FATE OF WATER BORNE THERAPEUTIC AGENTS AND ASSOCIATED EFFECTS ON NITRIFYING BIOFILTERS IN RECIRCULATING AQUACULTURE SYSTEMS

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FATE OF WATER BORNE THERAPEUTIC AGENTS AND ASSOCIATED EFFECTS ON NITRIFYING BIOFILTERS IN RECIRCULATING AQUACULTURE SYSTEMS

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1. PREFACE

This dissertation is submitted in partial fulfillment of the requirements for obtaining a degree of Doctor of Philosophy (Ph.D). The thesis has an introductory review and five papers. The studies were carried out at the Section of Aquaculture in Hirtshals, DTU-Aqua (formerly Danish Institute of Fisheries Research) and at the Section of Biotechnology, University of Aalborg. Part of the research was supported by the European Union, through the Financial Instrument for Fisheries Guidance and the Directorate for Food, Fisheries and Agri Business, Denmark, and was supervised by Per Halkjær Nielsen (AAU) and Per Bovbjerg Pedersen (DTU-Aqua).

I appreciate the privilege of having had the two inspiring supervisors – Per & Per – profound, enthusiastic and renowned in their respective fields. Thanks for the valuable ideas, comments and support during the process. Thanks to Jeppe L. Nielsen (AAU) for additional supervision, collaboration and support in the planning and analytical phases, to Artur T. Mielczarek for help and introduction to the FISH analysis and microscopy and to Marianne and Susanne for help in the AAU lab.

I would like to acknowledge my great colleagues in Hirtshals. A particular thanks to Ulla Sproegel for arriving just when the lab-facilities expanded. Thanks to Dorthe Frandsen for lab work assistance, Erik Poulsen, Ole M. Larsen, Rasmus F. Nielsen, for help and hints and great caretaking of fish and rearing facilities. And thanks to Alfred Jokumsen for being helpful and supportive from day one.

From outside the section of Aquaculture in Hirtshals, I thank Niels Henrik Henriksen, Villy Larsen and Peder Nielsen for also having shaped my conception of aquaculture; to Ole Sortkjær for interesting collaboration, nice company and comments to the thesis.

I also thank Julia L. Overton, Damian Moran, Jim Fish and Chris Good for comments and improvements to earlier manuscripts. Thanks to Marcel Noteboom for dropping by for a prolonged period of time, and to Martin Møller and Erik Arvin for good collaboration.

Exactly 20 years ago as I write this, I was finishing the final year in high school next to fishing and working at the local fish farm. I owe to thank my first aquaculture mentor Niels Raabjerg, Bisgaard for sharing his knowledge and practical experience with me, and thanks to my old friends and family for supporting my life in the vicinity of water.

Finally, thanks to my wife Julie for her love and understanding and to our two girls Laura Kamma and Frida Petrea for putting things in perspective.

CONTENTS

2. ENGLISH ABSTRACT

Recent discharge restrictions on antibiotics and chemotherapeutant residuals used in aquaculture have several implications to the aquaculture industry. Better management practices have to be adopted, and documentation and further knowledge of the chemical fate is required for proper administration and to support the ongoing development of a sustainable aquaculture industry.

A focal point of this thesis concerns formaldehyde (FA), a commonly used chemical additive with versatile aquaculture applications. FA is safe for use with fish and has a high treatment efficiency against fungal and parasite infections; however, current treatment practices have proven difficult to comply with existing discharge regulations. Hydrogen peroxide (HP) and peracetic acid (PAA) are potential candidates to replace FA, as they have similar antimicrobial effects and are more easily degradable than FA, but empirical aquaculture experience is limited.

The two main objectives of this Ph.D. project were to 1) investigate the fate of FA in nitrifying aquaculture biofilters, focusing on factors influencing degradation rates, and 2) investigate the fate of HP and PAA in nitrifying aquaculture biofilters and evaluate the effects of these agents on biofilter nitrification performance.

All experiments were conducted through addition of chemical additives to closed pilot scale recirculating aquaculture systems (RAS) with fixed media submerged biofilters under controlled operating conditions with rainbow trout (*Oncorhynchus mykiss*) in a factorial design with true replicates. Biofilter nitrification performances were evaluated by changes in chemical processes, and nitrifying populations were identified by fluorescence *in situ* hybridisation (FISH) analysis.

FA was degraded at a constant rate immediately after addition, and found to positively correlate to temperature, available biofilter surface-area, and the frequency of FA-exposure. Prolonged biofilter exposure to FA did not negatively affect nitrification, and could therefore be a method to optimize FA treatment in RAS and reduce FA discharge.
HP degradation was rapid and could be described as a concentration-dependent exponential decay. HP was found to be enzymatically eliminated by microorganisms, with degradation rates correlated to organic matter content and microbial abundance. Nitrification performance was not affected by HP when applied in dosages less than 30 mg/L, whereas prolonged multiple HP dosages at 10 mg/L were found to inhibit nitrite oxidation in systems with low organic loading.

PAA decay was found to be concentration-dependent. It had a considerable negative effect on nitrite oxidation over a prolonged period of time when applied at a dosage ≥2 mg/L. PAA and HP decay patterns were significantly affected by water quality parameters, i.e. at low organic matter content HP degradation was impeded due to microbial inhibition. FISH analysis on biofilm samples from two different types of RAS showed that *Nitrosomonas oligotropha* was the dominant ammonia oxidizing bacteria, whereas abundant nitrite oxidizing bacteria consisted of *Nitrospira* spp.

In conclusion, measures to reduce FA have been documented, and investigations of HP and PAA have reflected a relatively narrow safety margin when applied to biofilters.

3. DANSK RESUME

De nuværende vandkvalitetskriterier for dambrugs medicin og hjælpestoffer påvirker akvakultur industrien i betydelig grad. For at sikre en bæredygtig videre udvikling for erhvervet er der behov for øget dokumentation og kendskab til hjælpestoffernes omsætnings-forløb - dels med administrativt sigte og dels med henblik på forbedret driftspraksis.

Et centralt emne for denne afhandling er stoffet formaldehyd (F) som anvendes i betydelig udstrækning i akvakultur øjemed. F bekæmper effektivt svampe- og parasit infektion uden at påvirke fiskene under behandlingen, men denne praksis har vist sig at kunne medføre forhøjede udledningsværdier af formaldehyd til vandløb. Brintoverilte (B) og pereddikesyre (PS) er hjælpestoffer der potentielt kan erstatte F, da de begge har ønskede antimikrobielle egenskaber og nedbrydes relativt hurtigt. Brugen af disse stoffer er imidlertid beskeden i akvakultur sammenhæng og dermed er der et begrænset, praktisk erfaringsgrundlag.

Ph.D projektet har haft to hovedformål, dels 1) at undersøge omsætningen af F i akvakultur biofiltre og fastlægge nogle af de faktorer der påvirker nedbrydningshastigheden og dels 2) at undersøge henfaldsforløbet af B og PS i tilsvarende biofiltre og vurdere i hvilket omfang doseringen af disse påvirker filtrenes nitrifikationsevne.

Forsøgene er udført med tilsætning af hjælpestoffer til lukkede, fuldt recirkulerede pilot anlæg med dykkede fastnet biofiltre under en række kontrollerede forsøgsbetingelser. Forsøgene blev afviklet med regnbueørreder med veldefineret indfodring i enkeltfaktor forsøgsdesign og med brug af replikationer. Biofilter nitrifikationen blev vurderet ud fra vandkemiske ændringer, mens biofiltrets nitrifikanter blev belyst ved hjælp af fluorescence *in situ* hybridisation (FISH) analyser.

F blev omsat med en konstant hastighed lige efter tilsætning og var positiv korreleret med temperatur, biofilter overflade og hyppigheden af F tilsætninger. Længerevarende F opretholdelse i biofiltre påvirker ikke nitrifikationen, og biofiltre kan derved tænkes at indgå som et middel til at optimere vandbehandlinger og derved reducere F udledninger.

B nedbrydningen forløb eksponentielt ved en høj hastighed og afhang af doseringsmængden. B blev nedbrudt enzymatisk af mikroorganismer svarende til mængden af organisk materiale og den mikrobielle forekomst. Biofiltrets nitrifikationsevne blev ikke hæmmet som følge af B tilsætninger op til 30 mg/l, men forsøg med gentagen B dosering og opretholdelse af koncentrationer på 10 mg/l, viste sig i anlæg med lav forekomst af organisk materiale at påvirke nitrifikationen.

PS omsætningen var koncentrationsafhængig, og medførte langvarig hæmning af nitrit oxidationen ved dosering $\geq$ 2 mg/l PS. PS og B's omsætningsforløb var påvirket af vandkvaliteten, hvor det blev vist, at HP omsætningen aftog på grund af PS forårsaget mikrobiel hæmning. FISH analyser af biofilmprøver fra to forskellige typer recirkulations anlæg viste, at de dominerende ammonium oxiderende bakterier var *Nitrosomonas oligotropha,* mens de nitrite oxiderende bakterier bestod af *Nitrospira* spp.

Det kan uddrages, at metoder til nedbringelse af F er blevet dokumenteret, ligesom undersøgelserne med B og PS har dokumenteret omsætningsrater og vist, at sikkerheds-marginen for anvendelse af disse stoffer i anlæg med biofiltre er forholdsvis lille.

4. INTRODUCTION

As in all animal producing industries, antibiotics and chemical additives are commonly used in commercial fish farming, particularly to treat disease outbreaks and to control fungal and parasitic infections. Antibiotics are approved drugs with antibacterial effects requiring prescriptions by a veterinarian, and administered to the fish via the feed. Chemical additives can be used without a prescription, and are applied to the water phase to improve rearing conditions (e.g. to control ectoparasite outbreaks).

BACKGROUND

Formalin is a commonly applied chemical additive in aquaculture. The active agent in formalin solutions, formaldehyde, has a beneficial toxicological profile which allows effective pathogen control when added directly to the water without affecting the fish negatively during treatment. This water treatment practice has been adopted for several decades to control fungal and ectoparasite infections (Fish, 1932; Heinecke & Buchmann, 2009) but has recently been questioned due to the potential environmental consequences of discharging excessive formaldehyde (Masters, 2004).

Environmental Protection Agencies have tightened operation conditions by issuing severe drug-specific discharge thresholds (water quality criteria), thereby challenging current treatment practices. Different strategies can be pursued in order to adopt better management practices and hence reduce formaldehyde discharge (Fig. 1).

From an environmental perspective, the primary concern regards residual drug concentration in the effluent, as opposed to the amount of chemical added. In other words, a continuation of formalin application in aquaculture facilities requires documentation of either effective neutralization or adequate removal of formaldehyde in the effluent. There is limited information on the fate of formaldehyde and other aquaculture therapeutants in operating aquaculture systems, both in terms of the orders of magnitude of removal and in terms of factors determining the degradation rate.

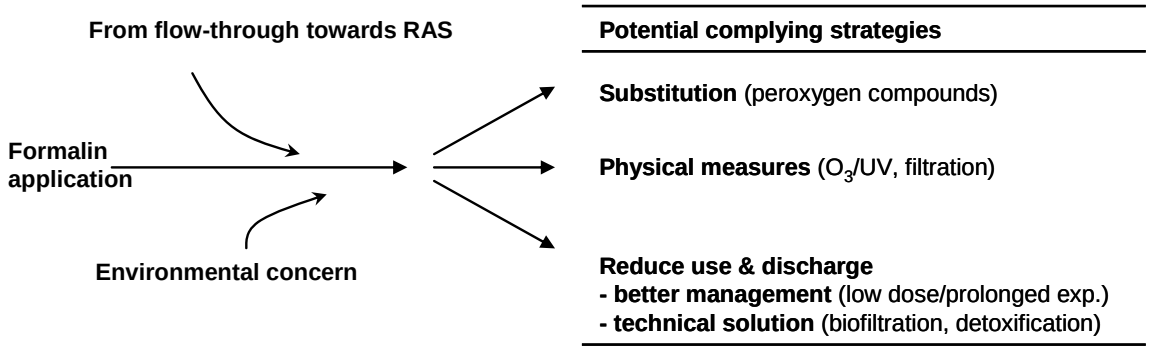

Fig. 1. A diagram illustrating the two main factors influencing formaldehyde application, and potential measures to comply with regulations. Biofilters are central treatment units in recirculating aquaculture systems (RAS), where water is recycled as opposed to traditional flow-through systems.

Therefore, there is a need to investigate and quantify the removal or degradation of formaldehyde, especially in biofilters as an essential component in RAS.

Investigations on alternative chemical additives to replace formalin also require studies of degradation kinetics in biofilters, but additionally require focus on the potential impact on the nitrification process. Peroxygens (i.e. hydrogen peroxide and peracetic acid) are considered potential aquaculture candidates as they have antimicrobial capabilities and degrade relatively quickly without producing toxic by-products.

AIM

Two main objectives have been pursued in the work presented in this thesis:

1. To investigate the fate of *formaldehyde* in biofilters, with specific focus on factors influencing degradation rates, and its effects on biofilter performance

2. To investigate the fate of *peroxygen compounds* in biofilters, with focus on factors influencing degradation rates, and their effects on nitrification performance

The experiments have been conducted in lab- and pilot-scale RAS under operating conditions with rainbow trout (*Oncorhynchus mykiss*) to mimic Danish aquaculture conditions. The experiments relied on true replicates and controlled factorial designs (Colt et al, 2006), and all experiments were conducted in fixed, submerged biofilters fitted with Bioblok® media, as this is the predominant type of filter material used in Danish RAS.

Nitrifying populations were identified by culture-independent molecular methods (fluorescent in situ hybridisation (FISH) and available gene probes). An additional aim was to develop methodologies and protocols to enhance experimental design and allow disinfectant experimentation with biofilter units from operating systems.

SCOPE OF THESIS

The research has basically been divided into three parts, with each section focusing on a specific chemotherapeutant: formaldehyde (FA), hydrogenperoxide (HP) and perecetic acid (PAA).

The first section deals with the decomposition of formaldehyde (FA) in two types of biofilters (PAPER I). Formaldehyde was applied to a by-passed full-scale biofilter at temperatures from 5 to 15°C, and experiments with reduced biofilter media volume were performed. Formaldehyde removal in six identical, independent pilot-scale RAS was also evaluated to assess surface-specific formaldehyde removal in different types of biofiltration systems. In the six pilot-scale RAS, effects of low dose and repetitive formaldehyde application was investigated, as well as nitrification performance and the screening and quantification of nitrifying populations (PAPER II).

The second section concerns the decomposition of peracetic acid and hydrogen peroxide, which was investigated in batch experiments and in 12 pilot- scale biofilter systems

(PAPER III). Effects on biofilter nitrification were assessed by spiking experiments, and ammonia- and nitrite-oxidizing bacteria were screened using FISH. Additional experiments examining PAA decay at various stocking densities, toxicological studies, and experiments with biofilter units were also carried out.

The third section describes different experiments with hydrogen peroxide. Sodium percarbonate (a hydrogen peroxide releasing product) was applied at different dosages to pilot-scale systems with two levels of organic loading, and decomposition and resulting water parameters were determined (PAPER IV). Additional multiple sodium percarbonate additions were made in pilot-scale systems, as well as temperature experiments.
Kinetic studies of HP degradation were also performed in two types of water (batch experiment), and multiple dosages of HP were administered to biofilter units and in a pilot-scale RAS, and nitrifying performance was evaluated (PAPER V).

This thesis is based on the five papers listed below, and an introductory review. The review includes an introduction to the issue of chemotherapeutant application in aquaculture, and related aspects of fish health management and biofiltration in aquaculture. Related studies, literature, and selected results concerning formaldehyde, peracetic acid and hydrogen peroxygen aquaculture application are presented in separate chapters, and specifically reviewed with regard to the decomposition of these agents in aquaculture biofilters. An environmental context is also presented, as well as a concluding section with potential ideas for future work.

5. LIST OF PAPERS

I. **Pedersen, L.-F**., Pedersen, P.B. & Sortkjær, O. 2007. Temperature-dependent and surface specific formaldehyde degradation in submerged biofilters. *Aquacultural Engineering* Vol. 36 pp 127-136.

II: **Pedersen, L.-F.,** Pedersen, P.B. Nielsen, J.L. & Nielsen, P.H. *In Press*. Long term/low dose formalin exposure to small-scale recirculated aquaculture systems. *Aquacultural Engineering (2009) doi:10.1016/aquaeng.2009.08.002.*

III: **Pedersen, L.-F.,** Pedersen, P.B. Nielsen, J.L. & Nielsen, P.H. 2009. Peracetic acid degradation and effects on nitrification in recirculating aquaculture systems. *Aquaculture, Vol. 296: 246-254.*

IV: **Pedersen, L.-F.,** Pedersen, P.B. & O. Sortkjær. 2006. Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale biofilters. *Aquacultural Engineering* Vol. 34(1): 8-15.

V: Møller, M.S., Arvin, E. & **Pedersen, L.-F**. *In Press*. Degradation and effect of hydrogen peroxide in small-scale recirculation aquaculture system biofilters. *Aquaculture Research (2009) doi: 10.1111/j.1365-2109.2009.02394.x*

6. ABBREVIATION

AOA	Ammonia oxidizing *Archaea*
AOB	Ammonia oxidizing bacteria
BOD	Biological oxygen demand
COD	Chemical oxygen demand
DBP	Disinfection by-products
EPA	Environmental protections agency
EPS	Exo-polymeric substances
FA	Formaldehyde
FD	Formaldehyde dehydrogenase
FCR	Feed conversion ratio
HP	Hydrogen peroxide
NOB	Nitrite oxidizing bacteria
NOEC	No observable effect concentration
PA+	Peraqua Plus, a commercial product
PAA	Peracetic acid
RAS	Recirculating aquaculture system
ROS	Reactive oxygen species
SGR	Specific growth rate
SPC	Sodium percarbonate
SSRr	Surface specific removal rate
TAN	Total ammonia-ammonium nitrogen
TGD	Technical guidance document
WFR	Water frame directive
WQC	Water quality criteria

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7. FATE OF FORMALDEHYDE, HYDROGEN PEROXIDE AND PERACETIC ACID AND ASSOCIATED EFFECTS ON NITRIFYING BIOFILTERS IN RECIRCULATING AQUACULTURE SYSTEMS

7.1. Introduction to current aquaculture issues

Aquaculture is an obvious solution to support the increasing global demand for fish and shellfish. The trends in the world aquaculture production are clear; aquaculture continues to grow more rapidly than all other animal food-producing industries with an average rate of 6.9 percent per year since 1970 (FAO, 2009). Annual global aquaculture has tripled within the last 15 years (Sapkota et al., 2008), almost half (45-47%) of the world's food fish now come from aquaculture (Diana, 2009; Subashinge et al., 2009).

The increased production has different environmental consequences, which beside competition for space concerns increased pressure on natural fish stocks as feed ingredients (Naylor et al., 2000; Hasan et al., 2007), water source competition and reallocation (Grommen & Verstrate, 2002; Verdegem et al,, 2006), risk of escapee (Naylor et al,, 2005; Morris et al,, 2008), disease transfer (Krkosek et al,, 2006), obstruction towards migrating fish (Aarestrup & Koed, 2003) and increased nutrient (Iwama, 1991; Bergheim & Brinker, 2003; Boyd, 2003) and biocide (Burka et al,, 1997; Schmidt et al,, 2000; Masters, 2004; Woodward, 2005) load to the receiving water courses. The aquaculture sector have made significant developmental progress during the past two decades in order to improve fish feed composition (e.g. Brinker, 2007; Glencross et al., 2007) and reduce environmental impact by various management and technical solutions (Cripps & Bergheim, 2000; Piedrahieta, 2003; Sindilariu, 2007; Svendsen et al., 2008).

Being mindful of the economically costs and investments, recirculation technology (water reuse aquaculture system) seems to be the technical revelation compared to traditional flow-through systems which solves the majority of the above listed concerns for fish production (Tal et al., 2009). The motivation to retrofit an existing system or to build a new recirculation aquaculture system (RAS) partly depends on the regulatory

severity and the enforcement of it. In Europe, particularly the Netherlands and Denmark (www.danskakvakultur.dk) have long ago pioneered the development and fully implementation of RAS technology, foreseeing the need of a sustainable development and forced by national legislation and restrictions (Bergheim & Brinker, 2003).

In Denmark, RAS now make up all the eel production and about 30% of the landbased trout production. According to Danish Aquaculture Organization annual trout production will double to 80.000 metric tonnes in 2020 and RAS will make up more than 90%.
The transition from fish farming in traditional flow-through systems with earthern ponds to RAS has been accelerated in Denmark recently, due to a combination of regulatory necessity and prospects, after years of stagnation, to increase production capacity.

RAS rely on reduced water consumption and a high degree of water reuse where all important water parameters are maintained, controlled and adjusted optimal. The core components typically include pumps or airlifts, mechanical screen filters (solid removal) and biofilters (organic matter removal, N-removal/nitrification) (Timmons et al., 2002). Oxygen cones, trickling filters for oxygen aeration and CO_2 stripping, denitrification units, UV and ozone equipment, sludge cones or separators can also be found in RAS, as well as end of pipe treatment in terms of chemical phosphorus removal, sludge deposition, geotextiles (Sharrer et al., 200A) and constructed wetlands (Sindilariu et al., 2008).

Current issues regarding water treatment in RAS

Management of RAS differs from traditional fish farming by the dependency on biofiltration, meaning both fish and (nitrifying-) microorganisms have to be maintained (Michaud et al., 2006). In this regard, it is important to ensure stable and optimal conditions, as fluctuations and disturbances in water quality parameters can jeopardize biofilter functioning (Noble & Summerfield, 1996; Botton et al., 2006). RAS can support the growth of bacteria, parasites, fungi, viruses and algae among which pathogens can accumulate. As health is a fundamental issue of welfare (Ashley, 2007), preventive or

curative therapeutic treatments are often necessary to reduce the risk of infections and disease outbreaks (Burka et al., 1997).

Antiparasitic treatment involves addition of chemicals directly to the water phase – hence exposing the biofilter and its microorganisms to the toxic chemicals. A relatively low number of water borne therapeutics are considered to be used for general aquaculture purposes (see section 3), and that number is even smaller when it comes to water treatment in RAS due to concerns of biofilter collapse. In addition, national EPA's have recently implemented stringent water quality criteria on aquaculture chemicals, based on the European Water Frame Directive [TGD, 2003], and hence again narrowed the potential choices for water treatment compounds.

The theoretical scope for adopting better management practice regarding chemical use and discharge follows at least three lines. One possibility is biosecurity (see section 3), improved treatment practice with existing chemicals (Sortkjær et al., 2008a) is a second solution, or thirdly, replacement of existing chemicals with more environmental neutral compounds (Clay, 2008). Knowledge of the fate and effect of therapeutics on biofilters under controlled conditions are important for all three strategies, collectively leading to a set of safe guidelines and ensuring acceptable levels of therapeutic residuals in aquaculture discharge (Gaikowski et al., 2004).

This review considers application of three common disinfectants used in aquaculture, in particular their application in RAS. The intension has been to extract empirical work and current knowledge of three selected aquaculture disinfectants with regard to treatment efficiency, mechanisms of action, decay kinetics, effect on biofilter nitrification and environmental consequences in order to make progress towards better management practice.

7.2. Aquaculture biofiltration

The real and perceived environmental benefits are important factors in the increasing popularity of RAS (Piedrahita, 2003). Water consumption per produced biomass (R-ratio) can for examples be reduced from more than 1000 L to 50 L/kg fish which require additional techniques and investments to avoid accumulation of unwanted substances and maintain acceptable conditions (Colt, 2006). Intensive fish farming with high degree of water recycling therefore demands high standards on control of water quality such as organic matter and nitrogenous control (Eding et al., 2006).

The organic input, apart from a minor potential input from the intake water, is derived solely from the amount of fish feed added to the system.
Metabolized feed and excretion leads to organic and nitrogenous waste products. The N-waste, beside the undigested part (~10 % of intake) included in the faeces, is by far dominated by TAN excretion (~80 %) via the gills and a minor part excreted as urea (< 10 %) (Timmons et al.. 2002). An additional amount of other dissolved nitrogenous waste products also exist (Kajimura et al., 2004). According to Timmons et al, (2002), ammonia-N generation rate can be estimated as approximately 10 percent of the protein content in the feed (i.e. 44 g TAN is produced from 1 kg feed with a protein content of 44 %). The amount of TAN released vary according to feeding regime and feed conversion, size of fish and species reared as well as feed composition and ingredients used. For example, 1.0 kg fish feed (44 % protein) provide 1.25 kg fish, assuming a 0.8 feed conversion ratio. Total N in the feed administered is 70.4 g N and 34.4 g N ends up in the fish, based on 16 % N in protein and 2.75 % N in fish biomass (Wik et al., 2009; Svendsen et al., 2008.). The difference, setting the undigested part to 7 g, is hence 70.4-7-34.4 = 29 g, predominately excreted as TAN (equalling some 23 g TAN/kg feed). A similar approach estimates 42.9 g N/kg feed (some 29 g as TAN) at an increased feed conversion ratio of 1.0.

Biofiltration, in this context the microbial degradation of organic matter, TAN, and nitrite, is facilitated by biofilter units connected to the rearing facilities. Various types of nitrifying biofilters have been developed for RAS (Fig. 3) all to control and degrade

ammonia and nitrite (Malone & Pfeiffer, 2006; Gutierrez-Wing & Malone, 2006). Aquaculture biofilters ideally maximize available surface area in a confined space while still ensuring oxygen and substrate transfer to support optimal conditions for the beneficial nitrifying microorganisms. Fixed film biofilter are far the most applied type in salmonid RAS, though suspended growth (biofloc technology) recently have gained new focus to non-salmonid species (Avnimelech, 2006; Crab et al., 2007; Kuhn et al., 2008).

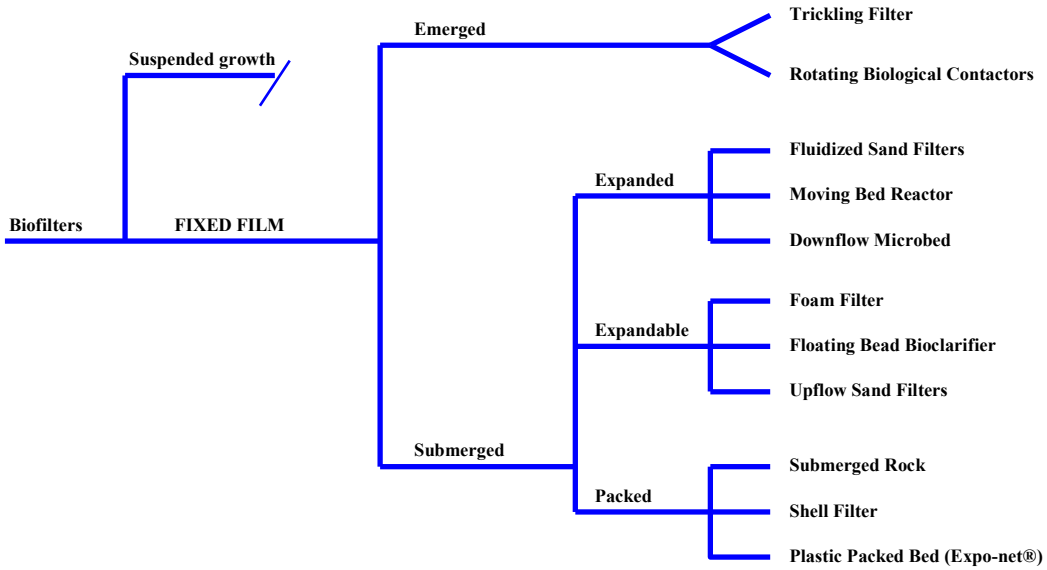


Fig. 1. Schematic representation of various types of nitrifying biofilters (modified after Malone & Pfeiffer, 2006).

Salmonids require water with relatively low levels of suspended solids, TAN and nitrite (*oligo- and mesotrophic systems*; Malone et al., 2006) and nitrifying bacteria in fixed biofilm systems generally ensure more stable water quality conditions compared to suspended growth or microbial flocs in suspension (Wik et al., 2009).

Fixed film biofilters can be either emerged (rotating disks or trickling filter) or submerged. Submerged filters have expanded media, i.e. fluidized sand or moving beds (e.g. Davidson et al., 2008; Suhr & Pedersen, submitted) or packed filter media, e.g. Bioblok or plastic beads (Fig.1; Malone & Pfeiffer, 2006). The dissolved substrates, such as ammonia are transported by diffusion from the bulk-phase into the biofilm leaving

hydraulics (especially in the boundary layer) and available surface area as shaping factors for the design and operation of biofilters. Media with a high surface:volume ratio have a large volumetric removal capacity and a low footprint, but often requires oxygen injection, increased maintenance and control than larger, more simple and robust biofilters (i.e. Wienbeck & Koops, 1990).

Beside the initial investment and operational costs in terms of potential expenses on oxygen transfer and biofilm management, robustness and stability are additional important factors to take into account when designing a biofilter. Fixed bed biofilters have been reported to convert 0.2-0.7 g TAN/m^2/day under aquaculture conditions (Janning et al., 2008; Suhr & Pedersen, submitted) but this figure is highly dependent on design, operation parameters, pH, temperature organic matter removal and the successive C/N ratio effect on heterotrophic competition with nitrifying microorganisms (Leonard et al., 2000; Chen et al., 2006, Michaud et al., 2006).

Nitrifying bacteria (nitrifiers) play a paramount role in RAS biofiltration as reviewed by Hagopian & Riley (1998). Nitrifiers carry out the fundamental two-step biochemical process referred to as nitrification process, which includes bio-oxidation of ammonia to nitrate via nitrite (after Henze et al., 2002)

Ammonia oxidation	*Nitrosomonas*	$NH_4^+ + 1½\ O_2 \rightarrow NO_2^- + H_2O + H^+$	$\Delta G° = -270$ kJ/mol NH_4^+-N
Nitrite oxidation	*Nitrospira*	$NO_2^- + ½\ O_2 \rightarrow NO_3^-$	$\Delta G° = -80$ kJ/mol NO_2^--N

The overall process $NH_4^+ + 1.86\ O_2 + 1.98\ HCO_3^- \rightarrow 0.020\ C_5H_7NO_2 + 0.98\ NO_3^- + 1.88\ H_2CO_3 + 1.04\ H_2O$

Nitrifying bacteria include ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). AOB oxidize NH_3 to NO_2 in two steps via hydroxylamine (NH_2OH) and NOB oxidize NO_2^- to NO_3^- (Koops & Pommerening-Röser, 2001). The growth rate of these chemolithotrophic, autotrophic microorganisms is very slow (doubling time in days) due to the low energy yield compared to growth of heterotrophic bacteria with doubling times in few hours (Gieseke et al., 2001).

Abundant AOB and NOB microorganisms in aquaculture are typically *Nitrosomonas oligotropha* and *Nitrospira sp*, respectively (Foesel et al., 2008). *N. oligotropha* is the dominant AOB in systems with low TAN due to a high ammonium affinity (low Ks) which generally favour their growth in freshwater aquaculture systems (Purkhold et al., 2000; Pedersen et al., Paper III). Different halophilic *Nitrosomonads* (*Nitrosomonas. sp*. nm143 linage and *Nitrosomonas marina* lineage) were found in a saltwater aquaculture system by Foesel et al. (2008). AOB and NOB were quantified by Pedersen et al. (Paper II) in relative abundances up to 5.4% and 3.3 % respectively of all EUB mix positive cells. Nitrifying bacteria have been characterized in other aquaculture studies, though in even lower numbers around or below level of quantification (Pedersen et al, Paper III; A. Cheatham, *pers. comm*.).

Only recently, with the implementation of culture independent molecular methods, the abundance and significance of *Nitrospira sp*. in stead of genus *Nitrobacter* as a major dominant NOB has been documented (Schramm et al., 2003; Maixner et al., 2006; Foesel et al. 2008; Pedersen et al., Paper II & III). *Nitrospira* spp. has, compared to *Nitrobacter* a competitive advantage at low nitrite levels under oligotrophic aquaculture conditions (Blackburne et al., 2007) due to a high nitrite affinity (low Ks).
AOB and NOB have been suggested to coexist in a beneficial, though fragile mutualism, where NOB strongly depends on AOB for its preferred electron donor and AOB depends on NOB to remove toxic nitrite (Graham et al., 2007). NOB is usually distributed/localized in a deeper layer behind AOB, and hence more prone to oxygen limitations due to the additional diffusion path. With sufficient oxygen present NO_2^- oxidation occurs at a faster rate than NH_4 oxidation (Schmidt et al., 2003), though with a lower energy yield per molecule. Beside AOB, the activity of ammonia oxidizing *Archaea* (AOA) and heterotrophic ammonium assimilation is also likely to contribute to the removal of TAN (Park et al., 2006).

Traditional *culture dependent* techniques for isolation or identification of microorganisms utilizing media with various substrates have been applied in microbiology for several decades, and are still used as a valuable tool in many applications (Michaud et al., 2006). Abundance of indicator (key) organisms can be used for monitoring purposes (i.e. *E.coli* for faecal pollution) and to assess disinfection efficiency in terms of log reduction on

diluted samples (e.g. CFU; Summerfelt et al., 2009). However, measures as most probable number (MPN) may range between 0.001 to 0.05%, meaning that only a minor fraction of the available microorganisms are actually detected. Culture independent methods including DAPI staining and Fluorescence in situ hybridization (FISH) techniques have gained considerable attention since the introduction of gene probes in the early 1990'ies (Amann, 1990; Wagner et al., 2003). The technique rely on the specificity (evolutionary conservation) of the ribosomal RNA sequences – which allows tailor made fluorescent probes sequences to be developed and used as visible markers (Amann & Fuchs, 2008).
Polymerase chain reaction (PCR), the amplification of specific DNA sequences can also be applied, e.g. targeting functional genes coding for AMO (Kowalchuk & Stephen, 2001).

A huge array of bacterial gene probes have been developed within the last decade, hierarchal covering species, guilds, functional groups and subphyla of micro organisms (Loy et al., 2007). With regard to AOB, more than 60 probes exist to cover and overlap phylogenetic groups, lineages, clusters, subspecies, or clones from the 16 AOB species (Koops et al., 2006). FISH can be extended and combined with other methods, i.e. FISH-MAR, where identification and relative activity can be assessed simultaneously (Wagner, 2004; Gieseke et al, 2005; Wagner et al., 2006).

Other culture-independent techniques include DAPI staining to count all bacteria (Kepner and Pratt 1994), while active bacteria can be estimated using the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride (CTC) that forms crystals in cells with an active metabolism (Rodriguez et al., 1992; Vollertsen et al., 2001). This theoretically allows distinguishing between active or inactive (potentially dead) cells similar to Live/dead bacterial viability kit with Syto 9 and propidium iodide reagents (www.invitrogen.com). However, both techniques have drawbacks, such as interference or species specific responses which might blur the results (Bredholt et al., 1999; Larsen et al., 2008).

Factors affecting biofilter function

An ideal biofilter should perform stable, have a high capacity and at the same time be flexible and tolerant to sudden changes in conditions (Gunderson, 2000; Botton et al., 2006). Biofilter nitrification performance is affected by a number of physical, chemical and biotic parameters (Chen et al., 2006). These include nitrogenous and organic loading rate, C/N ratio (Leonard et al, 2000; Michaud et al, 2006), hydraulic load, retention time and elevation velocity, changes in pH, alkalinity (Zhu & Chen, 2002; Chen et al., 2006; Lyssenko & Wheaton, 2006a, b), salinity (Grommen et al., 2005) oxygen concentration (Purkhold et al., 2000), metazoan grazing level (Boller et al., 1994) and photo-inhibition (Hagopian & Riley, 1998).

Beside those parameters, application of waterborne chemicals and antibiotics can be a pronounced stressor for the biofilter. If the applied biocide enters the biofilter, the nitrifying bacteria are likely to become affected, inhibited or killed. Bypassing the biofilter will, on the other hand, include the risk of pathogen refugee in the biofilter, sooner or later to spread into the system again. Biofilm kinetics and resistance against disinfectants are discussed in section 7.

AOB and NOB tolerance and recovery

NOBs are in general more sensitive to disturbances than AOB. This phenomenon has been observed in different studies in terms of unchanged ammonia level occurring while nitrite is accumulating (Keck & Blanc, 2002; Schwartz et al., 2000; Pedersen et al., Paper III).

Assuming AOB and NOB are affected or inhibited likewise, the differences in recovery can be due to slower growth rates of NOB compared to AOB. Furthermore, the evolution of recovery patterns can be affected by increased NH_3-levels, which can inhibit nitrite oxidation and hence create a delay in re-establishing a stable nitrite oxidation. If ammonia oxidizing *Archaea* are present in aquaculture biofilters and substantially contributes to ammonia oxidation, they might contribute considerably to the oxidation of TAN.

It can not be excluded that different biocides may pose various effects on the AOB/NOB recovery relation. When e.g. formaldehyde is applied, it has been hypothesized to act as a booster, i.e. providing favourable and degradable C-sources to AOB that previously have been reported to be able to use organic C instead of CO_2 (mixotrophic; facultative autotrophy). Furthermore, defence mechanisms may be different between AOB and NOB, though only few studies have compared protective enzymes, inactivation and recovery mode of nitrifiers (Wood & Sørensen; 2001; Antonelli et al., 2004).

7.3. Fish health management

Disease outbreaks occur both in wild fish populations and in domesticated stocks (Bergh, 2007). Recirculation technology has made it possible to avoid certain fish pathogens, but some pathogens, in particular the protozoan ciliate *I. multifiliis,* profit from the new conditions and present management strategies and can cause extensive economic losses.
Fish diseases can be categorized in two groups: infectious and non-infectious diseases. Infectious diseases are contagious and caused by pathogenic organisms whereas non-infectious diseases are not contagious caused by nutritional and environmental factors, often as symptoms of suboptimal rearing conditions (Noble & Summerfelt, 1996).

Infectious diseases can be sub-categorized into those caused by *obligate* (e.g. *Ichthyophthirius*, viruses, some bacteria) and *opportunistic* (e.g. *Flavobacteria*, *Saprolegnia*, etc.) pathogens. Obligate pathogens require a host to replicate and may only survive a very short time outside of the fish they infect thereby the most likely ways that they are spread to new areas is via infected fish. Opportunistic, or facultative pathogens are commonly found in all aquatic environments and may cause disease when a fish is under environmental stress, for example, from poor water quality, low oxygen, and other disease organisms. This distinction can have important ramification for disease control through biosecurity protocols (Delabbio et a, 2004). With high level biosecurity (fish-free water source, enclosed building, influent disinfection, and an all-in/all-out management strategy) it is possible to control pathogens that require a fish to continue their lifecycle, whereas with opportunistic pathogens it is more uncertain of their elimination through these means, and management strategies then include measures to minimize the likelihood of such pathogens causing losses through clinical disease.

The three main principle of biosecurity is 1) Reduce risk of pathogen introduction to the facility, 2) Reduce risk of pathogen spread throughout the facility, and 3) Reduce conditions within the facility that increase susceptibility to infection and disease (i.e. reduce stress). The extent of biosecurity measures of RAS varies from some mandatory requirements to a fundamental integrated part of the design and management strategy plan.

Different strategies can be followed in order to control pathogens. Biosecurity includes measures to avoid, eliminate or control pathogens in the operating system, by disinfection procedures, quarantine facilities and use of partial or fully shielded operations facilities in order to avoid birds and other predators and hence reduce loss and the risk of contamination (Bebak-Williams et al., 2007; Waldrop et al., 2009).

Use of UV in combination with ozone have proven to be a feasible solution to control pathogens and improve water quality in RAS (Summerfelt & Hochheimer, 1997; Summerfelt et al., 2009), but it is associated with a significant operational and investment cost. Alternatively, or in combination with UV and ozone technology, chemotherapeutic agents can be applied (Burka et al., 1997). This water treatment strategy, either prophylactic or curative is common and implies application of disinfectants (i.e. formaldehyde, peroxygens or Chloramine-T) directly to the water phase.
Beside the antiparasitic effects, the therapeutics may ease fish gill distress and promote better water quality and hence reduce the risk of environmentally related disease. Chemotherapeutants are hence particularly useful to remediate fish health episodes when disease prevention safeguards are overwhelmed, i.e. to change conditions and lessen stress related hyper-susceptibility among the reared fish.

In a recent survey of eight newly established commercial Danish RAS, *I. mulitifiliis* (white spot disease) was found to be far the most predominant cause of trout disease (Henriksen et al., 2008; Jørgensen et al., 2009). Almost inevitable, *Ich* outbreaks occur when new fish stocks are introduced to existing facilities without previous disinfection or measures to insure sufficient quarantine. An important issue is mixed immuno-competence in reuse raceway populations, i.e. without an all-in/all-out management approach, younger naïve fish entering a system will break with *Ich* such that theront levels in the system can raise to the a point where the older, more resistant fish even may succumb to the disease (Niels Henrik Henriksen, *Pers. comm.*).

Considerable work has been conducted in order to develop an efficient treatment strategy against I. *mulitifiliis* which has a characteristic multistage life cycle (Heinecke & Buchmann, 2009; Matthews, 2005). Treatment protocols can include prolonged salinity increase to 10-15 ppt for one-two weeks or consist of repetitive application of disinfectants, i.e. formaldehyde every second day over a 10 days period in order to eradicate the free swimming stages of *I. mulitifiliis.* Experience with use of HP or PAA is very limited in RAS with biofilters due to precautionary motives.

Recent findings also indicate that micro sieves with a mesh size below 80 µm efficiently can remove tomont stage from Ich, and thereby reduced successive proliferation (Heinecke & Buchmann, 2009). So far, Ich and other parasites pose a problem, and new management and treatment strategies are developing to replace existing routines. According to Angelucci et al., (2008), prophylaxis schedule could be conducted with environmentally friendly chemicals, considering the present environmental policies.

Water treatment strategies in RAS

Treatment strategies to chemically control parasites have to take several factors into account. Beside an effective treatment against the target organisms, the chemical should be non-toxic to the fish, it should not inhibit the activity of the nitrifying bacteria nor pose any work or environmental risks (Fig. 3). The extent of those compromises forms the safety margin of the particular chemical.

As disinfectants have variable effectiveness, depending on target organism and water quality, only general guidelines, if any, exist. Treatment practices are often system specific and developed empirically. Treatment practice should take measures to reduce the water volume and thereby the amount of chemical needed during treatment (Sortkjær et al., 2008a). Furthermore, a high degree of recirculation at least theoretically allows lower doses over a prolonged period of time, which then might benefit the fish, the biofilter and the environment.

PROCESS PARAMETER	OBJECTIVE AT A GIVEN EXPOSURE	RISK
TREATMENT EFFICIENCY	To eliminate pathogens	Under – or over-dosing
FISH HEALTH	No effects of exposure on fish	Sub-lethale or lethal effects on fish
BIOFILTER PERFORMANCE	No effects on nitrifying population	Biofilter collapse
WORK SAFETY	Harmless to workers	Explosive, corrosive or carcinogenetic agents
ENVIRONMENTAL CONSIDERATIONS	Comply with regulation	Excess discharge of toxic compounds
PRODUCT QUALITY	Unaffected	Residual build-up; reduced growth
ECONOMY	Price competitive	Expensive

Fig. 3. Treatment window – margin of effective and safe water treatment

A number of different chemicals can potentially be used to improve water quality and treat against diseases in RAS (Noble & Summerfelt, 1996; Burka et al., 1997). Malachite green, now banned in most countries due to its carcinogenetic properties, used to be a universal aquaculture agent and was previously considered to be practically irreplaceable (Srivastava et al., 2004; Sudova et al., 2007). When it comes to control and elimination of protozoan parasites, formaldehyde and sodium chloride are far the most applied chemicals used in Danish model fish farms (Henriksen et al., 2008). Both agents meet most of the important criteria listed in table 2, but as discharge regulation has become more severe (see chapter 8) present use might not imply with current discharge regulation (Masters, 2004, Sortkjær et al., 2008B).

Other antiparasitic agents include HP, PAA Chloramine-T, copper sulphate and potassium permanganate. Chloramine-T can be applied to RAS with biofilters though the safety margin is not known. Chl-T has fewer desirable attributes compared to peroxygen compounds, i.e. it has low degradation rate and release a complex, potential toxic intermediate compound, para-toluensulfonamide (Dawson et al., 2003). The use of copper sulphate has been steadily declining over the last decade and has the drawback of accumulation and very low levels of discharge are permitted. A new chemical additive - the peroxygen performic acid has not been tested in an aquaculture context yet (Gehr et al., 2009) but might be an antiparasitic candidate with desirable attributes.

7.4. Formaldehyde

Formalin, the trade name of formaldehyde solutions, is an important and very commonly used aquaculture chemical. According to EPA (2007), formalin was by far the most applied chemical additive in Danish Aquaculture systems, averaging 100.000 L per year (2001-2005). FA is often considered practically irreplaceable due to its high and versatile treatment efficiency with a wide safety margin, but also due to tradition and experience gained.

Physiochemical characteristics

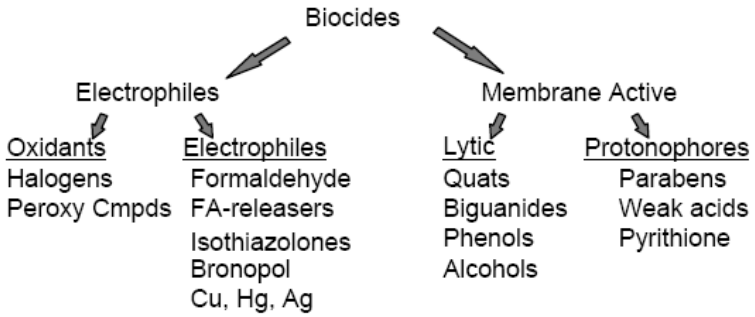
Formaldehyde (FA), the simplest aldehyde, is a pungent, reactive gas and the most abundant carbonyl compound in the ambient atmosphere (Chan & Lee, 1998). FA is very soluble in water and alcohols, and it is the active agent in formalin, typically containing 37 % FA. FA is unstable in its pure gaseous form and readily polymerizes to trioxane (a cyclic trimer) or para-formaldehyde (a poly-acetal). Para-formaldehyde, the white precipitate in formalin, is highly toxic and can be prevented by use of 10% methanol and other stabilizers normally added to formalin.

Antimicrobial properties and mode of action

Aqueous solutions of formaldehyde (2 %) are used to fix and preserve cells and tissues, and weaker dilutions have strong disinfective capacity. Formaldehyde has excellent bactericidal, fungicidal and parasitidal effects, and examples of virucidal and sporicidal activity have also been recorded (Power, 1997).

Formaldehyde is an organic electrophile agent with mechanisms of action similar to those of heavy metals. FA has been suggested to act by an alkylating effect – a nucleophil process where organic bound hydrogen proton is substituted and hence disrupt enzymes functions. FA also possesses cross-linking properties, affecting proteins, RNA and DNA (McDonell & Russell, 1999). Amines and sulfhydryl-groups are the main targets, affecting such amino acids as cystein, tripeptide gluthation and sulfhydryl dependant enzymens such as ATPases (Rossmore, 1991). Cellular productions of cystein and

glutathion have been suggested to be a resistance mechanism to inactivate or reduce biocidal activity form for example formaldehyde (Chapman, 2003).



(From Chapman, 2003)

Fig. 4: Classification of biocide according to mode of action; aquaculture chemical additives include formalin (formaldehyde) and peroxy compounds (hydrogen peroxide and peracetic acid)

Applications

Formaldehyde is ubiquitous in the environment. It is a common constituent in certain textile productions, plywood and carpet production and is included in resin compounds and paints (Chan & Lee, 1998). Formaldehyde is produced in the atmosphere due to the degradation of methane by sunlight. It is also released during the combustion of organic materials, and as such may be present in smoke from wood fires, automobile emissions and tobacco smoke. Natural production of FA arises from the troposphere where photochemical oxidation of methane and other simple hydrocarbons led to FA formation (IARC, 2004).

Formalin is an important and commonly used chemical in fish farming operations with high efficacy against ectoparasitic infections and has been applied for almost a century (Fish; 1932; Fish & Burrows, 1940). In commercial operating ponds, FA dosages are applied at around 100 mg/L FA (1:4000 ratio of 37%-formalin:water) during treatment (Sortkjær et al., 2000). Significantly reduced FA concentrations can be as effective

provided prolonged contact time (Heinecke & Buchmann, 2009), and in RAS, nominal formaldehyde concentration is often reduced to 20-30 mg/L (Henriksen et al., 2008). An advantage of applying FA to RAS is that biofilters are tolerant to the concentrations of FA used during a standard treatment (Pedersen et al., Paper II), and hence can be consider a relatively safe treatment compared to the use of other disinfectants.
The microbial degradation of FA by aquaculture application is described by Pedersen et al, 2007 and Pedersen et al, Paper II.

Antiparasitic treatments of especially *I. multifiliis* often include repetitive formaldehyde dosages, i.e. every second day over a two week period, and formalin may be used in combination with other chemicals (Matthews, 2003; Rintimaki-Kinnunen et al., 2005). FA are also efficiently applied to avoid fungal infection on spawners (Gieseker et al, 2006) and to prevent moulding in eggs (Rach et al., 1997c); it is also used as a surface disinfectant. Anecdotal records also include formaldehyde application to boost eel RAS; apparently able to improve water quality and as well as fish appetite.
The versatility of FA makes it suitable to be used for treating various diseases from many fish species, and the treatment margin (i.e safe concentration levels) is relative wide compared to other disinfectants.

Toxicity to fish

Toxicity of formaldehyde to salmonids is well known (Smith & Piper, 1972; Speare et al., 1997; Hochreiter & Riggs, 2002). Toxicity to formaldehyde increases with temperature (Piper & Smith, 1973) though caution should be taken at temperatures below 5 ° due to the increased risk of formation of the highly ichthyotoxic paraformaldehyde precipitate. Increasing water hardness have been found to lower FA toxicity as have the presence of organic matter (Meinelt et al., 2005). Formalin application reduces water oxygen content, and caution should hence be taken when formalin baths are used (Burka et al., 1997). Buchmann et al., (2004) describe acute reaction (sublethal effects) of rainbow trout in terms of impaired epithelial cells after 1 hrs contact time with 200-300 mg/L FA or after 24 hrs exposure with 50 mg/l formaldehyde.

Safety

Formaldehyde is a natural metabolic intermediate and is metabolised into formate, by formaldehyde dehydrogenase. FA does not accumulate in humans and is mainly eliminated by urinary excretion as formic acid or exhaled as carbon dioxide (IARC, 2004).

FA can lead to an allergic reactions (sensitisation) and lung dysfunction, and throat and nasal cancer like damage in humans have been related to formaldehyde exposure.. Exposure to lower levels FA for shorter periods is not considered to present any carcinogenic risk (www.hpa.org.uk). Wooster et al., (2005) found that ambient air FA concentration under aquaculture operation was below the recommended levels of FA exposure, in line with Lee & Radtke (1998; as cited in IACR, 2004) that measured up to 0.02 mg FA/m^3. To set in context, ambient FA value in new mobile homes has been recorded up to 0.5 mg/m^3 according to IARC (2004).

However, FA has been classified by the International Agency for Research on Cancer as carcinogenic to humans based on substantial evidences (IARC, 2004) hence it is a chemical that requires cautious handling.

Current status

Formalin use in aquaculture is still a controversial issue, and Danish Aquaculture announced recently an expected out-phasing of FA before 2014 (www.danskakvakultur.dk). The environmental context is highlighted by Hochheimer & Riggs, 2002; Master, 2004 Gearheart et al., 2006, and recent findings have documented FA discharge levels form certain types of fish farms exceeding limits set by the environmental agencies (Sortkjær et al., 2008b). Recent unofficial records indicate that formalin application has not declined (Søren Keller, EPA; *pers. comm.*) and a published survey of eight commercial freshwater RAS documents that approximately 14 L formalin has been used per metric ton of trout produced (Henriksen et al., 2008). The latter presumably without any environmental impact as the fish farms mentioned have huge biofilters and a high degree of recirculation i.e. long retention time that can facilitate complete internal microbial degradation of formaldehyde (Pedersen et al., 2007; Sortkjær et al., 2008b; section 7.8).

7.5. Hydrogen peroxide

Hydrogen peroxide is a relatively new candidate chemical in aquaculture compared to the traditional use of formalin. In Denmark, HP aquaculture applications averaged 10.000 kg/year of sodium percarbonate (contain 33 % HP) from 2001 to 2005 according to EPA (2007). Despite antimicrobial and environmental beneficial attributes, HP use and application is not yet commonly implemented and the experiences from practical application are accordingly modest (Sortkjær et al., 2008a).

Physiochemical characteristics

Hydrogen peroxide (H_2O_2) is the simplest stable peroxide. It is a pale blue liquid, which appears colorless in dilution. It contains oxygen in a state of oxidation midway between molecular oxygen and water, and it is a weak acid (pKa =11.6). HP is a powerful oxidant and is considered to belong to the highly reactive oxygen species. Through catalysis, HP can be converted into hydroxyl radicals ($^{\cdot}OH$) which react instantly and indiscriminately with virtually all organic molecules (Livingstone, 2003) with a reactivity second only to fluorine. The decomposition of H_2O_2, and hence the transient existence of hydroxyls, is beside enzymatic catalyzation also facilitated by the presence of certain metals, i.e. Fe^{++}. HP is most often stored in aqueous solution at a concentration less than 50%; typically 35% in technical solution (i.e. 35% PEROX-AID®), but is also applied via sodium percarbonate, HP-releasing granulated powder (Pedersen et al., 2006; Heinecke & Buchmann, 2009). See Sect. 6 for additional information on HP presence in quaternary peracetic acid solutions.

Antimicrobial properties and mechanisms of action

HP may be regarded as natures own disinfectant and preservative – naturally present in milk and honey – and a normal resident of tissue due to cellular metabolism (Block, 1991). HP has a broad antimicrobial spectrum, and has been recorded active against bacteria, yeast, fungi, viruses, spores, proto- and metazoan (Baldry, 1983; Schmidt et al., 2006). HP act synergistically in combination with peracetid acid (Alasri et al., 1991; Wagner et al., 2002) , and increased temperature and the presence of certain metal ions further increase the toxicity of HP.

HP is highly reactive and consequently short-lived, with transient highly reactive low molecule intermediates. The direct effect of HP is not described in details, but is expected to rely on the formation and action of ultra short lived highly toxic HP formed radicals (Block, 1991). These intermediate products – the reactive oxygen species (ROS) hydroxyl and superoxide radicals with unpaired electron pair (Fig. 5) is liberated during HP reactivity and decay (Derksen et al., 1999). HP is believed to do actual killing of the bacteria via hydroxyl ions attacking membrane lipids, and destroying essential cell components, i.e. oxidize sulfhydryl and methionyl groups proteins and DNA and inactivate certain enzymes (Block, 1991; Vroegop et al., 1995, Powell & Perry, 1997). The lipid peroxidation of cell membranes by ROS can alter membrane functions, for example changes permeability and impairs receptors and ionpumps. Lipid-protein cross bindings might also occur and negatively affect the cell or microorganism.

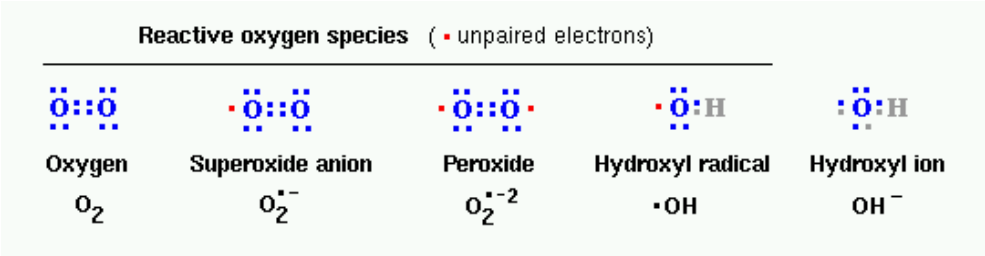


FIG. 5: Reactive oxygen species generated by HP.
(source:http://www.vivo.colostate.edu/hbooks/pathphys/misc_topics/radicals.html)

Application

Hydrogen peroxide has a long history of use, and has presently lots of applications. It is used as a bleaching agent (pulp & paper, textiles), it has environmental applications, e.g. in advanced oxidation processes and potable water treatment, is used as a bacterial disinfectant in food processing and it is included in detergent manufactures and the active agent in products as mouthwash and contact-lens cleaner. HP is also used as for odour control and to facilitate bioremediation.

Hydrogen peroxide is a relatively new candidate in aquaculture compared to the long traditional use of formalin. Within the last decade, however, numerous studies have been published regarding HP aquaculture applications (Schmidt et al., 2006). The main applications of HP are to control ectoparasites, to alleviate bacterial/ environmental gill disease and treat fungal outbreaks on eggs and spawners (Sortkjær et al., 2000). Just recently, has HP been approved in the US as a low regulatory compound for aquaculture purposes after thoroughly investigations (Schmidt et al., 2006).

A number of lab-studies with HP and fish pathogens have shown high treatment efficiency and documented the potential industrial applicability of HP (Table 5). Findings from clinical experimental conditions have not been sufficiently verified under commercial conditions and only few Danish fish farmers rely on water treatment management practice based on HP products only. Due to the fast breakdown of HP, there is a pertinent risk of under-dosing, resulting in inefficient treatment (Rach et al, 1997a; Rach & Ramsey, 2000; Saez & Bowser, 2001). At present, the upper safety margin of HP is just below 100 mg HP/L for short term exposure (Fig. 5.1) and caution should be taken at temperatures above 15°C where salmonids are extra vulnerable to HP (Rach et al., 1997b; Gaikowski et al., 1999) (Fig. 5.2).

Application of HP is commonly associated with oxygen liberation, so degassing/stripping is recommended in order to avoid prolonged hypersaturation of oxygen (Pedersen et al., 2006; Taylor & Ross, 1988). Use of HP-releasing products containing sodium-percarbonate (i.e. Oxyper® or Biocare®) should be done with caution in RAS, as the carbonate fraction results in a significant elevation on pH. Measures for correct HP dosage and monitoring during treatment still have to be developed and implemented in different aquaculture systems. General treatment recommendation is 500-1000 mg/l HP in 15 minutes for salmonid eggs and 50-100 mg/l HP for ½-1 hour for juvenile and adult salmonids (Schmidt et al., 2006).

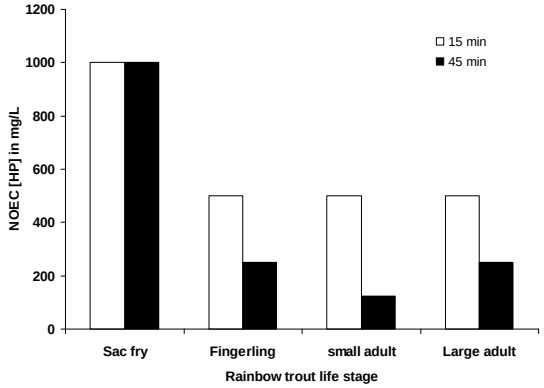


Fig. 5.1: No-observable-effect concentration for different life stages of *O. mykiss* treated with HP for 15 or 45 minutes every other day four consecutive times at 12 °C (after Rach et al, 1997). NOEC for fingerlings at same conditons after 1 and three hr exposure is 162 and 81 mg/L HP (Gaikowski et al, 1999).

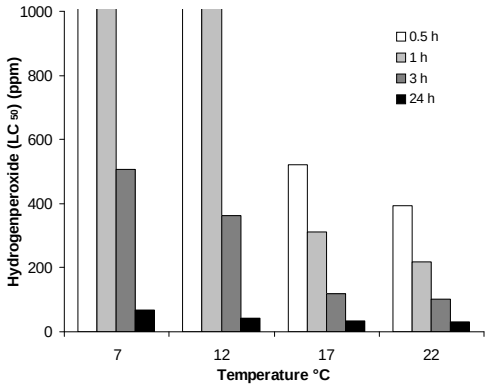


Fig. 5.2: Acute toxicity (LC_{50}) test with juvenile rainbow trout (~1 g) and hydrogen peroxide applied at four temperatures and with four different contact times. (after Rach et al, 1997).

Toxicity to fish

Several studies have confirmed the ability of rainbow trout to withstand acute exposure to 100 ppm (Powell & Perry, 1997; Derksen et al., 1999). Powell & Perry (1997) exposed rainbow trout to 100 and 500 ppm HP and noted decreased blood pH, respiratory acidosis and significant raised levels of plasma catecholamines in fish treated with 500 ppm, whereas fish in 100 ppm HP behaved similar to unexposed fish. High dosage HP > 400 ppm / 1 hr causes gill epithelia damage, which has been found to be reversible. The fish toxicity of HP is species specific and is related to fish size and temperature (Rach et al., 1997; Gaikowski et al., 1999). Generally, bath treatment for one hour should not exceed a HP concentration of 100mg/L for juvenile fish, while cold water fish eggs can withstand HP concentration five-ten times higher for a shorter period of time.

Safety

Hydrogen peroxide in weak dilutions as applied for water treatment in aquaculture in harmless, and does not raise work safety wise concern. As stock solution, hydrogen peroxide is a strong oxidizer, with corrosive ability and can cause burns to skin, eyes and respiratory tract. HP is harmful if swallowed or inhaled, and it can irritate the eyes, skin and mucous membranes. Exposure of the eyes to concentrations of 5% or more can result in permanent eye damage.

Current status

The protozoan gill and skin parasite *I. multifiliis* is particularly difficult to control in RAS and is often considered the major cause of fish mortality (Buchmann & Bresciani, 1997; Rintamaki-kinnunen, 2005; Jørgensen et al., 2009). Recent findings have clarified the dose/response correlation of FA and HP against I. multifiliis free living stages under clinical conditions (Heinecke & Buchmann, 2009). These finding (Fig. 5.3) shows that HP can eliminate it effectively at various concentrations, which opens up for testing and applying low dose HP application over prolonged period of time (Sortkjær et al., 2008a). When using new chemicals, caution should be taken as stated by Rach et al., (2000) "*Because fish sensitivity to a chemical is likely to vary, a preliminary bioassay should be conducted on a small number of fish*…"; a recommendable approach also to include with regards to biofilter units.

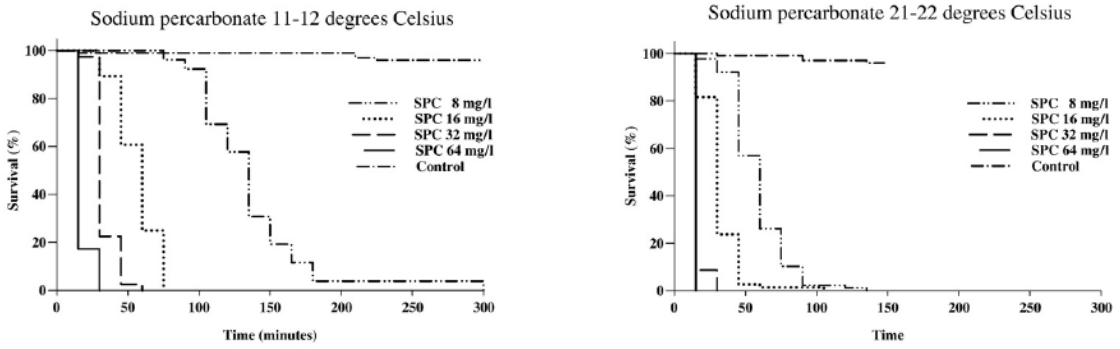


Fig. 5.3. Clinical dose/response experiments with *I. multifillies* theronts and SPC which liberated 0.33 g HP per gram. After Heinecke & Buchmann, 2009.

Table 5: Examples of freshwater salmonid aquaculture related studies with HP-products

TYPE OF STUDY	EVALUATION OF RESULTS	REFERENCES
Fish toxicity test	No mortality at 70 ppm HP @ 2 h or 280 ppm @ ½ h exposure; elevated mortality at 2 hr exposure to 170 ppm. Size effect, species effect and temperature effect.100 ppm safe for 45 min < 17°C; NOEC values and survival probability curves	Arndt & Wagner, 1997; Rach et al., 1997; Gaikowski et al., 1999; Taylor & Glenn, 2008
Antiparasitic effect *I. Multifiliis; G. derjavini; Ambiphrya Trichodina*	Dose related mortality; i.e. 5 ppm HP eradicates *Ich* theronts within 2 hours + temperature effect. Prevalence of *G. derjavini* infected fish reduced from 100 to 0 at dosage > 14 mg HP/L as well as significant reduction of parasites/fish Theronts killed after 3 hr. at 4 mg HP/l – but 89 % of tomocysts survived. Require repetitive treatment. Effective against Ambiphyra but not Trichodina	Rach et al., 2000 Buchmann & Kristensson, 2003; Buchmann et al., 2003; Heinecke & Buchmann, 2009
Antifungal effects on fish[A]	HP induced fin-index improvement; SGR and FQR not diff. from control	Speare & Arsenault, 1997; Gieseker et al., 2006
Gill effects	Clearence rate unaffected effect on gills > 100 ppm HP Confim the ability of RBT to withstand acute exposure to 100 ppm; structural damage on gill and induced mortality at high HP dose occurred initially (< 5 hr) – gill regenerates successively	Derksen et al., 1999 Tort et al., 2002
Physiological response	Disturbed acid-base balance, blood pH + catecholamine release	Powell & Perry, 1997
Full scale experiment	HP analytical verification i full scale; documentation of severely under dosing (< 60 % of expected peak values) HP fate during flow-through treatment and estimated T½ ~½ h.	Rach et al., 1997; Rach & Ramsey, 2000 Saez & Bowser, 2001
Biofilter exposure	Biofilter collapse in FSB biofilter Degradation kinetics in fixed, submerged biofilter with and without affecting biofilter performance. *See sect. 7 for further details*	Schwartz et al, 2000; Pedersen et al., 2006; Sortkjær et al., 2008; Møller et al., subm.

A/ studies including *Saproglenia* infection and treatment on eggs are excluded; see Schmidt et al., 2006 for numerous references.

7.6. Peracetic acid

Peracetic acid is a new aquaculture candidate. A commercial solution was merely introduced for aquaculture purposes at the end of last decade (Sortkjær et al., 2000) and use and practical experience is still modest. According to EPA (2007), aquaculture application of "liquid hydrogen peroxide products" PAA products averaged 6.400 L, among this a substantial part containing approximately 5% PAA. Despite great antimicrobial effects and benign environmental character, PAA application is limited and more knowledge is needed to uncover the potentials - weaknesses and strengths - of PAA.

Physiochemical characteristics

Peracetic acid (PAA) or peroxyacetic acid is a high-energy-state peroxygen derived from the peroxidation of acetic acid (Fig. 6.1). PAA is a strong oxidant and disinfectant due to its unpaired electrons (free radicals) and hydroxyl radical formation.

PAA is commercially available in the form of a quaternary equilibrium mixture containing acetic acid, hydrogen peroxide, PAA and water. Solutions are added different stabilizers and acidified to a pH below 2. It is a colorless liquid with no foaming capabilities, and has a very strong vinegar odour. At least half a dozen commercial products exist with different formulations; typically PAA comprises 4-20%, HP up to 35 %, and acetic acid up to 40 % and water. PAA is considerable less stable than HP, decaying up to 1-2 % per month, but commercially available PAA solutions with stabilizers and intermediate PAA concentrations are more stable. PAA destabilizes more rapidly in water/solution (half-life ~ 120 hours) and is also significantly degraded by photolysis.

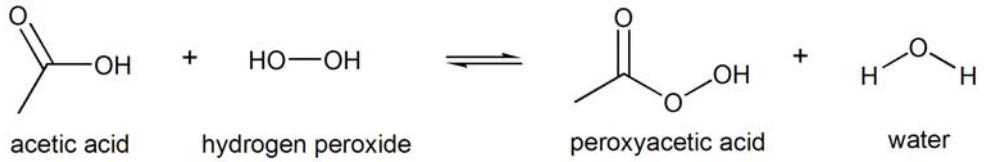


Fig.6.1. Quaternary equilibrium mixture of AA, HP, PAA and water.

The acid dissociation constant (pK_a) of PAA is 8.4.

PAA has very high water solubility, and compared to HP, greater lipid solubility and can not be deactivated by catalase or peroxidase (Block, 1991). The breakdown of PAA occurs spontaneously, by chemical oxidation and by hydrolysis (Yuan et al., 1997; Wagner et al., 2002). PAA is easy degradable and leave no noticeable disinfection by-products, and is for those reasons considered as an environmentally benign disinfection agent (Colgan & Gehr, 2001).

Antimicrobial properties and mechanisms of action

Peracetic acid is a more potent antimicrobial agent than HP, and is active at low concentrations against a wide spectrum of micro organisms (Block, 1991).

PAA are hence required in smaller amount than HP to achieve similar disinfective effect, and has synergistical effects in combination with HP (Alasri et al., 1991; Wagner et al., 2002; Pedersen et al., Paper III).

PAA has sterilizing capabilities and has been recorded efficient against virus, bacterial, fungus, algae, protozoan and metazoans and it has also germicidal and sporicidal effect (Kitis, 2004; Meinelt et al., 2007). Most of the reduction in microbial concentrations occurs during the first 10 min of contact time (Rajala-Mustonen et al., 1997; Antonelli et al., 2006).

Peracetic acid kills microorganisms by oxidation and subsequent disruption of their cell membrane, via the hydroxyl radical (·OH) which is an ultra short-lived intermediary metabolite. Free radicals and reactive oxygen species (Livingstone, 2003) can damage DNA; oxidize polyunsaturated fatty acids in lipids and amino acids in the membrane proteins and inactivate specific enzymes by oxidation of co-factors. PAA is also speculated to have intracellular effects, i.e. oxidize essential enzymes and destroy vital biochemical pathways, active transport across membranes, and intracellular solute levels are impaired. An important advantage of PAA is that it may inactivate catalase, an enzyme known to detoxify free hydroxyl radicals (Block, 1991) and hence affect concomitant HP removal (Pedersen et al., Paper III). The antimicrobial effect of PAA is amplified by the presence of HP (Alasri et al., 1991), and is it likely that acetic acid might also contribute to microbial inactivation (Balta et al., 2008; Taylor & Glenn, 2008). It

needs to be mentioned that the disinfection mechanism of PAA is still under investigation (Santoro et al., 2007).

Different factors affect the antimicrobial efficiency and decay of PAA. PAA is efficient at low temperatures, and is relatively unaffected by the presence of organic matter content as opposed to other disinfectants (Lefevre et al., 1992; Collivignarelli et al., 2000). Excess PAA can be quenched by sodium thio-sulphate though not reported from commercial operations. The water pH affects the antimicrobial efficiency of PAA, the lower pH the more efficient due to predominance of the undissociated, potent acid.

Applications

The powerful antimicrobial action of PAA in general, combined with high efficiency at low temperature, as vaporizer as well as the low risk of harmless by-product formation has left PAA an optimal disinfectant for several environmental and industrial applications (Colgan & Gehr, 2001). Peracetic acid has been used primarily as a sanitizer and water treatment compound in food and beverage processing, as well as a successful oxidizer for removing biofilms from food contact surfaces, as an outstanding odour suppressant, and as a bleaching agent. Peracetic acid degrades very quickly in the natural environment, and has no risk of bioaccumulation. It has been proven not to have carcinogenic, mutagenic, or toxicokinetic properties, and there no known hazardous or toxic by-products associated with its use (Kitis, 2004).

PAA is used to reduce faecal bacteria in waste water effluent, to decolour and control microbial growth in textile and pulp mill industry, as an additive in sanitizing solutions for the food and beverage industries, and as a terminal disinfectant or sterilant for stainless steel, glass tanks, pipes, tank trucks and ship ballast water (Baldry and French, 1989; Kitis, 2004; Santoro et al., 2007; de Lafontaine et al., 2008).

Application in aquaculture

PAA has not been used in the aquaculture industry and research until just recently, hence it is not mentioned in the excellent and comprehensive review by Burka et al. (1997).

Recent lab-experiments with *I. multifiliis* theronts have shown complete antiparasitic effects after short term exposure with 0.3 $mg \cdot L^{-1}$ PAA, and very significant reductions with 0.2 $mg \cdot L^{-1}$ PAA exposure (Meinelt et al., 2007a). Straus & Meinelt (2009) compared treatment efficiency against *I. multifiliis* with two commercial PAA solutions, and found effective concentrations (LC_{50} after 1 hr exposure) in the range 0.17 to 0.28 $mg \cdot L^{-1}$. Rintamaki-Kinnunen et al., (2005a,b) tested peracetic acid compounds against *I. multifiliis* in field trials and found that 1-1.3 $mg \cdot L^{-1}$ PAA could reduce the parasitic load on the fish. Differences between treatments were observed, and PAA were less effective in earthern ponds compared to concrete tanks. Other aquacultures related studies with PAA are summarised in table 6. A common feature is the acquired efficiency at very low dosages– typically below 0.5 mg PAA/L for short exposures. This underlines the importance of knowing the exact treatment concentration (stock solution strength) and factors affecting PAA decay.

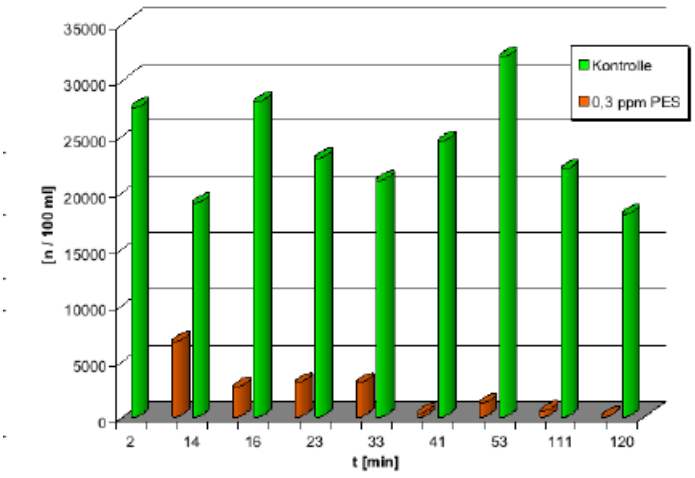


ABBILDUNG 3: *Überleben von I. multifiliis-Theronten bei Exposition mit 0,3 ppm PES.*

Fig. 6: Example of dose/response effects of PAA (~ PES) on *I. multifiliis* theronts, represented as # individual survivors in 100 ml without treatment (control) and after exposure to 0.3 mg/L PAA (*from Meinelt et al., 2007*)

From a practical point of view, treatment with PAA in RAS could include a temporary bypass of the biofilter which would allow PAA application in the rearing units for a predefined period of time and then redirection of the process water to a specific filter where potential PAA could be degraded without affecting the nitrifying microorganisms (Sect. 7). If possible, a temporary reduction in the treated water volume would further lead to reduced need for PA+ and hence reduce the potential risk of unwanted side effects.

Future use of PAA requires measures to ensure a correct nominal dosage (cf. the stability of the product) and monitor PAA concentration during treatment. Repetitive use of PAA also has to be tested, since this is a prerequisite to control some of the ecto-parasites.

Because of the high oxidation potential of PAA and the formation of free radical, measures of change in redox potential (ORP) during water treatment has been applied with success (Kees Kloot; *pers. comm*.). This method of ORP monitoring and regulation is already implemented in aquaculture ozone application as described by Summerfelt et al., 2009, and should also be tested with PAA under controlled conditions.

Toxicity to fish

PAA is relatively toxic to fish, which leaves a rather small treatment margin or window.

Meinelt et al., (2007) exposed walleyes (*Sander lucioperca*) to increasing PAA concentrations (0, 0.5, 0.9, 1.3 and 1.9 $mg \cdot L^{-1}$ PAA) for 24 hrs, and observed increasing mortality starting at 0.9 $mg \cdot L^{-1}$ and 100 % mortality was observed at 1.9 $mg \cdot L^{-1}$. Similar mortality of juvenile walleyes was observed the days after PAA water treatment, where blister-like bulges were observed on the gill lamella (Martin Vestergaard, AquaPri, *pers. comm*). Sortkjær et al., (2008a) made 1-hr toxicity tests on rainbow trout at 16 °C, and found that trout fully tolerated 1.3 and 2.6 $mg \cdot L^{-1}$ PAA, whereas 18% mortality was observed during the 24 h recovery period after exposure to 3.9 $mg \cdot L^{-1}$ PAA.

Apparently, PAA toxicity is less pronounced in saltwater species (according to manufacture; www.ecolab.com), though this has not been confirmed by other studies.

Safety

Safety sheets should be provided by the manufacturer; i.e. with advice on handling and storage conditions. PAA solutions should generally be store in original packaging and stored at room temperature or below. Concentrated PAA solutions have to be treated with caution. PAA decomposition may cause explosion if solutions are transferred to sealed containers, and caution should be taken not to add organic material or metal ions because the exothermic reaction can lead to ignition and produce fire. In aquaculture treatment dilutions, PAA dose not poses any noteworthy risks for humans.

Current status

In light of the restriction on formalin use and discharge, peracetic acid theoretically appears to be a good replacement candidate with its toxicological and environmental profile. The rapid degradation and the absence of harmful disinfection by-products make PAA an obvious benign candidate in flow though systems where the potential amount of excess PAA is very limited. Increased retention times by partial recirculation or discharge via constructed wetlands are options to eliminate PAA residual before entering the natural water source. The active agent PAA has however not been subjected to any water quality criteria as is the case for FA and HP.

A common feature is the acquired efficiency at very low dosages– typically below 0.5 mg PAA/L for short exposures. This underlines the importance of knowing the exact treatment concentration – but straight of stock solution and factors affecting PAA decay.

TYPE OF STUDY	EVALUATION OF RESULTS	REFERENCES
Fish toxicity test	Size specific tolerance of walleyes; NOEC level > 0.5 mg/L PAA. No mortality of rainbow trout ≤ 2.6 mg/L PAA (1 hr. exposure); Mortality observed after exposure of 100 µL Perotan/L.	Meinelt et al., 2007b Sortkjær et al., 2008a ; Lanhsteiner & Weismann, 2007
Antiparasitic effect (lab.) *I. multifiliis. I. necator*	Effective on theronts at 0.2 & 0.3 mg/L PAA and reduces trophonts at 0.5 & 1.0 mg/L PAA Effective concentration range were below 0.3 PAA mg/l. Positive effect against Costia – similar to FA.	Meinelt et al., 2007a,b; Straus & Meinelt, 2009; Holten et al., 2002 Lanhsteiner & Weismann, 2007
Antiparasitic effect (field) *I. multifiliis.*	Desirox/FA had good effect; lowest mortality groups. Per Aqua with some, but varying effect. Desirox in combination with formalin reduced parasitic burden; variation within treatment groups and effects of pond size, fish size and species	Rintamaki-Kinnunen et al., (2005a;b)
Antibacterial effect (field)	Reduced infection prevalence from 80 to 50 %,	Angelucci et al., 2008
Antifungal effects *Saproglenia ssp.*	Minor to good effect on controlling eggs fungal infection	Marking et al., 1994; Meinelt et al., 2006
Gill effects	Gill damage at PAA exposure >	Meinelt et al., 2007a Holten et al, 2002
Biofilter exposure	Dosage dependant decay; significantly impaired nitrification at 2.0 mg/L PAA	Pedersen et al., Paper III
Decay kinetic water quality stocking density	Simultaneous measures of PAA and HP residuals / kinetic Significant effects of fish density on PAA decay rate	Pedersen et al, Paper III

…

7.7. Degradation of water borne therapeutics in aquaculture biofilters

A major concern when using chemical water treatment in RAS is potential effects on biofilter performance. The biofilm-associated beneficial microorganisms are exposed to the disinfectants and the nitrifying bacteria may be inhibited or ultimately irreversible inactivated. Bypassing the biofilter during treatment excludes the risk of nitrifier inactivation; however bypassing is often not possible option in practice and it furthermore leaves compartments of RAS untreated. To achieve a pathogen efficient and a biofilter safe treatment is somehow different from *biofilm control* where disinfectants are used to reduce or destroy unwanted biofilm growth (Stewart and Franklin, 2008; Larsen et al., 2008b). A clear distinction should here be noted between central in-line biofilters in the RAS loop and end-of-pipe, effluent biofilters. Post treatment biofilters are detached from the fish rearing units and hence require less acute attention regarding potential collapse.

A number of reactions take place when biofilms are exposed to chemical additives. A mass transport diffusion of a chemical from the bulk-phase into the biofilm is associated with adsorption, microbial degradation and chemical oxidation of the compound. These processes and their contribution depend on the chemical's property, its concentration, the boundary layer, biofilm composition and thickness as well as oxygen availability, pH and temperature among others. Therefore, chemical consumption and biofilter robustness are most likely system specific and should be assessed as a bioassay.

Knowledge of the decomposition rate of the chemicals in the biofilm has two apparent applications: *i*) to be able to model and predict the amount of chemical removed and discharged, and *ii*) to obtain a more accurate treatment concentration and by that ensure adequate treatment efficiency (Saez & Bowser, 2001, Rach & Ramsey, 2000). As stated by Weavers & Wickramanayke, (1991). "… *the most important issue is not the amount of chemicals applied but the residual levels in the water during the treatment period*."

The fate of the chemical can be determined by temporal measurement of the residuals and by accounting for potential dilution removal rates can be calculated. The decomposition

kinetic can be complex and difficult to model/describe with all parameters and is therefore often described empirically as best linear fits (Falsanisi et al., 2007). Relatively few studies have intentionally studied and described decomposition rates of therapeutics in aquaculture biofilters (e.g. Pedersen et al., 2007), as focus have more often been on biofilter stability (Heinen et al., 1996; Schwartz et al., 2000). Fig.7 illustrate different types of decay patterns obtained from chemical addition to pilot scale RAS.

Biofilm and biofilter kinetics

The degradation of chemical additives undergoes transport similar to other substrates from the bulk phase via diffusion to the biofilm. Formaldehyde is a special case regarding the decay kinetics. As a C1-compound (see section 4) it can be utilized as a substrate by several groups of microorganisms (Pedersen et al., 2007). The decay kinetics is similar to nitrification kinetics in biofilm, as observed in different reaction order in the bulk-phase (Henze et al., 2002; Eding et al., 2006). This fixed film process involves mass transport via diffusion from the bulk phase into the biofilm and dependent on the penetration depth and concentration level will result in first, half and zero order process in the bulk phase (Henze et al., 2002; Chen et al., 2006). Experiments from Paper I & II with FA exposure to biofilters led to constant removal rates (zero order removal) indicating that the FA concentration did not limit the activity. The only exception was experiments with FA exposure in trickling filters where different decay patterns were observed (Pedersen & Pedersen, 2006; see below).

Table 7.1. Disinfectant decay kinetics and examples of studies with formaldehyde (FA), hydrogen peroxide (HP) or peracetic acid (PAA), modified from Falsanisi et al., 2007.
1/ Pedersen et al., 2007 2/ Pedersen et al; Paper II, 3/ Sortkjær et al, 2008, 4/ Pedersen et al, 2006, 5/ Sortkjær et al., 2008, 6/ Møller et al., Paper V, 7/ Santoro et al., 2006, 8/ Pedersen et al., Paper III, 9/ Antonelli et al., 2002; 10/ Falsanisi et al., 2007, 11/ Wagner et al., 2002.

	Empirical description	Empirical description with sign. initial decay (D≠0)	FA	HP	PAA
0° order	$C_t = C_0 - k_0 \cdot t$	$C_t = (C_0 - D) - k_0 \cdot t$	1-3		7
1° order	$C_t = C_0 \cdot e^{-k1 \cdot t}$	$C_t = (C_0 - D) \cdot e^{-k}1^{\cdot t}$		4-6	8-10
2° order	$C_t = C_0/(1+k_2 \cdot C_0 \cdot t)$	$C_t = (C_0 - D)/(1+k_2 \cdot C_0 \cdot t)$			11

Hydrogen peroxide is, as opposed to peracetic acid, broken down enzymatically by microbial catalase activity (Wood & Sørensen, 2001; Kitis, 2004; Pedersen et al., 2006). Both chemicals can be degraded via chemical oxidation (see section 5 & 6). HP and PAA decay kinetics do not reflect the removal kinetic of a substrate as was the case with FA.
A complex series of interdependent variables exist that influence the degradation of these chemicals e.g. concentration and physiochemical property, biofilm thickness, water pH, temperature etc.. The degradation rate can be estimated by linear curve fits by use of temporal data in closed systems (Table 7.1).

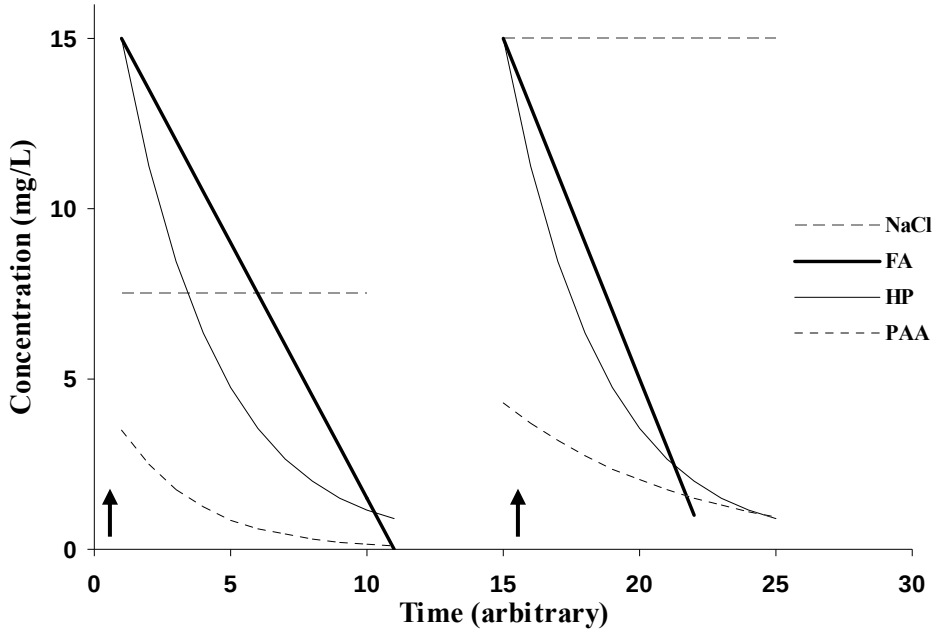


Fig. 7.1. Examples of different decomposition patterns of chemical added twice (marked with arrows) in closed RAS with fixed submerged biofilters assuming fast mixing and equilibrium. NaCl is an inert compound with no decay (constant) and no effect of repeated application (cumulative effects on concentration). Formaldehyde (FA) degrades with a constant decay that increases after repeated applications. Hydrogen peroxide degrades exponentially (1.st order) with similar rates regardless of repeated application. Peracetic acid degrades similar to HP, though reduced decay rate after repeated application (i.e. *k* = 0.30 and 0.15 respectively). Empiric description of chemical decay are here 0. and 1. order processes: $C_t = C_0 - k_0 * t$ (i.e. NaCl and FA) and $C_t = C_0 \cdot e^{-k1 \cdot t}$ (i.e. HP and PAA) where k_0 is a removal constant and k_1 is a removal rate constant. C_0 and C_t represent nominal and concentration at time t, respectively.

Table 7.2. Biofilter removal and response to chemical treatment

Chemical	Biofilter and treatment	Removal rate[A]	Effect on nitrification	Reference
Formalin	Trickling filter 15-20 °C	50 $g \cdot m^{-3} \cdot d^{-1}$	n.a.	Dickerson 1955
Formalin	Batch experiments shell	n.a.	No	Collins et al., 1975
Formalin	Comm. system Exponet-200 Subm. fixed BF, 20-22 °C	~ 45 $mg \cdot m^{-2} \cdot t^{-2}$ ~ 220 $g \cdot m^{-3} \cdot d^{-1}$	Yes	Wienbeck & Koops, 1990
Formalin	Fluidized sand bed filter	~ 220 $g \cdot m^{-3} \cdot d^{-1}$	Yes/ no	Heinen et al., 1996
Formalin	Fluidized sand bed filter		Yes/ no	Schwartz et al., 2000
Formalin	Commercial SW RAS	n.a.	Yes; Nitrite accumulation	Keck & Blanc, 2002
Formalin	Trickling filter Expo-Net 150, 5-15 °C	8-20 $mg \cdot m^{-2} \cdot t^{-1}$ ~ 30-70 $g \cdot m^{-3} \cdot d^{-1}$	(No)	Pedersen & Pedersen, 2006
Formalin	Different size of subm. fixed BF, Exponet 200, 5-16 °C	2-6 $mg \cdot m^{-2} \cdot t^{-1}$ ~10-30 $g \cdot m^{-3} \cdot d^{-1}$	No	Pedersen et al., 2007
Formalin	Commerical fish farm low dose/prolonged	25 $mg \cdot m^{-2} \cdot t^{-1}$ [B]	(No)	Sortkjær et al., 2008
Formalin	Fixed subm. BF Exponet 150 HD	3-26 $mg \cdot m^{-2} \cdot t^{-1}$ ~ 10-95 $g \cdot m^{-3} \cdot d^{-1}$	No	Pedersen et al., Paper III
Hydrogen peroxide	HP 100 ppm treatment in FSB filter		Yes	Schwartz et al., 2000
Hydrogen peroxide	Sodiumpercarbonate to subm. Fixed BF at 16° C		No	Pedersen et al., 2006
Hydrogen peroxide	Repetitive HP to subm. fixed Exponet 150 HD		Yes / no	Møller et al., Paper V
Hydrogen peroxide	Repetitive in submerged BF Unit and pilot RAS		no	Pedersen et al., Sortkjær et al., 2008
Peracetic acid	PerAquaPlus to subm. fixed BF Exponet 150 HD		Yes	Pedersen et al., Paper III
Chl.-T	Chl.-T		No	Gaikowski et al. 2004
Chl.-T	Chl.-T treatment to FSB filter		Yes	Schwartz et al., 2000

A: Removal rate of formaldehyde based on biofilter surface and volume specific removal
B: Estimated value; 60 kg 37 % formalin is removed in 17 hrs with 172.000 m^2 biofilter
n.a.: no data available;

Decay kinetics of disinfectants can theoretically be modelled taking a number of influencing parameters into account. Decay rates of disinfectants can also be empirically estimated by curve fitting data of concentration changes over time, typically linear or single exponential decay (Newman, 1995; Falsanisi et al., 2007). This approach can describe a specific system response, but not necessarily a uniform pattern for a given chemical additive. Figure 7.1 illustrate different decay kinetics and fate after repetitive exposure. In addition to constant decay or exponential decay, in one case with trickling filters, formaldehyde decay was found to fit square root transformation of FA (Fig. 7.2).

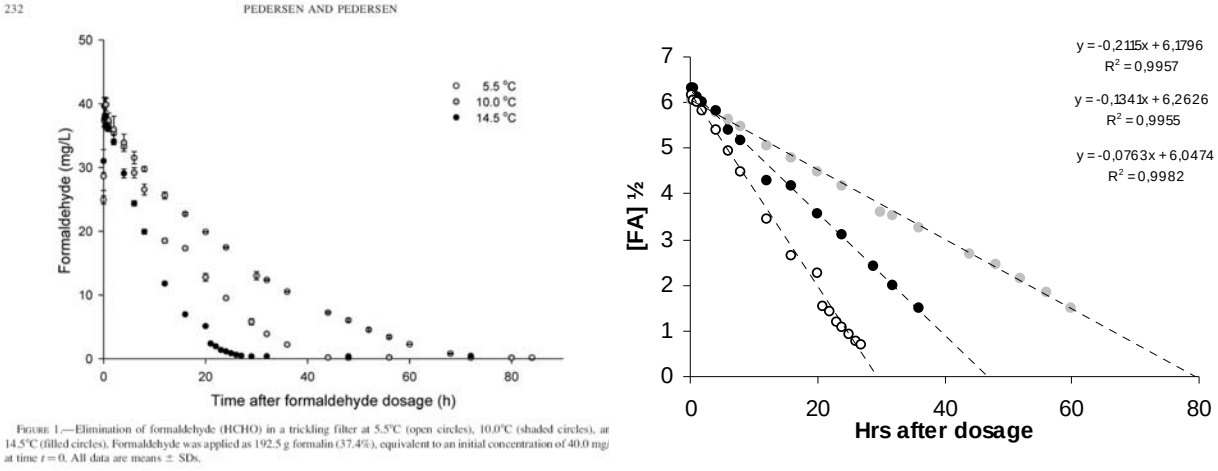


Fig. 7.2. Examples of FA decay in trickling filters with raw data, (left; Pedersen & Pedersen, 2006) and √-transformed values, right.

The water phase degradation of chemicals may contribute significantly to observed biofilter degradation, particularly if the system water has high organic content and microbial abundance. Formaldehyde removal in aquaculture system water is < 0.1 mg· h^{-1} FA, (Weinbeck & Koops, 1990; Sortkjær et al., 2008a). Occasionally rates are up to 0.2 mg· h^{-1} FA in RAS systems with high COD levels (Pedersen et al., 2008), but still orders of magnitude smaller than removal in activated sludge reaching well above >> 10 mg·l^{-1}·h^{-1} (Eiroa et al., 2005). Hydrogen peroxide and peracetic acid degradation in the water phase is significant, with contribution levels between 10 to 25% of the total removal in RAS (Møller et al, Paper V; Pedersen et al, Paper III).

The relative chemical consumption in the water phase, as well as the biofilter volume:total water volume are both important factors to consider when modeling the fate of chemicals in RAS.

RAS response to chemical treatment

Study approaches have typically measured or monitored changes in biofilter performance as changes in TAN or nitrite level after a treatment situation (Table 7.2). Some studies have been performed at commercial facilities where certain factors have been beyond experimental control, e.g. knowledge of biofilter properties or degree of dilution.

Biofilter performance can be assessed as changes in TAN inlet – TAN outlet and by multiplying with the biofilter flow an absolute rate can be estimated. A slightly different approach is to add a known amount of TAN or nitrite and measure removal rates. The method has some drawbacks: i) an addition of NH_4Cl or $NaNO_2$ can be considered an acute disturbance in itself to the fish and/or to the biofilter, ii) the biofilter status and activity can change on a diurnal basis and iii) high ammonia levels may inhibit NOB activity.

However, if true replicates are included in the experimental design, or if baseline experiments are repeated (before and after a disinfection event) this method can provide valuable information on the direct effect of a disinfectant on biofilter performance.

NOB is often found to be more sensitive to disturbances than AOB (Keck & Blanc, 2002); however few studies have spiked RAS biofilter systems with a nitrite salt. Direct spiking with chemical to RAS with fish has obvious limitations, but an experimental setup with sub-units of colonized biofilter allows both spiking and numerous replications, and can be very informative in single factor experiments (Møller et al., Paper V).

Biofilter performance – spiking experiments

The degree of chemically induced inactivation of nitrifiers can be estimated in different ways. Process measures of TAN and/or nitrite changes during a biofilter bypass (i.e. $\Delta TAN = [TAN]_{inlet} - [TAN]_{outlet}$) is a valid measure considering undisturbed baseline values, stable flow and retention time (Schwartz et al., 2000). Ammonium or nitrite spiking directly to the system can be an option although sometimes at certain

toxicological risks if fish are included in the experiments (Pedersen et al., 2008). A modified method is to remove a colonized biofilter unit from an operating biofilter and transfer it to a standardized experimental set-up. A tailor made cylinder reactor tube was developed specifically to fit individual Bioblok cylinders (Fig. 7; L.F. Pedersen, *Unpubl.*).

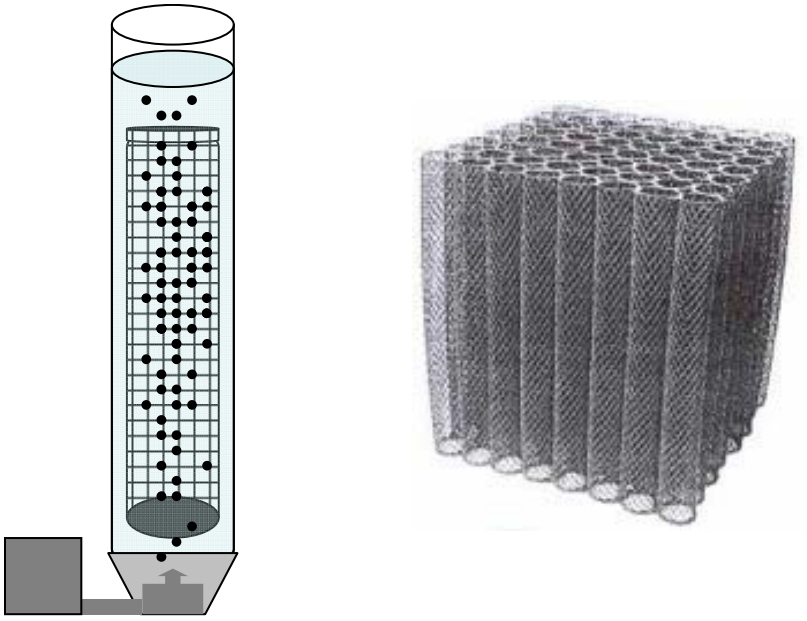


Fig. 7.3. Left: Illustration of a single unit of the experimental set-up to test biofilter nitrification performance. A detached Bioblok® biofilter cylinder (Ø 55mm; L 550 mm) can be submerged and provided with air by an airpump connected to a diffuser or optional internal flow from a circulation pump. Right: Bioblok 200 module; 55*55*55 cm; (www.expo-net.dk).

The experimental setup includes twelve identical tubes, which allow numerous factorial design and true replicates without the risk of system collapse. Preliminary experiments at DTU-Aqua showed significant conformity among filter elements and a high degree of similarity between surface specific TAN removal rates in filter units and in pilot scale RAS (M. Noteboom, *unpubl. data* ; L-F Pedersen, *unpubl. data*).

Preliminary experiments with peracetic acid were performed with the setup. Colonized biofilter units were transferred to individual tubes and after 1 h acclimatization, different dosages of PA+ was added. PAA decay was measured by sticks and HP according to Pedersen et al. (2006). After complete removal of peroxygen, ammonium chloride was added to all reactors and TAN and nitrite concentrations were measured over time. The study showed that 0.6 mg PAA/L (5 ml PA+/L) had no effect on TAN removal and only minor effect on nitrite removal (Fig. 7.4). In contrast, five time higher PA+ dose led to slightly lower TAN removal rate but a highly significantly inhibited nitrite oxidation. The investigation also proved the validity of the set up with low variation within treatment (triplicate).

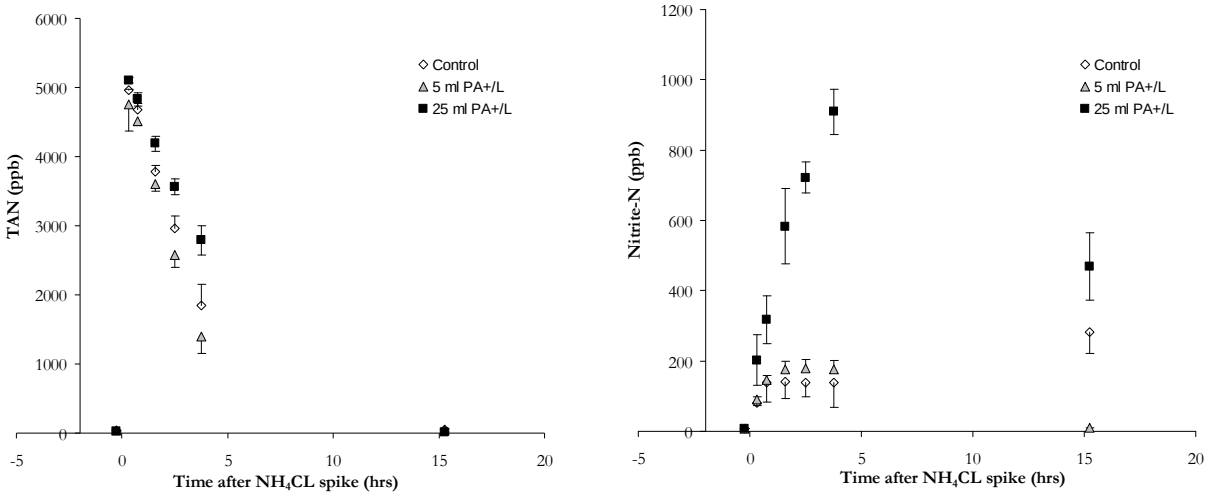


Fig. 7.4. Evaluation of biofilter performance after exposure to Peraqua Plus (PA+).
PA+ (containing 12.5 % PAA and 34 % HP) were added to individual bioblok units (cylinder tube) in individual reactors (N=9) and after complete disinfectant decay, reactors were spiked with NH_4Cl and simultaneous TAN and nitrite concentrations were measured. Ammonium removal ranged from 0.18-0.27 g TAN·m^{-2}·d^{-1} (L-F Pedersen; *unpubl. data*).

Nitrite spiking

As described above, and as often observed in other studies, it is the second step in the nitrification process that is most vulnerable to disturbances (Keck & Blanc, 2002). Nitrite levels have to be kept low in RAS and nitrite spiking is associated with pronounced risk to the fish. The experimental set up depicted in Fig. 7 was applied to quantify biofilter nitrite oxidation as part of an investigation of peracetic acid inhibition (Pedersen et al., Paper III). From full scale RAS biofilters exposed to 3 different levels of PAA two days

prior, colonized biofilter units were transferred to the test tubes with system water, acclimatized for 1 hr and spiked with sodium nitrite equivalent to 3 mg NO_2^--N/L (Fig. 7C).

The filters from the unexposed systems removed the added nitrite within 24 hrs. whereas no significant removal occurred with all filters from RAS exposed to medium or high PAA. Filter units from the same RAS exposed to low PAA showed a significant different pattern; in one biofilter nitrite removal was slightly lower compared to the control, whereas no nitrite was oxidized in the other two filters. An explanation for this pattern has not been found; however the experimental protocol (physical removal of filter from one to another system with air and light exposure) may have triggered an inactive state (on/off) on the susceptible biofilter.

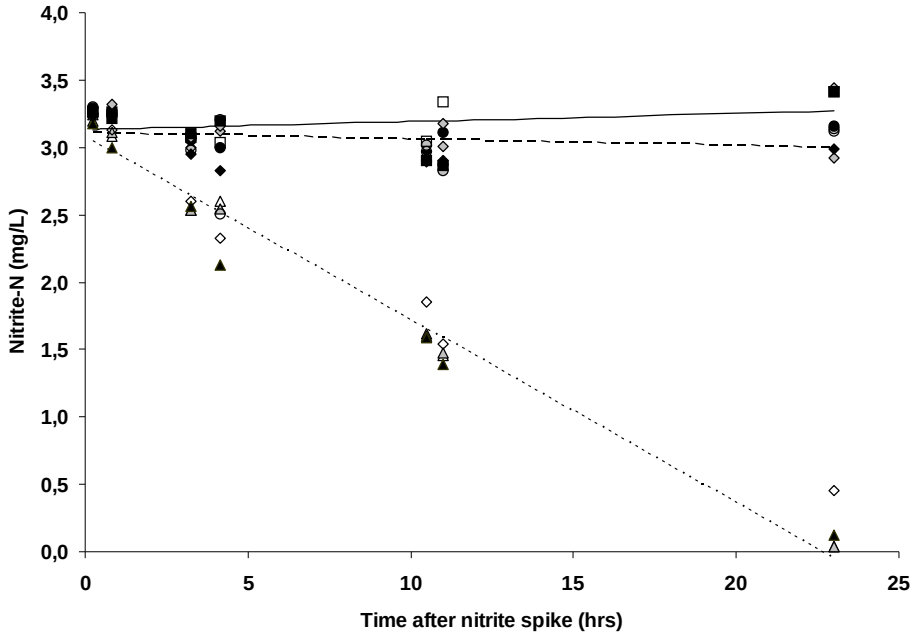


Fig. 7.5. Nitrite concentrations from experiments with biofilter units spiked with nitrite.
Biofilter units were transferred from RAS biofilters eight days after PAA exposure at a nominal dosage of 1.0 mg PAA/L (diamond symbols), 2.0 mg PAA/L (circles) or 3.0 mg PAA/L (squares) and unexposed control (triangles), (N=12). Trend lines are indicated for high and medium PAA (solid and broken lines) and the unexposed control groups (stippled); trend line for low dose PAA exposure not indicated due to a significant variation within the triplicate groups.

Antimicrobial resistance

Antimicrobial resistance of biofilms to antibiotics has been observed to increase considerably compared to what is normally seen with planktonic cells; 10-1000 fold increase in resistance has been observed (Mah & O'Toole, 2001). Microbial colonies are embedded in EPS which may serve as a physical barrier to reduce biocide penetration and action. EPS can adsorb and partly inactivate the biocide and the presence of organic matter contributes to a depletion of chemical reactive agents, i.e. oxidants. The diverse microenvironment in a biofilm can also promote phenotypic elasticity, and allow certain groups of inhabitants to respond differently to the biocide (stressor).

The potential existence of cells or organisms of various activity stages ("strategies"), e.g. persister cells and sporulating species, may also enhance the overall biofilm tolerance to a given antimicrobial. Due to the mechanisms of diffusion mediated substrate transport from bulk to biofilm, biofilm tolerance is positively correlated with biofilm thickness. The concentration of the disinfectant in the bulk-phase has a direct impact on the penetration depth, and as concentration levels decrease, only part of the biofilm will be affected. The underlying mechanisms of protection, inhibition and recovery of nitrifiers are not studied, but could provide important knowledge in understanding the ecophysiology and strength and weaknesses of biofilters.

In conclusion, the relatively few studies done with HP and PAA, indicate that treatment margins of these chemical to biofilter systems are narrower compared to formaldehyde. Formaldehyde, at concentrations below 30 mg/L, does not seem to have any negative effects on biofilter performance and is relative safe to use. The effect of biofilm thickness and robustness towards oxidants is not understood or described but may expand the treatment margin.

7.8. Environmental context

Application of water borne therapeutics in aquaculture can potentially have environmental impacts. The negative effects arise e.g. when excess disinfectant is discharged at levels inferring with or evenn toxic to the recipient fauna. Only recently this intricacy has been underscored and now chemical disinfectants and antibiotic-residuals pose a significant challenge to the aquaculture industry (Boyd et al., 2005; Bruun et al., 2007).

A consequential environmental assessment of aquaculture treatment practices is complex due to the numerous influential factors. Basically, a drug-specific threshold safety level has to be defined and discharge patterns (magnitude and duration) have to be predicted in a reliable way. In Europe, according to the Water Frame Directive, threshold levels are assessed by a standard procedure of evaluating toxicological data (TGD, 2003). Based on the lowest EC or NOEC from three trophical levels of animals, a concentration value is extracted and further reduced by a factor 20-1000 depending on the amount and quality of data available (EPA, 2004). This precautionary approach is recommended in order to ensure no living animals, irrespective of life stage or species is to be subjected to unfavourable conditions. The water quality criteria's (WQC) are defined either in terms of average discharge concentrations during treatment (typically 24 hours) called WQC or as a maximum acute value not to be exceed named AWQC (EPA, 2004). The respective values for formaldehyde are 9.2 µg/L and 46 µg/L, respectively, while hydrogen peroxide has values of 10 µg/L and 100 µg/L. No values have been proposed for peracetic acid and chloride so far.

Discharge patterns, normally bell shaped time-curves, are generally affected by system design and hydraulic conditions, whereas drug specific removal kinetics is variously affected by temperature, organic matter content, pH, water hardness and iron content, biofiltration, adsorption and precipitation. A simple dilution model can give fairly accurate estimates of drug discharge concentration (Gaikowski et al., 2004). However, calculated/predicted concentration values are typically higher than the actual measured

values due to significant consumption of the chemical immediately after dosage (Sortkjær et al, 2008a). The discrepancy between conservative estimates and actual concentrations increases with the (bio-) degradability of the chemicals, exemplified by a study with hydrogen peroxide which was hardly found outside the fish farm facility (Saez & Bowser, 2001). Fig. 8.1 illustrates the processes that affect FA concentration when applied to an earthen pond. The microbial FA degradation cause biofilters and retention time to become determining factors for the FA removal within the system (Sortkjær et al, 2008b). This imply that partial recirculation over a biofilter in some situations could be a technical solution to reduce FA.

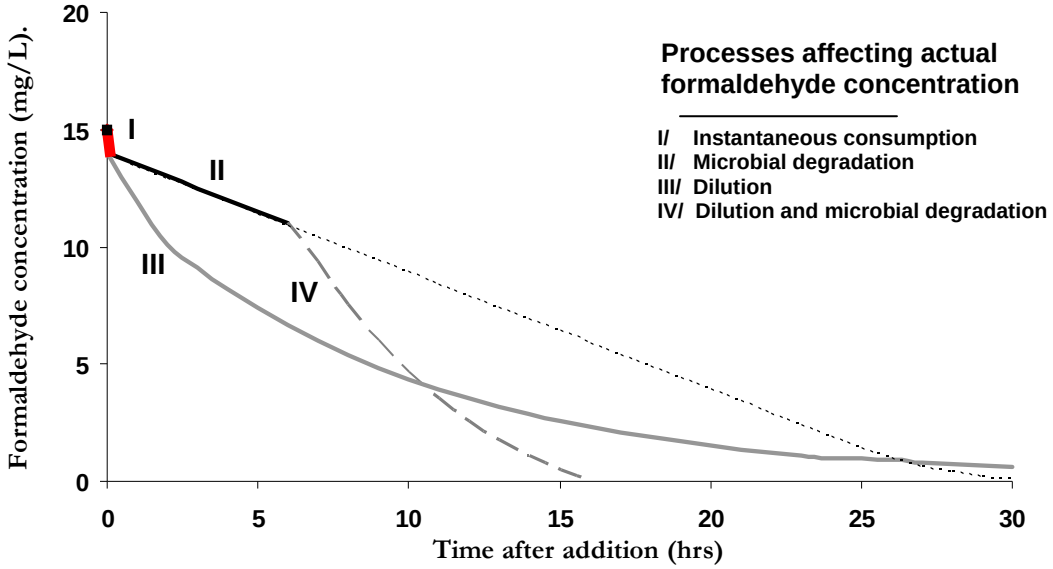


Fig. 8.1 Example of formalin application to RAS with adjustable retention time; C_0 = 15 FA mg/L (from Sortkjær et al., 2008).

Especially use and discharge of formaldehyde is a current issue of concern (Hochreiter & Riggs, 2001; Masters, 2004; Gearheart et al., 2006 Sortkjær et al., 2008). Different studies have documented significantly biodegradation of FA and means to increase microbial abundance and contact time is implicit related to aquaculture biofilters, and could be a technical solution to further reduce FA discharge (Pedersen & Pedersen, 2006; Pedersen et al., 2007).

The use of FA per ton produced fish may increase as production is intensified and water recirculated. In an older EIFAC report, average FA consumption per tons produced fish to be 0.7 kg was reported (Alabaster, 1982). Recent Danish data show that that 1.2-4.6 kg FA were used per tons fish produced during 2001 to 2005, further exceeded by FA consumptions of 13.6-13.8 kg per metric tons fish produced at newly established RAS. In an environmental context, FA use is only of concern if the amount used violates the discharge regulations. This implies that technical solutions can either neutralize or degrade formaldehyde to comply with the regulations.

Table 8.1. Account of chemicals applied according to reports from Danish fish farmers from 2001-2005 (EPA, 2006). Total trout production average 33.000 MT/y

Chemical[A]	2001	2002	2003	2004	2005
Formalin, 37% (L)	108.843	134.751	151.284	65.571	40.314
HP-products[B] (L)	4.178	7.210	5.271	7.561	1.961
Sodium percarbonate (kg)	11.696	23.703	3.598	9.503	2.333
Sodium chloride (kg)	400	67.100	41.200	31.525	63.881

[A] Records of other chemicals (lime, copper sulphate, Chloramine-T) or antibiotics are not included; see EPA 2007). [B] Various commercial peracetic acid solutions

Table 8.2. Account of chemicals applied in newly implemented RAS (DA, 2008) – representing approx. 10% of annual production i.e. 3.042 MT in 2006 and 3.544 MT in 2007.

Chemical	2006	2007
Formalin, 37% (L)	42.000	48.000
HP-products (L)	325	25
Sodium percarbonate (kg)	-	-
Sodium chloride (kg)	129.000	230.000

Treatment efficiency of FA against *Ich* has occasionally been reported to diminish over time (Niels Henrik Henriksen, *pers. comm.*). These observations have not been experimentally verified or falsified yet. A potential explanation could be either ascribed to an increased microbial FA consumption (underdosing) or to increased protozoan resistance.

Recent studies of FA discharge from nine Danish freshwater fish farms after a simulated FA treatment showed that only RAS with biofilters and prolonged retention time (Model trout farm type III) could comply with existing EPA values (Sortkjær et al., 2008B; Table 8.3).

Table 8.3. Summary of the resultant formaldehyde discharge concentration from nine different field experiments. The AWQC for maximal formaldehyde in the recipient is 46 µg/L;.

Type of aquaculture system	Flow-through with low retention time			Raceways with medium retention time			Raceways with BF[a] and high HRT		
Commerical fish farm ID	A	B	C	D	E	F	G	H	I
Maximal [FA] in effluent (µg/L)	540	250	330	7420	3850	640	19	280	< 1
Maximal [FA] in receiving water body (µg/L)	270	125	165	3090	1540	255	0.8	12	< 0.1
Surface specific FA removal in biofiltre ($mg/m^2/h$)							27.5	8.9	6.4

[a]: BF= fixed bed biofilter

The findings underline the fact that traditional water treatment practices with FA far from comply with the discharge regulations, and in worse case scenarios FA discharge exceed the maximum allowable value several times. In these cases, a revised and better management practice is required. In the cases with fish farm D-F, very large rearing units were treated, and therefore higher FA amounts added.

Better management

The term *best management practice* can with some advantage be replaced by *better management practice* and hence describe a dynamic process of measures to improve an industry, for example aquaculture (Clay, 2008). This ultimately allows comparison and certification of aquaculture facilities which is the anticipation of the recent launched WWF initiative regarding aquaculture dialogues (www.wwf.org).

Figure 8.2 depict a conceptual idea of management performance. By regulations or incentives to allow increased production, the average performance of the aquaculture unit improves, hence resulting in a performance shift. One index of performance could theoretically be gram chemical X discharged per MT fish produced.

Improved management in terms of replacing slowly degradable drugs with easy degradable and/or more environmental neutral chemicals is one option, another is to use less chemical while increasing contact time as exemplified by Heinecke & Buchmann, 2009. Alternatively, management strategies that deliberately abolish use of formalin now have en option to be certified as organic fish farmers. This type of production strategy presently appeal to smaller, more extensive entrepreneurs, which presently have succeeded in producing an attractive premier price niche product (Pedersen et al., 2005).

Present, no obvious chemical agents can replace formaldehyde, and though sodium chloride can reduce white spot disease and is used in increasingly amounts (> 240.000 kg in 2007), the environmental issues are not solved (Saez & Bowser, 2001). However, use of salt and the resulting salinity rise might also have potential detrimental consequences for the natural environment.

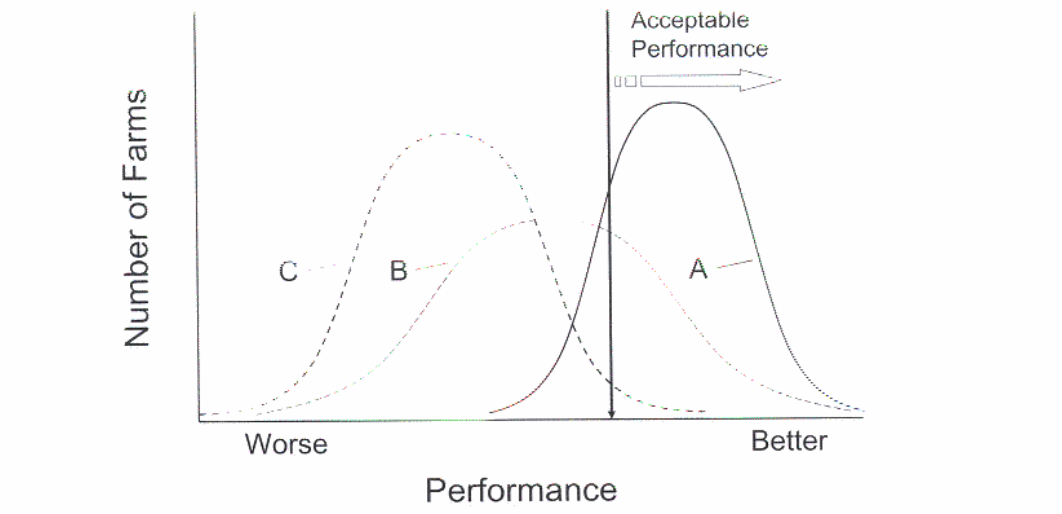


Fig. 8. 2. Example of different performance from three arbitrary types of aquaculture systems (Clay, 2008).

7.9. Conclusion and future needs

The literature reviewed gives a basis to identify future needs regarding chemical use in RAS. Only recently, issues regarding chemotherapeutic discharge from aquaculture have been raised, and still relatively few studies have focused on related aspects. However, in order for the industry to be at the cutting edge and anticipate future trends, it is important to continuously improve management practice.

The present thesis has documented potential low-tech solutions to reduce use and discharge of formaldehyde. The scope for additional improvement in terms of implementation of alternative environmental neutral disinfectants (e.g. hydrogen peroxide or peracetic acid) requires more research of the antiparasitic efficiency and associated risks. Increased knowledge and experience with the "new" therapeutic agents is required to address the question to which degree formaldehyde can be practically replaced.

When it comes to investigation of water disinfectants in RAS, several trade-offs have to be made. Studies have to reflect commercial conditions and at the same time accessed scientifically, which imply parameter control and experimental replication. Biofilter systems require relatively long experimental periods to be in steady state, and a number of interrelating factors often complicates interpretation from study to study. And the fact that chemotherapeutics potentially can cause biofilter collapse does not appeal to commercial scale testing due to the economical risks and costs of investigations. Therefore, further investigations on pilot scale level are needed before full scale testing.

Knowledge from municipal waste water technology should be transferred to aquaculture research. Molecular approaches have improved the understanding of microbial composition and their ecophysiology in activated sludge, but hardly utilised in aquaculture research. By addressing these microbial processes under controlled aquaculture conditions, there is a chance to shed more light into the black boxes of biofiltration

Table 9 lists a number of topics which could lead to an increased understanding of the interactions of different levels and ultimately support technical utilisation.

Table 9: Selected research issues concerning chemotherapeutants and RAS

Objective	Rationale
Biofilter process oriented experiments	
Disinfectant decay in different biofilter types	Documentation and modelling
Disinfectant kinetics; i.e. effect of biofilm thickness	Documentation and modelling
ORP and peroxygen correlations in water	Control during treatment
Nitrite spiking - bioassays	Bioassay development
Exposure/contact time and inactivation correlation	To define "tipping point" of biofilters
New chemicals – e.g. performic acid, potassium permanganate	Potential substitution for FA – or disinfection colour marker
Denitrification – FA removal capacity	Technical solution to RAS
Fish physiology experiments	
Physiological responses to (new) disinfectants	Biomarker development and fish welfare
Investigate TAN and nitrite thresholds under stable RAS conditions	Challenge existing paradigms and define safe values
Pilot to full scale experiments	
Disinfection strategy (all in/ system design)	Define effective protocols
Peroxygen treatment testing in full scale / commercial RAS (field implementation)	Investigate the practicability – identify guideline
Microbiological approaches	
Archaea function and abundance in RAS	Assess abundance and relative importance of AOA
To investigate potential FA metabolizing nitrifiers	Potential and plasticity
Microbial abundance in biofilter – correlation to robustness against chemical stress	Define a measure / index
Mechanisms of peroxygen inactivation; measures of catalase activity	Understand mechanisms and distinguish inactive from dead
HCHO microbial memory – acclimatization	Microbial ecophysiology
Technical developments	
Electrochemical water treatment	Technical preventive solution
Peroxygen dosage and regulation	Improve treatment efficiency
System specific assays – including biofilter testing	Routine testing at every RAS
Biosecurity and preventive measures	Proactive measures
Effluent monitoring	Documentation

7. 10. REFERENCER

1. Aarestrup,K. and Koed,A. 2003. Survival of migrating sea trout (Salmo trutta) and Atlantic salmon (Salmo salar) smolts negotiating weirs in small Danish rivers. Ecology of Freshwater Fish 12: 169-176.

2. Alabaster, J. S. 1982. EIFAC Technical Paper (FAO) , Report no. 41*; Workshop on Fish-Farm Effluents*, Silkeborg (Denmark) / FAO, Rome (Italy). Fisheries Dept. ISBN 92-5-101162-1, pp 165.

3. Alasri,A., Roques,C., Michel,G., Cabassud,C., and Aptel,P. 1992. Bactericidal properties of peracetic-acid and hydrogen-peroxide, alone and in combination, and chlorine and formaldehyde against bacterial wastewater. Canadian Journal of Microbiology 38: 635-642.

4. Amann,R. and Fuchs,B.M. 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6: 339-348.

5. Amann,R.I., Krumholz,L., and Stahl,D.A. 1990. Fluorescent-Oligonucleotide Probing of Whole Cells for Determinative, Phylogenetic, and Environmental-Studies in Microbiology. Journal of Bacteriology 172: 762-770.

6. Angelucci,G., Viale,I., Fenza,A., and Salati,F. 2008. Fish-farm application of a direct prophylaxis plan against flexibacteriosis and myxosporidiosis. Bulletin of the European Association of Fish Pathologists 28: 245-251.

7. Antonelli,M., Rossi,S., Mezzanotte,V., and Nurizzo,C. 2006. Secondary effluent disinfection: PAA long term efficiency. Environmental Science & Technology 40: 4771-4775.

8. Arndt,R.E. and Wagner,E.J. 1997. The toxicity of hydrogen peroxide to rainbow trout Oncorhynchus mykiss and cutthroat trout Oncorhynchus clarki fry and fingerlings. Journal of the World Aquaculture Society 28: 150-157.

9. Ashley,P.J. 2007. Fish welfare: Current issues in aquaculture. Applied Animal Behaviour Science 104: 199-235.

10. Avnimelech,Y. 2006. Bio-filters: The need for an new comprehensive approach. Aquacultural Engineering 34: 172-178.

11. Baldry,M.G.C. 1983. The Bactericidal, Fungicidal and Sporicidal Properties of Hydrogen-Peroxide and Peracetic-Acid. Journal of Applied Bacteriology 54: 417-423.

12. Baldry,M.G.C. and French,M.S. 1989. Activity of Peracetic-Acid Against Sewage Indicator Organisms. Water Science and Technology 21: 1747-1749.

13. Balta, F. Kayis S. & Altinok I. 2008. External protozoan parasites in three trout species in the eastern Black Sea region of the Turkey: intensity, seasonality and their treatments. Bulletin of the European Association of Fish Pathologists, Vol. 28(4): 157-162.

14. Bebak-Williams, J. Noble A. Bowser P. R. Wooster G. A. 2007. Fish Health Management. In: Timmons, M.B., Ebeling, J.M. (Eds.) Recirculating Aquaculture. Cayuga Aqua Ventures, Ithaca, 975pp.

15. Bergh,O. 2007. The dual myths of the healthy wild fish and the unhealthy farmed fish. Diseases of Aquatic Organisms 75: 159-164.

16. Bergheim,A. and Brinker,A. 2003. Effluent treatment for flow through systems and European Environmental Regulations. Aquacultural Engineering 27: 61-77.

17. Blackburne,R., Vadivelu,V.M., Yuan,Z.G., and Keller,J. 2007. Kinetic characterisation of an enriched Nitrospira culture with comparison to Nitrobacter. Water Research 41: 3033-3042.

18. Block,S.S. Peroxygen compounds *In* S. S. Block (ed.), Disinfection, sterilization, and preservation, 4th ed. Lea & Febiger, Philadelphia, Pa. ISBN 0-683-30740-1.

19. Boller,M., Gujer,W., and Tschui,M. 1994. Parameters Affecting Nitrifying Biofilm Reactors. Water Science and Technology 29: 1-11.

20. Botton,S., van Heusden,M., Parsons,J.R., Smidt,H., and van Straalen,N. 2006. Resilience of microbial systems towards disturbances. Critical Reviews in Microbiology 32: 101-112.

21. Boyd,C.E. and Massaut,L. 1999. Risks associated with the use of chemicals in pond aquaculture. Aquacultural Engineering 20: 113-132.

22. Boyd,C.E. 2003. Guidelines for aquaculture effluent management at the farm-level. Aquaculture 226: 101-112.

23. Boyd,C.E., McNevin,A.A., Clay,J., and Johnson,H.M. 2005. Certification Issues for Some Common Aquaculture Species. Reviews in Fisheries Science 13: 231-279.

24. Bredholt,S., Maukonen,J., Kujanpaa,K., Alanko,T., Olofson,U., Husmark,U., Sjoberg,A.M., and Wirtanen,G. 1999. Microbial methods for assessment of cleaning and disinfection of food-processing surfaces cleaned in a low-pressure system. European Food Research and Technology 209: 145-152.

25. Brinker,A. 2007. Guar gum in rainbow trout (*Oncorhynchus mykiss*) feed: The influence of quality and dose on stabilisation of faecal solids. Aquaculture 267: 315-327.

26. Bruun, M. S. Pedersen L. F. Dalsgaard I. Pedersen P. B. and Sortkjær O. 2007. The fate of chemical additives and antimicrobial agents applied in Danish freshwater fish farms. World of Aquaculture Magazine, 57-61.

27. Buchmann,K. and Bresciani,J. 1997. Parasitic infections in pond-reared rainbow trout Oncorhynchus mykiss in Denmark. Diseases of Aquatic Organisms 28: 125-138.

28. Buchmann,K., Bresciani,J., and Jappe,C. 2004. Effects of formalin treatment on epithelial structure and mucous cell densities in rainbow trout, Oncorhynchus mykiss (Walbaum), skin. Journal of Fish Diseases 27: 99-104.

29. Buchmann,K., Jensen,P.B., and Kruse,K.D. 2003. Effects of sodium percarbonate and garlic extract on Ichthyophthirius multifiliis theronts and tomocysts: In vitro experiments. North American Journal of Aquaculture 65: 21-24.

30. Buchmann,K. and Kristensson,R.T. 2003. Efficacy of sodium percarbonate and formaldehyde bath treatments against Gyrodactylus derjavini infestations of rainbow trout. North American Journal of Aquaculture 65: 25-27.

31. Burka,J.F., Hammell,K.L., Horsberg,T.E., Johnson,G.R., Rainnie,D.J., and Speare,D.J. 1997. Drugs in salmonid aquaculture - A review. Journal of Veterinary Pharmacology and Therapeutics 20: 333-349.

32. Chan, W-H. & and .S.C.Lee. 1998. Formaldehyde in the environment. In Encyclopedia of environmental analysis & remideation, Vol. 3, Ed. A. Meyer. ISBN 0-471-11708-0.

33. Chapman,J.S. 2003. Biocide resistance mechanisms. International Biodeterioration & Biodegradation 51: 133-138.

34. Chen,S.L., Ling,J., and Blancheton,J.P. 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacultural Engineering 34: 179-197.

35. Clay,J.W. and Eds.Tucker,C.S.&.H.J.A. 2008. The role of better management practices in environmental management. In Environmental best management practices for aquaculture. Blackwell Publishing, ISBN-13: 978-0-8138-2027-9.

36. Colgan,S.&.G.R. 2001. Disinfection. Water Environment and Technology, p.29-33.

37. Collins, M. T. Gratzek J. B. Dawe D. L. & Nemetz T. G. 1975. Effects of parasiticides on nitrification. Journal of Research Board Canada, Vol. 32:2033-2037.

38. Collivignarelli,C., Bertanza,G., and Pedrazzani,R. 2000. A comparison among different wastewater disinfection systems: Experimental results. Environmental Technology 21: 1-16.

39. Colt,J. 2006. Water quality requirements for reuse systems. Aquacultural Engineering 34: 143-156.

40. Colt,J., Lamoureux,J., Patterson,R., and Rogers,G. 2006. Reporting standards for biofilter performance studies. Aquacultural Engineering 34: 377-388.

41. Crab,R., Avnimelech,Y., Defoirdt,T., Bossier,P., and Verstraete,W. 2007. Nitrogen removal techniques in aquaculture for a sustainable production. Aquaculture 270: 1-14.

42. Cripps,S.J. and Bergheim,A. 2000. Solids management and removal for intensive land-based aquaculture production systems. Aquacultural Engineering 22: 33-56.

43. Davidson,J., Helwig,N., and Summerfelt,S.T. 2008. Fluidized sand biofilters used to remove ammonia, biochemical oxygen demand, total coliform bacteria, and suspended solids from an intensive aquaculture effluent. Aquacultural Engineering 39: 6-15.

44. Dawson,V.K., Meinertz,J.R., Schmidt,L.J., and Gingerich,W.H. 2003. A simple analytical procedure to replace HPLC for monitoring treatment concentrations of chloramine-T on fish culture facilities. Aquaculture 217: 61-72.

45. de Lafontaine,Y., Despatie,S.P., Veilleux,E., and Wiley,C. 2009. Onboard Ship Evaluation of the Effectiveness and the Potential Environmental Effects of PERACLEAN (R) Ocean for Ballast Water Treatment in Very Cold Conditions. Environmental Toxicology 24: 49-65.

46. Delabbio,J., Murphy,B.R., Johnson,G.R., and McMullin,S.L. 2004. An assessment of biosecurity utilization in the recirculation sector of finfish aquaculture in the United States and Canada. Aquaculture 242: 165-179.

47. Derksen,J.A., Ostland,V.E., and Ferguson,H.W. 1999. Effects of hydrogen peroxide on clearance of formalin-killed Flavobacterium branchiophilum front the gills of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases 22: 59-67.

48. Diana,J.S. 2009. Aquaculture Production and Biodiversity Conservation. Bioscience 59: 27-38.

49. Dickerson, B. W. 1955. High rate trickling filter operation on formaldehyde wastes. Sewage Indust. Wastes 22, 536-545.

50. Eding,E.H., Kamstra,A., Verreth,J.A.J., Huisman,E.A., and Klapwijk,A. 2006. Design and operation of nitrifying trickling filters in recirculating aquaculture: A review. Aquacultural Engineering 34: 234-260.

51. Eiroa,M., Kennes,C., and Veiga,M.C. 2005. Simultaneous nitrification and formaldehyde biodegradation in an activated sludge unit. Bioresource Technology 96: 1914-1918.

52. EPA 2007. Punkt kilder 2006. Orientering fra Miljøstyrelsen, , p. 154. www.blst.dk.

53. EPA. 2004. Principper for fastsættelse af vandkvalitetskriterier for stoffer i overfladevand. Vejledning fra Miljøstyrelsen [In Dansih]. No. 4 , p. 76. www.mst.dk.

54. Falsanisi,D., Gehr,R., Santoro,D., Dell'Erba,A., Notarnicola,M., and Liberti,L. 2006. Kinetics of PAA demand and its implications on disinfection of wastewaters. Water Quality Research Journal of Canada 41: 398-409.

55. FAO.2009.The state of world fisheries and aquaculture.FAO Fisheries and Aquaculture Department, Rome. ISBN 978-92-5-106029-2 pp 179. 2009.

56. Fish, F. 1932. The chemical disinfection of trout ponds. Transactions of the American Fisheries society, Vol. 63: 158-163.

57. Fish,F.F.&.Burrows, R.F. 1940. Experiments upon the control of trichodiniasis of salmonid fishes by the prolonged recirculation of formalin solutions. Trans.Am.Fish.Soc. 69: 94-100.

58. Foesel,B.U., Gieseke,A., Schwermer,C., Stief,P., Koch,L., Cytryn,E., de la Torre,J.R., van Rijn,J., Minz,D., Drake,H.L., and Schramm,A. 2008. Nitrosomonas Nm143-like ammonia oxidizers and Nitrospira marina-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. FEMS Microbiology Ecology 63: 192-204.

59. Gaikowski,M.P., Larson,W.J., Steuer,J.J., and Gingerich,W.H. 2004. Validation of two dilution models to predict chloramine-T concentrations in aquaculture facility effluent. Aquacultural Engineering 30: 127-140.

60. Gaikowski,M.P., Rach,J.J., and Ramsay,R.T. 1999. Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. Aquaculture 178: 191-207.

61. Gearheart,J.M., Masters,A.L., and Bebak-Williams,J. 2006. Application of methods for the detoxification and neutralization of formalin in fish hatchery effluents. North American Journal of Aquaculture 68: 256-263.

62. Gehr,R., Chen,D., and Moreau,M. 2009. Performic acid (PFA): tests on an advanced primary effluent show promising disinfection performance. Water Science and Technology 59: 89-96.

63. Gieseke,A., Nielsen,J.L., Amann,R., Nielsen,P.H., and de Beer,D. 2005. In situ substrate conversion and assimilation by nitrifying bacteria in a model biofilm. Environmental Microbiology 7: 1392-1404.

64. Gieseke,A., Purkhold,U., Wagner,M., Amann,R., and Schramm,A. 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Appl.Environ.Microbiol. 67: 1351-1362.

65. Gieseker,C.M., Serfling,S.G., and Reimschuessel,R. 2006. Formalin treatment to reduce mortality associated with Saprolegnia parasitica in rainbow trout, *Oncorhynchus mykiss*. Aquaculture 253: 120-129.

66. Glencross,B.D., Booth,M., and Allan,G.L. 2007. A feed is only as good as its ingredients - a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13: 17-34.

67. Graham,D.W., Knapp,C.W., Van Vleck,E.S., Bloor,K., Lane,T.B., and Graham,C.E. 2007. Experimental demonstration of chaotic instability in biological nitrification. Isme Journal 1: 385-393.

68. Grommen,R. and Verstraete,W. 2002. Environmental biotechnology: the ongoing quest. Journal of Biotechnology 98: 113-123.

69. Gunderson,L.H. 2000. Ecological resilience - in theory and application. Annual Review of Ecology and Systematics 31: 425-439.

70. Gutierrez-Wing,M.T. and Malone,R.F. 2006. Biological filters in aquaculture: Trends and research directions for freshwater and marine applications. Aquacultural Engineering 34: 163-171.

71. Hagopian,D.S. and Riley,J.G. 1998. A closer look at the bacteriology of nitrification. Aquacultural Engineering 18: 223-244.

72. Hall-Stoodley,L., Costerton,J.W., and Stoodley,P. 2004. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews Microbiology 2: 95-108.

73. Hasan MR, Hecht T De Silva SS Tacon ADJ eds 2007 497 p. 512. FAO Rome. 2007. Study and Analysis of Feeds and Fertilizers for Sustainable Aquaculture Development. FAO Technical Paper.

74. Heinecke,R.D. and Buchmann,K. 2009. Control of Ichthyophthirius multifiliis using a combination of water filtration and sodium percarbonate: Dose-response studies. Aquaculture 288: 32-35.

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

76. Henriksen, N. H. Michelsen K. & Plessner L. J. 2008. 2008. Drift og fiskesygdomme i modeldambrug (Master Management System) [Management and fish diseases in model fish farms]. Report, 43 p. Available at www.danskakvakultur.dk.

77. Henze, M.Harremoes, P., la Cour Jansen. J. & Arvin .E. 2002. Waste water treatment. Springer Verlag, Berlin. ISBN 3-540-42228-5, p. 430.

78. Hohreiter,D.W. and Rigg,D.K. 2001. Derivation of ambient water quality criteria for formaldehyde. Chemosphere 45: 471-486.

79. Holten, K. B. Skjelstad og A. Aunsmo. 2002. Effekt af Detarox ®AP og formalin mot Costia. VESO Projekt Rapport nr. 1592; p.12.

80. IARC 2004. Formaldehyde monograph. Report from the International Agency for Research on Cancer, p. 287. www.iarc.fr.

81. IWAMA,G.K. 1991. Interactions Between Aquaculture and the Environment. Critical Reviews in Environmental Control 21: 177-216.

82. Janning, K. Jørgensen P. E. Klausen M. M. Højgaard B. & Thomsen U. 2008. 2008. Rens-tek; Optimerede rensningsteknologier til forbedret drift af recirkulerede opdrætsanlæg [In Danish]; DHI rapport, p. 96.

83. Jørgensen,T.R., Larsen,T.B., and Buchmann,K. Parasite infections in recirculated rainbow trout (*Oncorhynchus mykiss*) farms. Aquaculture, Vol. 289: 91-94.

84. Kajimura,M., Croke,S.J., Glover,C.N., and Wood,C.M. 2004. Dogmas and controversies in the handling of nitrogenous wastes: The effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. Journal of Experimental Biology 207: 1993-2002.

85. Keck,N. and Blanc,G. 2002. Effects of formalin chemotherapeutic treatments on biofilter efficiency in a marine recirculating fish farming system. Aquatic Living Resources 15: 361-370.

86. Kepner,R.L. and Pratt,J.R. 1994. Use of Fluorochromes for Direct Enumeration of Total Bacteria in Environmental-Samples - Past and Present. Microbiological Reviews 58: 603-615.

87. Kitis,M. 2004. Disinfection of wastewater with peracetic acid: a review. Environment International 30: 47-55.

88. Koops,H.-P., Purkhold, U. Pommerening.Röser, A. Timmermann, G. & Wagner, M. 2006. The Lithoautotrophic Ammonia-Oxidizing Bacteria. Procaryotes 5: 778-811; DOI: 10.1007/0-387-30745-1_36

89. Koops, H.P. and Pommerening-Rõser,A. 2001. Distribution and ecophysiology of the nitrifying bacteria emphasizing cultured species. FEMS Microbiology Ecology 37: 1-9.

90. Kowalchuk,G.A. and Stephen,J.R. 2001. Ammonia-oxidizing bacteria: A model for molecular microbial ecology. Annual Review of Microbiology 55: 485-529.

91. Krkosek,M., Lewis,M.A., Morton,A., Frazer,L.N., and Volpe,J.P. 2006. Epizootics of wild fish induced by farm fish. Proceedings of the National Academy of Sciences of the United States of America 103: 15506-15510.

92. Kuhn,D.D., Boardman,G.D., Craig,S.R., Flick,G.J., and McLean,E. 2008. Use of microbial flocs generated from tilapia effluent as a nutritional supplement for shrimp, Litopenaeus vannamei, in recirculating aquaculture systems. Journal of the World Aquaculture Society 39: 72-82.

93. Lahnsteiner,F. and Weismann,T. 2007. Treatment of *ichthyophthiriasis* in rainbow trout and common carp with common and alternative therapeutics. Journal of Aquatic Animal Health 19: 186-194.

94. Larsen,P., Olesen,B.H., Nielsen,P.H., and Nielsen,J.L. 2008. Quantification of lipids and protein in thin biofilms by fluorescence staining. Biofouling 24: 241-250.

95. Lee, S. & Radtke T. 1998. Exposure to formaldehyde among fish hatchery workers. *Appl. occup.environ. Hyg*., 13, 3-6 (cited in IACR, 2004).

96. Lefevre,F., Audic,J.M., and Ferrand,F. 1992. Peracetic-Acid Disinfection of Secondary Effluents Discharged Off Coastal Seawater. Water Science and Technology 25: 155-164.

97. Leonard,N., Blancheton,J.P., and Guiraud,J.P. 2000. Populations of heterotrophic bacteria in an experimental recirculating aquaculture system. Aquacultural Engineering 22: 109-120.

98. Livingstone,D.R. 2003. Oxidative stress in aquatic organisms in relation to pollution and aquaculture. Revue de Medecine Veterinaire 154: 427-430.

99. Loy A, Maixner F Wagner M Horn M. 2007. probeBase - an online resource for rRNA-targeted oligonucleotide probes: new features 2007. *Nucleic Acids Res*. 35: D800-D804.

100. Lyssenko,C. and Wheaton,F. 2006a. Impact of positive ramp short-term operating disturbances on ammonia removal by trickling and submerged-upflow biofilters for intensive recirculating aquaculture. Aquacultural Engineering 35: 26-37.

101. Lyssenko,C. and Wheaton,F. 2006b. Impact of rapid impulse operating disturbances on ammonia removal by trickling and submerged-upflow biofilters for intensive recirculating aquaculture. Aquacultural Engineering 35: 38-50.

102. Mah,T.F.C. and O'Toole,G.A. 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in Microbiology 9: 34-39.

103. Maixner,F., Noguera,D.R., Anneser,B., Stoecker,K., Wegl,G., Wagner,M., and Daims,H. 2006. Nitrite concentration influences the population structure of Nitrospira-like bacteria. Environmental Microbiology 8: 1487-1495.

104. Malone,R.F., Bergeron,J., and Cristina,C.M. 2006. Linear versus Monod representation of ammonia oxidation rates in oligotrophic recirculating aquaculture systems. Aquacultural Engineering 34: 214-223.

105. Malone,R.F. and Pfeiffer,T.J. 2006. Rating fixed film nitrifying biofilters used in recirculating aquaculture systems. Aquacultural Engineering 34: 389-402.

106. Marking,L.L., Rach,J.J., and Schreier,T.M. 1994. Evaluation of Antifungal Agents for Fish Culture. Progressive Fish-Culturist 56: 225-231.

107. Masters,A.L. 2004. A review of methods for detoxification and neutralization of formalin in water. North American Journal of Aquaculture 66: 325-333.

108. Matthews,R.A. 2005. Ichthyophthirius multifiliis Fouquet and Ichthyophthiriosis in Freshwater Teleosts. *In* Advances in Parasitology. *Edited by* J.R.Baker. Academic Press, pp. 159-241.

109. McDonnell,G. and Russell,A.D. 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. Clin.Microbiol.Rev. 12: 147-179.

110. Meinelt,T., Pietrock,M., Burnison,K., and Steinberg,C. 2005. Formaldehyde toxicity is altered by calcium and organic matter. Journal of Applied Ichthyology 21: 121-124.

111. Meinelt,T., Richert,I., Stuber,A., and Braunig,I. 2007a. Application of peracetic acid to the parasite Ichthyophthirius-multifiliis in Sander (*Sander lucioperca*) breeding. Deutsche Tierarztliche Wochenschrift 114: 244-251.

112. Meinelt,T., Staaks,J., Staaks,G., Stueber,A., and Braunig,I. 2007b. Anti-parasitic effects of peracetic acid (PAA) to free infective stages (Theronts) of the white spot disease, *Ichthyophthirius multifiliis* in vitro. Deutsche Tierarztliche Wochenschrift 114: 384-387.

113. Michaud,L., Blancheton,J.P., Bruni,V., and Piedrahita,R. 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquacultural Engineering 34: 224-233.

114. Møller, M. Arvin E. & Pedersen L. F. 2222. Degradation and effect of hydrogen peroxide in small-scale recirculation aquaculture system biofilters. (*Submitted*).

115. Morris,M.R.J., Fraser,D.J., Heggelin,A.J., Whoriskey,F.G., Carr,J.W., O'Neil,S.F., and Hutchings,J.A. 2008. Prevalence and recurrence of escaped

farmed Atlantic salmon (*Salmo salar*) in eastern North American rivers. Canadian Journal of Fisheries and Aquatic Sciences 65: 2807-2826.

116. Naylor,R., Hindar,K., Fleming,I.A., Goldburg,R., Williams,S., Volpe,J., Whoriskey,F., Eagle,J., Kelso,D., and Mangel,M. 2005. Fugitive salmon: Assessing the risks of escaped fish from net-pen aquaculture. Bioscience 55: 427-437.

117. Naylor,R.L., Goldburg,R.J., Primavera,J.H., Kautsky,N., Beveridge,M.C.M., Clay,J., Folke,C., Lubchenco,J., Mooney,H., and Troell,M. 2000. Effect of aquaculture on world fish supplies. Nature 405: 1017-1024.

118. Newman, C. M. 1995. Quantitative Methods in Aquatic Ecotoxicology. Advances in Trace Substances Research. CRC Press, ISBN: 0-87371-622-1, p. 426.

119. Noble,A.C. and Summerfelt,S.T. 1996. Diseases encountered in rainbow trout cultured in recirculating systems. Annual Review of Fish Diseases 6: 65-92.

120. Park,H.D. and Noguera,D.R. 2007. Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. Journal of Applied Microbiology 102: 1401-1417.

121. Park,H.D., Wells,G.F., Bae,H., Criddle,C.S., and Francis,C.A. 2006. Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. Appl.Environ.Microbiol. 72: 5643-5647.

122. Pedersen, L-F Pedersen P. B. Nielsen J. L & Nielsen P. H. 2008. Formaldehyde induced variation in biofilter performance and microbial composition. Proceedings from the Seventh International Conference on Recirculating Aquaculture, Roanoke, Virginia, p. 430-439.

123. Pedersen,L.F. and Pedersen,P. 2006. Temperature-dependent formaldehyde degradation in trickling filters. North American Journal of Aquaculture 68: 230-234.

124. Pedersen,L.F., Pedersen,P.B., and Sortkjaer,O. 2006. Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale bio filters. Aquacultural Engineering 34: 8-15.

125. Pedersen, L.-F. Larsen V. J & Henriksen N. H. 2005. Developing organic aquaculture in Denmark. Eurofish Magazine (ISSN:1020-9956), Vol.: 556-558.

126. Pedersen,L.F., Pedersen,P.B., and Sortkjaer,O. 2007. Temperature-dependent and surface specific formaldehyde degradation in submerged biofilters. Aquacultural Engineering 36: 127-136.

127. Piedrahita,R.H. 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture 226: 35-44.

128. Piper,R.G. and Smith,C.E. 1973. Factors Influencing Formalin Toxicity in Trout. Progressive Fish-Culturist 35: 78-&.

129. PommereningRoser,A., RATH,G., and KOOPS,H.P. 1996. Phylogenetic diversity within the genus Nitrosomonas. Systematic and Applied Microbiology 19: 344-351.

130. Powell,M.D. and Clark,G.A. 2004. Efficacy and toxicity of oxidative disinfectants for the removal of gill amoebae from the gills of amoebic gill disease affected Atlantic salmon (Salmo salar L.) in freshwater. Aquaculture Research 35: 112-123.

131. Powell,M.D. and Perry,S.F. 1997. Respiratory and acid-base pathophysiology of hydrogen peroxide in rainbow trout (Oncorhynchus mykiss Walbaum). Aquatic Toxicology 37: 99-112.

132. Power,E.G.M. Aldehydes as biocides. Prog. Med. Chem. 34:149-201.

133. Purkhold,U., Pommerening-Roser,A., Juretschko,S., Schmid,M.C., KOOPS,H.P., and Wagner,M. 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: Implications for molecular diversity surveys. Appl.Environ.Microbiol. 66: 5368-5382.

134. Rach,J.J., Gaikowski,M.P., and Olson,J.J. 1997a. Importance of analytically verifying chemical treatments. Progressive Fish-Culturist 59: 222-228.

135. Rach,J.J., Gaikowski,M.P., and Ramsay,R.T. 2000. Efficacy of hydrogen peroxide to control parasitic infestations on hatchery-reared fish. Journal of Aquatic Animal Health 12: 267-273.

136. Rach,J.J. and Ramsay,R.T. 2000. Analytical verification of waterborne chemical treatment regimens in Hatchery raceways. North American Journal of Aquaculture 62: 60-66.

137. Rach,J.J., Schreier,T.M., Howe,G.E., and Redman,S.D. 1997b. Effect of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. Progressive Fish-Culturist 59: 41-46.

138. Rach,J.J., Howe,G.E., and Schreier,T.M. 1997c. Safety of formalin treatments on warm- and coolwater fish eggs. Aquaculture 149: 183-191.

139. RajalaMustonen,R.L., Toivola,P.S., and HeinonenTanski,H. 1997. Effects of peracetic acid and UV irradiation on the inactivation of coliphages in wastewater. Water Science and Technology 35: 237-241.

140. Rintamaki-Kinnunen,P., Rahkonen,M., Mannermaa-Keranen,A.L., Suomalainen,L.R., Mykra,H., and Valtonen,E.T. 2005a. Treatment of ichthyophthiriasis after malachite green. I. Concrete tanks at salmonid farms. Diseases of Aquatic Organisms 64: 69-76.

141. Rintamaki-Kinnunen,P., Rahkonen,M., Mykra,H., and Valtonen,E.T. 2005b. Treatment of ichthyophthiriasis after malachite green. II. Earth ponds at salmonid farms. Diseases of Aquatic Organisms 66: 15-20.

142. Rodriguez,G.G., Phipps,D., Ishiguro,K., and Ridgway,H.F. 1992. Use of A Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria. Appl.Environ.Microbiol. 58: 1801-1808.

143. Rossmore, H. W. 1991. Nitrogen compounds. *In* S. S. Block (ed.), Disinfection, sterilization, and preservation, 4th ed. Lea & Febiger, Philadelphia, Pa. ISBN 0-683-30740-1.

144. Saez,J.A. and Bowser,P.R. 2001. Hydrogen peroxide concentrations in hatchery culture units and effluent during and after treatment. North American Journal of Aquaculture 63: 74-78.

145. Santoro,D., Gehr,R., Bartrand,T.A., Liberti,L., Notarnicola,M., Dell'Erba,A., Falsanisi,D., and Haas,C.N. 2007. Wastewater disinfection by peracetic acid: Assessment of models for tracking residual measurements and inactivation. Water Environment Research 79: 775-787.

146. Sapkota,A., Sapkota,A.R., Kucharski,M., Burke,J., McKenzie,S., Walker,P., and Lawrence,R. 2008. Aquaculture practices and potential human health risks: Current knowledge and future priorities. Environment International 34: 1215-1226.

147. Schmidt,A.S., Bruun,M.S., Dalsgaard,I., Pedersen,K., and Larsen,J.L. 2000. Occurrence of antimicrobial resistance in fish-pathogenic and environmental bacteria associated with four Danish rainbow trout farms. Appl.Environ.Microbiol. 66: 4908-+.

148. Schmidt,I., Sliekers,O., Schmid,M., Bock,E., Fuerst,J., Kuenen,J.G., Jetten,M.S.M., and Strous,M. 2003. New concepts of microbial treatment processes for the nitrogen removal in wastewater. FEMS Microbiology Reviews 27: 481-492.

149. Schmidt, L. J. Gaikowski M. P. & Gingerich W. H. 2006. Environmental assessment for the use of hydrogen peroxide in aquaculture for treating external fungal and bacterial diseases of cultured fish and fish eggs. USGS Report, 180 pages.

150. Schramm,A. 2003. In situ analysis of structure and activity of the nitrifying community in biofilms, aggregates, and sediments. Geomicrobiology Journal 20: 313-333.

151. Schramm,A., de Beer,D., van den Heuvel,J.C., Ottengraf,S., and Amann,R. 1999. Microscale distribution of populations and activities of Nitrosospira and Nitrospira spp. along a macroscale gradient in a nitrifying bioreactor: Quantification by in situ hybridization and the use of microsensors. Appl.Environ.Microbiol. 65: 3690-3696.

152. Schwartz, M. F. G. L. Bullock J. A. Hankins S. T. Summerfelt and J. A. Mathias 2000. Effects of selected chemo- therapeutants on nitrification in fluidized-sand biofilters for coldwater fish production. International Journal of Recirculating Aquaculture. 1 (2000), pp. 61–81.

153. Sharrer,M.J., Rishel,K., and Summerfelt,S. 2009. Evaluation of geotextile filtration applying coagulant and flocculant amendments for aquaculture biosolids dewatering and phosphorus removal. Aquacultural Engineering 40: 1-10.

154. Sindilariu,P.D. 2007. Reduction in effluent nutrient loads from flow-through facilities for trout production: a review. Aquaculture Research 38: 1005-1036.

155. Sindilariu,P.D., Wolter,C., and Reiter,R. 2008. Constructed wetlands as a treatment method for effluents; from intensive trout farms. Aquaculture 277: 179-184.

156. Smith,C.E. and Piper,R.G. 1972. Pathological Effects in Formalin-Treated Rainbow-Trout (Salmo-Gairdneri). Journal of the Fisheries Research Board of Canada 29: 328-&.

157. Sortkjær, O. Henriksen N. H. Heinecke R. D. & Pedersen L-F. 2008a. Optimering af behandlingseffekten i akvakultur. Minimering af forbrug og udledning af hjælpestoffer. Danmarks Miljøundersøgelser, AarhusUniversitet. 124 s. - Faglig rapport fra DMU nr. 659. http://www.dmu.dk/Pub/FR659.pdf.

158. Sortkjær, O. P. B. Pedersen S. J. Steenfeldt M. S. Bruun I. Dalsgaard P. Nielsen & P. Aarup 2000. Undersøgelse af eventuelle miljøpåvirkninger ved anvendelse af hjælpestoffer og medicin i ferskvandsdambrug, samt metoder til at reducere /eliminere sådanne påvirkninger [*In Danish*]. DFU rapport nr. 79, p. 206.

159. Sortkjær, O. Pedersen L-F & Ovesen N. B. 2008b. Omsætning af formalin i danske dambrug. Danmarks Miljøundersøgelser, Aarhus Universitet. 122 s. - *Faglig rapport fra DMU* nr. 699. http://www.dmu.dk/Pub/FR699.pdf.

160. Speare,D.J., Arsenault,G., MacNair,N., and Powell,M.D. 1997. Branchial lesions associated with intermittent formalin bath treatment of Atlantic salmon, Salmo salar L, and rainbow trout, Oncorhynchus mykiss (Walbaum). Journal of Fish Diseases 20: 27-33.

161. Speare,D.J. and Arsenault,G.J. 1997. Effects of intermittent hydrogen peroxide exposure on growth and columnaris disease prevention of juvenile rainbow trout (Oncorhynchus mykiss). Canadian Journal of Fisheries and Aquatic Sciences 54: 2653-2658.

162. Srivastava,S., Sinha,R., and Roy,D. 2004. Toxicological effects of malachite green. Aquatic Toxicology 66: 319-329.

163. Stewart,P.S. and Franklin,M.J. 2008. Physiological heterogeneity in biofilms. Nature Reviews Microbiology 6: 199-210.

164. Strauss, D. L. & Meinelt T. 2009. Acute toxicity of peracetic acid (PAA) to *Ichthyophthirius* multifiliis theronts. Parasitology Research, DOI 10.1007/s00436-009-1361-9.

165. Subashinghe,R. and Sot,D.a.J.J. (*Editors*). 2009. Global Aquaculture and its role in sustainable development. Reviews in Aquaculture 1.

166. Sudova,E., Machova,J., Svobodova,Z., and Vesely,T. 2007. Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: a review. Veterinarni Medicina 52: 527-539.

167. Suhr, K. I. & Pedersen P. B. Comparison of nitrification rates of moving bed and fixed bed filters treating effluents from a commercial recirculating rainbow trout production facility (*Submitted*).

168. Summerfelt,S.T. and Hochheimer,J.N. 1997. Review of ozone processes and applications as an oxidizing agent in aquaculture. Progressive Fish-Culturist 59: 94-105.

169. Summerfelt,S.T., Sharrer,M.J., Tsukuda,S.M., and Gearheart,M. 2009. Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. Aquacultural Engineering 40: 17-27.

170. Svendsen, L. M. Sortkjær O. Ovesen N. B. Skriver J. Larsen S. E. Bouttrup S. Pedersen P. B. Rasmussen R. S. Dalsgaard A. J. T. Suhr K. 2008. Modeldambrug under forsøgsordningen. Faglig slutrapport for måle- og dokumentationsprojekt for modeldambrug (*in Danish*). DTU Aqua rapport nr. 193-08., 222 pp. ISBN: 978-87-7481-082-7.

171. Tal,Y., Schreier,H.J., Sowers,K.R., Stubblefield,J.D., Place,A.R., and Zohar,Y. 2009. Environmentally sustainable land-based marine aquaculture. Aquaculture 286: 28-35.

172. Taylor,N.I. and Ross,L.G. 1988. The Use of Hydrogen-Peroxide As A Source of Oxygen for the Transportation of Live Fish. Aquaculture 70: 183-192.

173. Taylor,P.W. and Glenn,R.A. 2008. Toxicity of five therapeutic compounds on juvenile salmonids. North American Journal of Aquaculture 70: 175-183.

174. TGD 2003. Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No. 1488/94 Available at http://ecb.jrc.it/Technical-Guidance-Document/.

175. Timmons, M. B. Ebeling J. M. Wheaton F. W. Summerfelt S. T. Vinci B. J. 2002. Recirculating Aquaculture Systems, Second ed. NRAC Publication No. 01-002, Itchaca, NY, USA, ISBN 0-9712646-1-9, p. 769.

176. Tort,M.J., Jennings-Bashore,C., Wilson,D., Wooster,G.A., and Bowser,P.R. 2002. Assessing the effects of increasing hydrogen peroxide dosage on rainbow trout gills utilizing a digitized scoring methodology. Journal of Aquatic Animal Health 14: 95-103.

177. Tucker, C. S. & Hargraves J. A. 2009. Environmental best management practices for aquaculture. Blackwell Publishing Ltd. ISBN:-10:0-8138-2027-8, p. 592.

178. Verdegem,M.C.J., Bosma,R.H., and Verreth,J.A.J. 2006. Reducing water use for animal production through aquaculture. International Journal of Water Resources Development 22: 101-113.

179. Vollertsen,J., Jahn,A., Nielsen,J.L., Hvitved-Jacobsen,T., and Nielsen,P.H. 2001. Comparison of methods for determination of microbial biomass in wastewater. Water Research 35: 1649-1658.

180. Vroegop,S.M., Decker,D.E., and Buxser,S.E. 1995. Localization of Damage-Induced by Reactive Oxygen Species in Cultured-Cells. Free Radical Biology and Medicine 18: 141-151.

181. Wagner,M. 2004. Deciphering functions of uncultured microorganisms. ASM News 70: 63-70.

182. Wagner,M., Brumelis,D., and Gehr,R. 2002. Disinfection of wastewater by hydrogen peroxide or peracetic acid: Development of procedures for measurement of residual disinfectant and application to a physicochemically treated municipal effluent. Water Environment Research 74: 33-50.

183. Wagner,M., Horn,M., and Daims,H. 2003. Fluorescence in situ hybridisation for the identification and characterisation of prokaryotes. Current Opinion in Microbiology 6: 302-309.

184. Wagner,M., Nielsen,P.H., Loy,A., Nielsen,J.L., and Daims,H. 2006. Linking microbial community structure with function: fluorescence in situ hybridization-microautoradiography and isotope arrays. Current Opinion in Biotechnology 17: 83-91.

185. Waldrop,T. and Gearheart,M.a.C.W. (*Editors*). 2009. Disinfecting Recirculating Aquaculture Systems: Post-harvest cleaning. Hatchery Internatinal Jan/Feb.

186. Weavers,L.K.&.W.G.B. Kinetics of the inactivation of microorganisms.

187. Wienbeck,H.K.H. Untersuchungen zum Formalinabbau in Kreislaufanlagen [Decomposition of formaldehyde in a recirculation fish farming system]. Archives für Fischereiwissenschaft, 40, 153-166.

188. Wik,T.E.I., Linden,B.T., and Wramner,P.I. 2009. Integrated dynamic aquaculture and wastewater treatment modelling for recirculating aquaculture systems. Aquaculture 287: 361-370.

189. Wood,N.J. and Sorensen,J. 2001. Catalase and superoxide dismutase activity in ammonia-oxidising bacteria. Fems Microbiology Ecology 38: 53-58.

190. Woodward,K.N. 2005. Veterinary pharmacovigilance. Part 3. Adverse effects of veterinary medicinal products in animals and on the environment. Journal of Veterinary Pharmacology and Therapeutics 28: 171-184.

191. Wooster,G.A., Martinez,C.M., and Bowser,P.R. 2005. Human health risks associated with formalin treatments used in aquaculture: Initial study. North American Journal of Aquaculture 67: 111-113.

192. Yuan,Z., Ni,Y., and VanHeiningen,A.R.P. 1997. Kinetics of peracetic acid decomposition .1. Spontaneous decomposition at typical pulp bleaching conditions. Canadian Journal of Chemical Engineering 75: 37-41.

193. Zhu,S. and Chen,S. 2002. The impact of temperature on nitrification rate in fixed film biofilters. Aquacultural Engineering 26: 221-237.

PAPER I

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Temperature-dependent and surface specific formaldehyde degradation in submerged biofilters

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Abstract

This study investigated formaldehyde removal in submerged fixed media biofilters in commercial and pilot scale recirculation aquaculture systems. Steady removal of formaldehyde (F) was observed immediately after simulated therapeutic treatment in closed systems and complete removal occurred within 1–4 days depending on water temperature. Formaldehyde removal was dependent on available biofilter surface area, and comparable rates of surface specific removal (SSR) were observed in two different systems. SSR was positively correlated to temperature (Q_{10} = 3.4) with estimates of 2.1 mg F/(m^2 h) at 5.7 °C to 6.5 ± 0.2 mg F/(m^2 h) at 14.5 °C. The estimates for SSR of formaldehyde can be used to predict actual treatment and effluent concentration with more accuracy. Furthermore, the results allow calculation on biofilter removal capacity of formaldehyde, applicable for developing biofilters ensuring sufficient formaldehyde removal in effluent water.

Keywords: Recirculation; Biofilter; Wastewater; Effluent; Formaldehyde; Removal kinetics

1. Introduction

Formaldehyde is an efficient liquid disinfectant and is widely used in aquaculture industry (Masters, 2004). Commercial solutions (i.e. Formalin-F, Paracide-F) typically contain 24–37% (w/w) formaldehyde (CH_2O) dissolved and stabilized in 10% (w/w) methanol (CH_3O). Water treatment with formaldehyde serves to suppress bacterial and fungal pathogens/diseases and to control parasites (Marking et al., 1994; Schreier et al., 1996; Madsen et al., 2000; Buchmann and Kristensson, 2003). Water treatment practices can result in concomitant discharge of excess chemicals, the duration and magnitude depending on various parameters. Recent concern on potentially negative impacts from periodical discharge of formaldehyde from fish farms have lead to discharge regulations and national water quality criteria of the receiving water bodies (Hohreiter and Rigg, 2001; Masters, 2004). Therefore, focus on methods to reduce formaldehyde discharge has emerged, either in terms of substitution (Madsen et al., 2000) or of removal and neutralization (Aitcheson et al., 2000; Masters, 2004; Gearheart et al., 2006; Pedersen and Pedersen, 2006).

Beside the bactericidal effect, formaldehyde also serves as an easy degradable organic energy source for some heterotrophic organisms (Adroer et al., 1990; Kaszycki et al., 2001). *Pseudomonas* strains, *Vibrio*, *Methyloccocus*, *Halomonas* and *Rhodococcus* strains

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and methylotrophic yeast, i.e. *Hansenula polymorpha* have been reported to be able to biodegrade formaldehyde (Kaszycki and Kołoczek, 2000; Hidalgo et al., 2002; Eiroa et al., 2004). *Pseudomanas putida* may be present in activated sludge and also colonize biofilm in recirculation aquaculture systems (RAS) as described by Adroer et al. (1990). Consequently, in RAS where active biological filters are central components, the concentration and efficacy of formaldehyde is likely to decrease due to bacterial degradation of the substance. After dosage of formaldehyde in RAS, the formaldehyde concentration is controlled by the degree of water exchange and the bacterial removal rate in the specific system. High concentration and prolonged exposure of formaldehyde may affect the microbial community, e.g. by temporarily or permanently impairing existing nitrifying biofilm (Keck and Blanc, 2002; Eiroa et al., 2004), or it may lead to increased oxygen demand as a result of the addition of easily degradable carbon sources such as formaldehyde and methanol.

Biological water treatment, where heterotrophic microbial processes reduce the amount of organic matter and chemoautotrophic nitrifying bacteria oxidize ammonium is central to intensive aquaculture systems (Timmons et al., 2002; Piedrahita, 2003; Michaud et al., 2006). Here, biofiltration partly takes place in suspension, but is primarily associated to biofilm on surfaces. Numerous designs of biofilters exist, considering ammonia removal capacity, available surface area and oxygen transfer capabilities (Malone and Pfeiffer, 2006). Biofilters can be categorized in many ways, e.g. by distinguishing between emerged and submerged types, as well as dynamic (fluidized) and fixed biofilters, direction of flow and packing media (Timmons et al., 2002).

Available surface area is the shaping parameter for removal of suspended solids and ammonia in biofilters. Removal kinetics is significantly affected by temperature, oxygen saturation, biofilm thickness, and diffusion conditions and by other variable production parameters such as pH, salinity, turbulence and C/N ratio (Avnimelech, 1999; Chen et al., 2006; Eding et al., 2006). Fixed submerged biofilter are widely used in Danish RAS, due to relatively low cost, high robustness and moderate maintenance, combined with a satisfying, stable performance with regard to organic matter content and ammonia removal. Packing media typically comprise PE/plastic modules with a high specific surface area and void space, i.e. Bioblok® (www.expo-net.dk).

Knowledge of biological formaldehyde removal and kinetics in submerged fixed biofilters will provide useful information to which extend low technological components can be used to diminish formaldehyde discharge. Estimates of surface specific removal rates (SSR) under various conditions can be used to achieve better estimates of actual concentrations upon treatment (Rach et al., 1997; Rach and Ramsey, 2000). Furthermore, estimates of SSR of formaldehyde will be valuable in design of biofilters with the intention of removing excess formaldehyde in effluents (Saez and Bowser, 2001; Gaikowski et al., 2004).

The purpose of this study was to investigate the potential formaldehyde removal efficiency from submerged fixed biofilters. Experiments were made in two different recirculation aquaculture systems with various sized biofilter to compare formaldehyde removal kinetic. Fixed amounts of formaldehyde, resulting in commercial treatment concentrations were applied to the two different systems operating at water temperatures from 5.7 to 15.5 °C. Effects of temperature and available biofilter surface area were evaluated subsequently.

2. Materials and methods

All experiments were conducted at the Danish Institute of Fisheries Research (DIFRES) aquaculture facility. Six full scale experiments were performed in an operating freshwater recirculation system, followed by experiments in six pilot scale freshwater recirculation systems in order to verify surface specific formaldehyde degradation.

2.1. Test facilities and set-up for full scale experiments

The full scale recirculation system included 12 separate tanks (1.5 m^3 each), a mechanical drum filter (40 μm), a biofilter, a trickling filter and a water reservoir (Fig. 1A). System water entered the reservoir after mechanical filtration, and was then pumped to the bottom of the biofilter (up-flow). Biofilter overflow entered a trickling filter 3.5 m above ground level, below which a 750 l reservoir with outlets to fish tanks and water reservoir was positioned. Flow averaged 8.3 ± 0.1 l/s and water elevation velocity in the biofilter was approximately 18 cm/min. The cylindrical biofilter (3.5 m high; diameter 2.0 m) was a submerged up-flow type packed with five layers (2.75 m) of BIO-BLOK® 200 plastic media (Expo-net, Hjørring, Denmark). BIO-BLOK® 200 consists of polyethylene net tubes with a specific surface area of 200 m^2/m^3. During dosage experiments, tanks and trickling filter were bypassed,

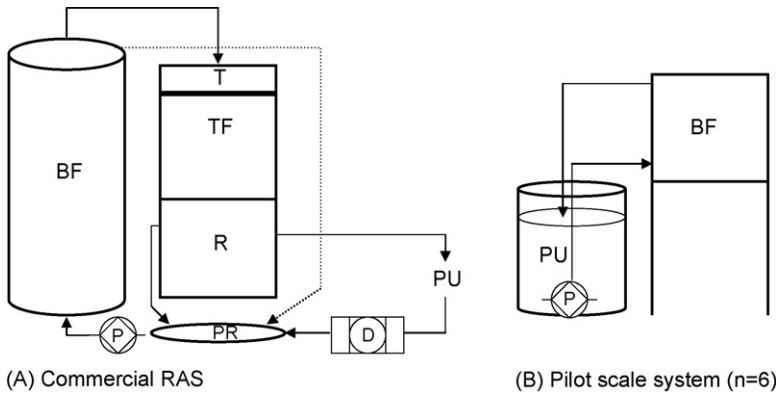

Fig. 1. Schematic presentation of experimental recirculation aquaculture systems; full scale (A) and pilot scale (B). PU, production units; D, drum filter; PR, pump reservoir; P, pump; BF, biofilter; T, perforated tray; TF, trickling filter; R, reservoir. Dotted line represents by-pass during dosage experiment.

and the closed system included recirculation between biofilter and water reservoir only.

Two months before the first dosage experiment, approximately 80 kg of 0.3–0.5 kg sized rainbow trout (*Oncorhynchus mykiss*) were evenly distributed in the tanks at a water temperature of 14 °C. Fish were fed daily with 1.6 kg of 3 mm high energy extruded fish feed pellets (Aller Aqua 576, Christiansfeld, Denmark). Daily water renewal in the system was approximately 2 m^3, equivalent to about 10% of the total volume. The acclimation period prior to experiments included stabilization of nitrification processes, with satisfactory equilibrium concentrations reached 5 weeks after commencement ($NH_3/NH_4^+ < 0.1$ mg/l, $NO_2^- < 0.1$ mg/l, and $NO_3^- < 50$ mg/l). After stabilization, formaldehyde was added in increasing amounts (C_0 approximately 10, 20, 30 and 40 mg/l) at four subsequent occasions in order to acclimatize the microbial community.

2.1.1. Experimental protocol for full scale experiment

Prior to each experiment, pumps were temporarily turned off and water supply lines were closed. A closed circuit was then established by recirculating water between biofilter and reservoir. The trickling filter was bypassed by use of a 160 mm PVC pipe.

Total water volume of the closed system was adjusted to 8500 l. At equilibrium, water samples from the outlet of the biofilter were collected for measuring organic matter content as 5-day biological oxygen demand (BOD_5), and used as control values.

With a magnetic diaphragm-dosing pump, formaldehyde (37.4% as formalin solution stabilized with 10% methanol) was added to the water surface of the reservoir tank over a 17 min period (equivalent to the calculated hydraulic residence time of the system). The nominal formaldehyde concentration (C_0) was calculated to be 40.0 mg l^{-1}. After addition of formaldehyde ($t = 0$), water samples were collected at fixed intervals ($t = 0.25$, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 60, 72 and 96 h).

Water samples were collected in triplicate from the outlet of the submerged biofilter in 10 ml tubes, capped and kept at <5 °C and analyzed for formaldehyde at the National Environmental Research Institute (NERI) within 3 days after final sampling. Preliminary investigations of storage conditions showed that formaldehyde concentration remained stable for at least 8 days at <5 °C, whereas, after 2 weeks a significant decomposition was observed (data not shown). Oxygen content, pH and temperature were monitored before and during the application experiments. Oxygen concentrations were measured with an Oxyguard® Handy β DO meter and Oxyguard® standard probes (Birkerød, Denmark), and pH was measured with an Oxyguard® Handy pH-meter (do).

2.1.2. Full scale experimental conditions

Experiments 1–4 (temperature variation): four similar dosage experiments in an operating biofilter were performed with temperature as the independent variable. Water temperature was kept stable by refrigeration at least 1 week prior to experiments with temperature fluctuations of less than 1 °C.

Experiment 5 (acute biofilter reduction): a similar dosage experiment at 15 °C was carried out in the same system but with reduction of biofilter material. Forty percent (two upper rows of BIO-BLOKs) of the filter media was removed and the system was maintained with similar water volume (adjusted by the use of air pockets). Immediately after removal and attained system equilibrium, formaldehyde was added as described formerly.

Experiment 6 (prolonged biofilter reduction): the dosage experiment was repeated in the same reduced biofilter system at May 12. Temperature and dosage as in experiment 5.

2.2. Test facilities and set-up for pilot scale experiments

Pilot scale recirculation systems (Fig. 1B) were established with a biofilter based on a BIO-BLOK® 200 standard module (Pedersen et al., 2006). Each of six identical systems had a 200-l tank for fish and an elevated up-flow biofilter containing a submerged BIO-BLOK® 200 module transferred from the operating full scale biofilter. Water temperature between and during experiments averaged 15.5 °C, with fluctuations being less than 1 °C. Approximately 1.5 kg rainbow trout (50 ± 5 g) were put into each of the six tanks/systems. The tanks were randomly assigned a daily amount of organic matter, defined as six different feeding levels. Thus, the fish (and thereby the biofilter) were given 0, 4, 8, 12, 16 or 20 g feed/day, respectively, of 3 mm high-energy extruded pellets (Aller Aqua 576). The initial feeding ration was equivalent to 0, 0.3, 0.5, 0.8, 1.1 and 1.3% feed per biomass, respectively. Feeding was continuous from 9 a.m. till 3 p.m. by automatic belt-feeder. Everyday, 10% of the water volume was replaced by tap water. The fish/systems were given the individual amount of feed for 10 days before formaldehyde application. Oxygen concentration, pH and temperature were measured daily in the pilot systems, and general biofilter status and performance was monitored regularly with colorimetric test-kit NH_4^+/NH_3 (Aquamerck® 1.11117.0001) and test strips measurements of NO_2^- and NO_3^- (Merckoquant 1.10020 and 1.10007, respectively).

2.2.1. Experimental protocol for pilot scale dosage experiment

Prior to experiments, pumps were temporarily turned off and removed, in order to capture and move the fish from the tanks. Total water volume was adjusted to 360 l by tap water. The pumps were turned on, and flow was adjusted to 1.0 l/s. At this equilibrium, water samples were taken for BOD_5 and background determinations. After application of formaldehyde ($t = 0$), water samples were collected at fixed intervals ($t = 5$, 10, 15, 30, 45 and 60 min and 1.25, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16, 24, 48 and 72 h). For sampling, see text above.

2.2.2. Pilot scale experimental conditions

Experiment 7: dosage experiments were made in six pilot scale RAS. An amount of 38.5 g formalin (T.A. Baker; 37.5 formaldehyde stabilised with 10% methanol), equivalent to a calculated C_0 of 40 mg F/l) was applied to each systems during 30 s.

2.3. Analysis

Formaldehyde concentration in water samples was analyzed as a photometric complex reaction with Spectroquant® test kit reagents (Merck 1.14678.0001 HCHO) in a spectrophotometer in 10 mm vials at 585 nm. Samples with expected high formaldehyde concentrations were diluted with distilled water, and compared to standard curves made from 0 to 2.992 mg/l formaldehyde. Formaldehyde concentrations were corrected for any background interference in RAS water sampled prior to the experiments. The organic content of the system water prior to experiments were measured as biological oxygen (BOD_5) demand over a 5-day period (DS/EN 1899-2, 2004) with use of a nitrification inhibitor (0.5 ml 1.0 g/l *N*-allylthiourea (purum $\geq$ 98%; Fluka)).

2.4. Statistics

Formaldehyde degradation was estimated from linear regression analyses on measured concentration values above 1 mg/l only. This arbitrary value was selected to obtain equal wide ranges of concentration and to exclude disproportionate weighting from low concentrations. Degradation rates at various temperatures were compared using *F*-tests for difference between slopes (Sokal and Rohlf, 1995) and significance defined as $P < 0.05$. Temperature coefficients were calculated according to van't Hoff–Arrhenius relationship (Metcalf and Eddy, 2003).

3. Results

3.1. Elimination of formaldehyde in full scale experiments (experiments 1–6)

Formalin dosing to the closed full scale biofilter system caused a rapid increase in formaldehyde concentration to approximately 40 mg/l at equilibrium (Fig. 2). Hereafter formaldehyde concentration decreased at an approximately constant rate towards complete elimination. Complete elimination were reached after 32–35 h at c. 15 °C (Fig. 2A, D), significantly faster than in experiments performed at temperatures of 10 and 5.7 °C, with elimination time exceeding 60 and 96 h, respectively (Fig. 2B, C). Degradation pattern obtained from experiments 2 and 3

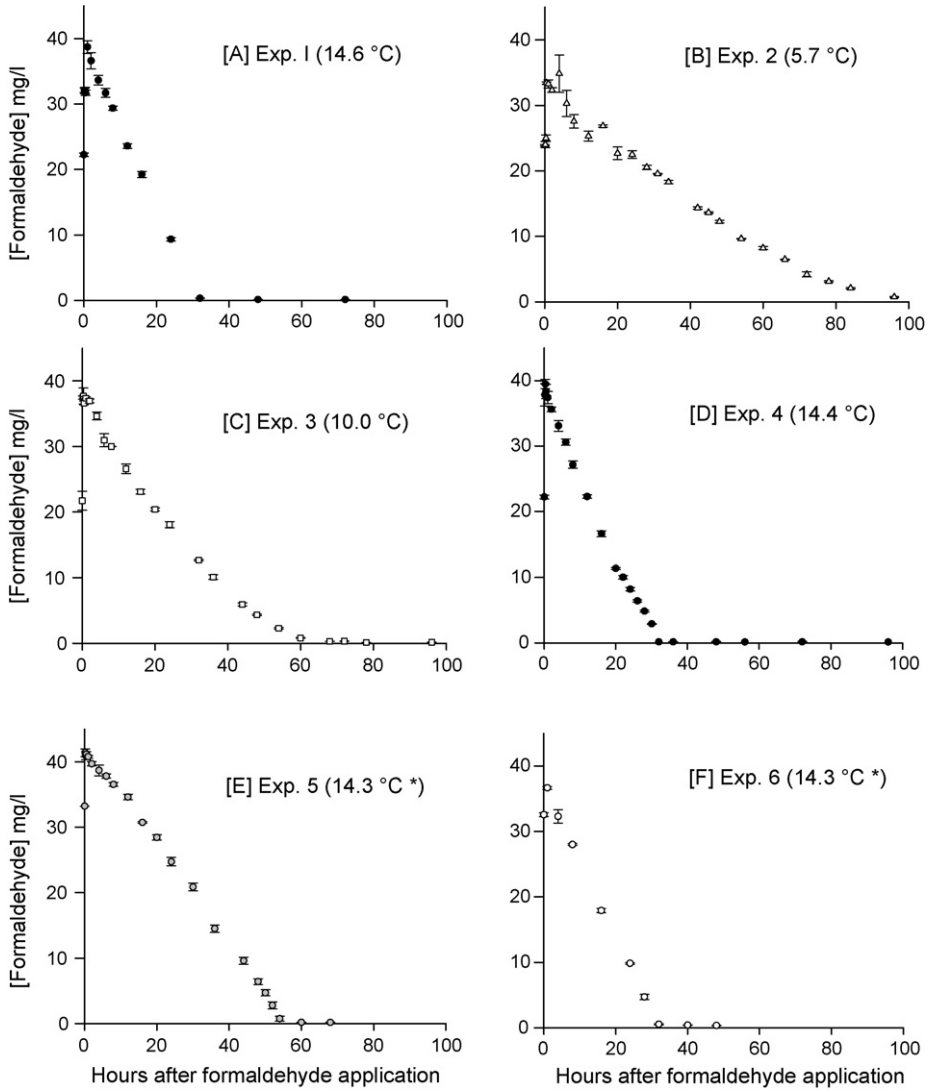

Fig. 2. Degradation of formaldehyde applied to a full scale biofilter at six different conditions. Temperature effects were investigated in experiments 1–4. (*) In experiments 5 and 6, 40% of the total biofiltermedia was removed, and formaldehyde was applied immediately after removal (experiment 5) and reapplied after 2 weeks (experiment 6). A total of 906.7 g formalin (37.5% formaldehyde in 10% methanol stabilized solution) was applied to the system.

Table 1
Elimination kinetics of formaldehyde following application to a full scale biofilter

Experiment	Temperature (°C)	pH	BOD_5[a] (mg O_2/l)	Linear regression	Removal[b] (g/h)	Removal[c] (mg/(m^2 h))
1	14.6 ± 0.8	8.05–8.35	2.9 ± 0.1	37.7–1.16t	9.86	6.32
2	5.7 ± 0.6	8.14–8.32	1.4 ± 0.1	32.2–0.39t	3.34	2.14
3	10.0 ± 0.5	8.08–8.32	2.2 ± 0.5	36.0–0.68t	5.79	3.71
4	14.4 ± 0.2	7.98–8.27	2.5 ± 0.2	37.7–1.22t	10.40	6.66
5	14.3 ± 0.3	7.94–8.36	1.6 ± 0.1	42.2–0.74t	6.32	6.75
6	14.3 ± 0.3	8.02–8.36	1.5 ± 0.1	37.2–1.16t	9.82	10.49

Linear elimination was calculated on measured HCHO concentrations from $t = 0.5$ h to [HCHO] < 1.0 mg/l; $R^2 \geq 0.9$. System volume was 8500 l with an estimated biofilter volume of 7.80 m^3. The calculated nominal formaldehyde treatment concentration was 40.0 mg/l.

[a] Biological oxygen demand over 5 days at 20 °C; measured prior to formaldehyde treatment.

[b] Gross/total removal rate of formaldehyde.

[c] Estimated surface specific removal rate (with trickling filter media of 200 m^2/m^3).

slightly digress from zero order kinetic, which may be ascribed to altered diffusion condition and transition to 1/2-order kinetic (Eding et al., 2006). The average removal of formaldehyde ranged from 3.3 g/h at low temperature (5.7 °C) to 10.4 g/h at high temperature (14.4 °C) equivalent to surface specific reductions (SSR) of 2.1 and 6.7 mg/(h m^2), respectively (Table 1, experiments 1–4, see below). Experiments with identical temperature conditions revealed similar elimination kinetics (Table 1, experiments 1 and 4).

The first application experiment with 40% reduced biofilter media (experiment 5) showed a reduced elimination capacity, as formaldehyde was present till 52 h after addition (Fig. 2E). The reduction of 37.6% correlates closely to the reduction in biofiltermedia of 40% (Table 1). The SSR was 6.75 mg/(h m^2), closely resembling the SSR found in experiments 1 and 4 of 6.32 and 6.66 mg/(h m^2). When the reduced biofilter was exposed to formaldehyde again (experiment 6) complete formaldehyde elimination occurred within 34 h, with a resulting removal of 9.8 g/h. With an estimated SSR of 10.5 mg/(h m^2) in experiment 6, the surface specific removal capacity estimated from experiments with intact biofilter was exceeded by approximately 62% (Table 1).

3.2. Elimination of formaldehyde in pilot scale experiments (experiments 7.1–7.6)

When applied to pilot scale biofilter, formaldehyde exhibited similar zero order elimination (Newman, 1995) as found in full scale experiments (Fig. 3). In five out of six experiments formaldehyde concentration was reduced to less than 1 mg/l within 42 h, whereas, experiment 7.1 reached that level after 48 h. The overall elimination equation from the six experiments is $y = -0.87x + 40.4$ ($R^2 = 0.95$), with y being the formaldehyde concentration in mg/l and x h elapsed after application to the specific system.

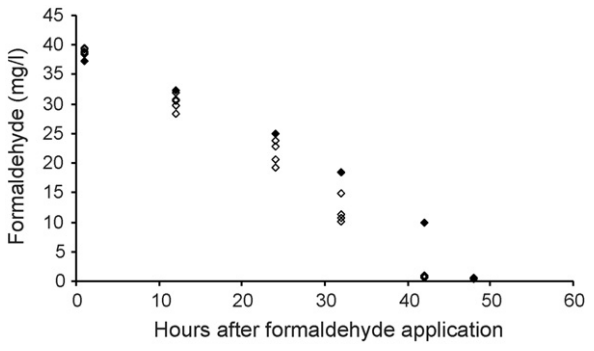

Fig. 3. Formaldehyde concentration in six closed pilot scale biofilters. Within 30 s (t_0) 38.5 g formalin (37.5% (w/w) formaldehyde) was applied to each system, with an expected initial concentration (C_0) of 40 mg formaldehyde/l. Solid symbols refer to experiment 7.1; open symbols to experiment 7.2–7.6 (cf. Table 2). Overall regression is described as $y = 40.4 - 0.87$ ($R^2 = 0.95$).

3.3. Surface dependent elimination of formaldehyde

Estimates of SSR of formaldehyde as a function of temperature from both experimental set-ups are depicted in Fig. 4. In full scale applications, SSR from the higher temperature range average 7.6 ± 1 mg/(h m^2), with SSR from experiment 6 deviating considerably. Average SSR of formaldehyde in pilot scale biofilter at 15.5–16.2 °C was 9.7 ± 0.3 mg/(h m^2) ranging from 8.2 to 10.1 mg/(h m^2). The general correlation between temperature and SSR was described by $Y = 0.88\ e^{0.15x}$ ($R^2 = 0.91$; $p < 0.01$), with Y being the SSR of formaldehyde in mg/(h m^2) and x is the water temperature.

3.4. Temperature dependent elimination of formaldehyde

Temperature activity coefficient (θ) in the full scale experiments was estimated to 1.13 according to SSR values obtained in experiments 1–4 (Fig. 1). The 13% increase in removal rate per degree Celsius equals a Q_{10}-value of 3.4 in the temperature range 5.7–14.5 °C. All experiments combined (Fig. 4) reveal a slightly higher θ_{total} of 1.16 in the temperature range 5.7–16.2 °C.

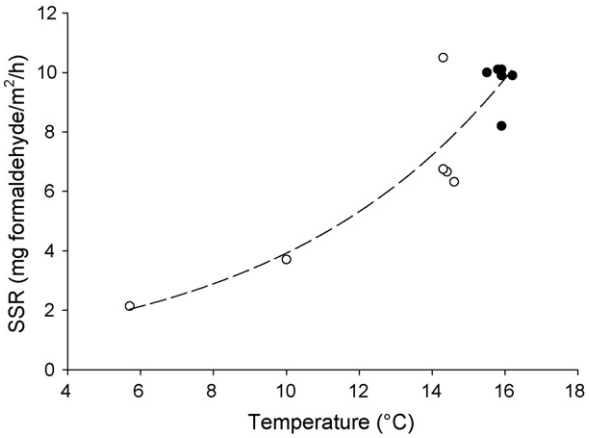

Fig. 4. Estimates of surface specific removal (SSR) rates of formaldehyde in commercial RAS (open symbols) and in pilot scale RAS (black symbols). The relationship between SSR and temperature can be described as $Y = 0.88\ e^{0.15x}$ ($R^2 = 0.91$; $p < 0.01$), where Y is the SSR of formaldehyde in mg/(m^2 h) and x is the water temperature.

Table 2
Elimination kinetics of formaldehyde following application to pilot scale biofilters

Experiment 7	Temperature (°C)	$[pH]_{start}$	BOD_5[a] (mg O_2/l)	Linear regression	Removal[b] (g/h)	Removal[c] (mg/(m^2 h))
7.1	15.9 ± 0.2	8.10	1.1 ± 0.2	−0.76t	0.274	8.2
7.2	16.2 ± 0.2	8.06	1.3 ± 0.4	−0.92t	0.331	9.9
7.3	15.9 ± 0.4	8.02	1.2 ± 0.1	−0.94t	0.336	10.1
7.4	15.5 ± 0.5	8.05	1.6 ± 0.1	−0.92t	0.332	10.0
7.5	15.8 ± 0.3	7.99	2.1 ± 0.1	−0.94t	0.337	10.1
7.6	15.9 ± 0.2	7.94	2.6 ± 0.1	−0.92t	0.329	9.9

Linear ($C_t = C_0 - kt$) elimination was calculated on measured HCHO concentrations over time to a [HCHO] < 1.0 mg/l; $R^2 \geq 0.9$. System volume was 360 l with an estimated biofilter volume of 0.166 m^3. The calculated nominal formaldehyde treatment concentration was 40.0 mg/l.
[a] Biological oxygen demand over 5 days at 20 °C; measured prior to formaldehyde treatment.
[b] Formaldehyde removal rate.
[c] Estimated surface specific removal rate (with biofilter media of 200 m^2/m^3).

3.5. System water quality

Formaldehyde was added to closed operating systems where fish had been removed just prior to. Due to intended variation in feeding regimes in small scale systems, water quality varied with regard to pH and organic load (Table 2). Biological oxygen demand over a 5-day period (BOD_5) ranged from 1.1 to 2.6 mg O_2/l, comparable to the BOD_5 range of 1.4–2.9 in full scale experiments. No correlation between temperature and BOD_5 or between SSR and BOD_5 were found ($p > 0.05$; $R^2 < 0.1$).

Increased oxygen consumption, measured as reduced O_2-concentration in water leaving the biofilter, occurred during the period of formaldehyde elimination and ceased abruptly when all formaldehyde was removed from the system (not shown). In full scale experiments, oxygen consumption reached approximately 0.3 mg/l during the period of formaldehyde removal compared to the steady state conditions, measured as difference in oxygen content between the inlet and the outlet of the biofilter (not shown). With a water-flow of 8 l/s, approximately 8.6 g O_2/h was additionally consumed, which is within the order of magnitude of the theoretical stochiometric fraction of O_2 needed for oxidation of formaldehyde and methanol.

4. Discussion

4.1. Surface dependent formaldehyde removal

This results show that formaldehyde removal is closely related to available biofilter surface area, and hence to the microbial abundance. An equal surface specific removal (SSR) rate has been demonstrated in operating systems with two different volumes of biofilter media, and in two different types of RAS. Biofilter surfaces were estimated from the manufacturer's information of 200 m^2/m^3 for Bioblok® 200 media, which, however, does not represent exact values. A fixed surface specific area used in all experiment, allow volumetric removal rates (removal per volume biofilter) to be estimated and compared. Additional ''passive'' surfaces (i.e. pipe and reservoir walls) were not accounted for, though biofilm were present and might have accounted for a certain contribution (Timmons et al., 2002). The measured values here derived from a standard recirculation aquaculture system are likely to be in the same order of magnitude as common commercial operating freshwater systems.

SSR of formaldehyde in trickling filters are found to be approximately 2.5 times higher compared to SSR in the submerged biofilter (Pedersen and Pedersen, 2006). This discrepancy between filter types can be explained by significant formaldehyde evaporation and/or by improved microbial conditions in terms of central parameters such as oxygen availability and hydraulic surface load (m^3/m^2). The biological treatment of system water, caused by microbial activity, are controlled and affected by several parameters among, which is available surface area.

The microbial composition and activity determine removal of primary effluent (nutrients), and has a latent capacity to degrade some secondary effluents (micropollutants) such as formaldehyde. Studies of secondary degradation performance and effects on process stability in different biofilter reactors will therefore provide important information of biofilter potentials.

4.2. Temperature dependent formaldehyde removal

Decomposition of formaldehyde significantly depended on the system temperature in biofilters. At operating temperatures of 5.7 °C, SSR of formaldehyde reached

2.14 mg F/(m^2 h), exceeded by 173% and 303% at 10 and 14.5 °C, respectively. A temperature activity coefficient (Metcalf and Eddy, 2003) describing the exponential increase in decomposition rate form the experiments in the full scale system was found to be 1.13 (experiments 1–4). This is equivalent to a Q_{10} of 3.4 (Sand-Jensen and Pedersen, 2005), in accordance to the temperature dependency of formaldehyde removal in trickling filters with a Q_{10} of 2.8 (Pedersen and Pedersen, 2006). Temperature coefficient of the nitrification process derived from coexisting nitrifying bacteria are in a range of 1.02–1.08 according to Eding et al. (2006), which may imply strain specific differences or abiotic input to formaldehyde degradation. Temperatures in the present study reflect temperature ranges realistic to outdoor fish farms in the northern hemisphere, but are lower than the majority of indoor/sheltered RAS. At higher temperatures, significantly increased decomposition rates are expected to occur (Zhu and Chen, 2002). The exponentially positive correlation found in the present temperature interval of 10° has to be extrapolated with caution, as a threshold level followed by an abruptly decrease is expected to occur as temperature increases.

The microbial activity was demonstrated by concomitant oxygen consumption in the biofilter during the entire period of formaldehyde presence and degradation. This study demonstrates formaldehyde removal; however, the specific underlying mechanisms are not evaluated neither in terms of active microorganisms nor other potential contributions, such as evaporation and bioadsorption. Evaporation is likely to be minimal due to low water surface/air exchange. Furthermore, results from experiments with reduced biofilter volume show a corresponding reduced formaldehyde removal, which implies evaporation is insignificant. Eiroa et al. (2006) reported significant bioadsorption of formaldehyde in activated sludge, indicating similar initial breakdown mechanisms to occur in fixed biofilm.

4.3. Organic load and filter colonization

When 40% of the colonized bio-blocks were removed, an equivalent reduction in the total system removal rate of formaldehyde was found. This implies that the surface specific removal rate was unchanged. Afterwards, a new system equilibrium was established, where identical daily organic load were now administered to a reduced biofilter volume. Application of formaldehyde to the new equilibrium demonstrated a significant increase in the surface specific removal rate as shown in experiment 6. This indicates that the activity and/or the amount of the heterotrophic bacteria able to degrade formaldehyde have increased at the relative increased load of organic matter.

Formaldehyde application in small scale RAS was investigated at various organic loads, measured as low range BOD_5 prior to formaldehyde application (Table 2). A markedly low SSR was observed in the set-up with unfed fish (experiment 7.1) although the measured BOD did not deviate significantly. No correlation between formaldehyde decomposition rate and (low range) BOD_5 in the six experiments was found. Permanent or recurring loading of formaldehyde is likely to change the microbial community, increasing the abundance and activity of heterotrophic microorganisms able to degradate formaldehyde (Dickerson, 1955; Kaszycki and Kołoczek, 2000). To which extend formaldehyde reduction rates are related to colonization status of biofilter, type of filter media or BOD_5 levels are not sufficiently investigated. As such, further research is required to develop optimised systems for controlling critical aquaculture effluent.

Nitrification processes was measured semi-quantitatively during the formaldehyde application and no significant changes to filter performance were observed. It should be noted, however, that the experimental set-up excluded the fish tanks during application, preventing more ammonium and organic matter from reaching the filter. As described by Keck and Blanc (2002), formaldehyde application might alter microbial composition and affect part of the nitrification processes in biofilters and trickling filters. It is therefore recommended to monitor oxygen, ammonium and nitrite concentrations during formaldehyde treatment in recirculating systems. In a marine recirculation fish farm system, Keck and Blanc (2002) found that prolonged formaldehyde exposures above 40 mg/l HCHO impaired the nitrite-oxidizing bacteria, leading to temporarily increased nitrite levels. Eiroa et al. (2004) reported inhibitory effects on the nitrification process by formaldehyde concentrations above 350 mg F/l when applied to activated sludge. Such concentrations are unlikely to be observed in RAS.

4.4. Applicability

This study has different fields of application, as the results can be used to estimate the formaldehyde capacity of a biofilter reactor and allow modelling during simulated treatment situations. The results provide an alternative option for complying with formaldehyde discharge limits beside dilution or a discontinuation of formaldehyde use as stated by

Gearheart et al. (2006). Discharge permissions of formaldehyde from fish farms operate in the lower ppb ranges, which are likely to be incompatible with normal aquaculture management. The way the discharge problem presents itself is well known from other industries, where waste water treatment plants have been introduced. Approaches and solutions for non-aquaculture industries (i.e. Oliveira et al., 2004) may therefore provide an important future interface to aquaculture operations.

The need to reduce formaldehyde discharge call upon management improvement in terms of disinfection procedures and/or use of post-treatment units to polish production water before it enters the receiving water bodies. In flow through ponds, formaldehyde treatment might be improved by the insert of a (portable) biofilter reactor to be established as a temporary (closed) recirculation system. The controlled condition makes it possible to predict treatment concentration and exposure time, which ultimately lead to efficient water treatment and reduced use of formaldehyde. Meanwhile, the reactor degrades formaldehyde and other suspended solids and ensure CO_2 stripping and satisfactory oxygen conditions.

Development of optimized post-treatment reactors includes research of various filter and media types of potential in aquaculture. Also new microbiological approaches in terms of understanding biofilter composition, function, versatility and resiliency will provide important knowledge to central components in recirculation aquaculture systems.

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References

Adroer, N., Casas, C., de Mas, C., Solà, C., 1990. Mechanism of formaldehyde biodegradation by Pseudomonas putida. Appl. Microbiol. Biotechnol. 33, 210–220.

Aitcheson, S.J., Arnett, J., Murray, K.R., Zhang, J., 2000. Removal of aquaculture therapeutants by carbon adsorption: 1. Equilibrium adsorption behaviour of single components. Aquaculture 183 (3-4), 269–284.

Avnimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176, 227–235.

Buchmann, K., Kristensson, R.T., 2003. Efficacy of sodium percarbonate and formaldehyde bath treatments against *Gyrodactylus derjavini* infestations of rainbow trout. N. Am. J. Aquacult. 65, 25–27.

Chen, S.L., Ling, J., Blancheton, J.P., 2006. Nitrification kinetics of biofilm as affected by water quality factors. Aquacult. Eng. 34, 179–197.

Dickerson, B.W., 1955. High rate trickling filter operation on formaldehyde wastes. Sewage Indust. Wastes 22, 536–545.

DS/EN 1899-2, 2004. Water quality – determination of biochemical oxygen demand after *n* days (BODn) – Part 2: method for undiluted samples (www.ds.dk).

Eding, E.H., Kamstra, A., Verreth, J.A.J., Huisman, E.A., Klapwijk, A., 2006. Design and operation of nitrifying trickling filters in recirculating aquaculture: a review. Aquacult. Eng. 34, 234–260.

Eiroa, M., Kennes, C., Veiga, M.C., 2004. Formaldehyde biodegradation and its inhibitory effect on nitrification. J. Chem. Technol. Biotechnol. 79, 499–504.

Eiroa, M., Vilar, A., Kennes, C., Veiga, M.C., 2006. Formaldehyde biodegradation in the presence of methanol under denitrifying conditions. J. Chem. Technol. Biotechnol. 81, 312–317.

Gaikowski, M.P., Larson, W.J., Steur, J.J., Gingerich, W.H., 2004. Validation of two models to predict chloramine-T concentrations in aquaculture facility effluent. Aquacult. Eng. 30, 127–140.

Gearheart, J.M., Masters, A.L., Bebak-Williams, J., 2006. Application of methods for the detoxification and neutralization of formalin in fish hatchery effluents. N. Am. J. Aquacul. 68, 256–263.

Hidalgo, A., Lopategi, A., Prieto, M., Serra, J.L., Llama, M.J., 2002. Formaldehyde removal in synthetic and industrial wastewater by Rhodococcus erythropolis UPV-1. Appl. Microbiol. Biotechnol. 58, 260–263.

Hohreiter, D.W., Rigg, D.K., 2001. Derivation of ambient water quality criteria for formaldehyde. Chemosphere 45, 471–486.

Kaszycki, P., Kołoczek, H., 2000. Formaldehyde and methanol biodegradation with methylotrophic yeast *Hansenula polymorpha* in a model wastewater system. Microbiol. Res. 154, 289–296.

Kaszycki, P., Tyszka, M., Malec, P., Koloczek, H., 2001. Formaldehyde and methanol biodegradation with the methylotrophic yeast *Hansenula polymorpha*. An application to real wastewater treatment. Biodegradation 12, 169–177.

Keck, N., Blanc, G., 2002. Effects of formalin chemotherapeutic treatments on biofilter efficiency in a marine recirculating fish farming system. Aquat. Living Resour. 15, 361–370.

Madsen, H.C.K., Buchmann, K., Mellergård, S., 2000. Treatment of trichodiniasis in eel (Anguilla anguilla) reared in recirculation systems in Denmark: alternatives to formaldehyde. Aquaculture 186, 221–231.

Malone, R.F., Pfeiffer, T.J., 2006. Rating fixed film nitrifying biofilters used in recirculating aquaculture systems. Aquacult. Eng. 34, 389–402.

Marking, L.L., Rach, J.J., Schreier, M., 1994. Evaluation of antifungal agents for fish culture. Progress. Fish Cultur. 56, 225–231.

Masters, A.L., 2004. A review of methods for detoxification and neutralization of formalin in water. N. Am. J. Aquacul. 66 (4), 325–333.

Metcalf and Eddy Inc., 2003. Waste Water Engineering: Treatment, Disposal Reuse, IV ed. McGraw-Hill Inc., New York, pp. 1819.

Michaud, L., Blancheton, J.P., Bruni, V., Piedrahita, R., 2006. Effect of particulate organic carbon on heterotrophic bacterial populations

and nitrification efficiency in biological filters. Aquacult. Eng. 34, 224–233.

Newman, C.M., 1995. Quantitative Methods in Aquatic Ecotoxicology. Advances in Trace Substances Research. CRC Press, ISBN: 0-87371-622-1, p. 426.

Oliveira, S.V.W.B., Moraes, E.M., Adorno, M.A.T., Varesche, M.B.A., Foresti, E., Zaiat, M., 2004. Formaldehyde degradation in an anaerobic packed-bed bioreactor. Water Res. 38 (7), 1685–1694.

Pedersen, L.-F., Pedersen, P.B., 2006. Temperature-dependent formaldehyde degradation in trickling filters. N. Am. J. Aquacul. 68, 230–234.

Pedersen, L.-F., Pedersen, P.B., Sortkjær, O., 2006. Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale biofilters. Aquacult. Eng. 34 (1), 8–15.

Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture 226, 35–44.

Rach, J.J., Gaikowski, M.P., Olson, J.J., 1997. Importance of analytically verifying chemical treatments. Progress. Fish Cultur. 59, 222–228.

Rach, J.J., Ramsey, R.T., 2000. Analytical verification of waterborne chemical treatment regimes in hatchery raceways. N. Am. J. Aquacul. 62, 60–66.

Saez, J.A., Bowser, P.R., 2001. Hydrogen peroxide concentrations in hatchery culture units and effluent during and after treatment. N. Am. J. Aquacul. 63, 74–78.

Sand-Jensen, K., Pedersen, N.L., 2005. Differences in temperature, organic carbon and oxygen consumption among lowland streams. Freshwater Biology 50, 1927–1937.

Schreier, T.M., Rach, J.J., Howe, G.E., 1996. Efficiency of formalin, hydrogen peroxide and sodium chloride on fungal-infected rainbow trout eggs. Aquaculture 140, 323–331.

Sokal, R.R., Rohlf, J.M., 1995. Biometry, Third ed. W.H. Freeman and Company, ISBN: 0-7167-2411-1, p. 850.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating Aquaculture Systems, Second ed. NRAC Publication No. 01-002, Itchaca, NY, USA, ISBN 0-9712646-1-9, p. 769.

Zhu, S., Chen, S., 2002. The impact of temperature on nitrification rate in fixed film biofilters. Aquacult. Eng. 26, 221–237.

PAPER II

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Long term/low dose formalin exposure to small-scale recirculation aquaculture systems

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ABSTRACT

Repetitive long term formalin application at low dose was investigated to determine the effect on formaldehyde removal rate, biofilter nitrification and the microbial composition in small-scale recirculation aquaculture biofilters. Six pilot-scale recirculation aquaculture systems holding rainbow trout (*Oncorhynchus mykiss*) were designated to formalin treatments (C_0 at 10 and 20 mg/L formaldehyde) on a daily or weekly basis. Formaldehyde removal rates were measured over 10 weeks, during which biofilter nitrification rates were measured in terms of standardized NH_4Cl spiking events. The rates were positively correlated to the amount and frequency of formalin treatment. In systems with regularly low formalin dosage, the formaldehyde removal rate increased up to tenfold from 0.19 ± 0.05 to 1.81 ± 0.13 mg/(L h). Biofilter nitrification was not impaired in systems treated with formalin on a daily basis as compared to untreated systems. In systems intermittently treated with formalin, increased variation and minor reductions of ammonium and nitrite oxidation rates were observed. Nitrifying bacteria were screened by specific gene probes using fluorescence in situ hybridization and quantified by digital image analysis. The relative abundance of ammonia-oxidizing bacteria (AOB) was up to 5.4% of all *Bacteria* (EUB) positive cells, predominantly *Nitrosomonas oligotropha*. Nitrite-oxidizing bacteria (NOB), mainly consisting of *Nitrospira* sp. were found in all biofilm samples up to 2.9%, whereas *Nitrobacter* sp. was not detected. The relative abundances of AOB and NOB in the untreated system were generally higher compared to the system exposed to formalin. Low dose formalin in recirculated aquaculture systems proved to be a possible treatment strategy, as the effect on nitrification was minimal. Since formaldehyde was steadily removed by microorganisms, available biofilter surface area, hydraulic retention time and temperature can be used to predict removal and hence estimate e.g. effluent concentration.

1. Introduction

The ongoing development from flow-through systems to recirculation aquaculture systems (RAS) has led to a number of environmental benefits, such as reduced water use and improved effluent treatment (Piedrahita, 2003). These water reuse systems rely on microbial biofiltration, as sufficient removal of ammonia and nitrite is a prerequisite to manage high fish loading densities (Noble and Summerfelt, 1996). Biofilter stability of RAS is therefore crucial and emphasis is taken not to disturb the microbial performance. One possible harmful disturbance is during fish disease prevention and control, where chemical agents are added to the bulk water phase. In such cases, biofilters are often exposed to a high concentration of the chemical with a risk of impairing the nitrifying microbial population and hence reduce biofilter performance (Schwartz et al., 2000).

Outbreaks of the ciliated protozoa *Ichthyophthirius multifiliis* (white spot disease) tend to occur in intensive freshwater recirculating systems and can cause high fish mortality if not treated timely (Rintamaki-Kinnunen et al., 2005; Heinecke and Buchmann, 2009; Shinn et al., in press). Curative treatments typically include short-term repetitive topical baths with formalin at concentrations as high as 100 mg/L. This treatment regime has shown to control the extent of infection, as formaldehyde (CH_2O; the active component in formalin) destroys the infective free living stage of *I. multifiliis* (Matthews, 2005). Formaldehyde is also efficacious against other ectoparasites, and it does not pose sublethal effects on the fish at short term exposure over a wide concentration range. Thus, formalin is an efficacious aquaculture therapeutic agent that is used in significant amounts in Denmark as well as worldwide, when proactive exclusion practices have failed.

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Future use of formalin is expected to be considerably reduced due to environmental considerations and work safety reasons (Hohreiter and Rigg, 2001; Masters, 2004; Wooster et al., 2005; EU Biocide Product Directive). Excess formaldehyde after a treatment situation is discharged to the receiving water body, where it may harm the natural micro-fauna. Despite focusing on more environmental friendly chemicals, no valid substitutes for formalin have so far been implemented in RAS, partly due to insufficient treatment efficacy and the risk of biofilter collapse (Schwartz et al., 2000; Madsen et al., 2000; Rintamaki-Kinnunen et al., 2005).

Recent lab-studies have shown acceptable treatment efficacy of formaldehyde against *I. multifiliis* and *Gyrodactylos* sp. when applied at low concentrations for prolonged periods of time (Sortkjær et al., 2008; Heinecke and Buchmann, 2009). These findings can positively affect present treatment practices by requiring lower overall disinfectant dosage which will ultimately lead to reduced discharges of the potentially harmful substance (Masters, 2004; Gearheart et al., 2006). This approach is reasonable in RAS where system design allow stable water quality with low dilution and high retention time. However, the effect on biofilter performance in closed RAS after prolonged, repetitive periods of formaldehyde exposure has not been investigated.

Additional information is needed on factors affecting formaldehyde degradation in biofilters as well as investigating formaldehyde tolerance of ammonia- and nitrite-oxidizing bacteria (Hagopian and Riley, 1998). Studies have shown biodegradation of formaldehyde in activated sludge (Eiroa et al., 2004, 2005) and biofilm reactors associated with wastewater treatment (Ong et al., 2006). Formalin has also been applied to different types of saltwater RAS (Keck and Blanc, 2002) and freshwater RAS (Wienbeck and Koops, 1990; Heinen et al., 1995; Schwartz et al., 2000), focusing on chemical measurements of the removal of ammonia and nitrite across the biofilter. Some of the studies showed significant impaired nitrification related to addition of the chemical. With formalin dosages above 100 mg/L, some experiments have indicated that especially nitrite-oxidizing bacteria were inhibited by the presence of formalin (Keck and Blanc, 2002), though explicit threshold levels for concentration and contact time does not exist (Noble and Summerfelt, 1996). Heinen et al. (1995) found that the upper permissible limits of formalin application were determined by the fish rather than by the biofilter. Regular use of formalin is expected to affect the microbial activity and composition in the biofilters of RAS. Studies have suggested microbial biofiltration as a potential measure to reduce excess formaldehyde to an acceptable level prior to aquaculture discharge either achieved in terms of an integrated biofilter system, a by-pass biofilter unit or an end-of-pipe measure (Pedersen and Pedersen, 2006).

The aim of the present study was to investigate the effect of different low dosage strategies of formalin on the nitrification performance of pilot-scale biofilters in recirculated aquaculture systems, the nitrifying populations and the fish growth. If prolonged, low-dose application comply with normal RAS operation, such an altered practice could lead to reduced net formalin use and discharge.

2. Materials and methods

2.1. Experimental setup and operating conditions

The experiments were carried out in six identical static freshwater reuse aquaculture systems (Pedersen et al., 2006) with a total volume of each system of 360 L. They included a submerged up-flow biofilter and a tank with 3–5 kg of rainbow trout held at 17 ± 0.3 °C. Biofilters were fitted with polyethylene media (Bioblok 150 HD®; Expo-Net, Hjørring, DK) with a total volume of 0.166 m^3 and a surface area of 25 m^2. The biofilters were fed with 1.0 ± 0.1 L/s water from the rearing tanks, resulting in an up-flow water velocity of approx. 20 cm/min in the filters and a water turnover time of 6 min. Twice a week, approx. 90 L from each system was manually siphoned to remove particulate organic matters from the bottom of the rearing tanks. Water exchange equaled 7–8% per day with a hydraulic retention time of two weeks. All systems were allocated a fixed amount of 50 g commercial fish feed per day (DAN-EX 1754 with 17% fat, 54% protein and 17.4 MJ/kg), leading to a relative water consumption of 500 L per kg feed. The pilot-scale systems had been in operation under similar conditions for several months prior to the present experiments in order to secure stable biofilter operation. To initiate the experiments, tanks were emptied and biomasses from previous experiments were determined and approximately 3 kg of rainbow trout was assigned to each tank (day number −26). Rearing conditions in terms of pH and oxygen content were monitored daily with Hach Lange HQd40 multimeter (Brønshøj, Denmark). Oxygen concentration was kept at 7.5–8.5 mg/L, and pH was adjusted to 7.2 ± 0.2 by sodium bicarbonate addition. Common water chemistry parameters were measured on a regular basis using Merck® test kit and sticks. Ammonium and nitrite levels were maintained below 0.2 mg N/L and nitrate levels between 60 and 80 mg N/L.

2.2. Biofilter exposure to formalin

Four pilot scale RAS were randomly chosen and exposed to formalin concentrations in accordance to recent finding by Heinecke and Buchmann (2009). The remaining two RAS served as control systems and were not exposed to formalin until the termination of the nine weeks lasting experiments. An amount of formalin (T.A. Baker; 37.4% formaldehyde stabilised with 10% methanol; 1.09 kg/L), equivalent to a nominal formaldehyde concentration of 10 mg/L was added directly to the rearing tanks in the RAS. The experiments were divided into two adaptation trials due to an accidentally mechanical breakdown of one of the control RAS pumps after four weeks after start. In the first experiment trial (Day 0–23) duplicate systems were randomly designated 10 mg/L formaldehyde once or twice a week. Following a three-week operation period without any formalin addition (Day 24–44), the second experimental trial (Day 45–80) included increased exposure frequency, formalin exposure being increased from twice weekly to daily exposure. Weekly formalin addition remained unchanged in the other two systems. At the end of the experiment (Day 68–80), formalin dosages were doubled in the respective RAS for 10 days, and the experiment was terminated by adding 20 mg/L to the two control RAS.

2.3. Biofilter performance: ammonium and nitrite removal

Nitrification performance was investigated in all systems by ammonium spiking experiments once a week from week six to ten. NH_4Cl equivalent to 3 mg/L total ammonia nitrogen (TAN) was added to the rearing tanks before (baseline performance measure of all six RAS) and during the formalin experimental trial, the TAN spiking was carried out when all formaldehyde was removed (by measurement) from the water in the systems. Water sampling was performed with half an hour intervals over a period of 3 h after TAN addition. Hereafter, approx. 25% of the process water was replaced by tap water and fish were fed as usual, though delayed.

2.4. Fish performance

Fish growth was evaluated by specific growth rate (SGR) and by achieved feed conversion ratio (FCR), relating total added amount of feed during the experimental period to gained biomass. The

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latter was determined as final biomass plus biomass of any dead fish minus initial biomass.

2.5. Chemical analyses

The water samples were analysed for TAN concentrations in accordance to Danish Standard 224, modified to include sodium salicylate catalyser reagent instead of phenol. Nitrite nitrogen (NO_2-N) as measured photometrically by diazotization of sulfanilamide and nitrite coupled with *N*-(1-naphthyl)-ethylenediamine according to Danish Standard 223. Formaldehyde concentrations were measured spectrophotometrically according to the Hantzch reaction as described by Nash (1953) on filtered or centrifuged water samples. Suspended solids content were determined by filtration using preweighed Whatman GF/A filter and reweighed after dehydration at 105 °C. Alkalinity was determined by titration with 0.1 M HCl. Chemical oxygen demand (COD) was measured via potassium dichromate and biological oxygen demand (BOD) was determined over a five days period in pure water samples and samples diluted 1:2 with Milli-Q water.

2.6. Identification and quantification of bacteria

The FISH technique was applied to identify and quantify selected ammonia- and nitrite-oxidizing bacteria in biofilm samples collected during the experimental period. Samples were fixed for 2 h in 4% paraformaldehyde at 4 °C and stored at −20 °C in 1:1 ethanol/PBS solution. The technique is culture-independent using fluorescently labeled oligonucleotide probes that bind to specific bacterial ribosomal RNA targets and thereby identify microbial presence and composition (Amann and Fuchs, 2008). The following probes were used: EUB338mix (equimolar concentrations of EUB338, EUB338II, and EUB338III) covering most *Bacteria* (Amann et al., 1990; Daims et al., 1999); Nso1225 covers many betaproteobacterial AOB (Mobarry et al., 1996). Ntspa662 (incl. competitor probe) covers the genus *Nitrospira* (Daims et al., 2001) and NIT3 (incl. competitor probe) covers *Nitrobacter* sp. (Wagner et al., 1996) Additional AOB probes used in this study and their targets are listed in Table 2. Details on oligonucleotide probes and hybridization conditions are available at probeBase (Loy et al., 2007). Positive cells were identified by an epifluorescence microscope and quantified by area measurement in thin, homogenized samples (relative to total number of EUBmix hybridizations) by processing of series of digital pictures with ImageJ (Papadopulos et al., 2007). Biofilm were sampled from Bioblok® cylinders. A detached cylinder (d = 55 mm; l: 55 cm) was removed from the biofilter, and biofilm from the bottom part of the cylinder was scraped off with a small, stiff sterile brush.

2.7. Statistics

Formaldehyde removal rate was estimated from linear regression analyses on measured concentration values above 1 mg/L only. This arbitrary value was selected to obtain equal wide ranges of concentration and to exclude disproportionate weighting from low concentrations. Degradation rates of formaldehyde were compared pair-wise by a *t*-test testing for difference between linear regression coefficients (Zar, 1999) and level of significance defined as $P < 0.05$.

3. Results

3.1. Removal of formaldehyde

Formaldehyde was rapidly mixed in the systems after addition of formalin, and it was immediately removed with a constant rate.

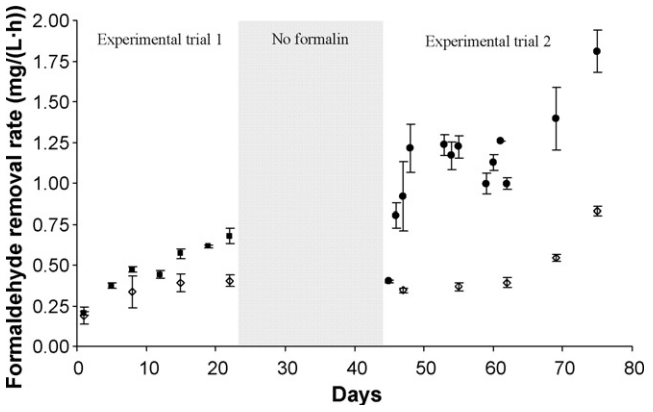

Fig. 1. Formaldehyde removal rates in mg/L per hour derived from formaldehyde spiking experiments in pilot-scale systems. Formaldehyde (C_0 = 10 mg/L) was added as formalin with different intervals in duplicate systems either weekly (◇) or twice a week (■) during the first experimental trial. From Day 24–44 no formalin was applied to any of the systems. Weekly or daily (●) treatment with formaldehyde equivalent to 10 mg/L were applied in the second experimental trial, where the final two treatment episodes includes double dosages equivalent to 20 mg/L. Values shown represent mean ± S.D.

Formaldehyde was initially removed at a rate of approx. 0.19 mg/(L h) (Fig. 1). This rate increased significantly the three following weeks, where rates were found to be 0.40 ± 0.04 and 0.68 ± 0.05 mg/(L h), respectively, in systems with one or two weekly additions of formalin. Formaldehyde was not degraded in autoclaved process water from the investigated RAS, and thus assumed primarily to be degraded by microbial activity.

When formalin was added again after three weeks without formaldehyde addition, the systems previously receiving weekly additions revealed similar removal rates as the initial unexposed systems (Fig. 1). This removal rate was maintained throughout the experimental period with the addition of 10 mg/L formaldehyde (Day 63). The two systems initially treated with formaldehyde twice a week obtained similar rates at Day 45 (0.40 ± 0.01 mg/(L h)) and within a week this was increased threefold (Fig. 1). Formaldehyde removal rates in both treatment groups were significantly increased when formalin dosages were doubled (from Day 70), and rates were measured up to 1.95 mg/(L h), approximately ten times higher than the initial removal rates. The surface specific formaldehyde removal rates were estimated to range from 2.8 to 26 mg/(h m^2). Systems treated similarly showed the most variance when new treatments commenced and tended to even out over time. Formaldehyde removal rates from the three treatment groups were all significantly distinct; the intermittent systems were significantly higher compared to the control groups, and significantly slower compared to the systems exposed to formalin daily ($P < 0.01$) (Fig. 2). Controlled batch experiments on water taken from each of the three FA treatment groups, revealed a water phase removal of FA ranging from 0.09 to 0.11 mg/L at 16 °C.

3.2. Biofilter tolerance—nitrification

Baseline ammonia removal and potential nitrite accumulation were determined in all six RAS with spikes of NH_4Cl before and during the second formaldehyde application period (Day 43–71). Prior to formalin addition, TAN was removed at constant and similar rates in all six systems, at approx. 0.85 ± 0.05 mg/(L h) (Fig. 3A). The following four weeks, the TAN spikes revealed similar patterns and rates in the control systems. Average TAN removal rates slightly decreased in the two systems treated with formalin once a week, but not in the RAS treated daily with formalin. Nitrite concentration was transiently build up from 0.1 to 0.5 mg/L, following TAN spiking. The accumulation of nitrite peaked and most

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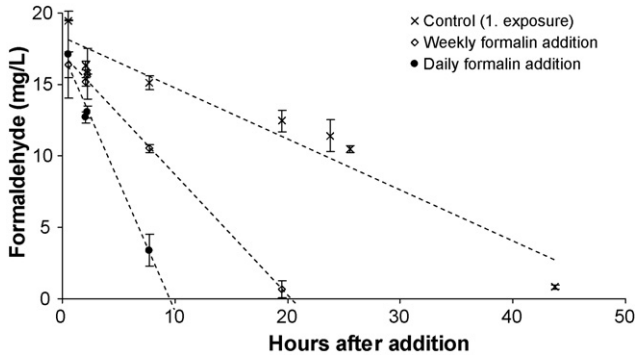

Fig. 2. Formaldehyde degradation in closed pilot-scale systems after the addition of formalin. Each value shown represents mean ± S.D. formaldehyde concentration of two systems added formaldehyde with different frequency. Control systems have not been exposed to formalin prior to experiments (×) in contrast to systems applied with formalin once a week (◇) or daily (■) over a four-week period. Expected C_0 and dosages prior to experiments was 20 mg/L formaldehyde.

often declined to original values within the period of measurement. Similarly to TAN patterns, nitrite removal was most affected in the treatment systems applied with formaldehyde once a week (Fig. 3B). Relatively large inter-variability within the treatment group in regard to nitrite removal was observed as opposed to the control group. Consecutive fish feeding never caused any prolonged nitrite accumulation that could be measured the following day, and no long term effects of formalin were detected.

3.3. Fish growth and performance

Average feed conversion ratios ranged from 0.81 ± 0.04 in the control groups to 0.84 ± 0.04 and 0.87 ± 0.01 in the groups treated with formalin weekly and twice weekly, respectively. The fish grew at similar rates in all systems during the second experimental period, and had equal feed conversion ratios of 0.98 ± 0.05 to 0.99 ± 0.02 feed/biomass. Correspondingly, control fish had a marginal improved SGR (1.53 ± 0.06%) after the first experimental trial compared to SGRs of 1.48 ± 0.06% and 1.42 ± 0.03% in the other two groups. SRG during the second experimental trial did not differ between groups (1.34 ± 0.01%; 1.32 ± 0.01% and 1.32 ± 0.03%). Fish health and shape was inspected visually, and the vast majority of fish were intact without fin erosion or minor bite. Four fish (<5%) had small bites and two fish had irregular/rough slime layer, both incidents not related to a given treatment. No fish mortality occurred during the first experimental trial until it was disrupted at 25 days by an overnight mechanical failure which led to the death of the fish in one of the control systems (not applied with formalin). All tanks were emptied and biomasses of fish were determined. Throughout the second experimental period no fish mortality was observed.

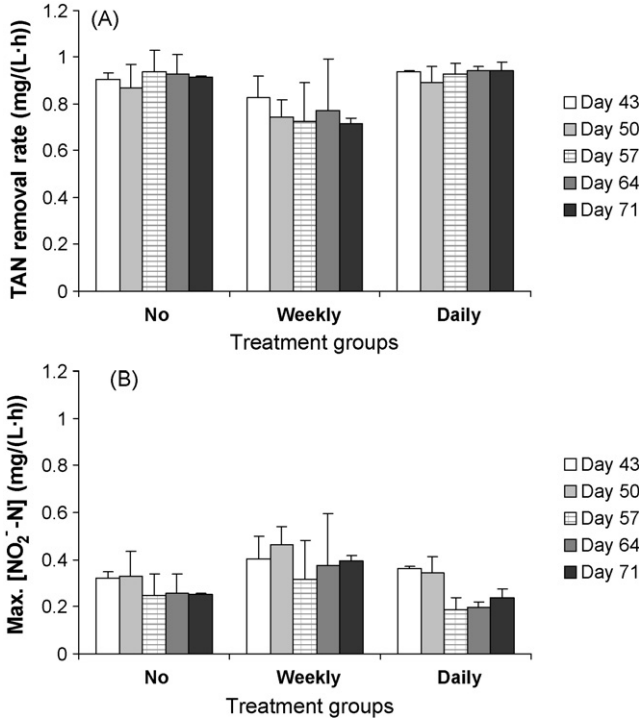

Fig. 3. Average ammonia removal rate (mean ± S.D.) in 360 L pilot-scale recirculated systems over a four-week spiking period (A). Formalin was added after the control experiment, either daily, weekly or not at all. Maximal nitrite concentrations were measured concomitantly for the same three treatment groups after the ammonia spike (B).

3.4. Water quality

Water quality did not change throughout the experimental period, and systems were similar with regards to alkalinity (ranging from 120 to 150 mequiv./L) total suspended solids (ranging from 25 to 30 mg/L) and organic matter content in terms of COD and BOD_5 (approximately 60 ± 5 mg and 11 ± 1 mg O_2/L, respectively; data not shown).

3.5. Microbial composition of biofilter samples

AOB were detected in all samples with relative abundances up to 5.4% of all *Bacteria* (EUBmix) using the probe Nso1225 (targeting most AOB belonging to the *Betaproteobacteria*) (Table 1). Compared to the untreated system, the AOB abundance was in general lower in the system treated with formaldehyde. One sample contained only few positive AOB microcolonies (<0.5% of the eubacterial count (EUBmix)). NOB as *Nitrospira* sp. were found in all biofilm samples up to 3.3% using probe Ntspa662 with the highest relative abundance in the control system. Samples from the systems eight days before initiation of formaldehyde addition were significantly different both in terms of relative AOB and NOB abundance (Table 1). *Nitrobacter* sp. was not found in the samples.

The samples harvested on Day 27 were screened with more specific AOB probes (Table 2). *Nitrosospira*, *Nitrosococcus* or *Nitrosomonas* (*europea*/*halophila*/*eutropha*) were not detected, but nitrosomonads sp. including *N. oligotropha* was targeted by five relatively broad probes. The screening indicated discrepancy in the relative AOB-species abundance between the two samples, though not quantified and compared statistically.

4. Discussion

4.1. Factors affecting formaldehyde removal

Formaldehyde is known to be consumed in RAS biofilter systems (Heinen et al., 1995) and this was also observed in this pilot-scale system. The removal rates depended on concentration and frequency of formalin dosage and our experiments showed that different steady state levels of formaldehyde removal occurred with a maximal rate of 5–25 mg/(m^2 h). The different levels indicate a difference in adaptation, either due to increasing specific microbial activities and/or the numbers of formaldehyde utilizing microorganisms. Interestingly, it was found that the biofilter had unaltered removal capacity during the more than three weeks period without formalin addition. This shows that the formaldehyde degrading microbes most likely stayed in the biofilm, probably growing on other organic compounds.

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Table 1
Quantification and partial community analysis of nitrifiers by FISH in biofilm samples from pilot-scale recirculated aquaculture system. Values reflect relative prevalence in % of EUBmix-positive cells based on digital image analysis ($n = 30$). Positive signals below quantification level are listed <0.5%. Treatment groups reflect a system not exposed to formalin and a system with application of 10 mg/L formaldehyde twice a week from Day 1 to 22.

Gene probes	Treatment groups	Experimental day of sampling in relation to treatment start (Day 1)					
		−8	6	13	21	27	45
Nso1225, broad probe targeting most ammonium oxidizing bacteria	No formalin (control)	1.2	<0.5	4.7	4.9	3.4	5.4
	Formalin twice a week	<0.5 (0.3)	2.9	1.7	2.8	1.4	2.8
Ntspa662 + comp., Specific probe targeting nitrite-oxidizing bacteria genus *Nitrospira*	No formalin (control)	1.1	1.2	3.3	3.2	2.7	2.9
	Formalin twice a week	<0.5 (0.3)	1.3	0.9	1.7	1.3	3.1

Table 2
Screening with different gene probes targeting ammonia-oxidizing bacteria. Biofilm samples were taken from two aquaculture systems; a system not exposed to formalin and a system exposed to low formalin application twice a week over a four-week period (Day 27).

Probe name	Specificity	Reference	FISH positive − formalin	FISH positive + formalin
Nso1225	AOB, Betaproteobacterial	Mobarry et al. (1996)	++	+
Cluster 6A192	*Nitrosomonas oligotropha*-lineage (Cluster 6a)	Adamczyk et al. (2003)	++	+
Nmo218	*N. oligotropha*-lineage	Gieseke et al. (2001)	+	–
Nsm156	*Nitrosomonas* sp. + *Nitrosococcus*	Mobarry et al. (1996)	+	+
Nsv443	*Nitrosospira*		–	–
NEU	Most halophilic and halotolerant *Nitrosomonas* sp.	Wagner et al. (1995)	–	–
Nse1472	*Nitrosomonas europea*, *N. halophila*, *N. eutropha*	Juretschko et al. (1998)	–	–
Nmv	*Nitrosococcus mobilis* lineage	Pommerening-Roeser et al. (1996)	–	–

–: not present.
+: 1–2% relative abundance of all *Bacteria* (EUB338mix: equimolar concentrations of EUB338, EUB338II, and EUB338III (Amann et al., 1990; Daims et al., 1999)).
++: 2–3% relative abundance of all *Bacteria* (as above).

Average surface specific removal rates from previous studies (Pedersen et al., 2007) with similar pilot-scale systems and temperature were approx. 10 mg/(m^2 h) which fits in the middle range of what was observed in this study. Experiments carried out in a commercial eel production RAS with similar biofilter media showed formaldehyde removal rates up to 47 mg/(m^2 h) (Wienbeck and Koops, 1990), although at a higher temperature and with more intense loading. Surface specific formaldehyde removal rates around 25 mg/(m^2 h) was obtained from a full-scale commercial Danish freshwater trout RAS maintained at 14 °C and with a similar filter type as used in this study but with indefinite formalin treatment (Sortkjær et al., 2008).

Formaldehyde is toxic and antimicrobial at high concentrations but can be an excellent substrate for microbial growth at low concentrations after adaptation (Chongcharoen et al., 2005; Klotz et al., 2006). The effects of repeated formalin addition on formaldehyde removal have not previously been studied in aquaculture systems, though the effects of adaptation have only been hypothesized (Wienbeck and Koops, 1990; Heinen et al., 1995). Microbial acclimatization has consequences not only for modelling the fate of formaldehyde, but can also be speculated to lead to specific microbial resistance towards formaldehyde.

4.2. Effects of formalin addition on nitrification

Frequent formalin exposure at low concentration did not impair biofilter nitrification performance significantly following TAN spikes. TAN removal ranged from 0.70 to 0.95 mg/(L h) (equivalent to 0.24–0.31 g/(m^2 day)) throughout the entire experimental period in all three treatment groups. However, one of the two systems treated with formalin once a week predominantly accumulated NO_2^--N during the period following NH_4Cl spiking indicating a minor impact on the nitrification performance. Temporarily impaired nitrification in RAS biofilter has been described previously when higher formalin concentration (110–160 mg/L) is used in indefinite treatment situations (Wienbeck and Koops, 1990; Heinen et al., 1995; Noble and Summerfelt, 1996; Keck and Blanc, 2002). The lack of a prolonged negative effect of formaldehyde on nitrification in our studies could indicate that the concentration level and the exposure time were below a critical level. The formaldehyde concentrations were two- to five times lower than normally applied, and due to the microbial formaldehyde removal, short exposure time occurred. A threshold exposure time and the amount of formalin to cause a biofilter collapse or at least to significantly affect the nitrifiers remain unanswered in the present experiments. We may expect that at a formalin dosage above a certain level and contact time, the degree of disturbance will pose effects on the biofilter performance according to the resiliency and robustness of the biofilters (Botton et al., 2006).

4.3. Microbial abundance in biofilters

Biofilm samples from the biofilters revealed presence of ammonia-oxidizing *Nitrosomonas* sp. (nitrosomonads) and nitrite-oxidizing *Nitrospira* sp. These species are also commonly found in wastewater treatment plants (Wagner et al., 1995; Daims et al., 2001) and also found in a saltwater recirculated aquaculture systems (Foesel et al., 2008). *Nitrobacter* sp. was not detected as also observed in other studies (e.g. Wagner et al., 1996). Aquaculture textbooks (e.g. Timmons et al., 2002; Lekang, 2007) refer to *Nitrosomonas* and *Nitrobacter* as the responsible groups of bacteria in the two step nitrification process. This paradigm is based on culture-dependent methods, by which artificial conditions prevail with high nitrite concentrations in order to obtain optimal growth. Under such conditions *Nitrobacter* outcompete other NOBs, in particular *Nitrospira* sp. (Schramm et al., 1999). *Nitrospira* sp. has been shown to be the dominant NOB at low nitrite concentrations due to it high affinity towards nitrite (Wagner et al., 2002; Maixner et al., 2006). This is likely to be the case for RAS where nitrite levels generally are kept below 0.5 mg/L NO_2^--N. The AOB community analysis demonstrated that the far majority of the populations were related to *Nitrosomonas* with no *Nitrosospira* detected. This is in line with previous findings (Dionisi et al., 2002; Lydmark, 2006). *N. oligotropha*'s dominance most likely reflects superior substrate affinity compared to *N. europea*, and *Nitrosospira* at the given conditions.

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The relative abundances of both AOB and NOB were generally higher in biofilm samples from the system not exposed to formalin, compared to the formalin treated system. Data from the first sample date may reflect that the systems were not in a microbial steady state, though chemical measurement did indicate stable nitrification (TAN and NO_2^--N < 0.2 mg/L). The pilot RAS systems were emptied and replaced with new fish and water four weeks before formalin exposure. Sampling date Day −8 hence reflect the systems less than three weeks after the disturbance, and from sampling date 13–45 relatively high and stable values of AOB and NOB abundances were found from the system not exposed to formalin. Opposite to the control group, the AOB and NOB level remained low in the formalin treated group, and after a prolonged period of time without formalin, AOB and NOB abundance increased markedly (Day 45). The findings indicate a minor formaldehyde suppression of AOB and NOB. The formaldehyde supplement accounted for less than 2% of the feed mass and hence not likely to increase C/N ratio significantly to explain the decreased relative abundance of nitrifiers.

A minor part of the formaldehyde removal occurred in the water phase in the present study. Due to the relative long hydraulic retention time, the microbial abundance in the process water was relatively high (Pedersen et al., 2008) compared to other aquaculture systems (Ebeling et al., 2006; Michaud et al., 2006). This has implications for the distribution of attached biofilm and suspended bacteria (Avnimelech, 2006), and hence the relative contribution of the formaldehyde removal in the water phase and the biofilter. The minor degradation in the water phase is likely to be even smaller in systems with UV control and/or more efficient solid removal.

4.4. Fish performance and water quality

The treatment regime used in the present study mimicked the recommended dose and contact time to a sufficiently control of white spot disease. The prolonged and repetitive formalin treatments did not have any apparent effects on fish health. All fish survived and no indications of epithelium damage were noted. Buchmann et al. (2004) observed a decrease in mucous cell density on rainbow trout after 24 h formalin exposure at 50 mg/L, and they noted clear disorganization of both cell layers and plasma membranes as a result of mucus emptying from the mucous cells. In the first experimental series, formalin dosage might have had a marginal negative effect on feed conversion rate and SGR, but these tendencies were not present in the second experimental trial with even more intensive formalin application. The significant difference in feed conversion ratio and SGR between the two experimental trials can only be explained by the increased fish size and the disturbing weekly application of TAN which differed between the trials. The spiking events in the systems were of short duration and limited to a weekly addition of 3 mg/L TAN in order to reduce temporarily disturbance of increased ammonia and nitrite for the experimental fish. During the excess TAN and nitrite, some fish showed restless behavior in terms of increased near-surface activity during these TAN additions, and potential sublethal effects are likely to have taken place.

4.5. Perspectives

The treatment practice of long term/low dose formalin in RAS has various implications. Treatment strategies can be improved in terms of using less amounts of formalin in biofilter systems and increase the hydraulic retention time during treatment. At low formalin concentrations the nitrification in the biofilters will not be disturbed on the process level, and predicting formaldehyde removal based on biofilter surface area can ensure that sufficient fractions of formaldehyde is removed before discharge. The mechanisms of microbial adaptation to formaldehyde exposure have to be taken into account when calculating, since formaldehyde removal rate can be boosted significantly. One of the potential drawbacks with the frequent use of formalin is, however, the risk of protozoan resistance, which would lead to inefficient treatment or require an increase of formalin dosage. Therefore, research into preventive measures or the use of alternative, more environmental friendly biocides such as peroxygen compounds (Schreier et al., 1996; Kitis, 2004; Pedersen et al., in press) seems to be a relevant future research field as long as parasitic outbreaks occur. Furthermore, biosecurity practices and measures to eliminate or reduce pathogens are proactive solutions (Shinn et al., in press; Summerfelt et al., 2009) not presently fully applied in Danish commercial systems.

In conclusion, a minor shift in AOB and NOB community structure was observed, and prolonged additions of low concentration formalin speeded up the formaldehyde removal rates of the pilot scale RAS without affecting the overall biofilter nitrification performance. This implies that prolonged water treatment with low formalin concentrations is compatible with active biofilter systems. The positive effect of formaldehyde adaptation can be utilized in terms of an end-of-pipe treatment reactor to facilitate a better aquaculture management.

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References

Adamczyk, J., Hesselsoe, M., Iversen, N., Horn, M., Lehner, A., Nielsen, P.H., Schloter, M., Roslev, P., Wagner, M., 2003. The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Applied and Environmental Microbiology 69, 6875–6887.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology 172, 762–770.

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6 (5), 339–348.

Avnimelech, Y., 2006. Bio-filters: the need for an new comprehensive approach. Aquacultural Engineering 34 (3), 172–178.

Botton, S., van Heusden, M., Parsons, J.R., Smidt, H., van Straalen, N., 2006. Resilience of microbial systems towards disturbances. Critical Reviews in Microbiology 32, 101–112.

Buchmann, K., Bresciani, J., Jappe, C., 2004. Effects of formalin treatment on epithelial structure and mucous cell densities in rainbow trout, *Oncorhynchus mykiss* (Walbaum), skin. Journal of Fish Diseases 27, 99–104.

Chongcharoen, R., Smith, T.J., Flint, K.P., Dalton, H., 2005. Adaptation and acclimatization to formaldehyde in methylotrophs capable of high-concentration formaldehyde detoxification. Microbiology-Sgm 151, 2615–2622.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 22, 434–444.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.Z., Wagner, M., 2001. In situ characterization of Nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. Applied and Environmental Microbiology 67 (11), 5273–5284.

Dionisi, H.M., Layton, A.C., Harms, G., Gregory, I.R., Robinson, K.G., Sayler, G.S., 2002. Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira spp.* from full-scale wastewater treatment plants by competitive PCR. Applied and Environmental Microbiology 68 (1), 245–253.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257, 346–358.

Eiroa, M., Kennes, C., Veiga, M.C., 2004. Formaldehyde biodegradation and its inhibitory effect on nitrification. Journal of Chemical Technology and Biotechnology 79, 499–504.

Eiroa, M., Kennes, C., Veiga, M.C., 2005. Simultaneous nitrification and formaldehyde biodegradation in an activated sludge unit. Bioresource Technology 96, 1914–1918.

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EU Biocide Product Directive. http://ec.europa.eu/environment/biocides/index.htm. Accessed October 14. 2008.
Foesel, B.U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., de la Torre, J.R., van Rijn, J., Minz, D., Drake, H.L., Schramm, A., 2008. Nitrosomonas Nm143-like ammonia oxidizers and Nitrospira marina-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. FEMS Microbiology Ecology 63, 192–204.
Gearheart, J.M., Masters, A.L., Bebak-Williams, J., 2006. Application of methods for the detoxification and neutralization of formalin in fish hatchery effluents. North American Journal of Aquaculture 68, 256–263.
Gieseke, A., Purkhold, U., Wagner, M., Amann, R., Schramm, A., 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Applied and Environmental Microbiology 67, 1351–1362.
Hagopian, D.S., Riley, J.G., 1998. A closer look at the bacteriology of nitrification. Aquacultural Engineering 18, 223–244.
Heinecke, R.D., Buchmann, K., 2009. Control of Ichthyophthirius multifiliis: dose–response trials using sodium percarbonate and water filtration. Aquaculture 288, 32–35.
Heinen, J.M., Weber, A.L., Noble, A.C., Morton, J.D., 1995. Tolerance to formalin by a fluidized-bed biofilter and rainbow trout Oncorhynchus mykiss in a recirculating culture system. Journal of World Aquacultural Society 26 (1995), 65–71.
Hohreiter, D.W., Rigg, D.K., 2001. Derivation of ambient water quality criteria for formaldehyde. Chemosphere 45, 471–486.
Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P., Wagner, M., 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: Nitrosococcus mobilis and Nitrospira-Like Bacteria as Dominant Populations. Applied and Environmental Microbiology 64, 3042–3051.
Keck, N., Blanc, G., 2002. Effects of formalin chemotherapeutic treatments on biofilter efficiency in a marine recirculating fish farming system. Aquatic Living Resources 15, 361–370.
Kitis, M., 2004. Disinfection of wastewater with peracetic acid: a review. Environment International 30, 47–55.
Klotz, M.G., Arp, D.J., Chain, P.S.G., El-Sheikh, A.F., Hauser, L.J., Hommes, N.G., Larimer, F.W., Malfatti, S.A., Norton, J.M., Poret-Peterson, A.T., Vergez, L.M., Ward, B.B., 2006. Complete genome sequence of the marine, chemolithoautotrophic, ammonia-oxidizing bacterium *Nitrosococcus oceani* ATCC 19707. Applied and Environmental Microbiology 72, 6299–6315.
Lekang, O.-I., 2007. Aquaculture Engineering. Blackwell Publishing Ltd., pp. 330.
Loy, A., Maixner, F., Wagner, M., Horn, M., 2007. Probebase—an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic Acids Research 35, D800–D804.
Lydmark, P., 2006. Population dynamics of nitrifying bacteria in biological wastewater treatment. Ph.D. Thesis, Department of Cell and Molecular Biology, Microbiology, Göteborg University, Sweden.
Madsen, H.C.K., Buchmann, K., Mellergaard, S., 2000. Treatment of trichodiniasis in eel (*Anguilla anguilla*) reared in recirculation systems in Denmark: alternatives to formaldehyde. Aquaculture 186 (3–4), 221–231.
Maixner, F., Noguera, D.R., Anneser, B., Stoecker, K., Wegl, G., Wagner, M., Daims, H., 2006. Nitrite concentration influences the population structure of Nitrospira-like bacteria. Environmental Microbiology 8, 1487–1495.
Masters, A.L., 2004. A review of methods for detoxification and neutralization of formalin in water. North American Journal of Aquaculture 66 (4), 325–333.
Matthews, R.A., 2005. Ichthyophthirius multifiliis Fouquet and ichthyophthiriosis in freshwater teleosts. In: Baker, J.R. (Ed.), Advances in Parasitology. Academic Press, pp. 159–241.
Michaud, L., Blancheton, J.P., Bruni, V., Piedrahita, R., 2006. Effect of particulate organic carbon on heterotrophic bacterial populations and nitrification efficiency in biological filters. Aquacultural Engineering 34, 224–233.
Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Applied and Environmental Microbiology 62, 2156–2162.
Nash, T., 1953. The colorimetric estimation of formaldehyde by means of the Hantzch reaction. Biochemical Journal 55, 416–421.
Noble, A.C., Summerfelt, S.S., 1996. Diseases encountered in rainbow trout cultured in recirculating systems. Annual Review of Fish Diseases 6, 65–92.
Ong, S.L., Sarkar, S.K., Lee, L.Y., Hu, J.Y., Ng, H.Y., van Loosdrecht, M., 2006. Effect of formaldehyde on biofilm activity and morphology in an ultracompact biofilm reactor for carbonaceous wastewater treatment. Water Environment Research 78, 372–380.
Papadopulos, F., Spinelli, M., Valente, S., Foroni, L., Orrico, C., Alviano, F., Pasquinelli, G., 2007. Common tasks in microscopic and ultrastructural image analysis using Image. Journal of Ultrastructural Pathology 31, 401–407.
Pedersen, L.F., Pedersen, P., 2006. Temperature-dependent formaldehyde degradation in trickling filters. North American Journal of Aquaculture 68, 230–234.
Pedersen, L.-F., Pedersen, P.B., Sortkjaer, O., 2006. Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale bio filters. Aquacultural Engineering 34 (1), 8–15.
Pedersen, L.-F., Pedersen, P.B., Sortkjær, 2007. Temperature-dependent and surface specific formaldehyde degradation in submerged biofilters. Aquacultural Engineering 36, 127–136.
Pedersen, L.-F., Pedersen, P.B., Nielsen, J.L, Nielsen, P.H., 2008. Formaldehyde induced variation in biofilter performance and microbial composition. In: Proceedings from the Seventh International Conference on Recirculating Aquaculture, Roanoke, Virginia, pp. 430–439.
Pedersen, L.-F., Pedersen, P.B. Nielsen, J.L., Nielsen, P.H., in press. Peracetic acid degradation and effects on nitrification in recirculating aquaculture systems. Aquaculture, doi:10.1016/j.aquaculture.2009.08.021
Piedrahita, R.H., 2003. Reducing the potential environmental impact of tank aquaculture effluents through intensification and recirculation. Aquaculture 226, 35–44.
Pommerening-Roeser, A., Rath, G., Koops, H.-P., 1996. Phylogenetic diversity within the genus Nitrosomonas. Systematic Applied Microbiology 19, 344–351.
Rintamaki-Kinnunen, P., Rahkonen, M., Mannermaa-Keranen, A.L., Suomalainen, L.R., Mykra, H., Valtonen, E.T., 2005. Treatment of ichthyophthiriasis after malachite green. I. Concrete tanks at salmonid farms. Diseases of Aquatic Organisms 64, 69–76.
Schramm, A., de Beer, D., van den Heuvel, J.C., Ottengraf, S., Amann, R., 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira spp*. along a macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Applied and Environmental Microbiology 65, 3690–3696.
Schreier, T.M., Rach, J.J., Howe, G.E., 1996. Efficiency of formalin, hydrogen peroxide and sodium chloride on fungal-infected rainbow trout eggs. Aquaculture 140, 323–331.
Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating Aquaculture Systems, 2nd ed. NRAC Publication No. 01-002, Itchaca, NY, USA, p. 769.
Schwartz, M.F., Bullock, G.L., Hankins, J.A., Summerfelt, S.T., Mathias, J.A., 2000. Effects of selected chemo-therapeutants on nitrification in fluidized-sand biofilters for coldwater fish production. International Journal Of Recirculation Aquaculture 1, 61–81.
Shinn, A.P., Picon-Camacho, S.M. Bawden, R., Taylor N.G.H., in press. Mechanical control of Ichthyophthirius multifiliis Fouquet, 1876 (Ciliophora) in a rainbow trout hatchery. Aquacultural Engineering.
Sortkjær, O., Henriksen, N.H., Heinecke, R.D., Pedersen, L-F. 2008: Optimering af behandlingseffekten i akvakultur. Minimering af forbrug og udledning af hjælpestoffer [Optimizing treatment efficacy in aquaculture]. Danmarks Miljøundersøgelser, Aarhus Universitet. 124s. Faglig rapport fra DMU nr. 659. [In Danish; English abstract] http://www.dmu.dk/Pub/FR659.pdf.
Summerfelt, S.T., Sharrer, M.J., Tsukuda, S.M., Gearheart, M., 2009. Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. Aquacultural Engineering 40 (1), 17–27.
Wagner, M., Rath, G., Amann, R., Koops, H.P., Schleifer, K.H., 1995. In-situ identification of ammonia-oxidizing bacteria. Systematic and Applied Microbiology 18, 251–264.
Wagner, M., Rath, G., Koops, H.P., Flood, J., Amann, R., 1996. In Situ analysis of nitrifying bacteria in sewage treatment plants. Water Science and Technology 34, 237–244.
Wagner, M.A. Loy, Nogueira, R., Purkhold, U., Lee, N., Daims, H., 2002. Microbial community composition and function in wastewater treatment plants. Antonie Van Leeuwenhoek International 81 (1–4), 665–680.
Wienbeck, H., Koops, H., 1990. Untersuchungen zum Formalinabbau in Kreislaufanlagen [Decomposition of formaldehyde in a recirculation fish farming system]. Archives für Fischereiwissenschaft 40, 153–166.
Wooster, G.A., Martinez, C.M., Bowser, P.R., 2005. Human health risks associated with formalin treatments used in aquaculture: initial study. North American Journal of Aquaculture 67, 111–113.
Zar, J.H., 1999. Biostatistical Analysis, 4th ed. Prentice-Hall.

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PAPER III

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Peracetic acid degradation and effects on nitrification in recirculating aquaculture systems

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ABSTRACT

Peracetic acid (PAA) is a powerful disinfectant with a wide spectrum of antimicrobial activity. PAA and hydrogen peroxide (HP) degrade easily to oxygen and water and have potential to replace formalin in aquaculture applications to control fish pathogens, for example the ectoparasite, *Ichthyophthirius multifiliis*. We studied water phase PAA and HP decay in three aquaculture situations, i) batch experiments with two types of system waters, ii) PAA decay at different fish densities, and iii) degradation of PAA in submerged biofilters of recirculating aquaculture systems (RAS). Furthermore, effect of PAA on the nitrification activity and the composition of the nitrifying population were investigated.

PAA and HP decay showed first order kinetics. High dosage PAA/HP in water with low COD inhibited HP removal, which was not observed in water having a higher COD content. PAA decay was significantly related to fish stocking density, with half life constants for PAA of 4.6 and 1.7 h at 12 and 63 kg m^{-3}, respectively. PAA application to RAS biofilter showed rapid exponentially decay with half life constants of less than 1 h, three to five times faster than the water phase decay rates.

Biofilter surface specific PAA removal rates ranged from 4.6 to 13.9 mg PAA $m^{-2} h^{-1}$ and was positively correlated to the nominal dosage. Low PAA additions (1.0 mg L^{-1}) caused only minor impaired nitrification, in contrast to PAA application of 2.0 and 3.0 mg L^{-1}, where nitrite levels were significantly increased over a prolonged period, albeit without fish mortality. The dominant ammonium oxidizer was *Nitrosomonas oligotropha* and the dominant nitrite oxidizer was *Nitrospira*. Based on the present findings and other recent results from field and in vitro studies, application perspectives of PAA are discussed.

1. Introduction

There is a pertinent use of chemical additives and antibiotics related to aquaculture in order to improve water quality and treat fish diseases, respectively (Buchmann and Bresciani, 1997; Jørgensen et al., 2009). The chemical sanitizers are generally used to control fish pathogens in the systems and are applied to the water phase (Noble and Summerfelt, 1996; Boyd and Massaut, 1999; Rintamaki-Kinnunen et al., 2005a). White spot disease, caused by the ectoparasite *Ichthyophthirius multifiliis* (Matthews, 2005) is one of the most common parasitic outbreaks in recirculating aquaculture systems (RAS) and cause significant loss if not treated timely and correctly. To control outbreaks of *I. multifiliis*, formaldehyde is most commonly used and it is in many ways an ideal chemical to add to RAS, as its treatment efficiency is very high, without harming the fish nor the biofilter at the concentrations used for treatment (Pedersen et al., 2007). However, recent concern on potential environmental effects of excess formaldehyde discharge, as well as worker safety issues, has lead to an intention of phasing out the use of formaldehyde. Therefore, there is an urgent need to find and implement suitable therapeutic candidates to replace formaldehyde. In order to facilitate a safe and fully sustainable implementation of a new chemical, an array of new additional information about the chemicals is required.

Use of UV in combination with ozone have proven to be a feasible solution to control pathogens (Summerfelt and Hochheimer, 1997; Summerfelt et al., 2009), but to date this approach is not commercially applied in full-scale open Danish RAS systems. Hydrogen peroxide is an obvious candidate due to the minor environmental footprint, and proven efficiency towards *I. multifiliis* infections (Heinecke and Buchmann, 2009). However, the rapid degradation and potential impact on biofilter performance in RAS cause caution in the use of HP (Schwartz et al., 2000; Møller et al., in press). Sodium chloride has also proven to be effective towards *I. multifiliis* infections, when used at concentrations of 10–15 kg m^{-3}. It too has obvious limitations however, when very large water volumes are to be treated, both in terms of economy and handling, as in terms of the resulting discharge to a water course. At present, alternatives to formaldehyde are thus needed in controlling white spot disease.

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Peracetic acid (PAA) is a strong disinfectant with a wide spectrum of antimicrobial activity (Baldry, 1983; Kitis, 2004). The oxidation potential of PAA is larger than that of chlorine, hypochlorite and hydrogen peroxide and hence, due to a very high treatment efficacy, the use of PAA as a disinfectant for municipal wastewater effluents has recently received considerable attention (Antonelli et al., 2006; Falsanisi et al., 2006; Rossi et al., 2007; Santoro et al., 2007). PAA is available in commercial solutions in an acidic quaternary equilibrium mixture of hydrogen peroxide (HP), acetic acid (AA) and water in the equilibrium:

$$CH_3COOH + H_2O_2 \rightleftharpoons CH_3CO_3H + H_2O$$

The equilibrium stability and decomposition of PAA is pH-dependent (Yuan et al., 1997), and the commercial products are often stabilized by acidification.

The biocidal active form is undissociated at pH less than 8.2 (Wagner et al., 2002). PAA has far more antimicrobial effect than HP (Block, 1991), but the combination of PAA and HP has been found to be synergistic (Alasri et al., 1992). Various commercial compounds exist (Peroxyacetic acid; proxitane, Divosan®, Wofasteril; Peraqua and PerAqua Plus®; Promox; Parasan™; Incimaxx Aquatic) with different combinations of PAA and HP concentrations. Commercial compounds have a PAA content ranging from 3 to 40% and an HP content from 14 to 35%. In the present study we used PerAqua Plus® (PA+) containing 12% PAA and 30% HP according to the manufacturer (Brenntag, DK).

PAA can be considered a promising therapeutic for use in aquaculture. PAA has excellent antimicrobial activity and parasitidal effects over a wide temperature range, including temperatures below 10 °C (Block, 1991; Colgan and Gehr, 2001). It is relatively stable at low organic matter content, and it is degraded into harmless, neutral residuals (acetic acid and H_2O_2 and eventually to water), easing the discharge into receiving water bodies. A prerequisite for using PAA in aquaculture though, is that PAA neither poses sublethal effects to the fish treated nor impair the nitrification process in the biofilter at the dosages applied. The nitrification issue has become essential, as intensive aquaculture systems rely on a high degree of water reuse and hence, the need for constant removal of ammonium and nitrite by means of biofiltration. PAA treatment efficacy towards fish pathogens has been evaluated in some aquaculture studies (Rintamaki-Kinnunen et al., 2005a,b; Meinelt et al., 2007a,b; Lahnsteiner and Weismann, 2007), but PAA degradation kinetics and effects on nitrification under aquaculture conditions have, to our knowledge, not been reported. In order to ensure an adequate treatment efficacy, the most important issue is not the amount of PAA applied but the residual levels in the water during the treatment period (Weavers and Wickramanayaka, 1991). This has to be kept in mind as an explanation for the very different treatment efficacies reported from different studies. Therefore, knowledge of the fate and decay rates of PAA and HP under aquaculture conditions is clearly needed.

The aim of this study was to investigate (i) degradation rates of PAA and HP in biofilters and process water from different RAS, (ii) PAA degradation in relation to the fish stocking (biomass), and (iii) effects of PAA and HP on biofilters and stability with regard to accumulation of ammonia or nitrite and activity of the nitrifying communities.

2. Material and methods

2.1. Experimental setup

The experiments were carried out in twelve identical freshwater RAS, each with a total volume of 1700 L (Fig. 1). Each system consisted of a glass fiber rearing tank with approximately 500 L, connected to a swirl separator via a central drain. From the swirl separator, water was lead to a reservoir and pumped (up-flow) to a submerged biofilter, after which it was passed through a trickling filter and returned to the fish tank. The submerged biofilter was filled with four vertically positioned polyethylene media modules (Bioblok 150 HD®; Expo-Net, DK) with a total volume of 0.667 m^3 and a total surface area of 100 m^2. The trickling filter was fitted with the same type of media arranged horizontally (0.167 m^3 and a surface area of 25 m^2). The pump delivered water with a rate of ca. 100 $L\,min^{-1}$, resulting in an elevation velocity in the biofilter of approx. 15 cm min^{-1}. Flow and hydraulics were measured and documented in preliminary experiments using sodium chloride as a tracer while logging electrical conductivity with an EC-probe attached to an HQd40 multimeter (Hach Lange APS, DK).

2.2. Management and operating conditions

All systems were operated at similar conditions for more than three months before the PAA application experiments. Rearing tanks were stocked with rainbow trout (*O. mykiss*) of an average weight of 150 g. Biomass in each tank during the experiment was kept near 12 kg (rearing density ~24 $kg\,m^{-3}$), by regularly removing excess biomass, with resulting biomass ranging from 10.5 to 13.5 kg per tank. Throughout the experiment, all systems were allocated a fixed amount of 100 g commercial fish feed (DAN-EX 1754) per day during a 6 h period using belt feeders. 40 L of water was manually tapped from the swirl separators and automatically replaced with new tap water, corresponding to a water exchange rate of 2.35% $day^{-1}$and a hydraulic retention time of six weeks in the systems. Any uneaten feed pellets were counted and recorded daily by manually emptying the swirl. The relative water renewal rate was 400 $L\,kg^{-1}$ feed.

The temperature was kept constant at 17.0 ± 0.3 °C. pH and dissolved oxygen were monitored daily with a Hach Lange HQd40. Oxygen concentrations ranged from 7.2 to 8.5 mg L^{-1} on a daily basis, and the pH was kept between 7.2 and 7.4 and was adjusted on a daily basis by addition of sodium bicarbonate to compensate for the alkalinity loss due to nitrification. Ammonium and nitrite levels were below 0.2 mg NL^{-1} and nitrate between 50 and 75 mg NL^{-1} as monitored on a daily basis. The biomass of all fish in each tank were measured every third week, and surplus biomass was removed to keep the density and the feeding ratio somewhat constant. Fish were visually inspected every day, and mortalities and moribund fish were recorded daily.

2.3. Experimental protocols

The degradation of PAA and HP from the commercial product PA+ (Brenntag, DK) was investigated in three separate experiments: degradation in biofilters from RAS, degradation in the water phase, and degradation in relation to fish densities.

2.3.1. PAA degradation in submerged aquaculture biofilters

This experiment was designed as a single factor experiment with nominal PAA concentration as the fixed factor and PAA degradation rate as the response parameter. Nine RAS were randomly chosen and exposed in triplicate to the different PA+ dosages, corresponding to nominal PAA concentrations of approximately 1, 2 and 3 mg L^{-1} hereinafter referred to as “low”, “medium” and “high” exposure, respectively. The remaining three RAS served as control systems and were not exposed to PA+. The PA+ addition and degradation experiments were performed in a closed circuit of the RAS (volume ~1170 L) by bypassing the rearing tank (and hence the fish) and the swirl separator during exposure (Fig. 1). Prior to and during these experiments, the fish were not fed. Sufficient dissolved oxygen was maintained in each tank by providing additional aeration with a Resun® multichannel airpump and air stones. The trickling filter provided sufficient dissolved oxygen for the biofilter units. Water samples were taken prior to PA+ addition and during frequent, regular intervals afterwards.

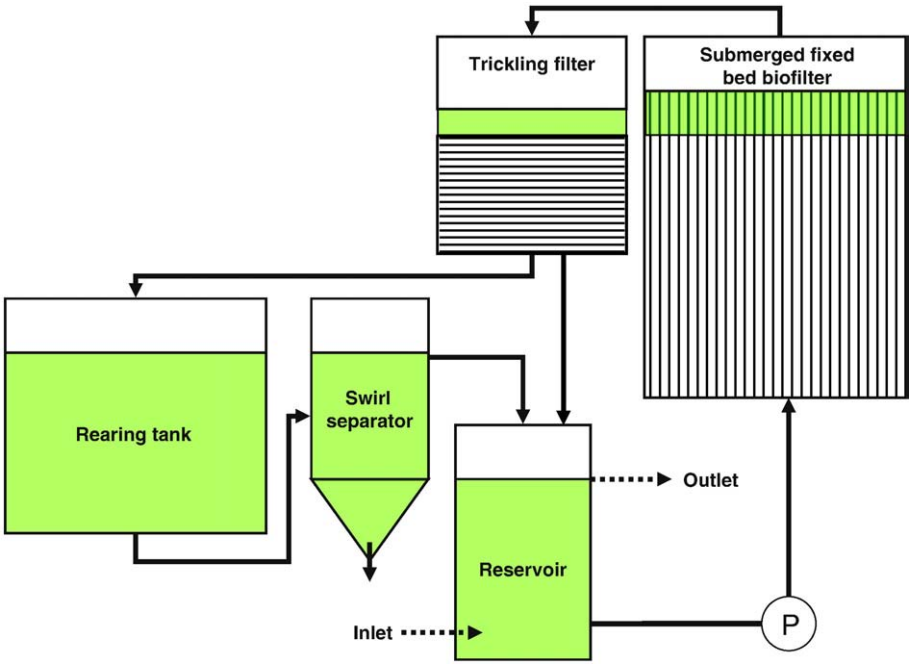

Fig. 1. The experimental recirculating aquaculture system ($N = 12$). Water circulation in the RAS is represented with thick arrows; dotted lines represent inlet and outlet water.

2.3.1.1. Biofilter performance: ammonium and nitrite removal. Nitrification performance in the biofilter of each system was investigated by ammonium spiking experiments the day before and the day after PA+ addition. In the short-circuited biofilter system, NH_4Cl equivalent to 3 mg L^{-1} total ammonia nitrogen (TAN) was added to the rearing reservoir. Baseline biofilter performance measured as TAN removal rate and nitrite build up were determined in all twelve RAS prior to PA+ addition, and repeated by an additional TAN spike the day after PA+ exposure. Water was sampled prior to TAN addition and with regular intervals during a period of 4 h after PA+ addition. Hereafter, the inlet valve to the rearing tank was reopened, and potential ammonium and nitrite accumulates were mixed and diluted into the full RAS. Nitrification performance was also followed by daily measurements of TAN and NO_2^-–N in the process water over a prolonged period of 28 days following the PAA addition.

2.3.2. PAA degradation in process water from two distinct RAS

Water was sampled from two different types of RAS. RAS I water originated from one of the twelve RAS (Exp. 2.3.1), and RAS II water from a closed pilot scale RAS (Møller et al., in press). The water samples, as well as controls with milli-Q water, were poured into 500 ml beakers ($N = 15$), supplemented with air via airstones and acclimatized to room temperature (21 °C) for 1 h. PA+ was added corresponding to C_0 of 1.0–5.0 mg PAA L^{-1}. Water samples were both fixed with regard to HP for later measurements and immediately analyzed for PAA content.

2.3.3. PAA degradation in relation to fish density

Six 200 L cylinder tanks were each filled with 100 L of dechlorinated tap water, supplemented with air via airstones and acclimatized overnight. Rainbow trout (200–400 g) from a nearby holding tank were introduced at densities of 10 to 60 kg m^{-3}). PAA was added to each tank after one hour at a concentration of 1 mg L^{-1}. Water samples were successively collected for determination of actual PAA concentration. Fish behaviour and any moribund fish were recorded during the experiment.

2.4. Fish performance

Fish growth was evaluated by feed conversion ratio (FCR), relating total added amount of feed (minus any uneaten feed) during the experimental period to biomass gained. The latter was determined as final biomass plus biomass of dead fish minus initial biomass. Fish behaviour and any moribund fish were recorded daily throughout the experiment.

2.5. Chemical analyses

Water samples were measured for TAN, nitrite-N and nitrate-N content, as well as for alkalinity, total suspended solids and COD as described by Pedersen et al, 2008.

Analysis of HP residual concentration was done as described by Pedersen et al., 2006. The residual PAA concentration was measured immediately after sampling according to Falsinisi et al., 2006. HP and PAA concentrations in the PA+ stock solution were analyzed by a two step titration according to the producer's protocol (Brenntag, DK).

2.6. Screening and identification of nitrifying microorgansims in biofilters

For culture-independent identification and enumeration of ammonia- and nitrite-oxidizing *Bacteria* and *Archaea* in biofilm samples collected during the experimental period the fluorescence in situ hybridisation (FISH) technique (Amann and Fuchs, 2008) was applied. Samples were fixed for 2 h in 4% paraformaldehyde at 4 °C, then stored at −20 °C in 1:1 ethanol/PBS solution. Different ribosomal RNA-targeted oligonucleotide probes were used: EUB338mix (equimolar concentrations of EUB338, EUB338II, and EUB338III) covering most *Bacteria* (Amann et al., 1990; Daims et al., 1999), Nso1225 which covers many betaproteobacterial AOB (Mobarry et al., 1996), Cluster 6A192 covering *Nitrosomonas oligotropha* lineage (Cluster 6a) (Adamczyk et al., 2003), Nmo218 which cover *N. oligotropha* lineage (Gieseke et al., 2001), Nsm156 which cover *Nitrosomonas* spp. and *Nitrosococcus* (Mobarry et al., 1996), Nsv443 covering *Nitrosospira*

(Mobarry et al., 1996), NEU, which cover most halophilic and halotolerant *Nitrosomonas* spp. (Wagner et al., 1995), Nse1472 which cover *Nitrosomonas europea*, *N. halophila* and *N. eutropha* (Juretschko et al., 1998) and Nmv covering *Nitrosococcus mobilis* lineage (Pommerening-Roser et al., 1996), Ntspa662 (incl. competitor probe) which covers the genus *Nitrospira* (Daims et al., 2001) NIT3 (incl. competitor probe) covering *Nitrobacter* spp. (Wagner et al., 1996) and three archaeal probes: Arch915 probe which target *Archaea*; probe CREN499 which target most *Crenarchaeota* and probe EURY498 which target most *Euryarchaeota*. A nonsense probe (NonEUB) was used as negative control. Details on oligonucleotide probes and hybridization conditions are available at probeBase (Loy et al., 2007).

2.7. Statistics

PAA and HP removal rate was estimated from exponential first order regression analyses on measured concentration values above 0.1 mg L^{-1} only. This value was arbitrarily selected to obtain equal wide ranges of concentration and to exclude disproportionate weighting from low concentrations. Testing for difference between two degradation rates was done by comparing linear regression coefficient (Zar, 1999).

3. Results

3.1. PAA removal in submerged aquaculture biofilters

In all three groups of biofilters tested (low, medium and high level of PA+ addition), a rapid decrease in PAA was observed after PA+ addition, which was found to fit a first order equation: $C_t\ C_0\ e^{-kt}$ (Fig. 2). Systems treated with equal amounts of PA+ showed response curves with only minor variation, whereas the removal rates were found to differ significantly between the treatment groups. The average half life constant ($\ln 2/k_r$) of 0.46 h at low PA+ dosage was half of the value of 0.93 h found from the experiments with high dosage (Table 1). Within 3.5 h after addition 93.3 to 99.9% of the PAA added was removed.

The initial decay constant, estimated as a constant rate within the first hour after PAA addition, was positively related to PAA dosage with 0.51, 0.97 and 1.48 mg PAA $L^{-1}h^{-1}$ removed, respectively. Based on available biofilter surface area, the surface specific removal rate was found to range from 4.6 to 13.9 mg PAA $m^{-2}h^{-1}$ (Table 1).

Hydrogen peroxide was found to be simultaneously eliminated after PA+ addition, with decay kinetics similar to PAA. For both chemical agents, significant effects of dosage on removal rates and initial decay constants were found. PAA and HP kinetics were similar at low and medium PA+ exposure, whereas the ratio between the two first order rates (PAA_{Kr} and HP_{Kr}) increased markedly to approx. 1.3 in the systems exposed to high PA+ ($p = 0.09$).

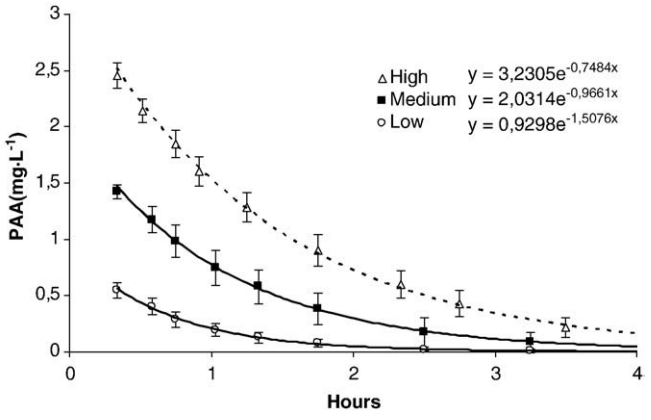

Fig. 2. Peracetic acid (PAA) decay after application to biofilters form RAS. Data represent PAA concentration (mean ± std.) of triplicate experiments with low, medium and high PAA dosage, equivalent to nominal concentrations of 1.0, 2.0 and 3.0 mg L^{-1}. Exponential regression equations listed, all have $R^2 \geq 0.995$.

3.2. PA+ effects on biofilter performance

Baseline biofilter performances after NH_4Cl spiking the day prior to PA+ addition followed zero order kinetics with regards to TAN removal. Based on linear regression of data from all 12 RAS, a total average TAN removal rate of 428 ± 17 μg TAN $L^{-1}h^{-1}$ ($R^2 = 0.94 \pm 0.04$) was found, equivalent to a surface specific removal rate (SSRr) of 96 ± 3.8 TAN mg m^{-2} day^{-1}. Average TAN removal rates were similar in all treatment groups before PA+ exposure (Day −1) but it was significantly reduced among systems treated with medium and high dose PAA the day after PA+ exposure (Fig. 3). After eight days with constant organic loading (feeding), all systems performed equally in ammonia removal (Fig. 4), and TAN levels were kept below 0.3 mg TAN L^{-1} throughout the remaining experimental period.

NH_4Cl spiking the day prior to PA+ addition led to short transient nitrite accumulation up to, 0.8–1.0 mg N L^{-1} for all 12 systems, and all returned below 0.2 mg N L^{-1}, when measured 20 h after spiking. After PA+ application, significant nitrite accumulations were observed in the systems treated with PAA (Fig. 5). A minor accumulation, both temporally and in magnitude, was observed in the three systems treated at the low PA+, dosage whereas medium and high dose PA+ led to an additional 4-to 5 fold increase in nitrite level. NO_2^-–N levels ≥5 mg L^{-1} were measured five and eight days after PA+ exposure, with a considerable variation within treatment groups (Fig. 5). Maximum nitrite levels of 5.56 mg N L^{-1} were measured in a medium dose exposed system, with corresponding values of 4.15 and 3.12 mg N L^{-1} in the other two systems treated likewise. All systems fully recovered with stable nitrite oxidation four weeks after PAA addition.

3.3. PAA and HP degradation in the process water from two distinct RAS

The PAA removal in the water phase from the batch experiments (Fig. 6) followed first order kinetics at all five nominal concentrations in both RAS-I and RAS II water (COD content 28.6 ± 0.1 and 48.8 ± 0.5 mg O_2 L^{-1}, respectively). T½ of PAA were significantly positively related to PA+ dosage ($T½_{RAS\text{-}I} = 1.142X$; $R = 0.90$; $T½_{RAS\text{-}II} = 0.963X$; $R = 0.98$; $p < 0.05$; with X being PAA concentration in mg L^{-1} and T½ is h), with similar response in both types of water ($p = 0.37$). An inferior PAA decay was found in batches with Millipore water, estimating half live constants in the range of 50 to 90 h. Hydrogen peroxide was also exponentially degraded in both types of water (Table 2), but as opposed to PAA, a highly significant effect of water quality was found ($p < 0.01$). In the water samples from RAS-II with higher organic matter content, HP removal rates were similar to PAA removal rates, whereas HP decay was reduced by up to a ten-fold when compared to PAA removal rates in RAS I water with lower organic matter content. HP decay in control experiments were negligible (T½ >> 100 h), with changes in HP concentration after 20–25 h being close to the detection level.

3.4. PAA degradation in relation to fish density

Nominal concentrations of PAA ranged between 1.05 and 1.15 mg L^{-1} and PAA was degraded exponentially immediately after addition. The decay rates were significantly positively related to fish biomass ($p < 0.001$), with T½ > 5 h in the system without fish, and being reduced to 100 min in the system with a fish density of 63 kg m^{-3} (Fig. 7).

3.5. Fish performance

During the experimental period no fish mortality occurred. Distinctly altered fish behaviour was observed during the days following PAA

Table 1
Summary of PAA and HP removal kinetics in RAS biofilters exposed to 8, 16 and 24 ml PA+ per m^3 (low, medium and high, respectively).

Treatment groups	Peracetic acid removal kinetics			Hydrogen peroxide removal kinetics		
	1st order removal rate constant k_r	Zero order removal constant k	Surface specific removal rate SSRr	1st order removal rate constant k_r	Zero order removal constant k	Surface specific removal rate SSRr
Low	1.419	0.491	4.60	1.475	1.68	15.7
	1.671	0.513	4.80	1.797	1.80	16.9
	1.242	0.536	5.02	1.100	1.80	16.9
Medium	1.182	1.04	9.73	1.129	3.23	30.2
	1.220	1.06	9.92	1.104	3.15	29.5
	0.723	0.83	7.77	0.797	2.66	24.9
High	0.918	1.48	13.9	0.768	4.35	40.7
	0.708	1.41	13.2	0.442	3.49	32.7
	0.717	1.42	13.3	0.623	4.18	39.1
R^2 range	0.99–1.00	0.95–1.00		0.99–1.00	0.95–1.00	

The removal rate constant k_r are derived from 1st order estimates (exponential linear regression) on measured samples ($PAA\,L^{-1}\,h^{-1}$) is calculated as an initial removal constant based on data from dosage to T = 1 h, and surface specific removal rate SSRr ($mg\,PAA\,m^{-2}\,h^{-1}$) is calculated based on volume and biofilter surface area.

addition. Within two systems treated with a high PAA dosage, trout altered behaviour and became stationary close to the surface for 1 to 2 days. Reduced feeding activity was also noticed, which was reflected by an abrupt increase in the number of uneaten pellets. This pattern was observed within only a small number of the systems at the medium or high dose treated PA+. After 3–4 days all fish were actively eating again, and were observed to gather under to the belt feeder.

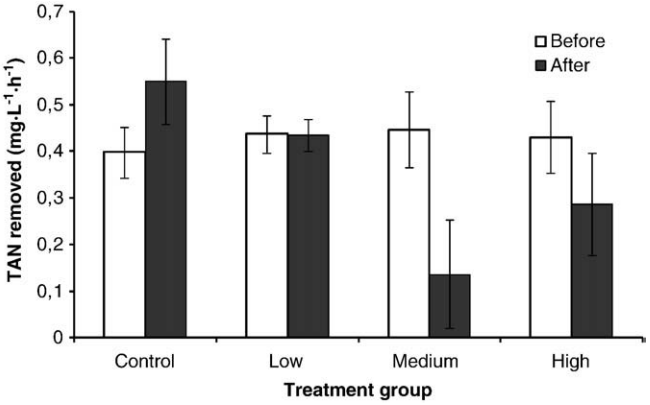

Fig. 3. TAN removal rate in biofilters from RAS spiked with NH_4Cl before and after PA+ addition (Day −1 and Day +1). Values represent mean ± std based on linear regression of triplicate experiments of each four treatment group ($N = 12$).

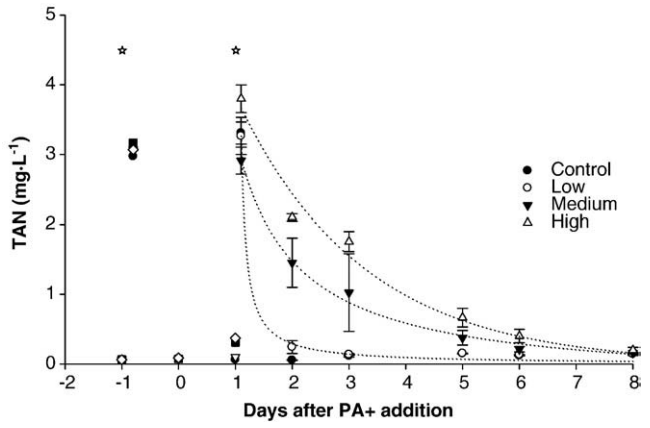

Fig. 4. TAN concentration in RAS before and after PA+ addition at Day 0. PA+ was added at dosages equivalent to 8, 16 and 24 ml PA+ per m^3 (low, medium and high, respectively). Asterisks indicate events (Day −1 and 1) where NH_4Cl was added to the systems.

No differences were observed in biomass growth between treatment groups, and no deviating visual cues were observed. Feed conversion ratios ranged from 1.10–1.14 during period II, 1.20–1.29 in period III and 1.02–1.14 after PAA during period IV, with SGR ranging from 0.64 to 0.76%·day^{-1}. PA+ facilitated water quality deterioration did not appear to affect FCR or SGR (data not shown).

3.6. Nitrifying bacteria and Archaea in biofilter samples

AOB were detected by the FISH technique in all samples using probe Nso1225, which target most AOB belonging to the *Betaproteobacteria*. Additional samples (unexposed control system and a high PA+ exposed system harvested at Day −1 and Day 14) were screened with more specific AOB probes, and four probes (Cluster6a_192, Nsm156, Nmo218, and NEU) but not Nse1472 targeting nitrosomonads were found positive. *Nitrosospira* spp. were not detected using probe Nsv443, neither were *Nitrosococcus* spp. detected by probe NmV.

NOB as *Nitrospira* spp. were found in all biofilm samples as single cells and microcolony assemblies using the probe Ntspa662. *Nitrobacter* spp. was targeted in two out of twenty-two samples but only as a few single positive cells.

Samples screened with selected archaeal probes, were found to contain several *Archaea* cells (as detected by the Arch915 probe), and the use of the more specific probe CREN499 revealed that this group consisted mainly of *Crenarchaeota*. No *Archaea* cells were found to belong to the phylum *Euryarchaeota* (EURY498). Common for all of the hybridizations above was that positive cells were present in relative low numbers (typically less than 0.5% of total EUB mix positive cells) hence no further quantification was performed.

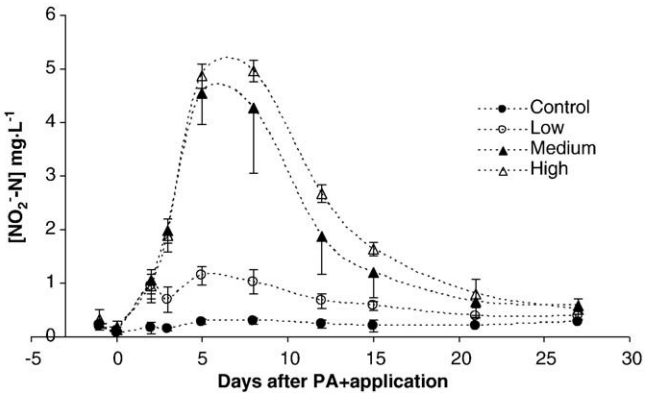

Fig. 5. Nitrite level in RAS before and after PA+ addition to the biofilter at Day 0.

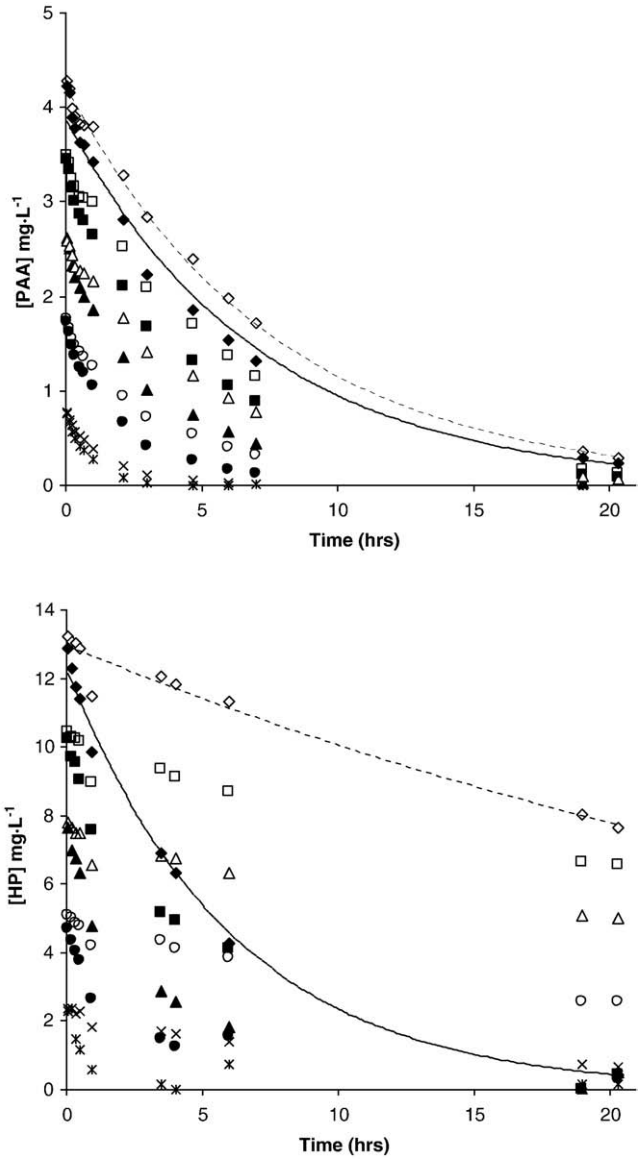

Fig. 6. Peracetic acid (PAA) and hydrogen peroxide (HP) decay in two types of aquaculture water with different COD levels of 28.6 mg $O_2 L^{-1}$ (open symbols, broken trend lines) and 48.8 mg $O_2 L^{-1}$ (filled symbols, solid trend lines). PA+ were added corresponding to nominal concentrations ranging from 1.0 to 5.0 mg L^{-1} PAA and 2.6 to 13.0 mg L^{-1} HP.

Table 2
Summary of PAA and HP 1 order removal kinetics in batch experiments with two types of system water (low: COD = 28.6 mg $O_2 L^{-1}$, high: COD = 48.8 mg $O_2 L^{-1}$) and milli-Q water.

PA+ exp.		Peracetic acid removal kinetics		Hydrogen peroxide removal kinetics	
Treatment groups	[PAA] C_0	1st order removal rate constant k_r	Half life T½ (h)	1st order removal rate constant k_r	Half life T½ (h)
Low organic matter RAS I	1.0	0.559	1.2	0.059	12
	2.0	0.238	2.9	0.032	22
	3.0	0.174	4.0	0.020	35
	4.0	0.162	4.3	0.022	32
	5.0	0.130	5.3	0.026	27
High organic matter RAS II	1.0	1.174	0.6	0.782	0.9
	2.0	0.382	1.8	0.321	2.2
	3.0	0.247	2.8	0.239	2.9
	4.0	0.179	3.9	0.158	4.4
	5.0	0.139	4.9	0.177	3.9
Milli-Q water	1.0	0.014	50	0.0005	>>100
	2.0	0.009	75	0.0009	–
	3.0	0.008	88	0.0002	–
	4.0	0.010	73	0.0015	–
	5.0	0.008	92	0.0018	–
R^2 range[a]		>0.98		0.99–1.00	

Exponential linear regression is made on measured PAA and HP concentrations ($N = 10$–12 per. trial). R^2 values for exp. regression analysis for all PAA and HP trials in system water ≥0.98.
[a] R^2 values for PAA decay in Milli-Q ≥ 0.95, with an exception at 0.86.

4. Discussion

A safe and reliable aquaculture guideline for water treatment with PAA does not exist and is difficult to extract from current literature. Interpretation of various studies is complicated by the complexity of pathogen infections, the diversity of aquaculture systems and rearing conditions, as well as different antimicrobial strength of available commercial peracetic acid compounds. The present findings of PAA degradation kinetics and RAS biofilter tolerance under one set of controlled aquaculture conditions is an attempt to identify important parameters for the use of PA+ in a well-described aquaculture system and to identify further needs for applied research.

4.1. Factors affecting PAA and HP degradation

PAA is primarily degraded by chemical oxidation in contrast to the microbial removal of HP by catalase activity (Block, 1991). Temperature, therefore, has a much more pronounced effect on the rate of HP degradation than on PAA degradation. The decay of PAA was found to fit exponential first order kinetics in accordance with other studies (Antonelli et al., 2006; Falsanisi et al., 2006), although zero order processes with short contact time have also been reported (Santoro et al., 2007). Typically, low dosage PAA from 0.5 to 2 mg L^{-1} and short contact times have been found sufficient to obtain satisfactory disinfecting of secondary and tertiary effluent from municipal wastewater systems (Baldry and French, 1989; Stampi et al., 2001; Wagner et al., 2002).

In our study we found that the degradation of PAA depended on the amount of organic matter in the system. The decay rate in the water phase was low (low COD level) and it increased with increasing amounts of fish biomass and with the presence of a biofilter. Thus, these factors are important for the use of PAA as an antiparasitic agent in aquaculture. The relation between biofilter surface area and total water volume is the primary influence for the absolute PAA consumption

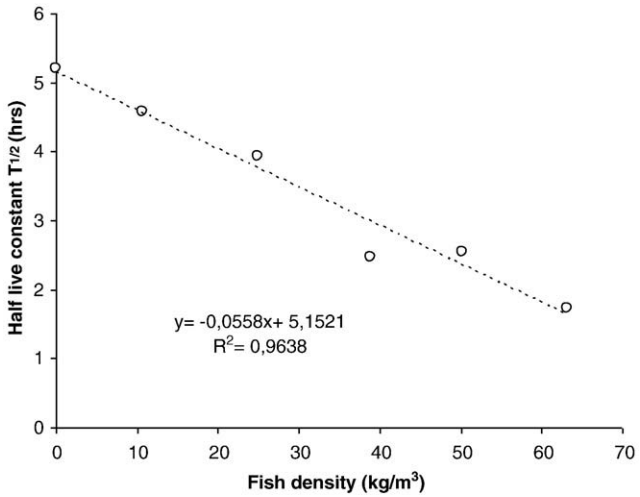

Fig. 7. PAA degradation according to stocking density; nominal peracetic acid concentration corresponded to ~1.0 mg L^{-1}.

during the resulting contact time. PAA decay rates in the water phase were found relatively unaffected by different low COD levels (25 and 45 mg $O_2 L^{-1}$), whereas other studies have shown an increased PA+ consumption and a reduced microbial inactivation at increasing levels of organic matter content (Stampi et al., 2001; Koivunen and Heinonen-Tanski, 2005). This relation was observed with HP concentration, which declined exponentially, as also found in other studies (Wagner et al., 2002; Pedersen et al., 2006; Møller et al., in press). However, in combination with a high dose PA+ and low organic matter content (~low microbial abundance), the resulting HP decay ceased, which is indirect evidence that PAA facilitated microbial inactivation, causing an inhibition of microbial catalase activity.

4.2. PA+ effects on biofilter nitrification

The magnitude of biofilter disturbance was related to the amount of PA+ added, i.e. when PA+ was applied at a dosage equivalent to 1.0 mg L^{-1} PAA, only transient disturbance of the nitrification process was observed. Based on the daily addition of feed and the protein content therein, approx. 3 g of TAN should be added to the system water (Timmons et al., 2002) resulting in a daily TAN addition ($\Delta_{[Tan]}$) of 1.7 mg L^{-1} when accounting for the minimal water exchange. If the biofilter were fully inactivated in regard to ammonia oxidizing bacteria, TAN concentration would have increased further on top of the TAN addition the day following PA+ application. And though TAN removal capacity was reduced compared to performance prior to PA+ addition, no signs of ammonia accumulation were observed in any of the systems.

Nitrite was partially accumulated with a delay reflecting the TAN removal pattern. However, if NOB were fully inactivated and hence nitrite oxidation stopped, nitrite levels would have exceeded 10 mg N L^{-1} on Day 5, which was not observed and indicated that the NOB were not completely inactivated. A full recuperation of NOB lasted four weeks, and underscores the vulnerability and slow colonization of NOB (Henze et al., 2002; Keck and Blanc, 2002). Very few studies have investigated PAA or HP effect on biofilter performance and stability. Schwartz et al. (2000) challenged a fluidised sand biofilter with 100 mg L^{-1} HP and observed a severe short term impairment of the nitrification as TAN removal efficiency, and found recovery lasted 10 days. Other studies have investigated application of HP in closed RAS, and dosages equivalent to 35 mg L^{-1} HP were found to cause only insignificant changes in TAN or nitrite removal efficiency (Pedersen et al., 2006; Sortkjær et al., 2008).

The present results should be interpreted in the light of the relatively low organic loading rate of the system. The negative effects of PA+ observed might reflect a worse-case scenario. When PA+ was added in equivalent 0.15, 0.8 or 2.0 mg L^{-1} PAA to different types of closed RAS systems described elsewhere (Sortkjær et al., 2008), neither TAN nor nitrite-N exceed 0.5 mg L^{-1} on the consecutive days during normal operation where daily feeding resulted in nitrogenous loading at 4.2 mg TAN L^{-1} (data not shown). This is presumably a result of a faster PAA and HP breakdown, and hence a reduced contact time, as well as a more robust biofilm composition due to a higher organic load.

4.3. Microbial composition

Biofilm samples from the biofilter revealed presence of ammonia oxidizing *Nitrosomonas* spp. (nitrosomonads) and nitrite-oxidizing *Nitrospira* spp. These species are commonly found in municipal wastewater treatment plants (Wagner et al., 1995; Daims et al., 2001) and were also found in a salt water recirculated aquaculture systems (Foesel et al., 2008, Pedersen et al., 2008). The FISH analysis demonstrated that the majority of the populations were related to *Nitrosomonas* spp., with no *Nitrosospira* spp. or *Nitrosococcus* spp. detected. The screening indicated *N. oligotropha* to be the dominant AOB. *N. oligotropha* has a strikingly low affinity constant (Koops et al., 2006) and hence benefits from the low level ammonia found in the freshwater aquaculture system. *Nitrobacter* sp. was just detected in two out of twenty-two samples, and is apparently of no or minor importance to total nitrite oxidation. *Nitrospira* spp. made up the most abundant NOB, which has also been observed in other studies with highly diluted water and low nitrogenous content, and corroborate with the findings that *Nitrospira* has a higher substrate affinity compared to *Nitrobacter* (Schramm et al., 1999; Blackburne et al., 2007).

The relative abundance of AOB and NOB were not quantified as both AOB and NOB were found in low abundances. The presence of similar AOBs before and two weeks after PA+ addition, from both control and exposed systems, did not indicate any significant changes in microbial composition in terms of altered presence or absence.

Crenarchaea and other *Archaea* were found in biofilm samples in relative low numbers (<1%) and could potentially have contributed to the ammonia oxidation as described in other studies (Könneke et al., 2005; Park et al., 2006; Urakawa et al., 2008).

4.4. Implications of PAA use in aquaculture systems

In an aquaculture context, chemical disinfection is a compromise between the benefits of destroying pathogens and the potential toxicity to fish, as well as environmental issues of the risk of discharging residuals or undesirable disinfection by-products. Furthermore, if the aquaculture system recycles water and depends on biological filtration to keep ammonia and nitrite levels low, caution should be taken on the tolerance and vulnerability of the nitrifying units when using disinfectants.

Information of disinfection efficiency and PAA consumption from municipal wastewater studies with secondary and tertiary effluents can be transferred to aquaculture systems. A high disinfection efficiency has been reported with dosages from 0.5 to 2 mg L^{-1} PAA (Stampi et al., 2001; Wagner et al., 2002; Koivunen and Heinonen-Tanski, 2005). Koivunen and Heinonen-Tanski (2005) found that most of the microbial reduction occurred within 15 min of contact time, and demonstrated further that extended contact time with a low PAA dosage increased reductions of enteric bacteria. Recommendations from authorized veterinarians on PAA application in traditional flow through systems would typically be in the range of 0.2–2.0 mg L^{-1} PA+. In RAS, none or highly precautionary lower levels would presumably be recommended.

Meinelt et al. (2007a) exposed walleyes (*Sander lucioperca*) to increasing PAA concentrations (0, 0.5, 0.9, 1.3 and 1.9 mg L^{-1} PAA) for 24 h, and observed increasing mortality starting at 0.9 mg L^{-1} and 100% mortality was observed at 1.9 mg L^{-1}. Sortkjær et al. (2008) made 1-h toxicity tests on rainbow trout, and found that trout fully tolerated 1.3 and 2.6 mg L^{-1} PAA, whereas 18% mortality was observed during the 24 h recovery period after exposure to 3.9 mg L^{-1} PAA. In vitro experiments with *I. multifiliis* theronts have shown complete antiparasitic effects by short term exposure with 0.3 mg L^{-1} PAA, and very significant reductions with 0.2 mg L^{-1} PAA exposure (Meinelt et al., 2007b). Strauss and Meinelt (2009) compared treatment efficiency against *I. multifiliis* with two commercial PAA solutions, and found effective concentrations (LC_{50} after 1 h exposure) in the range from 0.17 to 0.28 mg L^{-1}. Rintamaki-Kinnunen et al. (2005a,b) tested peracetic acid compounds against *I. multifiliis* in field trials and found that 1–1.3 mg L^{-1} PAA could reduce the parasitic load on the fish. Differences between treatments were observed, and PAA were less effective in earth ponds compared to concrete tanks. It was generally also less effective than formalin and malachite treatments.

From a practical point of view, treatment with PAA in RAS could include a temporary bypass of the biofilter which would allow PAA application in the rearing units for a predefined period of time and then redirect the process water to a specific filter where potential excess PAA could be degraded without affecting the nitrifying

microorganisms. If possible, a temporary reduction in the treated water volume would further lead to reduced need for PA+ and hence reduce the potential risk of unwanted side effects. In general, simple preliminary tests such as PA+ decay in water from a given fish farm would help circumvent incidences where the commercial product or the water quality lead to unexpected levels of PAA residues. TAN and nitrite levels should also be monitored regularly, in order to make corrected action, i.e. cease of feeding or addition of salt. In the present study only fish behavioural responses to the sudden increase in TAN and nitrite levels were observed. Prolonged increased levels of nitrite, without corrected actions did not cause mortality, and trout growth performance was unaffected compared to unexposed controls.

In order to ensure adequate treatment efficiency, the most important issue is not the amount of PAA applied but the residual levels in the water during the treatment period (Weavers and Wickramanayaka, 1991). Future studies would be improved if PAA residuals were analytically verified during the treatment period as emphasised by Rach et al. (1997), and repeated PAA/HP application under controlled commercial conditions would provide valuable information.

In conclusion, the potency and potential, but also the risks of PAA as a candidate agent in aquaculture, have been documented in the presented study. Further work should elaborate on the perspectives for optimizing the treatment strategy within the safe treatment zones for peracetic acid.

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References

Adamczyk, J., Hesselsoe, M., Iversen, N., Horn, M., Lehner, A., Nielsen, P.H., Schloter, M., Roslev, P., Wagner, M., 2003. The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. Applied Environmental Microbiology 69, 6875–6887.

Alasri, A., Roques, C., Michel, G., Cabassu, C., Aptel, P., 1992. Bactericidal properties of peracetic-acid and hydrogen-peroxide, alone and in combination, and chlorine and formaldehyde against bacterial wastewater. Canadian Journal of Microbiology 38, 635–642.

Amann, R., Fuchs, B.M., 2008. Single-cell identification in microbial communities by improved fluorescence in situ hybridization techniques. Nature Reviews Microbiology 6, 339–348.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology 172, 762–770.

Antonelli, M., Rossi, S., Mezzanotte, V., Nurizzo, C., 2006. Secondary effluent disinfection: PAA long term efficiency. Environmental Science & Technology 40, 4771–4775.

Baldry, M.G.C., 1983. The bactericidal, fungicidal and sporicidal properties of hydrogen-peroxide and peracetic-acid. Journal of Applied Bacteriology 54, 417–423.

Baldry, M.G.C., French, M.S., 1989. Activity of peracetic-acid against sewage indicator organisms. Water Science and Technology 21 (12), 1747–1749.

Blackburne, R., Vadivelu, V.M., Yuan, Z.G., Keller, J., 2007. Kinetic characterisation of an enriched *Nitrospira* culture with comparison to *Nitrobacter*. Water Research 41, 3033–3042.

Block, S.S., 1991. Peroxygen compounds, In: Block, S.S. (Ed.), Disinfection, sterilization, and preservation, fifth ed. Pa, Philadelphia. ISBN: 0-683-30740-1, pp. 185–205.

Boyd, C.E., Massaut, L., 1999. Risks associated with the use of chemicals in pond aquaculture. Aquacultural Engineering 20, 113–132.

Buchmann, K., Bresciani, J., 1997. Parasitic infections in pond-reared rainbow trout *Oncorhynchus mykiss* in Denmark. Diseases of Aquatic Organisms 28, 125–138.

Colgan, S., Gehr, R., 2001. Disinfection. Water Environment and Technology 13 (11), 29–33.

Daims, H., Bruhl, A., Amann, R., Schleifer, K.H., Wagner, M., 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe set. Systematic and Applied Microbiology 22, 434–444.

Daims, H., Nielsen, J.L., Nielsen, P.H., Schleifer, K.H., Wagner, M., 2001. In situ characterization of nitrospira-like nitrite-oxidizing bacteria active in wastewater treatment plants. Applied Environmental Microbiology 67 (11), 5273–5284.

Falsanisi, D., Gehr, R., Santoro, D., Dell'Erba, A., Notarnicola, M., Liberti, L., 2006. Kinetics of PAA demand and its implications on disinfection of wastewaters. Water Quality Research Journal of Canada 41, 398–409.

Foesel, B.U., Gieseke, A., Schwermer, C., Stief, P., Koch, L., Cytryn, E., de la Torre, J.R., van Rijn, J., Minz, D., Drake, H.L., Schramm, A., 2008. *Nitrosomonas* Nm143-like ammonia oxidizers and *Nitrospira marina*-like nitrite oxidizers dominate the nitrifier community in a marine aquaculture biofilm. FEMS Microbiology Ecology 63, 192–204.

Gieseke, A., Purkhold, U., Wagner, M., Amann, R., Schramm, A., 2001. Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. Applied Environmental Microbiology 67, 1351–1362.

Heinecke, R.D., Buchmann, K., 2009. Control of *Ichthyophthirius multifiliis*: dose–response trials using sodium percarbonate and water filtration. Aquaculture 288 (1), 32–35.

Henze, M., Harremoes, P., la Cour Jansen, J., Arvin, E., 2002. Waste water treatment. Springer Verlag, Berlin. ISBN: 3-540-42228-5, p. 430.

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P., Wagner, M., 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Applied Environmental Microbiology 64, 3042–3051.

Jørgensen, T.R., Larsen, T.B., Buchmann, K., 2009. Parasite infections in recirculated rainbow trout (*Oncorhynchus mykiss*) farms. Aquaculture 289 (1), 91–94.

Keck, N., Blanc, G., 2002. Effects of formalin chemotherapeutic treatments on biofilter efficiency in a marine recirculating fish farming system. Aquatic Living Resources 15, 361–370.

Kitis, M., 2004. Disinfection of wastewater with peracetic acid: a review. Environment International 30, 47–55.

Koivunen, J., Heinonen-Tanski, H., 2005. Peracetic acid (PAA) disinfection of primary, secondary and tertiary treated municipal wastewaters. Water Research 39, 4445–4453.

Koops, H.P., Purkhold, U., Pommerening-Roeser, A., Timmermann, G., Wagner, M., 2006. The lithoautotrophic ammonia-oxidizing bacteria, In: Stackebrandt, E. (Ed.), The Prokaryotes, third ed. Springer-Verlag, New York, pp. 778–811. doi:10.1007/0-387-30745-1_36.

Könneke, M., Bernhard, A.E., de la Torre, J.R., Walker, C.B., Waterbury, J.B., Stahl, D.A., 2005. Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437, 543–546.

Lahnsteiner, F., Weismann, T., 2007. Treatment of ichthyophthiriasis in rainbow trout and common carp with common and alternative therapeutics. Journal of Aquatic Animal Health 19, 186–194.

Loy, A., Maixner, F., Wagner, M., Horn, M., 2007. probeBase—an online resource for rRNA-targeted oligonucleotide probes: new features 2007. Nucleic Acids Research 35, D800–D804.

Matthews, R.A., 2005. *Ichthyophthirius multifiliis* Fouquet and *ichthyophthiriosis* in freshwater teleosts. Advances in Parasitology 59, 159–241.

Meinelt, T., Staaks, J., Staaks, G., Stueber, A., Braunig, I., 2007a. Anti-parasitic effects of peracetic acid (PAA) to free infective stages (Theronts) of the white spot disease, *Ichthyophthirius multifiliis* in vitro. Deutsche Tierarztliche Wochenschrift 114, 384–387.

Meinelt, T., Richert, I., Stuber, A., Braunig, I., 2007b. Application of peracetic acid to the parasite *Ichthyophthirius-multifiliis* in Sander (*Sander lucioperca*) breeding. Deutsche Tierarztliche Wochenschrift 114, 244–251.

Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E., Stahl, D.A., 1996. Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. Applied Environmental Microbiology. 62, 2156–2162.

Møller, M. Arvin, E. & Pedersen, L.F.L.F., in press. Degradation and effect of hydrogen peroxide in small-scale recirculation aquaculture system biofilters Aquaculture Research.

Noble, A.C., Summerfelt, S.T., 1996. Diseases encountered in rainbow trout cultured in recirculating systems. Annual Review of Fish Diseases 6, 65–92.

Park, H.D., Wells, G.F., Bae, H., Criddle, C.S., Francis, C.A., 2006. Occurrence of ammonia-oxidizing archaea in wastewater treatment plant bioreactors. Applied Environmental Microbiology 72, 5643–5647.

Pedersen, L.F., Pedersen, P.B., Sortkjaer, O., 2006. Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale bio filters. Aquacultural Engineering 34, 8–15.

Pedersen, L.F., Pedersen, P.B., Sortkjaer, O., 2007. Temperature-dependent and surface specific formaldehyde degradation in submerged biofilters. Aquacultural Engineering 36, 127–136.

Pedersen, L-F, Pedersen, P.B. Nielsen, J.L & Nielsen, P.H. 2008. Formaldehyde induced variation in biofilter performance and microbial composition. *Proceedings from the Seventh International Conference on Recirculating Aquaculture*, Roanoke, Virginia, p. 430–439.

PommereningRoser, A., Rath, G., Koops, H.P., 1996. Phylogenetic diversity within the genus *Nitrosomonas*. Systematic and Applied Microbiology 19, 344–351.

Rach, J.J., Gaikowski, M.P., Olson, J.J., 1997. Importance of analytically verifying chemical treatments. Progressive Fish-Culturist 59, 222–228.

Rintamaki-Kinnunen, P., Rahkonen, M., Mannermaa-Keranen, A.L., Suomalainen, L.R., Mykra, H., Valtonen, E.T., 2005a. Treatment of ichthyophthiriasis after malachite green. I. Concrete tanks at salmonid farms. Diseases of Aquatic Organisms 64, 69–76.

Rintamaki-Kinnunen, P., Rahkonen, M., Mykra, H., Valtonen, E.T., 2005b. Treatment of ichthyophthiriasis after malachite green. II. Earth ponds at salmonid farms. Diseases of Aquatic Organisms 66, 15–20.

Rossi, S., Antonelli, M., Mezzanotte, V., Nurizzo, C., 2007. Peracetic acid disinfection: a feasible alternative to wastewater chlorination. Water Environment Research 79, 341–350.

Santoro, D., Gehr, R., Bartrand, T.A., Liberti, L., Notarnicola, M., Dell'Erba, A., Falsanisi, D., Haas, C.N., 2007. Wastewater disinfection by peracetic acid: assessment of models for tracking residual measurements and inactivation. Water Environment Research 79, 775–787.

Schramm, A., de Beer, D., van den Heuvel, J.C., Ottengraf, S., Amann, R., 1999. Microscale distribution of populations and activities of *Nitrosospira* and *Nitrospira* spp. along a

macroscale gradient in a nitrifying bioreactor: quantification by in situ hybridization and the use of microsensors. Applied Environmental Microbiology 65, 3690–3696.
Schwartz, M.F., Bullock, G.L., Hankins, J.A., Summerfelt, S.T., Mathias, J.A., 2000. Effects of selected chemo- therapeutants on nitrification in fluidized-sand biofilters for cold-water fish production. Internatinoal Journal of Recirculation Aquaculture 1, 61–81.
Sortkjær, O., Henriksen, N.H., Heinecke, R.D., Pedersen, L.-F., 2008. Optimizing treatment efficacy in aquaculture. DMU, Aarhus Universitet. Report 659. [In Danish; English abstract], http://www.dmu.dk/Pub/FR659.pdf.
Stampi, S., De Luca, G., Zanetti, F., 2001. Evaluation of the efficiency of peracetic acid in the disinfection of sewage effluents. Journal of Applied Microbiology 91 (5), 833–838.
Strauss, D., Meinelt, T., 2009. Acute toxicity of peracetic acid (PAA) formulations to *Ichthyophthirius multifiliis* theronts. Parasitology Research. doi:10.1007/s00436-009-1361-9.
Summerfelt, S.T., Hochheimer, J.N., 1997. Review of ozone processes and applications as an oxidizing agent in aquaculture. Progressive Fish-Culturist 59, 94–105.
Summerfelt, S.T., Sharrer, M.J., Tsukuda, S.M., Gearheart, M., 2009. Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. Aquacultural Engineering 40 (1), 17–27.
Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating Aquaculture Systems, Second ed. 2002. ISBN: 0-9712646-1-9, p. 769. NRAC Publication No. 01-002, Itchaca, NY, USA.
Urakawa, H., Tajima, Y., Numata, Y., Tsuneda, S., 2008. Low temperature decreases the phylogenetic diversity of ammonia-oxidizing archaea and bacteria in aquarium biofiltration systems. Applied Environmental Microbiology 74, 894–900.
Wagner, M., Brumelis, D., Gehr, R., 2002. Disinfection of wastewater by hydrogen peroxide or peracetic acid: development of procedures for measurement of residual disinfectant and application to a physicochemically treated municipal effluent. Water Environment Research 74, 33–50.
Wagner, M., Rath, G., Amann, R., Koops, H.P., Schleifer, K.H., 1995. In-situ identification of ammonia-oxidizing bacteria. Systematic and Applied Microbiology 18, 251–264.
Wagner, M., Rath, G., Koops, H.P., Flood, J., Amann, R., 1996. In Situ analysis of nitrifying bacteria in sewage treatment plants. Water Science and Technology 34, 237–244.
Weavers, L.K., Wickramanayaka, G.B., 1991. Kinetics of the inactivation of microorganisms, In: Block, S.S. (Ed.), Disinfection, sterilization, and preservation, fourth ed. Lea & Febiger, Philadelphia, Pa, pp. 65–78.
Yuan, Z., Ni, Y., VanHeiningen, A.R.P., 1997. Kinetics of peracetic acid decomposition.1. Spontaneous decomposition at typical pulp bleaching conditions. Canadian Journal of Chemical Engineering 75, 37–41.
Zar, J.H., 1999. Biostatistical Analysis, fourth ed. Prentice-Hall International, London. ISBN: 0-13-081542-X.

PAPER IV

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Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale bio filters

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Abstract

This study investigated rates of hydrogen peroxide (H_2O_2) degradation in biofilters, to provide information for more accurate treatment regimes in recirculation systems and more accurate prediction of effluent H_2O_2 concentrations. Sodium percarbonate ($2Na_2CO_3{\cdot}3H_2O_2$) was applied to small-scale recirculation systems with active bio filters. Three different treatment dosages corresponding to an initial hydrogen peroxide (H_2O_2) concentration (C_0) of 13.0, 26.0 or 39.0 ppm were used ($N = 18$). Decomposition rate constants (k_e) of H_2O_2 were identified by exponential regression analysis of recurrent water samples from treatment start to complete decomposition. The chemical fate of H_2O_2 obeyed first order kinetics with half-lives inversely correlated with C_0. Decomposition rate constants were significantly related to the amount of organic matter (BOD_5) and initial dosage of H_2O_2, and ranged from $k_e = 0.451$ ($C_0 = 26$ ppm; $BOD_5 = 2.0$ mg O_2/l) to $k_e = 3.686\ h^{-1}$($C_0 = 13$ ppm; $BOD_5 = 16.1$ mg O_2/l).

Surface specific reduction (SSR) of H_2O_2 in biofilters was positively related to dosage concentration for both levels of BOD_5, where SSR from 55 to 220 mg $H_2O_2\ m^{-2}\ t^{-1}$ were found.

Oxygen liberation was positive correlated to C_0 and BOD_5, indicating that hyperoxic conditions can arise if large amounts of sodium percarbonate are added to water with high organic matter content.

This study assesses the environmental fate of H_2O_2 in a closed recirculation system with biofilters, simulating recirculation aquaculture systems. The information can be applied to hydraulic model to predict actual treatment concentrations in aquaculture facilities and to assess effluent pulse from simulated treatment regimes.

Keywords: Aquaculture; Degradation; Recirculation; Hydrogen peroxide; BOD_5; Dosage

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1. Introduction

Prophylactic treatments used in aquaculture can result in drug contaminated discharges in effluents. The discharge pulse (excess released to the recipient) is a consequence of several interacting factors, i.e. hydraulics, geometry of culture units, treatment regimes and specific drug/chemical kinetics (Rach and Ramsey, 2000).

Due to potential ecotoxicological effects, aquaculture effluents have come under increasing scrutiny in recent years (Gaikowski et al., 2004; Hohreiter and Rigg, 2001). In order to regulate aquaculture operations, the Danish Environmental Protection Agency has proposed national legislation with regard to water quality criteria (WQC), as required by EU water quality directives (TGD, 2003). WQC are assessed for individual aquaculture-related drugs, as the cut-off value of a given maximal discharge concentration. A prerequisite for environmental approval of the aquaculture industry is that common treatment practices do not conflict with WQC and, therefore, in some cases alternative treatments must be applied.

Therapeutic control of water quality and prevention of fish disease in aquaculture has primarily been conducted with formaldehyde, chloramine-T and copper-sulphate. These compounds are applied preventively to disinfect, to diminish parasite infections or to impede bacterial gill disease (From, 1980; Schreier et al., 1996; Madsen et al., 2000). The dose–response and treatment efficiency are well known, but treatment regimes combined with relatively slow decomposition rates might lead to significant discharges of these potentially toxic chemicals.

Hydrogen peroxide (H_2O_2) is a powerful oxidizing agent and has potential for use as a therapeutic compound for fish (Buchmann and Kristensson, 2003; Buchmann et al., 2003; Rach et al., 1997b; Rach et al., 2000a,b). Various H_2O_2 releasing products have, therefore, been developed recently as substitutes for traditional agents. H_2O_2 decomposes rapidly into H_2O and CO_2 and does not, therefore, accumulate or give rise to complex reversible toxic metabolites. In these respects, as well as for its compatibility of cost and handling, H_2O_2 appears to be a universal treatment candidate. However, disinfection and/or treatment efficiency are often inconsistent in practice, and treatment regimes have to be tailor-made to specific situations.

Decomposition of H_2O_2 depends on several factors, including light intensity, temperature, available organic matter, presence of catalysing metals and microbial activity (Saez and Bowser, 2001; Liltved, 2000; http://www.H2O2.org/). Knowledge of H_2O_2 reactivity in practical treatment situations is limited, so further investigations are needed.

The purpose of this study was to assess the degradation rates of H_2O_2 in a closed recirculation system with biofilters, simulating recirculation aquaculture systems. The elimination kinetics was investigated at three different initial concentrations of H_2O_2, based on recommendations from practical treatment situations. Furthermore, correlations between decomposition of H_2O_2 and dosage, BOD_5 and temperature were described.

The information can be applied to hydraulic model to predict actual treatment concentrations in aquaculture facilities and to assess effluent pulse from simulated treatment regimes. Ultimately, treatment efficiency can be improved and a treatment practice ensured where maximal discharge concentrations of H_2O_2 comply with WQC set.

2. Materials and methods

2.1. Test facility and experimental set-up

All experiments were conducted at the Danish Institute of Fisheries Research (DIFRES) aquaculture facility. This facility comprises three full-scale recirculation systems, each with 12 separate tanks (1.5 m^3) mechanical drum filters, biofilters and trickling filters. The biofilter (ca. 8.5 m^3), providing biofilter elements for testing was a submerged up flow type packed with solid plastic media (BIO-BLOK® 200). A BIO-BLOK® 200 unit is a cube of 55 cm, made of polyethylene net tubes with a specific surface area of 200 m^2/m^3.

Six identical pilot scale recirculation systems were established at DIFRES, based on the dimension of one BIO-BLOK® 200 unit. Each system had a 200 l tank for fish and an elevated up flow biofilter containing a submerged BIO-BLOK® 200 unit. Biofilter overflow entered the fish-tank, from which a submerged pump redelivered the water to the bottom of the biofilter. Water leaving the biofilter was aerated continuously

by vigorous mix with the surface water. Flow averaged 1.0 l/s; total volume was 330 l and a residence time in the biofilter of approximately 5.5 min. Water velocity in the biofilter was 3.3–3.5 mm/s. The experimental set ups were installed in a refrigerated room to control temperature. Water temperature between and during experiments averaged 15.5 °C, with minimal fluctuations of less than 1 °C.

2.2. Filter colonization and maintenance

Active BIO-BLOK® 200 units for the pilot systems were removed from an operating full-scale biofilter. At the time of removal, biofilter elements were colonized by biofilm and nitrification rates were stable (Wheaton et al., 1994). Twenty liters of system water were added to each pilot system and the remainder filled with tap water. Successively, ca. 1.5 kg rainbow trout (50 ± 5 g) were released into each of the six tanks. The tanks were randomly assigned a specific daily amount of organic matter, defined as two different feeding levels. Thus, the fish (and thereby the biofilter) were given either 7.5 or 30.0 g feed/day of 3 mm high energy extruded pellet (Aller Aqua 576), initially equivalent to 0.5 and 2% feed per biomass, respectively. Feeding occurred continuously from 9 a.m. till 3 p.m. by automatic belt-feeder. Every day, 10% of the water volume was replaced by tap water. The systems were kept constant by fixed feeding for 4 weeks before sodium percarbonate ($2Na_2CO_3{\cdot}3H_2O_2$; BioCare SPC; CAS #15630-89-4) application. Oxygen concentration, pH and temperature were measured daily in the pilot systems. Biofilter status was monitored regularly by semi quantitative measurements of NH_4^+, NO_2^- and NO_3 (Merck).

2.3. Experimental protocol

Prior to each experiment, pumps were temporarily turned off and removed, in order to capture the fish in the tanks. Total volume was adjusted to 360 l by tap water. At this new equilibrium, water samples were taken for BOD_5 determinations and control values. Water chemistry parameters were measured and flow was adjusted to 1.0 l/s.

A given amount of sodium percarbonate was applied over 30 s to the water surface of the tank. After application ($t = 0$), water samples were collected at fixed intervals (t = 5, 10, 15, 30, 45, 60 min and 1.25, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 16 and 24 h). Sodium percarbonate was applied to the pilot systems with two types of active biofilters; low organic content (LOC) and high organic content (HOC). Initial sodium percarbonate concentrations were fixed at: 40 ppm ($n = 6$), 80 ppm ($n = 6$) and 120 ppm ($n = 6$) equivalent to 13.0, 26.0 and 39.0 ppm H_2O_2, respectively.

Samples were collected from the outlet of the biofilter and 100 ml were transferred to new storage bottles each containing 10.0 ml of 4A reagents (see analysis). Bottles were capped and kept at <5 °C until they were analyzed at the National Environmental Research Institute (NERI).

Oxygen content (% relative saturation and mg/l), pH and temperature were monitored before and during the application experiments. Oxygen was manually measured with a calibrated Oxyguard® Handy Beta portable DO meter as well as automatically logged by a Picolog® data logger connected to Oxyguard® standard probes.

2.4. Analysis

Analysis of H_2O_2 was performed as described by Tanner and Wong (1998). Reagent A (NH_4VO_3 and Na_2EDTA a.o.) was added at four times the prescribed dosage, in order to provide fixation of H_2O_2 in water samples containing organic matter.

Standard solutions were sampled prior to the experiment (100 ml in 10 ml 4A), and corrections for background interference were made with samples without reagent 4A, measured with a spectrophotometer at 432 nm.

Organic content were measured as the biological oxygen demand over a 5-day period (BOD_5). Water samples were aerated and adjusted to 20.0 °C and poured into sterile 500 ml incubation bottles with glass stoppers. The BOD bottles contained 0.5 ml 1.0 g/l allylthiourea (ALU), to inhibit potential oxygen consumption related to nitrification (European standard, 1998). Samples expected to contain high organic content were diluted with micropore water (1:2). Control measurements were made with samples of micropore water.

Oxygen content was measured in BOD bottles with a WTW oxygen sensor (model AK 340/B) before and after incubation in darkness at 20.0 °C for 5 days ±4 h.

2.5. Statistics

Degradation kinetics of H_2O_2 was estimated from linear and exponential regression analyses. Linear reduction was determined in the initial phase ($t < 2$ h), whereas elimination constant rates and half-life constants were estimated from equilibrium to concentration range down to 1 ppm.

Functional relationship between BOD_5 or dosage and elimination rate constants were described by linear regression analysis. Regression lines were compared using F-tests for difference between regression coefficients (Sokal and Rohlf, 1995). A probability value of less than 5% ($p < 0.05$) was taken as the fiducial level of statistical significance.

3. Results

Typical patterns of exponential H_2O_2 reduction immediately after application are shown in Fig. 1. In the systems with low organic content, 50% reduction occurred after ca. 1.5 h, whereas the corresponding reduction at high organic content was achieved within less than 45 min (Fig. 1). The monoexponential elimination equation, $C_t = C_0 e^{-k_e t}$, was used to estimate the elimination rate constant (k_e) based on initial H_2O_2 concentrations (C_0) and H_2O_2 concentration after t hours (C_t). The elimination rate constant, k_e, describes the fractional change in concentration per hour (h^{-1}), and is the value relevant for modelling.

A significant rise in oxygen concentration occurred simultaneous to the elimination of H_2O_2 (Fig. 1). Oxygen liberation occurred immediately and peaked within 1 h, with a prolonged elimination period compared to H_2O_2. Maximum oxygen concentrations were significantly positively dependent to organic matter content, measured as BOD_5. In systems with low content of organic matter ($BOD_5 = 5.1 \pm 0.3$ mg O_2/l) oxygen levels changed from 99 to 128%, whereas systems with high organic matter content ($BOD_5 = 16.5 \pm 3.3$ mg O_2/l) had a rise in oxygen level from 93 to 140%.

The elimination equation from individual experiments was based on measured H_2O_2 concentrations at intervals following application (Table 1). k_e was derived as the numerical value of the exponent, ranging from 0.451 ($C_0 = 26$ ppm; $BOD_5 = 2.0$ mg O_2/l) to 3.686 h^{-1} ($C_0 = 13$ ppm; $BOD_5 = 16.1$ mg O_2/l), with half-lives ($t_{1/2} = \ln 2/k_e$) of 92.2 min down to 15.5 min, respectively.

There was a significant, positive correlation between k_e and BOD_5 within treatment regimes ($p < 0.01$; Fig. 2), as elimination rate constants rose

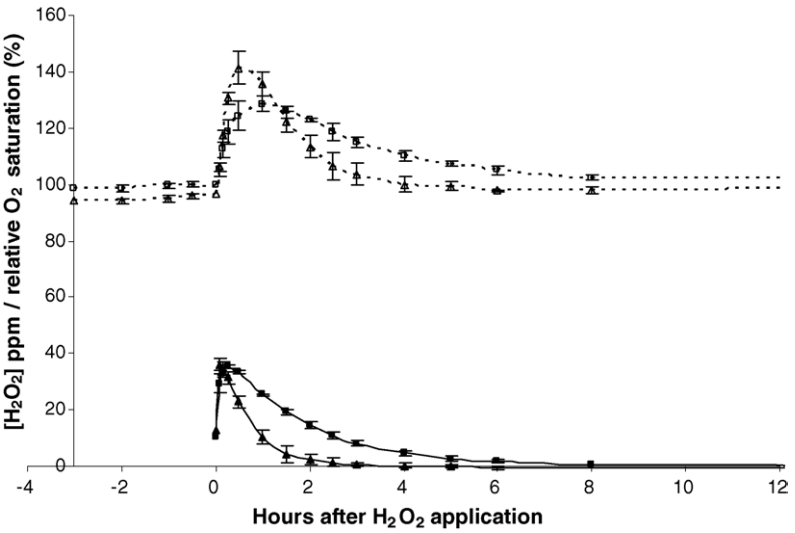

Fig. 1. Elimination fate of hydrogen peroxide (solid lines) applied to two types of pilot systems with low (■) or high (▲) organic content. Corresponding oxygen liberation (broken lines) are expressed as percent relative saturation at 15.5 °C. Hydrogen peroxide was applied as sodium percarbonate, equivalent to initial concentration of 39.0 ppm at $t = 0$. All data represent mean ± S.D. from triplicate trials.

Table 1
Elimination kinetics of hydrogen peroxide applied to pilot systems with low or high organic content (BOD_5 in mg O_2/l)

	$C_{0[H_2O_2]}$ = 13.0 ppm			$C_{0[H_2O_2]}$ = 26.0 ppm			$C_{0[H_2O_2]}$ = 39.0 ppm		
	BOD_5	Exp. regr.	R^2	BOD_5	Exp. regr.	R^2	BOD_5	Exp. regr.	R^2
Low BOD_5	2.8	$13.53e^{-0.807t}$	0.99	2.0	$21.02e^{-0.451t}$	0.98	5.2	$44.99e^{-0.537t}$	1.0
	1.7	$13.97e^{-0.806t}$	0.99	1.3	$21.15e^{-0.529t}$	1.0	4.6	$41.02e^{-0.596t}$	0.99
	1.5	$15.25e^{-0.555t}$	0.99	2.0	$22.77e^{-0.677t}$	0.99	5.4	$45.31e^{-0.566t}$	0.99
High BOD_5	5.9	$13.19e^{-1.536t}$	0.98	8.4	$18.98e^{-1.139t}$	0.95	10.0	$41.56e^{-0.979t}$	0.98
	7.9	$12.39e^{-1.502t}$	0.96	7.6	$17.61e^{-1.189t}$	0.95	19.3	$47.65e^{-1.836t}$	0.98
	16.1	$24.11e^{-3.686t}$	0.98	16.7	$20.91e^{-2.501t}$	0.99	20.1	$43.99e^{-2.027t}$	0.99

Exponential regression analysis was based on measured H_2O_2 concentration from equilibrium to $[H_2O_2] < 0.5$ ppm.

with increasing BOD_5. Furthermore, the dosage dependant correlation of k_e and BOD_5 was significantly different between treatment regimes ($p < 0.05$). k_e was affected by BOD_5 to a higher extend when applied in low doses. The three types of treatment regime (dosage at 13.0, 26.0 and 39.0 ppm H_2O_2), each with significant positive correlations between K_e and BOD_5, revealed that low initial dosages of H_2O_2 increased relative rates of elimination (Fig. 2). Assuming inert conditions in distilled water (k_e and $BOD_5 = 0$), the dosage specific correlations between k_e and BOD_5 were $k_{e13} = 0.229 \times (R^2 = 0.94)$, $k_{e26} = 0.152 \times (R^2 = 0.89)$ and $k_{e39} = 0.099 \times (R^2 = 0.99)$, all significant at $p < 0.05$.

Parallel experiments ($n = 5$) were performed at reduced water temperature (10–11 °C) with dosages of 40 ppm sodium percarbonate (equivalent to 13 ppm H_2O_2). This showed a significant positive correlation ($k_e = 0.156 \times BOD_5$; $R^2 = 0.82$; $p < 0.05$) between k_e and BOD_5 in the range from 4.0 to 18.5 mg O_2/l.

For this experimental set-up, the relation between α ($k_e \times BOD_5^{-1}$) and dosage can be expressed as $\alpha = 0.349e^{-0.032x}$ ($R^2 = 0.99$), x being C_0 of H_2O_2.

Surface specific reduction (SSR) of H_2O_2 was estimated by relating elimination constants of H_2O_2 to the total biofilter surface area of 33 m^2. SSR was positively related to dosage concentration for both levels of BOD_5. SSR was between 55 mg H_2O_2 m^{-2} t^{-1} (13.0 ppm dosage; low BOD_5) and 220 mg H_2O_2 m^{-2} t^{-1} (39.0 ppm dosage; high BOD_5).

The decomposition of H_2O_2 resulted in liberation of O_2, which temporarily caused hyperoxic conditions in the closed recirculating system (Fig. 3).

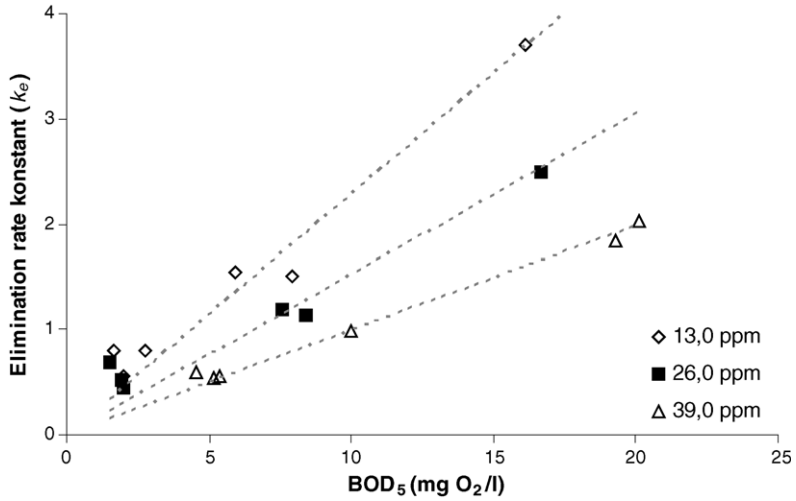

Fig. 2. Relation between elimination rate constant (k_e) and organic matter content measured as BOD_5 in experiments with three different initial H_2O concentrations.

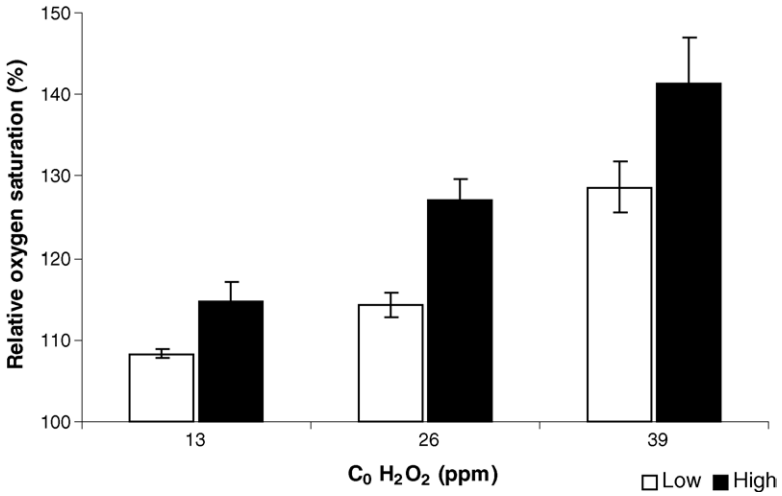

Fig. 3. Maximum oxygen concentration (%) measured in pilot system with various initial concentrations (C_0) of hydrogen peroxide. Each application was made in triplicate to systems with low and high organic matter content ($N = 18$).

In the present study, significant correlations were found between O_2 concentration, BOD_5 and dosage of H_2O_2. Maximum O_2 concentrations were significantly correlated to C_0 at both low BOD_5 (percent of $O_2 = 100 + 0.676\ C_0$; $R^2 = 0.93$; $p < 0.05$) and high BOD_5 (% $O_2 = 100 + 1.059\ C_0$; $R^2 = 0.99$; $p < 0.01$) levels.

4. Discussion

The degradation of H_2O_2 obeyed first order kinetics in all experiments (Newman, 1995). This is in accordance with studies by Saez and Bowser (2001). They reported half-lives of elimination from 18.2 to 28.4 min in kinetic hatchery discharge trials, but were unable to differentiate between breakdown of H_2O_2 and dilution by hatchery water. Rach et al. (1997a) reported H_2O_2 fates following application to various flow through egg incubators, and concluded that dilution was the most plausible explanation for the observed concentrations being lower than expected. In our study, the breakdown could be entirely ascribed to redox processes, as no dilution occurred. Furthermore, oxygen concentrations were monitored during breakdown, which resulted in oxygen supersaturation in the recirculating system. With half-lives of elimination being less than 20 min, in accordance to Saez and Bowser (2001), it is evident that actual effective treatment concentrations in recirculation systems might often be overestimated.

The chemical fate of therapeautants depends on both physical and biological factors. The reduction rate constant for H_2O_2 increased significantly with organic matter content. Available bio film surface is expected to enhance H_2O_2 breakdown, due to chemical and enzymatic reactions. Biofilter related bacteria, i.e. *Pseudomonas* spp. possess catalase enzymes that can degrade H_2O_2 (Anderson and Miller, 2001). Evidence of microbial activity was indicated by a 33% reduction in k_e/BOD_5 correlation in experiments at 10.5 ± 0.5 and 15.5 ± 0.5 °C (0.156× and 0.229×, respectively). The functional relationship between degradation rate constant and BOD are expected to decrease with lower temperature, though the present study cannot differentiate between chemical and microbial breakdown.

Modelling treatment situations and concomitant discharge can be a useful tool for administrative application, if the model can predict discharge concentrations with high accuracy (Gaikowski et al., 2004; Saez and Bowser, 2001). Aquaculture models need valid reduction factors for more accurate predictions of treatment concentrations and discharge pulses of a given therapeautant. This requires knowledge of degradation kinetics and parameter dependency in different compartments (pond, bio filter, laguna, etc.) for readily degradable agents. As shown

in the present study, significant effect of dosage on half-lives of H_2O_2-elimination, which necessitates the use of dose specific decomposition rate constants, rather than a universal value for H_2O_2.

For more conservative agents, modelling has primarily to take hydraulics and dilution rates into account when predicting concentration levels at a given site or time. On the other hand, decomposition rate of fast degradable waterborne chemicals, like H_2O_2, is an important additional factor when used in aquaculture applications. Effective treatment regimes for optimal pathogenic control generally require a minimum effective concentration to be maintained for a specific length of time (Rach et al., 1997a). Treatment regimes either below or above the minimum effective concentration area might lead to inadequate treatments or become toxic to the fish (Rach et al., 1997a).

The degradation rates of H_2O_2 in our study were also quantified as surface specific reduction based on estimates from short term zero-order kinetics. These estimates (mg H_2O_2 m^{-2} t^{-1}) can be useful in modelling flow-though systems (low retention time) with biofilters of given dimensions. However, estimates will be affected by the chosen end-time of linear regressions and will generally be less accurate. Modelling with either relative terms (i.e. rate constants, k_e) or absolute terms (SSR) depends on the purpose and specific system, and will have to be a compromise between complexity and the desired level of predictive accuracy. The majority of Danish fish farms are land-based systems, having moderate water retention time. Modelling and analytically verifying chemical treatments with any chemical under these circumstances require exact measures of flow as well as knowledge of decomposition kinetics in water and sediment. In closed recirculation systems, on the contrary, H_2O_2 kinetics will be dependant on the various components involved, such as the biofilter.

This study deviates from the other studies regarding analytical verification (Rach and Ramsey, 2000; Rach et al., 1997a) and modelling of aquaculture therapeautant effluents (Gaikowski et al., 2004; Saez and Bowser, 2001). In contrast to these studies, the present study included: (1) considerable oxideable material; (2) large fixed biofilm surface; and (3) a static, closed set-up with corresponding oxygen measurements.

As such, the advantage in this study was that flow and geometry of involved units were fixed, and total degradation was described. Decomposition rate constants were deduced from true replicate experiments fitting close to linear first order kinetics ($R^2 > 0.93$) in systems devoid of fish. A disadvantage is the use of BOD_5 as an indicator of organic matter content, which is a comprehensive indirect measure that cannot be quantified in advance of experiments. The study showed accurate rates and interactions within the specific systems, which are applicable by extrapolation to large-scale recirculation systems. The results indicate the magnitudes of H_2O_2 degradation rates and oxygen liberation when large amounts of organic matter are oxidized.

The liberation of oxygen when applying H_2O_2 to organic loaded systems can cause treatment failures and fish mortalities. If H_2O_2 is applied to a raceway system as a bolus of sodium percarbonate, local pockets of organic material ($BOD_5 \gg 10$ mg O_2/l) will result in super saturation. Common use of sodium percarbonate at a concentration of 100 mg/l have been recorded to increase water oxygen content to >200% relative saturation (N.H. Henriksen, Danish Aquaculture, personal communication).

Use of H_2O_2 can give raise to an increase in BOD_5, as slowly degradable organic matter, such as humus or biofilm can be oxidized to more readily degradable compounds (Neyens et al., 2002).

Several studies have dealt with alternative drug-agents of lower regulatory priority status as potential substitutes for formaldehyde (Marking et al., 1994; Schreier et al., 1996).

Various agents, including H_2O_2 solution and and Detarox®AP were investigated as potential alternatives to formaldehyde against trichodinisis in eels in a recirculating system (Madsen et al., 2000). Paraciticidal effects were investigated on infected eels in aquaria at 25 °C with 5–15 mg organic dry matter. Treatment with H_2O_2 subsequently resulted in 60% mortality of the eels, and no significant treatment effects were observed. This might have been due to hyperoxic conditions, if H_2O_2 decomposed rapidly into O_2 (not measured in the study) and thereby not being available in a concentration sufficient for disinfection. The H_2O_2-releasing product, Detarox AP, had significant parasitidal effects and caused no mortality among the eels, indicating effect of

stabilization and/or additional parasitical effects of the peracetic acid (Kitis, 2004). Care should be taken when high concentrations of disinfectants such as sodium percarbonate are recirculated in biofilters for prolonged periods. If loading rates of H_2O_2 exceed the microbial degradation capacity, the biofilm will eventually be oxidized resulting in a crash of the biofilter.

Future research should focus on further detection of effective treatment regimes and response areas for H_2O_2 (Rach et al., 1997a), by identifying factors that influence and determine H_2O_2 kinetics. Presently, the efficiency of waterborne chemicals is determined visually without verification (Rach and Ramsey, 2000), which leaves much space for optimizing treatment regimes and reducing discharge of chemicals from aquaculture.

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References

Anderson, A.J., Miller, C.D., 2001. Catalase activity and the survival of *Pseudomonas putida*, a root colonizer, upon treatment with peracetic acid. Can. J. Microbiol. 47 (3), 222–228.

Buchmann, K., Jensen, P.B., Kruse, K.D., 2003. Effects of sodium percarbonate and garlic extract on *Ichthyophthirius multifiliis* theronts and tomocysts: in vitro experiments. N. Am. J. Aquaculture 65 (1), 21–24.

Buchmann, K., Kristensson, R.T., 2003. Efficacy of sodium percarbonate and formaldehyde bath treatments against *Gyrodactylus derjavini* infestations of rainbow trout. N. Am. J. Aquaculture 65 (1), 25–27.

European standard EN 1899-2. 1998. Water quality—determination of biochemical oxygen demand after *n* days (BOD_n).

From, J., 1980. Chloramine-T for control of bacterial gill disease. Prog. Fish Culturist 42 (2), 85–86.

Gaikowski, M.P., Larson, W.J., Steur, J.J., Gingerich, W.H., 2004. Validation of two models to predict chloramine-T concentrations in aquaculture facility effluent. Aquacultural Eng. 30, 127–140.

Hohreiter, D.W., Rigg, D.K., 2001. Derivation of ambient water quality criteria for formaldehyde. Chemosphere 45, 471–486.

Liltved, H., 2000. Disinfection of water in aquaculture: factors affecting the physical and chemical inactivation of microorganisms. D.S. thesis, University of Tromsoe, Norway.

Madsen, H.C.K., Buchmann, K., Mellergård, S., 2000. Treatment of trichodiniasis in eel (*Anguilla anguilla*) reared in recirculation systems in Denmark: alternatives to formaldehyde. Aquaculture 186, 221–231.

Marking, L.L., Rach, J.J., Schreier, M., 1994. Evaluation of antifungal agents for fish culture. Prog. Fish Culturist 56, 225–231.

Newman, C.M., 1995. Quantitative methods in aquatic ecotoxicology. In: Advances in Trace Substances Research, CRC Press, ISBN: 0-87371-622-1, p. 426.

Neyens, E., Baeyens, J., Weemas, M., De Heyder, B., 2002. Advanced biosolids treatment using H_2O_2 oxidation. Environ. Eng. Sci. 19 (1), 27–35.

Kitis, M., 2004. Disinfection of wastewater with peracetic acid: a review. Environ. Int. 30 (1), 47–55.

Rach, J.J., Ramsey, R.T., 2000. Analytical verification of waterborne chemical treatment regimes in hatchery raceways. N. Am. J. Aquaculture 62, 60–66.

Rach, J.J., Gaikowski, M.P., Olson, J.J., 1997a. Importance of analytically verifying chemical treatments. Prog. Fish Culturist 59, 222–228.

Rach, J.J., Schreier, T.M., Howe, G.E., Redman, S.D., 1997b. Effects of species, life stage, and water temperature on the toxicity of hydrogen peroxide to fish. Prog. Fish Culturist 59, 41–46.

Rach, J.J., Gaikowski, M.P., Ramsay, R.T., 2000a. Efficacy of hydrogen peroxide to control parasitic infestations on hatchery-reared fish. J. Aquat. Anim. Health 12 (4), 267–273.

Rach, J.J., Gaikowski, M.P., Ramsay, R.T., 2000b. Efficacy of hydrogen peroxide to control mortalities associated with bacterial gill disease infections on hatchery-reared salmonids. J. Aquat. Anim. Health 12, 119–127.

Saez, J.A., Bowser, P.R., 2001. Hydrogen peroxide concentrations in hatchery culture units and effluent during and after treatment. N. Am. J. Aquaculture 63, 74–78.

Schreier, T.M., Rach, J.J., Howe, G.E., 1996. Efficiency of formalin, hydrogen peroxide and sodium chloride on fungal-infected rainbow trout eggs. Aquaculture 140, 323–331.

Sokal, R.R., Rohlf, J.M., 1995. Biometry, third ed. W.H. Freeman and Company, ISBN: 0-7167-2411-1, p. 850.

Tanner, P.A., Wong, A.Y.S., 1998. Spectrophotometric determination of hydrogen peroxide in rainwater. Anal. Chim. Acta 370, 279–287.

TGD, 2003. Technical Guidance Document in support of Commission Directive 93/67/EEC on Risk Assessment for new notified substances, Commission Regulation (EC) No. 1488/94 on Risk Assessment for existing substances and Directive 98/8/EC of the European Parliament and of the Council concerning the placing of biocidal products on the market. EUR 20418 EN/2. Available at http://ecb.jrc.it/Technical-Guidance-Document/.

Wheaton, F.W., Hochheimer, J., Kaiser, G.E., Malone, R.F., Krones, M.J., Libey, G.S., Easter, C.C., 1994. Nitrification filter design methods. In: Timmons, M.B., Losardo, T.M. (Eds), Aquaculture Water Reuse Systems: Engineering Design and Management. Developments in Aquaculture and Fisheries Science, vol. 27, p. 333.

PAPER V

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Degradation and effect of hydrogen peroxide in small-scale recirculation aquaculture system biofilters

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Abstract

From an environmental point of view, hydrogen peroxide (HP) has beneficial attributes compared with other disinfectants in terms of its ready degradation and neutral by-products. The rapid degradation of HP can, however, cause difficulties with regard to safe and efficient water treatment when applied in different systems. In this study, we investigated the degradation kinetics of HP in biofilters from water recirculating aquaculture systems (RAS). The potential effect of HP on the nitrification process in the biofilters was also examined. Biofilter elements from two different pilot-scale RAS were exposed to various HP treatments in batch experiments, and the HP concentration was found to follow an exponential decay. The biofilter ammonia and nitrite oxidation processes showed quick recuperation after exposure to a single dose of HP up to 30 mg L^{-1}. An average HP concentration of 10–13 mg L^{-1} maintained over 3 h had a moderate inhibitory effect on the biofilter elements from one of the RAS with relatively high organic loading, while the nitrification was severely inhibited in the pilot-scale biofilters from the other RAS with a relatively low organic loading. A pilot-scale RAS, equipped with two biofilter units, both a moving-bed (Biomedia) and a fixed-bed (BIO-BLOK®) biofilter, was subjected to an average HP concentration of $\sim$ 12 mg L^{-1} for 3 h. The ammonium- and nitrite-degrading efficiencies of both the Biomedia and the BIO-BLOK® filters were drastically reduced. The filters had not reverted to pre-HP exposure efficiency after 24 h, suggesting a possible long-term impact on the biofilters.

Keywords: aquaculture, biofilter, hydrogen peroxide, degradation, nitrification, reaction kinetics

Introduction

Disinfectant agents are widely used in aquaculture to control problems with parasites, fungus and other pathogens (Heinecke & Buchmann 2009; Summerfelt, Sharrer, Tsukuda & Gearheart 2009). Formalin is currently one of the most commonly used therapeutic agents because of its high treatment efficiency and substantial knowledge on the dose–response effect. Furthermore, formalin does not appear to be harmful for fish or biofilters in recirculating aquaculture systems (RAS) in the doses relevant for treatment (Rintamäki-Kinnunen, Rahkonen, Mannermaa-Keränen, Suomalainen, Mykrä & Valtonen 2005; Pedersen, Pedersen & Sortkjær 2007).

There are, however, growing concerns that the excess formaldehyde imposes an ecological problem in the recipient water bodies (Masters 2004; Gearheart, Masters & Bebak-Williams 2006; Pedersen & Pedersen 2006), and recent studies show that formalin is connected with worker safety issues (IARC 2004; Cogliano, Grosse, Baan, Straif, Secretan, El Ghissassi & The Working Group for Volume 88, 2005; Shangina, Brennan, Szeszenia-Dabrowska, Mates, Fabianova, Fletcher, t'Mannetje, Boffetta & Zaridze 2006). Therefore, a need for alternative disinfectants has emerged. Hydrogen peroxide (HP) has shown promising results in the treatment of a number of different parasites and fungus on fish and fish eggs (Lilley & Inglis 1997; Montgomery-Brock, Sato, Brock & Tamaru 2001; Rach, Valentine, Schreier, Gaikowski & Crawford 2004; Heinecke & Buchmann 2009). An advantage of using HP over other chemotherapeutic agents is that the end-products consist of non-toxic substances (Block 1991). Furthermore, as HP decomposes relatively fast in the aquaculture system, it has

the potential to be sufficiently eliminated before discharge and complies with discharge regulations (Schmidt, Gaikowski & Gingerich 2006).

Hydrogen peroxide has, until now, primarily been used as a sanitizer in flow-through aquaculture systems. The studies conducted with HP have mostly concentrated on the parasite treatment efficiency and the tolerance of different fish species to HP in bath treatments. The bath treatments have all been conducted in flow-through systems or fish tanks, where the entire water volume was changed after the treatment period. Treatments typically consist of a high dose of HP (50–100 mg L^{-1} for fish; for eggs up to 1000 mg L^{-1}) over a relatively short period, normally from 15 min to 2 h (Arndt & Wagner 1997; Gaikowski, Rach & Ramsay 1999; Montgomery-Brock *et al.*, 2001; Tripi & Bowser 2001; Avendaño-Herrera, Magariños, Irgang & Toranzo 2006). Knowledge on treatment efficiency and treatment regimes in partial and fully RAS is limited, and potential damaging effects on nitrifying populations in biofilters have impeded the use of HP in RAS so far.

Schwartz, Bullock, Hankins, Summerfelt and Mathias (2000) is one of few studies dealing with the effects of HP on the nitrifying performance of biofilters in a RAS. Here, a severe inhibition of nitrification in terms of significantly reduced total ammonium nitrogen (TAN) removal was demonstrated following a static bath treatment with 100 mg L^{-1} HP. The unwanted side effect of HP on nitrification provides a relatively narrow treatment margin, and underscores the need for additional information on HP kinetics in RAS in order to set guidelines for safe treatment protocols.

The effect of multiple doses of HP on the nitrification performance in RAS biofilters was investigated by Sortkjær, Henriksen, Heinecke and Pedersen (2008). In general, biofilter nitrification performance was found to be unaffected by HP application below 30 mg L^{-1}, but repetitive HP dosage caused transient nitrite accumulation. It is difficult to determine a safe threshold level for HP dose and contact time in relation to inhibition of biofilter nitrification as it depends on a number of parameters. Further research is thus needed to describe the underlying mechanisms of HP degradation and inhibition, and it is not fully understood, e.g. which parameters determine the HP degradation rate and how nitrifying microorganisms are affected by different treatment regimes.

The purpose of this study, was to examine the degradation kinetics of HP in biofilters from aquaculture systems as well as to investigate the effect of HP on the nitrification process in a RAS biofilter. The degradation was studied at varying HP concentrations in batch experiments with biofilter elements from two pilot-scale RAS, as well as experiments in an entire small-scale RAS.

Materials and methods

Test systems

All experiments were conducted at DTU Aqua, National Institute of Aquatic Resources in Hirtshals, Denmark. The experiments were conducted using water and biofilters from two different freshwater small-scale RAS.

RAS-A: The system consisted of a circular 200 L PE fish tank, which contained between 4 and 6 kg rainbow trout *(Oncorhyncus mykiss)* during the test period. The water was pumped from the bottom of the fish tank to the bottom of the biofilter, which was located just above the fish tank. The water was then led back to the fish tank from the top of the biofilter. The submerged biofilter consisted of a single BIO-BLOK® 150 HD module (http://www.expo-net.dk, Hjørring, Denmark) with a volume of 0.165 m^3 and an area of $\sim 25\ m^2$. A BIO-BLOK® 150 HD module is a cube made out of 100 PE cylinders which, for the experiments, were deliberately separated (Pedersen *et al.* 2009). The water volume of the entire system was 0.36 m^3 and 90 L system water was replaced by tap water twice a week, which provided a daily water replacement of approximately 7%. The fish were fed daily with 50 g commercial fish feed (DAN-EX 1754; 4 mm pellets), using belt feeders.

RAS-B: This system consisted of a square glassfibre-fish tank holding approximately 1 m^3 of water and about 40 kg of rainbow trout. The water was drained centrally and pumped to the bottom of two parallel biofilters installed above the fish tank. One of the biofilters consisted of two BIO-BLOK® 200 units with a total volume of 0.33 m^3 and an estimated surface area of 66 m^2. The other biofilter was an aerated moving bed filter fitted with Biomedia 850 (2H Kunststoff, Wettringen, Germany) with an approximate volume of 0.4 m^3 and a surface area of approximately 340 m^2. The water flow through each of the filters was approximately 1200 L h^{-1}. The water temperature in the system was 15.5 ± 1 °C. Oxygen saturation was maintained above 7 mg O_2 L^{-1} by aeration and the pH was approximately 7.5. The total system water volume was approximately 2 m^3 and the daily water exchange with non-chlorinated tap water (Hirtshals, groundwater-based water supply) was approximately 150%. Both RAS-A and RAS-B were operated under the experimental conditions for more than 3 months before experimentation.

Experimental approach

Experimental set-up and protocol for baseline nitrification

A series of six tailormade plexi glass reactor tubes (length ~ 65 cm; diameter ~ 8 cm), each fitted with an air diffuser at the centre of the conical-shaped bottom, were used as a supplementary experimental set-up (Fig. 1). Individual BIO-BLOK® tubes from the biofilter of RAS-A system were placed in each of the reactor tubes. Subsequently, 2.80 L water from the RAS-A fish tank was poured in the reactor tubes, and the BIO-BLOK® tube was positioned below the water surface. Aeration was provided by a Resun® LP-40 (Guangdong, China) airpump and airflow was adjusted in each reactor by a valve to the air diffusers. The set-up was acclimated for 2 h, and the water temperature was 20 ± 1 °C during the experiments.

To determine the nitrification rate of the BIO-BLOK® tubes, the water in the reactor tubes was spiked with ammonium chloride to an initial ammonium-N concentration of ~ 2.5 mg L^{-1}. The ammonium and nitrite levels were subsequently monitored until the concentrations had declined to the levels present before the spike with ammonium chloride. Hereinafter, this treatment is referred to as the 'baseline'.

Single HP dose

After the baseline nitrification had been established, the system water was changed, while the biofilter tubes remained in the reactor tubes. A single dose of HP (technical grade 35%) was added equivalent to an initial concentration of 15 mg L^{-1}. After the HP was completely degraded, the reactor tubes were again spiked with ammonium chloride. The TAN and nitrite concentrations were monitored until they had declined to the levels before the ammonium chloride spiking.

The dry weight of the organic matter on the BIO-BLOK® tubes was determined after the experiment (see 'Analysis').

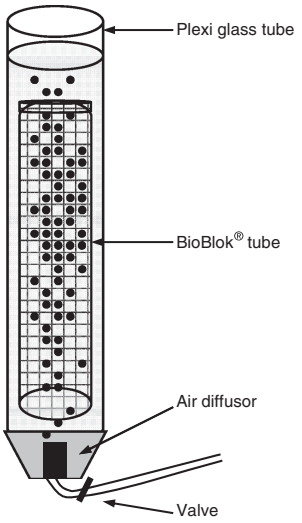

Figure 1 The set-up for single unit experiments with BIO-BLOK® biofilter cylinder elements from different recirculating aquaculture systems.

Multiple HP doses

The effects of a prolonged HP treatment on the nitrification process in the biofilter were assessed by exposing biofilter tubes ($N = 6$) from RAS-A over a 4-h period. The experiment was carried out in the reactor tubes described in 'Experimental set-up and protocol for baseline nitrification'. Supplementary amounts of HP were added every 20 min for a period of 3 h to maintain an average HP concentration of 10 mg L^{-1} in the reactor tubes.

The reactor tubes were spiked with ammonium chloride after complete HP decay equivalent to nominal TAN concentrations of 2.5 mg L^{-1}. The TAN and nitrite levels were subsequently monitored for 3 h.

Experiments similar to those described in 'Experimental set-up and protocol for baseline nitrification'–'Multiple HP doses' were carried out using BIO-BLOK® tubes and water from the RAS-B system for comparison.

Varying HP concentrations

Biofilter tubes ($N = 6$) from the RAS-A were placed in the reactor tubes with system water, and allowed to adjust to room temperature for 1 h. Hydrogen peroxide was added at nominal concentrations of 0, 5, 10, 20, 25 and 30 mg L^{-1}. Simultaneously, the reactor tubes were spiked with ammonium chloride to an initial ammonium–N concentration of ~ 2.5 mg L^{-1}. Hydrogen peroxide, TAN and nitrite levels were measured over a 4-h period until the levels were the same as before spiking. A new dose of ammonium chloride was provided after 4 h and the ammonium and nitrite levels were monitored for an additional 4 h.

Addition of HP in RAS-B

The effect of HP on nitrification was examined in RAS-B by adding HP directly to the entire system. The efficiency of the filters was measured before the

experiment by subtracting the concentration of ammonium in the outlet from the inlet and dividing with the inlet concentration, as carried out by Schwartz *et al.* (2000). During the experiment, inlet water was turned off to avoid dilution.

An average concentration of 10 mg HP L^{-1} over 3 h was achieved by repeated addition of stock solution HP to the fish tank. The first three doses (equivalent to 12, 12 and 8 g HP) showed a higher HP concentration than expected, due to a slower HP degradation in the particular system, and the HP dosages were subsequently reduced to 2 g 35% HP per dosage.

Hydrogen peroxide, TAN and nitrite concentrations were measured in the fish tank and in the inlet and outlet of both biofilters. Oxygen and pH were logged in the fish tank every 15 min. After 4 h, the addition of new cool water was restarted to a constant level.

Analysis

Hydrogen peroxide was measured spectrophotometrically using a modified version of the method described in Tanner and Wong (1998) and Pedersen, Sortkjær and Pedersen (2006).

Total ammonium nitrogen and nitrite-N were measured spectrophotometrically (Danish Standard 224, 1975; Danish Standard 223, 1991). Chemical oxygen demand (COD) was measured using either a Hach–Lange test kit LCK 314 (15–150 mg O_2 L^{-1}, Brønshøj, Denmark) or a Hach–Lange test kit LCK 414 (5–60 mg O_2 L^{-1}).

Organic matter dry weight on the BIO-BLOK® tubes was determined by cleaning each of the tubes thoroughly with a stiff brush on the exterior and a bottle cleaner in the interior in a plastic bucket containing 10 L tap water. Representative sub samples were subsequently filtered through pre-weighted Whatman GFC filters, heated at 105 °C for 10 h and reweighed.

Results

Effect of a single dose of HP

The degradation of a single dose of HP in biofilter elements from two different RAS is presented in Fig. 2. The degradation of HP followed a first-order exponential pattern, $C_t = C_0 e^{-k_e t}$, in filter elements from both systems. The highest elimination rate constant, k_e, was found with filters from RAS-A with a half-life five times shorter compared with the RAS-B filters (Table 1). The surface specific removal rate (SSR) of the RAS-A filters was approximately twice as high as in the RAS-B filters, when estimated as a constant removal during the first 30 min (Table 1).

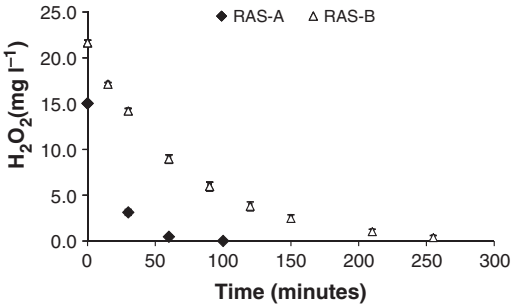

Figure 2 Degradation of hydrogen peroxide (HP) following a single-dose HP in experiments with biofilter elements forms recirculating aquaculture systems (RAS)-A and RAS-B. Each series is the average of six biofilter tubes and the error bars represent the 95% confidence interval (not visible in RAS-A results).

Table 1 Hydrogen peroxide degradation in biofilter tubes from two different systems: RAS-A and RAS-B

	RAS-A	RAS-B	Unit
Exp. regression	$22.06e^{-4.38h}$	$21.55e^{-0.86h}$	
R^2 value	1.0	0.98	
$t_{1/2}$ ($\ln(2)/k_e$)	9.5	48.4	min
SSR	95	45	mg m^{-2} h^{-1}
Biomass dw	272	33	mg
Biomass dw per m^2	1090	100	mg m^{-2}

Values are based on six replicate experiments for both filter types. The biofilter tube area was calculated on the basis of the surface/volume ratio given by the manufacturer (RAS-A = 150 m^2 m^{-3}, RAS-B = 200 m^2 m^{-3}; http://www.expo-net.com).
SSR, surface specific removal rate; RAS, recirculating aquaculture systems.

The baseline TAN removal in biofilter elements from RAS-A compared with the TAN removal in the same tubes after 15 mg L^{-1} of HP had been added and completely degraded is shown in Fig. 3. The baseline TAN removal was marginally faster than the TAN removal rate following HP exposure. Likewise, only minor differences were found between the baseline nitrite level and the nitrite level after HP treatment of biofilter tubes from RAS-A (data not shown).

Effect of multiple HP doses on RAS-A biofilter

During 3 h of multiple HP spiking to biofilter tubes from RAS-A, the HP concentration ranged from 7.5

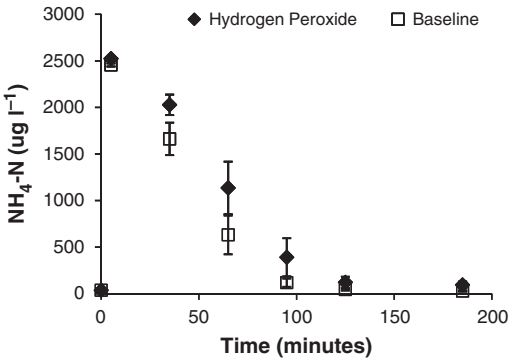

Figure 3 Total ammonium nitrogen (TAN) concentration after a single hydrogen peroxide (HP) dose. The 'baseline' shows TAN changed in unexposed recirculating aquaculture system-A biofilter elements, whereas 'HP' shows TAN following an addition and complete degradation of 15 mg L^{-1} of HP. The data are the average from all six reactor tubes and the error bars represent the 95% confidence interval.

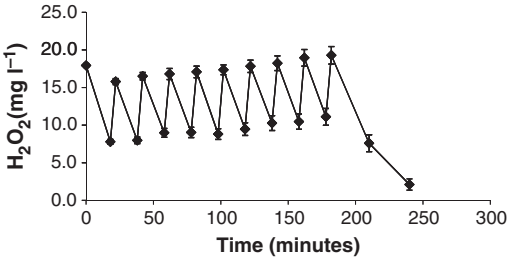

Figure 4 The concentration of hydrogen peroxide (HP) over time in the six reactor tubes containing recirculating aquaculture system-A biofilters. The reactor tubes were spiked with HP every 20 min. The error bars represent 95% confidence interval.

to 19 mg L^{-1}, averaging 12.8 mg L^{-1} (Fig. 4). The SSR of HP only decreased 15% from $t = 20$ min (243 mg m^{-2} h^{-1}) to $t = 160$ min after the second last spike (206 mg m^{-2} h^{-1}).

The TAN removal rate after the prolonged HP treatment was significantly decreased compared with the baseline ammonium degradation (Fig. 5).

Table 2 summarizes TAN removal efficiencies from single and multiple HP addition, where reductions of 24% and 59% were found compared with the baseline rates.

Prolonged HP exposure significantly impaired nitrite conversion compared with baseline conditions (Fig. 6). Nitrite concentrations stabilized at approximately 0.5 mg L^{-1} for at least 2 h, and were measured to be around $\sim$ 5 μg L^{-1} after 12 h.

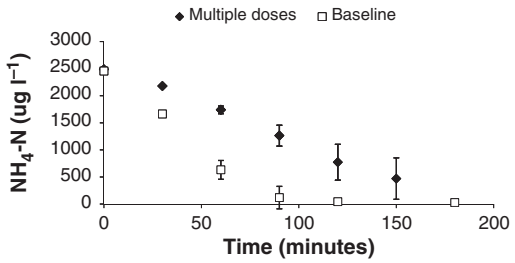

Figure 5 Total ammonium nitrogen concentration in unexposed biofilters from recirculating aquaculture system-A and after multiple doses of hydrogen peroxide.

Table 2 Surface specific TAN removal rates in biofilter tubes from RAS-A after different HP treatments

	Unit	Baseline	Single dose	Multiple doses
Degradation rate	mg NH_4-N m^{-2} day^{-1}	0.31	0.24	0.13
Efficiency	%	100	76	41

The values are based on replicated experiments ($N = 6$).
RAS, recirculating aquaculture systems; HP, hydrogen peroxide; TAN, total ammonium nitrogen.

Effect of multiple HP doses on RAS-B biofilter

Because of slower HP degradation, only three additional spikes were needed to maintain an average HP concentration of 10 mg L^{-1} for the 3 h in the biofilter tubes from RAS-B (data not shown). The SSR was 99 mg m^{-2} h^{-1} over the first 45 min after the initial HP spike and 77 mg m^{-2} h^{-1} the 25 min following the last spike at $t = 145$ min.

The prolonged HP exposure had a large effect on the ammonia-oxidizing process in the biofilters from RAS-B (Fig. 7). The TAN concentration remained unaltered up till 17 h after HP addition. As hardly any ammonium was oxidized to nitrite, comparisons with baseline nitrite removal rates were not made.

Effect of different initial HP concentrations added to RAS-A biofilters

The SSR of HP during the first 15 min was significantly correlated with the amount of HP added, which indicates that the HP degradation is a first-order reaction (Fig. 8).

The degree of inhibition on the TAN removal was positively correlated with the amount of HP added (Fig. 9). This effect was reduced when HP was added

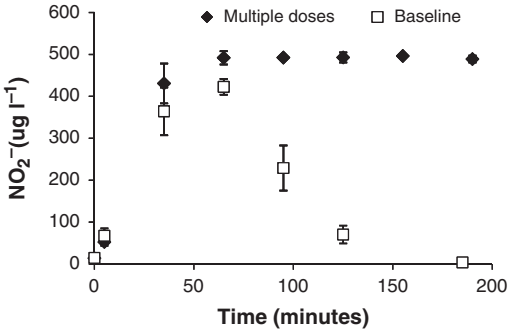

Figure 6 Nitrite-N levels after multiple doses of hydrogen peroxide (HP) in recirculating aquaculture system (RAS)-A biofilter tubes. The 'baseline' shows the nitrite concentration in unexposed RAS-A biofilters and 'multiple H_2O_2 doses' shows the nitrite concentration after multiple HP doses and complete HP removal. The data are the average from all six reactor tubes and the error bars represent the 95% confidence interval.

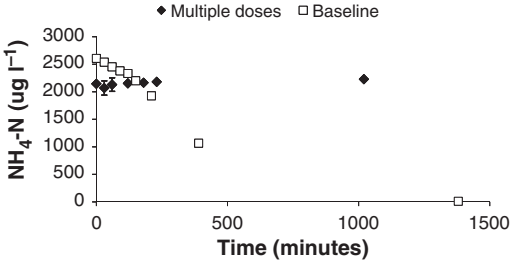

Figure 7 Total ammonium nitrogen (TAN) concentration in recirculating aquaculture systems (RAS-B) biofilter tubes after multiple hydrogen peroxide (HP) exposure. The 'baseline' shows the TAN concentration in unexposed RAS-B biofilters, while 'multiple H_2O_2 doses' shows the TAN concentration shortly after multiple HP doses and complete HP removal. The data are the average from all six reactor tubes and the error bars represent the 95% confidence interval.

again, and TAN removal rates became more uniform. Nitrite accumulation and removal rates were not affected as much TAN removal rates by the initial HP concentration. Temporal discrepancies and delays in degradation patterns were observed but can, to a large extent, be explained by differences in the ammonia oxidation rate (Fig. 10).

HP degradation and effect on nitrification in RAS-B

The degradation of HP in the different parts of the RAS-B system is presented in Fig. 11. The initial three dosages of HP proved to be too high, which increased the HP concentration two to three times the intended

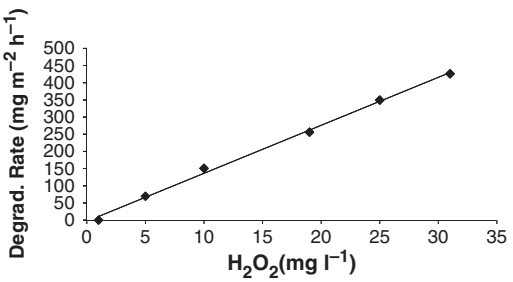

Figure 8 Surface specific removal of hydrogen peroxide (HP) in recirculating aquaculture system-A biofilter tubes, based on linear regression analysis of the initial HP concentration (t = 0–15 min) at different dosages.

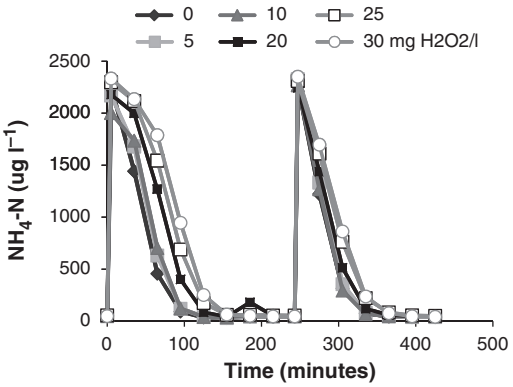

Figure 9 Total ammonium nitrogen concentration in six biofilter tubes from recirculating aquaculture system-A after spiking with both NH_4Cl and different initial hydrogen peroxide doses. NH_4Cl was respiked at t = 240 min.

average concentration of 10 mg L^{-1}. When the doses were adjusted it proved feasible to maintain an average concentration of about 10 mg L^{-1} for the rest of the period. The fluidized-bed filter removed more HP than the submerged filter during the first 5 h after HP addition.

After HP addition, TAN levels increased steadily, reaching levels up to 1.3–1.45 mg L^{-1} after 6 h and 24 h later the TAN concentration in the system was still 1.4 mg L^{-1}. Total ammonium nitrogen removal efficiencies in the two types of biofiltres were significantly reduced after HP addition. Before HP addition, TAN removal efficiency was 27% for the fixed bed biofilter (BIO-BLOK®) and 46% for the fluidized biofilter with biomedia. One and half hours after HP addition, the efficiencies of the filters had decreased to 7% and 10% respectively (Fig. 12). Prolonged nitrite accumulation occurred after HP exposure, as a consequence of inhibition of nitrite-oxidizing bacteria.

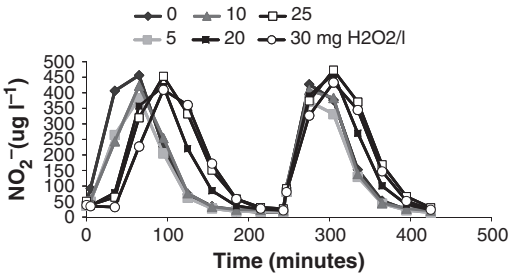

Figure 10 Nitrite-N concentration in six biofilter tubes from recirculating aquaculture system-A after spiking with both NH_4Cl and different initial hydrogen peroxide dosages. NH_4Cl was respiked at $t = 240$ min.

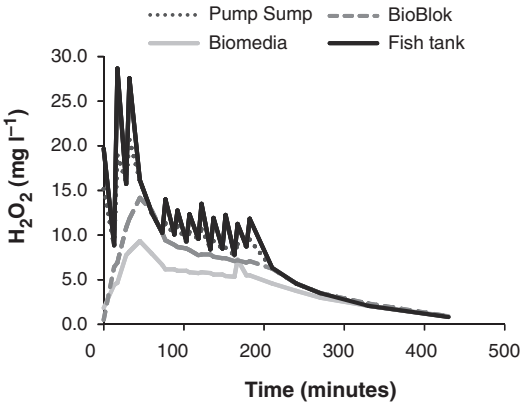

Figure 11 Hydrogen peroxide (HP) application in recirculating aquaculture system-B. The pump sump represents the inlet to the two biofilters (submerged, fitted with BIO-BLOK®, and fluidized, Biomedia); HP measured at the outlets from the biofilters and in the rearing tank.

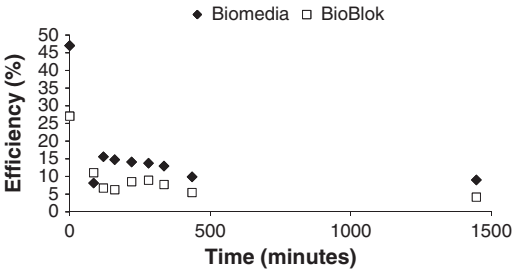

Figure 12 Total ammonium nitrogen removal efficiency of Biomedia and BIO-BLOK® filters following multiple hydrogen peroxide (HP) additions. Initial HP application at $t = 0$. NH_4-N degrading efficiency was calculated by (Outlet–Inlet)/Inlet.

Discussion

The HP degradation rates found in the present study are considerably higher than the degradation rates reported by Pedersen *et al.* (2006), who studied the degradation rates in small-scale biofilters similar to RAS-A. The SSRs in Pedersen *et al.* (2006) varied between 55 mg m^{-2} h^{-1} at an initial HP concentration of 13 mg L^{-1} and 220 mg m^{-2} h^{-1} at an initial HP concentration of 39 mg L^{-1}. These values were, however, calculated on the basis of zero-order functions over a longer period of time (0–2 h) after HP had been added. This will result in an underestimate compared with the present study with a shorter time interval, due to the first-order nature of the HP degradation. The difference can also partly be explained by a lower water temperature, as Pedersen *et al.* (2006) conducted the experiments at 15.5 ± 1 °C, while the water temperature was 20 ± 1 °C in the present study. According to Sortkjær *et al.* (2008), the degradation rate of HP increases rapidly with higher water temperature.

The degradation of HP cannot be ascribed to the bacteria on the biofilter alone, as a considerable amount was degraded by the bacteria suspended in the water as well (Møller 2008). In batch experiments carried out with water from the RAS-A, a strong positive correlation was found between the HP degradation rate and the COD concentration, which most likely is proportional to the number of bacteria (Møller 2008). With a COD concentration of 32 mg O_2 L^{-1} in RAS-A water, HP degradation rate of ~ 6 mg L^{-1} h^{-1} with an initial HP concentration of 20 mg L^{-1} has been reported. This corresponds to around 25% of the overall HP removal from microorganisms in suspension and the remaining 75% degraded by the microorganisms associated with the biofilm in the biofilter.

The results from the experiments with the biofilter tubes from both the RAS systems showed that a single dose of HP has only a limited effect on the nitrification, as the biofilters, after HP exposure, maintained their ability to degrade ammonium and nitrite at almost the same rate as in the non-treated biofilters (baseline). In contrast, the prolonged HP treatment regime of single biofilter elements from RAS-B as well as experiments in the full system led to a severe inhibition of the nitrification process. If the concentration during the first hour had been maintained in the intended range in the experiment with the entire RAS-B system, the nitrification would presumably have been inhibited to a lesser extent. Great care should therefore be exercised when dosing the HP and an accurate estimate of the water volume is important. When possible, HP should be added to rearing units and these should be kept isolated from the biofilters during the majority of the treatment period, and only allow excess HP to

enter the biofilter. This safe approach to reduce pathogen pressure has the built-in drawback that the biofilter potential becomes a retreat.

A long-term effect of HP was recorded in Schwartz *et al.* (2000), where the ammonium-degrading efficiency of a fluidized-sand biofilter in a small-scale RAS was measured before and after HP exposure. The nitrification efficiency declined from 85% to 1% within the first 24 h after a 1-h exposure of 100 mg L^{-1} HP. Subsequently, it increased steadily to around 20% on day 8, and finally increased to approximately 65% on day 10. A long-term inhibition of the biofilters in a RAS would pose a management problem, as a build-up of unionized ammonia and nitrite could result in inhibited fish growth or fish mortality (Jensen 2003; Siikavuopio & Sæther 2006; Rodrigues, Schwarz, Delbos & Sampai 2007). This would require reduced feed load or taking other precautions, such as adding sodium chloride to reduce the detrimental effects of nitrite.

There are indications that the affinity of a biofilter might be increased by pre-treatments with smaller doses of HP, whereby the immense decrease in biofilter efficiency might be avoided. Tripi and Bowser (2001) found that young-of-the-year walleye (*Stizostedion vitreum*) had a higher affinity towards HP under hard water conditions when pre-treated with 10 mg L^{-1} for 1 h. These results are consistent with the findings of Tort, Hurley, Fernandez-Cobas, Wooster and Bowser (2005), who recorded significantly higher catalase levels in the gills of walleye (*Sander vitreum*) that had been pre-exposed to three 10 mg L^{-1} 1-h HP baths than in the walleyes in the control group not exposed to HP. Decreased treatment efficacy of HP on sea lice was observed by Treasurer, Wadsworth and Grant (2000), which was explained by sea lice resistance most likely developed during low-HP dose exposure.

Schmidt *et al.* (2006) mentioned that a number of organisms, among them bacteria, have the ability to increase their resistance towards HP. If pre-exposed to non-lethal HP doses, the organisms will begin to increase the production of antioxidant enzymes, such as catalase, over time which will degrade the HP in the cell. This phenomenon would most likely apply not only to the beneficial microorganisms but also pathogens, which would require alternating chemicals in order to obtain a high treatment efficacy.

In the present study, both the ammonium- and the nitrite-oxidizing bacteria proved to possess the ability to recuperate rather quickly from a short-term/single HP exposure as high as 30 mg H_2O_2 L^{-1}. As the performance of the filter was not severely affected by these single doses, a series of pre-treatments within this range might help reduce the susceptibility of the nitrifying bacteria towards HP in an actual prolonged treatment regime.

Limited research has been published on whether biofilm can increase its tolerance towards HP by pre-treatments. This is an important aspect to examine in order to fecilitate the implementation of HP as a chemotherapeutic agent in the recirculation aquaculture sector.

The tolerance towards HP in the present study was found to be higher in the biofilters with the thickest biofilm and a high feed:water exchange ratio. The biofilm on the biofilters used in this study was, however, relatively thin, compared with the biofilm in most commercial aquaculture system biofilters. It is likely that the biofilters in commercial RAS with a thicker biofilm layer are more tolerant towards HP and might only experience a moderate, if any, ammonia or nitrite accumulation.

In conclusion, the findings of this study have shown that the scope for safe water treatment with HP is somehow narrow. Low dosage/exposure time will decrease treatment efficacy (Heinecke & Buchmann 2009), whereas high dosage/exposure time may impair the important microbial processes in biofilters. In order for HP to partially or fully substitute formalin, future research should include measures to regulate HP concentration during treatment, as well as determine the main parameters of biofilter tolerance and robustness towards HP in order to provide safe guidelines about the practical use of HP.

Acknowledgment

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References

Arndt R.E. & Wagner E.J. (1997) The toxicity of hydrogen peroxide to rainbow trout *Oncorhynchus mykiss* and cutthroat trout *Oncorhynchus clarki* fry and fingerlings. *Journal of the World Aquaculture Society* **28**, 150–157.

Avendaño-Herrera R., Magariños B., Irgang R. & Toranzo A.E. (2006) Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophtalamus maximus*). *Aquaculture* **257**, 104–110.

Block S.S. (1991) Peroxygen compounds. In: *Disinfection, Sterilization, and Preservation. ISBN 0-683-30740-1*, 4th edn. (ed. by S.S. Block) pp. 185–204. Lea & Febiger, Philadelphia, PA, USA.

Cogliano V.J., Grosse Y., Baan R.A., Straif K., Secretan M.B. & El Ghissassi F. The Working Group for Volume 88 (2005) Meeting report: summary of IARC monographs on formaldehyde, 2-butoxyethanol, and 1-tert-butoxy-2-propanol. International Agency for Research on Cancer, Lyon, France. *Environmental Health Perspectives* **3**, 1205–1208.

Danish Standard 223 (1991) *Water Analysis – Determination of the Sum of Nitrite- and Nitrate-Nitrogen*, 3rd edn. Danish Standard, Copenhagen, Denmark.

Danish Standard 224 (1975) *Water Analysis – Determination of Ammonium–Nitrogen*, 1st edn. Danish Standard, Copenhagen, Denmark. p. 6.

Gaikowski M.P., Rach J.J. & Ramsay R.T. (1999) Acute toxicity of hydrogen peroxide treatments to selected lifestages of cold-, cool-, and warmwater fish. *Aquaculture* **178**, 191–207.

Gearheart J.M., Masters A.L. & Bebak-Williams J. (2006) Application of methods for the detoxification and neutralization of formalin in fish hatchery effluents. *North American Journal of Aquaculture* **68**, 256–263.

Heinecke R.D. & Buchmann K. (2009) Control of *Ichthyophthirius multifiliis* using a combination of water filtration and sodium percarbonate: Doseresponse studies. *Aquaculture* **288**, 32–35.

IARC (2004) *Formaldehyde monograph*. Report from the International Agency for Research on Cancer, p. 287. Available at http://monographs.iarc.fr/ENG/Monographs/vol88/mono88-6.pdf (accessed 8 September 2009).

Jensen F.B. (2003) Nitrite disrupts multiple physiological functions in aquatic animals. *Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology* **135**, 9–24.

Lilley J.H. & Inglis V. (1997) Comparative effects of various antibiotics, fungicides and disinfectants on *Aphanomyces invaderis* and other saprolegniaceous fungi. *Aquaculture Research* **28**, 461–469.

Masters A.L. (2004) A review of methods for detoxification and neutralization of formalin in water. *North American Journal of Aquaculture* **66**, 325–333.

Møller M.S. (2008) *Degradation of hydrogen peroxide in recirculation aquaculture system water*. MSc thesis, Department of Environmental Engineering, Technical University of Denmark, 2800 Kgs, Lyngby.

Montgomery-Brock D., Sato V. T., Brock J.A. & Tamaru C.S. (2001) The application of hydrogen peroxide as a treatment for the ectoparasite *Amyloodinium ocellatum* (Brown 1931) on the pacific threadfin polydactylus sexfilis. *World Aquaculture Society* **32**, 250–254.

Pedersen L.F. & Pedersen P. (2006) Temperature-dependent formaldehyde degradation in trickling filters. *North American Journal of Aquaculture* **68**, 230–234.

Pedersen L.-F., Sortkjær O. & Pedersen P.B. (2006) Dose-dependent decomposition rate constants of hydrogen peroxide in small-scale bio filters. *Aquacultural Engineering* **34**, 8–15.

Pedersen L.-F., Pedersen P.B. & Sortkjær O. (2007) Temperature-dependent and surface specific formaldehyde degradation in submerged biofilters. *Aquacultural Engineering* **36**, 127–136.

Pedersen L.-F., Pedersen P.B., Nielsen J.L. & Nielsen P.H. (2009) Long term/low dose formalin exposure to small-scale recirculated aquaculture systems. *Aquacultural Engineering*, doi: 10.1016/j.aquaeng.2009.08.002.

Rach J.J., Valentine J.J., Schreier T.M., Gaikowski M.P. & Crawford T.G. (2004) Efficacy of hydrogen peroxide to control *saprolegniasis* on channel catfish (*Ictalarus punctatus*) eggs. *Aquaculture* **238**, 135–142.

Rintamäki-Kinnunen P., Rahkonen M., Mannermaa-Keränen A.-L., Suomalainen L.-R., Mykrä H. & Valtonen E.T. (2005) Treatment of ichthyophthiriasis after malachite green. I. Concrete tanks at salmonid farms. *Diseases of Aquatic Organisms* **64**, 69–76.

Rodrigues R.V., Schwarz M.H., Delbos B.C. & Sampaio L.A. (2007) Acute toxicity and sublethal effects of ammonia and nitrite for juvenile cobia *Rachycentron canadum*. *Aquaculture* **271**, 553–557.

Schwartz M.F., Bullock G.L., Hankins J.A., Summerfelt S.T. & Mathias J.A. (2000) Effects of selected chemotherapeutants on nitrification in fluidized-sand biofilters for coldwater fish production. *International Journal of Recirculating Aquaculture* **1**, 61–81.

Shangina O., Brennan P., Szeszenia-Dabrowska N., Mates D., Fabianova E., Fletcher T., t'Mannetje A., Boffetta P. & Zaridze D. (2006) Occupational exposure and laryngeal and hypopharyngeal cancer risk in Central and Eastern *Europe*. *American Journal of Epidemiology* **164**, 367–375.

Siikavuopio S.I. & Sæther B-S. (2006) Effects of chronic nitrite exposure on growth in juvenile Atlantic cod, *Gadus morhua*. *Aquaculture* **255**, 351–356.

Sortkjær O., Henriksen N.H., Heinecke R.D. & Pedersen L-F. (2008) *Optimering af behandlingseffekten i akvakultur. Minimering af forbrug og udledning af hjælpestoffer*. 124 s. – Faglig rapport fra DMU nr. 659. Danmarks Miljøundersøgelser, Aarhus Universitet. Available at http://www.dmu.dk/Pub/FR659.pdf (accessed 8 September 2009).

Tanner P.A. & Wong A.Y.S. (1998) Spectrophotometric determination of hydrogen peroxide in rainwater. *Analytica Chimica Acta* **370**, 279–287.

Tort M.J., Hurley D., Fernandez-Cobas C., Wooster G.A. & Bowser P.R. (2005) Effects of hydrogen peroxide treatments on catalase and glutathione activity in walleye Sander vitreus. *Journal of the World Aquaculture Society* **36**, 577–585.

Treasurer J.W., Wadsworth S. & Grant A. (2000) Resistance of sea lice, Lepeophtheirus salmonis (Kroyer), to hydrogen

peroxide on farmed Atlantic salmon, *Salmo salar L. Aquaculture Research* **31**, 855–860.

Tripi C. & Bowser P.R. (2001) Toxicity of hydrogen peroxide to pre-exposed young-of-the-year walleye: effects of water hardness and age of fish. *Journal of the World Aquaculture Society* **32**, 416–421.

Schmidt L.J., Gaikowski M.P. & Gingerich W.H. (2006) *Environmental assessment for the use of hydrogen peroxide in aquaculture for treating external fungal and bacterial diseases of cultured fish and fish eggs*. USGS report, 180pp. Available at http://www.fda.gov (accessed June 2009).

Summerfelt S.T., Sharrer M.J., Tsukuda S.M. & Gearheart M. (2009) Process requirements for achieving full-flow disinfection of recirculating water using ozonation and UV irradiation. *Aquacultural Engineering* **40**, 17–27.