SEA GRANT PROJECT SUMMARY FORM (90-2)

TITLE: Development of an efficient approach to quantify and control a fish disease caused by *Saprolegnia* sp..

PRINCIPAL INVESTIGATOR: Vipaporn Phuntumart

AFFILIATION: Bowling Green State University

EFFORT: Two months

ASSOCIATE INVESTIGATOR: Christopher Good

AFFILIATION: Aquatic Veterinary Research, Freshwater Institute, National Headquarters (WV)

EFFORT: One month

ASSOCIATE INVESTIGATOR: David Straus

AFFILIATION: U.S. Dept. of Agriculture, Agricultural Research Service (AR)

EFFORT: One month

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PROJECT NUMBER:

SEA GRANT STRATEGIC PLAN CLASSIFICATION:

KEYWORDS: *Saprolegnia*, detection, quantification, peracetic acid, Recirculating Aquaculture Systems, LAMP, qPCR

OBJECTIVES: Aquaculture is considered to be the world's fastest growing source of food production and could account for almost 50 percent of the world's food fish (Food and Agriculture Organization of the United Nations Fisheries & Aquaculture). The aquaculture industry is utilizing an ever-increasing amount of recirculating aquaculture systems (RAS) to meet the demand of a growing fish market. While RAS offers the benefit of a controlled- and optimized rearing environment for site-selection, enhanced biosecurity and reducing water consumption, among other benefits, the risk of introducing and spreading opportunistic pathogens such as *Saprolegnia* spp. during unfavorable conditions could occur. Saprolegniasis is one of the major problems in RAS, especially in the Atlantic salmon, *Salmo salar* industry where it is estimated to cause 10% mortality.

The overall objective of the project is to develop an effective method to detect, quantify, identify and treat *Saprolegnia* spp. in the RAS. Specific aims are
Aim1) To develop molecular approaches to detect, identify and quantify *Saprolegnia* spp. from water samples.
Aim 2) To investigate the efficacy of peracetic acid (PAA) in reducing growth of *Saprolegnia* spp. in Atlantic salmon cultured in a RAS

METHODOLOGY:

Aim1) To develop molecular approaches to detect, identify and quantify Saprolegnia spp. *from water samples (this research will be conducted entirely by Dr. Phuntumart at Bowling Green State University [BGSU])*

Waterborne pathogens in the genus *Saprolegnia* presents challenges in aquaculture managements. This research is focused on developing sensitive, rapid, accurate and low cost methods to help fish hatcheries and farm managers to monitor pathogens in this genus in their culture systems. Our experiments in year one will involve 1) development of rapid, accurate and sensitive specific techniques to detect, identify and quantify this fish pathogen. We will adopt the recently developed molecular technology used to amplify DNA, Loop Mediated Isothermal Amplification (LAMP) to detect and quantify *Saprolegnia* spp. LAMP is a rapid and sensitive molecular techniques that provides results that can be seen by eye within 5 - 10 minutes or measured with an inexpensive spectrophotometer for quantification. The consumable cost for LAMP is approximately $2 per reaction and can be

done at a single temperature at 65°C in a single tube; farmers can apply this technique on site by using a heat block. This approach would enable for on-site detection and treatment of the pathogen. LAMP has been developed and successfully employed to detect soil borne plant pathogens, not only the oomycetes (*Pythium* and *Phytophthora*) but also some fungi in the genus *Fusarium* (Dai, et al. 2012, Fukuta, et al. 2013, Niessen, 2013). However, this approach has not been developed to be used with the water- borne pathogens due to the difficulties in designing a set of primer specific to *Saprolegnia* spp. This project will adopt the LAMP method to be used in aquaculture systems including fish ponds and RAS. In preparation for this project, we have already established these specific primers and a PCR program. In addition, we will also develop a quantitative PCR (qPCR) method for comparison to the LAMP method. qPCR, also known as real-time PCR is a technique that combines conventional PCR amplification and detection into a single tube. The results of qPCR can be evaluated without gel electrophoresis. The major advantages of qPCR include the measurements of PCR products in real time while conventional PCR measure the products at the end points. Thus qPCR enables the initial quantification of the DNA molecules in the samples.

In our previous experiments, we successfully developed PCR and LAMP protocols using the collection of isolates from the Phuntumart lab. To further establish these protocols, 1) we will initially quantify Saprolegnia spp. zoospores from water samples by counting them under a microscope via a hemocytometer. 2) Next, we will be applying the LAMP technique using the four or six sets of primers and the qPCR program that we developed to the samples containing zoospores. To standardize this step, the known number of zoospores will be added to sterilized water samples at different concentrations followed by LAMP and qPCR. Our approach is significant because we will directly detect the pathogens from water samples and bypass the DNA purification step. 3) Once the LAMP method is developed, we will apply these techniques to different water samples collected by the Co-I's from various fish ponds (Straus) and from RAS (Good). These water samples will be used to detect the presence of *Saprolegnia* spp. 4) In a separate experiment, we will spike each of the samples with a known amount of zoospores before subjecting them to LAMP and qPCR.

Aim 2) To investigate the efficacy of peracetic acid (PAA) in reducing growth of Saprolegnia sp. on Atlantic salmon cultured in the RAS (this experiment will be conducted by all three collaborators):

To accomplish Aim 2, we will start by determining the half-life of PAA in different water samples. The PAA degradation study will be carried-out using a commercial PAA test kit (CHEMetrics, Inc., Midland, VA). Initial data for this study was obtained using different water samples collected from local sources including 1) a ten gallon-fish tank containing a cichlid, 2) a small recirculating water system containing crayfish for research at BGSU, and 3) two ponds located within the BGSU campus; two types of sterilized waters were used as controls. Each of these water samples were treated with PAA to give a final concentration of 4 mg/L. The PAA concentrations were measured at 10 minute-interval for 60 minutes. As expected, PAA degradation occurred much faster in water that had live animals than in the controls. We will continue this study by collecting additional water samples from RAS (Good), fish ponds from Arkansas (Straus) and local creeks and rivers near BGSU (Phuntumart). PAA will be added to each sample to give final concentrations of 2, 4 and 6 mg/L and the degradation will be measured as above. This will allow the evaluation of the degradation of PAA in various environments.

As previously discussed, RAS has become an attractive system for industrial production of fish and is also used in some hatcheries. The ubiquitous presence of *Saprolegnia* spp. has been reported as one of the major problems in RAS. The susceptibility of the fish to Saprolegniasis is exacerbated by stress such as vaccination or, as below, smoltification. Peracetic acid (PAA) is an organic compound that has been recently registered by the EPA to use as a disinfectant in aquaculture. PAA's degradation products are acetic acid (vinegar) and hydrogen peroxide (which breaks down into oxygen and water) therefore it could represent a potential environmental friendly chemical for treating Saprolegniasis on fish. This research will be conducted in year two after completing *Aim 1*. Atlantic salmon will be procured as live eggs from an international commercial breeder, and be raised by Dr. Good at the Freshwater Institute (Shepherdsville, West Virginia). Dr. Good will start this study using Atlantic salmon parr (~40g). The study will consist of twelve replicated flow-through $0.5m^3$ circular tanks. A total of 200 fish per tank will be used. The fish will undergo a 6-week of photoperiod manipulation (12 hours-light, 12 hours-dark) to mimicking winter light conditions, followed by a return to constant lighting to induce smoltification.

During this period, the salmon will be vulnerable to opportunistic infections, primarily (as noted through experience) Saprolegniasis. Three tanks without PAA treatment will serve as controls. Treatments will be provided by daily bath treatments of either 0.2 mg/L or 0.5 mg/L PAA for a period of 3 weeks. Observation will include visible signs of Saprolegniasis (confirmed via wet-mount light microscopy showing the typical hyphae)) and daily mortalities. Survival curves for each treatment will be developed and compared statistically via Cox proportional hazards survival analysis. Twice weekly, a random sample of three fish per tank will be euthanized and gill and skin tissue will be collected for histopathology in order to assess fish health under the treatment conditions. Histopathology will be scored (0-5) based on extent and severity of lesions observed; data will be analyzed via ordered logit regression. At the end of the smoltification period, all remaining fish will be euthanized, and tanks disinfected.

For LAMP and qPCR analyses, water samples will be collected before and during PAA treatments (every three days during treatment until the end of the experiment). A 100 ml water sample from each tank will be filtered through a 47 mm diameter filter with 5-micron pore size, frozen at -18°C and sent to the Phuntumart lab for testing by LAMP and qPCR methods. Concurrently, a 100 ml water sample from each tank will be filtered through a 90 mm diameter filter with 5-micron pore size, the filter will then be inverted on a culture plate containing Yeast Peptone Sucrose (YPS) media to transfer *Saprolegnia* spp. spores and the filter paper will be removed; the plates will be chilled and shipped overnight to the Straus lab where they will incubate at room temperature (21°C-23°C) for two days and colony growth will be recorded. Total colony counts will be compared between samples. Statistical Analysis will be performed using JMP 7.0 (SAS Institute, Inc, Cary, NC) software. Several colonies from each plate will be isolated and subject to PCR using ITS primers for identification of the isolates.

RATIONALE: This project addresses aquaculture and seeks to address barriers or hurdles impeding the advancement of aquaculture initiatives, one of the Ohio Sea Grant's main priorities in the aquaculture area. *Saprolegnia* spp. are ubiquitous in the freshwater environment and are associated with significant losses in the global aquaculture industry, including here in Ohio. This will be the first study to develop the rapid LAMP method to quantify *Saprolegnia* spp. from water samples, and to examine the efficacy of PAA, an environmental friendly agent to prevent Saprolegniasis in RAS. This research will use a combination of molecular tools, histopathology and traditional plate counting for the presence of the pathogen. This approach will enable development of a rapid and accurate method to detect and quantify *Saprolegnia* spp. Ultimately, this project will benefit the development of not only the rapid quantification of Saprolegniasis, but also other diseases, thereby enhancing the future of RAS management efforts. The techniques developed will also have important implications for other oomycete pathogens. Additionally, it can be used to assist in improving the economic viability of RASs to promote fish population health, and to minimize losses to disease without an impact on RAS biofilter performance. Our outreach program includes the participation in BGSU's Kids Tech University and recruitment of the minority undergraduates to participate in this research through BGSU's AIMS (Academic Investment in Mathematics and Science) Program and Center for Undergraduate Research. Results of this work will be disseminated through presentations at international, national and regional conferences.