Bath Treatments/Alternate Days)		
Before	0.96 ± 0.03**	0.83 ± 0.06
After (24 h)	0.74 ± 0.10	0.56 ± 0.11
Before	0.71 ± 0.10	0.53 ± 0.07
After (24 h)	0.54 ± 0.15	0.46 ± 0.08
Before	0.52 ± 0.15	0.43 ± 0.08
After (24 h)	0.33 ± 0.12	0.37 ± 0.07

Table 4. Summary of chloramine-T treatments at different concentrations and treatment modes and their effect on biofilter nitrification efficiency. Values are means of six biofilters with the standard error. Single asterisks indicate a significant difference (p<0.05) between before and after values for one particular test, while double asterisks indicate a highly significant difference (p<0.01). For the 12 ppm chloramine-T tests asterisks indicate significant difference between initial and final values.

	Removal Efficiency	
Treatment	**Ambient TAN**	**Challenged TAN**
9 ppm Chloramine-T ***Static Bath***		
Before	0.71 ± 0.03	0.65 ± 0.06*
After (24 h)	0.91 ± 0.02	0.60 ± 0.05
9 ppm Chloramine-T ***Recycle Bath***		
Before	0.80 ± 0.03**	0.89 ± 0.03**
After (24 h)	0.70 ± 0.01	0.80 ± 0.05
12 ppm Chloramine-T (Three Static Bath Treatments/Alternate Days)		
Before	0.85 ± 0.03	0.81 ± 0.06**
After (24 h)	0.94 ± 0.01	0.79 ± 0.07
Before	0.85 ± 0.01	0.82 ± 0.08
After (24 h)	0.77 ± 0.04	0.83 ± 0.08
Before	0.99 ± 0.01	0.74 ± 0.09
After (24 h)	0.84 ± 0.02	0.73 ± 0.08

Table 5. Summary of effects of static bath treatment with hydrogen peroxide on biofilter nitrification efficiency. Values are means of six biofilters with the standard error. Double asterisks indicate a highly significant difference (p<0.01) between before and after values for one particular test.

	Removal Efficiency	
Treatment	**Ambient TAN**	**Challenged TAN**
100 ppm Hydrogen Peroxide		
Before	0.85 ± 0.03**	0.61 ± 0.08**
After (24 h)	0.01 ± 0.01	0.04 ± 0.06

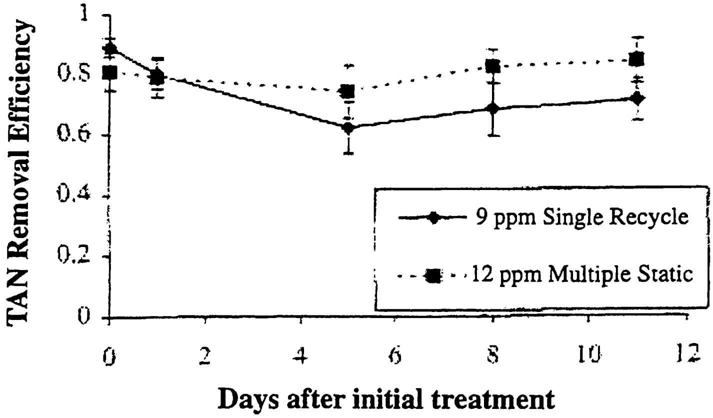

Figure 3. Effect of single and multiple chloramine-T treatments on challenged biofilter TAN removal efficiency. The multiple treatments ended on day 5. Values are means ± standard errors of six biofilters.

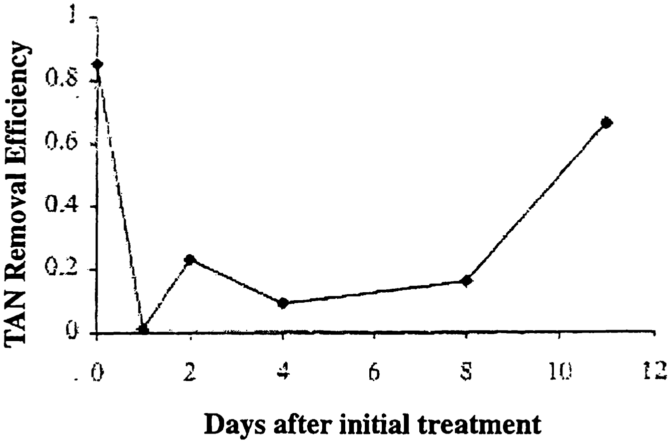

Figure 4. Effect of single 100 ppm hydrogen peroxide static bath treatment on ambient biofilter TAN removal efficiency. Values are means of six biofilters. Ambient values were used because ambient TAN values rose high enough after the treatment to render biofilter challenge superfluous.

DISCUSSION

The primary goal in this research was to determine which of the chemotherapeutants evaluated affect biofilter performance, and to what effect, with the overall concern being the preservation of adequate water quality for fish rearing. As long as adequate water quality can be maintained, minor drops in biofilter efficiency can be tolerated. Within the recirculating aquaculture system used in these experiments, biofilter nitrification efficiency often fluctuates from 5-10% over a period of several days without significant effects on water quality (unpublished data). The authors chose to make the distinction between significant ($p<0.05$) and highly significant ($p<0.01$) statistical differences in biofilter efficiency because it was assumed that only highly significant differences would have a biologically significant effect on biofiltration and the resulting water quality. The effect of changes in nitrification efficiency on TAN concentrations can be illustrated using Liao and Mayo's (1972) equation for calculating steady-state concentrations in recirculating systems. For example: assuming an initial removal efficiency of 90%, a 10% decrease in TAN removal efficiency will increase the tank TAN concentration by 12%; a 20% decrease will increase it by 27%; while a 60% decrease will increase it by 170%.

$$\text{TAN} = \left\{ \frac{1}{1 - R + (R \cdot f_{\text{rem}})} \right\} \cdot \frac{P_{\text{TAN}}}{Q}$$

TAN= total ammonia-nitrogen, mg/L
R= recycle fraction, decimal
f_{rem}= TAN removal efficiency, decimal
P_{TAN}= daily production of TAN, kg/d
Q= system flow, L/min

Of the two formalin treatments, only the single 300 ppm formalin recycle bath treatment caused significant impairment of TAN removal and only within the CAR component. In comparison, Weinbeck and Koops (1990) found that indefinite treatments with 149 ppm formalin had an adverse affect on nitrification in their biofilters, while Heinen et al. (1995) found indefinite treatments at 120 ppm, and one hour treatments at 167 ppm, had no effect on nitrification.

Both of the single benzalkonium chloride recycle bath treatments (1 and 2 ppm) caused significant impairment of CAR. Of these two tests only the 2 ppm test caused a significant reduction of AAR. This impairment of nitrification would be expected, since this test resulted in the highest concentration of benzalkonium chloride that the biofilters were exposed to. With multiple benzalkonium chloride treatments, only the 2 ppm recycle bath treatments significantly impaired TAN removal. Similarly, Noble and Summerfelt (1996) reported that 2 ppm benzalkonium chloride treatments had a negative impact on biofilters at a coldwater hatchery.

All of the chloramine-T treatments caused a significant reduction of CAR, while only the single 9 ppm chloramine-T recycle bath treatment caused a significant reduction of both AAR and CAR. In contrast, Noble and Summerfelt (1996) reported that treatment with 12 ppm of chloramine-T had no effect on biofilters at a coldwater hatchery.

The single 100 ppm hydrogen peroxide treatment caused significant reduction of both AAR and CAR. As there was limited literature available on the effect of hydrogen peroxide treatments on biofilters, the authors had to rely on anecdotal information for comparison. As such, Bullock and others at the Freshwater Institute have observed hydrogen peroxide treatments at 100 ppm to cause a major impairment of biofilter efficiency (unpublished data).

The tests having a highly significant impact on both AAR and CAR were: multiple 2 ppm benzalkonium chloride recycle bath; single 9 ppm chloramine-T recycle bath; and single 100 ppm hydrogen peroxide static bath. These treatments should be avoided in recirculation systems as they could cause major changes in water quality.

Treatments having a highly significant effect on CAR only were: single 300 ppm formalin recycle bath; single 2 ppm benzalkonium chloride recycle bath; single 1 ppm benzalkonium chloride recycle bath; and multiple 12 ppm chloramine-T static bath. These treatments could also cause significant impairment of water quality in a recirculating system.

Regardless of treatment type or concentration, chloramine-T and hydrogen peroxide consistently impaired nitrification. The severe impact of hydrogen peroxide would make it suitable for use as a chemotherapeutant in recirculating systems only if completely flushed out of the system or completely inactivated before resuming normal operations. Chloramine-T could possibly be used with caution in a static bath treatment at the lowest concentration possible. Both formalin and benzalkonium chloride did not cause impairment of nitrification at lower concentrations, hence they could probably be used safely at these concentrations with a static bath treatment being the preferred mode of application. Aquaculturists considering the use of chemotherapeutants in recirculating systems should exercise caution as it is difficult to predict the potential effects of these chemotherapeutants on other types of recirculating systems. They should also be aware that the effect of a given chemotherapeutant on water quality will depend on the type of biofilter and ambient water quality. Our experience has been that fluidized-sand biofilters are particularly resilient to perturbations. The maintenance of good water quality should provide an adequate buffer in the event of a low-level, temporary biofilter impairment caused by a chemotherapeutant treatment.

The results of this research support the hypothesis that impairment of nitrification in fluidized-sand biofilters can be determined through challenging the biofilters with high concentrations of TAN. In all cases where AAR was significantly impaired, CAR was also significantly impaired. In two of the tests only CAR was significantly impaired, indicating that the nitrifiers within the biofilter were affected by the chemotherapeutant without any apparent effect on AAR. Excluding hydrogen peroxide, the results of this research show that while biofilters were all impaired to a certain extent by the chemicals used, the effect was primarily related to concentration. It should be kept in mind that the static bath treatments with the same concentration as recycle bath treatments resulted in lower biofilter exposure concentrations. This brings up the need for further research to test the efficacy of longer duration disease treatments at lower concentrations and the effect of these long duration treatments on biofilters.

The fact that chemotherapeutants are effective at reducing or eliminating fish pathogens and relatively ineffective at eliminating microbes within a biofilter is not surprising. In order to become permanently established within a biofilter nitrifying bacteria must form biofilms (Hagopian 1998). Bacteria can protect itself from antimicrobial agents through the production of a film of hydrated exopolysaccharides (EPS) and are inherently more resistant to antimicrobials than their planktonic forms (Costerton et al. 1995). Since the concentrations required for disinfection with benzalkonium chloride and formalin are 200 ppm and 10,000 ppm respectively (Stoskopf 1993), it is not surprising that the concentrations used in these experiments did not have a greater effect on the biofilters with their well-established populations of nitrifiers. Such high concentrations are necessary because disinfection is generally directed against biofilms (Block 1991). LeChevallier (1991) reports that it generally requires 150 times the CT factor (concentration x time) of hypochlorous acid to achieve the same reduction in activity in a biofilm as against planktonic forms. In addition, microbes use calcium and magnesium ions in the production of EPS (Costerton et al. 1995). Anderson (1985) reported that Roccal®, a quaternary ammonium compound analogous to benzalkonium chloride, was more toxic to biofilters in soft waters than in hard waters. The water used in this experiment was hard, which may explain why benzalkonium chloride was not observed to cause greater impairment of the biofilter. While the resistance of bacteria within biofilms to antimicrobials allows a margin of safety, the aquaculturist who uses antimicrobials should still exercise caution, as it appears that repeated use may have serious consequences on biofilter performance.

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REFERENCES

American Public Health Association (APHA). **1989**. Standard Methods for the Examination of Water and Wastewater. APHA, Washington, D.C.

Anderson, A.R. Effects of Therapeutic Disease Treatments on Nitrification in Model Water Recycle Systems: A Preliminary Study **1985**. Technology Transfer Series No. 84-1. U.S. Fish and Wildlife Service.

Arndt, R.E., Wagner, E.J. The Toxicity of Hydrogen Peroxide to Rainbow Trout and Cutthroat Trout Fry and Fingerlings. *Journal of the World Aquaculture Society* **1997**, 28(2), 150-157.

Bills, T.D., Marking, L.L., Dawson, V.K., Rach, J.J. Effects of Environmental Factors on the Toxicity of Chloramine-T to Fish. *Investigations in Fish Control* No. 96 **1988**. U.S. Fish and Wildlife Service.

Block, S.S. **1991**. Disinfection, Sterilization and Preservation. Lea & Febiger, Philadelphia, Pennsylvania.

Bullock, G.L. Bacterial Gill Disease of Freshwater Fishes. Fish Disease Leaflet 84 **1990**. U.S. Fish and Wildlife Service.

Bullock, G.L., Herman, R.L., Waggy C. Hatchery Efficacy Trials with Chloramine-T for Control of Bacterial Gill Disease. *Journal of Aquatic Animal Health* **1991**, 3, 48-50.

Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., Lappin-Scott, H. M. Microbial Biofilms. *Annual Review of Microbiology* **1995**, 49, 711-745.

Hagopian, D. S., Riley, J. G. A Closer Look at the Bacteriology of Nitrification. *Aquacultural Engineering* **1998**, 18(4), 223-244.

Heinen, J. M., Weber, A.L., Noble, A.C., Morton, J.D. Tolerance to Formalin by a Fluidized-Bed Biofilter and Rainbow Trout in a Recirculating Culture System. *Journal of the World Aquaculture Society* **1995**, 26(1), 65-71.

Klontz, G.W. Environmental Requirements and Environmental Diseases of Salmonids. In: Fish Medicine, Stoskopf, M.K. (ed.), W.B. Saunders, Philadelphia, Pennsylvania, **1993**.

LeChevallier, M.W. Biocides and the Current Status of Biofouling Control in Water Systems. In: Biofouling and Biocorrosion in Industrial Water Systems, Flemming, H.-C., and Geesey, G.G., (eds.), Springer-Verlag, Berlin, Heidelberg, **1991**.

Liao, P.B., Mayo, R.D. Salmonid Hatchery Water Reuse Systems. *Aquaculture* **1972**, 1, 317-335.

Noble, A.C., Summerfelt, S.T. Diseases Encountered in Rainbow Trout Cultured in Recirculating Systems. *Annual Review of Fish Diseases* **1996**, 6, 65-92.

Noga, E.J. **1996**. Fish Disease: Diagnosis and Treatment. Mosby-Year Book, St. Louis, Missouri.

Rach, J.J., Schreiber, T.M., Howe, G.E., Redman, S.D. Effect of Species, Life Stage, and Water Temperature on the Toxicity of Hydrogen Peroxide to Fish. *Progressive Fish-Culturist* **1997**, 59, 41-46.

Schlotfeldt, H.J. Current Practices of Chemotherapy in Fish Culture. In: Chemotherapy in Aquaculture: From Theory to Reality, Michel, C., Alderman, D. (eds.), Office International des Epizooties, Paris, France,**1990**.

Stoskopf, M.K. **1993**. Fish Medicine. W.B. Saunders, Philadelphia, PA.

Summerfelt, S.T. Engineering Design of a Water Reuse System. In: Walleye Culture Manual, Summerfelt, R.C. (ed.), NCRAC Culture Series 101. North Central Regional Aquaculture Center Publications Office, Iowa State University, Ames, Iowa, **1996**.

Summerfelt, S.T., Cleasby, J.L. A Review of Hydraulics in Fluidized-Bed Biological Filters. *Transactions of the ASAE.* **1996**, 39, 1161-1173.

Weinbeck, H., Koops, H. Decomposition of Formaldehyde in Recirculation Systems. *Archiv fur Fischereiwissenschaft* **1990**, 40, 153-166.

Zar, J.H. **1974**. Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ.

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