

# **Potential and limitations of ozone in marine recirculating aquaculture systems**

**- Guidelines and thresholds for a safe application -**

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## SUMMARY

The aim of the present thesis was to assess the potential and limitations of ozonation in marine recirculating aquaculture systems (RAS) while particularly focussing on the toxicity, formation and removal of ozone-produced oxidants (OPO) in order to develop guidelines and thresholds for a reasonable and safe ozone application.

In the first two chapters the toxicity of OPO was investigated for two different marine aquaculture species and maximum safe levels were determined for both species.

In **Chapter I** the acute and chronic toxicity of OPO to juvenile Pacific white shrimp (*Litopenaeus vannamei*) was investigated in 96-hour to 21-day exposure experiments by analysing mortality data and incidence of diseases.

In **Chapter II** juvenile turbot (*Psetta maxima*) were exposed to three different sublethal OPO concentrations for up to 21 days. Fish were sampled after 1, 7 and 21 days of exposure to cover short-term, intermediate and long-term OPO effects. A range of biological indices such as gill morphology, hemoglobin, hematocrit and plasma cortisol were evaluated in order to characterize potential chronic impairments of fish health.

Despite their strong differences in biology, both investigated species possess a similar sensitivity towards OPO. Results demonstrate that OPO concentrations  $\geq 0.10$  mg/l cause adverse effects in both species. An OPO concentration of 0.06 mg/l was determined as the maximum safe exposure level for rearing juvenile *L. vannamei* and *P. maxima*. Furthermore, we proved this safe level to be sufficient to control and reduce bacterial biomass in the recirculating process water (**Chapter I**).

To improve the control of toxic OPO, the removal performance of activated carbon filtration was tested for different oxidant species (free bromine, bromamines, free chlorine and chloramines) (**Chapter III**). Results proved activated carbon filtration to be very efficient in removing the dominating oxidant species free bromine and bromamines formed during the ozonation of natural and most artificial seawaters. In contrast, removability of chloramines, sometimes present in ozonated bromide-free artificial seawater, was shown to be significantly lower.

Finally the suitability of ozone for water quality improvement was evaluated by investigating the ozone-based removal of nitrite, ammonia, yellow substances and total bacterial biomass with regard to feasibility, efficiency as well as safety for the cultivated organisms (**Chapter IV**). Results demonstrate that ozone can be efficiently utilized to simultaneously remove nitrite and yellow substances from process water in RAS without risking the formation of toxic OPO concentrations. Although ammonia oxidation in seawater by ozonation is independent from pH and enables almost the complete removal of ammonia-nitrogen from the aquaculture system with nitrogen gas as the primary end product, it presupposes an initial accumulation of OPO to highly toxic amounts, restricting a safe application in aquaculture.

This thesis provides new information in ozone research representing an important precondition for a reasonable and safe application of ozone in marine RAS.



## ZUSAMMENFASSUNG

Ziel dieser Arbeit war es, Potential und Grenzen einer Ozonbehandlung in marinen Aquakultur-Kreislaufsystemen unter besonderer Berücksichtigung der Entstehung, Toxizität und Entfernbarkeit Ozon-generierter Oxidantien aufzuzeigen und Richtlinien für eine sinnvolle und sichere Ozon-Anwendung zu erarbeiten.

In **Kapitel I und II** wurde anhand zweier Ozon-Verträglichkeitsstudien die Toxizität Ozon-generierter Oxidantien für zwei marine aquakulturrelevante Arten untersucht, um auf Basis dessen Grenzwerte für eine maximale unbedenkliche Oxidantien-Konzentration für beide Arten zu ermitteln.

Um sowohl die akute als auch die chronische Toxizität von Ozon-generierten Oxidantien auf juvenile Shrimps der Art *Litopenaeus vannamei* zu untersuchen, wurden Expositionsversuche unterschiedlicher Dauer (96 Stunden, 21 Tage) durchgeführt, bei denen die jeweiligen Mortalitäten sowie das Auftreten von Folgeerkrankungen analysiert wurden (**Kapitel I**). Des Weiteren wurden juvenile Steinbutte (*Psetta maxima*) drei verschiedenen subletalen Oxidantien-Konzentrationen für 21 Tage ausgesetzt und nach 1, 7 und 21 Tagen beprobt.

Neben histologischen Untersuchungen der Kiemen wurden Hämoglobin-, Hämatokrit- und Cortisol-Gehalte im Blut bestimmt, um mögliche Beeinträchtigungen der Fische aufzuzeigen zu können (**Kapitel II**).

Beide Arten zeigten trotz ihrer völlig unterschiedlichen Biologie eine ähnliche Empfindlichkeit gegenüber Ozon-generierten Oxidantien. Während Oxidantien-Konzentrationen  $\geq 0,10 \text{ mg/l}$  nachweisbare chronische Beeinträchtigungen der Gesundheit beider Arten bewirkten, konnte dagegen eine Restoxidantien-Konzentration von  $0,06 \text{ mg/l}$  als Grenzwert für eine sichere Ozonbehandlung bestimmt werden. Darauf aufbauende Untersuchungen zeigten, dass bereits dieser Sicherheits-Grenzwert durchaus eine effektive Keimreduktion des Kreislaufwassers gewährleisten kann (**Kapitel I**).

Zur Verbesserung der Kontrolle toxischer Restoxidantien wurde die Entfernungsleistung von Aktivkohle-Filtration für vier verschiedene Ozon-generierte Oxidantien (freies Brom, Bromamine, freies Chlor, Chloramine) getestet (**Kapitel III**). So konnte der Aktivkohle-Filtration eine effektive Abbau-Leistung für die dominierenden Oxidantien eines ozonisierten Meerwassersystems (freies Brom, Bromamine) nachgewiesen werden. Die Entfernbarkeit von Chloraminen, welche bei der Ozonisierung von bromidfreiem künstlichem Meerwasser entstehen können, stellte sich dagegen im Vergleich geringer dar.

Um das Potential von Ozon zur Verbesserung der Wasserqualität zu evaluieren, wurde in **Kapitel IV** die Ozon-basierte Entfernung von Nitrit, Ammonium, organischen Gelbstoffen sowie Gesamt-Bakterien unter Berücksichtigung von Machbarkeit, Effizienz, sowie Sicherheit für die kultivierten Organismen untersucht.

Die Ergebnisse zeigen, dass Ozon sehr effizient in marinen Kreislaufsystemen zur schnellen und simultanen Entfernung von Gelbstoffen und Nitrit ohne das Risiko einer Akkumulation schädlicher Oxidantien-Konzentrationen eingesetzt werden kann.

Zudem konnte nachgewiesen werden, dass die Ozon-basierte Oxidation von Ammonium in Meerwasser pH-unabhängig verläuft, bei der ein überwiegender Teil des Ammonium-

Stickstoffs in Form molekularen Stickstoffs komplett aus dem System entfernt werden kann. Allerdings stellte sich die vorangehende Anreicherung von als Zwischenprodukt fungierender toxischer Ozon-generierter Oxidantien als limitierender Faktor für eine unbedenkliche Ammonium-Oxidation mittels Ozon heraus.

Mit den gewonnenen Ergebnissen liefert diese Arbeit neue Erkenntnisse als wichtige Voraussetzung für eine sinnvolle und unbedenkliche Ozon-Anwendung in marin Kreislaufsystemen.

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## GENERAL INTRODUCTION

### The state of fisheries and aquaculture

According to the Food and Agriculture Organisation of the United Nations (FAO) around 80% of the marine fish stocks for which information is available are either fully exploited, overexploited or even collapsed (FAO, 2009a). Since 1995 the worldwide fisheries yield has stagnated at around 90 – 95 million tonnes per year (Fig. 1). No short-term recovery from the current situation can be expected in the future.

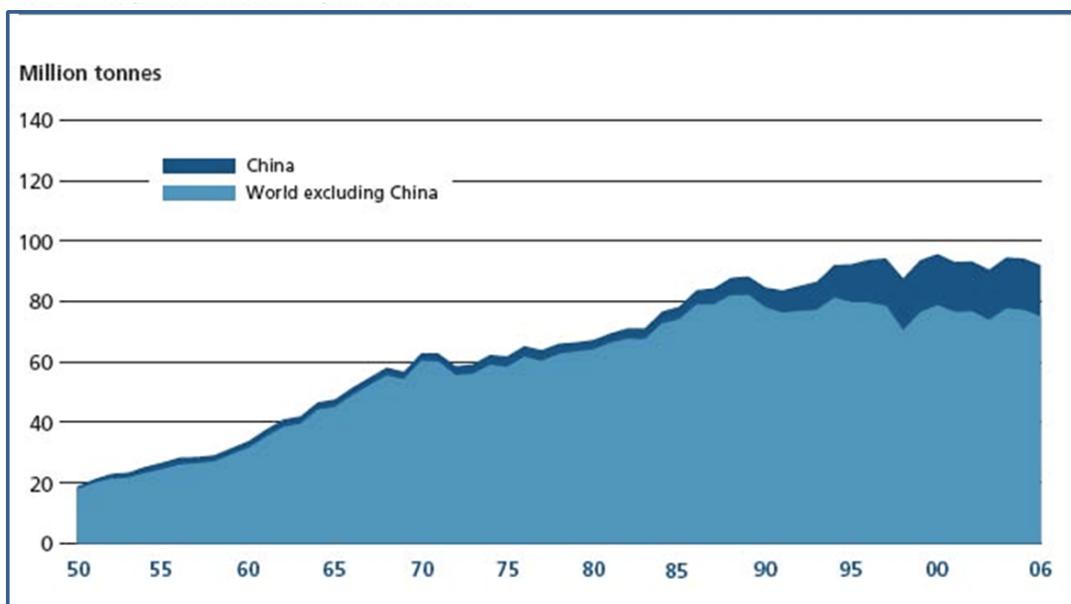


Fig. 1: World capture fisheries production (source: FAO, 2009a)

In contrast, the consumer demand for high quality fish and shellfish products is continuously rising. Global consumption of fish has doubled since the early 1970s and will continue to grow with human population increase, income and urban growth (Delgado et al., 2003; Cahu et al. 2004). Capture fisheries cannot cover the increasing demand for aquatic animal protein for human consumption anymore (FAO, 2009a). This lack of aquatic animal protein represents one of the greatest challenges of the seafood industry and can only be counteracted by cultivation methods. Hence, aquaculture is gaining more and more relevance, representing nowadays the world's fastest growing food sector. After a steady growth phase, particularly in the last four decades, aquaculture nowadays contributes almost half of the fish consumed by the human population worldwide (FAO, 2009a).

However, the expansion of conventional aquaculture practices represents a potential risk for adjacent ecosystems. The resources land and water become more and more limited in many regions. Hence, an intensification of cultivation techniques is indispensable for a further expansion of aquaculture. Especially in Europe, aquaculture production is shifting more and more towards the marine sector. Conventional mariculture practices such as net cages and flow-through ponds are heavily criticized, as they comprise potential risks for the adjacent ecosystems such as eutrophication of coastal waters due to an elevated nutrient contamination, the potential transfer of diseases into the environment as well as the escape of cultivated organisms (Braaten, 1992; Ackefors and Enell, 1994). Hence, there is an increasing demand for sustainable and environmentally friendly production systems.

### Recirculating aquaculture systems

Closed recirculating aquaculture systems (RAS) could meet this required demand, as they represent independent systems, preventing significant interactions with the environment. RAS provide potential advantages over pond or cage-based forms of aquaculture. These include reduced water usage, lower effluent volumes, better environmental control, flexibility in site selection, and higher intensity of production.

However, intensive stocking densities and high levels of water re-use cause an accumulation of inorganic and organic wastes in the process water. Furthermore, the increased nutrient loads create an ideal environment for fish pathogens. Especially bacterial and viral infections pose a serious problem for an intensive production in RAS (Liltved et al., 2006). Hence, prophylactic disinfection units such as ultraviolet radiation and ozone are widely used to reduce pathogen loads (Summerfelt, 2003). However, the effectiveness of ultraviolet radiation is often restricted in aquaculture process water due to turbidity (Honn and Chavin, 1976) and its potential for water quality improvement is limited. As the fish's health depends not only on the pathogen pressure but also on the water quality in general, a disinfection unit is beneficial, which additionally contributes to the improvement of water quality by removing toxic metabolites and organic wastes. Ozone, a powerful oxidant, is capable of providing both, an effective disinfection as well as a significant improvement in water quality.

## Ozone in aquaculture

Ozone ( $O_3$ ) is a clear blue coloured gas that is formed when an oxygen molecule ( $O_2$ ) is forced to bond with a third oxygen atom (O). The third atom is only loosely bound to the molecule, making ozone highly unstable. This property makes ozone an excellent oxidizing agent and ideal for use in water treatment.

Ozone is widely used for drinking water processing and oxidation of sewage and industrial wastewaters (Katzenelson and Biedermann, 1976; von Gunten, 2003). Hubbs (1930) first discussed the potential utilization of ozone in aquaculture. Since several decades, ozone is increasingly being used in aquaculture as a strong oxidant for disinfection and improvement of water quality (Summerfelt and Hochheimer, 1997). Ozone has been proven to be effective in a range of aquaculture applications over the years.

Being a strong oxidizing agent, ozone has a high germicidal effectiveness against a wide range of pathogenic organisms including bacteria, viruses, fungi, and protozoa (Colberg and Lingg, 1978; Danald et al., 1979; Liltved et al., 2006; Schneider et al., 1990). The effectiveness of ozone treatment for disinfection depends on ozone concentration, contact time, pathogen loads and levels of organic matter. Due to its high reactivity, ozone attacks microorganisms extremely fast while producing primarily oxygen as an end product, thus making it more environmentally-friendly than most other chemical disinfectants. Besides direct oxidation ozone can destroy harmful microorganisms indirectly by the formation of germicidal by-products, particularly in seawater.

Ozonation is used for disinfection of exchange water in order to prevent the entry of pathogen loads to the aquaculture system. As ozonation of the incoming water alone cannot counteract the build-up of bacterial biomass, dissolved organics and toxic metabolites within the system, ozone is additionally introduced into the process water stream to contribute in different ways to the improvement of process water quality.

Besides reducing bacterial biomass in the process water, ozone promotes microflocculation of organic matter, resulting in an improved filtration and skimming of colloids and suspended matter (Sander and Rosenthal, 1975; Otte and Rosenthal, 1979; Williams et al., 1982).

Furthermore, ozone is used to remove color and taste (Otte et al., 1977; Westerhoff et al., 2006; Liang et al., 2007), as it oxidizes several dissolved organics by breaking up their carbon double bonds (Hoigne and Bader, 1983). Several organic substances are non-biodegradable

and accumulate according to feed input, water exchange rate and suspended solid removal rate. Ozone partially oxidizes non-biodegradable organics in the water to biodegradable compounds that can be removed by biological filtration (Krumins et al., 2001).

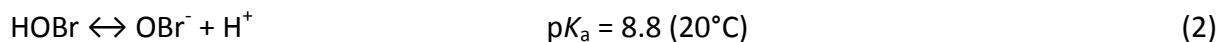
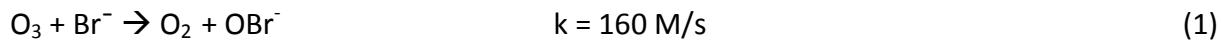
Additionally, ozonation has been reported to be highly effective for the removal of nitrite (Colberg and Lingg, 1978; Rosenthal and Otte, 1979). With a rate constant of  $3.7 \times 10^5$  M/s, ozone reacts almost instantaneously with nitrite to nitrate (Hoigne et al., 1985; Lin and Wu, 1996).

Ozone's ability to remove ammonia has been controversially discussed in previous studies (Singer and Zilli, 1975; Colberg and Lingg, 1978; Lin and Wu, 1996; Krumins et al., 2001). Whereas the oxidation of ammonia by ozone is reported to be very slow ( $k = 5$  M/s) in freshwater and only reasonably obtainable in an alkaline medium ( $\text{pH} > 8$ ) (Singer and Zilli, 1975; Lin and Wu, 1996), not much is known about the efficiency and the pH-dependence of the ozone-based ammonia-removal in marine aquaculture systems.

### Ozonation of seawater

During the ozonation of seawater, additional side reactions occur, making chemical reaction processes much more complicated. In seawater, unreacted ozone has a very short half life of only a few seconds (Haag and Hoigne, 1983), as it reacts with different chemical seawater compounds instantaneously, resulting in the formation of several reactive species. The produced secondary oxidants are summed up by the term 'Ozone-produced oxidants' (OPO). In seawater, particularly halogen ions are oxidized by ozone to halo-oxides. Hoigne et al. (1985) showed specific formation potentials of halo-oxides being highest for iodide followed by bromide and lowest for chloride species. Low concentrations of iodide ions in typical seawater as well as the slow first order rate constant for ozone's reaction with the chloride ion ( $k = 3.0 \times 10^{-3}$  M/s) (Hoigne et al., 1985) make these respective oxidation products less important. With a relatively high bromide-ion concentration of 60-70 mg/l and an ozone reaction rate constant of approximately 160 M/s, there is high formation potential for brominated oxidants in ozonated seawater (Hoigne et al., 1985).

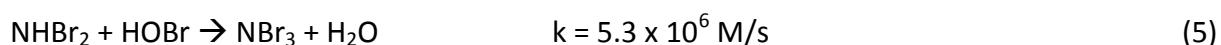
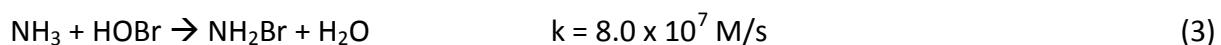
The oxidation of bromide ions by ozone leads to the formation of hypobromite ( $\text{OBr}^-$ ) and hypobromous acid ( $\text{HOBr}$ ), as given by the following reaction equations (1,2) (Haag and Hoigne, 1983).



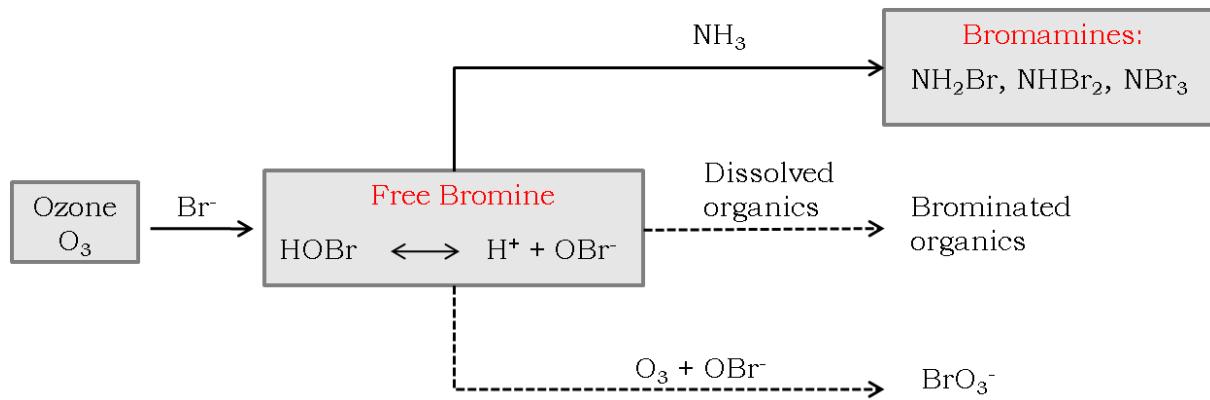
As long as bromide is in the water, the equivalent reaction of ozone with chloride to hypochlorite and hypochlorous acid ( $\text{OCl}^- / \text{HOCl}$ ) is unlikely to be significant as it is much slower ( $k = 3.0 \times 10^{-3} \text{ M/s}$ ) than the reaction with bromide.

The sum of  $\text{HOBr}$  and  $\text{OBr}^-$  is termed as 'free bromine'. Free bromine is itself a strong oxidizing agent and acts as a secondary disinfectant (Johnson and Overby, 1971).

Free bromine rapidly reacts with ammonia contained in most aquaculture facilities to form different bromamines ( $\text{NH}_2\text{Br}$ ,  $\text{NHBr}_2$ ,  $\text{NBr}_3$ ) (Wajon and Morris, 1979). The reaction of hypobromous acid with ammonia results in the formation of bromamines as follows (3,4,5):

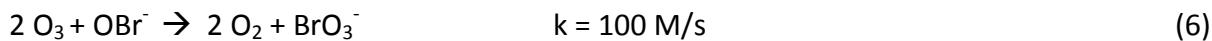


Bromamines are excellent bactericides and exhibit activity similar to hypobromous acid. Hence, bromamines are as effective as free bromine for disinfection (Johnson and Overby, 1971; Fisher et al., 1999).



**Fig. 2: Formation of different brominated oxidants during ozonation of seawater. Continuous lines and boxes indicate the dominant reaction pathways and products in ozonated aquacultural seawater, respectively.**

The hypobromite ion can also be oxidized by ozone to bromate ion ( $BrO_3^-$ ) (6) (Haag and Hoigne, 1983).



Hypobromous acid may further react with organic substances present in natural and aquaculture process water, to form brominated organic compounds, particularly bromoform ( $CHBr_3$ ) (Glaze et al., 1993).

Although bromate and bromoform have been reported to be not acutely toxic to aquatic animals (Liltved et al., 2006), they are considered to be carcinogens (USEPA, 2004) and the formation of these compounds should be strongly avoided. However, in most RAS bromate and brominated organics are only present in trace amounts due to several factors minimizing their formation, primarily due to the preferred reaction of free bromine with ammonia (3) (von Gunten and Hoigne, 1994; Pinkernell and von Gunten, 2001; Sun et al., 2009).

Hence, the reactive oxidants free bromine and bromamines are much more prominent and represent the majority of OPO in marine RAS. Due to ozone's high instability in seawater, free bromine and bromamines are suggested to be primarily responsible for disinfection. However, as these compounds are also toxic to many cultured species (Jones et al., 2006; Meunpol et al., 2003; Richardson et al., 1983), critical concentrations have to be avoided for use in aquaculture.

## Toxicity of ozone and its by-products

The tolerance towards ozone and its by-products has been reported to vary considerably between species and life history stages (Reid and Arnold, 1994).

Ozone is toxic to a wide range of freshwater organisms at very low levels (Coler and Asbury, 1980; Fukunaga et al., 1991; Fukunaga et al., 1992a, 1992b; Hébert et al., 2008; Leynen et al., 1998; Ollenschläger, 1981; Paller and Heidinger, 1979; Ritola et al., 2000; Wedemeyer et al., 1979). With its high reactivity ozone damages membrane-bound enzymes and lipids. Due to severe gill lamellar epithelial tissue destruction ozone causes an impairment of respiration and osmoregulation of organisms (Wedemeyer et al., 1979). Ozone has also a deleterious impact on fish red blood cells (Fukunaga et al., 1992a, 1992b).

In seawater, toxicity results rather from OPO (mostly free bromine and bromamines) than from ozone itself, as ozone decomposes in seawater immediately. These toxic OPO are much more stable than ozone and can accumulate in the system, leading to deleterious impacts on the cultivated organisms, often culminating in mortalities. Although evidence exists that the toxicity differs between ozonated freshwater and seawater due to differences in the reactivity of the predominant oxidants, investigations on the toxic effects of OPO to marine and estuarine organisms are limited. Moreover, the existing studies on OPO toxicity are mostly limited to short-term exposures, allowing only interpretation of acute toxicity (Richardson and Burton, 1981; Richardson et al., 1983; Hall et al., 1981; Meunpol et al., 2003; Reid and Arnold, 1994; Jiang et al., 2001).

However, before reliable safe limits can be established it is necessary to determine the chronic effects of sublethal concentrations. Only comprehensive studies including long-term exposures can provide the information necessary to establish biologically realistic guidelines. As the toxicity of ozone or its by-products to aquatic organisms strongly depends upon species (Reid and Arnold, 1994), guidelines for the maximum safe exposure level have to be determined for the respective species of interest.

## Removal of OPO

Due to their high toxicity, an effective control of residual OPO in the circulating water is very important for the protection of the cultured animals. Although the minimization of OPO formation to the minimum required amount is rather preferable than a subsequent removal,

## General Introduction

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OPO concentrations might exceed the maximum safe exposure level in some applications. Whereas in freshwater residual ozone and secondarily produced radical species dissociate within seconds, OPO formed in seawater are much more stable and have to be removed actively before reaching the fish tanks.

Residual OPO can be removed from the water by addition of reducing agents, UV irradiation, air stripping or by activated carbon filtration. Activated carbon filtration has been established as the most reliable method for removing OPO in aquaculture (Ozawa et al., 1991).

However, the composition of OPO often varies with different water characteristics and oxidation processes. As removability by activated carbon filtration may differ among single oxidant species, removability of the most abundant OPO has to be investigated separately to allow a better assessment of activated carbon efficiency.

## **AIM AND OUTLINE OF THIS THESIS**

The aim of this thesis was to identify the potential and limitations of ozonation in marine recirculating aquaculture systems (RAS), particularly focussing on the toxicity of ozone-produced oxidants (OPO) including their formation and removability. Besides including an evaluation of ozone's suitability for the improvement of different water quality parameters, the toxicity of OPO to different marine aquaculture species was assessed and maximum safe levels were determined. Moreover, removability of different OPO by activated carbon filtration was evaluated. Information derived from the present investigations are valuable, in order to optimize ozone's utilization for water treatment while preventing the deleterious impacts of toxic OPO on the cultured organisms during ozonation of aquacultural seawater.

This thesis is divided into the following chapters:

### **Chapter I**

#### **The toxicity of ozone-produced oxidants to the Pacific white shrimp *Litopenaeus vannamei*.**

The aim of this study was to work out a reliable guideline for the maximum safe exposure level of OPO for juvenile *L. vannamei*. Based on acute toxicity data, determined in a standard 96 h LC<sub>50</sub> test, a maximum safe OPO concentration was calculated and further verified by a chronic exposure-experiment. Furthermore, the determined safe level was tested for its disinfection capacity. The overall objective was to determine an oxidant concentration, efficient in reducing bacterial biomass while simultaneously being nonhazardous for *L. vannamei*, even under chronic exposure.

### **Chapter II**

#### **Histological and physiological alterations in juvenile turbot (*Psetta maxima*) exposed to sublethal concentrations of ozone-produced oxidants in ozonated seawater.**

In this study the biological impacts of three different sublethal OPO concentrations (0.06, 0.10 and 0.15 mg/l) on juvenile turbot (*Psetta maxima*, L.) were investigated to ultimately define a safe, non-hazardous OPO concentration for juvenile *P. maxima* upon chronic

exposure. To characterize the impact of chronic exposure to different sublethal OPO concentrations, morphology of the gills, cortisol as well as hemoglobin and hematocrit levels in turbot blood were analyzed.

### **Chapter III**

#### **A comparative study on the removability of different ozone-produced oxidants by activated carbon filtration.**

The removability of four different OPO (free bromine, bromamines, free chlorine and chloramines) was determined using a standardized experimental procedure under constant conditions to comparatively assess removability of single oxidants, prominent at different water properties, by activated carbon filtration. A further objective of this study was to evaluate three different activated carbon types for their removal capacity on the most persistent oxidant in order to improve removal of OPO by activated carbon filtration.

### **Chapter IV**

#### **Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems.**

In order to assess ozone's potential and limitations for water quality improvement in marine RAS, ozone's efficiency in removing yellow substances, nitrite, ammonia and total bacterial biomass has been comparatively tested in this study, considering relevant aspects such as reaction preferences and the formation of toxic OPO. In particular ozone's suitability to remove ammonia in seawater was evaluated by investigating the dominating reaction pathways and end-products, the effect of pH on removal efficiency as well as the formation of harmful OPO as intermediates.

Each of the four chapters represents a manuscript that is published or submitted for publication in a peer-reviewed scientific journal.

The nomenclature “ozone-produced oxidants” (OPO) is used throughout this thesis to refer to the total residual oxidant concentration, measured spectrophotometrically as chlorine equivalent (mg/l Cl<sub>2</sub>).

# CHAPTER I

## The toxicity of ozone-produced oxidants to the Pacific white shrimp *Litopenaeus vannamei*.

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### Abstract

In marine recirculating aquaculture systems ozone, as a strong oxidant, is often used to improve water quality by reducing the pathogen load and removing inorganic and organic wastes. However, mainly when disinfection of recirculating water is desired, high ozone dosage is required, which may lead to toxicity problems for the cultured species. Acute toxicity of ozone-produced oxidants (OPO) to juvenile Pacific white shrimp, *Litopenaeus vannamei*, was assessed by determining the medium lethal concentration ( $LC_{50}$ ). Shrimp were exposed to a series of OPO concentrations for 96 h. Toxicity was analysed using standard probit regression. The 24, 48, 72 and 96 h  $LC_{50}$  values were 0.84, 0.61, 0.54 and 0.50 mg/l, respectively. A safe level for residual OPO concentration was calculated and further verified by chronic exposure experiments. While long-term exposure of juvenile white shrimp to an OPO concentration of 0.06 mg/l revealed no observable effect, long-term exposures to 0.10 and 0.15 mg/l induced incidence of soft shell syndrome which led to mortalities due to cannibalism. Thus, an OPO concentration of 0.06 mg/l is suggested to be the maximum safe exposure level for rearing juvenile *L. vannamei*. Furthermore, we proved this safe level to be sufficient to control and reduce bacterial biomass in the recirculating process water.

## Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is nowadays the most important shrimp species for aquaculture, replacing *Penaeus monodon* and *Penaeus chinensis* to an increasing degree (FAO, 2009a). In addition to different semi-intensive to intensive pond culture methods (Teichert-Coddington and Rodriguez, 1995; Wyban et al., 1988), the production of *L. vannamei* in land-based recirculating aquaculture systems (RAS) has become more and more popular (Reid and Arnold, 1992). But despite major advantages regarding the control of culture conditions, especially bacterial and viral infections pose a serious problem for an intensive production in closed RAS (Liltved et al., 2006). As a consequence, ozone, a powerful oxidizing agent, is widely used for both water quality improvements and disinfection of the influent as well as recirculating water (Rosenthal and Otte, 1979; Summerfelt and Hochheimer, 1997; Tango and Gagnon, 2003). Ozone can effectively inactivate a range of bacterial, viral, fungal and protozoan fish pathogens (Colberg and Lingg, 1978; Danald et al., 1979; Liltved et al., 2006; Schneider et al., 1990). However, bactericidal activity of ozonated saltwater substantially differs from ozonated fresh water (Liltved et al., 1995; Sugita et al., 1992). It has been shown that persistent oxidative by-products occur in seawater after ozonation which do not occur in freshwater. In saltwater ozone reacts with halides, mainly the bromide ion, generating more stable secondary oxidants, such as free bromine and bromoamines (Crecelius, 1979; Tango and Gagnon, 2003). Due to the high instability of ozone in seawater, these persistent ozone-produced oxidants (OPO) are primarily responsible for disinfection. However, as these compounds are also toxic to many cultured species (Jones et al., 2006; Meunpol et al., 2003; Richardson et al., 1983), critical concentrations have to be avoided in aquaculture.

The tolerance towards ozone and its by-products has been reported to vary considerably between species and life history stages (Reid and Arnold, 1994).

The toxicity of ozone has so far mainly been studied for freshwater finfish species (Coler and Asbury, 1980; Fukunaga et al., 1991; Fukunaga et al., 1992a, 1992b; Hébert et al., 2008; Leynen et al., 1998; Ollenschläger, 1981; Paller and Heidinger, 1979; Ritola et al., 2000; Wedemeyer et al., 1979). Although toxicity is suggested to differ between ozonated fresh- and saltwater due to differences in reactivity of predominant oxidants, investigations on the toxic effects of OPO to marine and estuarine fish and crustaceans are limited.

Classical LC<sub>50</sub> tests were performed with blue crab (*Callinectes sapidus*) and Atlantic menhaden (*Brevoortia tyrannus*) (Richardson and Burton, 1981), white perch (*Morone americana*) (Richardson et al., 1983) and striped bass (*Morone saxatilis*) (Hall et al., 1981) to investigate the toxicity of ozonated waste water for estuarine species. Mariculture related toxicity analyses of OPO have so far been published for black tiger shrimp (*P. monodon*) (Meunpol et al., 2003), Pacific white shrimp (*L. vannamei*) and red drum (*Sciaenops ocellatus*) (Reid and Arnold, 1994), fleshy prawn (*P. chinensis*) and bastard halibut (*Paralichthys olivaceus*) (Jiang et al., 2001). However, these studies were limited to short-term exposures of less than 48 h and rarely followed standardized toxicity test procedures, allowing only conservative interpretation of acute toxicity.

For an appropriate toxicity testing on shrimp, the dependence of crustacean's sensitivity on molt stage has to be considered.

Before, during and immediately after molt, shrimp were shown to be significantly more sensitive (Kibria, 1993). Juvenile penaeid shrimp molt at intervals of a few days (Kibria, 1993). Hence, a bioassay of at least 96 h duration is needed to ensure the inclusion of different molt stages to the observation period - an important precondition for a serious evaluation of toxicity levels (Wajsbrot et al., 1990).

The aim of this study was to work out a reliable guideline for the maximum safe exposure level of OPO for juvenile *L. vannamei*. Based on acute toxicity data, determined in a standard 96 h LC<sub>50</sub> test, a maximum safe OPO concentration was calculated and further verified by a chronic exposure experiment. Furthermore we tested the determined safe level for its disinfection capacity. Our overall objective was to determine an oxidant concentration, efficient in reducing bacterial biomass while simultaneously being nonhazardous for *L. vannamei*, even under chronic exposure.

## Material and Methods

Juvenile Pacific white shrimp were obtained from Ecomares GmbH (Germany) and stocked to 12 identical recirculation systems 7 days prior to experiments. Juvenile shrimp were randomly distributed to the tanks in order to ensure a uniform size- and molt stage frequency distribution across the treatment groups. No further individual molt staging was conducted prior to toxicity tests to avoid additional handling stress after acclimatisation.

Shrimp were kept under a 12 h light - 12 h dark photoperiod and fed with special shrimp pellet feed (DanaFeed DAN-EX 1344, 2mm).

### *Experimental Setup*

Both acute and chronic toxicity tests were performed in 12 identical experimental recirculation systems, located in a temperature controlled lab to ensure constant environmental conditions. Each experimental system consisted of a 200 l fiberglass tank with biofiltration and foam fractionation operated in by-pass. Tanks were filled with approximately 150 l of filtered natural seawater and covered with transparent acrylic glass to avoid animal losses. Ozone gas was produced by electrical discharge ozonators (Model C 200, Erwin Sander Elektroapparatebau GmbH) using compressed air. The gas flow was held constant at 70 l/h. The ozone-enriched air was injected into the seawater through a porous lime stone diffuser at the bottom of the foam fractionator (Model 1 AH 1100, Erwin Sander Elektroapparatebau GmbH), which served as a contact chamber between water and gaseous ozone. Recirculating water entered the foam fractionator at the top and flowed downward - past the uprising bubbles - creating a counter current exchanger which maximized diffusion of ozone into the water. The retention time in the foam fractionator was set to approximately 1 min.

Ozone-supply was controlled and regulated automatically by linking a redox potential controller (Erwin Sander Elektroapparatebau GmbH) to each ozone generator. Desired OPO concentrations were maintained by monitoring oxidant concentrations spectrophotometrically in regular time intervals and adjusting redox potential setpoints if necessary. Especially at higher concentrations frequent measurements and adjustments of concentrations were necessary to maintain a constant concentration of residual OPO. By continuously measuring and adjusting OPO concentrations in very short intervals for 24 h a day, the presence of peaks which may have inflicted disproportionate damage in a short period of time could be avoided. Ozonated water was discharged into the shrimp-tanks with high flow rates (600 l/h) and dispersed by perforated pipes over the whole water column. The induced circular current caused a complete mixing of inflow-water and therefore enabled identical OPO concentrations over the whole water body. The off-gas from foam fractionation was passed into an ozone-decomposer in which residual ozone was adsorbed

via activated carbon in order to maintain the ambient ozone level in the lab room always in safe limits. Identical setups without an ozonation unit were used as reference.

### *Analysis*

OPO were measured spectrophotometrically by a DR/2800 Spectrophotometer (Hach Lange GmbH) as equivalent total residual chlorine (TRC) using the colorimetric *N,N*-diethyl-*p*-phenylenediamine (DPD) method as recommended for the measurement of total residual oxidants (TRO) in seawater (Buchan et al., 2005). As it is not possible to distinguish between single oxidative species methodically, the used DPD Total Chlorine test (Hach) reports concentrations of total oxidants standardized as mg/l Cl<sub>2</sub>, using the molecular weight of chlorine to express concentrations in a mass-volume unit.

Salinity and temperature were measured by a conductivity meter, model 315i (WTW), pH was determined with a pH meter, type CyberScan pH310 series (Eutech Instruments). Dissolved oxygen concentration was measured with an oxygen meter CellOx 325 (WTW). For the spectrophotometrical measurement of total ammonia-N and nitrite-N the Ammonia Salicylate Method and Diazotization Method were applied, respectively, using a DR/2800 photometer (Hach Lange GmbH) and powder pillow detection kits (Hach).

### *Acute toxicity test*

Acute toxicity studies were conducted to define dose-mortality relationships for juvenile Pacific white shrimp exposed to OPO concentrations ( $\pm$  SD) of 0.00, 0.15 ( $\pm$  0.014), 0.30 ( $\pm$  0.026), 0.60 ( $\pm$  0.030), 0.90 ( $\pm$  0.037) and 1.20 ( $\pm$  0.039) mg/l for 96 h. Every concentration was tested with two replicates of 15 shrimp each.

Individuals had a mean ( $\pm$  SD) total wet weight of 6.10 ( $\pm$  1.55) g across all treatment groups. During the acute toxicity test shrimp were fed ad libitum after 24 h and 72 h of exposure. Measurements of pH, salinity, temperature and dissolved oxygen were carried out at 24 h intervals, whereas measurements of dissolved nutrients (total ammonia-N, nitrite-N) were conducted after 48 h and 96 h of exposure.

Mean water quality ( $\pm$  SD) during the acute toxicity test was: temperature: 27.4 ( $\pm$  0.30) °C, salinity: 17.6 ( $\pm$  0.27) ppt, pH: 7.4 ( $\pm$  0.22), dissolved oxygen concentration: 8.6 ( $\pm$  0.13) mg/l, total ammonia-N (TAN): 0.238 ( $\pm$  0.499) mg/l and nitrite-N: 0.087 ( $\pm$  0.199) mg/l.

Feeding activity of shrimp was assessed by monitoring the shrimp's response during and within 20 minutes after feeding.

Death was considered as cessation of respiration and failure to respond to tactile stimuli. Dead animals were removed regularly and classified in postmolt- or intermolt stage by monitoring shell hardness.

Toxicity data were statistically analysed using standard probit regression. A BioStat probit analysis was used to calculate standard LC<sub>50</sub> values and their 95% confidence limits. LC<sub>50</sub> values at different exposure times were statistically compared according to the method described by Natrella (1963).

A "safe" concentration level for rearing *L. vannamei* juveniles was calculated by multiplying the LC<sub>50</sub> value by a factor of 0.1 (Sprague, 1971). This factor is statistically derived as the result of experiments where no perceptible impact on parameters like growth, reproduction, respiration and disease had been observed.

#### *Chronic toxicity test*

The effect of chronic exposure of juvenile *L. vannamei* to sublethal OPO concentrations was investigated in a long-term toxicity study. For the chronic exposure experiment OPO concentrations sublethal to juvenile *L. vannamei* were selected based upon results of the acute toxicity study. Two replicates of 15 shrimp each were exposed to each of the three OPO concentrations ( $\pm$  SD) of 0.06 ( $\pm$  0.010), 0.10 ( $\pm$  0.013) and 0.15 ( $\pm$  0.015) mg/l for 21 days. For control 30 individuals, divided in two replicate groups, were maintained under identical conditions but without ozonation for the same period of time.

Physical and chemical parameters (pH, salinity, temperature, dissolved oxygen, total ammonia-N and nitrite-N) were measured at five day intervals. Mean water quality ( $\pm$  SD) during the chronic toxicity test was: temperature: 27.3 ( $\pm$  0.27) °C, salinity: 18.5 ( $\pm$  0.30) ppt, pH: 7.4 ( $\pm$  0.21), dissolved oxygen concentration: 8.4 ( $\pm$  0.16) mg/l, total ammonia-N (TAN): 0.135 ( $\pm$  0.096) mg/l and nitrite-N: 0.105 ( $\pm$  0.124) mg/l.

Shrimp were fed ad libitum in 48 h intervals to minimize fluctuations in ozone demand and therefore stabilize exposure levels.

Shrimp were checked for behavioural alterations such as loss of equilibrium and lethargy by observing behaviour in regular time intervals. Feeding activity of shrimp was assessed by monitoring the shrimp's response during and within 20 minutes after feeding.

Dead animals were removed regularly, but could not be analysed for physiological and histological alterations due to rapid decomposition caused by cannibalism.

Surviving shrimp were killed in ice water and checked for morphological abnormality. Especially shell conditions were monitored. A scalpel was used to cut the shrimp across the tail. Cross-sections were inspected under a binocular for indications of soft shell syndrome.

#### *Microbial analyses*

To investigate the disinfection potential of continuous exposure to different OPO levels (0.00, 0.06; 0.10; 0.15 mg/l), water samples taken from each experimental recirculation system used in the chronic toxicity test were analysed for viable bacteria at the end of the 21 day exposure.

In addition, bacterial biomass was analysed prior to and after a continuous exposure to 0.06 mg/l. Therefore, in three replicate recirculation systems a constant OPO concentration of 0.06 ( $\pm$  0.010) mg/l was maintained for 21 days. Simultaneously three additional recirculation systems were used as replicate controls without ozonation. All tanks were stocked with juvenile *L. vannamei*. Experimental conditions were identical to those in the chronic toxicity test. Prior to and after the 21 day exposure water samples of replicate control- and treatment-tanks were analysed for total viable cell counts.

Water samples were plated in a series of dilution on TSB 3 agar (15 g agar, 3 g tryptic soy broth (Difco), 10 g NaCl in 1 L of deionized water) supplemented with 1% NaCl. Triplicate platings were done for each sample and dilution. Colony forming units (CFU) per plate were recorded after 48 h of incubation at room temperature. Survival rates of bacteria were calculated from the number of colonies grown.

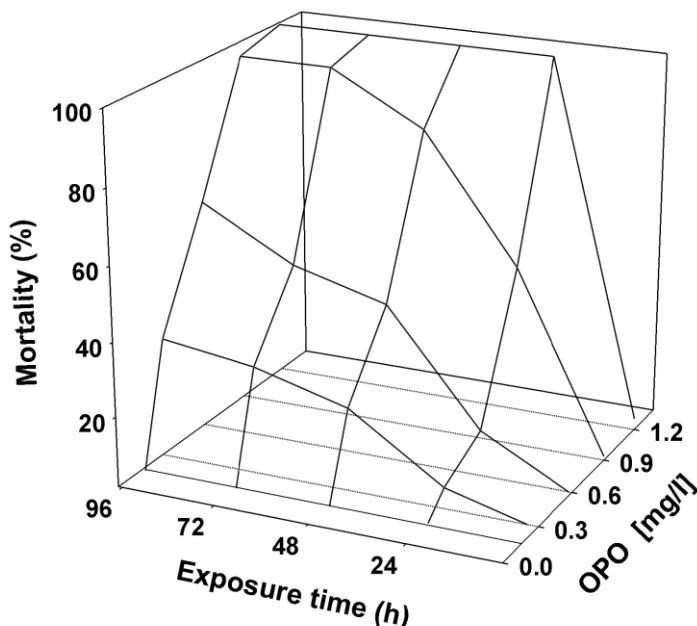
As the variances were not homogeneous, data were analysed using the nonparametric Scheirer-Ray-Hare extension of the Kruskal-Wallis test (Sokal and Rohlf, 1995).

## Results

### Acute toxicity test

Mortality increased with both exposure time and OPO concentration in juvenile *L. vannamei* during 96 h exposure (Fig. I-1). While all shrimp survived in the control group and in the 0.15 mg/l treatment, mortalities of 33%, 63%, 97% and 100% were observed at 0.3, 0.6, 0.9 and 1.2 mg/l within 96 h exposure time, respectively. At OPO concentrations of 0.9 and 1.2 mg/l 50% and 100% of specimens died already within the first 24 h of exposure, respectively.

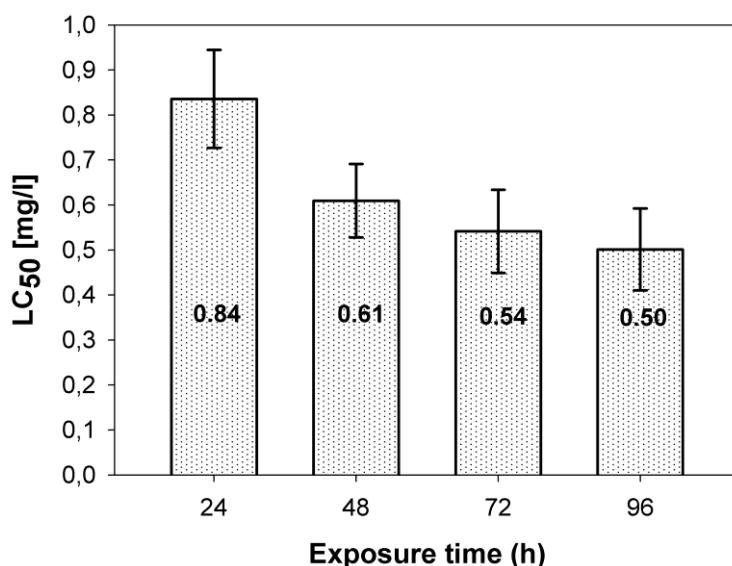
At 0.3 mg/l only individuals in postmolt stage died. At 0.6 mg/l all individuals that died during the first 24 h were in postmolt stage, while after 24 h also an increasing number of shrimp in intermolt stages died.



**Fig. I-1: Mortality response surface for juvenile *Litopenaeus vannamei* exposed to a series of OPO concentrations (measured as chlorine equivalent) for periods up to 96 hours.**

Feeding activity of shrimp in the ozone-exposed groups decreased noticeably with increasing OPO concentration. At the lowest OPO level (0.15 mg/l) only a slight reduction in feeding activity even after 96 h exposure could be observed, while feeding ceased at concentrations  $\geq 0.6$  mg/l.

Mortality data obtained in the 96 h bioassay were used to compute dose-response curves. The 24, 48, 72 and 96 h LC<sub>50</sub> concentrations ( $\pm$  95% confidence limits) were 0.84 (0.727 – 0.945), 0.61 (0.528 – 0.691), 0.54 (0.449 – 0.634) and 0.50 (0.410 – 0.592) mg/l, respectively (Fig. I-2).



**Fig. I-2: LC<sub>50</sub> values and 95% confidence limits for OPO (measured as chlorine equivalent) in *Litopenaeus vannamei* juveniles exposed for 24 to 96 hours. Data are based on results of one standard acute toxicity test.**

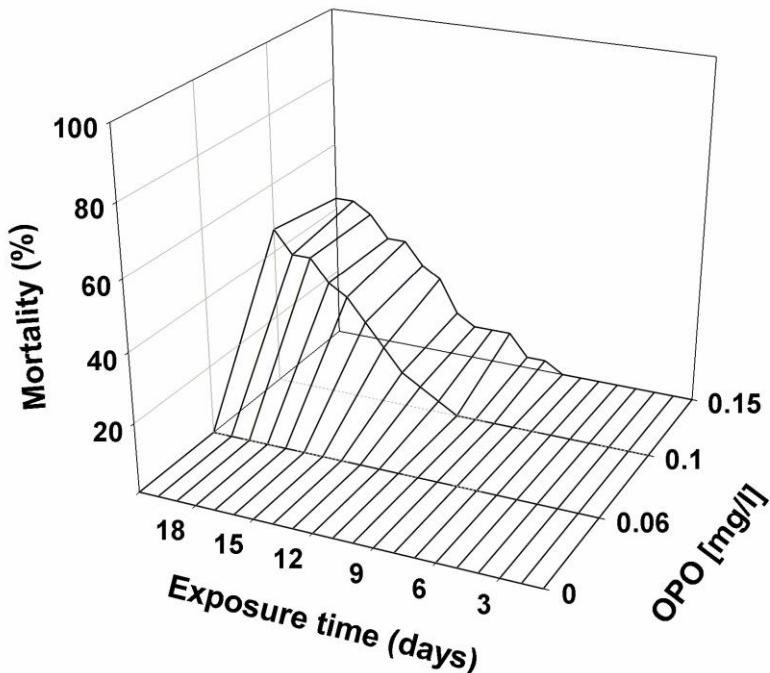
Statistical analysis indicated only a significant effect of exposure time on lethal concentrations during very short-term exposure. Statistical comparison of LC<sub>50</sub> values at different exposure times revealed significant differences ( $p<0.05$ ) between values obtained at 24 h and 48 h, 24 h and 72 h, and 24 h and 96 h. There was no significant difference ( $P<0.05$ ) between LC<sub>50</sub> values at 48 h and 72 h, 48 h and 96 h, and 72 h and 96 h.

Based on the 96 h LC<sub>50</sub> of 0.50 mg/l a “safe” OPO limit of 0.05 mg/l was calculated for rearing juvenile *L. vannamei* (Sprague, 1971).

As there was no significant difference between 48 h and 96 h LC<sub>50</sub> values, a calculated safe limit of 0.06 mg/l based on the 48 h LC<sub>50</sub> (0.61 mg/l) can be used as subject for further verification by chronic exposure experiments.

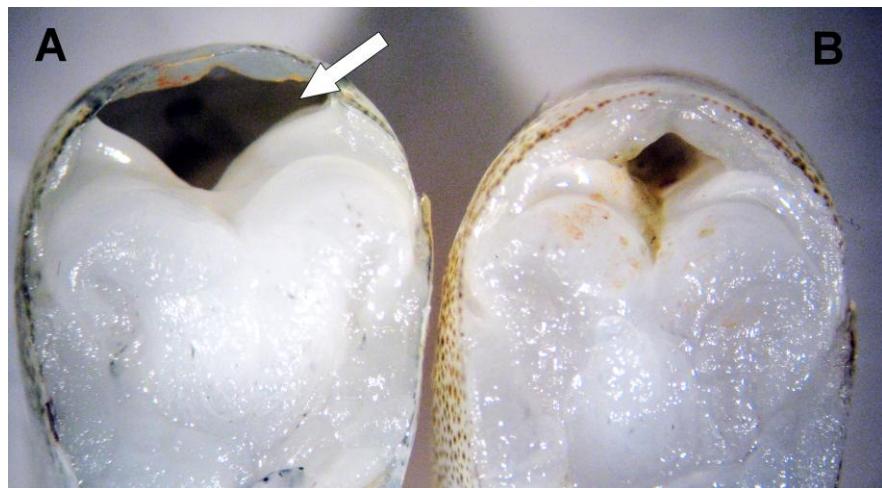
### Chronic toxicity test

Throughout the long-term toxicity test all shrimp in control and 0.06 mg/l tanks survived and did not show any obvious behavioural abnormalities. Even at higher OPO concentrations no behavioural impairment such as loss of equilibrium, lethargy or reduced feeding activity could be observed. However, an obvious increase of cannibalistic behaviour in shrimp exposed to the 0.10 and 0.15 mg/l treatments was evident and mortality levels reached 47% and 43% after 21 days of exposure, respectively. However, mortality did not appear until day 12 and 9 in 0.10 and 0.15 mg/l treatments, respectively. The mortality response surface is shown in Fig. I-3.



**Fig. I-3: Mortality response surface for juvenile *Litopenaeus vannamei* exposed to a series of OPO concentrations (measured as chlorine equivalent) for a long-term period up to 21 days.**

After the 21 day exposure 69% and 35% of the survivors showed clear indications of soft shell syndrome at OPO concentrations of 0.10 and 0.15 mg/l, respectively. The affected shrimp had a soft, paper-like carapace with a gap between muscle tissue and exoskeleton (Fig. I-4).

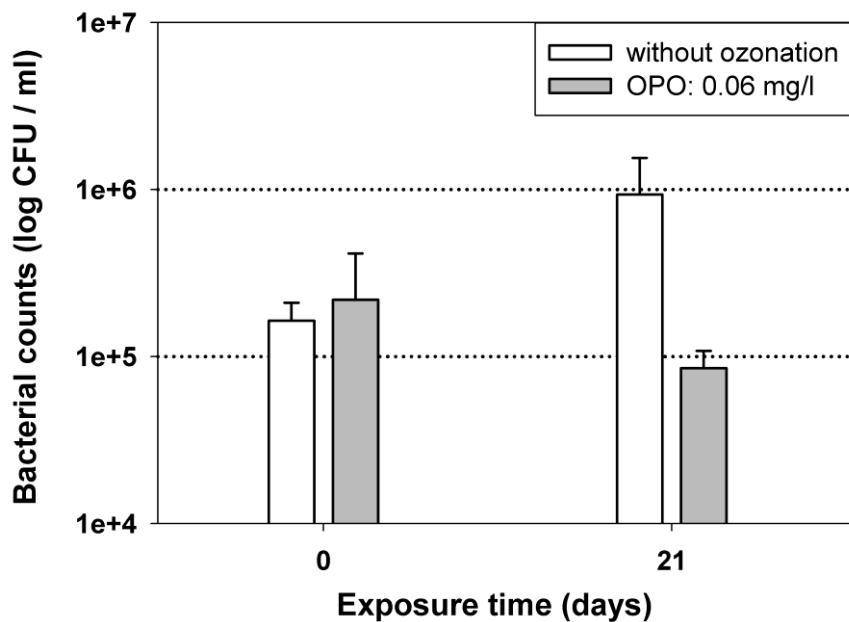


**Fig. I-4:** Tail cross section of juvenile *Litopenaeus vannamei*. A: Soft shell syndrome-affected *L. vannamei* with a gap between muscle tissue and exoskeleton (indicated by arrow). B: Healthy *L. vannamei* without space between muscle tissue and exoskeleton.

### Microbiology

After a 21 day exposure to OPO concentrations of 0.06, 0.10 and 0.15 mg/l, total viable cell counts were only 9.1, 1.1 and 1.7% of control values, respectively. Hence, compared to the number of viable cells in control tanks, at 0.06 mg/l a 10-fold decline (91%), at 0.10 and 0.15 mg/l nearly a 100-fold decline (99 and 98%) in viable cell counts were observed.

For the determination of actual bacterial biomass reduction during continuous exposure to an OPO concentration of 0.06 mg/l, CFU were analysed prior to and after a 21 day exposure. According to CFU, bacterial biomass significantly ( $p < 0.01$ ) decreased at an OPO exposure level of 0.06 mg/l. Viable cells were reduced from  $22 \times 10^4$  CFU/ml to  $85 \times 10^3$  CFU/ml, a reduction of 61%. In contrast, viable cells in control tanks without ozonation increased significantly ( $p < 0.01$ ) from  $16 \times 10^4$  CFU/ml to  $93 \times 10^4$  CFU/ml. Mean viable cell counts prior to and after 21 days of exposure to 0 and 0.06 mg/l are shown in Fig. I-5.



**Fig. I-5:** Viable cell counts (log CFU/ml) before and after a 21 day period of shrimp culture with and without exposure to an OPO concentration of 0.06 mg/l (measured as chlorine equivalent). Columns and error bars represent means and standard deviations, respectively.

## Discussion

Up to now, only few studies investigated the tolerance of marine species to OPO.

Embryos of the two shrimp species *Penaeus japonicus* and *P. monodon* tolerate OPO concentrations well above those reported to inactivate viral and bacterial pathogens (Sellars et al., 2005; Coman and Sellars, 2007). However, as the egg chorion reduces the impact of ozone and its by-products on the embryos, unprotected life history stages such as larvae and juveniles are suggested to be much more sensitive towards oxidants.

Juvenile Pacific white shrimp (*L. vannamei*) were found to tolerate OPO concentrations of up to 1.0 mg/l for 24 h while juvenile red drum (*S. ocellatus*) were only tolerant to OPO levels up to 0.1 mg/l for the same period of time (Reid and Arnold, 1994). The shrimp species *P. chinensis* survived up to 48 hours at OPO concentrations of more than 1 mg/l, while Bastard halibut (*P. olivaceus*) died when exposed for more than three hours to 1 mg/l in the same study (Jiang et al., 2001). These two studies indicate at least a ten times higher sensitivity to OPO of fish compared to crustaceans. However, results are derived from toxicity tests based on very short exposure times of up to 48 hours and rarely following any standardized toxicity test procedures. In the present study acute toxicity to Pacific white shrimp was investigated by means of LC<sub>50</sub> tests, as standard lethal concentration assays are widely accepted and

facilitate cross-comparisons. Compared to those for adult white perch (*M. americana*) (Richardson et al., 1983) and striped bass fingerlings (*M. saxatilis*) (Hall et al., 1981), we found much higher LC<sub>50</sub> values for *L. vannamei*, supporting the higher tolerance of crustaceans to OPO compared to fish. Due to accordance of our results with previous findings, the higher tolerance of crustaceans compared to fish is suggested to be a generalized pattern which may be attributed to the crustacean's chitinous outer layer on the gills, protecting crustaceans from oxidative epithelial tissue destruction.

However, at the time of molting (ecdysis stage) and immediately after molt (postmolt stage) shrimp are more sensitive than in intermolt stages due to an increased permeability of the soft and uncalcified cuticle to the passage of toxic substances (Passano, 1960). Wajsbrot et al. (1990) found a significant effect of molt stage on the sensitivity of juvenile green tiger prawn (*Penaeus semisulcatus*) to ammonia. Results of the present study clearly show that the sensitivity of juvenile *L. vannamei* towards OPO is highly dependent on molt stage, too. In the present study, the LC<sub>50</sub> of OPO declined significantly from 24 to 48 h, while no further significant decrease could be observed between 48, 72 and 96 h LC<sub>50</sub> values. Hence, the threshold for acute toxicity has not been reached until 48 h of exposure.

Accordingly, the limitation of exposure time to 24 hours in the study of Reid and Arnold (1994) might be the reason for the discrepancy in dose-response relationships of OPO in juvenile *L. vannamei* compared to the findings in the present study.

48 to 96 h exposures are generally accepted as covering the period of acute lethal action (Sprague, 1969). Although a significant difference between 48, 72 and 96 h LC<sub>50</sub> values could not be revealed in juvenile *L. vannamei* in our study, we recommend a minimum of 96 h exposure for the determination of acute toxicity levels in shrimp in order to ensure the inclusion of different molt stages to the observation period.

An acute toxicity test provides information about the relative toxicity of a substance but can hardly predict a threshold for a "safe" level (defined as the concentration of a potentially harmful substance that has no adverse effect on organisms even under chronic exposure). However, when information about chronic effects is lacking, a "safe" level is often estimated as a fraction of the lethal concentration. It is suggested that keeping the concentration of a toxicant under 10% of the determined median lethal concentration (LC<sub>50</sub>) should avoid any chronic impacts (Sprague, 1971). Hence, to calculate a safe level by multiplying a 48 to 96 h LC<sub>50</sub> value by an application factor of 0.1 is widely accepted. This factor is an empirical value,

based on experimental exposures without any perceptible damage. As acute toxicity does not significantly differ between 48 and 96 h exposure times in the present study, calculated safe levels lie very close to each other.

Results from our chronic exposure experiment fit well to the calculated safe levels based on results of the acute toxicity experiment, supporting the recommendations by Sprague (1971) for safe level calculations. Juvenile *L. vannamei* tested against 10% concentrations of the 48 h LC<sub>50</sub> value of OPO survived the 21 day exposure and did not show any observable impairment. In contrast, long term exposure of juvenile *L. vannamei* to OPO concentrations of 16% and 25% of the 48 h LC<sub>50</sub> for 21 days induced shell-softening and caused mortality in almost half of the test population.

Soft shell syndrome is a result of chronically toxic conditions, nutritional deficiency, chemical intoxication (e.g. pesticides) or other adverse environmental conditions in culture systems (Baticados et al., 1986). Several pesticides were found to inhibit the chitin synthesis (Corbett, 1974; Nagesh et al., 1999). However, no information is available on the effect of oxidants on shell formation. Reactive oxidant-species might interfere directly with the process of shell formation. Since calcium and phosphorous are the major components required for shell hardening, reactive oxidants might impair the mobilization of these elements from the hepatopancreas.

Nutritional deficiencies were also noted to be a prevalent cause for soft-shelling (Mayavu et al., 2003). However, as in the control group and the 0.06 mg/l treatment no soft shelling was detectable at all, inadequate food and feeding practices can be excluded. Nevertheless, feeding response of shrimp exposed to OPO concentrations of 0.10 and 0.15 mg/l may have been reduced due to oxidative stress caused by chronic exposure to these sublethal OPO concentrations.

Observed mortalities in the soft shell-affected treatments (0.10 and 0.15 mg/l) could be clearly attributed to cannibalism. Soft shelled shrimps are more susceptible to cannibalism due to the soft shell's significant thinness and softness (Baticados et al., 1986).

Considering particularly the deleterious impact of chronic exposure to OPO concentrations ≥0.10 mg/l, sensitivity of *L. vannamei* to OPO seems to be much higher than it was expected for a crustacean species. In many marine RAS ozonation is applied continuously in order to avoid heavy fluctuations in water quality. However, even if batch ozonation is applied, in terms of safety, residual OPO levels should be limited to concentrations that have no

deleterious impact even at continuous exposure, as toxicity of oxidants is more dependant on concentration than time (Coman et al., 2005). Hence, for rearing juvenile *L. vannamei* residual OPO concentration should not exceed the determined safe level of 0.06 mg/l in cultivation tanks in order to avoid any risk to the animal.

If disinfection of recirculating water is the prior intention of ozonation, bactericidal action of safe OPO concentrations becomes of particular importance. Sugita et al. (1992) reported a high inactivation of bacterial fish pathogens in seawater at OPO levels as low as 0.06 mg/l, which corresponds to the discovered safe level for juvenile *L. vannamei*. A 99% reduction was achieved at concentrations of 0.063 mg/l for *P. piscicida* and 0.064 mg/l for *V. anguillarum* within 1 min treatment time. However, a disinfection experiment with pure cultures is not the same as working with a mixed bacteria population, as bacterial communities comprises many species with different tolerances to toxicants. Due to the beneficial effects of a rich bacteria community in RAS it is questionable whether a complete disinfection of water is desirable. However, a thorough control of bacterial biomass is without doubt necessary. Thus, we proved the determined OPO concentration, nonhazardous to juvenile *L. vannamei* even at continuous exposure, to be adequate to control total bacterial biomass of a mixed bacteria population in the recirculating water. While bacterial biomass in control tanks increased with time, a bacterial reduction of 61% was observed within a 21 day exposure to 0.06 mg/l. Further experimental data (Chapter IV) proved ozonation resulting in OPO concentrations  $\leq$  0.06 mg/l to be very efficient in reducing total bacterial counts even at short-term ozonation.

In conclusion, residual OPO should be reduced to at least 0.06 mg/l to eliminate adverse impacts on cultivated juvenile Pacific white shrimp. Since our results show clearly that a satisfactory reduction of bacteria is possible even at low OPO concentrations, a minimization of OPO rather than a subsequent removal should be taken into account. OPO concentrations exceeding the determined safe level are, according to the current results, not necessary, risky and economically questionable.

## Acknowledgements

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## CHAPTER II

# Histological and physiological alterations in juvenile turbot (*Psetta maxima*) exposed to sublethal concentrations of ozone-produced oxidants in ozonated seawater

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## Abstract

Ozone is a powerful oxidizing agent and widely used for disinfection and improvement of the water quality in aquaculture plants. However, the formation of ozone-produced oxidants (OPO) during ozonation of seawater may lead to toxicity impacts on the cultivated organisms. To determine adverse effects of continuous exposure to sublethal OPO concentrations, juvenile turbot (*Psetta maxima*) were exposed to three different OPO concentrations (0.06, 0.10 and 0.15 mg/l) for up to 21 days. Fish were sampled after 1, 7 and 21 days of exposure to cover short-term, intermediate and long-term OPO effects. Gills were analyzed for morphological alterations, hemoglobin and hematocrit were quantified to assess a loss in gill functionality. Plasma cortisol was measured as physiologic stress marker. Gill histology revealed significant histopathological alterations with increasing OPO concentration and prolonged time of exposure. However, hemoglobin concentrations were only elevated during short-term exposure at the highest OPO concentration. Hematocrit values did not show any differences between OPO exposed specimens and the control group. At 0.15 mg/l, plasma cortisol was elevated after 24 hours. The results demonstrate that sublethal OPO concentrations of 0.10 and 0.15 mg/l cause histological and physiological alterations in juvenile turbot, characterising OPO as substantial stressor in recirculating aquaculture systems. At an OPO concentration of 0.06 mg/l just slight alterations in the tested parameters were observed, suggesting concentrations of  $\leq 0.06$  mg/l as acceptable OPO levels for the rearing of juvenile turbot.

## Introduction

Within the aquaculture sector, production of turbot (*Psetta maxima*) passed through a rapid expansion during the last few years featuring turbot as one of the most promising species for the European aquaculture industry (Alonzo Gonzales, 1994; Brown, 2002). Turbot holds different attributes like high feed conversion efficiency (Imsland et al., 1995, 1996), high stress tolerance (Waring et al., 1996, 1997; Mugnier et al., 1998; van Ham et al., 2003), moderate water quality requirements (Person Le-Ruyet et al., 2003) and low susceptibility to diseases (Mulcahy, 2002), all favourable characteristics for the intensive production in land-based aquaculture. Hence, the production of turbot in land-based systems becomes more and more popular (Brown, 2002).

However, bacterial and viral infections are one of the most important issues in the economic context of intensive land-based aquaculture (Liltved et al., 2006). The high stocking densities found in recirculating aquaculture systems (RAS) are mostly associated with fish stress and high nutrient loads resulting in an ideal environment for fish pathogen proliferation. To counteract this, ozone, as a powerful oxidizing agent, has seen wide use in aquaculture applications for achieving several water quality improvements (Summerfelt and Hochheimer, 1997). As ozone can effectively inactivate a range of bacterial, viral, fungal and protozoan fish pathogens (Tipping, 1988; Bablon et al., 1991; Liltved et al., 1995; Tango and Gagnon, 2003; Liltved et al., 2006), it is successfully used for the disinfection of recirculating and influent water in RAS in order to control pathogen loads and ultimately prevent diseases.

Beside these positive attributes, ozone is also known to attack biological membranes of the cultured organisms thereby, causing physiological deteriorations like impaired respiration and osmoregulation (Block et al., 1978; Wedemeyer et al., 1979; Paller and Heidinger, 1980; Ollenschläger, 1981; Richardson et al., 1983). Up to now, toxic effects of ozone on aquaculture relevant finfish species have mostly been studied in freshwater species such as rainbow trout (*Oncorhynchus mykiss*) (Wedemeyer et al., 1979; Morita et al., 1995; Ritola et al., 2002a, 2002b), charr (*Salvelinus* spp.) (Fukunaga et al., 1991, 1992a, 1992b; Ritola et al., 2000) and bluegill (*Lepomis macrochirus*) (Paller and Heidinger, 1980). As the chemical reactions of ozone in marine and estuarine waters differ substantially from those in freshwater (Hoigné et al., 1985; Oemcke and van Leeuwen, 1998), findings from freshwater cannot be simply projected to saltwater conditions. In seawater, ozone reacts with halogen ions, mainly the bromide-ion, producing persistent oxidative by-products (Blogoslawski et

al., 1976; Crecelius, 1979). These ozone-produced oxidants (OPO) are more stable compared to ozone and its radicals in freshwater. Thus, the reprocessed seawater might still contain toxic amounts of residual oxidants when recirculated into the cultivation units again.

Hitherto, investigations on toxic effects of OPO to estuarine and marine fish are limited and have mostly been conducted on different model species like Atlantic silverside (*Menidia menidia*) (Toner and Brooks, 1975), juvenile Atlantic menhaden (*Brevoortia tyrannus*) (Richardson and Burton, 1981), juvenile sheepshead minnow (*Cyprinodon variegatus*) and larval topsmelt (*Atherinops affinis*) (Jones et al., 2006). Only few studies addressed aquaculture relevant species so far, like white perch (*Morone americana*) (Block et al., 1978; Hall et al., 1981; Richardson et al., 1983), red drum (*Sciaenops ocellatus*) (Reid and Arnold, 1994) and olive flounder (*Paralichthys olivaceus*) (Jiang et al., 2001). In addition, most of the previous studies investigated only acute toxicity by exposure to lethal OPO concentrations, neglecting chronic effects of sublethal concentrations. Referring to the permanent increase of ozone application in marine aquaculture production, biological effects of OPO have to be determined even for chronic exposure to provide guidelines and thresholds for a safe ozonation avoiding deleterious impacts on the cultured fish. Obviously, as pointed out by Sprague (1971) toxicants have to be evaluated in a species and life-stage specific manner. In the present study, we investigated the biological effects of three different sublethal OPO concentrations (0.06, 0.10 and 0.15 mg/l) on juvenile turbot (*P. maxima*) to ultimately define a safe, non-hazardous OPO concentration for juvenile *P. maxima* upon chronic exposure.

## Material and Methods

### *Experimental Setup*

Juvenile turbot were purchased from a commercial turbot hatchery (Maximus Fry, Denmark), randomly stocked to 12 identical recirculation systems and kept under a 12 h light - 12 h dark photoperiod (Pichavant et al., 1998). Prior to the experiment, fish were acclimatized to the experimental conditions for 10 days. Initially, fish had a mean total length ( $\pm$  SD) of  $12.2 \pm 1.0$  cm and a mean total wet weight ( $\pm$  SD) of  $36.4 \pm 8.2$  g. Tanks were equally stocked with regard to total wet weight at the start of the experiment. Fish were fed with a commercial pellet feed (DAN-EX 1562, 1.5 mm; Dana Feed) ad libitum in 48 h intervals to minimize fluctuations in ozone demand.

The 12 individual recirculation systems were assigned to three treatments and a control group with three replicates each. Recirculation systems were set up in a temperature controlled lab in order to ensure constant environmental conditions, considering suggestions on the arrangement by Hurlbert (1984). Each unit comprised a 200 l fiberglass tank (Chemowerk) filled with approximately 150 l of filtered natural seawater, with an individual biofilter and foam fractionator (by-pass operated).

Ozone gas was generated from compressed air by electrical discharge ozone generators (Model C 200, Erwin Sander Elektroapparatebau GmbH) and ozone-enriched air was injected into the seawater through a porous lime stone diffuser at the bottom of the foam fractionator (Model 1 AH 1100, Erwin Sander Elektroapparatebau GmbH), which served as a contactor for water and gaseous ozone. For a maximized diffusion of ozone into the water, a counter-flow mode was created by directing the recirculating water downward - past the uprising bubbles. The retention time in the foam fractionator was set to approximately 1 min. Ozone-supply was controlled and regulated by linking a redox potential controller (Erwin Sander Elektroapparatebau GmbH) to each ozone generator using the redox potential as proxy for the total oxidant concentration (Buchan et al. 2005). Nominal OPO concentrations were controlled in 2 h intervals over the entire experimental period by monitoring oxidant concentrations spectrophotometrically and redox potential setpoints were adjusted if necessary. Ozonated water was introduced into the tanks at high flow rates (600 l/h) and dispersed evenly by vertical spray bars. Juvenile turbot were exposed to OPO concentrations ( $\pm$  SD) of 0.00, 0.06  $\pm$  0.01, 0.10  $\pm$  0.01 and 0.15  $\pm$  0.02 mg/l for 21 days. Concentrations were tested with three replicates of 23 fish each.

Physical and chemical water parameters (pH, salinity, temperature, dissolved oxygen, total ammonia-N and nitrite-N) were measured at 2 day intervals. Mean water quality ( $\pm$  SD) during the exposure experiment was: temperature: 14.29  $\pm$  0.45 °C, salinity: 18.10  $\pm$  0.25, pH: 7.57  $\pm$  0.07, dissolved oxygen concentration: 10.48  $\pm$  0.16 mg/l, total ammonia-N (TAN): 0.93  $\pm$  0.53 mg/l and nitrite-N: 0.41  $\pm$  0.84 mg/l.

### *Water Analysis*

OPO were measured spectrophotometrically using the colorimetric *N,N*-diethyl-*p*-phenylenediamine (DPD) method (DPD Total Chlorine powder pillows, Hach), recommended for the quantification of OPO in seawater (American Public Health Association, 1989; Buchan et al., 2005). Extinction was measured at 530 nm with a DR/2800 Spectrophotometer (Hach Lange GmbH) and OPO concentrations were expressed as chlorine equivalents.

Physical and chemical water parameters were monitored by measuring dissolved oxygen concentration (CellOx 325, WTW), pH (CyberScan pH310, Eutech Instruments) salinity and temperature (315 i, WTW). Total ammonia-N and nitrite-N were determined spectrophotometrically using a DR/2800 photometer (Hach Lange GmbH) and powder pillow detection kits (Hach) based on the Ammonia Salicylate Method and Diazotization Method, respectively.

### *Sampling*

Fish were randomly sampled after 1, 7 and 21 days of OPO exposure by dip-netting and immediately killed in icewater as recommended by the FAO (2009b). The experiment was conducted in compliance with the institutional guidelines for the care and use of animals and the national legislation.

Blood was sampled via cardiac puncture through the gill chamber using broken heparinized micro-hematocrit capillaries (Hirschmann) and was collected in ice chilled 1.3 ml heparinized blood collection vials (Li-Heparin LH/1.3; Sarstedt). Subsamples for hemoglobin and hematocrit quantification were removed before the remaining blood was centrifuged (2 min, 10000g, 4°C) to separate plasma. Plasma was frozen at -20°C for subsequent cortisol analysis. The gill apparatus was dissected, gill arches were separated and the second right gill arch was stored in 4% sodiumtetra-borate buffered formalin prior to histological analysis.

### *Histology*

Gill arches were preserved in 4% phosphate buffered formaldehyde, dehydrated in a graded series of ethanol, cleared with xylene and subsequently infiltrated and embedded in paraffin (Wuertz, 2005). Cross-sections (2 µm) were stained with haematoxylin-eosin and analyzed at 400 x magnification using a Leica DMRBE microscope equipped with a digital camera (DFC320, Leica). Considering five primary filaments, a total of 100 secondary lamellae was

analysed per fish. Histopathological alterations were recorded as percentage of secondary lamellae affected, assigned to the categories not affected, necrotic, lamellar fusion, epithelial lifting, clubbed lamellar tips, hypertrophy and hyperplasia following Mallat (1985), Crespo et al. (1987), Frances et al. (1998) and Gisbert et al. (2004).

*Blood parameters - Hemoglobin, hematocrit and plasma cortisol*

Hematocrit was determined in micro-hematocrit capillaries (Na-heparin, 75 mm/60 µl; Hirschmann) upon centrifugation (12000 g, 8 min) with a micro-hematocrit centrifuge (Microfuge HG 101; Heraeus Crist). Hemoglobin was determined by the cyanmethemoglobin method using a Hemoglobin FS reagent kit (DiaSys). Before photometric quantification at 540 nm (UV-1202 UV-Vis; Shimadzu), erythrocyte nuclei and cellular debris were removed by centrifugation (6 min, 3000 g) (Stoskopf, 1993; Borges et al., 2004).

Plasma cortisol was quantified by a solid-phase Enzyme-linked Immunosorbent Assay (ELISA) kit (RE52611; IBL). As a prerequisite, four different protocols (diethyl ether; ethanol; heat treatment modified after Antonopoulou et al. (1999) and Sakar et al. (2007); plasma as reference) were evaluated by cold spiking with cortisol ( $40 \text{ ng ml}^{-1}$ ) to determine the recovery. Therefore 100 µl of plasma were (1) extracted in 3 ml of diethyl ether (Roth), vortexed (30 s), incubated at room temperature (RT) for 10 min, frozen for 30 min at -80°C for separation of the organic phase, followed by a second extraction with 3 ml diethylether; (2) extracted in 1.5 ml pure ethanol, vortexed for 1 min, incubated for 10 min at RT, centrifuged (12000 g, 1 min) and subsequent separation of the organic phase, followed by a second extraction with 1.5 ml ethanol; (3) heat denatured at 80°C for 1 h, subsequently vortexed (20 s), diluted with 1 x PBS, vortexed for 20 s, centrifuged (13000g, 20 min), for separation of the supernatant and (4) compared to cold spiked plasma. Upon evaluation, heat denaturation was applied before ELISA.

*Statistical analysis*

Data presented as mean ± standard deviation were analysed using STATISTICA 6.1 (StatSoft). Data were calculated as mean for each single tank and compared between replicates and treatments (Hurlbert, 1984). Percentage data were arcsin square-root transformed and outliers were detected using Nalimov outlier test. Normality of the data was determined by Shapiro-Wilk's W Test. When conformed, factorial ANOVAs were conducted and significance

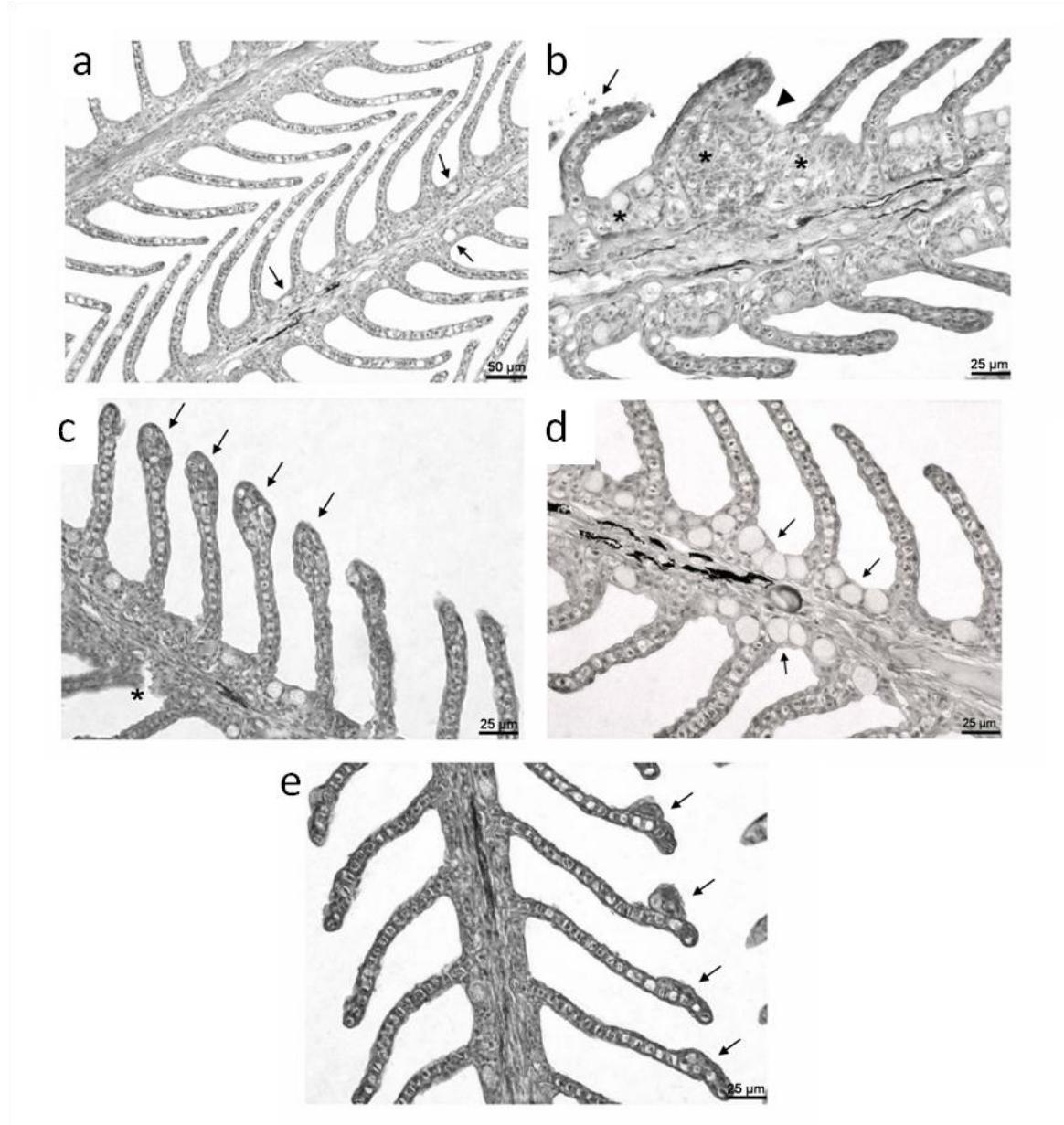
was analysed by Tukey HSD test for equal sample sizes or unequal N HSD test, respectively. If a significant interaction between the two factors exposure time and OPO concentration was observed, the effect of each single factor was investigated by one-way ANOVA. If normality or homogeneity of variances was not confirmed, even though transformed, data were analyzed with the Scheirer-Ray-Hare test (Sokal and Rohlf, 1995). Here, analyses within levels of each factor were carried out using Kruskal-Wallis test afterwards.

## Results

During the acclimation period and the entire experiment, no mortalities were recorded. Turbot showed typical behavioural patterns including balanced periods of resting and moderate swimming activity. After the beginning of the experiment, behavioural alterations were observed at higher OPO concentrations: During the first 4 days of exposure to 0.15 mg/l reduced swimming activity and prolonged resting periods were observed. Additionally, differences in feeding behaviour were recorded between treatment groups: Control specimens and fish exposed to 0.06 mg/l surfaced while feeding, typical for fish adapted to aquaculture conditions. In contrast fish exposed to 0.10 mg/l utilized only 60% of the water column. At 0.15 mg/l specimen even remained close to the bottom during feeding, utilizing only 30% of the water column. For these groups, reduced appetite was observed, indicated by shorter time spent in feeding, finishing feeding earlier than turbot subjected to 0.06 mg/l and control specimens. However, after 21 days of OPO exposure, fish sampled from the different treatments reached similar weights ( $\pm$ SD) with  $51.7 \pm 9.2$  g in controls,  $51.7 \pm 7.3$  g in the 0.06 mg/l,  $49.8 \pm 5.8$  g in the 0.10 mg/l and  $50.5 \pm 10.3$  g in the 0.15 mg/l treatment. No significant difference in Fulton's condition factor could be observed between OPO concentrations (two-way ANOVA,  $F(3, 24) = 1.03$ ,  $p>0.05$ ) as well as between different times of exposure (two-way ANOVA,  $F(2, 24) = 2.92$ ,  $p>0.05$ ).

### *Histology*

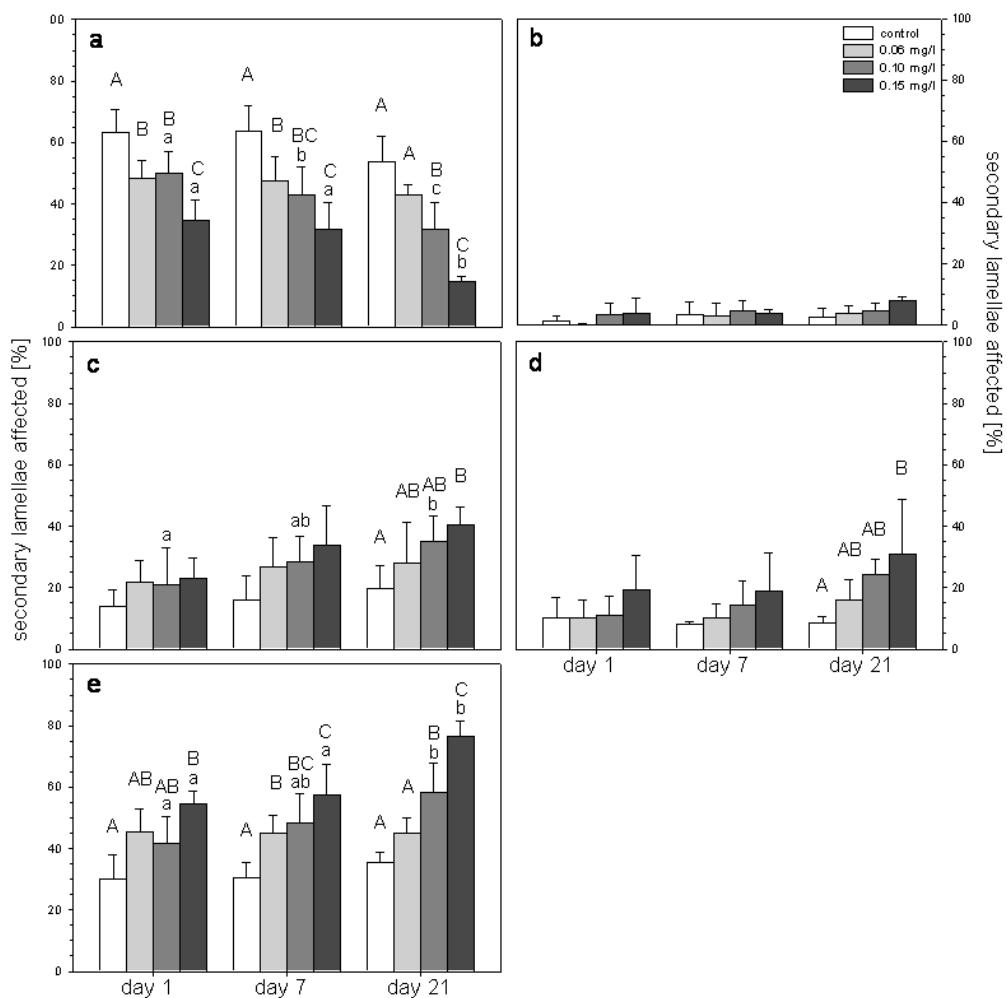
Several histopathological alterations were recorded in the gills, categorised as not affected, necrosis, lamellar fusion, epithelial lifting, lamellar clubbing, hypertrophy and hyperplasia (Fig. II-1).



**Fig. II-1: Morphology of the gills in control specimens and turbot exposed to different OPO concentrations.**  
**(a)** Control specimen with nearly unaffected gills, 200x. Note the slight hypertrophy of chloride cells in the primary filament (arrows). **(b) - (d)** Histo-pathological alterations in gills of juvenile turbot exposed to OPO. **(b)** Hyperplasia (asterisks) with partial lamellar fusion (arrowhead) and necrosis (arrow), 400x. **(c)** Hyperplasia on the tip of secondary lamellae (clubbed lamellae) (arrows), 400x. The disruption in the lower part of the primary lamella is an artefact (asterisk). **(d)** Massive hypertrophy combined with hyperplasia of chloride cells in the primary filament (arrows), 400x. **(e)** Hyperplasia on the distal part of secondary lamellae (arrows), 400x.

Lamellar fusion and epithelial lifting were detected in only four and three specimens, respectively, not correlated to the OPO concentration and thus excluded from further analysis. Lamellar clubbing, hypertrophy and hyperplasia occurred in all specimens analysed, necrotic abnormalities were observed in 69.4% of the investigated specimens.

Few necroses occurred in OPO treated specimens and increased just slightly with increasing OPO concentration and prolonged time of exposure (Fig. II-2 b).



**Fig. II-2:** (a) - (e) Mean percentage of affected secondary lamellae in gills of juvenile *P. maxima* control specimens and fish exposed to OPO concentrations of 0.06, 0.10 and 0.15 mg/l at days 1, 7 and 21 ( $n$  per day = 24). The major histopathological categories analyzed were: (a) not affected, (b) necrosis, (c) lamellar clubbing, (d) hypertrophy and (e) hyperplasia. Error bars denote standard deviations. At the same exposure time, columns with distinct upper case letters indicate significant differences among concentrations. Within the same concentration, different lower case letters indicate significant differences between exposure times. Means not sharing a common letter were significant different. If there was no significant difference within one sampling day or between sampling days for one concentration, letters are not shown.

Compared to control specimen, no significant increase of necrosis was observed between treatments (Scheirer-Ray-Hare two-way ANOVA,  $H(3) = 3.13$ ;  $p>0.05$ ) over time (Scheirer-Ray-Hare two-way ANOVA,  $H(2) = 3.47$ ;  $p>0.05$ ). The amount of clubbed lamellar tips in OPO treated turbot increased slightly compared to the control, with only minor differences between OPO treatments (Fig. II-2 c) and was correlated to OPO concentrations (two-way

ANOVA,  $F(3, 23) = 16.85$ ,  $p<0.01$ ) as well as time of exposure (two-way ANOVA,  $F(2, 23) = 9.18$ ,  $p<0.01$ ). No interaction was detected (two-way ANOVA,  $F(6, 23) = 1.82$ ,  $p>0.1$ ). Hypertrophic abnormalities followed a similar pattern as for clubbed lamellae but the abundance of the secondary lamellae affected was lower (Fig. II-2 d). However, differences were only significant in terms of OPO concentration (Scheirer-Ray-Hare two-way ANOVA,  $H(3) = 10.92$ ,  $p<0.01$ ) but not for exposure time (Scheirer-Ray-Hare two-way ANOVA,  $H(2) = 4.82$ ,  $p>0.05$ ). Hyperplasia was the most prevalent histopathological abnormality (Fig. II-2 e) observed in juvenile turbot. Even in the control, hyperplastic filaments were detected frequently (day 1:  $30.16 \pm 7.76\%$ ; day 7:  $30.50 \pm 5.13\%$ ; day 21:  $35.60 \pm 3.05\%$ ). However, OPO concentration (two-way ANOVA,  $F(3, 24) = 82.67$ ,  $p<0.01$ ) as well as time of exposure (two-way ANOVA,  $F(2, 24) = 24.00$ ,  $p<0.01$ ) had a statistical impact on the development of hyperplasia. A significant interaction between time of exposure and OPO concentration was observed (two-way ANOVA,  $F(6, 24) = 5.52$ ,  $p<0.01$ ). Subsequent one-way ANOVAS revealed OPO to effect the amount of hyperplastic filaments at day 1 (Kruskal-Wallis ANOVA,  $H(3) = 8.23$ ,  $p<0.05$ ), day 7 (one-way ANOVA,  $F(3, 8) = 23.97$ ,  $p<0.01$ ) and day 21 (one-way ANOVA,  $F(3, 8) = 77.83$ ,  $p<0.01$ ) as well as time of exposure within the 0.10 mg/l (one-way ANOVA,  $F(2, 6) = 13.12$ ,  $p<0.01$ ) and the 0.15 mg/l treatment (one-way ANOVA,  $F(2, 6) = 49.91$ ,  $p<0.01$ ). Consistently, the amount of gill filaments showing no histopathological abnormalities decreased significantly with increasing OPO concentration (two-way ANOVA,  $F(3, 24) = 81.25$ ,  $p<0.01$ ) and exposure time (two-way ANOVA,  $F(2, 24) = 30.07$ ,  $p<0.01$ ). Again, due to a significant interaction between exposure time and OPO concentration (two-way ANOVA,  $F(6, 24) = 2.60$ ,  $p<0.05$ ), one-way analysis of variance was performed revealing a significant impact of OPO concentration on the amount of non-affected filaments at day 1 ( $F(3, 8) = 17.75$ ,  $p<0.01$ ), day 7 ( $F(3, 8) = 20.83$ ,  $p<0.01$ ) and day 21 ( $F(3, 8) = 55.10$ ,  $p<0.01$ ) as well as a significant impact of OPO exposure within the 0.10 mg/l ( $F(2, 6) = 46.24$ ,  $p<0.01$ ) and the 0.15 mg/l treatment ( $F(2, 6) = 24.68$ ,  $p<0.01$ ).

#### *Blood parameters - Hemoglobin, hematocrit and cortisol*

At day 1, hemoglobin was slightly elevated in the OPO exposed specimen compared to control animals (Tab. II-1). At day 7, hemoglobin in OPO exposed turbot levelled off and returned to normal. A significant effect on hemoglobin concentration was not detected,

neither for OPO concentration (two-way ANOVA,  $F(3, 24) = 3.07$ ,  $p \geq 0.05$ ) nor for exposure time (two-way ANOVA,  $F(2, 24) = 0.92$ ,  $p > 0.05$ ).

**Tab. II-1: Mean blood hemoglobin ( $\pm$  SD) [g 100 ml $^{-1}$ ] in juvenile *P. maxima* exposed to different OPO concentrations ( $n$  per day = 72).**

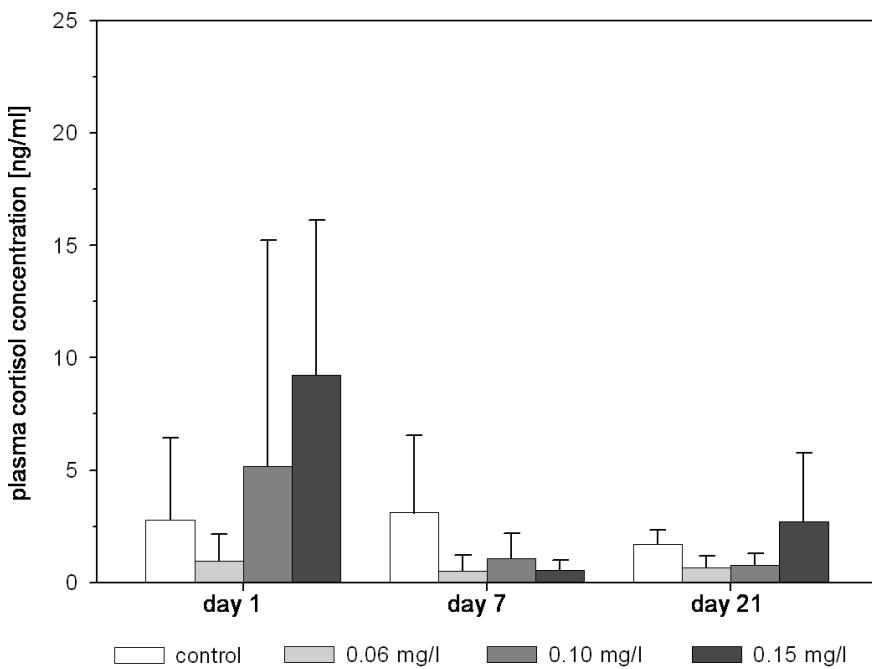
	day 1	day 7	day 21
control	2.19 $\pm$ 0.35	2.13 $\pm$ 0.30	2.25 $\pm$ 0.27
0.06 mg/l	2.31 $\pm$ 0.18	2.49 $\pm$ 0.33	2.40 $\pm$ 0.31
0.10 mg/l	2.49 $\pm$ 0.45	2.35 $\pm$ 0.62	2.64 $\pm$ 0.29
0.15 mg/l	2.71 $\pm$ 0.44	2.26 $\pm$ 0.68	2.51 $\pm$ 0.35

Only minor variations were observed in the hematocrit, ranging from  $17.63 \pm 1.19\%$  to  $21.22 \pm 1.48\%$  (Tab. II-2). No significant differences were detected between OPO treatments (two-way ANOVA,  $F(3, 24) = 0.27$ ,  $p > 0.05$ ) as well as between exposure times (two-way ANOVA,  $F(2, 24) = 3.69$ ,  $p > 0.05$ ).

**Tab. II-2: Mean blood hematocrit ( $\pm$  SD) [% packed cell volume] in juvenile *P. maxima* exposed to different OPO concentrations ( $n$  per day = 72).**

	day 1	day 7	day 21
control	17.63 $\pm$ 1.19	19.11 $\pm$ 2.03	19.11 $\pm$ 1.90
0.06 mg/l	19.22 $\pm$ 1.39	18.78 $\pm$ 2.17	20.00 $\pm$ 1.58
0.10 mg/l	19.22 $\pm$ 1.92	18.89 $\pm$ 3.10	21.22 $\pm$ 1.48
0.15 mg/l	19.44 $\pm$ 1.59	19.22 $\pm$ 1.99	20.00 $\pm$ 1.58

After 1 day of OPO exposure to 0.10 mg/l and 0.15 mg/l, plasma cortisol increased to  $5.16 \pm 10.08$  ng/ml and  $9.22 \pm 6.89$  ng/ml, respectively (Fig. II-3). However, concentrations returned to levels of control animals until day 7. The impact of OPO on plasma cortisol seemed to be concentration dependent, although statistical analysis revealed no significant differences between OPO concentrations (Scheirer-Ray-Hare two-way ANOVA,  $H(3) = 8.46$ ,  $p > 0.05$ ). In contrast, exposure time seemed to have a significant effect on cortisol levels (Scheirer-Ray-Hare two-way ANOVA,  $H(2) = 1.10$ ,  $p < 0.05$ ). However, subsequent non-parametric analysis of variance (Kruskal-Wallis ANOVA) to determine the effect of each single factor did not detect any differences in terms of OPO concentration nor of exposure duration (day 1:  $H(3) = 7.21$ ,  $p > 0.05$ ; day 7:  $H(3) = 6.08$ ,  $p > 0.1$ ; day 21:  $H(3) = 3.82$ ,  $p > 0.2$ ; control:  $H(2) = 0.62$ ,  $p > 0.7$ ; 0.06 mg/l:  $H(2) = 1.16$ ,  $p > 0.5$ ; 0.10 mg/l:  $H(2) = 0.36$ ,  $p > 0.8$ ; 0.15 mg/l:  $H(2) = 5.96$ ,  $p > 0.05$ ).



**Fig. II-3: Mean cortisol values in juvenile *P. maxima* control specimens and fish exposed to OPO concentrations of 0.06, 0.10 and 0.15 mg/l at days 1, 7 and 21 ( $n$  per day = 36). Error bars denote standard deviations.**

## Discussion

Several studies addressed the histological and physiological effects of ozone on different freshwater finfish species (Wedemeyer et al., 1979; Paller and Heidinger, 1980; Fukunaga et al., 1992b; Morita et al., 1995; Ritola et al., 2000, 2002b). In contrast, just few studies investigated the biological impact of ozonation on fish species in estuarine and marine waters. These studies focused on the consequences of acute OPO toxicity following only short exposures (Toner and Brooks, 1975; Hall et al., 1981; Richardson and Burton, 1981; Richardson et al., 1983; Jiang et al., 2001; Jones et al., 2006). Indeed, for aquaculture purposes the physiological consequences of chronic sublethal OPO impacts are of special concern as they might affect overall fish condition, impair wellbeing of the cultured animals and influence total aquaculture production. Hence, in the present study we analyzed the effects of sublethal OPO concentrations on juvenile turbot addressing behavioural observations, histopathology and blood functionality considering hemoglobin and hematocrit as well as plasma cortisol as physiologic stress parameter to ultimately determine a safe OPO concentration for a routine ozone application in turbot on-growing.

The alterations in turbots' feeding behaviour observed in the present study, although not assessed quantitatively, strongly suggest that OPO concentrations  $\geq 0.10$  mg/l can reduce feed uptake and may alter growth performance. Altered behaviour and reduced appetite can generally be observed in fish exposed to toxicants and have previously been reported for both, freshwater and marine fish following ozone exposure (Wedemeyer et al., 1979; Richardson et al., 1983). However, the observed alteration in feed uptake between the different OPO exposure groups in the present study did not result in any differences in growth or condition. This might be attributed to the relative short duration of the experiment (21 days) and significant differences might have been manifested in a longer exposition. Consistently to this study, Richardson et al. (1983) observed first behavioural abnormalities at OPO concentrations  $\geq 0.10$  mg/l in white perch in brackish water. However, the behavioural alterations observed in white perch were more severe, reporting extreme lethargy and insensitivity to tactile stimulation (Richardson et al., 1983). In the present study, lethargic behaviour could only be observed during the first four days at the highest OPO concentration of 0.15 mg/l. After 4 days of exposure, no lethargic behaviour was observed, potentially reflecting a high adaptability and robustness of turbot towards OPO as previously reported for other stressors like moderate water quality (Person-Le Ruyet et al., 2003), handling (van Ham, 2003), photoperiod and temperature (Imsland and Jonassen, 2002).

As gill tissue comprises the largest surface area of the fish in direct contact with the surrounding medium (Evans et al., 2005) the gills represent the most sensitive organ towards dissolved toxicants. Hence, histological analyses of the gills were performed to monitor histopathologic alterations upon OPO exposure. So far, several studies addressed the analysis of gill histopathology as biomarker for the harmful impact of oxidants like ozone and its derived by-products on freshwater and marine fish, thereby revealing the gills as the main target site of oxidative toxicity (Wedemeyer et al., 1979; Middaugh et al., 1980; Ollenschläger, 1981; Paller and Heidinger, 1980; Richardson et al., 1983). Concordantly, in the present study we observed gradual alterations of the gill epithelium with increasing OPO concentration and prolonged time of exposure. According to Mallatt (1985), the here observed alterations, i.e. hyperplastic and hypertrophic changes, can be considered adaptive since these histopathological alterations increase the distance between the surrounding medium and the blood vessels and therefore protect the organism due to reduced uptake of

the toxicant. In contrast, necroses reflect a direct and deleterious effect of an irritant, which represents a severe damage of the gill lamellae, thereby affecting gill's functionality (Temmink et al., 1983; Mallatt, 1985). However, the here observed necrosis were just weak compared to previous studies (Middaugh et al., 1980; Richardson et al., 1983) and were even present in controls. Indeed, although we detected a considerable increase in morphological abnormalities, these changes were less severe as those observed in white perch at comparable OPO concentrations (Richardson et al., 1983). For example, Richardson et al. (1983) observed "massive clumping" in fish subjected to an OPO concentration of 0.05 mg/l for 96 h and described a "loss of epithelial cells" in the gills of fish exposed to 0.10 mg/l as well as complete disruption of the secondary lamellae, extensive loss of epithelial tissue, and severe hyperplasia and edema in fish subjected to 0.15 mg/l for 48 h. These symptoms were further accompanied by an increase in hematocrit, compensating the loss in gill functionality (Richardson et al., 1983). In the present study, we could only observe an increase in hemoglobin at day 1 in fish subjected to the highest OPO concentration (0.15 mg/l). Prolonged OPO exposure, however, did not result in any long-term alteration of hemoglobin. Hematocrit did not show any alteration between treatment groups and exposure times, suggesting a higher robustness of turbot compared to white perch. This might be due to the akinetic lifestyle of the benthic turbot compared to the highly mobile, demersal white perch, which requires higher oxygen concentrations. Still, in the present study, hemoglobin of the control specimens were slightly lower ( $2.13 \pm 0.30$  g/dl and  $2.25 \pm 0.27$  g/dl) than values reported in previous studies, ranging from  $5.5 \pm 0.2$  g/dl (Quentel and Obach, 1992),  $3.0 \pm 0.2$  g/dl (Waring et al., 1996) and  $4.0 \pm 0.5$  g/dl (Waring et al., 1997). Even in OPO treated specimens hemoglobin never exceeded a maximum of  $2.71 \pm 0.44$  g/dl, detected in turbot subjected to 0.15 mg/l at day 1. Hematocrit values detected in the present study were higher compared to those of other studies conducted on turbot reporting around  $11.0 \pm 1.3$  to  $16.7 \pm 0.7$  % PCV (Quentel and Obach, 1992; Waring et al., 1996; Mugnier et al., 1998) and lower compared to those of further studies which reported slightly higher hematocrit between ~20 to ~25 % PCV (Staurnes, 1994; Person-Le Ruyet et al., 1998; Pichavant et al., 2001; Person-Le Ruyet et al., 2003). The hematocrit in the present study, spanning  $17.63 \pm 1.19$  % PCV to  $19.11 \pm 2.03$  % PCV in controls and  $18.78 \pm 2.17$  % PCV to  $21.22 \pm 1.48$  % PCV in OPO exposed turbot, can be considered intermediate compared to these reports. However, hemoglobin and hematocrit are age and size-dependent and beyond that influenced by the

rearing conditions (Quentel and Obach, 1992). This might explain the here observed differences. In addition, Romestand et al. (1983) pointed out that hematocrit values increase progressively with increasing salinity, representing an adaptation to the reduced dissolved oxygen concentration in more saline environments. Furthermore, the studies cited above did not report whether erythrocyte nuclei were removed prior to photometrical hemoglobin quantification as recommended by Stoskopf (1993), Borges et al. (2004) and Thrall et al. (2004). It is not known to which extent this might alter the hemoglobin value.

Although turbot demonstrated the previously described robustness towards sublethal OPO concentrations, cortisol increase at day 1 in 0.10 and 0.15 mg/l treatments suggests an induction of the HPI axis. Here an OPO concentration of 0.10 mg/l increased plasma cortisol slightly to  $5.16 \pm 10.08$  ng/ml and 0.15 mg/l to  $9.22 \pm 6.89$  ng/ml, respectively, indicating stressful conditions for juvenile turbot. However, the cortisol levels observed in the present study, returned to levels of control animals until day 7. Cortisol resting levels around 2.5 ng/ml were in good agreement with those reported for turbot by Pichavant et al. (2001) and were lower compared to others: Waring et al. (1996, 1997) reported resting levels around 5-8 ng/ml, congruently with Mugnier et al. (1998) who reported 4-5 ng/ml. Waring et al. (1996, 1997) reported cortisol peak levels of ~62 ng/ml and ~80 ng/ml in turbot following a recovery period of 1 h after single net-confinement and 60-80 ng/ml following multiple net-confinement, respectively. In the present study fish exposed to an OPO concentration of 0.15 mg/l for 1 day exhibited a maximum in cortisol with around  $9.22 \pm 6.90$  ng/ml. This is probably indicative for a mild stress. Chronic stress usually results in slight increases in cortisol (Vijayan and Leatherland, 1990; Wendelaar Bonga, 1997; Wuertz et al., 2006). On the other hand, increased cortisol might be attributed to the central role of corticosteroid hormones in metabolism and several major physiological functions in general. Following a chronic exposure towards a long term stressor for up to several weeks, cortisol levels are generally reported to return to initial values feigning homeostasis (Strange et al., 1978; Pickering and Steward, 1984), partly due to the down-regulation of ACTH and cortisol receptors (Wendelaar Bonga, 1997). Therefore, cortisol is not the most suitable parameter to evaluate the effect of a chronic stressor. This pattern might underlay the observation in the present study, and may also explain the aberrations to previous studies conducted on turbot.

In conclusion, the multidimensional approach used in this study, addressing different physiological parameters, clearly demonstrates that sublethal OPO concentrations of  $\geq 0.10$  mg/l can affect behaviour, gill functionality and the physiological status in juvenile turbot. In contrast, at an OPO concentration of 0.06 mg/l only slight alterations in the tested parameters could be detected suggesting  $\leq 0.06$  mg/l as a safe level for the rearing of juvenile turbot. As previous studies reported a high inactivation of bacterial fish pathogens in seawater at OPO levels as low as 0.06 mg/l (Sugita et al., 1992; Chapter I), residual OPO can be reduced to at least 0.06 mg/l to prevent adverse impacts on cultivated juvenile turbot while maintaining an effective reduction of bacterial biomass. Our findings are highly valuable for ozone application in daily aquaculture routine, although it would be desirable to validate these results by extending exposure time to an even longer period. Despite, results provide a first insight concerning the effect of OPO on fish welfare in aquaculture production which is becoming increasingly important in terms of marketing and consumer perception towards farmed fish.

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## CHAPTER III

# A comparative study on the removability of different ozone-produced oxidants by activated carbon filtration

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### Abstract

A comparative study about the removability of four potentially harmful ozone-produced oxidants (free bromine, bromamines, free chlorine and chloramines) by activated carbon filtration was performed under identical conditions in small-scale filter experiments. Removability was high and similar among the oxidants instead of chloramines showing the least removability. Results proved activated carbon filtration to be very efficient in removing the dominating brominated oxidants formed during the ozonation of natural and most artificial seawaters. In contrast, removability of chloramines, sometimes present in ozonated bromide-free artificial seawater, was shown to be significantly lower. To improve removal of persistent chloramines by activated carbon filtration, a comparative evaluation of three different activated carbon types was conducted. Granulated activated carbon on coal basis was suggested to be the most cost-effective carbon for removing chloramines as it possessed highest removal capacity while being the cheapest of the carbons tested.

## Introduction

Ozone, as a powerful oxidizing agent is widely used in recirculating aquaculture systems (RAS) and public aquaria to improve water quality while reducing the pathogenous load and removing inorganic and organic wastes (Liltved et al., 1995; Rosenthal, 1981; Summerfelt and Hochheimer, 1997). Particularly in seawater systems the application of ozone in combination with foam fractionation is widespread (Sander and Rosenthal, 1975; Orellana, 2006). In seawater ozone oxidizes several halogen ions to reactive by-products (Williams et al., 1978) generally termed as ozone-produced oxidants (OPO). Although most of these OPO are desirable and effective disinfectants (Johnson and Overby, 1971; Butterfield, 1946) they are also toxic to many cultured species and critical concentrations have to be avoided in aquaculture (Richardson et al., 1983; Chapter I and II). Mainly when an exhaustive disinfection of recirculating water is desired, high ozone dosage is needed, which may lead to toxicity problems for the cultured species. In those cases, residual OPO have to be removed from the recirculating water.

Activated carbon filtration, known for its excellent adsorption capacity, has been established as a reliable method for the removal of offensive tastes, odors, turbidity and even pesticides in process water treatment. Besides its adsorption capacity (Urano et al., 1976) activated carbon shows high capacity for a catalyzed reduction of oxidants (Asami et al., 1999; Kirisits et al., 2000). It has been shown that granular activated carbon is able to reduce residual OPO to acceptable levels in ozonated seawater (Ozawa et al., 1991). However, the composition of residual OPO varies in dependence of different water parameters like salt mixture and concentration of ammonia and dissolved organics. Therefore, removal efficiency of activated carbon filtration for OPO is non-transferable among discriminative water properties, as single oxidant species might differ in their removability. Accordingly, detailed information is needed on the removability of different OPO.

So far, several studies have proven activated carbon filtration to be effective in removing persistent and potentially carcinogenic disinfection by-products like bromate (Siddiqui et al., 1996; Bao et al., 1999; Yang et al., 2000; Liltved et al., 2006) and trihalomethanes (Yang et al., 2000; Liltved et al., 2006; Amy et al., 1991; Nakamura et al., 2001) in fresh- and seawater. In most RAS, however, bromate and trihalomethanes are only present in trace amounts due to several factors minimizing their formation, primarily due to a preferred reaction of intermediates with ammonia (von Gunten and Hoigne, 1994; Pinkernell and von Gunten,

2001; Sun et al., 2009). Much more prominent is the formation of reactive intermediates like hypohalides and haloamines. Those acutely toxic oxidants may accumulate to harmful concentrations during ozonation of aquacultural seawater really fast.

Brominated oxidants have highest formation potential, as the oxidation of bromide ion to free bromine by ozone ( $k = 160 \pm 20 \text{ M}^{-1}\text{s}^{-1}$ ) (Hoigne et al., 1985) is the most dominant reaction process during ozonation of seawater (Liltved et al., 2006; Blogoslawski et al., 1976). Although chloride is by far the most frequent ion in seawater with about 19 g/l (Horne, 1969), there is much less formation potential for chlorinated oxidants, as the first order rate constant  $k$  for the reaction of ozone with chloride ion to free chlorine is very low ( $< 3,0 \times 10^{-3} [\text{M}^{-1}\text{s}^{-1}]$ ) (Hoigne et al., 1985). As long as bromide ions are present in the water, the equivalent reaction of ozone with chloride ions is unlikely to occur in significant quantities. However, bromide ions are often excluded from artificial seawater mixes to avoid formation of carcinogenic and bioaccumulating ozonation by-products like bromate and bromoform (Grguric et al., 1994). In those cases, the oxidation of the chloride ion to free chlorine by ozonation may become more important.

High stocking densities in RAS naturally implicate significant amounts of ammonia. As free bromine and free chlorine react with ammonia very fast to form bromamines and chloramines, respectively (Johnson and Overby, 1971), these haloamines usually represent the most dominant residual OPO in RAS.

However, only a few studies have investigated the removability of those reactive intermediates by activated carbon filtration. Whereas activated carbon filtration has been shown to remove free chlorine and chloramines (Bauer and Snoeyink, 1973; Seegert and Brooks, 1978), there is currently a lack of information on free bromine and bromamine removal via activated carbon. Furthermore, variations in factors affecting the efficiency of oxidant reduction by activated carbon, like type and age of the carbon, solution pH, and the presence of natural organic matter prohibit a direct comparison of data based on different studies. For the first time, the removability of free bromine, bromamines, free chlorine and chloramines was determined using a standardized experimental procedure under constant conditions to comparatively assess removability of different OPO prominent at discriminate water properties by activated carbon filtration.

As a wide variety of activated carbon products are available, exhibiting markedly different characteristics and removal performances, a further objective of this study was to evaluate

three different activated carbon types for their removal capacity on the most persistent oxidant in order to improve removal of OPO by activated carbon filtration.

## Material and methods

Two different experiments were performed. In experiment A four different oxidant species (free bromine, bromamines, free chlorine and chloramines) were compared for their removability by activated carbon filtration. New granulated activated carbon on coal basis (AC 1) (MDLindinger) was used as filter medium, which is applied most frequently in process water treatment.

In experiment B three different types of activated carbon (MDLindinger) were comparatively evaluated for their chloramine removal capacity: (AC 1) Granulated activated carbon on coal basis as used in experiment A, (AC 2) Granulated activated carbon on coconut shell basis – food safe and therefore suitable for treatment of drinking water, (AC 3) Extruded activated carbon on coal basis – pH neutral, acid washed and designed for removing drug and pesticide residues in aquaria. The main characteristics of the tested activated carbons are summarized in Tab. III-1.

**Tab. III-1: Manufacturer's specifications of tested activated carbon types.**

	<b>AC 1</b>	<b>AC 2</b>	<b>AC 3</b>
<b>Origin</b>	coal	coconut	coal
<b>Bulk density [kg/m³]</b>	± 530/40	± 510/40	510 g/l
<b>Grain diameter [mm]</b>	0.6-2.4	0.6-2.36	3
<b>Surface area [m²/g]</b>	> 950	> 1050	> 1000
<b>Iodine number [mg/g]</b>	> 900	> 1000	> 950
<b>Cl4 activity (CTC) [%]</b>	> 60	> 55	> 60
<b>Ash [%]</b>	max. 8	max. 2	max. 10
<b>Moisture packed [%]</b>	max. 5	max. 3	3-5
<b>Hardness [%]</b>	> 92	> 99	> 95

Both experiments were conducted at 20°C in a temperature controlled lab to ensure isothermal conditions.

### *Test facility*

The experimental system consisted of a 10 l polyethylene tank with activated carbon filtration running in by-pass. A polyethylene tube was used as a filter column. Its underpart was capped with a 100 µm mesh to prevent small-sized carbon particles to get lost. For feeding the filter column with test solution a small aquarium pump was used. The water flow rate was held constant via a plug valve. A centrifugal pump inside the tank was installed for mixing the test solution continuously enabling identical oxidant concentrations over the whole water body.

### *Small scale filtration experiments*

Prior to each filtration experiment a fixed volume (~ 160 ml, 70 g) of new activated carbon was placed in the filter column resulting in a carbon bed of 20 cm height and rinsed exhaustively with deionized water to remove fines. Prior to each experimental run the tank was rinsed thoroughly and filled with 6 l of oxidant test solution. Oxidant test solutions were prepared by adding predefined volumes of concentrated oxidant stock solutions to 6 l of deionized water. Deionized water for test solutions was buffered by a 5 mM phosphate buffer system and adjusted to an initial pH of 8 using analytical grade sodium hydroxide (NaOH). As impurities of bromide ions are present in trace amounts even in analytical grade sodium chloride (Haag et al., 1982), deionized water was used for test solutions instead of artificial saltwater in order to avoid formation of brominated oxidants in mixed chlorine solutions. For a better comparison of removability, initial concentrations of different oxidant test solutions were consistently adjusted to approximately 1.0 mg/l. Prior to the filtration experiment, test solutions were mixed for 1 min via a centrifugal pump to assure homogeneity in oxidant concentration. Subsequently, test solutions were passed through the filter column for 10 min with a continuous flow rate of 0.8 l/min. Prior to and after filtration, initial and residual oxidant concentrations were measured, respectively, to determine the removal of oxidants.

As oxidants naturally decay with time due to molecular instability and photochemical destruction, control experiments were performed under identical conditions as described above but without carbon filtration. In control experiments the activated carbon in filter columns was replaced by PE granulate, which is typically used in fluidized bed reactors for biofiltration. Before use, the PE granulate was submerged in sodium hypochlorite solution to

prevent reduction of oxidants by oxidant demanding surfaces and finally rinsed with deionized water.

For each tested oxidant species and carbon type small scale filtration experiments as well as corresponding control runs were replicated 10 times under identical conditions.

#### *Stock solutions*

Chlorine stock solution was prepared by diluting 0.14 M sodium hypochlorite (NaOCl) (Sigma Aldrich, 10-13% free chlorine) with deionized water. For the preparation of bromine stock solution, sodium hypochlorite was added to a sodium bromide (NaBr) solution, as bromide ions react rapidly with free chlorine to form free bromine. Sodium bromide were added 2 times the stoichiometric concentration of chlorine, in order to assure complete conversion of free chlorine into free bromine. The chloramine stock solution was prepared by adding sodium hypochlorite to an ammonium chloride solution in a NH<sub>4</sub> : Cl molar ratio of 2 : 1. At pH 8, this stoichiometric ratio of ammonia to chlorine allows full conversion of free chlorine into monochloramine. Bromamine stock solution was prepared by adding an appropriate excess of ammonium chloride (NH<sub>4</sub>Cl) to bromine stock solution to convert all free bromine into bromamines. At pH 8, this bromamine solution contains monobromamine and dibromamine.

#### *Analyses*

Oxidants were measured spectrophotometrically with the *N,N*-Diethyl-*p*-Phenylenediamine (DPD) method as equivalent total residual chlorine (TRC), using a DPD Total Chlorine detection kit (Hach). The DPD reagent is suitable to detect free bromine, bromamines and free chlorine. As the used Total Chlorine detection kit contained potassium iodide additionally, even chloramines could be detected in a stoichiometric ratio of 1:1 (Sollo et al., 1971). Colour intensity was measured at 530 nm by a DR/2800 Spectrophotometer (Hach Lange GmbH). The pH of the test solutions was determined using a pH meter, type CyberScan pH 310 series (Eutech Instruments).

#### *Statistical Analysis*

As initial oxidant concentration could not be adjusted consistently to a unified value, the respective difference of initial and final oxidant concentration was used for data analysis. For

statistical calculations relative values were used, setting the initial concentration to 1.0. Data were arcsin square-root transformed and outliers were detected using Thompson's outlier test. Normality of the data was determined by Shapiro-Wilk's W Test.

For a statistical comparison of oxidant removal by activated carbon filtration with oxidants' natural decay in control experiments, a two-tailed t-test for independent samples was performed for each oxidant.

In order to test for significant differences between single oxidants in oxidants' natural decay as well as oxidants' removability by activated carbon filtration, a Kruskal-Wallis ANOVA with subsequent multiple comparison of mean ranks as post-hoc was conducted, respectively. To evaluate exclusively the carbons' effect on the removal of oxidant species excluding oxidants' natural decay, the mean of control measurements was subtracted from activated carbon filtration data.

The chloramine removal capacity of different carbon types were comparatively analysed for significant differences using a Kruskal-Wallis ANOVA and multiple comparison of mean ranks.

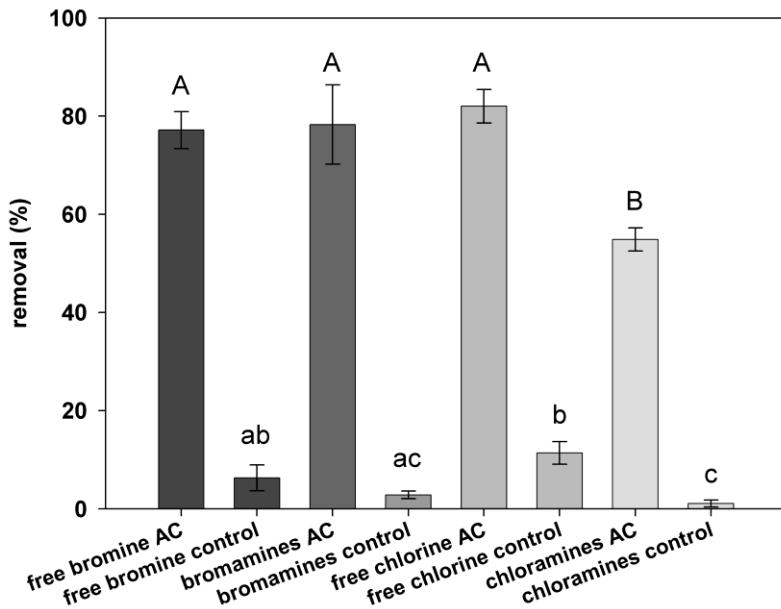
## Results

### *Removability of different oxidant species by activated carbon filtration*

In small-scale filtration experiments initial concentrations of all tested oxidants decreased during 10 minutes of activated carbon filtration. A slight decrease of initial concentrations was also observable for all tested oxidant species in control experiments without carbon filtration. The decline of initial oxidant concentrations during control runs without activated carbon filtration depicts the natural decay of the tested oxidant species. Control experiments showed highest oxidant loss for free chlorine ( $11.4 \pm 2.3\%$ ) followed by free bromine ( $6.3 \pm 2.6\%$ ) and bromamines ( $2.8 \pm 0.8\%$ ). Least oxidant loss was observed for chloramines ( $1.1 \pm 0.7\%$ ) (Fig. III-1). Statistical analysis showed significant differences in natural decay for free chlorine and chloramines ( $p<0.0001$ ), free chlorine and bromamines ( $p<0.001$ ) and free bromine and chloramines ( $p<0.001$ ).

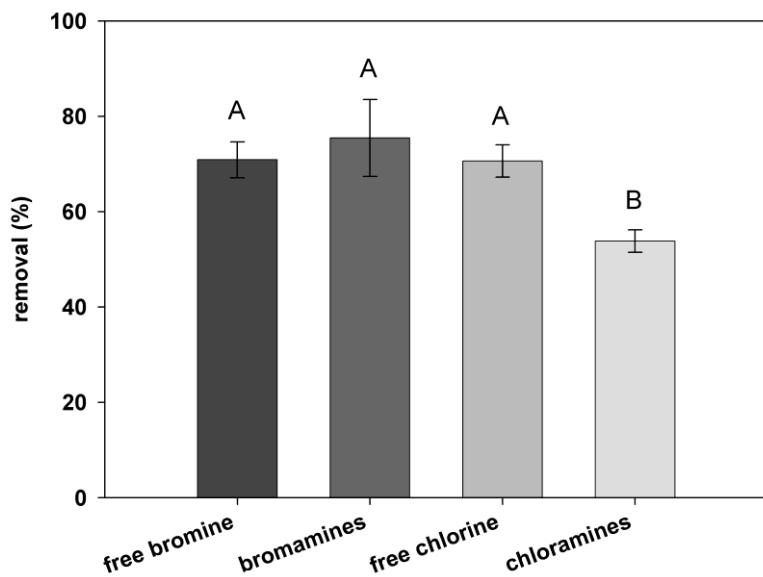
Compared to the oxidant loss in control experiments due to natural decay, the use of activated carbon filtration effected a higher decrease of all oxidants. This was verified by t-test for free chlorine ( $p < 0,0001$ ) and free bromine ( $p < 0,0001$ ) as well as by Welch-test for chloramines ( $p < 0,0001$ ) and bromamines ( $p < 0,0001$ ) showing a significant improvement of oxidant removal by using activated carbon filtration. Overall removal including the natural

decay was highest for free chlorine ( $82.0 \pm 3.4\%$ ) followed by bromamines ( $78.3 \pm 8.1\%$ ) and free bromine ( $77.2 \pm 3.8\%$ ). For chloramines least removal ( $54.9 \pm 2.4\%$ ) was observed (Fig. III-1). Statistical analysis showed significant differences in removability between free bromine and chloramines ( $p<0.05$ ), bromamines and chloramines ( $p<0.01$ ) as well as between free chlorine and chloramines ( $p<0.0001$ ).



**Fig. III-1: Mean removal of free bromine, bromamines, free chlorine and chloramines by activated carbon filtration (AC1) compared to their natural loss during control experiments. Error bars denote standard deviations. Within activated carbon filtration experiments different upper case letters and within control experiments different lower case letters indicate significant differences between oxidants. Bars not sharing a common letter were significantly different.**

To exclusively evaluate the carbon's effect on the removal of oxidant species excluding oxidants' natural decay, the mean oxidant losses of control experiments were subtracted from oxidant removal rates of activated carbon filtration experiments. Pure carbon removal effect was highest for bromamines ( $75.5 \pm 8.1\%$ ) followed by free bromine ( $70.9 \pm 3.8\%$ ) and free chlorine ( $70.6 \pm 3.4\%$ ) (Fig. III-2). Least removal was observed for chloramines ( $53.8 \pm 2.4\%$ ). Statistical analysis of pure carbon removal efficiency reproduced and verified results of activated carbon filtration analysis including natural decay by showing significant differences in removability between free bromine and chloramines ( $p<0.01$ ), bromamines and chloramines ( $p<0.0001$ ) as well as between free chlorine and chloramines ( $p<0.01$ ).

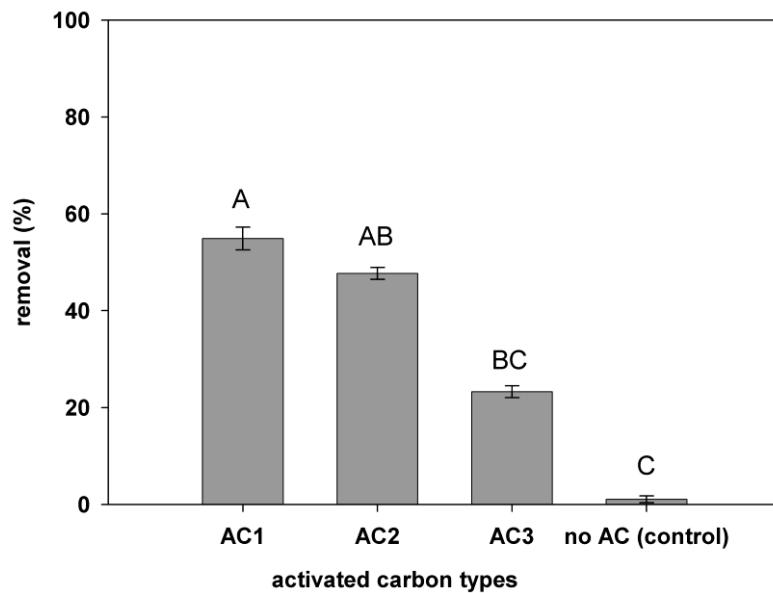


**Fig. III-2:** Mean oxidant removal of free bromine, bromamines, free chlorine and chloramines exclusively caused by activated carbon filtration (AC1). The mean oxidant loss due to natural decay was subtracted from removal data. Error bars denote standard deviations. Different upper case letters indicate significant difference. Bars sharing no common letter were significantly different.

#### *Removal efficiency of different activated carbon types*

A comparative evaluation of the removal of chloramines by activated carbon filtration revealed differences in removal performance of different types of activated carbon.

Compared to the natural decay of chloramines during control experiments (1% of initial concentrations), with all of the three tested carbons an improved decrease of chloramine concentration was achieved during ten minutes of carbon filtration (Fig. III-3). However, statistical analysis detected a significant effect in chloramine removal only for AC 1 ( $p < 0.0001$ ) and AC 2 ( $p < 0.001$ ) but not for AC 3 ( $p > 0.26$ ). Best removal of chloramines was achieved with AC 1 on coal basis ( $54.9 \pm 2.4\%$ ). AC 2 on coconut shell basis showed second best removal ( $47.7 \pm 1.2\%$ ) while AC 3 for aquaria use removed only a small amount ( $23.3 \pm 1.2\%$ ) of chloramines. Statistical analysis detected a significant difference in removal efficiency between AC 1 and AC 3 ( $p < 0.01$ ). No significant difference has been found between AC 1 and AC 2 ( $p > 0.51$ ) as well as between AC 2 and AC 3 ( $p > 0.37$ ).



**Fig. III-3: Mean chloramine removal by activated carbon filtration using three different carbon types (AC1, AC2, AC3), and without activated carbon filtration (control). Error bars denote standard deviations. Different upper case letters indicate significant difference. Bars sharing no common letter were significantly different.**

## Discussion

### *Removability of different oxidant species by activated carbon filtration*

The composition of OPO might differ between discriminative water characteristics and ozonation strategies. The presence or absence of bromide ions in artificial seawater formula determines the formation of brominated or chlorinated oxidants, the concentration of ammonia affects the ratio of hypohalides to haloamines in the ozonated seawater. Thus, removability of free bromine, free chlorine, bromamines and chloramines by activated carbon filtration was investigated in a comparative study, to allow a better assessment of activated carbon efficiency for discriminative water properties.

Small scale filtration experiments showed the tested brominated oxidants, free bromine and bromamines, being oxidants easy to remove with activated carbon filtration. As no significant difference in the removability of free bromine and bromamines by activated carbon filtration has been found, ammonia concentration seems to have no effect on the removability of residual OPO in ozonated bromide-containing water. Although bromamine loss due to natural decay was shown to be little slower compared to that of free bromine, bromamines featured a slight better removability by activated carbon filtration, causing a compensational effect for the overall removability of bromamines. As free bromine and

bromamines are the dominant oxidant species formed during ozonation of natural and most artificial seawaters (Liltved et al., 2006; Grguric et al., 1994), results of the present study proved activated carbon filtration to be suitable as OPO removal unit for marine RAS.

However, in artificial seawater systems bromide ions are sometimes excluded from the salt mix in order to prevent formation of potentially harmful brominated by-products. As the trace amounts of bromide ions, mostly present from impurities in technical grade NaCl (Grguric et al., 1994), may be removed with time from those artificial seawater mixes in a closed system by biological filtration, skimming or through the metabolic process of the cultivated animals, chloride ions might become the predominant reactant during ozonation. Whereas the direct reaction of ozone with chloride ions to free chlorine is slow, the oxidation of chloride ion via the hydroxyl radical pathway is much faster, as hydroxyl radicals, formed as oxidative intermediates during the ozonation of water, are much more reactive compared to ozone itself (Hoigner and Bader, 1975). While significant amounts of free chlorine were shown to accumulate during ozonation of unbuffered NaCl-solution (Grguric and Egli, 2001), formation of free chlorine is low during ozonation of carbonate buffered NaCl-solution (Grguric and Egli, 2001), presumably due to the inhibition of the hydroxyl radical pathway, as bicarbonate and carbonate may serve as hydroxyl radical scavengers. Hence, particularly during ozonation of bromide-free saltwater with low carbonate alkalinity significant quantities of free chlorine might be produced.

Results of small scale filtration experiments proved free chlorine to be as easy to remove via activated carbon filtration as free bromine and bromamines. In accordance with Cooper et al. (2007) who studied photochemical decay of different oxidants due to sunlight irradiation, natural decay of free chlorine was found to be even faster than that of free bromine. Due to this faster natural decay of free chlorine compared to free bromine and bromamines, the overall removability of free chlorine is actually highest.

However, in RAS significant quantities of ammonia are present, effecting a rapid conversion of ozone-produced chlorine into chloramines. In accordance with findings of Cooper et al. (2007) control experiments without activated carbon filtration clearly demonstrated chloramines decaying significantly slower compared to free chlorine, free bromine and even slower compared to bromamines, due to their low reactivity. Hence, chloramines have high potential for accumulation and have to be removed actively from the recirculating water. However, in activated carbon filtration experiments chloramines showed by far the least

removability of tested oxidant species suggesting the low reactivity of stable chloramines decelerating catalytic reactions at the carbon surface.

Although chloramines are weak biocides (Butterfield, 1946), they have a toxic effect to several aquatic organisms (Capuzzo et al., 1977; Larson et al., 1977) due to oxidation of hemoglobin to methemoglobin in blood, resulting in tissue anoxia (Grothe and Eaton, 1975). For a few species toxicity of chloramines was proven to be even greater than that of free chlorine (Capuzzo et al., 1976). Therefore, a rapid and effective removal of persistent chloramines from water is of particular importance to avoid contamination of cultivation tanks. Due to chloramines low removability by activated carbon filtration, one must make sure that chloramines come in contact with the carbon's surface for quite a long time. However, this might be difficult in a high flow application such as a foam fractionator's effluent.

As the present study proved free chlorine and chloramines to have a significant difference in their removability by activated carbon filtration, ammonia concentration has a considerable effect on the removability of residual OPO when chlorinated oxidants have been produced during ozonation of bromide-free saltwater. In closed RAS, where significant amounts of ammonia are mostly present in the recirculating water, the removability of OPO might depend on the existence of bromide-ions, as bromamines were shown to be easier to remove by activated carbon filtration compared to chloramines.

Although its original intention is the reduction of harmful ozonation by-products, the use of bromide-free salt mixes for the production of artificial seawater might involve the risk of an increased formation of harmful chloramines, which are more stable and harder to remove by activated carbon filtration than corresponding bromine compounds. More information is needed on the formation processes of chlorinated oxidants during ozonation of bromide-free artificial seawaters based on different seawater formulae.

Unless a potential formation of chlorinated oxidants during ozonation of bromide-free seawater can be definitely eliminated, the use of bromide-containing seawater for marine RAS is suggested to provide a better control of harmful oxidants by activated carbon filtration. The transfer of results to conditions deviating strongly in their pH from that in the present study (pH 8), however, has to be done with caution, as tested oxidants exist in different chemical forms depending on the pH and therefore might vary in their removability with changing pH.

Beyond oxidants' removability further factors such as empty bed contact time, type and age of the carbon affect the oxidant removal efficiency of activated carbon filtration. For example, some preliminary tests indicated a considerably temporal limitation of activated carbon performance (data not shown). Although OPO are catalytically reduced by activated carbon and do not accumulate in the carbon structure (Asami et al., 1999), organics may occupy portions of the carbon's surface with time and therefore inhibit oxidant reduction. In aquaculture practise residual OPO concentration should be monitored in regular time intervals in order to notice when the saturation capacity of the carbon is reached.

#### *Removal efficiency of different activated carbon types*

A further factor that has impact on the removal of oxidants by activated carbon filtration is the type of carbon used. While free chlorine, free bromine and bromamines were removed well by granular activated carbon on coal basis (AC 1), low removability of chloramines was tried to improve by testing two discriminative carbon types additionally. A higher removal efficiency compared to AC 1 was expected particularly for granular activated carbon on coconut shell basis (AC 2), as this carbon type possessed a higher surface area while having the same small particle size as AC 1. However, removal of chloramines was even lower using AC 2, although differences in removal efficiency were not significant. Extruded activated carbon for aquaria use (AC 3) showed by far the least removal capacity for chloramines. Unlike for AC 1 and AC 2 statistical analyses revealed no significant effect in chloramine removal for this type of carbon compared to control experiments without activated carbon filtration.

No further improvement of chloramine removal was accomplished with AC 2 and AC 3, as AC 1 on coal basis actually showed best removal performance for chloramines among all three carbon types, although having the smallest active surface area of tested carbons. As AC 3 exceeds AC 1 in its active surface area, low removal performance of AC 3 has to be attributed to its greater particle size, resulting in a reduced accessibility of active surface area.

Results revealed particle size of activated carbon to be an important parameter affecting the removal capacity. A small particle size of activated carbon provides a better access to the surface area and therefore a faster rate of oxidant reduction.

If the exclusive aim of activated carbon filtration is the removal of prominent OPO, costs can be minimized by using conventional granulated activated carbon on coal basis, as AC 1 represented the cheapest but most effective carbon for oxidant removal tested in the present study.

### Conclusion

In conclusion, activated carbon filtration may be characterized as suitable for the elimination of the main OPO, free bromine and bromamines, in natural and even most artificial seawaters. However, using artificial seawater formulae excluding bromide-ions might result in a limited OPO removal rate due to the lower removability of produced chloramines by activated carbon filtration. The reduced efficiency of activated carbon filtration for chloramine removal should be taken into considerations when designing seawater formulae and ozonation strategies. Furthermore, ozonation as well as oxidant removal strategies have to be adapted to the respective oxidant composition of the ozonated water in order to assure an optimized management of residual OPO. Conventional granulated activated carbon on coal basis was proven to be a cost-effective carbon for removing persistent OPO as it possessed highest removal capacity for chloramines while being the cheapest of the carbons tested.

### Acknowledgements

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## CHAPTER IV

Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems.

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### Abstract

The high levels of water-reuse in intensive recirculating aquaculture systems (RAS) require an effective water treatment in order to maintain good water quality. In order to reveal the potential and limitations of ozonation for water quality improvement in marine RAS, we tested ozone's ability to remove nitrite, ammonia, yellow substances and total bacterial biomass in seawater, considering aspects such as efficiency, pH-dependency as well as the formation of toxic ozone-produced oxidants (OPO). Our results demonstrate that ozone can be efficiently utilized to simultaneously remove nitrite and yellow substances from process water in RAS without risking the formation of toxic OPO concentrations. Contemporaneously, an effective reduction of bacterial biomass was achieved by ozonation in combination with foam fractionation. In contrast, ammonia is not oxidized by ozone so long as nitrite and yellow substances are present in the water, as the dominant reaction of the ozone-based ammonia-oxidation in seawater requires the previous formation of OPO as intermediates. The oxidation of ammonia in seawater by ozone is basically a bromide-catalyzed reaction with nitrogen gas as end product, enabling an almost complete removal of ammonia-nitrogen from the aquaculture system. Results further show that pH has no effect on the ozone-based ammonia oxidation in seawater. Unlike in freshwater, an effective removal of ammonia even at pH-values as low as 6.5 has been shown to be feasible in seawater. However, as the predominant reaction pathway involves an initial accumulation of OPO to toxic amounts, we consider the ozone-based removal of ammonia in marine RAS as risky for animal health and economically unviable.

## Introduction

In recirculating aquaculture systems (RAS), high levels of water re-use cause an accumulation of toxic metabolites and dissolved organics in the process water.

Due to their high toxicity to fish, the accumulation of ammonia and nitrite to harmful concentrations is one of the most notorious problems in RAS (Hargreaves, 1998) and removal of those nitrogenous compounds is of high importance. Biological nitrification has been established in RAS as the most reliable method for the removal of ammonia and nitrite. However, biological nitrification beds are subject to great fluctuations in efficiency, as nitrifying bacteria in biofilter beds are sensitive to environmental perturbations and changes in operating conditions (Graham et al., 2007). Besides nitrogenous compounds several non-biodegradable organics accumulate in closed RAS, which often implicate colour and odour problems and moreover impair biofilter function. Among those dissolved organics particularly the colour causing yellow substances are highly resistant against bacterial degradation and rapidly accumulate in RAS, often resulting in a significant reduction of water clarity.

For safeguarding high water quality, a chemical water treatment is often required, that counteracts the accumulation of non-biodegradable organics as well as supplements biological nitrification at peak loads or during biofilter dysfunction.

Ozone, generally known as an effective disinfection agent, has been proven to efficiently improve water quality in RAS (Summerfelt and Hochheimer, 1997; Summerfelt, 2003; Tango and Gagnon, 2003).

Besides its germicidal action, ozone oxidizes several dissolved organics by effectively breaking up their carbon double bonds. Various studies have determined the rate constant of reactions of ozone with different organic compounds in water (Hoigne and Bader, 1983; Colberg and Lingg, 1978; Takahashi, 1990), revealing some organic species to be more reactive with respect to oxidation by ozone than others. In particular, the non-biodegradable yellow substances are efficiently oxidized by ozone resulting in a significant improvement of water clarity (Otte et al., 1977).

Furthermore, ozonation has been proven to be highly effective for the removal of nitrite (Colberg and Lingg, 1978; Rosenthal and Otte, 1979). With a rate constant of  $3.7 \times 10^5$  M/s, ozone reacts almost instantaneously with nitrite to nitrate (Hoigne et al., 1985; Lin and Wu, 1996).

In contrast, ozone's ability to remove ammonia has been controversially discussed in previous studies due to the complexity of existing reaction pathways (Singer and Zilli, 1975; Colberg and Lingg, 1978; Lin and Wu, 1996; Krumins et al., 2001).

During ozonation of freshwater, ammonia is oxidized by ozone to nitrate, but the rate of this reaction is very slow ( $k = 5 \text{ M/s}$ ). Besides, the direct oxidation of ammonia by ozone is strongly pH-dependent and proceeds only in an alkaline medium ( $\text{pH} > 8$ ) (Singer and Zilli, 1975; Lin and Wu, 1996).

In bromide-containing seawater, oxidation of ammonia by ozone is reported to be enhanced (Haag et al., 1984; Kobayashi et al., 1993; Tanaka and Matsumura, 2003). As the direct oxidation reaction of ammonia to nitrate by ozone is negligible at pH-values typical for aquacultural seawater, in particular the occurrence of two additional reaction pathways is suggested to contribute mainly to the improved removal of ammonia by ozonation in seawater. Both reaction pathways are based on the oxidation of ammonia by brominated ozone-produced oxidants (OPO) as intermediates:

(1) During ozonation of seawater, ozone reacts with bromide ions to form free bromine ( $\text{HOBr} / \text{OBr}^-$ ) (Williams et al., 1978; Crecelius, 1979) which in turn rapidly reacts with ammonia to form monobromamine ( $\text{NH}_2\text{Br}$ ) (Wajon and Morris, 1979). The monobromamine is slowly oxidized by ozone to nitrate and bromide (Haag et al., 1984). However, with a rate constant of  $40 \text{ M/s}$  the conversion of ammonia to nitrate via the  $\text{NH}_2\text{Br}$  intermediate is relatively slow (Haag et al., 1984).

(2) Dependent on the free bromine / ammonia ratio and pH, in addition to monobromamine two further species of bromamines might be formed by the stepwise addition of HOBr to ammonia: dibromamine ( $\text{NHBr}_2$ ), and tribromamine ( $\text{NBr}_3$ ) (Hofmann and Andrews, 2001). A fast breakpoint reaction between dibromamine and tribromamine may account for a very rapid bromamine decay, leading to the conversion of ammonia into nitrogen gas (Haag et al., 1984; Yang et al., 1999; Tanaka and Matsumura, 2002; Tanaka and Matsumura, 2003).

Both reaction pathways are catalyzed by the bromide-ion in which bromamines serve as intermediates in a cyclic process where ammonia is oxidized to nitrate or nitrogen gas, respectively.

As those oxidation processes have mostly been described for waste water treatment, serious gaps remain in the basic understanding of ozone-based ammonia oxidation under marine aquaculture conditions.

Besides ozone's benefits for water quality improvement, toxicity of ozone and its by-products is of extreme importance in aquaculture. As OPO have been proven to be harmful to different marine aquaculture species (Richardson et al., 1983; Chapter I and II), the accumulation of oxidative intermediates to high amounts during ozone treatment is not tolerable in aquaculture practice.

In order to assess ozone's potential and limitations for water quality improvement in marine RAS, ozone's efficiency in removing yellow substances, nitrite, ammonia and total bacterial biomass has been comparatively tested in the present study, considering relevant aspects such as reaction preferences and the formation of toxic OPO.

In particular we evaluated ozone's suitability to remove ammonia in seawater by investigating the dominating reaction pathways and end-products, the effect of pH on removal efficiency as well as the formation of harmful OPO as intermediates.

## Material and methods

Three different experiments were conducted at 27°C in a temperature controlled lab:

In experiment A, an initial evaluation on ozone's efficiency in reducing yellow substances, nitrite, ammonia, and total bacterial biomass was conducted under aquaculture conditions.

Based on the results of experiment A, in experiment B and C more detailed investigations concerning the ozone-based ammonia removal in seawater were performed. While the effect of pH on the efficiency of the ozone-based ammonia oxidation was investigated in experiment B, the amount of OPO required for an effective ammonia removal was determined in experiment C.

### *Experiment A*

Short-term ozonation over a period of 10 h was performed at a constant ozone dose of 250 mg O<sub>3</sub>/h in an experimental recirculation system stocked with juvenile Pacific white shrimp (*Litopenaeus vannamei*) in which metabolites and organic substances had accumulated.

The experimental recirculation system consisted of a 200 l fiberglass tank, filled with approximately 150 l of filtered seawater, a biofilter and a foam fractionator each operating in by-pass. During the ozonation experiment the biofiltration unit was not operating. In order to ensure an appropriate accumulation of significant amounts of ammonia and nitrite, biofiltration was already restrained from water treatment 5 days prior to the experiment.

Ozone gas was produced by an electrical discharge ozonator (Model C 300, Erwin Sander Elektroapparatebau GmbH) using compressed air. Before short-term ozonation was started, the applied ozone dose was set to 250 mg O<sub>3</sub>/h by monitoring ozone concentration in the gas stream and adjusting the ozone generator's output accordingly. The gas flow was held constant at 67 l/h by using a gas flowmeter (Vögtlin Instruments). Seawater was ozonated by ozone-enriched air passing through a porous lime stone diffuser at the bottom of the foam fractionator (Model 1 AH 1100, Erwin Sander Elektroapparatebau GmbH), which served as a contact chamber between water and gaseous ozone. Recirculating water entered the foam fractionator at the top and flowed downward - past the uprising bubbles - creating a counter current exchanger which maximized diffusion of ozone into the water. The retention time in the foam fractionator was set to 50 sec by adjusting the water flow rate to 600 l/h.

During short-term ozonation analysis of the following water quality parameters was carried out on an hourly basis: nitrite-N, nitrate-N, total ammonia-N, OPO and oxidation reduction potential (ORP). The degradation of yellow substances was continuously monitored by on-line UV spectrophotometry. Temperature, salinity and pH were measured prior to and after the 10 h ozonation period and were found to be constant at 27.3°C, 17.6 ppt and 7.3, respectively. Reduction of viable bacteria during short-term ozonation was determined by analysing total viable cell counts prior to and after 8.5 hours of continuous ozonation by applying the spread plate method on TSB 3 agar (15 g agar, 3 g tryptic soy broth (Difco), 10 g NaCl in 1 L of deionized water), supplemented with 1% NaCl. Serial dilutions of each water sample were plated in triplicates. Colony forming units (CFU) per plate were recorded after 48 h of incubation at room temperature. Survival rates of bacteria were calculated from the number of colonies grown.

#### *Experiment B*

To investigate the effect of pH on the ozone-based ammonia-oxidation in seawater, ammonia test solutions were ozonated at different pH-values (6.5, 7.5, 8.5) in a small-scale batch reactor. Therefore, a small foam fractionator (Model 1 AH 700, Erwin Sander Elektroapparatebau GmbH) was used as ozonation column.

Ammonia test solutions with an initial concentration of approximately 1.8 mg/l total ammonia-N were prepared by dissolving the required amounts of ammonium chloride (p.a.) in filtered natural seawater. For each experimental run a 4 l volume of ammonia test

solution was buffered by a 5 mM phosphate buffer system, adjusted to the desired pH using analytical grade sodium hydroxide (NaOH) or hydrochloric acid (HCl) and finally filled in the ozonation column. After each experimental run, the test solution was exchanged.

Ozone gas was produced by an electrical discharge ozonator (Model C 200, Erwin Sander Elektroapparatebau GmbH) using compressed air and injected into the test solution through a porous lime stone diffuser at the bottom of the foam fractionator, which served as a contact chamber between water and gaseous ozone.

For each pH-value (6.5, 7.5, 8.5) ozonation experiments were performed with ozone doses of 65, 90 and 122 mg O<sub>3</sub>/h, respectively. Prior to each experimental run the applied ozone dose was adjusted to the desired level by measuring ozone concentration in the gas stream and regulating ozone generator's output accordingly. The gas flow was held constant at 32 l/h by using a gas flow meter (Vögtlin Instruments). Ozonation was stopped after all ammonia-nitrogen had been oxidized.

During ozonation total ammonia-N, nitrate-N and the amount of OPO were continuously monitored spectrophotometrically.

#### *Experiment C*

To determine the required amount of OPO for an effective ammonia removal, ammonia test solutions were treated continuously for 154 h with different constant OPO concentrations ( $\pm$  SD) of 0.1 ( $\pm$  0.02), 0.3 ( $\pm$  0.03), 0.6 ( $\pm$  0.09) and 1.0 ( $\pm$  0.30) mg/l in four experimental recirculation systems, identical to the one used for experiment A. Two additional experimental systems without an ozonation unit were used as control, in order to quantify natural decay of ammonia in untreated natural and artificial seawater. Biofiltration units of experimental systems were not operating during the experiment.

Ammonia test solutions with an initial concentration of approximately 1.8 mg/l total ammonia-N were prepared by dissolving the required amounts of ammonium chloride (p.a.) in 150 l of filtered natural seawater. As additional control, another 150 l of ammonia test solution was made up of artificial seawater, prepared by dissolving artificial sea salt (Instant Ocean) in deionized water. The pH of test solutions was adjusted to 8.3.

After transferring ammonia test solutions to the experimental recirculation systems, the initial concentrations of total ammonia-N were measured and ozonation was started.

Ozone-enriched air was injected into the foam fractionators. Considering the effect of ammonia stripping during foam fractionation, compressed air was injected into the foam fractionators of control tanks and gas flow rates were set equal among all experimental systems and held constant using a flow meter (Vögtlin Instruments).

The retention time in the foam fractionator was set to 50 sec by adjusting the water flow rate to 600 l/h. Ozone-supply was controlled and regulated automatically by linking a redox potential controller (Erwin Sander Elektroapparatebau GmbH) to each ozone generator. Desired OPO concentrations were maintained by monitoring oxidant concentrations spectrophotometrically and adjusting redox potential set points if necessary.

During the experiment total ammonia-N was determined spectrophotometrically in regular time intervals.

### *Analysis*

OPO were measured spectrophotometrically by a DR/2800 Spectrophotometer (Hach Lange GmbH) as equivalent total residual chlorine (TRC) using the colorimetric *N,N*-diethyl-*p*-phenylenediamine (DPD) method (DPD Total Chlorine powder pillows, Hach) as recommended for measurement of total residual oxidants (TRO) in seawater (Buchan et al., 2005).

For the spectrophotometrical measurement of total ammonia-N, nitrite-N and nitrate-N, the Ammonia Salicylate Method, Diazotization Method and Cadmium Reduction Method were applied, respectively, using the DR/2800 photometer (Hach Lange GmbH) and powder pillow detection kits (Hach). The applied test kits had been proven to be a reliable method for standard analysis in aquaculture (Ormaza-Gonzalez and Villalba-Flor, 1994).

ORP was measured by a redox potential controller (Erwin Sander Elektroapparatebau GmbH). Salinity and temperature were measured by a conductivity meter, model 315i (WTW), pH was determined with a pH meter, type CyberScan pH310 series (Eutech Instruments).

The degradation of yellow substances was monitored continuously during experiment A by on-line UV spectrophotometry using a high-sensitivity miniature fiber optic spectrometer system. Therefore, tank water was continuously pumped via a peristaltic pump (Rainin) to the spectrometer system. Before passing a flow-through cuvette (Ocean Optics), water samples were filtered through 0.2 µm membrane filters in order to remove turbidity. The

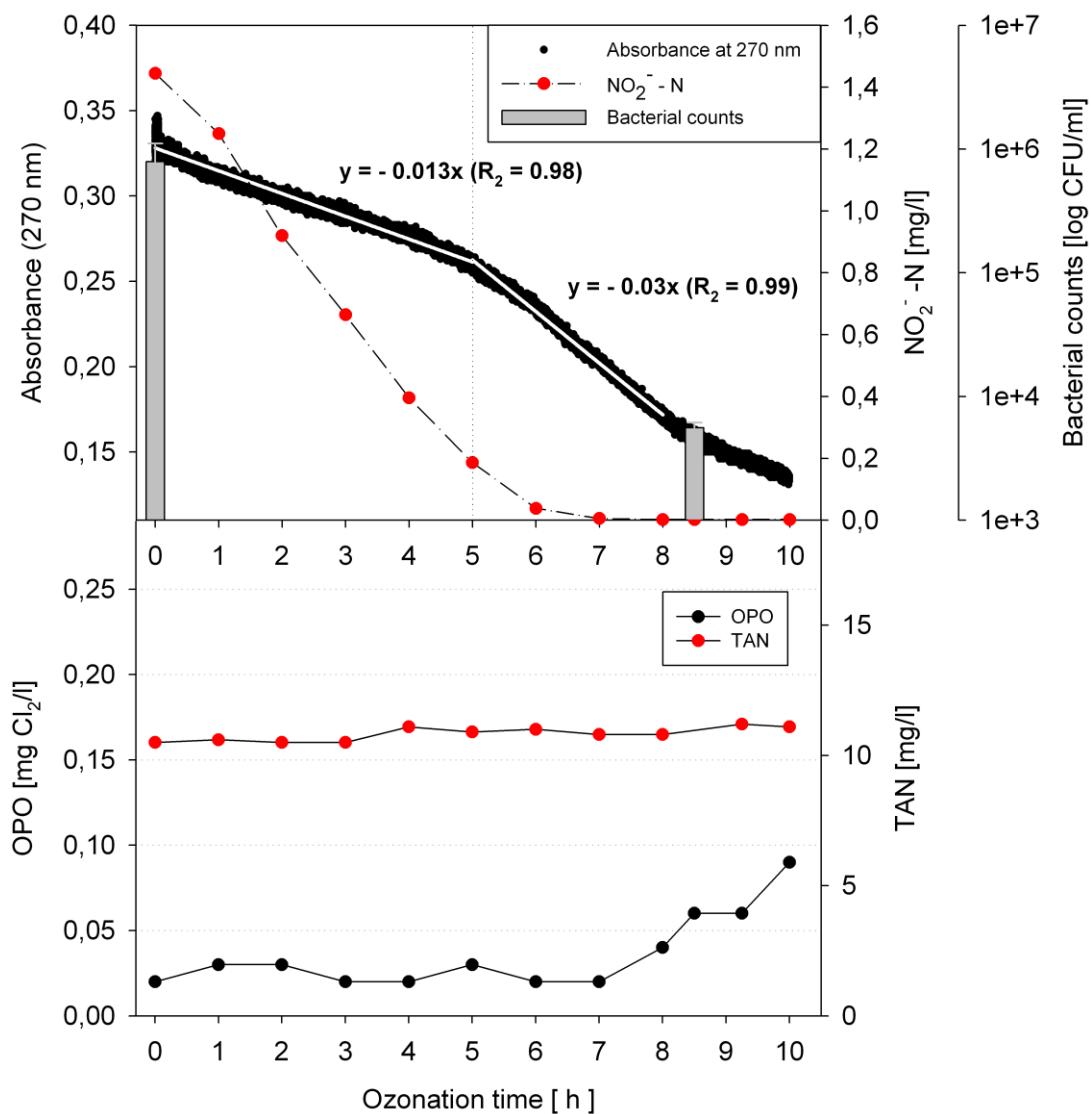
flow through cuvette of 1 cm path length was fixed in a cuvette holder and connected via optical fiber cables to a miniature deuterium tungsten halogen light source (DT-Mini-2-GS, Ocean Optics Inc.) and an USB Fiber Optic Spectrometer (USB2000-UV-VIS spectrometer, Ocean Optics). The light source sends light via an input fiber into the quartz cuvette. The output fiber carries residual light from the sample to the spectrometer connected to the PC. As different organic substances contribute to the yellowish colour, the determination of absolute concentrations is impossible. The degradation of yellow substances was therefore observed by continuously monitoring the decrease in direct UV absorbance of the samples at 270 nm, using filtered seawater as blank, as comparison of absorption spectra of sample and blank identified the absorption peak of yellow substances at the wave length of 270 nm.

Ozone concentration in the gas stream was determined by direct UV absorption measurements at a wavelength of 258 nm using the high-sensitivity miniature fiber optic spectrometer system as described above. Therefore, ozone enriched air was continuously passed through the flow through cuvette and absorbance of UV light was detected at ozone's absorption peak at 258 nm. Ozone-free air was used as blank. For calculation of ozone concentrations a molar absorptivity of  $2950 \text{ M}^{-1} \text{ cm}^{-1}$  was used.

## Results

### *Experiment A*

Short-term ozonation resulted in an effective colour removal. Whereas in the beginning of the experiment water had a strong yellowish colour, after 10 hours of ozonation water had become completely clear. As can be seen from Fig. IV-1, ozonation over a 10 hour period resulted in a continuous decrease of UV absorbance at 270 nm, indicating a rapid oxidation of the colour causing substances. Simultaneous to the decline of yellow substances, the concentration of nitrite-N decreased continuously from 1.45 mg/l to almost zero within 6 hours (Fig. IV-1).



**Fig. IV-1: Effect of a 10 h ozonation period on the amount of yellow substances (indirectly quantified by absorbance measurement at 270 nm), nitrite-N, viable bacterial counts (log CFU/ml), total ammonia-N (TAN) and ozone-produced oxidants (OPO) (measured as chlorine equivalent). Columns and error bars represent means and standard deviations of viable cell counts, respectively.**

Monitoring the concentration of nitrate-N during the ozonation experiment revealed a 100% conversion of nitrite-N to nitrate-N (data not shown). After the complete oxidation of nitrite, the decomposition of yellow substances accelerated significantly in its reaction rate. At the applied ozone dose of 250 mg/h and a retention time of 50 sec, a reduction of yellow substances by 4 % of the initial concentration was reached within 1 h under the presence of nitrite. In contrast, reduction of yellow substances increased to 9 % per hour since nitrite

was absent in the water. At the end of the degradation curve, oxidation of yellow substances was slowed down.

Total ammonia-N did not change significantly during the whole experimental period.

Within 8.5 hours of ozonation, total viable cell counts were reduced from  $79 \times 10^4$  CFU/ml to  $56 \times 10^2$  CFU/ml, a reduction of more than 99 %. Mean viable cell counts prior and after 8.5 h of ozonation are shown in Fig. IV-1. Furthermore, an increased foam production in the foam fractionator could be observed as soon as ozonation was started.

At the applied retention time of 50 sec in the foam fractionator, the formation of OPO to significant amounts was delayed as long as nitrite and yellow substances were still present in the ozonated water.

During the first 7 hours of ozonation, formation of OPO was negligible resulting in OPO concentrations  $\leq 0.03$  mg/l. After the complete removal of nitrite and a strong increase in water clarity, OPO increased slowly to 0.06 mg/l during the last 3 hours of ozonation, finally reaching a concentration of 0.09 mg/l at the end of the experiment (Fig. IV-1). The ORP was raised exponentially with decreasing concentration of nitrite and yellow substances but remained below critical values ( $< 350$  mV) until all nitrite and most of the yellow substances were removed (data not shown).

### *Experiment B*

In small scale batch experiments total ammonia-N was effectively oxidized by ozone.

Removal rates of total ammonia-N increased with increasing ozone supply. In contrast, no differences in the removal rates of total ammonia-N were observed among the different pH-values tested (6.5, 7.5, 8.5) (Fig. IV-2).

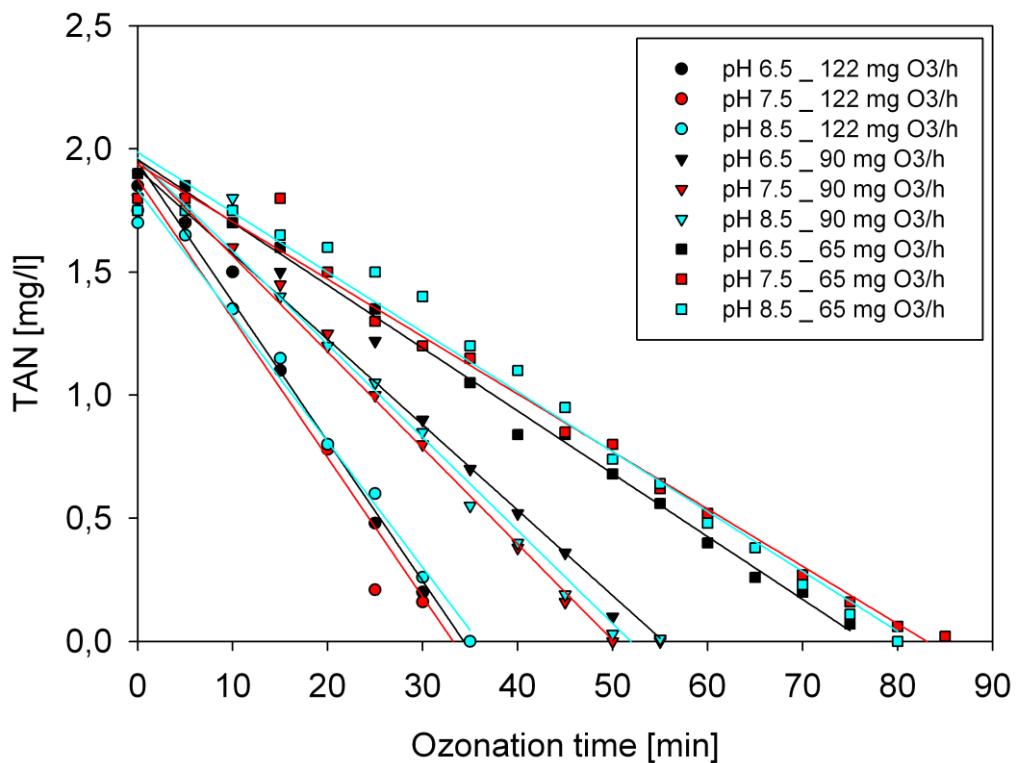


Fig. IV-2: Effect of pH and ozone dosage on the removal of total ammonia-N (TAN) during ozonation of seawater. Black: pH 6.5, red: pH 7.5, blue: pH 8.5; circles: 122 mg/h ozone, triangle down: 90 mg/h ozone, squares: 65 mg/h ozone.

During continuous ozonation, changes in concentrations of total ammonia-N and OPO proceeded in the same following manner at all pH-values tested:

Ozonation of ammonia test solutions resulted in an almost constant decrease of total ammonia-N along with a fast initial increase in OPO, followed by a short plateau phase fading to a sudden decrease in OPO. After complete removal of total ammonia-N, OPO started to rapidly increase again (Fig. IV-3 a, b).

A continuous measurement of nitrate-N detected a slow but almost constant increase in nitrate-N concentration along with the constant decrease of total ammonia-N during ozonation.

Like removal rates of total ammonia-N, formation of nitrate-N showed no differences among discriminative pH-values. However, during the complete removal of 1.8 mg/l total ammonia-N via ozonation only 0.3 mg/l of nitrate-N was produced, which is less than 17% of the initial concentration of total ammonia-N (Fig. IV-3 a).

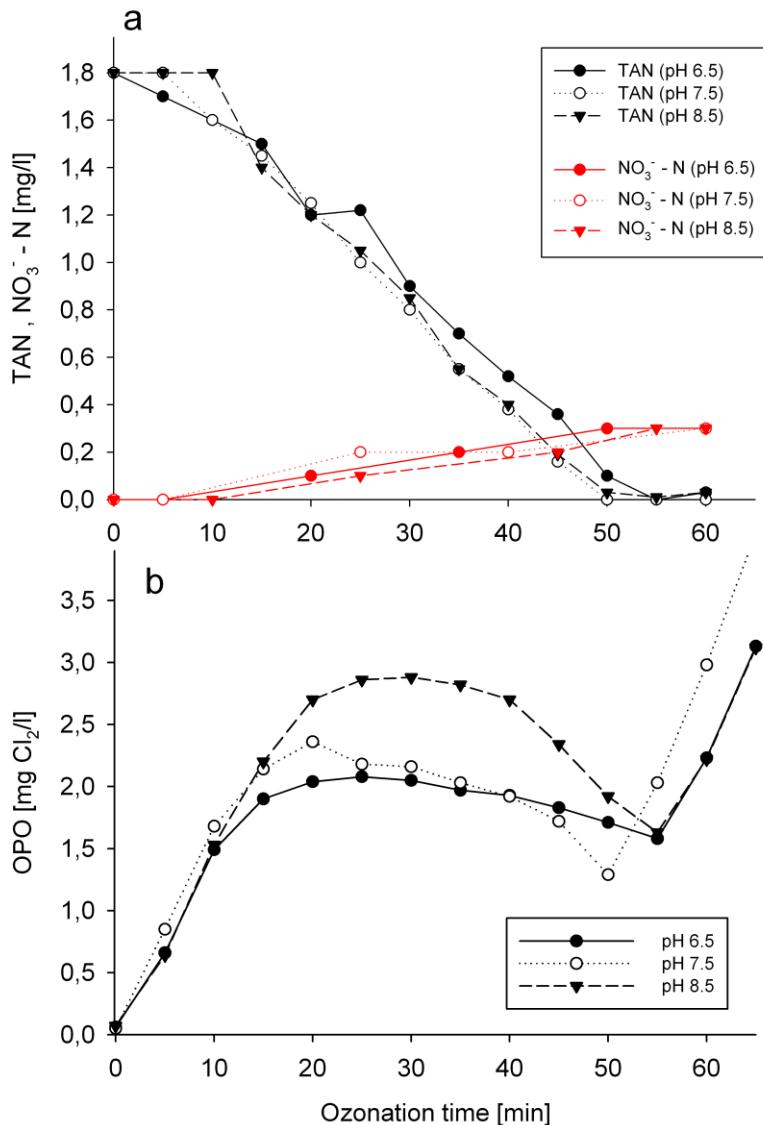


Fig. IV-3: Ammonia removal in seawater by ozonation (90 mg/h ozone) at three different pH values (filled circles: pH 6.5, empty circles: pH 7.5, triangles down: pH 8.5). (a) Concentration of nitrogen compounds: black: total ammonia-N (TAN), red: nitrate-N. (b) Concentration of ozone-produced oxidants (OPO) (measured as chlorine equivalent).

### Experiment C

At all OPO treatments applied, total ammonia-N concentrations decreased with time during ozonation. Removal rate of total ammonia-N was positive correlated to the amount of OPO. At constant OPO concentrations of 0.10, 0.30, 0.60 and 1.00 mg/l, the time required to completely remove the initial amount of 1.8 mg/l total ammonia-N was >154, 154, 80 and 47 h, respectively. The degradation rate of total ammonia-N during control runs without ozonation differed strongly between the two discriminative controls. Whereas in artificial seawater total ammonia-N decreased only little from its initial concentration of 1.8 to 1.6

mg/l within the whole experimental period of 154 h, in natural seawater a complete degradation of the initial concentration of total ammonia-N (1.9 mg/l) occurred in only 110 hours. Hence, the decrease of total ammonia-N in natural seawater without ozonation was faster than the removal rate at a continuous treatment with OPO concentrations of 0.10 and even 0.30 mg/l (Fig. IV-4). An OPO concentration of  $\geq 0.60$  mg/l was required to exceed the loss of ammonia in the natural seawater control.

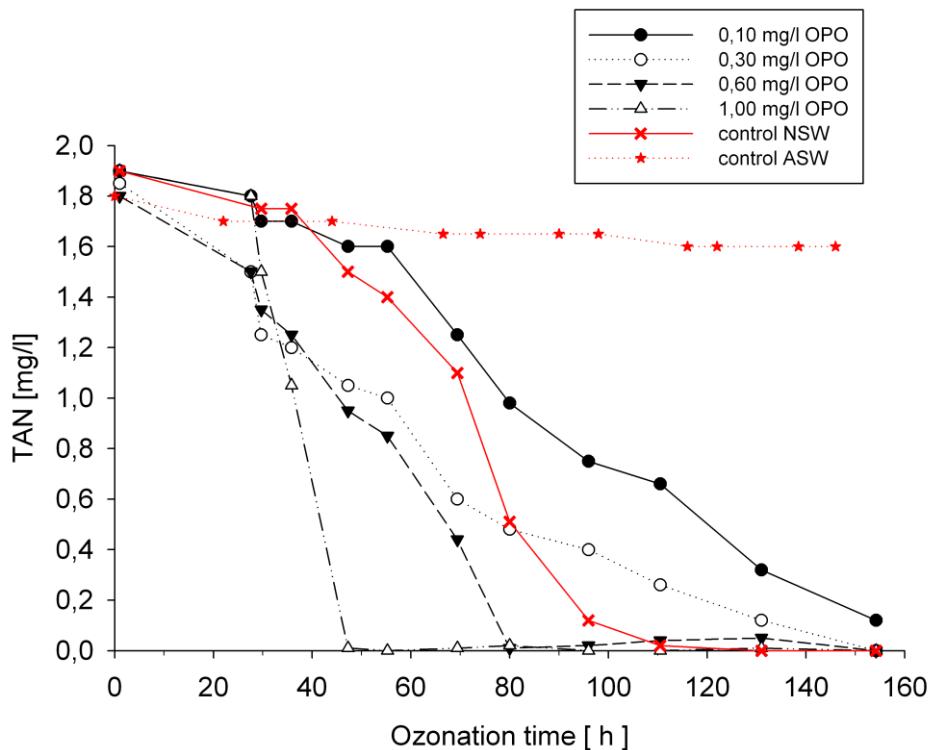


Fig. IV-4: Removal rates of total ammonia-N (TAN) during 154 h of continuous treatment with different concentrations of ozone-produced oxidants (OPO). Red lines demonstrate ammonia degradation at control treatments without ozonation. X: Natural seawater control; stars: Artificial seawater control.

## Discussion

### Removal of nitrite and yellow substances

Results of the present study demonstrate ozone's high potential to efficiently counteract the accumulation of nitrite and yellow substances in intensive RAS. Removal of non-biodegradable yellow substances was reflected in a fast improvement of water clarity, detectable by monitoring the decrease in absorbance at 270 nm in the process water. Furthermore, toxic nitrite was rapidly oxidized by ozone to nitrate, which is tolerable to most aquatic organisms in high concentrations (Bromage et al., 1988).

The suitability of ozonation for the removal of yellow substances and nitrite has been already demonstrated in previous research (Otte et al., 1977; Colberg and Lingg, 1978). However, until now, not much is known about preferences and interactions of corresponding oxidation reactions responsible for the ozone-based removal of nitrite and yellow substances. Our results emphasize the potential of ozonation to remove yellow substances and nitrite simultaneously, as rapid oxidation of both substances occurred at the same time. Moreover, results revealed a competition between nitrite and yellow substances for the reaction with ozone, resulting in a reduced removal rate of yellow substances in the presence of nitrite.

Whereas the removal of yellow substances by ozonation is highly desirable, as it is the only way to counteract the accumulation of those non-biodegradable organics, a continuous nitrite oxidation by ozonation competes with the process of biofiltration, resulting in a reduced efficiency of biofilter performance. Due to the competition of nitrite with yellow substances for the reaction with ozone, a continuous application of ozone might be economically questionable. However, a rapid nitrite oxidation via short-term ozonation is highly beneficial to temporarily support the second step of nitrification in a biofilter, as this step is particularly sensitive to changes in environmental conditions such as pH, organic load and substrate concentration (Krüner and Rosenthal, 1983), and tends to vary in its efficiency. Although there is evidence for continuous ozonation as best practice to sustain stable water quality (Brazil et al., 1996), batch ozonation can be timed when demand is highest, avoiding redundancy of water treatment units and maximizing efficiency of ozonation for water quality improvement.

Until now, not much is known about the potential formation of toxic OPO during the ozone-based removal of nitrite and yellow substances, as most of the previous studies quantified or calculated the ozone generator's output but didn't measure OPO in the treated water.

Our results demonstrate that at high flow rates formation of OPO is negligible as long as significant amounts of nitrite and yellow substances are present in the process water. With a rate constant of 160 M/s for the oxidation of bromide-ion by ozone (Haag and Hoigne, 1983), free bromine ( $\text{HOBr} / \text{OBr}^-$ ) has highest formation potential among OPO in seawater. However, with a reaction rate of  $3.7 \times 10^5$  M/s (Hoigne et al., 1985; Lin and Wu, 1996) nitrite reacts much faster with ozone, outcompeting the bromide-ion for the reaction with ozone. Our results further revealed a higher chemical affinity of ozone for the reaction with yellow

substances than with bromide-ions, as a significant amount of OPO only formed after almost all yellow substances had been removed. Until then, OPO did not exceed a concentration of 0.06 mg/l, which has been proven to be non-hazardous for different marine aquaculture species (Chapter I and II).

Although tolerable to various aquaculture species, previous studies reported OPO levels as low as 0.06 mg/l to be able to effectively inactivate bacteria in seawater (Sugita et al., 1992; Chapter I). In the present study, a 100-fold decline (99%) in viable cell counts was reached after 8.5 hours of ozonation, proving moderate ozonation resulting in OPO concentrations  $\leq$  0.06 mg/l to be very efficient in reducing total bacterial biomass even at short-term ozonation. As Kobayashi et al. (1993) found highly elevated bacterial counts in the discharged foam of foam fractionators compared to bacterial counts in recirculating water, it might be suggested that, besides ozone's bactericidal effect, the observed improvement in skimming performance of the foam fractionator due to ozonation might have contributed to the reduction of total viable cell counts.

#### *Removal of ammonia*

The ammonia concentration did not decrease during the whole ozonation period of experiment A. Hence, it can be suggested that ammonia is not oxidized by ozone as long as nitrite or yellow substances are available. This observation is supported by the study of Rosenthal and Krüner (1985) in which ammonia was oxidized with an initial delay as long as nitrite and dissolved organics were present in the system. As the bromide-catalyzed ammonia oxidation, in which free bromine and bromamines serve as intermediates, represents the main reaction pathway in seawater, formation rates of those intermediates dictate the removal rate of ammonia in seawater. As ozone's preference for the reaction with nitrite and yellow substances inhibits the formation of brominated oxidants, ammonia-oxidation by ozonation is negligible as long as those ozone-demanding substances are available for the reaction with ozone. Hence, due to high flow rates the low retention time in the ozonation column (foam fractionator) of experiment A prevented a complete removal of nitrite and yellow substances from the ozonation column, avoiding a subsequent accumulation of brominated oxidants and with it the potential of an ozone-based ammonia oxidation. However, extending the retention time by reducing the flow rate might cause a complete removal of nitrite and yellow substances from the ozonation column, resulting in a

subsequent formation of significant amounts of brominated oxidants potentially enabling an ozone-based ammonia oxidation.

In order to assess ozone's suitability to remove ammonia at marine aquaculture conditions, subsequent experiments were performed investigating the preconditions for an effective ammonia removal via ozonation.

Results of experiment B indicate that ozone-based ammonia oxidation in aquacultural seawater is independent of pH, as ammonia removal rates varied only among different ozone dosages, but were identical over the tested range of pH values (6.5, 7.5, 8.5). The pH in RAS is often lower than natural surface seawater pH due to biological processes and moreover fluctuates depending upon environmental conditions. Unlike the direct ozonation of ammonia in freshwater, the bromide-catalyzed process in seawater was shown not to be decelerated at pH values as low as 6.5. Hence, ammonia removal by ozonation is achieved much more readily in marine applications, without the necessity of pH adjustment.

A nitrogen mass balance analysis indicated that less than 17% of the total ammonia-N added to the test solutions was converted into nitrate-N, suggesting ammonia to be primarily oxidized to nitrogen gas rather than being oxidized to nitrate. Together with the characteristic run of OPO concentration typically observed during breakpoint bromination, the strong discrepancy in ammonia-nitrate mass balance reveals the bromine-ammonia breakpoint reaction to be the dominant reaction pathway for the tested pH-range (6.5 – 8.5). This breakpoint reaction converts total ammonia-N to nitrogen gas via ozone-produced intermediates. Although nitrate is far less toxic to most aquatic organisms than ammonia and nitrite (Bromage et al., 1988), it rapidly accumulates in RAS to very high concentrations and has to be controlled by partial water exchange. To allow high levels of water re-use, a complete nitrogen removal via the conversion of total ammonia-N to the end-product nitrogen gas is desirable for RAS. Unlike biological nitrogen-removal, which strongly depends on environmental conditions and normally requires various reactors for different biological reactions (nitrification – denitrification), ozonation of seawater has been proven in the present study to decompose total ammonia-N to nitrogen gas in a single ozonation reactor (foam fractionator).

However, as the amount of intermediate OPO increased to highly toxic concentrations ( $> 1.0 \text{ mg/l}$ ) during ammonia removal at all tested pH-values, an additional experiment was performed in which ammonia test solutions were continuously treated with different

constant OPO concentrations (experiment C), in order to determine the minimum OPO concentration needed for an effective ammonia-removal. Although ammonia removal rates increased with increasing OPO concentration, results further indicate that high OPO concentrations of  $\geq 0.6$  mg/l are required to exceed ammonia decomposition in natural seawater control. At constant OPO concentrations  $\leq 0.30$  mg/l removal rates of ammonia were slower compared to the ammonia-loss in natural seawater without ozonation. The loss of ammonia in natural seawater without ozonation is suggested to result from bacterial degradation, as air-stripping considerably affects the rate of ammonia-removal only at pH-values higher than 8.5 (Tanaka and Matsumura, 2002). This assumption is supported by the small loss of ammonia in the artificial seawater control, as bacterial biomass is considered to be much lower in freshly mixed artificial seawater based on deionized water than in natural seawater. It might be suggested, that OPO concentrations  $\leq 0.30$  mg/l are not effective for an adequate ammonia oxidation, but high enough to significantly reduce the amount of nitrifying bacteria in the water, resulting in a decreased ammonia-loss due to bacterial degradation. In contrast, to achieve a higher ammonia removal compared to the ammonia-loss due to biological degradation, an OPO concentration of  $\geq 0.60$  mg/l is needed. This concentration is highly toxic to aquatic life and has to be avoided in aquaculture practice. Hence, the formation of high OPO concentrations required for an effective ammonia removal restricts the application of strong ozonation for ammonia removal in aquaculture. Although it is possible to effectively remove OPO by activated carbon filtration (Ozawa et al., 1991; Chapter III), strong ozonation remains risky and is economically questionable.

In conclusion, ozone can be efficiently utilized to simultaneously remove nitrite and yellow substances from recirculating water in RAS without risking the formation of toxic concentrations of OPO when retention time in the ozone reactor is low. Due to the competition between ozonation and biofiltration for nitrite oxidation, batch ozonation at times when demand is highest is recommendable in order to avoid redundancy of water treatment units. Although ammonia oxidation in seawater by ozonation is pH-independent and enables the complete removal of total ammonia-N from the aquaculture system with nitrogen gas as the primary end product, it presupposes an initial accumulation of OPO to highly toxic concentrations, restricting a safe application in aquaculture.

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## GENERAL DISCUSSION

In the past, several authors have reported ozone to be an effective oxidizing agent for disinfection and improvement of water quality in recirculating aquaculture systems (RAS). However, the toxicity of ozone and its by-products is also well-known and dreaded by many aquaculturists. Until now, both, ozone's potential for water treatment as well as its limitations due to toxicity have so far been mostly investigated in an isolated context. One of the innovative approaches of this thesis is the combined consideration of ozone's effectiveness in water treatment and the toxicity of its by-products. This study aims to add knowledge on several aspects of ozonation in marine RAS and its influence on water quality and fish health, in order to contribute to the development of a reasonable and harmless ozonation strategy for marine RAS.

In order to determine thresholds for a safe ozone application in marine RAS, the toxicity of ozone-produced oxidants (OPO) to two different marine aquaculture species, a shrimp species (*Litopenaeus vannamei*) and a fish species (*Psetta maxima*), was investigated in the first two sections (**Chapter I and II**) of this thesis. Previous studies reported a higher tolerance to OPO of crustaceans compared to fish (Reid and Arnold, 1994; Jiang et al., 2001). However, results of the present studies revealed that, despite their strong differences in biology, the investigated species show a similar sensitivity towards OPO at least when exposed for a long-term period. Considering the deleterious impact of chronic exposure to OPO concentrations  $\geq 0.10$  mg/l, sensitivity of *L. vannamei* to OPO was found to be much higher than expected for a crustacean species. As the susceptibility of crustaceans is increased during and immediately after molting due to the temporary absence of chitinous protection layers, an exposure period long enough to ensure the coverage of different molt stages, is recommendable. The limitation of exposure time to  $\leq 48$  h in previous studies might be one reason for the overestimation of crustacean's tolerance towards OPO in the past.

In contrast, the sensitivity of turbot (*P. maxima*) was found to be lower than expected. Compared to the findings of Richardson et al. (1983), juvenile turbot was proven to be less sensitive towards OPO than adult white perch (*Morone americana*). Behavioural and histological alterations observed in turbot were less severe compared to those in white perch, suggesting a higher resistance of turbot, potentially due to the akinetic lifestyle of this

benthic flatfish species. Since the tolerance towards ozone and its by-products seems to be species-dependent, the maximum safe exposure level has to be determined for each species of interest.

Results of the here presented toxicity studies revealed a surprisingly similar tolerance of both investigated species towards OPO, even resulting in the same threshold for a safe OPO exposure. Whereas OPO concentrations of 0.10 mg/l and 0.15 mg/l, as they may arise during routine operation of RAS, were shown to have deleterious impacts on both, *L. vannamei* and *P. maxima*, a long-term exposure to an OPO concentration of 0.06 mg/l revealed no observable effect in both species. Hence, a maximum safe exposure level of 0.06 mg/l for the rearing of juvenile turbot and Pacific white shrimp can be recommended. OPO concentrations within tanks should not exceed this safe level in order to safeguard animal welfare.

Since the use of very low residual OPO concentrations (0.06 mg/l) was proven to provide a satisfactory reduction in bacterial loads (**Chapter I**), adequate microbiological control is still feasible even under safe OPO levels. However, if an exhaustive disinfection is the primary goal of ozonation, high ozone dosages are necessary in order to provide sufficient amounts of residual OPO, enabling higher contact times for an exhaustive germicidal action. Due to their stability, residual OPO may reach cultivation tanks and become harmful to the cultivated species. Whereas in freshwater residual ozone degrades rapidly on its way to the cultivation tanks, the persistent OPO formed during ozonation of seawater have to be removed actively.

Results of **Chapter III** revealed activated carbon filtration to be applicable as a de-ozonation unit in marine RAS, as activated carbon could be shown to efficiently remove free bromine and bromamines, the most abundant OPO formed during the ozonation of natural and most artificial seawaters. In contrast, the removability of chloramines, potentially produced during ozonation of bromide-free artificial seawater, by activated carbon filtration was significantly lower. The limited carbon performance for the removal of harmful chloramines should be taken into account when excluding bromide-ions from the artificial seawater formula in order to prevent formation of brominated by-products.

Conventional granulated activated carbon on coal basis in small particle sizes was revealed to be the most cost-effective carbon type for an effective removal of OPO. However, as high organic loadings of typical process water in RAS temporally limits removal capacity of activated carbon, a safer and more economic way to avert adverse effects on cultured species is to reduce formation of OPO to safe levels rather than to remove them after formation.

Therefore, in **Chapter IV** different ozone applications were evaluated for their reasonability and safety, considering the formation of toxic residual OPO with regard to the determined maximum safe exposure level. The most common purpose for the utilization of ozone is its germicidal action providing disinfection of treated process water. Due to the short half-life of reactive ozone in marine aquaculture process water, the more persistent residual OPO are suggested to be primarily responsible for disinfection. As the formation of OPO was shown to be restrained by the ozone-based oxidation of yellow substances and nitrite, the amount of ozone necessary to achieve an exhaustive disinfection might be largely dependent on the presence of those ozone demanding substances in the treated water. However, results of the present study proved that an adequate reduction in bacterial loads (up to 99% reduction) can be achieved even using moderate ozone dosages in combination with foam fractionation, without exceeding the determined safety level for OPO.

Besides the germicidal control, the most useful effect of ozonation is the degradation of dissolved organics, as several organic compounds are non-biodegradable and accumulate to high concentrations resulting in a significant impairment of process water due to colour and taste. The oxidative activity and selectivity of ozone depends on the organic compounds that have to be oxidized. Whereas removal rate of the taste and odour causing compounds by ozonation is low (Westerhoff et al., 2006), removal of colour causing compounds (yellow substances) by ozonation is highly effective (Otte et al., 1977). Results of the present study demonstrate ozone's suitability for an efficient removal of those yellow substances without risking animal health, as a contemporaneous formation of residual OPO is negligible due to the high preference of ozone for the reaction with yellow substances.

In order to assess ozone's potential for a temporary support of biofiltration, ozone's ability to remove ammonia and nitrite was further evaluated. Results proved nitrite to be at least as easy to remove by ozonation as yellow substances, allowing an efficient and simultaneous

removal of both substances without risking the formation of toxic OPO concentrations. Whereas a continuous nitrite oxidation by ozonation is economically questionable, as it competes with the process of biofiltration resulting in a reduced efficiency of biofilter performance, a rapid nitrite oxidation via batch-ozonation can be highly beneficial to temporarily support the second step of nitrification at times of biofilter malfunction.

Unlike the removal of nitrite and yellow substances, the removal of ammonia by ozonation in seawater is more complicated and involves the formation of OPO as intermediates. Results proved that unlike in freshwater, the ozone-based ammonia oxidation in seawater is pH-independent and enables an almost complete conversion of total ammonia-N to nitrogen gas, making this process highly interesting for marine RAS, as it might be able to replace both, biological nitrification and denitrification.

However, as the OPO concentration required for an effective ammonia removal is ten-fold higher than the determined maximum safe exposure level, a safe application in aquaculture seems to be limited. As activated carbon was shown to effectively remove those intermediate OPO (free bromine and bromamines) (**Chapter III**), an ozone-based ammonia removal would be applicable only in combination with an activated carbon filtration. However, as activated carbon performance is temporarily limited depending on the amount of organic substances in the process water, the ozone-based ammonia oxidation in seawater remains risky and economically questionable.

## Conclusion and Outlook

An adequate bacterial reduction as well as an efficient removal of colour causing compounds and nitrite can be achieved by moderate process water ozonation without risking deleterious effects on the cultivated organisms.

In order to avoid redundancy of biofiltration, an intermittent ozone application is preferable and economically recommendable. As a serial batch ozonation was shown to be adequate to temporarily counteract the accumulation of colour causing compounds and reduce total bacterial biomass, a demand-orientated ozonation strategy is economically reasonable. Unlike the removal of nitrite and yellow substances, the ozone-based removal of ammonia in aquacultural seawater is not recommendable, as it involves a high risk for the health of cultivated organisms.

For the rearing of turbot and Pacific white shrimp OPO should not exceed a concentration of 0.06 mg/l in order to avoid deleterious impacts on the cultivated organisms. As a reliable and practical device for an automated control and regulation of OPO in aquaculture routine is still lacking, OPO have yet to be monitored manually in regular time intervals by using photometric DPD test kits.

If ozonation strategy and system design bear the risk of temporarily exceeding the determined threshold, an activated carbon filtration might be installed as safety unit, but should not be used as a permanent removal unit of high OPO concentrations. Moreover, the optimization of ozonation conditions in order to minimize the formation of OPO rather than to remove them after formation should be a focus for future studies.

As several chemical reaction pathways that occur during the ozonation of aquaculture process water are only poorly understood, the chemical processes deserve more attention in future aquaculture research, in order to significantly improve the application of ozone in RAS. With its multidisciplinary approach, the present thesis takes an important step into the right direction, comprehensively considering chemical, biological and technical aspects. While characterizing both, potential and limitations of ozonation in seawater aquaculture, this thesis could contribute to the development of a reasonable and safe ozone application in marine RAS.

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## LIST OF PUBLICATIONS

The chapters of this thesis are partly published (Chapters I and II) or submitted (Chapters III and IV) to peer-reviewed scientific journals with multiple authorship.

### **Chapter I:**

**The toxicity of ozone-produced oxidants to the Pacific white shrimp *Litopenaeus vannamei*.**

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### **Chapter II:**

**Histological and physiological alterations in juvenile turbot (*Psetta maxima*) exposed to sublethal concentrations of ozone-produced oxidants in ozonated seawater.**

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**A comparative study on the removability of different ozone-produced oxidants by activated carbon filtration.**

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**Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems.**

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## **DESCRIPTION OF THE INDIVIDUAL CONTRIBUTION TO THE MULTIPLE-AUTHOR PAPERS**

This list serves as a clarification of the contributions of the candidate, Jan Schröder, on each publication.

### **Chapter I:**

#### **The toxicity of ozone-produced oxidants to the Pacific white shrimp *Litopenaeus vannamei*.**

Contributions: Experiments were designed and performed by Jan Schröder. Microbiological analyses were conducted by Jan Schröder and Andrea Gärtner. All data analyses, graphical presentations and text writing were done by Jan Schröder. All Co-authors provided helpful comments to improve earlier versions of the manuscript.

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### **Chapter IV:**

#### **Potential and limitations of ozone for the removal of ammonia, nitrite, and yellow substances in marine recirculating aquaculture systems.**

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08/2004	Diploma in Fisheries Biology at the Christian-Albrechts-University, Kiel.
09/2003 – 08/2004	Diploma Thesis “Der Einfluss von Umweltfaktoren auf die chemische Mikro-Struktur von Fischotolithen” at IFM-GEOMAR.
10/1998 – 08/2004	Student in Biology at the Christian-Albrechts-University, Kiel
1997 - 1998	Civilian service at the Northern German Epilepsy Center, Raisdorf
1988 – 1997	High-School-Student at the “Ricarda-Huch-Gymnasium”, Kiel
1984 – 1988	Primary-School, Kiel

## WORK EXPERIENCE

Since 08/2009	Scientist at the Gesellschaft für Marine Aquakultur mbH in Büsum
01/2006 – 04/2009	Scientist at the Leibniz Institute of Marine Sciences (IFM-GEOMAR), Kiel. Project “Ozon in marinen Kreislaufsystemen”
08/2006 – 01/2007	Staff member in a commercial fish farm (RAS / ‘Holsten-Stör’).
10/2005 – 12/2005	Scientist at the Leibniz Institute of Marine Sciences (IFM-GEOMAR), Kiel for contract research on behalf of company LINDE GmbH. Investigations on amperometric measurement techniques for ozone determination in seawater.



## ERKLÄRUNG

Hiermit erkläre ich, dass die vorliegende Dissertation selbständig von mir angefertigt wurde. Die Dissertation ist nach Form und Inhalt meine eigene Arbeit und es wurden keine anderen als die angegebenen Hilfsmittel verwendet. Diese Arbeit wurde weder ganz noch zum Teil einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Die Arbeit ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden. Dies ist mein einziges und bisher erstes Promotionsverfahren. Die Promotion soll im Fach Fischereibiologie erfolgen.

Kiel, den 24. November 2010

Jan Schröder