Dissertation Summary

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学 位 論 文 題 目

Title of dissertation

Studies on the Control of Water Mold Infection in Fish Eggs Using Glutaraldehyde

（グルタルアルデヒドを用いた魚卵におけるミズカビ病防除に関する研究）

Introduction

Salmonid aquaculture and stock enhancement are critical components of global food security and economic development, with salmon and trout consistently ranking among the highest-value commodities in international trade. However, the sustainable large-scale production of these species is continually challenged by water mold diseases caused by oomycetes, particularly *Saprolegniaceae* (*Saprolegnia, Aphanomyces, Achlya*) and *Pythiaceae* (*Pythium*). These pathogens are characterized by rapid reproduction and the formation of cotton-like hyphae that can rapidly spread across incubating eggs and cause substantial losses. Outbreaks are a persistent issue in hatcheries across major producing nations, including Japan, Norway, and Canada, making the development of effective management strategies indispensable to the industry's stability. Current control measures face significant regulatory and operational challenges. Malachite Green was banned globally due to carcinogenicity, while formalin is increasingly restricted for similar health risks. Although bronopol serves as an alternative, it suffers from unstable supply chains.

Glutaraldehyde (GA), a broad-spectrum dialdehyde widely used in medical and industrial disinfection, presents a potential alternative. Unlike conventional treatments, GA is not commonly classified as carcinogenic under major regulatory frameworks and acts by cross-linking microbial proteins, demonstrating potent biocidal activity against bacteria, fungi, and viruses. Early studies suggest GA can inhibit Saprolegnia growth without harming fish eggs, indicating a comparatively improved safety profile relative to historical treatments. Despite its potential, the environmental toxicity of GA residues in hatchery effluent remains a barrier to widespread adoption. To address this, current research highlights the necessity of developing practical neutralization strategies, specifically using cost-effective food-grade additives like sulfites, to inactivate residual GA and reduce toxicity prior to environmental discharge, thereby ensuring biosecurity without compromising ecosystem safety.

This dissertation addresses these challenges by systematically evaluating GA as a control strategy for oomycete infections. Chapter I assessed the in vitro efficacy of GA against major water mold pathogens in comparison with conventional treatments (malachite green, formalin, and bronopol). Chapter II evaluated its effectiveness in preventing infections in incubating salmonid eggs under laboratory conditions and investigated methods for neutralizing residual GA. Finally, Chapter III validated the efficacy and toxicity of GA in both a commercial salmonid hatchery and in ayu (Plecoglossus altivelis) eggs under laboratory conditions. Collectively, this research aims to establish GA as a practical and environmentally responsible disinfectant for hatchery management.

Chapter1: Fungicidal Activity of Glutaraldehyde against Causative Agents of Saprolegniasis in Fish Eggs: *Saprolegnia parasitica*, *S. diclina*, and *S. hypogyna* Strains

Saprolegniasis remains a critical cause of mass mortality in aquaculture, a challenge exacerbated by the global ban on malachite green and the operational limitations of remaining treatments like formalin and bronopol. Addressing the urgent need for effective, non-carcinogenic alternatives, this study evaluates the potential GA, a broad-spectrum dialdehyde disinfectant. Specifically, we investigated the *in vitro* efficacy of GA against seven pathogenic *Saprolegnia* strains under varying exposure durations and temperatures. By benchmarking GA against historical and conventional fungicides, this research aims to validate GA as a high-potency, viable therapeutic for controlling water mold diseases in aquaculture.

Methods

Methodologically, seven pathogenic strains of *Saprolegnia* (*S. parasitica, S. diclina,* and *S. hypogyna*) were subjected to *in vitro* susceptibility assays. To determine the Minimum Fungicidal Concentration (MFC), fungal hyphae were exposed to graded concentrations of GA, traditional disinfectants (malachite green, formalin, bronopol), and five structurally related aldehydes. Experiments assessed efficacy under varying exposure durations (15, 30, 60 min) and temperatures (10–20°C) to replicate realistic hatchery conditions and elucidate the influence of chemical structure on fungicidal activity.

Results and Discussion

To validate GA as a practical alternative for controlling saprolegniasis in cold-water hatcheries (10–20°C), this study assessed its efficacy against seven *Saprolegnia* strains relative to standard therapeutants and structurally related aldehydes. While malachite green and formalin demonstrated high potency, a single 30-minute exposure to bronopol proved insufficient at commercial doses, suggesting a need for repeated applications. In contrast, GA exhibited robust broad-spectrum fungicidal activity against *S. parasitica*, *S. diclina*, and *S. hypogyna*, achieving complete inhibition with Minimum Fungicidal Concentrations (MFCs) of 53–212 mg/L within 30 minutes. Importantly, these effective concentrations fall within the established safety margins for salmonid eggs, mitigating toxicity concerns. Furthermore, a comparative structural analysis indicated that the superior potency of GA and formaldehyde over five other aldehyde derivatives is likely attributable to their smaller molecular side chains, which facilitate enhanced fungicidal action. Collectively, these findings establish GA as a high-potency, operationally feasible candidate for replacing restricted chemicals in aquaculture biosecurity.

Chapter 2: Efficacy of Glutaraldehyde Against Oomycete Infections in Salmonid Eggs and Post-Application Neutralization Strategies

Building on the potent *in vitro* fungicidal activity of GA established in Chapter 1, this study advances to hatchery-scale validation in salmonids. We evaluated the prophylactic efficacy of GA against water mold infections in rainbow trout (*Oncorhynchus mykiss*) and chum salmon (*O. keta*) eggs, benchmarking it against traditional chemical reagents. Furthermore, to address the critical need for environmental safety (Leung, 2001), we investigated post-treatment neutralization protocols and rigorously assessed the potential toxicity of neutralized residues to early developmental stages.

Methods

To evaluate the practical application of GA in aquaculture, *in vivo* efficacy trials were conducted on fertilized rainbow trout and chum salmon eggs. Specimens were treated twice weekly with GA, malachite green, formaldehyde, or bronopol to assess infection control and embryo survival. GA was quantified analytically by HPLC after DNPH derivatization. Concurrently, a neutralization protocol was developed by screening various food-grade additives, optimizing sodium bisulfite for temperature and time-dependent efficacy. Finally, to ensure environmental safety, the acute toxicity of sodium bisulfite was assessed on chum salmon eyed eggs, larvae, and fry following OECD guidelines, with statistical significance determined via ANOVA and Probit analysis.

Result and discussion

This study validated GA as a potent alternative to conventional antimycotics for salmonid aquaculture, demonstrating that treatments of 100 mg/L (biweekly) or 50 mg/L (alternate days) effectively controlled oomycete infections in rainbow trout and chum salmon eggs with efficacy comparable to malachite green and bronopol. To mitigate environmental risks, sodium bisulfite was identified as an effective neutralizer, rapidly inactivating 106 mg/L GA (5 min) under the tested conditions. Notably, a 15.11-fold molar excess relative to GA (1,700 mg/L) was required for complete neutralization under acidic conditions, which is higher than ratios reported in alkaline environments due to pH-dependent chemical speciation. Toxicity assessments revealed that while eyed eggs exhibit high tolerance to sodium bisulfite—permitting direct in-tank neutralization—larvae and fry are sensitive, necessitating effluent dilution prior to downstream discharge. Collectively, these findings establish a comprehensive protocol combining effective GA disinfection with practical chemical neutralization.

Chapter 3: Use of Glutaraldehyde to Manage Oomycete Infections in Rainbow Trout (*Oncorhynchus mykiss*) Eggs under Hatchery Conditions and Preliminary Laboratory Assessment on Ayu (*Plecoglossus altivelis*) Eggs

While Chapter 2 confirmed the laboratory-scale efficacy and safety of GA for salmonids, extrapolating these results to commercial hatcheries is complicated by operational variables such as continuous flow dynamics and high egg densities, which can alter chemical performance compared to controlled environments. To bridge this gap, this chapter validates the practical efficacy of GA in a commercial rainbow trout facility. Furthermore, given that traditional substrate-based incubation in ayu hatcheries creates static, high-density conditions prone to rapid fungal proliferation, this study extends the investigation to evaluate GA's antifungal efficacy and acute toxicity in ayu eggs under laboratory conditions, aiming to broaden the potential scope of this alternative disinfectant.

Methods

To evaluate the efficacy and toxicity of GA against water mold, experiments were conducted on rainbow trout eggs at the Fuji Trout Hatchery (11.5°C, flow-through system) and ayu eggs at the Fisheries Experiment Station (17°C, static glass slide system). Commercial bronopol was used as a comparative benchmark. Efficacy trials involved periodic exposure of trout (starting 3 days post-fertilization (dpf)) and ayu (starting 1 dpf) to varying GA concentrations (25–500 mg/L) and durations, monitoring infection rates, survival, and hatching success. Concurrent toxicity assessments exposed trout eggs to high-intensity GA regimens (up to 1,000 mg/L) and monitored ayu mortality under efficacy conditions. Statistical significance was determined using one-way ANOVA followed by Tukey's (efficacy) or Dunnett's (toxicity) post hoc tests.

Result and discussion

This study validated the field efficacy of GA as a practical alternative to bronopol for rainbow trout hatcheries, while identifying critical species-specific limitations for ayu. In commercial rainbow trout settings, intermittent GA treatments (50–100 mg/L) effectively suppressed water mold infections with minimal toxicity, establishing a regimen of 100 mg/L for 30 minutes every two days as the optimal protocol for high-density, flow-through systems. Toxicity assessments defined a clear safety window for trout, with severe mortality occurring only at concentrations ≥300 mg/L. In sharp contrast, GA proved unsuitable for ayu eggs, in which effective antifungal doses invariably resulted in complete embryonic mortality. This hypersensitivity is attributed to the unique ultrastructure of the ayu egg envelope, specifically its thin chorion and microporous adhesive membrane, which likely facilitates excessive chemical uptake compared to the robust chorion of trout eggs. Consequently, while GA is a safe and effective solution for salmonids, it is not suitable for ayu culture under the tested conditions.