MANAGEMENT OF ENVIRONMENTALLY-DERIVED OFF-FLAVORS IN WARMWATER FISH PONDS

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Year 3	229,266
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Year 5	56,100
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PROJECT OBJECTIVES

1. Evaluate the feasibility of decreasing the incidence of fish off-flavors by reducing the amount of phosphorus available to support phytoplankton growth.

    a. Evaluate methods of reducing phosphorus input by diet modification by determining the minimum phosphorus requirement for food-sized channel catfish and quantifying the reduction in waste phosphorus generation by food-sized catfish fed "low-phosphorus" feeds relative to presently available feeds.

b. Evaluate methods of removing phosphorus from pond waters by studying methods of enhancing rates of phosphorus removal from pond waters by pond bottom soils and determining the feasibility of precipitating phosphorus from pond waters as sparingly soluble aluminum or calcium salts.

2. Evaluate the feasibility of reducing the incidence of fish off-flavors by manipulating pond phytoplankton biomass and taxonomic composition using biological and chemical control measures.

   a. Evaluate the effect of filter-feeding fishes on water quality and reduction or elimination of off-flavor in pond-raised channel catfish.

   b. Develop microbial pathogens to control blue-green algal abundance.

   c Determine whether plant phenolics (tannins) can control growth of microorganisms that produce odorous compounds in warmwater fish ponds.

   d. Evaluate the effect of routine, low-level treatments of ponds with copper sulfate on phytoplankton communities, off-flavor incidence, and water quality in channel catfish ponds.

3. Determine the feasibility of managing fish off-flavors by reducing rates of 2-methylisoborneol (MIB) uptake by fish and/or enhancing rates of MIB elimination from fish.

4. Develop statistical models describing the within-pond variation in the degree of off-flavor in fish populations under various conditions.

5. Develop analytical techniques for assessing flavor qualities in fish.

6. Develop publications to educate producers and processors on the ecology of environmentally-derived off-flavors, off-flavor management, and the results of this project.

ANTICIPATED BENEFITS

The overall goal of this project is to reduce the incidence of unacceptable flavor quality in pond-cultured fish. If this goal is accomplished, the aquaculture industry will benefit from increased farm profits and market expansion resulting from improved consumer attitude toward aquaculture products.

Use of the revised phosphorus allowance in commercial catfish feeds should reduce the phosphorus input to catfish ponds and thus reduce nutrients available to support algae growth. Similarly, use of alternative phosphorus supplements or phytase enzymes to increase utilization of phytate phosphorus in the feed may be beneficial in reducing phytoplankton growth and thus reduce occurrence of off-flavor. Regardless of the impact on algal communities, these studies will lead to more efficient use of phosphorus from feeds.

The use of chemical substances for reducing phosphorus levels in pond water could provide a simple procedure for channel catfish farmers and other aquaculturists to use in reducing the amount of phosphorus in waters of ponds to which large amounts of feed are applied. The benefits of the compounds (aluminum sulfate, calcium oxide, and calcium sulfate) chosen for use in this research are that they are common compounds, they are relatively inexpensive, they are environmentally safe and would not pose a food safety risk, and they would be easy to apply. If one or more of these compounds can reduce phytoplankton blooms, and especially blooms of blue-green algae, there does not appear to be any reason that farmers would not accept them readily.

The two biological control measures under investigation (use of filter-feeding animals and use of natural algal pathogens) are particularly attractive because they avoid the use of chemical control measures. In the case of control measures using filter-feeding fish or clams, economic returns from harvest of the animals stocked for algae control may be an added benefit. Other improvements in water quality may also occur. For example, a state fish hatchery is participating in the large-scale evaluation of planktivorous fish with the hopes of addressing chronic problems with low dissolved oxygen levels.

Several chemical control measures are being investigated, including the use of copper sulfate and natural compounds such as plant phenolics. These studies should also lead to the development of one or more novel chemical treatments that can be used to control noxious phytoplankton blooms.

Additional studies focus on enhancing the elimination of MIB from channel catfish, so that off-flavor fish may be purged more effectively prior to processing. These approaches may be of significant economic value to the aquacultural industry. Also, improved methods of analysis for geosmin and MIB that are comparable or better than sensory methods with regards to sensitivity, and comparable or better than GC analysis in terms of objectivity will be developed. Immunoassay methods have these benefits and can also be formatted into rapid and simple test kits for industry. These methods will provide the industry with a better tool for quality control and fish grading as well as the research community with a better tool to study off-flavor development and control.

PROGRESS AND PRINCIPAL ACCOMPLISHMENTS

Objective 1A. *Evaluate methods of reducing phosphorus input by diet modification by determining the minimum phosphorus requirement for food-sized channel catfish and quantifying the reduction in waste phosphorus generation by food-sized catfish fed "low-phosphorus" feeds relative to presently available feeds.*

The minimum dietary available phosphorus requirement for food-size channel catfish fed commercial type diets was determined in a 7-month pond study. The available phosphorus requirement based on subclinical measurements (bone breaking strength and alkaline phosphatase activity) was found to be 0.3% of the diet, although the requirement for maximum growth was less than this. These data indicate that the available phosphorus requirement for commercial catfish feeds should be 0.3% of the diet, which is approximately 25% lower than the

present National Research Council recommendation.

The availability of different forms of phosphorus in practical feed ingredients was determined for channel catfish. A reference diet and test diets containing either menhaden fish meal, fish meal analog, meat and bone meal, soybean meal, cottonseed meal, corn, rice bran, wheat, and wheat middlings were fed to channel catfish after which fecal samples were collected and analyzed to determine the availability of different forms of phosphorus from the ingredients. The various feed ingredients varied considerably in terms of their phosphorus composition and availability to the fish. Wheat, sorghum, and cottonseed meal had the greatest phosphorus availability of the plant feedstuffs while fish meal analog had the greatest availability of the animal feedstuffs. Also in this study, the uptake and mineralization of different forms of phosphorus and nitrogen in feces from channel catfish fed the various ingredients were determined.

Digestibility trials were also conducted with channel catfish to evaluate several different feedstuffs which have been genetically modified to contain low concentrations of phytic acid. The phytic acid which is typically found in reasonably high concentrations in plant feedstuffs is indigestible and thus excreted by fish. The low-phytate varieties of barley, corn, and soybean meal which have been evaluated have elevated phosphorus availability to channel catfish. Thus, as these feedstuffs become more readily available, they may provide a dietary means of reducing phosphorus excretion by this species.

Another study was conducted to evaluate diet formulations and feeding strategies to marginally meet the phosphorus requirement of channel catfish while minimizing dietary phosphorus input. Fingerling channel catfish were fed one of three practical diets with either no phosphorus supplementation (approximately 0.2% available phosphorus), minimal phosphorus supplementation from dicalcium phosphate (0.3% available phosphorus), or standard phosphorus supplementation from dicalcium phosphate (0.5% available phosphorus) for 8 weeks in aquaria. Two additional treatments included feeding the diet containing 0.2% available phosphorus with intermittent feeding (two days every other week or every fourth week) of the diet containing 0.5% available phosphorus. Samples of pectoral spines and whole-body tissues indicated adequate phosphorus status of channel catfish could be maintained with minimal phosphorus input by feeding the unsupplemented diet in conjunction with the phosphorus-supplemented diet for two days every fourth week.

Results at a glance...

- ☆ *Low-phytate varieties of barley, corn and soybean meal elevate phosphorus availability to channel catfish so they may provide a dietary means of reducing phosphorus excretion as these feed stuffs become more readily available.*

A pond study was conducted to quantify the reduction in waste phosphorus generation by food-size channel catfish fed experimental diets formulated to contain 28% protein and 0.4% available phosphorus from either dicalcium phosphate (water-soluble) or defluorinated phosphate (water-insoluble) phosphate. No statistical differences were observed in weight gain, feed conversion, survival, bone ash, and bone phosphorus between fish fed the two diets. There were no significant differences in total phosphorus, soluble phosphorus, and chlorophyll *a*

concentrations in pond water between the two dietary treatments.

A pond study was conducted in Mississippi to quantify the reduction in waste phosphorus generation by food-sized channel catfish fed low phosphorus diets. A basel diet was formulated to contain 32% protein without supplemental phosphorus (0.2% available phosphorus). Supplemental phosphorus was added to the basal diet to provide available phosphorus of 0.3 and 0.4%, respectively, using dicalcium phosphate. There were no significant differences in total phosphorus, soluble phosphorus, and chlorophyll *a* concentrations in pond water between the two dietary treatments. However, in a similar study conducted in Alabama where phosphorus in catfish feed was increased from 0.6% (0.2% bioavailable) to 1.0% (0.6% bioavailable), there were significant increases in total phosphorus and phytoplankton production.

Objective 1B. *Evaluate methods of removing phosphorus from pond waters by studying methods of enhancing rates of phosphorus removal from pond waters by pond bottom soils and determining the feasibility of precipitating phosphorus from pond waters as sparingly soluble aluminum or calcium salts.*

In 1996, laboratory and pond studies were conducted to determine the feasibility of precipitating phosphorus from waters as sparingly soluble aluminum or calcium salts through the application of aluminum sulfate (alum), calcium oxide (lime), or calcium sulfate (gypsum). The gypsum application had the greatest effect, and the treatment was repeated in 1997 at a higher application rate. At the higher rate (increasing total hardness to 200 ppm), gypsum significantly reduced total phosphorus and chlorophyll *a* levels. More frequent applications of alum to pond water (1998) showed distinct short-term effects but little long-term change in the pond water quality. Repeated applications of 7 pounds/acre of agricultural limestone at 2-day intervals also reduced soluble phosphorus concentrations but had little effect on phytoplankton. A 120-day pond trial was completed which evaluated the effects of periodic additions of alum on the availability of phosphorus in the pond environment. Addition of alum at 50 ppm every other week did not affect total production or feed conversion of channel catfish in 0.1-acre ponds. In addition, waterborne phosphorus concentration, primary productivity, algal species composition and sediment oxygen demand generally were not affected by alum addition; however, there was an obvious reduction in the amount of filamentous algae and other vegetation in ponds treated with alum.

Results at a glance...

- *Of several treatments tried, the treatment that resulted in less phosphorus availability and phytoplankton abundance combined pond bottom drying and tilling with gypsum application to the pond.*

In 1997 and 1998, drying and tilling empty pond bottoms before filling the ponds resulted in lower phosphorus levels in the water during the production season. Incorporation of alum, agricultural limestone, or sodium nitrate in the tilled soil did not reduce phosphorus levels or improve water quality above drying and tilling alone.

In 1999, the treatments were as follows: control, dry tilling of pond bottoms, and dry tilling of pond bottoms followed by periodic gypsum applications to maintain 200 ppm total hardness. The 1999 study showed that the dry-till with gypsum treatment was superior to dry-till alone in lessening phosphorus availability and phytoplankton abundance.

The experiment to evaluate the benefits of water circulation on pond water quality was completed in October 2000. This study revealed no added benefit over dry-till with gypsum treatment of providing water movement with a 0.5-hp mechanical circulator. This experiment completed the trials to be conducted in this project.

During 2001, soil analyses and phytoplankton evaluations were completed on all samples from previous studies, and the findings of the research are being described in several manuscripts.

Objective 2A. *Evaluate the effect of filter-feeding fishes on water quality and reduction or elimination of off-flavor in pond-raised channel catfish.*

Six species of filter-feeding macroorganisms were identified as candidates for biological control and tested in mesocosms in 1997 for their ability to filter off-flavor algae. Nile tilapia, blue tilapia, and silver carp significantly reduced numbers of *Oscillatoria perornata* and *Anabaena circinalis* (major producers of MIB and geosmin, respectively). Threadfin shad significantly reduced numbers of *A. circinalis*. Two species of local, unionid clams did not produce measurable effects. Based on these findings, a silver carp system was evaluated in 0.25-acre ponds in 1998. Abundance of *O. perornata* was reduced, but the alga was not eliminated. In addition, difficulty was encountered in adapting a silver carp system to channel catfish production ponds.

Testing of seven filter-feeding organisms and at several densities found a system based on Nile tilapia stocked at 2000 fish/acre in cages provided control of spiked *O. perornata* in 0.1-acre experimental ponds in one month. In two 2.5-acre ponds at a state fish hatchery, this system again eliminated the alga or reduced its abundance to below problem in one month. Genetically male tilapia grew from 3 inch stockers to 0.25 pounds in the 3 month evaluation, which is 10% faster growth than mixed-sex Nile tilapia. Additional water quality improvements (other than improving fish flavor) were also observed. For example, the incidence of low oxygen levels was reduced by 60-70% in ponds with the filter fish system compared to a control pond without the system.

Objective 2B. *Develop microbial pathogens to control blue-green algal abundance.*

Fungal and bacterial pathogens of *Anabaena* and *Oscillatoria* were isolated from commercial catfish ponds. In laboratory studies these agents lysed cells of *Anabaena* and *Oscillatoria*, and selectively removed these species from mixed cultures containing beneficial algae and blue-green algae.

A fungal pathogen was selected for evaluation in replicated tests that were conducted in 210-gallon tanks to study control of *Oscillatoria*

perornata. The tanks were filled with water from a commercial catfish pond, stocked with catfish fingerlings, and treated with preparations of a fungus. The fungus controlled *O. perornata*, but high oxygen demands were observed. Preparations of the fungus are being developed that will minimize the oxygen demand.

A bacterium that is pathogenic to species of *Anabaena* and *Oscillatoria* was isolated from pond water. When comparisons were made using a number of databases, no definitive match for the DNA sequence of the 16S rRNA gene for the bacterium could be established at the genus or species level. Therefore, the bacterium could represent a genus that has not been described. When the bacterium was evaluated as a biological control agent in laboratory studies, the average reductions in chlorophyll *a* were 94 to 98% for *Oscillatoria* spp. and 13 to 98% for *Anabaena* spp. No significant reductions in chlorophyll *a* were noted for *Chlorella vulgaris*, *Scenedesmus subspicatus, Selenastrum capricornutum, Microcystis aeruginosa*, or *Plectonema boryanum*. Shake flask cultures of the bacterium produced approximately 3 billion plaque-forming units/milliliter of culture broth. Therefore, 1 liter of culture broth, when uniformly distributed in one acre-foot of pond water, would result in an initial concentration of approximately 2,400 plaque-forming units/milliliter of pond water. In replicated tests conducted in 210-gallon tanks containing water from commercial catfish ponds, the bacterium selectively removed species of *Anabaena* and *Oscillatoria*. When the bacterium was inoculated to pond water, *Oscillatoria perornata* was reduced from an initial density of 2,700 filaments/milliliter to 0 filaments/milliliter after 48 hours. The blue-green alga *Microcystis* became dominant as the species composition of the phytoplankton changed in the treated pond water. Results of laboratory and tank tests indicated that the bacterium did not adversely affect channel catfish fingerlings.

Results at a glance...

☆ *A bacterium isolated from pond water selectively attacks odor-producing blue-green algae while having no effect on beneficial algae or catfish. The bacterium shows promise as a biological control agent for the algae that cause off-flavors.*

The bacterium was tested in ponds. Analyses of pond water following inoculation indicated that the bacterium was present in the water for up to 5 days after inoculation. While the results of preliminary pond experiments were encouraging, future tests in ponds need to be conducted under more uniform environmental conditions to facilitate interpretation of results.

Research is underway to optimize production of the bacterium and to produce preparations that have a shelf life of over 1 year. Three patents or patent applications related to this technology have been assigned to the Louisiana Tech Research Foundation. Negotiations are underway with two corporations that have expressed interest in commercial development of the bacterium.

Objective 2C. *Determine whether plant phenolics (tannins) can control growth of microorganisms that produce odorous compounds in warmwater fish ponds.*

The bacterium *Streptomyces tendae* is known to synthesize geosmin, an earthy off-flavor contaminant of aquatic products. Experiments were conducted to determine the antimicrobial effects

of tannic acid and related compounds such as propyl gallate, methyl gallate, and gallic acid on the growth of *Streptomyces tendae*. Well-diffusion assays and biomass determinations were performed. The biomass determination method is more sensitive than the well-diffusion assay. The results of these experiments indicate that tannic acid is inhibitory to *S. tendae* at levels as low as 0.3 ppt. Propyl gallate is inhibitory at higher concentrations, but methyl gallate and gallic acid have no inhibitory effects at concentrations up to 1 ppt. Olfactory evidence suggests that tannic acid may inhibit geosmin synthesis.

It was also demonstrated that tannic acid and related compounds are inhibitory to the growth and pigment synthesis of off-flavor producing *Nostoc* sp. strain MAC. The minimum inhibitory concentrations of tannic acid, propyl gallate, and gallic acid in augmented pond water were 320, 240, and 500 micrograms/disk, respectively. Tannic acid, propyl gallate, and gallic acid also exhibited inhibitory activity to *Cytophaga columnaris*, a ubiquitous, gliding fish pathogen, at 150, 300, and 300 ppm. Methyl gallate was effective at 500 ppm. The protein precipitation and polysaccharide binding capacities, lipophilicity and other physico-chemical properties of these compounds were measured in order to understand possible mechanisms for their antibacterial action. Tannic acid, a polymeric compound with multiple hydroxyl groups, had at least a nine times greater capacity for binding protein and glycogen than the other test compounds. These results suggest that the hydroxyl group availability of tannic acid is essential for antibacterial activity. Therefore, it is likely that these compounds may have some beneficial effect in controlling the microbial population in ponds and may have impact on the phytoplankton biomass.

Objective 2D. *Evaluate the effect of routine, low-level treatments of ponds with copper sulfate on phytoplankton communities, off-flavor incidence, and water quality in channel catfish ponds.*

Eighteen, 0.4-ha earthen ponds in northwest Mississippi were used in a 3-year study to evaluate the effect of weekly copper sulfate applications on the incidence and economic impact of environment-derived off-flavors in channel catfish. Each spring when water temperatures increased above 70°F, nine of the ponds were treated weekly with 5 pounds/acre copper sulfate by placing the required amount of copper sulfate crystals in a burlap bag which was then placed in the current produced by a paddlewheel aerator. Copper treatments were discontinued each fall when water temperatures fell below 70°F. Overall prevalence of off-flavor was reduced by 80% for ponds treated with copper sulfate relative to control ponds, and episodes of off-flavor were of shorter duration in treated ponds. Off-flavors never delayed fish harvest from treated ponds, whereas off-flavors delayed fish harvest on ten occasions in control ponds. Average annual fish harvest was 5,250 pounds/acre from ponds treated with copper sulfate and 4,760 pounds/acre from control ponds. The 9%

Results at a glance...

- ☆ *Weekly, low-level treatments of catfish ponds with copper sulfate reduced the incidence of off-flavor by 80% and increased net revenues by over 40% compared to untreated ponds.*

reduction in fish harvest from control ponds was due to infectious disease outbreaks in one or two ponds each year where harvest was delayed due to off-flavor. Enterprise budgets showed that average net returns above variable costs were $770/acre for control ponds and $1,100/acre for ponds treated with copper sulfate. Variation in net returns was twice as great for control ponds as for treated ponds, indicating increased stability in production and economic returns when off-flavors were managed using copper sulfate. High variation in annual economic performance on control ponds resulted from one or more ponds having high net returns while one or more ponds had extremely poor returns due to protracted episodes of off-flavor. Stability in production and costs is a means of reducing risk and is a positive factor farmers can use to better plan their cash flow needs throughout the production season and in the longer term.

Objective 3. *Determine the feasibility of managing fish off-flavors by reducing rates of 2-methylisoborneol (MIB) uptake by fish and/or enhancing rates of MIB elimination from fish.*

Three compounds were initially identified as potential enhancers of MIB elimination based on their ability to increase the activity of cytochrome P450, the enzyme system thought to be involved in metabolizing the off-flavor compound, 2-methylisoborneol (MIB). One of the three compounds (3-methylcholanthrene) significantly increased the residence time of MIB in channel catfish. After initial success with clofibric acid, further investigation only showed a trend toward enhanced elimination. The last compound, ethanol, provided the best results observed in enhancing MIB elimination, but only following MIB exposure. Pretreatment with each chemical did not affect MIB uptake or elimination. Treatment with ethanol following MIB uptake nearly doubled the rate of MIB elimination. Although a direct correlation was observed between temperature and MIB elimination, temperature failed to have any synergistic effect on the enhanced elimination by any of the three compounds.

Analysis of MIB elimination in the Uvalde strain of channel catfish indicated tremendous variation in the response of fish and their ability to eliminate MIB. Variation between individuals is nearly 35%. The factors controlling the variation in response are unclear. No relationship has been observed between individual isoforms of cytochrome P450 and MIB elimination in this particular strain. Examination of MIB metabolism in Uvalde strain channel catfish with induced enzymes indicated that MIB is not metabolized.

To determine whether extrahepatic (tissues other than the liver) biotransformation of MIB may be occurring, the metabolism and disposition of radio-labeled MIB was examined in Uvalde channel catfish as well as another strain of channel catfish (USDA 103) and the channel catfish x blue catfish hybrid. No metabolites were observed in plasma from animals treated with an intra-arterial dose of radio-labeled MIB. Elimination of MIB from the two strains and hybrid was accurately predicted using a three compartment pharmacokinetic model. There was no significant difference in terminal half-lives between strains, but significant differences in other predicted pharmacokinetic parameters, such as total clearance, were observed with the hybrid strain, which had a 10-fold greater clearance.

Objective 4. *Develop statistical models describing the within-pond variation in the degree of off-flavor in fish populations under various conditions.*

A study was conducted in summer and fall in ten commercial catfish farms to determine the proportion of off-flavor fish in ponds. Flavor was assessed by sensory evaluation. A larger occurrence of off-flavor was found in summer than in fall. Proportion of off-flavor fish varied from 0% to 54% depending on the pond and the acceptance criterion used. Between-pond variance of the proportion of unacceptable fish was greater than within-pond variance. Therefore, Bayesian sampling should be used instead of conventional sampling based on the binomial function. Different sampling plans are proposed depending on levels chosen for producer and consumer risks. It was recommended to consider a sample size of at least 30 fish.

Objective 5. *Develop analytical techniques for assessing flavor qualities in fish.*

Monoclonal antibodies have been produced that bind to 2-methylisoborneol (MIB). This led to the development of immunochemical methods (ELISA) to detect MIB down to levels of 0.01 ppb, low enough to be comparable to the human sensory threshold for MIB. Zeolite was tested as a material for MIB absorption and concentration. Using ELISA, zeolite was shown to absorb small molecules (glycoalkaloids) but not antibodies. Zeolite, however, was shown to be less efficient for MIB absorption than activated carbons, when compared using a purge and trap apparatus.

An eight-member sensory panel was trained using the Sensory Spectrum Method. A preliminary study was conducted to evaluate the effectiveness of various processing procedures in reducing off-flavor in catfish. Fillets of each flavor rating were either dipped or vacuum tumbled in water, dairy whey or 3% lemon juice. The panel gave significantly higher scores for the geosmin note and lower scores for the chicken-like note for off-flavor level 5 compared to level 1, regardless of the treatment. Lemon juice significantly increased the geosmin note, whereas dairy whey reduced it. Vacuum tumbling with lemon juice reduced the green/corn note (considered a desirable note) compared to dipping in lemon juice.

Objective 6. *Develop publications to educate producers and processors on the ecology of environmentally-derived off-flavors, off-flavor management, and the results of this project.*

See list of publications on pages 25-28.

WORK PLANNED

Work on all objectives is proceeding on schedule and no changes in the project have occurred this year.

IMPACTS

Information generated in two of the project objectives is already being used in the aquaculture industry. First, work to investigate phosphorus availability of various feedstuffs has been used by feed manufacturers to refine commercial diet formulations, with a cost savings to the farmer. Second, the efficacy of routine, low-level copper sulfate treatments for preventing algae-related off-flavors has been verified in large-scale field studies and the practice has been adopted by many commercial producers in Mississippi and Arkansas.

Although other results of this project are too preliminary to have had an impact on the aquaculture industry, several of the treatments and practices being investigated show promise. For example, phosphorus levels in ponds can be reduced by precipitating phosphorus as aluminum or calcium salts, or by treating the pond bottom to reduce phosphorus flux from soils to water. These practices could be an important management procedure for improving quality of pond water and effluents and in combating off-flavor.

Another example of a potentially effective practice is the use of filter-feeding fishes, which has been shown to be effective in controlling odor-producing algae in small-scale systems and in pond trials. Use of fast-growing, genetically male tilapia may help avoid problems of uncontrolled reproduction encountered when mixed-sex tilapia are used in polyculture with catfish.

Perhaps the most intriguing result is the success achieved using bacterial pathogens of odor-producing blue-green algae. If these results can be transferred to pond-scale ecosystems, the work may lead to a novel, safe, and effective method of controlling flavor problems in fish.

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED

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