Leibniz-Institut für Meereswissenschaften

The integration of microalgae photobioreactors in a recirculation system for low water discharge mariculture

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Foreword

The manuscripts included in this thesis are prepared for submission to peer-reviewed journals as listed below:

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MARE – Marine Artificial Recirculated Ecosystem: feasibility and modelling of a novel integrated recirculation system.
(manuscript)

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Cultivation of microalgae using a continuous photobioreactor system based on dissolved nutrients of a recirculation system for low water discharge mariculture
(manuscript)

Kube N. And Rosenthal H.
Ozonation and foam fractionation used for the removal of bacteria and particles in a marine recirculation system for microalgae cultivation
(manuscript)

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This thesis has been realised with the help of several collegues. The contributions in particular are listed below:

Chapter 2: MARE I

MARE was designed, constructed and daily maintained by Adrian A. Bischoff, Bert Wecker and Nicole Kube. Sampling and analyzing was done by Nicole Kube (daily maintenance of the recirculation system, fish biomass, dissolved nutrients, foam fractionation and supporting help for worm biomass), Adrian Bischoff (daily maintenance of the recirculation system, detritivorous tank sampling, fish biomass, dissolved nutrients, supporting help for foam fractionation) and Bert Wecker (macroalgae biomass, supporting maintenance of the recirculation system). Bert Wecker developed the model and made the figures. Nicole Kube wrote the manuscript, supported by Adrian Bischoff. Dr. Martina Blümel and Dr. Uwe Waller reviewed the manuscript.

Chapter 3: Photobioreactorsystem

Nicole Kube designed the photobioreactor system, did the sampling and analyzing, supported by Adrian A. Bischoff and Bert Wecker. The manuscript was written by Nicole Kube, reviewed by Dr. Uwe Waller.

Chapter 4: Foam fractionation

Nicole Kube did the sampling and analyzing of the data. Nicole Kube wrote the manuscript, supported by Prof. Dr. Harald Rosenthal.

Chapter 5: MARE II

Nicole Kube and Adrian A. Bischoff did the sampling, analyzing and daily maintenance of the system. The manuscript was written by Nicole Kube, supported by Adrian A. Bischoff and Dr. Martina Blümel. Bert Wecker supported the modelling of the data. Uwe Waller reviewed the manuscript.

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Summary

The development of mariculture recirculation systems increasingly comprehends not only reprocessing of the water body but also an enhanced nutrient recycling by integration of secondary modules like macro- and microalgae and detritivorous organisms. These modules are applied to utilize dissolved and particulate waste derived from fish cultivation in an environmental-friendly manner.

In this thesis a potential conceptual design of such a recirculation system is presented, developed by the mariculture group at IFM-GEOMAR. The underlying principle of the recirculation system was based on that of an artificial ecosystem and combined several trophic levels (fish, macroalgae, microalgae, worms) (MARE = Marine Artificial Recirculated Ecosystem). Corresponding investigations showed that this type of recirculation system becomes feasible, if the dimensions of the secondary modules are adapted to the biotic and abiotic culture requirements of the target species. The results of the experiments provided substantiated knowledge regarding nutrient cycles within the recirculation system, which could be described by a numeric model.

The main goal of this thesis was the development of a continuous photobioreactor system for cultivation of *Nannochloropsis* spec. based on dissolved nutrients derived from a marine recirculation system. From the results it became evident, that it is possible to gain additional biomass and to return almost 100 percent of the derived water back to the main water cycle. The data also revealed that the biofilter capacity of photobioreactor system remains strictly limited to a low level.

Furthermore, the efficacy of ozone and foam fractionation was investigated regarding removal of bacteria and particles from effluents of a marine recirculation system. Interestingly, it could be proved by using a specific staining method that most of the living bacteria are attached to particles with the size of up to 50 µm which protects these organisms efficiently from being killed

by treatment with ozone. Thus, reduction of total bacteria numbers could be achieved by the removal of the particles by foam fractionation.

A second trial of the MARE-system with fish, worms and microalgae revealed, that recirculation systems with several trophic levels require a proper management. If worms are not removed from the recirculation system before natural spawning, nitrogen cycle of the recirculation system can be negatively influenced. This leads to an impaired growth of microalgae and unstable culture conditions for fish.

Taken together, this work demonstrated for the first time, that a marine ecosystem could be artificially mimicked by installation of a cycle of nutrients over several trophic levels. Hence, these results may contribute to reduce environmental impacts of maricultures in the future.

Zusammenfassung

Die Entwicklung von Marikultur Kreislaufsystemen beinhaltet zunehmend nicht nur die vollständige Wiederaufbereitung von Wasser, sondern auch ein erweitertes Nährstoffrecycling durch die Integration von sekundären Modulen wie Makro- und Mikroalgen und detritivoren Organismen. Diese Module werden benutzt, um die anfallenden gelösten und partikulären Abfallstoffe der Fischproduktion umweltgerecht weiterzuverwerten.

In dieser Dissertation wird ein mögliches Konzept einer solchen Anlage vorgestellt, die von der Marikulturgruppe am IFM-GEOMAR entwickelt wurde. Das Prinzip der Anlage beruhte auf einem künstlichen Ökosystem und kombinierte verschiedene trophische Stufen (Fische, Makroalgen, Mikroalgen, Würmer) (MARE = Marine Artificial Recirculated Ecosystem). Die Untersuchungen haben gezeigt, dass die Machbarkeit einer solchen Anlage gegeben ist, wenn die Dimensionierungen der sekundären Module an die biotischen und abiotischen Bedingungen der Zielart angepasst sind. Die Ergebnisse der Experimente lieferten fundierte Kenntnisse in Bezug auf die Nährstoffkreisläufe innerhalb des Kreislaufsystems, die durch ein numerisches Modell beschrieben werden konnten.

Zentrales Thema dieser Dissertation ist die Entwicklung eines kontinuierlichen Photobioreaktorsystems zur Kultivierung von *Nannochloropsis* sp. auf der Basis gelöster Nährstoffe aus einem marinen Kreislaufsystem. Die Versuche haben gezeigt, dass die Gewinnung von zusätzlicher Mikroalgenbiomasse grundsätzlich möglich ist und das gereinigte Wasser zu fast 100% wieder an den Hauptkreislauf zurückgegeben werden kann. Die Ergebnisse wiesen aber auch darauf hin, dass das entwickelte Photobioreactorsystem nur eine beschränkte Biofilterleistung erbringen kann.

Des weiteren wurde die Effizienz von Ozon und Abschäumung im Hinblick auf die Entfernung von Bakterien und Partikeln aus dem Abwasser einer marinen Kreislaufanlage untersucht. Interessanterweise konnte durch die Anwendung einer spezifischen Färbemethode nachgewiesen werden, dass die

meisten lebenden Bakterien an Partikeln der Größe bis 50µm haften, wodurch sie den Behandlungsprozess mit Ozon überstehen können. Dennoch war es möglich, die Gesamtbakterienzahl durch die Entfernung der Partikel mittels Abschäumung zu reduzieren.

In einem zweiten Versuchslauf der MARE-Anlage mit Fischen, Würmern und Mikroalgen wurde deutlich, dass eine Kreislaufanlage mit verschiedenen trophischen Ebenen eines korrekten Managements bedarf. Werden Würmer vor ihrer natürlichen Reproduktion nicht aus dem System entfernt, kommt es zu einer Anreicherung von organischem Material im Kreislaufsystem, das den Stickstoffkreislauf des Kreislaufsystem nachhaltig negativ beeinflussen kann. Das führte zu einer Limitierung des Mikroalgenwachstums und instabilen Kulturbedingungen für die Fische.

Zusammenfassend wurde mit dieser Arbeit erstmalig dargelegt, dass durch den Aufbau eines erweiterten Nährstoffkreislaufs über verschiedene trophische Stufen ein marines Ökosystem artifiziell nachgebildet werden kann. Daher können die Ergebnisse einen Beitrag leisten, in Zukunft die Umwelteinflüsse von Marikulturen zu reduzieren.

Chapter 1

General introduction and thesis outline

1.1 Environmental impacts of open mariculture systems

The gradual decline in supplies of seafood from the ocean is one of the greatest challenges of the seafood industry nowadays. For the past decade the worldwide capture fishery industry has been stagnant due to overfishing. No short-term recovery from the current situation can be expected in the future.

However, consumer demand for high quality seafood at reasonable prices is increasing. Often a year-round availability of fresh seafood is expected. Hence these factors directly lead to a rapid expansion of aquaculture: this sector is showing an above-average economic growth. Estimations of a total aquaculture output of 62 millions tons per year in 2025 are published (FAO 2002), representing a duplication of today´s production (Fig. 1). The Food and Agriculture Organization of the United Nations (FAO) defines aquaculture as the farming of aquatic organisms including fish, crustaceans, molluscs and aquatic plants. Aquaculture can thus be very diverse. To date, commercial aquaculture is dominated by cultivation of kelp, carp, oysters and tiger prawns (FAO, 2005). The majority of organisms are cultivated within freshwater aquaculture systems (57%, Davenport, 2003). Until now, marine aquaculture (mariculture) accounts for a smaller proportion (43%) but is of growing importance because of the stagnating fishery industry. The application of a marine recirculation system for cultivation of Gilthead seabream (*Sparus aurata*) will therefore be the focus of this work.

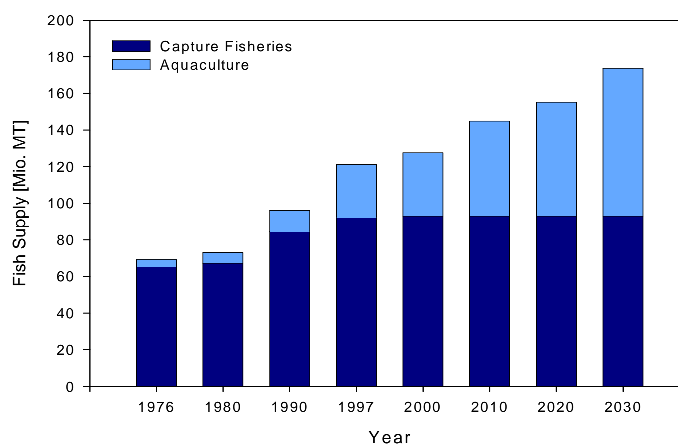

Fig. 1 Global fish supply, data presented from 1976 till 2000 as recorded data, 2010 to 2030 as projection (FAO, 2002).

In Europe, mariculture is dominated by the cultivation of Atlantic salmon (*Salmo salar*), Gilthead seabream (*Sparus aurata*) and Sea bass (*Dicentrachus labrax*) (Cross, 2003). In total, 80.000 tons of seabream were cultivated in 2002 mainly in the Mediterranean region. Greece was the major contributor to seabream mariculture (49%), followed by Turkey (15%), Spain (14%) and Italy (6%) (FAO, 2005) (Fig. 2b). The intensive culture of carnivorous fish species like salmon causes greater environmental impacts than extensive cultivation methods for herbivorous species like carp, oysters and mussels.

a)

b)

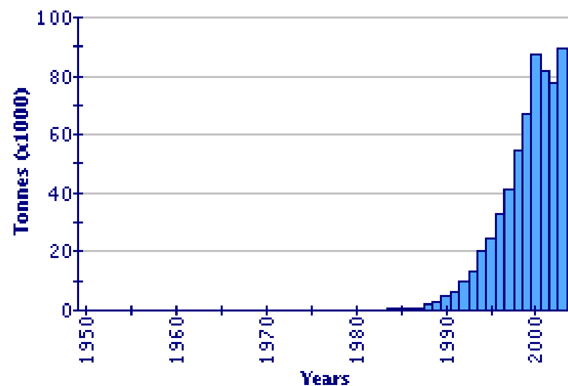

Fig. 2a) Net cages in Greece (Photo by H. Thetmeyer) **b)** Annual production of seabream in the EU-region by mariculture (FAO, 2005). The production was stagnant in recent years because enlargement of the cultivation units was not possible due to regulations of environmental impacts and conflicts with tourism (Cross, 2003)

In Europe, mariculture is mainly organized using cages along the coastline (Fig. 2a). Breeding in these systems is simple and economical, but the cages are directly connected with the environment. In most cases the cages are installed in wind protected areas, e.g. bays or fjords. The high load of organic (faeces and feed remains) and inorganic waste (fish excretory products) are directly leading to eutrophication in the surrounding environment (primary effects, Fig. 3) (Ackefors and Enell, 1994; Beveridge *et al.*, 1991). Additionally, trace elements and micronutrients (e.g. vitamins from the feed) or residual pharmaceuticals can be found in the environment surrounding the cages. Furthermore these open systems are easily affected by economical damages generated by the transfer of diseases, parasites and toxic substances (Braaten *et al.*, 1983; 1992; Weston 1991).

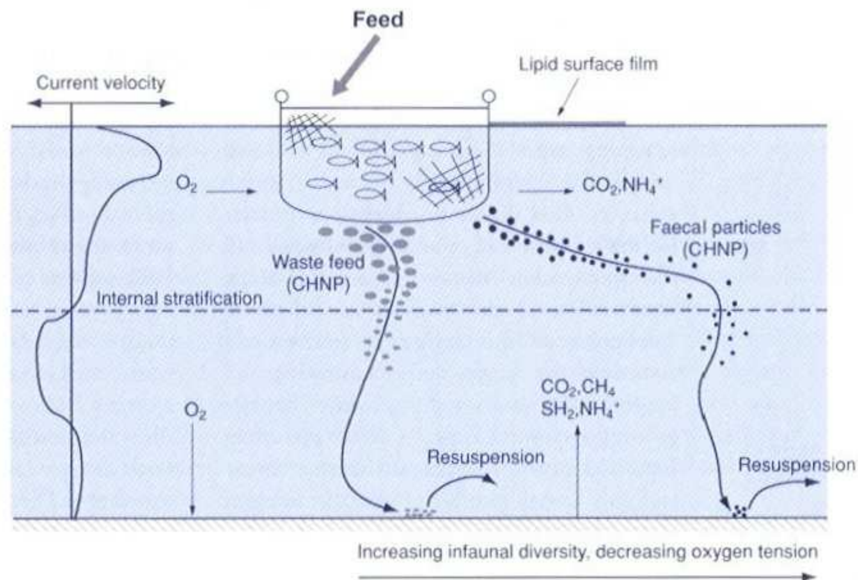

Fig. 3 Inputs and outputs from a fish cage culture system (from Davenport et al., 2003): Feed intake causes an increased demand of oxygen and releases a lipid film at the water surface. Due to the fish metabolism CO_2 and ammonia-nitrogen are excreted. Particulate waste, consisting of faeces material and uneaten fish feed, deposit at the sediment around the net cages. The organic load causes an oxygen drain due to bacteria activity. When the demand of oxygen exceeds the oxygen diffusion rate from overlying waters, sediment becomes anaerobic. This has on one hand a strong influence of the benthos ecosystem due to decreasing oxygen tension. Also a range of microbial processes can follow up: nitrification (ammonium oxidation to nitrite and nitrite oxidation to nitrate) is not taking place, denitrification (producing dinitrogen from nitrate) is competing with nitrate reduction (producing again toxic ammonia from nitrate). Also toxic hydrogen sulfide can be produced by sulfate reduction and under most reducing conditions also methane (via methanogenesis).

A second problem is the interaction with wild fish populations: unplanned releases of farmed fish (e.g. by destroyed nets) and/or gametes or fertile eggs can interfere with the genetic pool of wild stocks, resulting in interbreeding and reduced wild stock fitness and fertility. Furthermore, artificial structures used in mariculture affect local ecosystems: nets, anchors, mooring lines etc. can cause entanglement of the marine wildlife and provide substrates favouring fouling. Other species of fish, birds, marine mammals and also reptiles are attracted by the mass of cultivated fish (Nash *et al.*, 2005).

Very often mariculture activities are in conflict with other human interests. Competition for land and water are often the main targets. Especially conflicts with tourism limits growth of mariculture (Cross, 2003). Using coastlines for mariculture additionally destroys original biotopes and ruins fish breeding and nursery areas. Besides, local ecosystems (e.g. coral reefs) are affected by increased erosion, which secondarily effects the local fisheries. Modifications of the natural food web due to the fishing of wild juvenile fish

for breeding/mast, the consumption of planktonic organisms due to large-scale bivalve farming and increased fishing pressure on small pelagic fish populations for aquaculture feed (Nash *et al.*, 2005) have to be considered as long-term effects resulting from this form of aquaculture.

Therefore, a sustainable development of the aquaculture sector is required "to ensure the attainment and continued satisfaction of human needs for present and future generations. Such sustainable development (in the agriculture, forestry and fisheries sector) conserves land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable" (FAO, 1988). In particular, a sustainable management in the aquaculture sector comprises efficient use of land, water, energy and animal nutrition with simultaneous consideration of bearing capacity of the aquatic environment and adjacent ecosystems.

In recent years great efforts have been made to reduce the environmental impacts of net cages: improvements in siting, design, technology, and management at farm level including improved feeding with a lower waste discharge, better fish health management including disease and stock control at individual farm and sector level and the investigation of social and economical aspects are only a few issues (GESAMP, 2001).

Nevertheless, net cages are still open systems with negative impacts on the natural surrounding environment. The development of recirculating systems with partly or complete reuse of water (Losordo, 1999; Waller, 2000) has been promoted within the past decades, especially due to improvements in system technology (Rosenthal and Grimaldi, 1990; Losordo *et al.*, 1999; Summerfelt, 2002; Waller *et al.*, 2003).

These closed cultivation systems can ensure the constant market availability of the cultured marine organisms by providing controlled system conditions. They can be a potential alternative to conventional production systems when failure-free operation and profitability are assured. To date, intensive re-

search is carried out dealing with closed recirculation systems. Further applications for recirculation systems are ornamental/tropical fish culture, mature and brood stock culture, fry and fingerling production and niche markets for high price food fish.

1.2 Requirements of recirculation systems

Within a recirculation system a suitable environment and good water quality has to be maintained. The recirculating water (process water) is subject to several treatment processes leading to an elimination of harmful substances or enriched nutrients (anoxic conditions are favoured by nutrient enrichment). Primary conditions to ensure survival of the fish are: sufficient concentration of dissolved oxygen (>5mg/L or 50% DOT), low concentrations of undissociated ammonia-nitrogen (<1mg/L), nitrite-nitrogen (< 1mg/L) and carbon dioxide (<0.2 ppm) (Losordo *et al.*, 1998). Additionally, pH-values are required to be in a range from 6 to 9 (optimum for seawater: 7 to 8, Losordo, 1999).

If less than 10% d^{-1} of the total system volume are exchanged, a recirculation system is called closed recirculation system (EIFAC, 1986), although modern systems can reach << 1% d^{-1}. water exchange rate. A system meeting these requirements has been developed at IFM-GEOMAR (Waller *et al.*, 2003).

Recirculating systems are biologically complex systems and need a sophisticated planning. The critical parameters vary according to biological, chemical and physical interactions between the different system components, especially with intensive fish rearing (0.04 kg L^{-1} , Losordo *et al.* 1998). According to this study, an 80 L commercial home aquarium can be stocked with 5 kilograms of fish.

The key to an efficient mariculture in a closed recirculating system is the use of high-effective water treatment components and a good daily maintenance. In poorly managed systems the main problem is insufficient water quality

because of inappropriate components and failures. This leads to stress, diseases and high mortalities among the fishes. Uniform flow rates of water and air/oxygen, fixed water levels and continuous operation are of primary importance. Especially the amount of exchanged water is decisive: the lower the amount of discharged water, the more important are detailed knowledge of and experience with recirculating systems. An introduction dealing with the critical parameters for maintenance of a recirculating system is given in the following chapter.

1.2.1 Feed uptake

It is necessary to raise a fish from 5g body weight to market size of approximately 350-400g in about one year to generate a profit. To ensure such growth, fish are fed using high-protein pellets at rates from 0.8 to 10 percent of body weight depending on size and species. The feed uptake has an enormous influence on the environmental conditions of the recirculation system. The first effect appears directly after the feeding: due to fish digestion, the respiration rate increases dramatically associated by a decrease of dissolved oxygen concentrations to critical levels. Especially after intensive feeding (2 or 3 portions of the total daily feed) (Masser *et al.,* 1999) this effect can be observed. A longer feeding period up to 15 hours e.g. by installation of automatic feeders is therefore preferred. There are also secondary effects of intensive feeding. The fish do not use the whole content of nitrogen and phosphorus of the feed for biomass production: for example max. 30% of the total nitrogen of the feed remains in the fish body (Krom et al., 1985; Krom and Neori, 1989; Hall et al., 1992; Lupatsch and Kissil, 1998; Hargreaves, 1998). Thus, 70% of the feed nitrogen content is excreted as organic waste: it can be classified into settling and suspended solids and excreted dissolved nutrients. The main challenge of a successful maintenance of a recirculation system is the control of the high organic waste. A constant feeding is therefore considered as decisive for the system management.

1.2.2 Biogeochemical cycles

Two biogeochemical cycles are of major importance for recirculation systems: the nitrogen cycle and the phosphorus cycle. The nutrition of the fish is en-

riched in nitrogen as well as in phosphorus. Feed remains are subject to biogeochemical recycling within a recirculation system as well as the excretion products of the cultivated organisms. So it is important to understand the impact of the biogeochemical nutrient cycling in natural as well as in artificial systems. In the following chapters, the nitrogen as well as the phosphorus cycle are firstly presented in natural systems; then implications and applications for aquaculture systems are described.

Nitrogen cycle

The nitrogen cycle is a complex biogeochemical cycle in natural systems (Fig. 4). The nitrogen cycle is also of special interest in aquacultural systems in order to observe and control the bacterial nitrogen metabolism because toxic intermediate products (e.g. ammonia, nitrite) can form and be enriched in the circulation water. There are two major processes of nitrogen conversion: nitrification and denitrification.

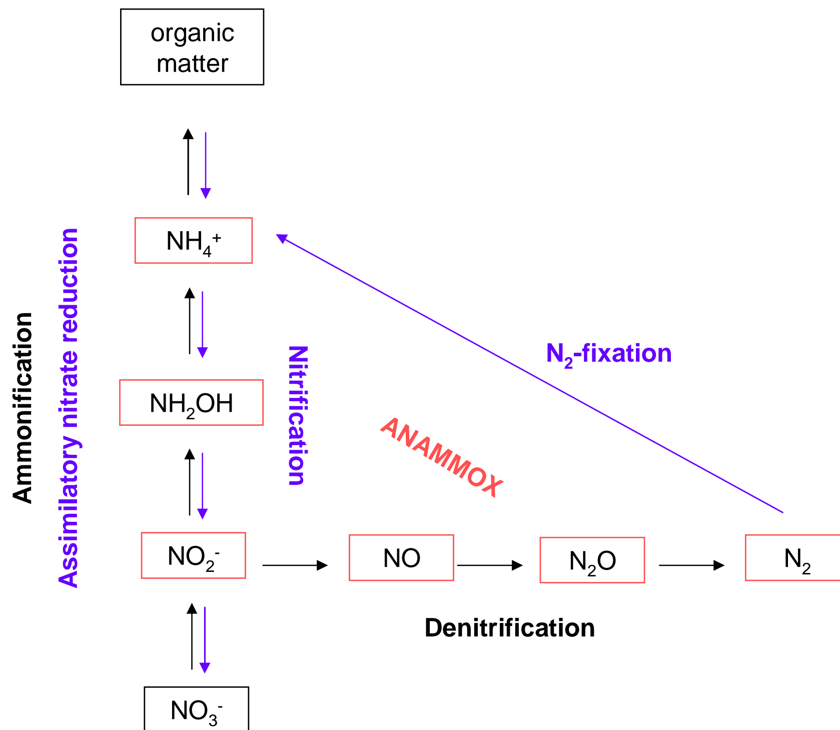

Fig. 4: Nitrogen cycle. Aerobic processes are marked in blue arrows, anaerobic processes are marked in black arrows. The ANAMMOX process is marked with red boxes.

Nitrification

The oxidation of organic or inorganic nitrogen compounds is called nitrification. It is an aerobic process resulting in the formation of nitrate ($NO3^{2-}$). Nitrification is performed by nitrifying bacteria (nitrifiers) being widely distributed in the terrestric and marine realm. Nitrifiers can be divided into ammonia-oxidising bacteria (AOB, e.g. *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus, Nitrosovibrio*) converting ammonia to nitrite and nitrite-oxidising bacteria (NOB, e.g. *Nitrobacter, Nitrospina, Nitrococcus, Nitrospira*) converting nitrite to nitrate (Bock *et al.,* 1986; Bothe, 2000). Nitrification can be performed both by autotrophic and heterotrophic bacteria. Autotrophic nitrifiers are able to combine nitrification with denitrification converting the NO_2^- produced by ammonia-oxidation to N_2 (nitrifier-denitrification, Wrage *et al.*, 2001). Heterotrophic nitrifiers gain no energy performing nitrification (Schmidt *et al.*, 1999) and therefore may have selective advantages under suboxic conditions.

In aquaculture systems the nitrification process is of major importance because of the removal of toxic compounds. When organic material is degraded by bacteria, ammonia is formed. Ammonia is also excreted by the fish. Two compounds of ammonia are present: dissociated and undissociated ammonia. The undissociated ammonia can rapidly reach toxic concentrations for fish and therefore needs to be removed. Therefore, nitrification is an important process. This also accounts for nitrite being toxic for fish in comparably low concentrations. In contrast, nitrate, the final product of the nitrification process is not known to be a toxic nitrogen compound for fish, even in elevated concentrations. However, nitrification can also be performed in the reverse direction, producing ammonia by nitrate reduction. Reduction equivalents from fermentation processes are oxidised during nitrite reduction resulting in the formation of ammonia (nitrate ammonification). Additionally, nitrate reduction can be used for an assimilatory process (assimilatory nitrate reduction, Schlegel, 1992). Nitrate ammonification is performed by facultative anaerobic bacteria and occurs during anoxic conditions. In contrast, the majority of bacteria carries the enzymes for assimilatory nitrate reduction. This process is performed during oxic as well as anoxic conditions

when nitrate is the only available nitrogen source. Assimilatory nitrate reduction is inhibited by elevated ammonia concentrations (Schlegel, 1992).

Another recently discovered process, the anaerobic ammonia oxidation (ANAMMOX; Strous, 1999), removes ammonia via the formation of nitrite forming N_2. However, the ANAMMOX process is an anaerobic process. Anaerobic conditions are not desired to develop in aquaculture systems and therefore the ANAMMOX process is not likely to play a major role. Due to the high particle load in a recirculation system (fish excretions), suboxic/ anoxic conditions are likely to occur at the particle surfaces and the ANAMMOX process therefore may contribute to the removal of ammonia.

Denitrification

The stepwise reduction of nitrate via nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) is called denitrification. The final product of the denitrification process is atmospheric nitrogen (N_2). *Pseudomonas* sp., *Bacillus* sp., *Thiobacillus* sp. and *Propionibacterium* sp. are known to be denitrifying bacterial genera (Zumft, 1997; Wrage *et al.*, 2001). Also among the *Archaea*, genera possessing the enzymes for denitrification have been detected (e.g. *Haloarcula marismortui,* Zumft 1997). The majority of denitrifying bacteria are aerobic heterotrophic organisms using nitrate (nitrite, nitric oxide, nitrous oxide) as terminal electron acceptor molecules in the respiratory chain. Denitrification occurs when suboxic/anoxic conditions are prevailing, because the enzymes responsible for this process (e.g. nitrate reductase, nitrite reductase) are sensitive to elevated oxygen concentrations. Like nitrifiers, denitrifying bacteria are widely distributed in the terrestric as well as in the marine realm. Factors affecting denitrification rates are: nitrate concentration, concentration of electron donators (e.g. organic carbon compounds, reduced sulphur compounds, hydrogen) and the presence/absence of oxygen being considered as the decisive criterion (Tiedje 1988). Recently, aerobic denitrification was also observed for several bacterial species. The oxygen-concentration tolerated by these bacteria is varying (Zumft 1997, Wrage 2001). In aquaculture systems, denitrification is restricted to special units where suboxic/anoxic conditions can be maintained (Chapter 3,5).

Phosphorus cycle

Compared to the nitrogen cycle, the biogeochemical cycling of phosphorus is rather simple. Inorganic phosphate is mainly brought into the marine realm by weathering of rocks. Inorganic phosphate often is a limiting factor for primary production in the oceans and is easily integrated into phytoplankton biomass by primary production. The phosphorus is then transferred biologically via the classical food chain to higher trophic levels. Particulate and dissolved phosphate is excreted by larger organisms of all trophic levels and particulate phosphate is produced during decay of organic matter. The detritus sinks to the seafloor and in geological timescales, is fixed in rock material.

1.2.3 Applications of biogeochemical cycles in aquaculture systems

Nitrogen cycle

Total ammonia-nitrogen (TAN) is a by-product of the protein-metabolism of fish (Bone and Marshall, 1995). TAN is excreted by the fish via the gills as digestion product and is also produced by bacteria converting organic waste into dissolved nutrients. TAN consists of undissociated ammonia (NH_3) and dissociated ammonia (NH_4^+). The undissociated form is extremely toxic to most fish species. The relative proportions of undissociated and dissociated ammonia are strongly depending on pH and temperature and also, to a minor extent, on the salinity of the system water (Trussel 1972, Bower 1978). At pH 7.0 the majority of ammonia occurs in the dissociated form, but at pH 8.5 more than 30 percent of the TAN is detected as NH_3. Thus, it is of major importance to keep TAN concentrations as low as possible coinciding with the maintenance of pH values <8.5. In general, the concentration of dissociated ammonia-nitrogen should not reach more than 0.05mg/L (Losordo, 1998), although lethal concentrations can vary from species to species. Only few studies have been conducted examining the influence of sub-lethal TAN concentrations on fish, but effects on growth and immune defence, skin damages and a reduced fertility was observed (Colt and Armstrong, 1979; Russo and Thruston, 1991; Hargreaves, 1998).

Thus, it is one of the primary objectives during maintenance of a recirculation system to avoid TAN accumulation and to eliminate this component from the system as soon as possible. To achieve TAN reduction/elimination, biological filtration is the preferred treatment step in aquaculture. There are several forms of biofilters, e.g. fluidized bed filters, mixed bed filters or trickling filters (Losordo *et al.*, 1999, Fig. 5a). Within a biofilter a suitable substrate (e.g. plastic pellets, Fig. 5c) providing an enlarged surface for attachment especially for nitrifying bacteria (e.g. *Nitrosomonas* sp.) is supplied.

The first product of the nitrification process is nitrite (NO_2^-) being also a toxic component for the fish. Nitrite ions are taken up into the fish by the chloride cells of the gills, oxidising the Fe^{2+} ion in the haemoglobin molecule (Boyd and Tucker, 1998). Methaemoglobin as the resulting product, is not able to reversibly bind oxygen (Colt and Armstrong, 1979; Russo and Thurston, 1991; Hargreaves, 1998; Kioussis *et al.*, 2000) being crucial for respiration. Therefore, with increasing nitrite concentrations in the water and hence in the blood circulation system the oxygen binding capacity of the blood decreases. Fish are forced to elevate the ventilation rate of the gills. Although the oxygen concentration of the water is sufficient for fish survival, suffocation is possible due to elevated nitrite concentrations. Toxicity of nitrite does not only depend on the fish species, but also on the pH and concentration of Cl^- and Ca^{2+} ions in the water (Colt and Armstrong, 1979; Kioussis *et al.*, 2000). The recommended concentrations of Nitrite-N for intensive aquaculture is $<0,1 mgL^{-1}$ NO_2-N in freshwater and $<1 mg\ L^{-1}$ NO_2-N in seawater (Wickins, 1980).

Assuming stable conditions in a recirculation system, nitrite is rapidly converted to the nontoxic nitrate (NO_3^{2-}). In biofilters used in aquaculture systems also bacterial species (e.g. *Nitrobacter* sp.) converting either nitrite or both ammonia and nitrite to nitrate are present, resulting in a complete removal of the toxic nitrogen compounds by the process of nitrification (Rheinheimer *et al.*, 1988; Hagopian and Riley, 1998). High concentrations of nitrate are not supposed to be of major importance for fish survival, aquatic species can tolerate extremely high levels (>200mg/L). Therefore, only few

studies are published discussing toxic levels and effects of nitrate (Russo and Thurston, 1991). However, there are indications of effects on the osmo-regulation and oxygen transport in the blood as reaction to very high nitrate concentrations (Colt and Armstrong, 1979; Kioussis *et al.*, 2000).

Within a completely closed recirculation system, as it is operated at the IFM-GEOMAR (see Subsection 1.3), nitrate is accumulating in the system. The installation of a denitrification reactor can help to control the nitrate concentration (Fig. 5b). In the denitrification reactor, anoxic conditions are maintained due to low flow rates. Additionally, methanol is added as a C-source for the bacterial metabolism according to the redox potential in the reactor (Wecker, 2002).

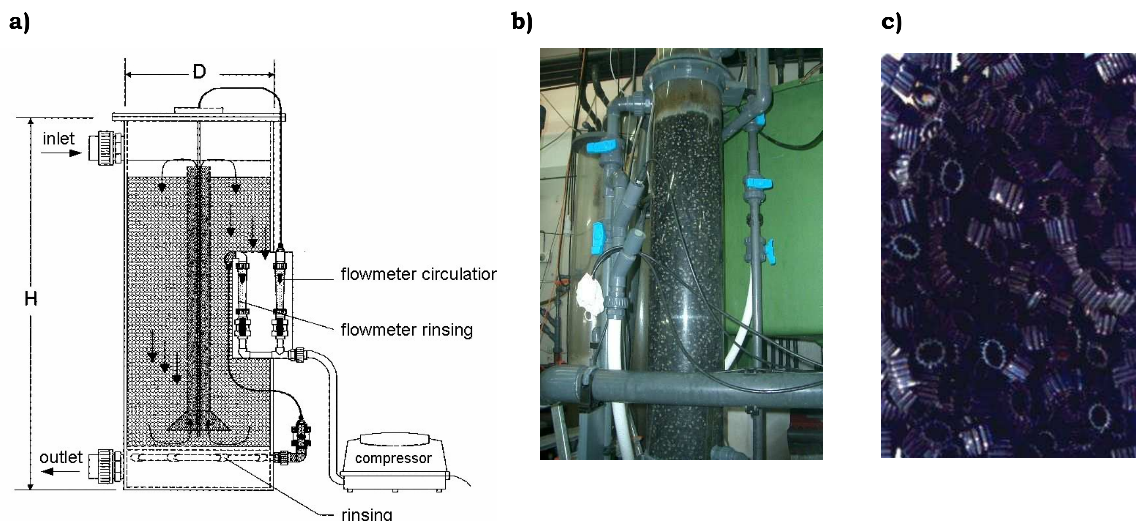

Fig. 5.a) Contiwash biofilter with internal counter-flow principle. The arrows show the flow direction of the pellets (Sander). **b)** Denitrification reactor, working analogous
c) Plastic pellets provided as substrate for bacteria. They are used both in the biofilter and the denitrification reactor.

Phosphorus cycle

The nutrition of the fish is enriched in phosphate. Around 70 to 90% of the phosphate provided with the feed is released to the system environment as PO_4^{3-}. A proportion of approximately 20% thereof is released as dissolved phosphate, whereas the major proportion (80%) is excreted as particulate matter (Bodvin *et al.*, 1996). Phosphate is a nontoxic compound and high concentrations can be tolerated by the fish. In a recirculation system, accumulation of phosphate can easily be avoided by proper water treatment, because most of the phosphate can be discharged with the organic solids or is

used in bacterial metabolism during denitrification. (Barak and van Rijn, 2000). If the accumulation exceeds concentrations of more than 100 mg L^{-1} a water exchange can be considered, but the integration of phototrophic organisms can help to control the phosphate levels.

1.2.4 Suspended and settable solids (Particles)

Especially the suspended (particles <50µm) and settable solids (particles >50µm, fish faeces) need to be removed rapidly from the system (Cripps and Bergheim, 2000). The fish faeces alloy the water quality: due to bacterial activity and chemico-physical conditions leaching (= extraction of nutrients) can be observed. Decomposition of remaining solids results especially in an increase of ammonia-nitrogen concentrations and a decrease in dissolved oxygen tension (DOT) in the system (Welch and Lindell, 1992). Just like during fish cultivation in an open system using net cages toxic compounds (e.g. hydrogen sulfide) can accumulate. The removal of particles is very important to control bacterial abundances in the recirculation water, because the majority of living bacteria can be found attached to particles (Kube & Rosenthal 2006, Chapter 4; Braaten, 1986; Litveld and Cripps, 1992).

Assuming undisturbed water conditions (no flow), larger particles (settable solids) will settle down soon after release. These particles can be removed using a sedimentation tank, a mechanical filtration or a swirl separator. However, the suspended solids (smaller particle sizes) will not settle down and can cause turbidity. When these particles are not efficiently removed, the resulting turbidity can cause stress and irritations of the fish gills and consequently influence fish health (Rosenthal *et al.*, 1982). The use of foam fractionators is a proper and economical way for removing suspended solids. An air/ozone mixture is discharged into a closed cylinder at the bottom of the cylinder. The water is let in following the counter-flow-principle (Fig. 6). The bubbles rise through the water column, their active surface binds proteins and particles, forming foam at the top of the cylinder. The foam is then collected in a waste tank. The proportion of ozone supports the foaming process: ozonation – through electrostatic loading and polarization of the hydrophobic-hydrophilic ends - foster settling characteristics of sus-

pended solids while also assisting in forming aggregates that attach to water-air interfaces, producing a stable foam which can be removed by counter-current stripping. Ozone is able to break up organic compounds, long-chain molecules and especially lipids forming a thin film on the water surface after feeding.

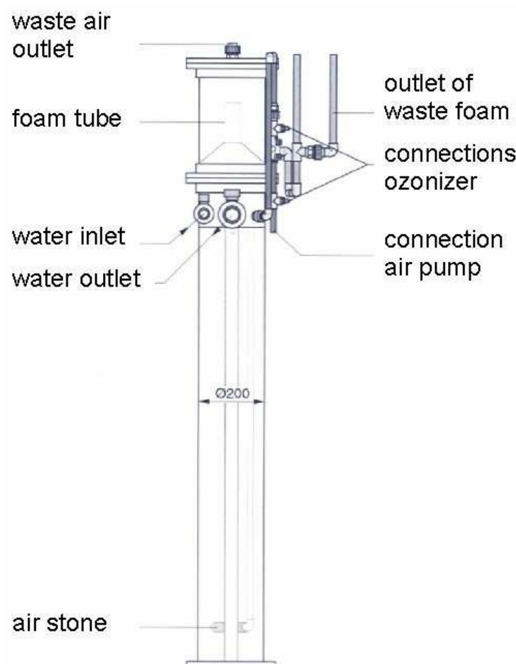

Fig. 6

A Fresh Skim 200 (Sander) foam fractionator: water is pumped in following the counter-flow principle. Through the airstone an air/ozone mixture is let in. A surface active layer is generated around the discharged bubbles. Proteins are accumulating with their hydrophobic end to the bubble. The hydrophilic end is catching particles esp. of organic origin. Due to the buoyancy of the bubbles and the following foam formation all particles are removed and collected in the upper foam collector.

1.2.5 pH and alkalinity

The pH (concentration of H^+ ions) of water does effect numerous other water parameters (e.g. ammonia-nitrogen). It also influences conversion rates of other biological and chemical processes. Thus, pH must be monitored to ensure optimal conditions for fish growth in recirculation systems (Losordo, 1998). The optimal pH range for fish health is 5-9 with an optimum from pH 7 to 8. Inadequate pH concentrations also influence metabolic rates of other important organisms in the system, e.g. below pH 7 the activity of nitrifying bacteria is reduced (Bischoff and Kube, unpubl. data)

Alkalinity is defined as the capacity of water for acidity neutralization. Bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) are the predominant compounds determining alkalinity in seawater. The higher the alkalinity, the higher is the buffering capacity of the water against pH change. pH values can fluctuate

or cycle daily due to respiration, whereas alkalinity is relatively stable but also alkalinity can change over longer time periods (Wurts *et al.*, 1992).

Nitrification is an acid-producing process; the transfer of ammonia-nitrogen to nitrate-nitrogen produces H^+ ions. Due to the reactions with hydroxide ions (OH^-), carbonate and bicarbonate, alkalinity and pH values decrease. In order to maintain alkalinity and pH-values, lime and sodium bicarbonate ($NaHCO_3$) can be added to the water.

1.2.6 Oxygen and CO_2

The concentration of dissolved oxygen (DO) is the main limiting factor for the fish carrying capacity of a recirculation system. The overall rate of oxygen consumption in a recirculation system is determined by the respiration rate of the fish, the oxygen demand of bacteria consuming organic waste and food remains and the oxygen demand in the nitrifying biofilters (BOD – Biological oxygen demand) (Losordo, 1998). DO values should be above 60% of saturation (DOT).

During intensified cultivation and increasing feeding rates additional aeration of the system is necessary. Required concentrations of dissolved oxygen can be maintained through continuous aeration either with atmospheric air or pure gaseous oxygen (Losordo, 1998). As mentioned before (subsession 1.2.3), the proper removal of solids can drastically reduce the oxygen demand of the system.

1.3 Technical recirculation system at IFM-GEOMAR

Modern recirculation systems allow the intensive culture of marine organisms at almost optimal living conditions und minimized water exchange (Liao and May 1974; Otte and Rosenthal 1979, Bovendeur *et al.*, 1987, Blancheton 2000, Waller *et al.*, 2001).

One of these systems is a technical recirculation system installed at the Fishery Biology Department of IFM-GEOMAR. Considering the special re-

quirements of such a system discussed in the preceding chapters the following components were installed (Fig. 6): in two circular fishtanks Gilthead Seabream (*Sparus aurata*) was cultivated (1). Solid waste was removed by a two-step separation process: larger particles sedimented in the swirl separator (2, Fig. 7b,c) and particles <50µm were removed with a foam fractionator Helgoland 700 with ozone addition (4, Fig. 7a). Ammonia-nitrogen and nitrite was converted to nitrate in a Contiwash-biofilter, the nitrate was removed by a denitrification bioreactor (5). Online measurements and automatically regulations of oxidation reduction potential (ORP), pH and oxygen levels were monitored with a control module (KM 2000). To maintain pH a CaO dosage unit was installed at the swirl separator (Fig. 7c).

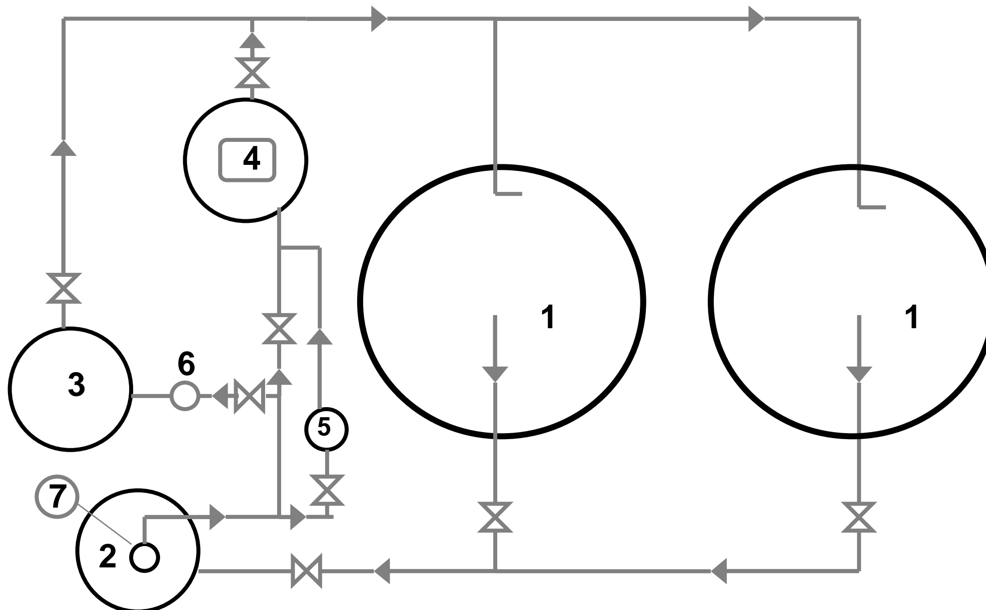

Fig. 6 Schematic drawing of the technical recirculation system at IFM-GEOMAR. Arrows show the flow direction of water. (1) fish tanks with aeration, (2) Swirl separator, (3) Contiwash-Biofilter, (4) Foam fractionator, (5) denitrification, (6) Airlift, double triangle = tap

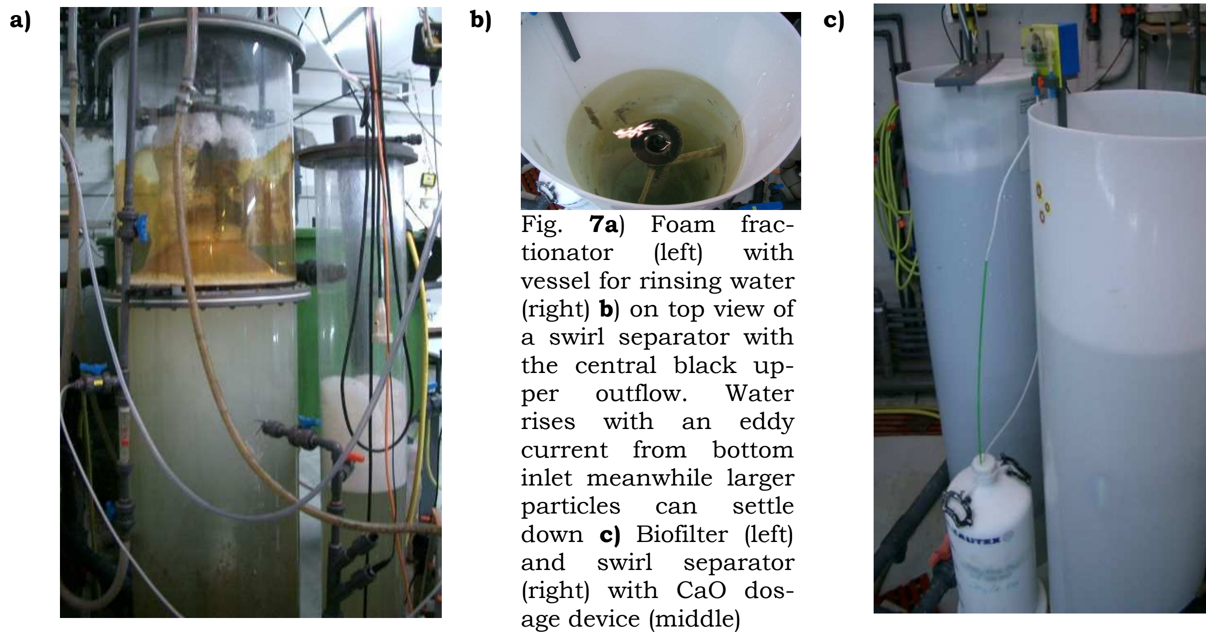

Fig. **7a**) Foam fractionator (left) with vessel for rinsing water (right) **b**) on top view of a swirl separator with the central black upper outflow. Water rises with an eddy current from bottom inlet meanwhile larger particles can settle down **c)** Biofilter (left) and swirl separator (right) with CaO dosage device (middle)

Water is aerated in the tanks using compressed air, but the foam fractionator and the biofilter water are enriched with oxygen as well. Due to effective treatment steps water discharge rates <<1% per day could be achieved (Waller *et al.,* 2003b). Based on this knowledge and research a commercial scale recirculation system with 120m³ water volume and 10 tons of fish biomass production was set up near Hanover (PISA – PolyIntegrated Seawater Aquaculture) (Waller *et al.*, 2005).

1.4 Recirculation systems with different trophic levels

To date, in closed recirculation systems waste produced by fish was either removed or nontoxic nutrients, e.g. nitrate (resulting from biofiltration) and phosphate (leaching of solids) were accumulating. The daily amount of waste is very high: results from different aquacultural cultivation systems showed that only 20-30% of the nitrogen from the feed are used by the fish for biomass synthesis (Krom *et al.*, 1985; Krom and Neori, 1989; Hall et al., 1992; Lupatsch and Kissil, 1998; Hargreaves, 1998). Utilization of phosphate is even lower: published values range from 10 to 30% of feed uptake (Krom et al., 1985; Krom and Neori, 1989; Barak and van Rij, 2000; Lupatsch and

Kissil, 1998) in turn meaning, that 70-90% are excreted either in dissolved or in particulate form.

Until recently, recirculation systems were designed to recycle the water only. Important nutrients like nitrogen, phosphorus or carbon compounds are therefore eliminated either by biological conversion or due to water exchange (Losordo *et al.*, 1999; Waller *et al.*, 2005). Thus, these valuable organic compounds are lost for the system. In modern aquaculture systems a comprehensive nutrient recycling should be maintained in order to reduce the waste produced by the system. Future production systems without any nutrient and energy recycling are not supposed to be economically and ecologically successful (Troell *et al.* 2003).

Integration in terms of aquaculture is defined as the controlled cultivation of aquatic organisms of different trophic levels and one of the key prerequisites for fulfilling the demands for sustainability.

Waste from fish cultivation can be regarded as „new resources“ (Chamberlain and Rosenthal, 1995). This topic increasingly attracts international attention. Due to the better utilization of these resources by joining different trophic levels the profitability of recirculation systems can be enhanced. Waste from the production of fish is used to create high quality biomass. Thus, the environmental impacts of a recirculation system can be reduced and resources can be used more efficiently (Asgard *et al.*, 1999; Schneider *et al.*, 2005). The additional biomass supports the economical diversification and the benefit per cultivation unit (Chopin *et al.*, 2001).

However, the idea of integration is not new. In the aquaculture sector dealing with freshwater and brackish systems Asian countries have been practising integrated aquaculture for centuries (Chopin *et al.*, 2001). Marine seaweed cultivation is the favoured integration step in open (Petrell et al., 1993; Newkirk, 1996; Chopin *et al.* 1999a; b) as well as land-based systems (Neori *et al.*, 1991; 2000; Krom *et al.,* 1995; Vandermeulen and Gordin, 1990; Buschmann, 1996) and is widely used. An overview about the benefits of the

application of integrated systems in aquaculture is given in Chopin *et al.*, 2001.

Several studies have been performed to investigate the usage of macro- and microalgae, hydroponics (growing plants without soil), artificial wetlands, filtering or detritivorous organisms as secondary steps in aquacultural systems (Schneider *et al.,* 2005). However, the systems discussed in Schneider et al. (2005) are open systems. Thus, the research enhancing integration in aquaculture systems needs to be intensified (Costa-Pierce, 2002). To date, no successful attempts have been made to set up a completely closed integrated seawater system, probably because of the lack of experience in running completely closed marine systems.

Based on the established knowledge and experience in marine recirculation systems at IFM-GEOMAR the first attempt of running a completely closed integrated seawater system over a longer time period (MARE = Marine Artificial Recirculation System) was made (see Chapter 2 and 5) (Bischoff *et al.*, 2005).

According to the requirements for recirculation systems all necessary treatment steps needed to be included. The function of the swirl separator was transferred to a sedimentation tank filled with sediment. The sedimented particles could be directly used by the common ragworm *Nereis diversicolor* living in the sediment. Instead of a conventional biofilter a macroalgae tank was included in the first experimental phase (Wecker *et al.*, 2005) and replaced by a photobioreactor system for microalgae cultivation in the second experimental phase (Kube *et al.*, 2005; see Chapter 3 and 4) (Fig. 8). The remaining technical components in MARE were: a pump and two foam fractionators for the removal of the suspended solids <50µm. A drawing of the system is included in Chapter 2.

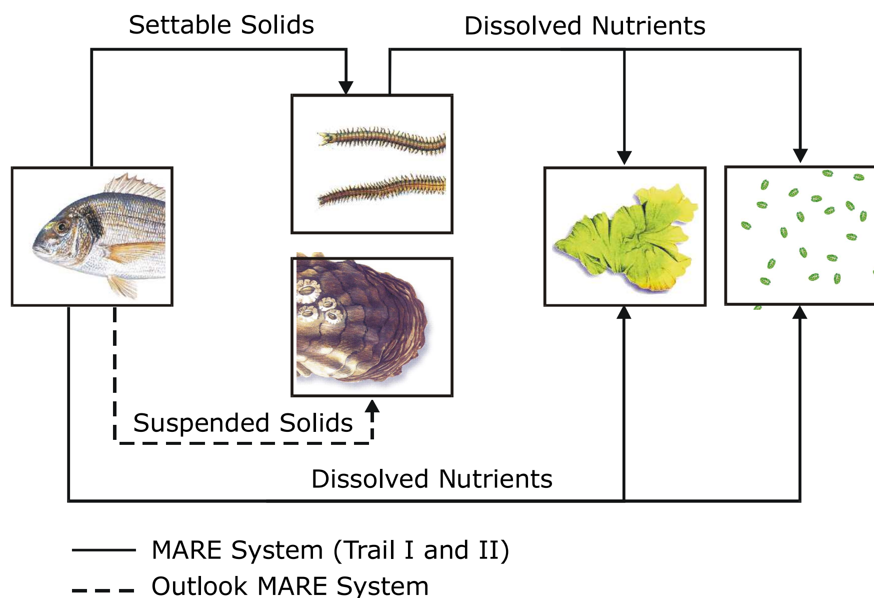

Fig. 8 Concept of the MARE-system (Marine Artificial Recirculating Ecosystem): The target species *Sparus aurata* provides the waste (dissolved and particulate matter) being used by the other biological system components. The common ragworm (*Nereis diversicolor)* is used for utilization of the particulate waste, dissolved nutrients are recycled by macroalgae (e.g. *Solieria chordalis*) (Phase I) and microalgae (*Nannochloropsis* spec.) (Phase II). In the present MARE system suspended solids are still removed by foam fractionators. But future perspectives also include for example bivalves (e.g. *Mytilus edulis*) for removal of particles >50µm.

1.5 Organisms

Gilthead Seabream (Sparus aurata)

The Gilthead seabream taxonomically can be assigned to the family *Sparidae* (Percoidei, Perciformes, Acanthopterygii, Teleostei, Neopterygii, Actinopterygei, Osteichthyes). The natural habitat of *Sparus aurata* are sea grass beds or sandy bottoms of the coastal waters of the Mediterranean Sea and the Eastern Atlantic Ocean. *Sparus aurata* is a bottom dwelling species and usually lives solitary or in small shoals. This fish species reaches an average size of approx. 35 cm. In nature, *Sparus aurata* prefers invertebrates as food (shellfish, bivalves), but they are also considered to casually feed on plants or phytoplankton. This fish species reaches maturity after approx. one year and is a protandric hermaphrodite (changes sex from ♂♂ to ♀). In nature, spawning is restricted to the winter season but in aquacultures, perennial breeding can be observed providing controlled conditions (changes on the

specific gravity of the water, temperature and photoperiod). *Sparus aurata* has planktonic larvae and the larval phase takes approx. 50 days (temperature 17 - 18° C).

The Gilthead seabream is a highly appreciated food fish, which is widely cultivated all over the Mediterranean Sea in well organised aquacultures.

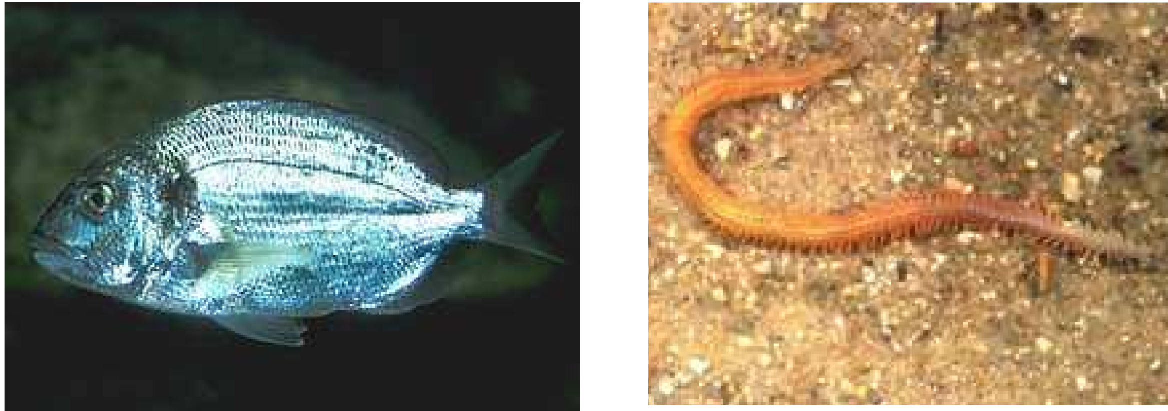

Fig. 9 Gilthead seabream (*Sparus aurata* Linnaeus, 1758, left); naturally distributed in the Mediterranean and the Eastern Atlantic Ocean and common ragworm (*Nereis diversicolor* Linnaeus, 1758, right).

Common ragworm (Nereis diversicolor)

The common ragworm can be classified as a member of the family Nereididae (Phyllodocida, Aciculata, Palpata, Polychaeta, Annelida).

Nereis diversicolor reaches a maximum size of approx. 10 cm and is coloured reddish-brown to greenish (when reaching maturity). It is characterized by the possession of numerous paddling feet and a characteristic red line on the dorsal side. *Nereis diversicolor* lives along the coasts of the North Atlantic Ocean and is adjacent seas. Adaptation to brackish water and freshwater (river estuaries) has also been observed for this polychaete species. *Nereis diversicolor* exhibits an endobenthic way of living and mainly can be found in living tubes reaching up to 10 cm sediment depth. Food can be acquired using different methods. For capture of sinking particles/planktonic organisms, a web composed of mucus is excreted into the living tube and water is pumped through this web. Thus, the mucus web acts as a sieve enriching particles/planktonic organisms. *Nereis diversicolor* also is able to graze on benthic algae and small benthic animals using his mandibles. Concerning

reproduction strategy, *Nereis* shows a monotelic reproduction (adult worms die after reproduction).

Due to its nutrition, *Nereis* is especially suitable for the integration into a closed recirculation system. Particulate matter (fish faeces, food remains) can thus be removed using the natural nutrition of this worm species.

Solieria chordalis

Distribution of *Solieria chordalis* (Solieriaceae, Rhodophyta) ranges from the Mediterranean to areas in the North Atlantic, influenced by the Gulf stream. Solieria is well adapted to temperatures demanded by the target species Gilthead seabream (*Sparus aurata*). Results about growth, reproduction and commercial cultivation were derived from an algae farm in Sylt, Germany. In contrast to other macroalgae, *Solieria chordalis* is well growing during the summer and resistant against biofouling.

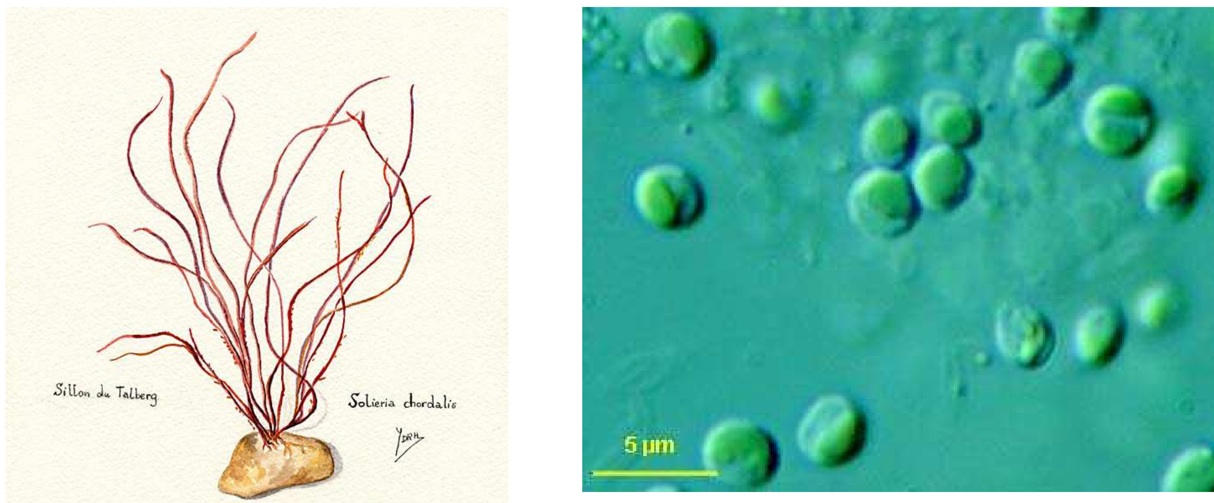

Fig. 10 Solieria chordalis (left) (Source:www.ifremer.fr) and Nannochloropsis sp. (right) (source:www.sb-roscoff.fr).

Nannochloropsis sp.

Nannochloropsis (Eustigmatophyceae) is generally described as a component of the picoeukaryotic plankton because of its size range of 2-5µm (Hu and Gao, 2003). They stand at the beginning of the food chain in aquatic ecosystems. Picoeukaryotic plankton are found throughout the world´s ocean at concentrations between 10^2 to 10^4 cells per cm^3 in the upper photic zone (Caron *et al.*, 1999), playing significant roles in global carbon and mineral

cycles (Fogg, 1995). Furthermore this microalgae contains highly nutritional compounds (e.g. sterols, polyunsaturated fatty acids; Verón *et al.*, 1998; Rocha et al., 2003) and is therefore used for feeding fish larvae in mariculture. It contains only chlorophyll a (Hibbet, 1988) but also valuable pigments such as zeaxanthin, canthaxanthin and astaxanthin at high levels (Lubián *et al.*, 2000).

1.6 Thesis outline

Based on the established knowledge and experience in marine recirculation systems at IFM-GEOMAR the first attempt of running an seawater recirculation system with different trophic levels over a longer time period (MARE = Marine Artificial Recirculation System) was made (see Chapter 2 and 5) (Bischoff *et al.*, 2005).

The system was set up and maintained by the members of the mariculture working group. The aim of the project was to test the feasibility and the operation ranges of such a novel recirculation system. The performance and applicability of the system should be investigated as well as the dynamics of the single modules. This work was split into three different topics (PhD theses). The combination of these data (system and modules) are used to obtain comprehensive information about the nutrient cycling based on the daily feeding in order to evaluate a nutrient budget for the system. Hereby the following topics were in focus of the experiments: the available nutrient concentrations in each module and the extent of nutrients being used for incorporation into biomass. Further investigations dealt with the system´s limitations and the potential dangers for the safe operation of the system. Main topic of this thesis was the development of a continuous photobioreactorsystem based on dissolved nutrients of a marine recirculation system.

The thesis is divided into five chapters (including the introduction), each focused on a different scientific objective.

Major scientific objectives of this thesis were:

- to investigate the applicability of a seawater recirculation system with several trophic levels (water exchange < 1% total water volume/day) for cultivation of *Sparus aurata* over a longer time period (chapters 2 and 5)

- to determine biotic and abiotic factors limiting the performance of this system (chapters 3 and 5)

- to evaluate the performance and practical applicability of the photobio-reactors for *Nannochloropsis* sp. cultivation integrated into the system (chapter 5)

- to evaluate the applicability of the foam fractionation technique for removal of bacteria and particles in a marine recirculation system suitable for microalgae cultivation (chapter 4)

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Chapter 2

MARE – Marine Artificial Recirculated Ecosystem: feasibility and modelling of a novel integrated re-circulation system

Wecker B., Kube N., Bischoff A.A., and Waller U. (2006)

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MARE – Marine Artificial Recirculated Ecosystem: feasibility and modelling of a novel integrated recirculation system

Wecker, B., Kube N., Bischoff A.A., Waller U.

Abstract

In conventional recirculating systems most of the nutrients supplied with the food are discharged because such installations do not include treatment steps for nutrient recycling. An advanced farming design for low water discharge (<< 1 % d^{-1} system volume) was investigated. The MARE-system is based on the concept of a closed biological integrated recirculating system not only with low water discharge but also with nutrient recycling. The target organism was Gilthead seabream (*Sparus aurata*). In secondary production compartments of the MARE system particulate matter was used by detritivorous worms (*Nereis diversicolor*) and dissolved nutrients were used by macroalgae (*Solieria chordalis*). There was no additional technical biofilter system.

This paper describes the feasibility of the MARE-system in order to maintain the water quality within safe limits and support adequate fish growth. Secondly, a mathematical model was developed to understand the nutrient flows and biomass developments within the MARE-System. It was possible to establish a nutrient budget for MARE. The results show that nitrogen is mostly converted microbiologically by nitrification and denitrification. Phosphorus was mainly taken up by macroalgae. The scientific concept for the MARE system was shown to be feasible: a simplified recirculated ecosystem can be used to recycle nutrients and to maintain water quality. However, the secondary modules (*Nereis, Solieria*) were found to be less important for the nutrient retention, but increased valorisation of the system. Thus, integration of different trophic levels led to an increased nutrient utilization.

Keywords: marine recirculation system, integration, nutrient budget model, *Nereis diversicolor*, *Sparus aurata*, *Solieria chordalis*, artificial ecosystem

2.1 Introduction

In closed recirculation systems waste is usually concentrated and discharged (solids in sinks) or dissolved nutrients are accumulating (nitrate and phosphate). The daily amount of waste is rather high: results from different aquacultural production systems showed that only 20-30% of nitrogen in the feed are used by fish to synthesize biomass (Krom *et al.*, 1985; Krom and Neori, 1989; Hall et al., 1992; Lupatsch and Kissil, 1998; Hargreaves, 1998). Utilization of phosphorus is between 10-30% of feed intake (Krom et al., 1985; Krom and Neori, 1989; Barak and van Rijn, 2000; Lupatsch and Kissil, 1998). Thus, 70-90% of the phosphorus provided with the feed are excreted either in dissolved or particulate form.

To date, the operation of recirculation systems is focused on recycling the water only. Nutrients like nitrogen, phosphorus or carbon compounds are eliminated by water treatment steps either biologically (biofiltration) or by water exchange, (Losordo *et al.*, 1999; Waller *et al.*, 2005). Thus, these valuable organic compounds are lost for the system and released having enormous impacts on the environment (e.g. eutrophication). In modern aquaculture systems a comprehensive nutrient recycling should be maintained in order to reduce these environmental impacts. Waste from fish production should be understood as „new resources“ (Chamberlain and Rosenthal, 1995). Due to the better utilization of these resources by integration of different trophic levels, the profitability of recirculation systems can be enhanced (Asgard *et al.*, 1999; Schneider *et al.*, 2005). Additional biomass supports the economical diversification and the benefit per production unit (Chopin *et al.*, 2001). Production systems without any nutrient and energy recycling are supposed to have less chances in future (Troell *et al.* 2003).

However, integration is not a new idea because in freshwater and brackish aquaculture systems integrated aquaculture has been practiced for centuries (Chopin *et al.*, 2001). Marine seaweed production is the mainly used integration step in open marine (Petrell et al., 1996; Newkirk, 1996; Chopin *et al.* 1999a; b) as well in land-based systems (Neori *et al.*, 1991; 2000; Krom *et*

al., 1995; Vandermeulen and Gordin, 1990; Buschmann, 1996). An overview about the current discussion concerning the integration of macroalgae into these systems is given in Chopin *et al.* (2001). Several studies describe the usage of macro- and microalgae, hydroponics (growing plants without soil), artificial wetlands, filtering or detritivorous organisms as secondary steps (Schneider *et al.,* 2005 and references therein), but these systems are characterized by larger water exchanging rates.

Based on the knowledge of low discharge (<1% per day) recirculation systems, the goal of this study was to develop a completely new recirculation system being characterized by a closed water recirculation as well as by nutrient recycling via the integration of secondary steps (*Nereis, Soleria*).

2.2 Material and Methods

2.2.1 MARE-System

MARE (4.5m^3) consisted of different units (Fig. 1): two fishtanks (700L volume) (1) each stocked with 85 juvenile Gilthead seabream (*Sparus aurata*) with an average weight of 66.5 ± 11g. A circular current was established by airlifts. Gilthead Seabream was chosen due to its comparatively easy cultivation and its wide range of tolerated salt concentrations and temperatures. Water from the fish tanks was completely transferred to the detritivorous reactor (2). The bioreactor for detritivorous organisms (2.1m^2 sediment surface, water column 0.7m) was filled with a 0.1m deep sand layer (grain size ≤ 2mm) and stocked with *Nereis diversicolor* at a density of approx. 900-950 individuals per m^2 on 18.6.04. Additional walls were installed within the tank to elongated the water route through the tank and thus to enhance the settling of suspended solids. For the removal of dissolved nutrients a macroalgae cultivation unit (2.3 m^2 surface area) was included with a stocking density of 4.4 kg/m^2 surface (3) of floating *Solieria chordalis* in an aerated circular tank with 400 µE m^{-2} s^{-1} illumination (day:night 16:8 hours).

A pump (type AG8, ITT Hydroair international, Denmark) (4) provided two foam fractionators (type Outside Skimmer III; Erwin Sander Elektroapparate

GmbH; Uetze-Eltze, Germany) with an average flow rate of 1000L*h^{-1} each (6) as well as the two fishtanks with about 600 to 800 L*h^{-1} respectively. The protein skimmers were aerated with compressed air and an additional ozone addition, produced by an ozone generator (Sander, Ozonizer A2000). In a separate bypass an additional tank filled with 50 litres of freshwater was attached to the foam fractionators for rinsing the foam collectors automatically every 15 minutes. Removed foam was collected in the freshwater tank.

When the MARE experiment started, Gilthead seabream were already cultured for 182 days within the MARE-system. Also *Nereis diversicolor* was stocked one month before the start of the experiments at 11.11.04. At the beginning, a biofilter for nitrification was included for safety reasons, but it was shut down during the experimental period (11.11.2004 – 01.06.2005).

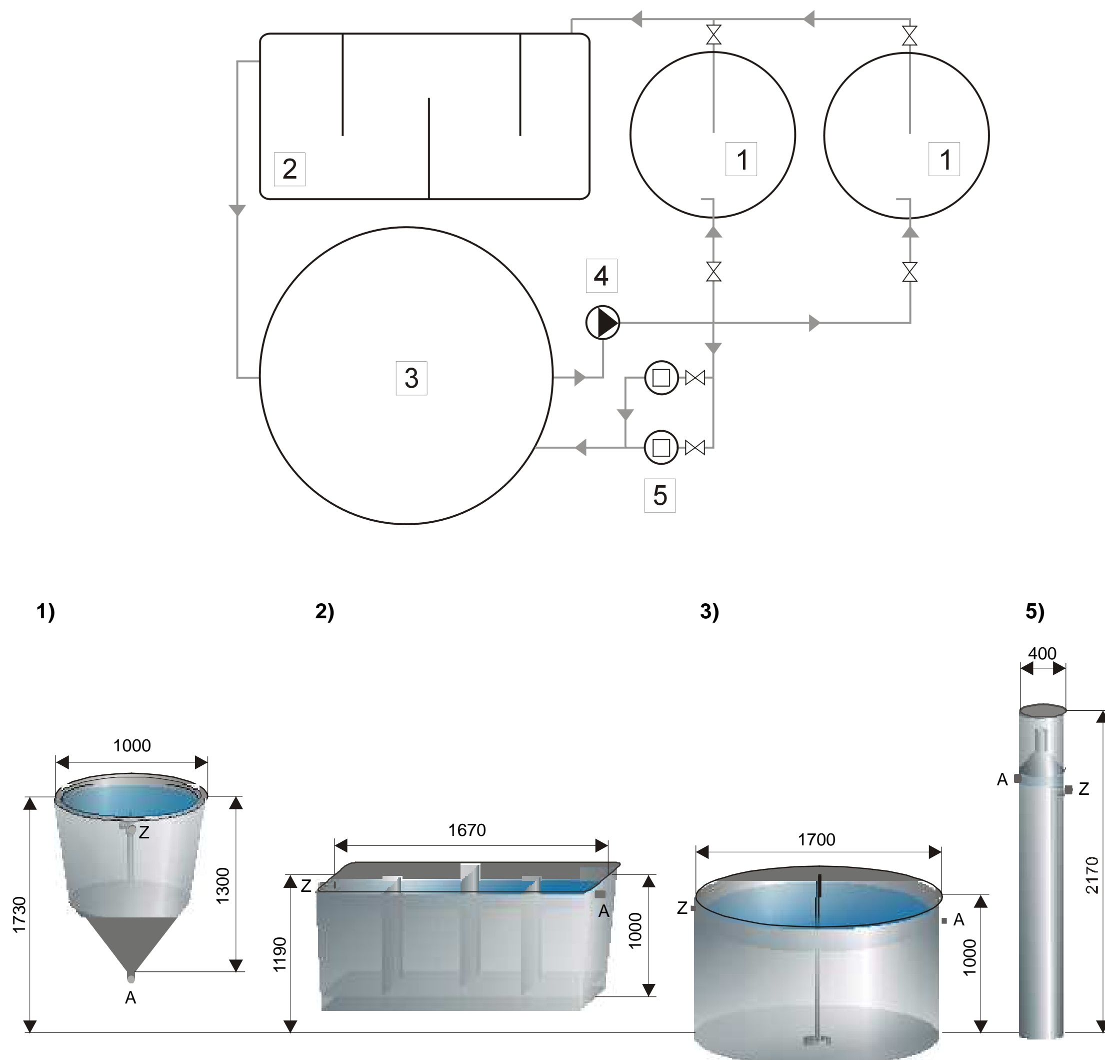

Fig. 1 Flow-chart of the MARE-system (Marine Artificial Recirculating Ecosystem) and 3D-figures of the modules. All units of the module are given in mm. (1) = fishtanks (*Sparus aurata*), (2) = detritivorous culture tank (*Nereis diversicolor*) filled with sediment and additional wall segments; (3) = macroalgae tank (*Solieria chordalis*) with a central aeration; (4) = pump; (5) = foam fractionators, arrows indicate water flow, double triangle = valve, Z = inlet, A = outlet

2.2.2 Measurements and Methods

Biomass determination

Fish were fed with pellets of 4.5mm size (Biomar, Aqualife 17, see Table 1). The daily food rate was adjusted following the results of fish biomass determination (wet weight of at least 30 individuals per tank) at intervals of appr. 3 weeks.

The *Nereis* biomass was also determined (number and wet weight of worms) using four subsamples. Therefore, the detritivorous tank was divided into four parts. From each part, a core of approx. 800 cm^3 was sampled. To remove the sediment and to enrich worms, a sieve of 1 mm mesh size was used for sampling.

Wet weight of *Solieria* was determined at an average of 1 to 2 weeks. Yield of biomass was remained in the tank until reaching stocking density of 8kg/m^2 at week 20 of the experimental period. Afterwards the stocking density was remained constant at this value by removing the weekly gain from the system.

Water parameters

Water samples for analysis of dissolved nutrients were taken daily at three different points of the MARE-system: outlet fishtanks, outlet *Nereis*-tank and outlet algae tank. Water samples were stored at –20°C and analysed with an AA3 Autoanalyzer (Method no. G-016-91 for Nitrate-N, G-029-92 for Nitrite-N, G-102-93 for Ammonia-N, G-103-93 for Phosphate, Bran-Lübbe GmbH; Norderstedt, Germany).

Flow rates through fish tanks and foam fractionators were recorded and adjusted to 600L h^{-1} and 1000L h^{-1}, respectively. Online measurements of redox potential, pH and dissolved oxygen were recorded with a control module KM 2000 (Meinsberg) and a portable measuring device (WTW multi 350).

System water level was controlled daily and if necessary, adjusted with filtered brackish seawater. Salinity of the system water was 24.1 ± 0.8 psu. If needed, artificial sea salt was used to increase salinity. The foam collectors were cleaned daily and the freshwater for rinsing was changed. The daily loss of water via the foam fractionators was recorded by the increased water volume in the freshwater tank.

Solid components

The analysis of solid waste, tissues and sediment parameters were performed as follows: dry matter content was determined by drying the sample in a drying oven at 60°C overnight. Organic content was measured by incineration of organic matter in a muffle furnace. C/N ratio was determined by an element analyzer (type NA 1500 series 2, FISONS). Calorific value was measured by complete sample combustion using an IKA calorimeter C4000. Weighing was performed using with a Sartorius A 210 P (max. 200g) and a Sartorius U 4600 P (max. 4000g).

Every week a sample of the rinsing water of the foam fractionators was taken to determine the proportion of suspended solids removed from the system. 12 tubes of 10ml sample were centrifuged and the supernatant was stored for later water analysis. Dry matter and C/N ratio analysis of the pellets resulting from centrifugation were performed analogous to the analysis of solid components. The organic matter content of the sediment also was analysed weekly with 5 subsamples of appr. 10 cm^3 sediment according to this principle

2.2.3 Modelling

The aim of this study was not only to test the feasibility of the MARE concept but also to process the experimental data within a mathematical model in order to evaluate the nutrient flows and biomass developments within the MARE system. Mathematical models are integrative and important tools of interdisciplinary work in order to combine knowledge and scientific methods from different subjects or data from diverse sources. A model can simplify complex processes for better understanding and helps to concentrate on the essential processes. In summary, developing a model demands comprehension but also augments knowledge (Ebenhöh, 2004).

Fig. 2 shows the concept of the numeric model developed for MARE. Variables and parameters are listed in Tab. 1 and indicate the connections among the respective modules.

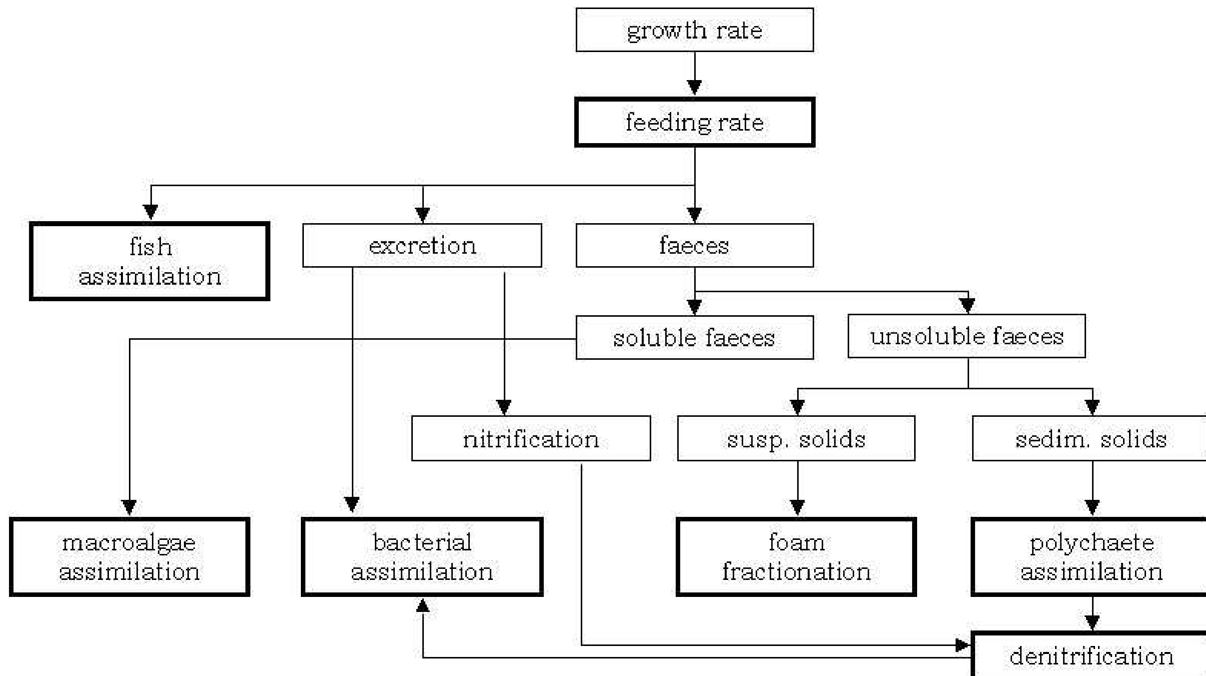

Fig. 2 Flow-chart of the nutrient fluxes in the MARE system. The concept of the numeric model and the connections of the single modules are shown schematically. The marked boxes indicate the final values of the nutrient budget. The model was designed to analyse nitrogen and phosphorus as nutrients of major importance for the system.

Tab. 1 Denomination of variables and parameters, dimensions, description and source used for numeric MARE model, **t denotes production time in days, x denotes kind of nutrient**

Denomination	Dimension	Description	Source
$W_{fish,t}$	g	individual weight of fish t denotes time in days (d)	experiment
$\mu_{fish,t}$	d^{-1}	specific growth rate	calculated
$Y_{fish,t}$	$g\ d^{-1}$	yield, weight increment	calculated
$F_{,t}$	$g\ d^{-1}$	weight of given aquafeed per day	calculated
FCR_t		feed conversation ratio	calculated
$intake_{nutrients}$	$g\ d^{-1}$	total amount of nutrients introduced to recirculation system per day	calculated
$C_{x,\ feed}$	%	percentage of nutrient in given feed x denotes the kind of nutrient	literature
$Faeces_{x,t}$	g	amount of nutrients in faeces	literature
ADC_x	%	apparent digestibility coefficient	literature
faeces $soluble_{x,t}$	g	dissolved fraction of faeces due to leaching	literature
faeces $unsoluble_{x,t}$	g	unsoluble fraction of faeces after leaching	literature
$RM_{mean,x}$	%	remaining matter of faeces after leaching	literature
$excretion_{x,t}$	g	amount of dissolved nutrient from fish metabolism	calculated
$retention_{x,t}$	g	amount of nutrient remained in body tissues	calculated
suspended $solids_{x,t}$	g	amount of nutrient in particles separated by foam fractionation	experiment
efficiency foam fractionator	%	share solids removed by foam fractionation process	calculated
$SettableSolids_{x,t}$	%	share of solids removed by swirl separation	calculated
$\mu_{worm,t}$	d^{-1}	growth rate of polychaetes depending on the energy supplied with fish faeces	literature
$E_{settableSolids,t}$	$kJ\ d^{-1}\ worm^{-1}$	daily amount of energy available from settable solids for each individual worm	literature
$C_{E,settableSolids}$	kJ	Energy content of settable solids	experiment
$Y_{worm,t}$	g	yield, weight increment of worms in the detritivorous reactor	calculated
$W_{worm,t}$	g	individual weight of worms	experiment
$n_{worm,t}$		number of worms in the detritivorous reactor	experiment
$M_{N,worm,t}$	$n_{worm,t}\ d^{-1}$	natural worm mortality	calculated
$EnergyloadTotal_t$	$KJ\ d^{-1}$	total energy available for production in the detritivorous reactor derived from faeces and worm mortality	
$c_{DM,\ worm}$	$g\ kg^{-1}$	dry matter of worm	literature
E_{worm}	$KJ\ g^{-1}$ DW	energy content of worm	literature
$M_{C,worm,t}$	$n_{worm,\ t}\ d^{-1}$	worm mortality due to cannibalistic behaviour	calculated
$c_{TAN,t}$	$mg\ L^{-1}$	TAN concentration in the recirculation water	calculated
$r_{TAN,algae,t}$	$g\ TAN\ h^{-1}\ m^{-2}$	daily TAN uptake by macroalgae	experiment
$r_{max,TAN,algae}$	$g\ TAN\ h^{-1}\ m^{-2}$	maximum daily TAN uptake by macroalgae	experiment
$r_{TAN\%,algae,t}$	$\%\ TAN\ h^{-1}\ m^{-2}$	relative daily TAN uptake by macroalgae	experiment
$r_{max,TAN\%,algae}$	$\%\ TAN\ h^{-1}\ m^{-2}$	maximum relative daily TAN uptake by macroalgae	experiment
$B_{TAN,t}$	$g\ m^{-2}\ h^{-1}$	amount of ammonia available in the macroalgae reactor	calculated
$y_{algae,t}$	g	yield, biomass increase in the macroalgae reactor	calculated
$r_{P,algae,t}$	$g\ P\ h^{-1}\ m^{-2}$	phosphorus uptake of algae	calculated
$r_{nitrification,t}$	$mg\ h^{-1}\ m^{-2}$	microbial TAN oxidation	calculated
$B_{OM,t}$	g	organic matter available for denitrification	calculated
$r_{denitrification,t}$	$mg\ h^{-1}\ m^{-2}$	microbial removal of nitrate	calculated
z		ratio of required organic matter to reduce nitrate to nitrogen gas	calculated
c_t	$mg\ L^{-1}$	nitrate concentration in the system water	calculated
$r_{N,bacteria,t}$	$mg\ NO_3\ h^{-1}\ m^{-2}$	nitrate assimilation by bacteria	calculated
$r_{P,bacteria,t}$	$mg\ PO_4\ h^{-1}\ m^{-2}$	phosphorous assimilation by bacteria	calculated
$c_{P,t}$	$mg\ L^{-1}$	phosphate concentration in the system water	calculated

Module Fish

a) Fish growth and feeding rate

Firstly, fish assimilation results in an increase of fish biomass. In the model, the specific growth rate per day ($\mu_{fish,\ t}$) was determined by

$$\mu_{fish,t} = Ln \frac{W_{fish,t+1}}{W_{fish,t}} \quad \text{(Equ. 1)}$$

where $W_{fish,t}$ = wet weight fish, t day of experiment
$\mu_{fish,t}$ was derived from experimental data of wet fish weight (see section 3.1 *fish growth*).

The yield of fish ($Y_{fish,t}$) per day can be described by

$$Y_{fish,t} = \mu_{fish,t} \times W_{fish,t} \quad \text{(Equ. 2)}$$

The relationship between feeding rate (F_t) and specific growth rate ($\mu_{fish,t}$) can be described as feed conversion ratio (FCR_t)

$$FCR_t = \frac{F_t}{Y_{t,Fisch}} \quad \text{(Equ. 3)}$$

and hence, daily feeding rate (F_t) is calculated by

$$F_t = FCR_t \times Y_{t,fish} \quad \text{(Equ. 4)}$$

b) Fish faeces

Fish do not use the complete amount of N and P of the provided feed for synthesis of biomass. Over 70% are excreted as dissolved nutrients (esp. ammonia-nitrogen) and solids. These unused nutrients form the basis for the integrated modules “detritivorous culture tank” and “macroalgae”.

The total nutrient entry ($entry_{nutrients}$) into the recirculation system can be calculated from the feeding rate per day (F_t) and the nutrient concentration of the feed ($c_{x,feed}$):

$$entry_{nutrients,x} = \frac{F_t \times c_{x,feed}}{100} \quad \text{(Equ. 5)}$$

where c_x is the fraction of each nutrient component (protein, PON, POP, organic matter (OM) or dry weight (DW) of the feed, Tab. 1).

Tab. 1 Feed composition (BIOMAR Ecostart 17), apparent digestibility coefficients (ADC, 20-24°C) and nutrient concentrations of Seabream (*Sparus aurata*) (Lupatsch & Kissil, 1998)

nutrient [x]	fraction in feed [$c_{x,feed}$ in %]	Digestibility [ADC_x in %]	fraction in fish [$c_{x,fish}$ in %]
protein	50	82.8	17.8
nitrogen (PON)	8	82.8	2.85
Phosphor (POP)	1.4	47.8	0.72
org. matter (OM)	79.9	68.9	
dry weight (DW)	90.4	60.7	

Faeces are defined as undigested remains of the feed. Based on experimentally derived apparent digestibility coefficients (ADC) for Gilthead seabream (Lupatsch & Kissil, 1998) and the composition of the feed (Tab. 1) the amount of expected solid faeces per day ($faeces_{x,t}$) for each nutrient was calculated by

$$faeces_{x,t} = entry_{nutrients,x} \times \frac{100 - ADC_x}{100} \quad \text{(Equ. 6)}$$

Once released into water, the excreted faeces are subject to gradual changing of nutrient composition with time due to passive leaching. Leaching processes are considered to be terminated after 6 hours retention time in water (Lupatsch & Kissil, 1998; Tab. 2). For the numeric model average numbers for the remaining matter (RM_{mean}) were used.

Tab. 2 Remaining matter (RM, %) of the solid faeces for each nutrient (x) after 6h, 24h and 48h water retention time for Gilthead seabream (following Lupatsch & Kissil, 1998)

nutrient [x]	RM after 6 hours	RM after 24 hours	RM after 48 hours	RM mean
nitrogen (PON)	57.4	54.9	56.8	56.4
phosphor (POP)	88.3	81.5	84.0	84.6
org. matter (OM)	59.0	57.8	57.8	58.2
dry weight (DW)	65.0	63.7	64.6	64.4

Due to leaching, faeces can be divided into a soluble part ($faeces_{soluble}$) and unsoluble part ($faeces_{unsoluble}$) by

$$faecesSoluble_{x,t} = \frac{faeces_{t,x} \times (100 - RM_{mean})}{100} \quad \text{(Equ. 7)}$$

$$faecesUnsoluble_{x,t} = \frac{faeces_{t,x} \times RM_{mean}}{100} \text{, respectively} \quad \text{(Equ. 8)}$$

c) fish biomass synthesis (= retention)

Retention of N and P in fish biomass was assessed by considering the yield of fish ($Y_{t,fish}$) and the nutrient fraction in fish ($c_{x,fish}$; Tab. 1) by

$$retention_{x,t} = \frac{Y_{fish,t} \times c_{x,fish}}{100} \quad \text{(Equ. 9)}$$

d) excretion

In contrast to faeces, all metabolic products from fish are termed as excretion. The excreted dissolved proportions of N and P in form of total ammonia nitrogen (TAN) and orthophosphate were determined by the following equation:

$$excretion_{x,t} = \frac{F_t \times c_{x,feed}}{100} - retention_{x,t} - faeces_{x,t} \quad \text{(Equ. 10)}$$

For the model, a minor importance of excreted urea or the rapid conversion of urea to ammonia was assumed. Consequently, total ammonia-nitrogen (TAN) is considered to be the only excreted dissolved nitrogen compound.

Module Foam Fractionation

Total daily amount of faeces (dry weight, DW) in relation to total nutrient entry (DW) (Equ. 5) is 39.3% calculated according to Equ. 6 ($faeces_{DW,t}$) and Tab. 1 (digestibility of DW). The unsoluble fraction can be determined according to Equ. 8 ($faeces_{unsoluble_{DW,t}}$) and Tab. 2 ($RM_{mean}$ DW) 25,3%. The amount of unsoluble faeces can be divided into a fraction of settable solids and a fraction of suspended solids.

The fraction of suspended solids is removed from the process water by foam fractionation. This process is considered to be very effective, because the turbidity in a closed recirculation system with a foam fractionator for removal of suspended solids is at constant low levels of <7mg dry weight L^{-1} (Waller et al., 2003).

The efficiency of the foam fractionators was used to determine the amount of suspended solids as fraction of nutrient entry ($entry_{nutrients}$). Assuming the amount of suspended solids separated by foam fractionation to correspond to the daily load of suspended solids in the recirculation system (experimental observations, this study), the following equation can be used:

$$SuspendedSolids_{x,t} = F_t \times \frac{c_{x,feed}}{100} \times \frac{EfficiencyFoamFractionators}{100} \qquad \text{(Equ. 11)}$$

Data concerning dry weight and C/N ratio of removed suspended solids were available from rinsing water samples of the foam fractionators: regression analysis of experimental data (removed suspended solids vs. nutrient uptake) resulted in 9.5% for the efficiency of foam fractionation (see Fig. 9, subsection 3.2.1 *Module foam fractionator*).

Module Detritivorous Reactor

The proportion of settable solids can be estimated according to Equ. 11 assuming 100% of settable solids to be found in the detritivorous reactor:

$$SettableSolids_{x,t} = F_t \times \frac{c_{x,Futter}}{100} \times \frac{25{,}3 - EfficiencyFoamFractionators}{100} \quad \text{(Equ. 12)}$$

This proportion is used as nutrition source by *Nereis diversicolor*. The relative growth rate of the polychaete is basically depending on the amount, the nutrient composition and the energy content of the fish faeces (Bischoff, 2003). Growth experiments with faeces from a swirl separator of a closed recirculation system showed that growth rates up to 2,8% d^{-1} are possible, if the feed energy content is sufficient (Bischoff, 2003).
Growth of worms ($\mu_{worm,t}$) can be described by

$$\mu_{worm,t} = \frac{(\mu_{\max} - \mu_{\min}) \times E_{settablesSolids,t}}{K_s \pm E_{settableSolids,t}} + \mu_{\min} \quad \text{(Equ. 13)}$$

where $E_{settableSolids,t}$ is the daily energy load derived from settable solids per individual worm [KJ d^{-1} $worm^{-1}$], μ_{max} the maximum growth rate [at $E_{settableSolids,t} = \infty$ in % d^{-1}], μ_{min} the minimum growth rate [at $E_{settableSolids,t} = 0$ in % d^{-1}] and K_S is the Michaelis constant [KJ d^{-1} $worm^{-1}$].

The energy load of settable solids (DW) ($E_{settableSolids_{DM,t}}$) reaching the detritivorous reactor is defined by:

$$E_{settableSolids,t} = \frac{SettableSolids_{DM,t} \times c_{E,settableSolids}}{n_{worm,t}} \quad \text{(Equ. 14)}$$

where c_E is the energy content of the settable solids ($C_{E,settableSolids}$) and the number of worms ($n_{worm,t}$) in the detritivorous tank.

The yield (weight increment) of worm ($Y_{worm,t}$) can be calculated by using Equ. 13 and 14:

$$Y_{worm,t} = \frac{\mu_{worm,t} \times (W_{worm,t} \cdot n_{worm}, t)}{100} \qquad \text{(Equ. 15)}$$

The average wet worm weight ($W_{worm,t}$) at any production day (t) is:

$$W_{worm,t} = W_{worm,t-1} + \frac{\left| \frac{W_{worm,t-1} \times \mu_{worm,t}}{100} \right| + \frac{W_{worm,t} \times \mu_{worm,t}}{100}}{2} \qquad \text{(Equ. 16)}$$

Equ. 16 contains a mathematical operation, if μ is negative: the daily yield is summed with the absolute value and afterwards divided by 2 to ensure that the average worm weight can not decrease. Thus, in the model the smallest Y is 0. This approximation was performed because it can be assumed that *Nereis* sp. does not build up considerable biological reserves (e.g. lipids) to endure starvation periods and therefore die after rather short starvation periods. Accordingly, the model converts negative μ into a natural mortality rate per day ($M_{N,worm,t}$):

$$M_{N,worm,t} = \frac{Y_{worm,t} - |Y_{worm,t}|}{2} \times \frac{1}{W_{worm,t}} \qquad \text{(Equ. 17)}$$

The same mathematical operation ensures, that only $\mu<0$ are converted to dead individuals per day. In this case $n_{worm,t}$ is decreasing calculated by:

$$n_{Worm,t} = n_{worm,t-1} - M_{N,t-1} \qquad \text{(Equ. 18)}$$

The model ensures that the worm numbers are decreasing, if $\mu_{worm,t}<0$. This process is stopped, when the energy load of settable solids per worm ($E_{settable-solids,t}$) is sufficient to ensure worm metabolism ($\mu_{worm,t}\geq0$). Additionally, the

model assumes the dead worms to be consumed by the living worms. So total energy load per day can be described as follows:

$$EnergyloadTotal_t = E_{settableSolids,t} + M_{N,worm,t} \times W_{worm,t} \times c_{DM,worm} \times E_{worm} \qquad \text{(Equ. 19)}$$

Data for $c_{DM,\,worm}$ (0.14 ± 0.002 g kg^{-1}) and E_{worm} (15.0 ± 0.8 KJ g^{-1}) were derived from literature data (Bischoff, 2003).

Cannibalism is described for this worm species (Hartmann-Schröder, 1996) and therefore has to be considered in the model. This option only has to be taken into account, when the natural mortality is lower than the mortality by cannibalism ($M_{C,worm,t}$). It appears to be unlikely, that the worms exhibit cannibalism when there are enough dead individuals available. If the $M_{N,worm,t}$ = 0, it is replaced in Equ.19 by $M_{C,worm,t}$ at the same rate (1.3% d^{-1}).

Module Macroalgae Assimilation

TAN (= total ammonia nitrogen) uptake by macroalgae was determined by the daily amount of TAN in the macroalgae tank ($B_{TAN,t}$) of the MARE-system. The uptake rates of TAN by macroalgae ($r_{TAN,algae,t}$) can be described following Michaelis-Menten-kinetics:

$$r_{TAN,algae,t} = \frac{r_{max,TAN,algae} \times B_{TAN,t}}{K_s + B_{TAN,t}} \qquad \text{(Equ. 20)}$$

where $r_{max,TAN,algae}$ is the maximum uptake rate, $B_{TAN,t}$ the amount of TAN available in the macroalgae tank and K_s the half saturation constant. Relative uptake of TAN in relation to $B_{TAN,t}$ can be described by the same kinetics:

$$r_{TAN\%,algae,t} = \frac{r_{max,TAN\%,algae} \times B_{TAN,t}}{K_s + B_{TAN,t}} \cdot \frac{100}{B_{TAN,t}} \qquad \text{(Equ. 21)}$$

$B_{TAN,t}$ can be calculated by using the average TAN concentration in the system water ($c_{TAN,t}$) and the flow rate (R) through the macroalgae tank:

$$B_{TAN,t} = c_{TAN,t} \times R \quad \text{(Equ. 22)}$$

The yield (biomass increase in the macroalgae tank) is described by N-assimilation of macroalgae determined by the amount of nitrogen in the macroalgae biomass ($c_{N,algae}$):

$$Y_{algae,t} = r_{t,TAN,algae} \times \frac{100}{c_{N,algae}} \times \frac{100}{c_{DW,algae}} \quad \text{(Equ. 23)}$$

where $c_{N,algae}$ is the content of N in the algae biomass and $C_{DW,algae}$ the dry weight of the algae. For the modelling the used values were for c_{Nalgae} 6% N DW^{-1} and for c_{DW} 14% of the fresh weight.

The uptake of phosphorus was estimated via the TAN uptake with respect to a Redfield ratio of 16. In consideration of the molar weight of the atoms the ratio is 7.3, so it is

$$r_{t,P,algae} = \frac{r_{t,TAN,algae}}{7.3} \quad \text{(Equ. 24)}$$

Module Nitrification

In contrast to conventional recirculation systems the MARE-system was operated without any additional nitrifying biofilter. However, nitrification was performed because no significant increase in TAN or nitrite was observed. This ammonia-converting process plays an important role, because on the one hand, it competes with the ammonia uptake of the macroalgae tank. On the other hand these two processes may be cumulative, leading to an efficient ammonia removal. The sediment of the detritivorous reactor increased the biologically active surface probably enhancing settlement of nitrifying

bacteria. Thus, the integration of an additional nitrifying biofilter was not necessary.

Experimental results of the performance of a fluized-bed biofilter from a technical recirculation system were used for modelling the daily nitrifying activity of these additional “nitrifying surfaces” (Fig. 3). Temperature, salinity and turbidity (factors which could influence nitrification) were comparable in both systems.

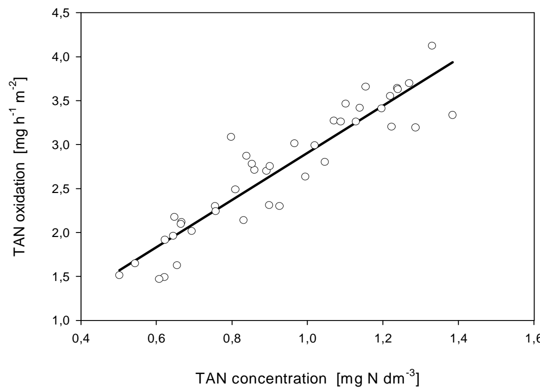

Fig. 3 Performance of a nitrifying fluized-bed biofilter of a closed recirculation system, stocked with Sea Bass (*Dicentrarchus labrax*), in relation to the inflow ammonia concentration (Wecker and Orellana, unpub.). The nitrifying activity was determined by the concentration difference of inflow and outflow. It only considers the oxidation from ammonia to nitrate and is given by the slope (s=2,68; r^2=0,86).
These data do not include data of zero order kinetic, but ammonia values in the system did not reach more than 1mgL^{-1} TAN in average in the MARE-system.

For simplifying reasons the model assumed ammonia to be completely converted to nitrate. This was reasonable, because nitrite concentrations were rather low during the experimental period in the MARE-system.

Via the slope (s) from Fig. 3 nitrification was calculated as follows:

$$r_{nitrification,t} = s \times c_{TAN,t} \qquad \text{(Equ. 25)}$$

where $c_{TAN,t}$ is total ammonia nitrogen (TAN) concentrations of the MARE-system. It is were calculated by:

$$c_{TAN,t} = \frac{excretion_{TAN,t} + \left(c_{TAN,t-1} \times V - r_{nitrification,t-1} - r_{TAN,a\lg ae,t-1}\right)}{V} \qquad \text{(Equ. 26)}$$

where $c_{TAN,t-1}$ is the total ammonia-nitrogen (TAN) concentration in the MARE-system from the previous day, V is MARE-system volume (4500l) and $r_{nitrification,t-1}$ is the nitrifying activity of bacteria potentially attached to walls and tubes from the previous day and $r_{TAN,algae,t-1}$ is the uptake rate of TAN by macroalgae from the previous day.

Total surface of MARE was calculated with 60m², (30m² walls and tubes, 30m² sediment). Experimental results of ammonia oxidation rates within the sediment yielded 1,78±0,77g d^{-1} (Bischoff, unpub.).

Module Denitrification

Denitrification is an anaerobic process and plays an important role for the nitrogen removal from the recirculation system. Within the MARE system anaerobic conditions are expected to be prevalent in the lower sediment centimetres of the detritivorous reactor.

Denitrification can be calculated based on of the amount of organic matter ($B_{OM,t}$) left by the worms for denitrifying bacteria. Assuming that no organic matter is accumulating (experimental data: organic matter of sediment during MARE-experiment: 2.4 ± 0.5%), $B_{t,OM}$ is calculated on a daily base by:

$$B_{OM,t} = SettableSolids_{OM,t} - Y_{worm,} \times c_{OM,worm} \qquad \text{(Equ. 27)}$$

Furthermore, for practical reasons it was assumed that the bacteria are utilizing the complete amount of $B_{t,OM}$ for the reduction of nitrate to N_2. Thus, denitrification rates were calculated as follows:

$$r_{denitirfication,t} = z \cdot B_{t,OM} \qquad \text{if } c_{nitrate,t} \geq 0 \qquad \text{(Equ. 28)}$$

where z is the ratio between organic matter needed for the reduction of 1mg nitrate-N. OM:N ratio was calculated to be 3.

The nitrate concentration of the recirculation water in the MARE system was calculated by:

$$c_{t,nitrate} = \frac{c_{t-1,nitrate} \cdot V + 0.98 \cdot r_{t-1,nitrification} - r_{t-1,denitrification}}{V} \quad \text{(Equ. 29)}$$

Module Bacterial Assimilation

During nitrification and denitrification bacteria are assimilating N and P for biomass production. Heterotrophic denitrifying bacteria can produce more biomass than autotrophic nitrifying bacteria, because energy yield per mol of electron donator (NH_4^+, NO_2^-) from nitrification is relatively low (Rheinheimer *et al.,* 1988). Utilization of ammonia-nitrogen by *Nitrosomonas* provides 0,015mg organic N per mg utilized N, while oxidation of nitrite-N by *Nitrobacter* gives 0,005mg organic N (Rheinheimer *et al.*, 1988). During denitrification 0,065mg organic N is formed by the complete reduction of 1mg nitrate-N with methanol as hydrogen donator (McCarty *et al.*, 1969). So N- assimilation by bacteria can be calculated as follows:

$$r_{N,bacteria,t} = 0.020 \times r_{t,nitrification} + 0.065 \times r_{t,denitrification} \quad \text{(Equ. 30)}$$

For the calculation of P-assimilation the stoichiometric ratio between P and N in bacterial biomass was used. The empirical formula $C_{250}H_{611}O_{77}N_{55}P_6S$ describes the average chemical composition of bacteria, independent from the species (Rheinheimer *et al.*, 1988). Thus, the stoichiometric N:P ratio is 9.2. Considering the molar mass of the atoms, the weight ratio is given by 4.2.

So P-Assimilation is calculated by:

$$r_{P,bacteria,t} = \frac{r_{N,bacteria,t}}{4.2} \quad \text{(Equ. 31)}$$

Combination of all modules – simulation of the MARE-system

Modelling the nutrient budget in the MARE-system was performed with the objective to understand the biological processes and crosslinks in the experimental recirculation system. All presented modules individually showed a good correlation between experimental and modelled data. So it can be supposed that all essential processes were adequately modelled. In a simulation of the complete model a coupling of all stated modules (Fig. 2) was tested.

Nitrate and phosphate concentrations are chosen to be the monitored values because of the following reasons: (1) they are simple to analyse, (2) they show very low daily variations, (3) concentration changes happen very slowly and (4) they are influenced by all individual modules.

Nitrate concentrations were calculated including nitrification, denitrification and bacterial assimilation:

$$c_{t,nitrate} = \frac{c_{t-1,nitrate} \cdot V + 0.98 \cdot r_{t-1,nitrification} - r_{t-1,denitrification}}{V} \qquad \text{(Equ. 32)}$$

Phosphate concentrations can be tolerated by fish at high concentrations, so removal of phosphate plays a minor role in recirculation systems. However, the removal of phosphate must be taken into account, because over a longer period it will accumulate in the system and may result in a water exchange and can be mainly found in unsoluble faeces and excretory products. Considering phosphate uptake by algae and bacteria, phosphate concentrations were calculated by:

$$c_{t,P} = \frac{excretion_{P,t} + faecesUnsoluble_{P,t} + \left(c_{P,t-1} \cdot V - r_{P,bacteria,t-1} - r_{P,algae,t-1}\right)}{V} \qquad \text{(Equ. 33)}$$

2.3 Results

2.3.1 Feasibility of the MARE-system

Water parameters

During the 5-months MARE experiment primary water quality parameters were always within safe limits for all cultured organisms: concentrations of toxic substances like nitrite and ammonia were at tolerable concentrations (0.36 ± 0.33 mg L^{-1} for ammonia and 0.009 ± 0.12 mg L^{-1} for nitrite during the entire experimental period (11.11.2004 – 1.6.2005). Nitrate was decreasing from 49.40 mgL^{-1} to less than 1.45 ±0.03 mg L^{-1}, respectively. pH was stable at 7.97 ± 0.24 but showed a slight decrease at the very end of the experimental period. However, pH did never reach critical values below 7. Oxygen levels averaged 5.73 ± 1.20 mg L^{-1} during the entire experimental period.

Additionally, MARE fulfilled the demands of a closed recirculation system (less than 10% water exchange per day) (Losordo *et al.*, 1999) The daily water replacement rate due to evaporation, foam fractionation and losses by maintenance or biomass determination averaged 0.8% of the total system volume (Fig. 4a). No water was exchanged during the entire recirculation system operation period from April 2004 to December 2005.

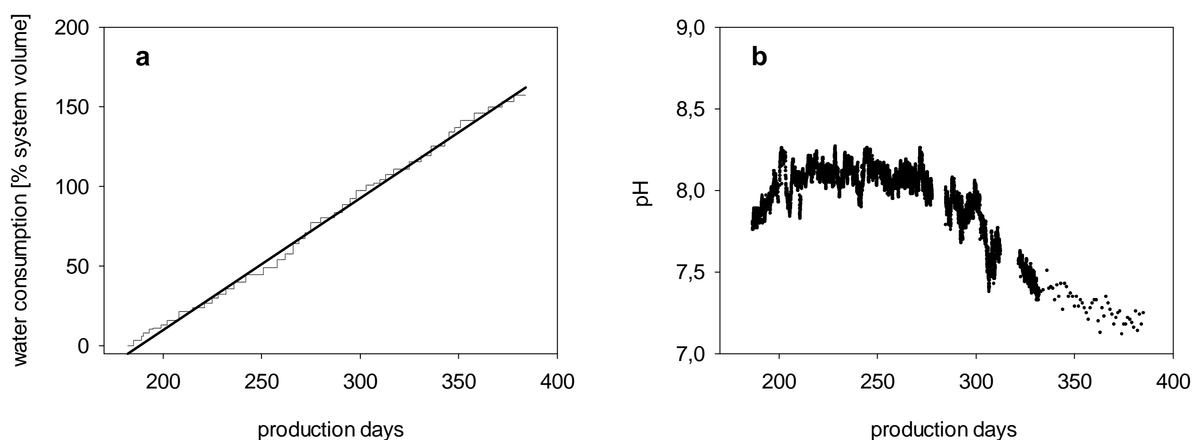

Fig. 4 (a) Presentation of the cumulative water replacement of the MARE system during the experimental phase. The daily replacement results from the slope of linear regression, which was 0.8% per day. (r^2 = 0.996). (b) Development of pH value during the MARE trial.

Fish growth

Fig. 5 shows the growth of *Sparus aurata* in the MARE-system over the experimental phase of 400 days (data shown from Tank 1). The experimental period started at after *Sparus aurata* was already cultured for 182 days in the tank showing weights of 71 ± 12g (Tab. 3). Table 3 shows the data from the biomass determinations during the MARE trial. For the numeric modelling experimental data of fish weight (Equ. 1) were taken from fish tank 1. Average feed conversion ratio (FCR) for tank 1 was 1.09 ± 0.32 and ranged from 0.71 to 1.64.

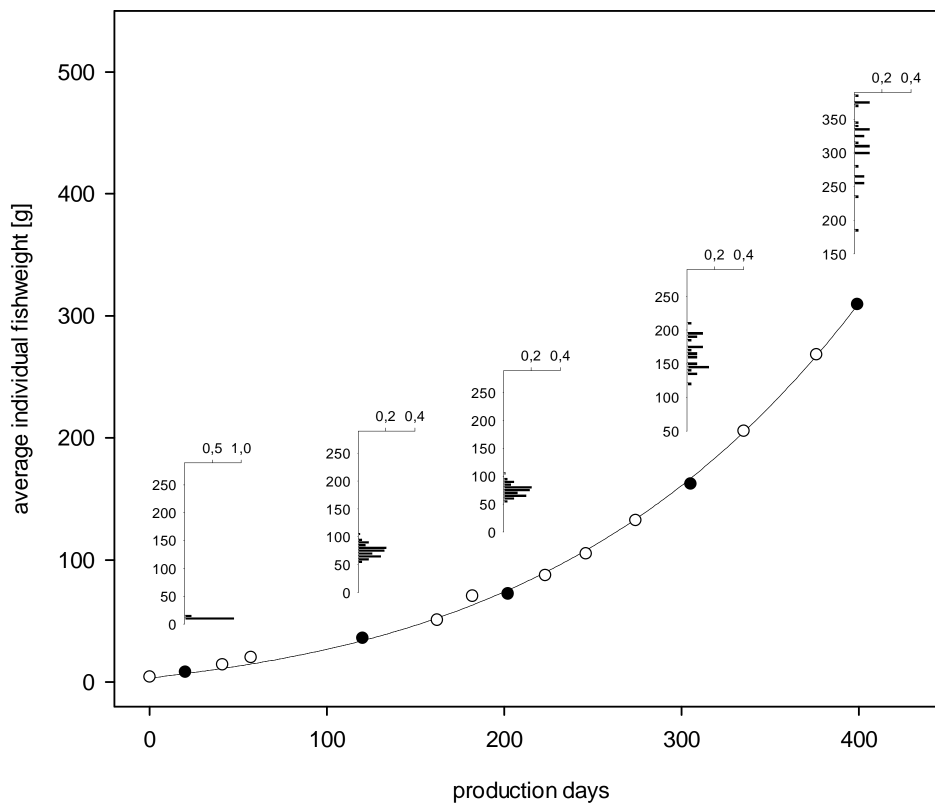

Fig. 5 Growth of *Sparus aurata* over a cultivation period of 400 days (tank 1). Average individual fishweights are shown in relation to time. Size distributions of *Sparus aurata* are indicated for the black spots. Growth is described by $\ln G_t = a + b \cdot t^{0,5}$. Regression analysis gave a=0.840; b=0.245; r^2=1.00.

Tab. 3 Average fresh weight, total length (TL) and stocking densities of *Sparus aurata* cultured in the MARE system during the experimental phase per m³ tank volume. A subsample of at least 20 fishes from each tank was taken for measurements.

date	production day	Tank 1 av. weight [g]	av. TL [mm]	stocking [kg m⁻³]	Tank 2 av. weight [g]	TL [mm]	stocking [kg m⁻³]
11.11.04	182	71±12	160±10	3.6	62±10	155±12	3.2
01.12.04	202	73±12	161±8	3.7	78±12	167±9	4.0
22.12.04	223	88±13	174±8	4.5	90±13	179±7	4.6
14.01.04	246	105±15	185±9	5.4	104±15	186±10	5.3
11.02.05	274	133±16	200±8	6.8	132±16	201±10	6.7
14.03.05	305	162±24	213±10	8.3	159±17	211±9	8.1
13.04.05	335	206±27	226±11	10.5	190±15	222±12	9.7
24.05.05	376	268±36	244±11	13.7	242±10	242±11	12.4
16.06.05	399	310±48	259±13	15.8	280±10	251±11	14.3

2.3.2 Modelling the nutrient budget

VALIDATION OF SINGLE MODULES

The nutrient budget plays an important role for comprehensive analysis of the system and potential future upscaling. For size optimization of the single modules with respect to the biological and physico-chemical interactions within the system a model was developed in order to be able to trace the pathways of the nutrients provided by the feed.

Module Fish

a) fish growth and feeding rate

Experimental data for feeding rate, absolute and relative growth of fish and FCR are given in Fig. 6a-d and show a good correlation to model data (solid line).

The experimental feeding rate was below the recommendation of the feed producer (Fig. 6a, dashed line). However, absolute and relative growth of the fish followed the model predictions (Fig. 6b,c): with increasing fishweight absolute growth rate is increasing. However, relative growth is decreasing because the specific metabolic rate of the fish is also decreasing with increasing weight (Wehner and Gehring, 1995). Therefore, the proportion of feed used for biomass synthesis also decreases. Consequently, the feed conversion ratio must be increasing with increasing fish weight (Fig. 6d). Experimental data were in accordance to model predictions; the model can therefore be considered as suitable for the derivation of these physiological interactions.

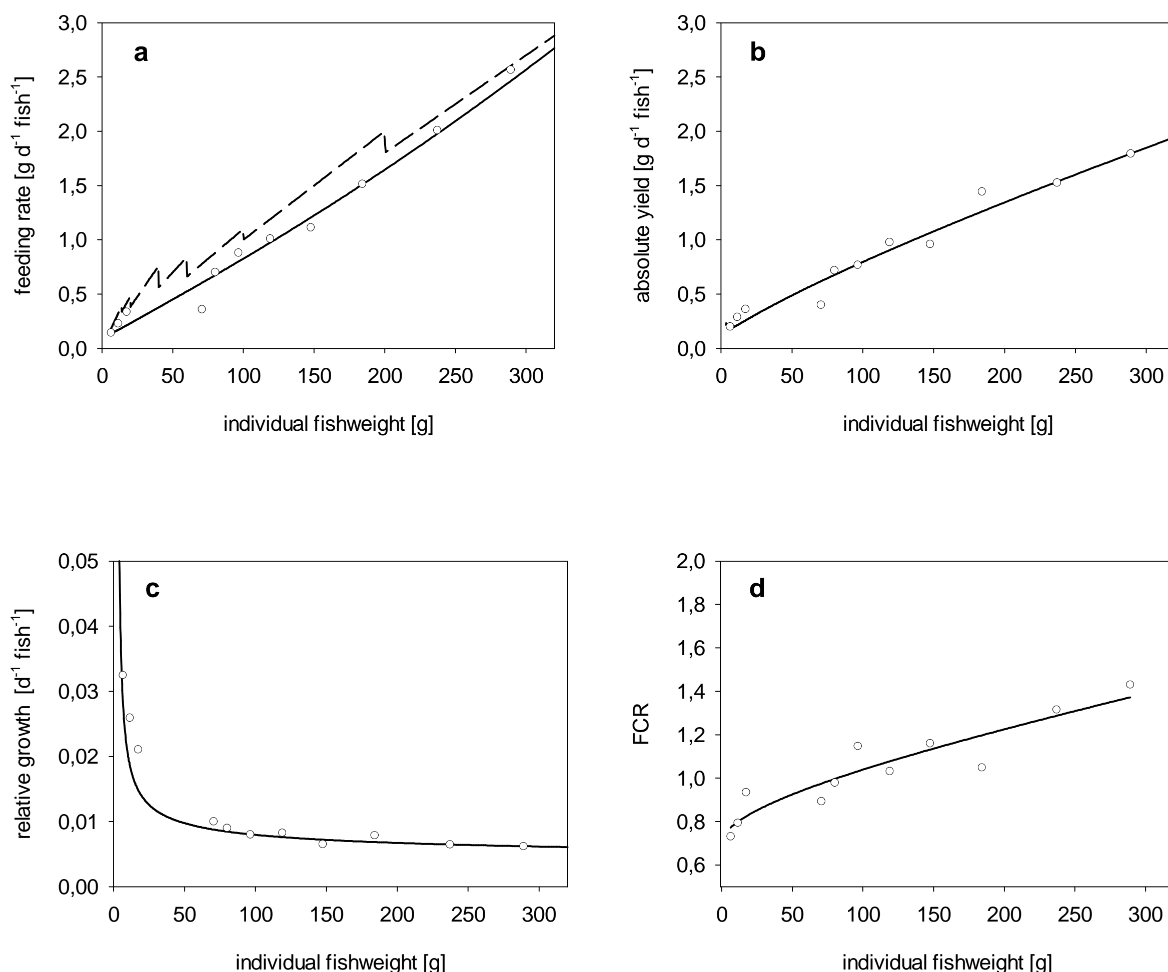

Fig. 6 Validation of the numeric modelling (line) with experimental data from fish biomass determinations (symbols). The dashed line in (a) indicate the recommended feeding rate from the fish feed producer (feeding rate at 20°C). r^2 for (a) 0.98; (b) 0.96; (c) 0.95; (d) 0.86.

b) Fractions of solid faeces, excretion and retention

The proportion of N and P retained in the fish biomass and the proportion of N and P released to the system environment is of major importance for the evaluation of the system concerning fish yield. Fig. 7 shows the modelled, individual nutrient balance per fish in relation to fish weight. According to physiological principles, the feed proportion used for biomass synthesis decreases with increasing fish weight. This leads to a percentage decrease of feed remaining in the fish body. Consequently, the proportion of excreted substances (e.g. urea, ammonia resulting from protein metabolism) is increasing. Accordingly, the proportion of excreted solid nutrients is also increasing.

The relative contributions of the different nutrients to the excretory products vary. Nitrogen is mainly released to the water by excretion processes (urea, ammonia) whereas phosphate is a compound of fish faeces (settable solids, Fig. 7a,b).

Relative proportions of excreted nutrients in relation to total amount of feed nutrients varied over the experimental period according to the changing utilisation efficiency: for example N-excretion rates increased from appr. 50% N d^{-1} nutrient uptake from feed (at 50g fish weight) to appr. 73% N d^{-1} nutrient uptake from feed (at 400g fish weight) (Fig. 7a).

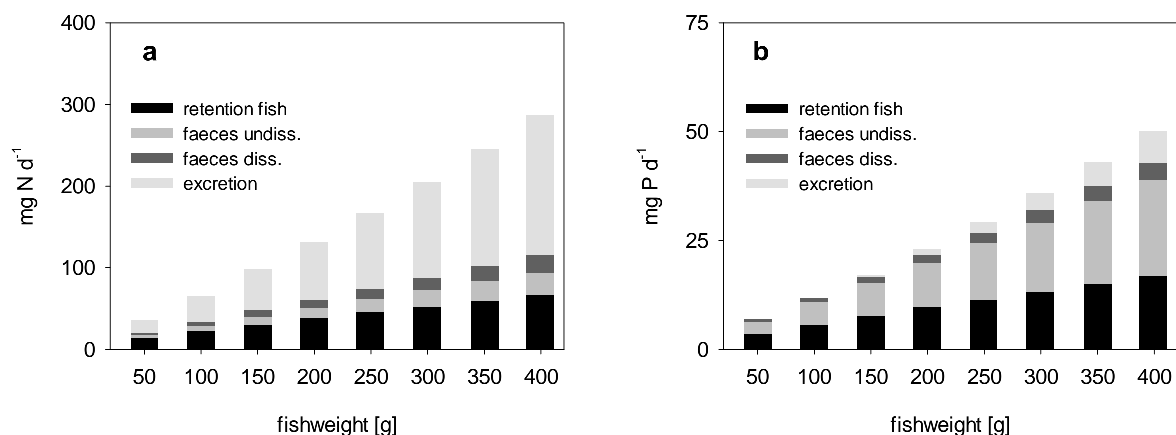

Fig. 7 Modelled nutrient balances for fish in the MARE-system for Nitrogen (a) and Phosphorus (b). The height of the bars corresponds to the feeding rate of each nutrient. The relative fractions are changing with increasing fish weight because of a different utilisation efficiency.

Experimental data for validation of the model are only available for nitrogen. Fig. 8 shows a good agreement between modelled and experimental data for feeding rate (Fig. 6a), feed retention and excretion (Fig. 7a,b).

Experimental data for excretion are derived from the difference of TAN concentrations between inflow and outflow samples of the fishtanks. Raw data of TAN concentrations were expected to be underestimated, because samples were taken before the feeding, but peaks of TAN concentrations occur at least 8 hours after feeding due to diurnal variations of TAN concentrations. 24-hour experiments yielded the factor 2.5 to equalize this underestimation. Therefore, data were corrected with this value. Thus, all model data except the particulate fraction can be validated by experimental values (Fig. 7).

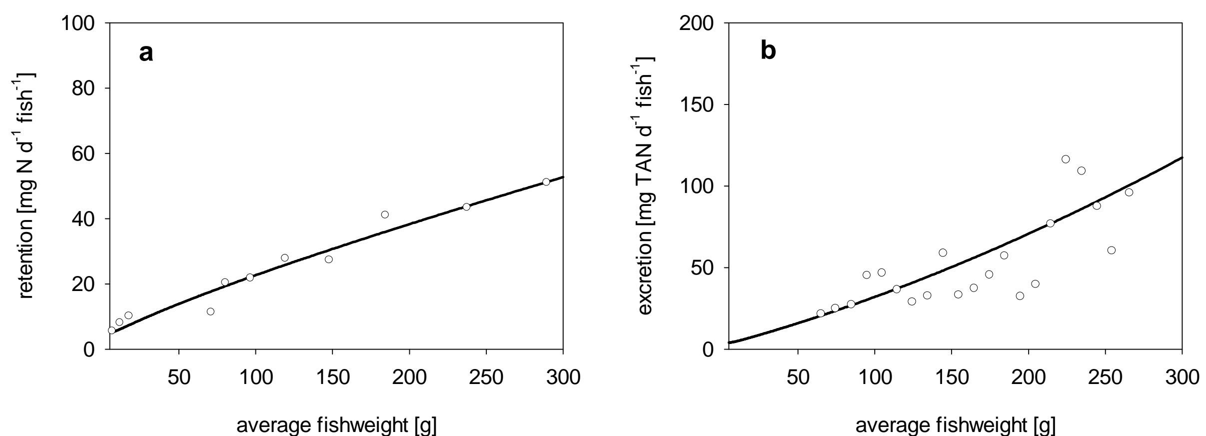

Fig. 8 Validation of the model for nitrogen (line) by available experimental data (symbols): (a) retention (= feeding rate x $Y_{fish,t}$ x fraction in fish, Tab. 1) r^2=0,96; (b) excretion (= concentration difference of TAN between inflow and outflow x flow rate x 2,5); r^2=0,59. Values for fish weight were summarized in 10g weight classes; TAN concentrations are average values of all experimental data from that time period where each weight class was measured (n>5).

It was not possible to validate the model data for phosphorus, because the sensitivity of the orthophosphate analysis for dissolved nutrients was not sensitive enough to measure a difference between inflow and outflow concentrations of the fishtanks. Thus, the applicability of the model for predicting phosphate concentrations can only be tested over the entire experimental MARE-phase (see subection 2.2.3.2).

Module foam fractionation

Foam fractionation removes all suspended particles from the process water. Data from the rinsing water of the foam fractionators were used to determine the foam fractionation efficiency by regression analysis. Maximum values for r^2 were calculated by using Microsoft Excel Add-In "Solver": 9,5% of the feed entry (DW) can be found as suspended solids in the MARE system (Fig. 9). As a result the fraction of settable faeces is 15.8% of feed entry (DW), calculated by Equ. 13.

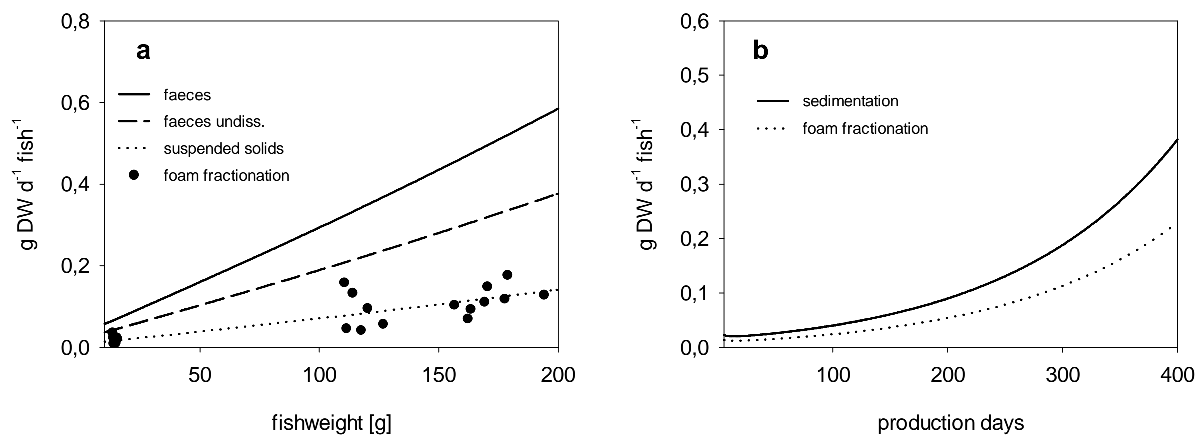

Fig. 9 (a) Modelled total amount of faeces (Equ.7, solid line), fractions of undissolved faeces (Equ. 9, dashed line) and suspended solids (Equ. 12, spotted line) in relation to fish weight. Experimental values (symbols) from the rinsing water of foam fractionation show a good correlation (r^2 = 0,71). (b) Calculated daily amounts of faeces transferred to the detritivorous reactor (sedimentation) and the foam fractionators for the entire production period.

Module Detritivorous reactor

a) Worm growth and biomass development

The daily amount of settable solids (sedimentation from Fig. 9b) is transferred to the detritivorous reactor for utilization by *Nereis diversicolor.*

Fig. 10a indicates that the growth of *Nereis* can be well described by Equ. 13 ($p<0,001$) (data from Bischoff, 2003). Thus, the energy content of the faeces (B_E) is an adequate variable to describe the growth performance of the worms. Gross energy content of the feed in MARE is given as 20.5MJ kg^{-1} by the fish feed producer, digestible energy content was 17MJ kg^{-1} (given by the feed producer), hence the non-digestible proportion was 3.5MJ kg^{-1}. Accord-

ing to Equ. 9 most of the non digestible proportion was detected as undissolved faeces and the estimated energy content of the faeces was 15KJ g^{-1} DW. This value could be confirmed by calorific value measurements (14.6 ± 0.5 KJ g DW, data not shown) of the faeces.

The individual energy load is influenced by the worm number living in the sediment (Equ. 14). The bioreactor was stocked with appr. 2000 individuals of *Nereis* at day 36 with a total biomass of 1.8 ± 0.5kg. The average individual worm weight was 890 ± 260mg (n= 270). Biomass determination at the start of the MARE experimental phase (day 182) resulted in worm numbers approximating 50.000 with a total biomass of 4.4 ±3.7kg (Fig. 10b). Individual worm weight averaged 87 ± 73mg. This clearly indicates a reproduction event between day 36 and 182 (data not shown). After spawning *Nereis diversicolor* dies (monotelic reproduction). The high standard deviation values in the individual weight (± 84%) at the beginning of the MARE experiment indicates that there were still larger *Nereis* individuals which had not yet spawned. Biomass and total number of worms in the bioreactor showed a continuous decrease during the experimental period (Fig. 10b, d). The average worm weight was nearly constant until day 340 and increased at the end of the experimental phase (Fig. 10c).

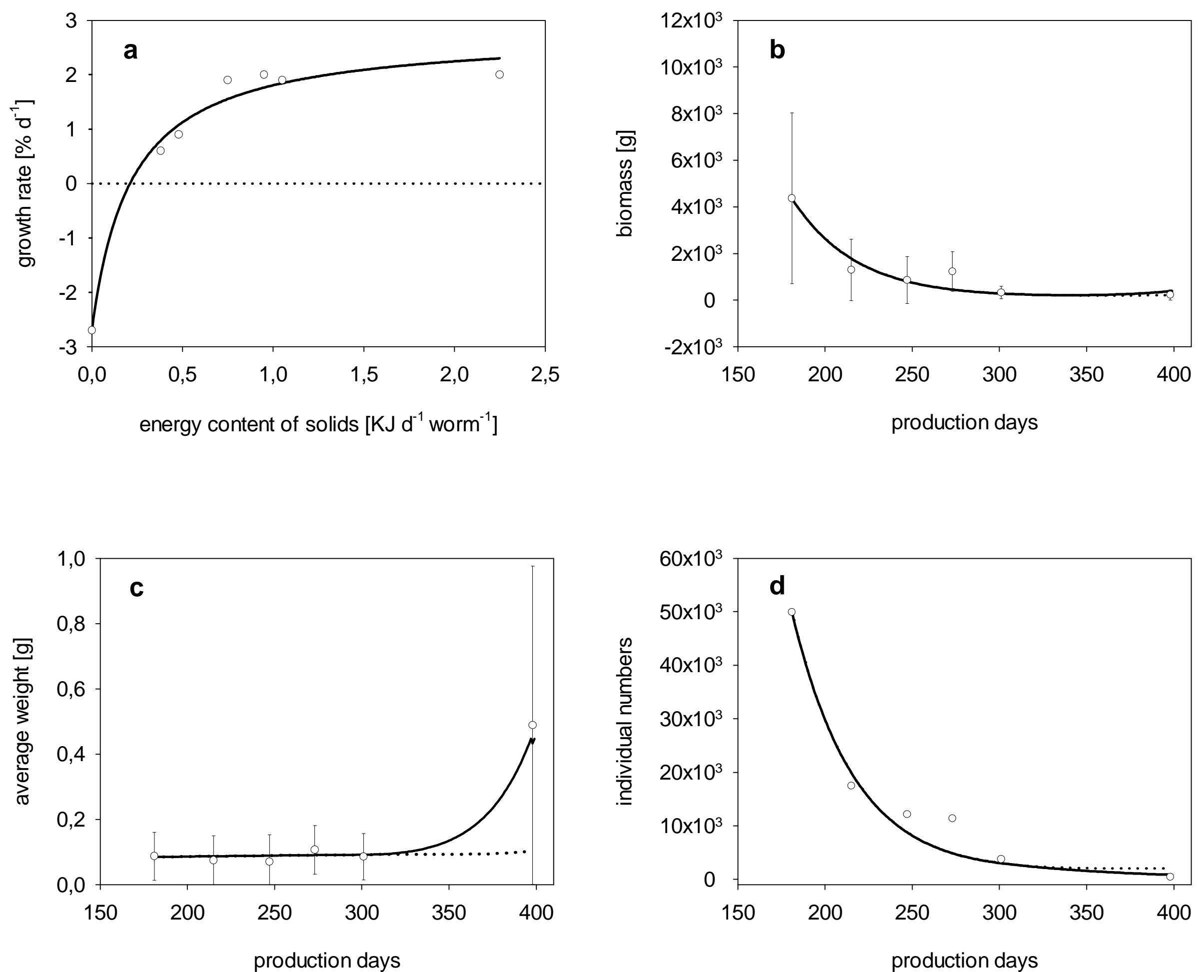

Fig. 10 (a) Growth performance of *Nereis diversicolor* in relation to energy content of the solids (Bischoff, 2003). Regression analysis gave: μ_{max} = 2.78; μ_{min} = -2.72; K_s = 0.22. (b-d) Modelled and experimental data of (a) total biomass, (b) average worm weight and (c) individual numbers of *Nereis diversicolor* in the detritivorous reactor in the MARE system. Dashed lines are calculations without cannibalism, solid line includes a relative mortality of 1.3% d^{-1}. Validation with experimental data gave for (a) r^2 = 0.98 and (b) r^2 = 0.93 and (c) r^2 = 0.99 and (d) r^2 = 0.97.

Considering elevated energy input by dead worms (Equ. 19) and potential cannibalism under certain conditions, model data show a good correlation to the experimental data. By using Equ. 14 relative and absolute worm growth rates can be calculated for the experimental period. The results indicate a feed undersupply of the worms because of the elevated numbers of individuals due to the reproduction event before the start of the experimental period. The energy load provided by the feed (fish faeces) turned out to be not sufficient to satisfy the basic metabolic needs (Fig. 11a). This may explain the negative absolute and relative growth rates until experimental day 340 (Fig.

11b,c), and the increase afterwards, resulting in the increased average worm weight at the end (Fig. 10c).

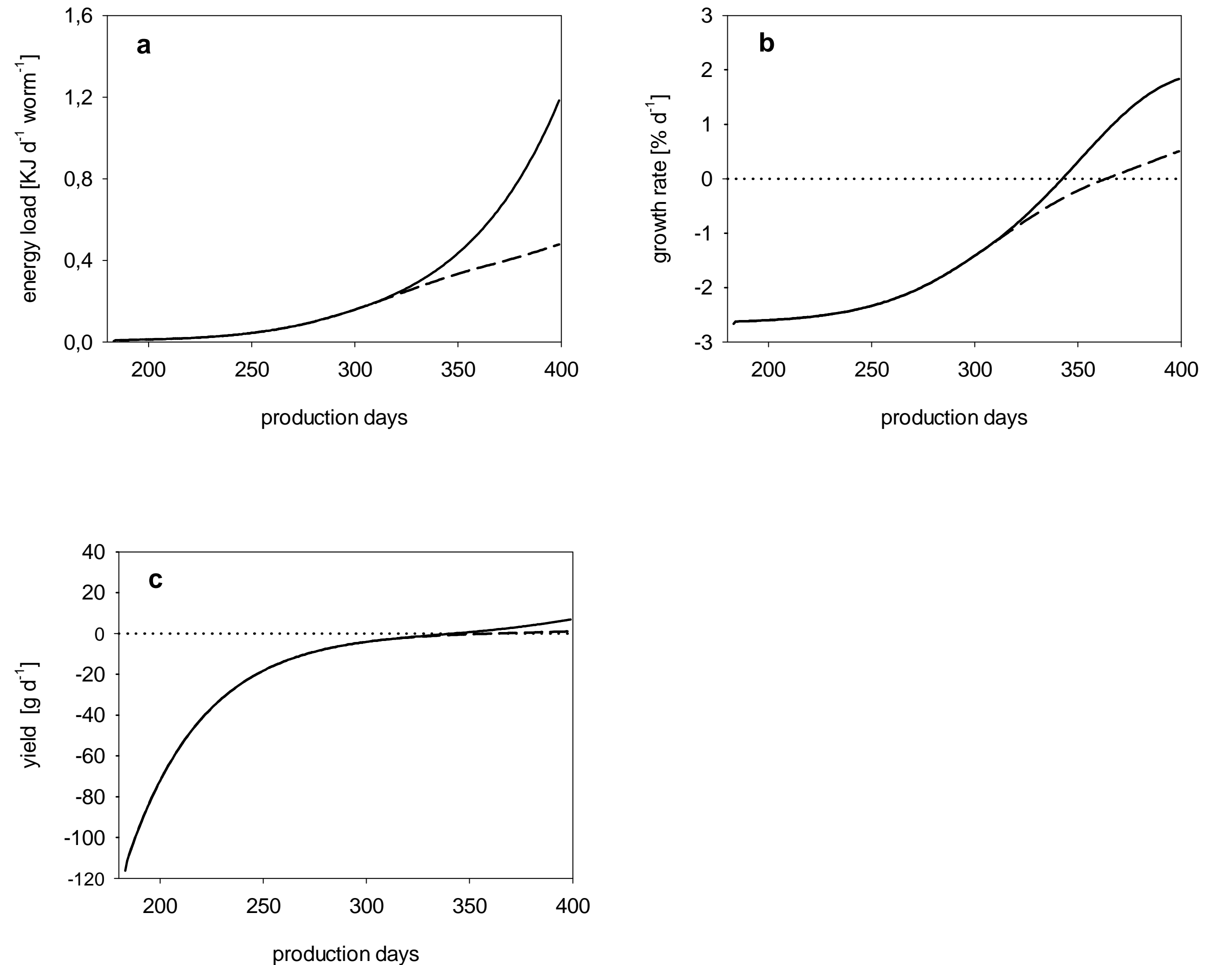

Fig. 11 Modelling of the energy load (a), relative growth rate (b) and absolute growth rate (c) in the detritivorous reactor during the complete MARE experiment. The model was calculated without mortality by cannibalism (dashed line) and regarding a relative mortality of 1.3% d^{-1} (solid line).

Module Macroalgae Filter

Ammonia uptake by macroalgae is described by a Michaelis-Menten-kinetic (Equ. 20). Experimental values show a better correlation to Equ. 21 (Fig. 12b, dashed line, r^2 = 0.75), caused by a better adaptation to low nutrient concentrations in contrast to the absolute uptake rates according to Equ. 20 (solid line). Absolute uptake rates were used for model calculations, because an underestimation of maximum uptake rate in Equ. 21 was expected (r_{max} = 0,06g TAN h^{-1} m^{-2}, Fig. 9a).

Fig. 12c compares modelled and experimental data of ammonia uptake during the MARE trial, which did not show a good correlation due to daily variations of the TAN concentrations and increasing modelled TAN concentrations.

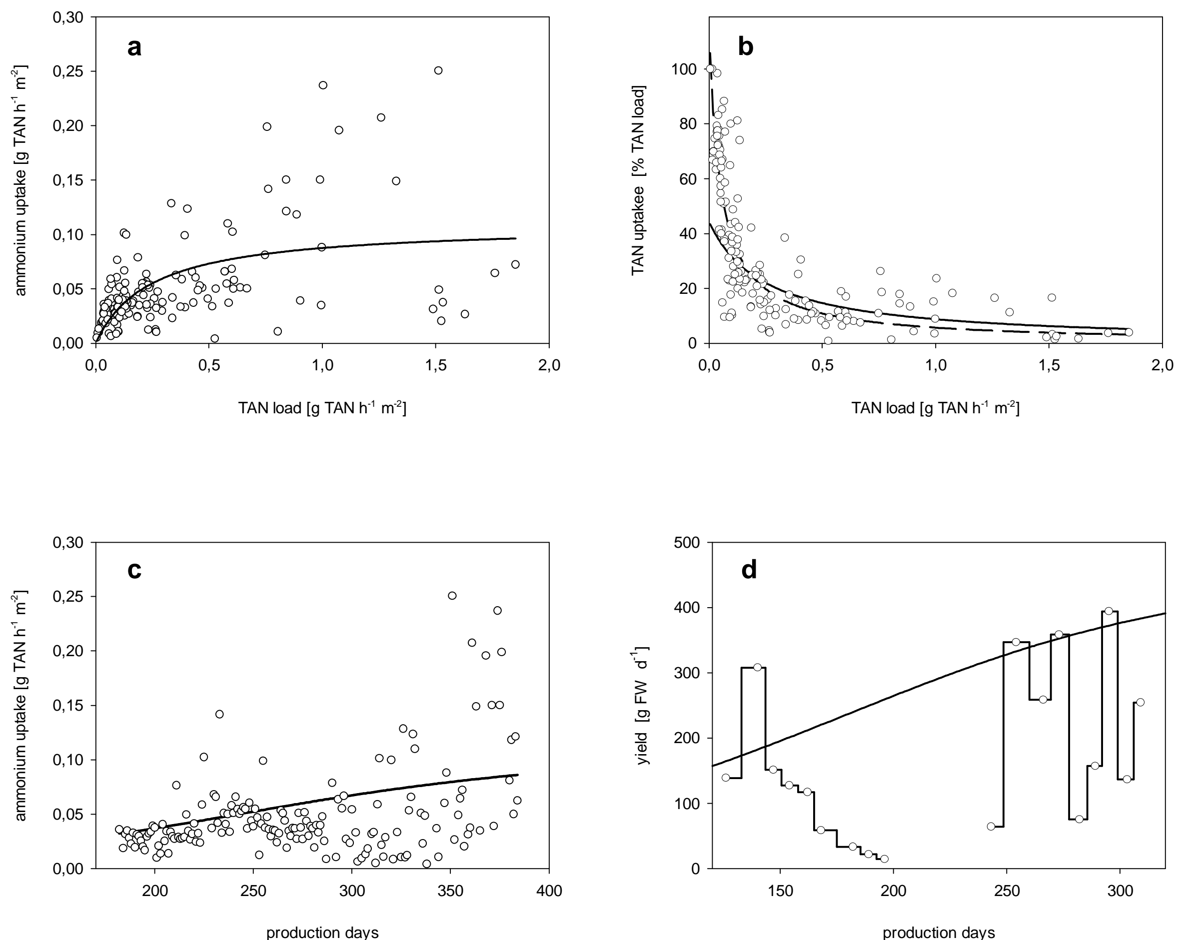

Fig. 12 Macroalgae filter: (a) absolute TAN-uptake in relation to TAN load. Numeric analysis gave for Equ. 26: r_{max} = 0.11; K_s = 0.25; r^2 = 0.28; (b) relative TAN uptake in relation of TAN load. Solid line represents zero-order-kinetic from Fig. 9a (r^2 = 0,64), dashed line from Equ. 25. Regression analysis gave: r_{max} = 0.06; K_s = 0.05; r^2 = 0,75. c) modelled and experimental data of ammonia uptake during the MARE trial. Experimental data are representing the concentration difference between inflow and outflow of the macroalgae tank. d) Modelled and experimental growth yield in the macroalgae tank during the experimental time.

In Table 4 the growth yield of *Solieria chordalis* in the MARE system is shown. At experimental day 125 (15.09.2004) *Solieria* was stocked for the first time. Recommended stocking density was 8kg FW m^{-2} surface area (Sylter Algenfarm, pers. comm.). Therefore, for the MARE-tank with a surface area of 2.3m² a total biomass of 18kg FW was needed. Technical problems

(delivery bottlenecks of the algae farm) resulted in a total stock of only 10kg FW and 11.2kg FW, respectively.

Tab. 4 Start and end stockings of *Solieria chordalis* in the MARE system during the experimental trial. Numbers present fresh weight.

experimental period	days	start stocking density [g]	end stocking density [g]	growth rate FW d^{-1} m^{-2}	specific growth rate [100 d^{-1}]
Solieria chordalis I first stocking					
15.09.04 – 29.09.04	13	10000	11806	60	1.28
29.09.04 – 06.10.04	6	11806	13653	134	2.42
06.10.04 – 13.10.04	6	13653	14561	66	1.07
13.10.04 – 21.10.04	7	14561	15454	56	0.85
21.10.04 – 27.10.04	5	15454	16039	51	0.74
27.10.04 – 10.11.04	13	16039	16800	26	0.36
10.11.04 – 17.11.04	6	16800	17000	15	0.20
17.11.04 – 24.11.04	6	15600	15731	10	0.14
24.11.04 – 10.01.05	46	14331	15000	6	0.10
Solieria chordalis II second stocking					
10.01.05 – 21.01.05	10	11195	11836	28	0.56
21.01.05 – 02.02.05	11	11836	15649	151	2.54
02.02.05 – 09.02.05	6	15649	17201	113	1.58
09.02.05 – 18.02.05	8	17201	20069	156	1.93
18.02.05 – 25.02.05	6	18000	18453	33	0.41
25.02.05 – 03.03.05	5	18000	18785	68	0.85
03.03.05 – 11.03.05	7	18000	20758	171	2.04
11.03.05 – 17.03.05	5	18000	18682	59	0.74
17.03.05 – 30.03.05	12	18000	21054	111	1.31

During the first experimental phase (*Solieria chordalis* I) recommended stocking densities were not reached due to decreasing growth rates. Therefore, a second stocking (*Solieria chordalis II*) was necessary. Additionally, a heavy fouling of epiphytes could be observed and the *Solieria* thalli were in-

terspersed with the green algae *Enteromorpha* sp.. Because of delivery bottlenecks the second stocking could not be performed before 10th January 2005. During the second experimental phase growth rates increased, but strong fouling of epiphytes and *Enteromorpha* growth occurred again after a few weeks.

COMBINATION OF ALL MODULES – SIMULATION OF THE MARE-SYSTEM

The prediction of nitrate and phosphate concentrations in the research recirculation system needs a comprehensive understanding of all biological processes in the MARE system. The presented modules were combined according to Equ. 29 und 30. Modelled and experimental nitrate and phosphate concentrations are shown in Fig. 13a,b.

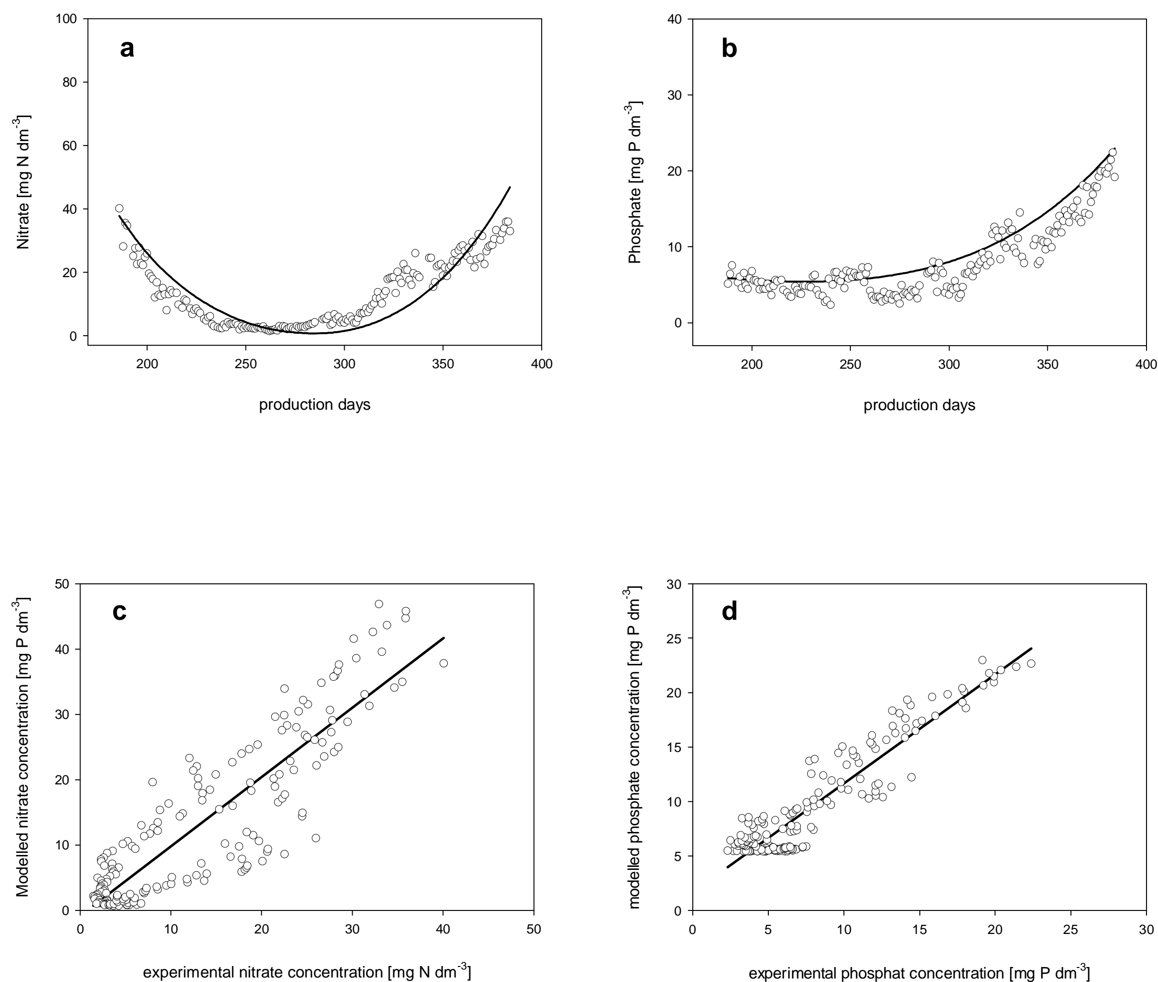

Fig. 13 Experimental (symbols) and modelled data (solid line) of (a) nitrate concentrations (b) phosphate concentrations in the process water of MARE over the experimental period. (c-d) linear regression analysis: Modelled vs. experimental nutrient concentrations (c) $y = 1.05x + 1.38$; $r^2 = 0.84$ (d) $y = 0.92x + 2.04$; $r^2 = 0.87$

Linear regression analysis (Fig. 14c,d) underlines the suitable predictions of the model over the entire experimental period. Standard error of regression was 5.7mg N dm^{-3} for nitrate and 1.8mg P dm^{-3} for phosphate.

The modelled N:P ratio was compared to the experimental data (Fig. 14) and also showed a very good correlation. Both results underline that the MARE model describes the process in the experimental system adequately.

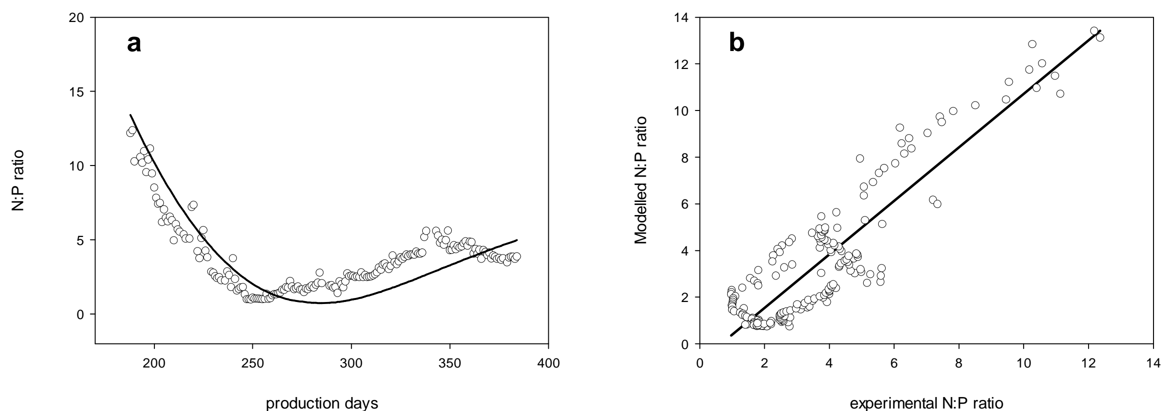

Fig. 14 (a) Modelled (solid line) and experimental data (symbols) of the stoichiometric N:P ratio in the process water over the entire experimental period (b) linear regression analysis: Modelled vs. Experimental stoichiometric N:P ratio: y= 1.04x + 0.30, r^2=0.87.

2.4 Discussion

The major aims of the present study were: a) the operation of the integrated system within safe limits for all cultured organisms (e.g. keeping ammonia and nitrite concentrations low, maintain pH values and oxygen saturation at constant levels, stabilizing nitrate and phosphate concentrations or even reduce them through culture of suitable secondary organisms). b) to test the feasibility of recycling nutrients within the different components of the system. c) achieve a long experimental period to obtain sufficient data for modelling. The different aims are discussed in detail in the following chapters.

2.4.1 Feasibility of the MARE system

The primary aim of this study was to ensure a safe operation of the system. Compared to conventional recirculating aquaculture systems the stock density of fish was low: at the end of the trial it was 7kg m^{-3} based on the system volume, 15kg m^{-3} based on the fish tank volume. Although the concentrations of inorganic dissolved nutrients (ammonia and nitrite) as well as oxygen levels would have allowed a biomass increase, the volume of the fish tanks limited the numbers of fish regarding the welfare of the animals. Larger tanks could not be used due to the localisation and technical features of the laboratory.

Fish grew well in the system, they reached commercial size after 14-15 months. The reduced growth compared to other systems (12-13 months) (Lupatsch and Kissil, 1998; Lupatsch *et al.,* 2003; Colloca and Cerasi, 2005; Fig. 16) was supposed to be caused by lower temperatures, a reduced feeding rate, irregular feeding before and on biomass determination days, larger maintenance/construction work or foam fractionator failures, which occurred especially at the beginning of the experiment. However, in comparison to cultivation in net cages observed growth was faster. Fish culture in net cages resulted in rearing periods of 15-16 months due to lower water temperatures surrounding the cages (Porter *et al.*, 1986; Colloca and Cerasi, 2005).

Noticeable, food conversion ratio was very good (1.0 – 1.3) during the complete experimental period and in the range described for commercial farms (Colloca and Cerasi, 2005). The measured value of 1.1 is below the FCR (1.5) described by Lupatsch and Kissil (1998, Fig 16b) and Porter *et al.* (1986) FCR = 2.7. This might be due to enhanced efforts of fish feed producers in the last few years to adapt feed compositions better to the requirements of the cultured species.

To ensure safe system operation the control of the faeces played a key role, due to the potential of influencing water quality severely (Cripps and Bergheim, 2000). MARE included a two step solid separation (sedimentation and

foam fractionation) to ensure best water quality (Losordo, 1999; Summerfelt, 2002). In contrast to conventional systems the particulate waste (fish faeces and remaining food pellets) was not eliminated from the system, consequently leaching occurred within the first 6 hours (Lupatsch and Kissil, 1998) and could not be influenced. However, concentrations of toxic nutrients and oxygen saturation did not reach critical values in the MARE-system at any time due to efficient macroalgae filter.

The decrease of pH at the end of the experimental period can be explained due to the lower growth performance of the macroalgae (further discussed in subsection 2.4.2). Because of the reduced growth rates of macroalgae the uptake of essential CO_2 for photoautotrophic production resulting from the fish metabolism was not efficient enough. Consequently, carbon dioxide (CO_2) can react in the water to form bicarbonate ions (HCO_3^-) as well as carbonate ions (CO_3^{2-}), depending on the pH value of the water.

2.4.2 Nutrient recycling by integration of secondary organisms (*Solieria, Nereis*)

Second aim of the study was to investigate the feasibility of an enhanced nutrient recycling by integration of biological secondary steps (*Solieria chordata, Nereis diversicolor*). Fig. 17 shows the modelled daily retention of N and P for all cultured organisms during the entire experimental period in the MARE system.

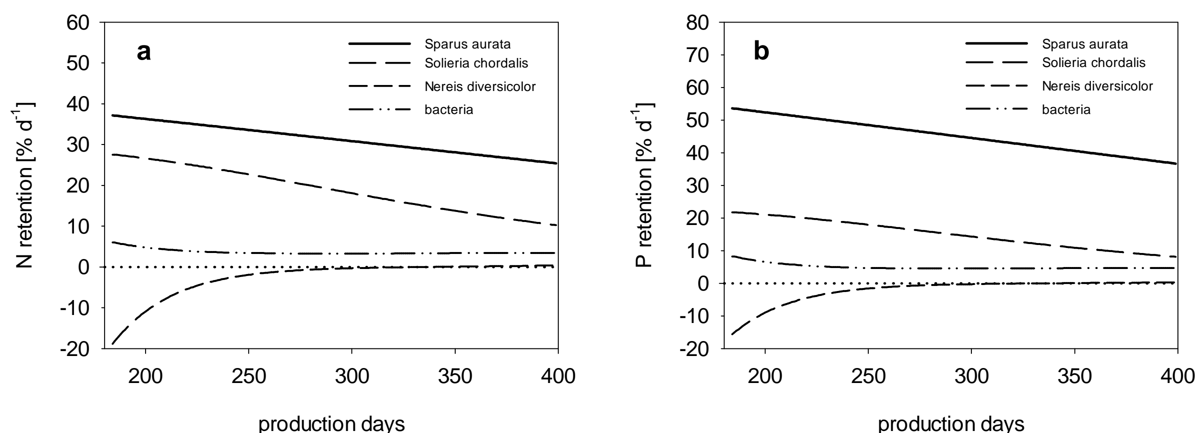

Fig. 15 Daily retention of (a) nitrogen and (b) phosphorus of the nutrient intake by feed for all cultivated organisms in the MARE system during the entire experimental period.

a) Fish unit

As expected, nutrient retention in fish decreased over the cultivation period due to decreased feed utilization with increasing fish size (Lupatsch and Kissil, 1998, Fig. 15a, b). In this experiment the determined values for N retention in fish (31±3%) are within values described in the literature (11-36%, Hargreaves 1998; Paspatis *et al.*, 2000; Van Ham *et al.*, 2003). For P retention in fish (45±5%) values obtained from experiments are higher than described in literature (21-29%; Krom *et al.*, 1985; Porter *et al.*, 1987b; Krom and Neori, 1989; Krom *et al.* 1995). This can be caused by the selected kind of feed, as relative P-rentention depends on type, concentration, availability and digestibility of this nutrient, which can vary due to different sources (fishmeal, fishoil, crops etc.) (Hua and Bureau, 2005).

b) Macroalgae unit

Around 17% of N and 13% of P were additionally retained in macroalgae biomass (Tab. 6). The macroalgae filter was sufficient to ensure low concentrations of nitrite and ammonia in the recirculation water of the MARE-system. However, growth performance of the macroalgae was inadequate and can be explained by the following reasons: concentrations of ammonia were very often only sufficient to cover the minimum physiological requirements of the algae but not enough for significant growth. Additionally, artificial illumination results in lower nutrient uptake rates compared to natural light. *Solieria chordalis* did not switch to nitrate uptake because of the preference for ammonia, which was permanently available at low levels in the tank. It takes at least a 12 hour absence of this nutrient to induce nitrate uptake in *Soleria chordalis* (Lüning, pers. comm.), but such a situation is unlikely to happen in recirculation systems due to a constant availability of ammonia caused by fish metabolism. Although concurrent uptake of ammonia and nitrate is known for macroalgae (Ahn *et al.,* 1998), the measurements clearly showed that *Solieria chordalis* did not take up nitrate. Biofouling of epiphytes occurred as a consequence of the poor growth and insufficient stock density of macroalgae at the beginning of the experiment (recommended stocking density approx. 18 kg m^{-2}). In conclusion, a smaller macroalgae cultivation

unit might have yielded better results with respect to these conditions. Another explanation for the insufficient performance of the macroalgae unit could be a limitation by micronutrients, although it is assumed that sufficient amounts of micronutrients are provided to the system by fish feed (Metaxa *et al.*, 2006). Nevertheless, it might be possible that a negative effect on some essential ions occurred (e.g. oxidizing Fe^{3+} to Fe^{2+}) as a result of the utilization of ozone. Thus, some essential ions may not further be available for the macroalgae. Addition of micronutrients was performed for a limited time period, but results showed no better growth performance of the macroalgae and addition was therefore terminated.

c) Worm unit

Caused by the spontaneous reproduction of *Nereis diversicolor* a biomass loss due to the monotelic behaviour (death of mature worms after the spawning incident) appeared. This resulted in negative growth rates of *Nereis diversicolor* since mature individuals possess a higher bodyweight than juveniles. This further leads to a negative nutrient retention. Simultaneously the reproduction incident increased stock density within the detritivorous tank up to about 50 000 individuals/m^2. The available energy load per worm decreased far below minimum requirements of an individual (Bischoff, 2003). MARE has to be considered as a simplified ecosystem. A destabilisation of this “artificial ecosystem” due to insufficient energy uptake by the worms and high individual worm numbers did not happen because these processes were regulated by decreased survival of juvenile worms. The number of worms decreased until sufficient energy was provided for each worm, and subsequently growth rates and average weight increased to positive values at the end of the experiment. In later experiments positive growth of worms up to spawning size could be observed (see Chapter 5).

In summary, the nutrient budget gives an overview of the retention of N and P in each module of the system for the entire experimental period (production day 182 – 399) in relation to the feed uptake (Tab. 6).

Tab.6 Nutrient budget for nitrogen (N) and phosphorus (P) according to the modelled data. They are presented as sum of absolute and relative amount from the MARE experiment.

	Nitrogen [g]	Nitrogen [%]	Phosphorus [g]	Phosphors [%]
Sparus aurata	1194	30	302	43
Nereis diversicolor	-50	-1	-7	-1
Solieria chordalis	666	17	92	13
Foam fractionation	145	4	62	9
Bacteria	134	3	32	5
Denitrification	1417	35		
Process water	259	6	118	17
Sum	3765	94	599	86
Feed intake	4003	100	701	100
Difference	238	6	102	14

d) Nitrogen recycling

It seems obvious that one of the major processes within the system was denitrification, possibly mainly located in the sediment of the detritivorous reactor: bacterial degradation of the organic matter could create anaerobic conditions in the sediment. Combined with observed nitrate concentrations and organic content in the sediment favourable conditions for denitrification can occur. Nitrate will be reduced to elementary nitrogen during denitrification; ascending gas bubbles could be observed during sediment sampling processes.

Another considerable conversion process of nitrate is the bacterial assimilatory nitrate reduction. This process may therefore also be responsible for a certain part of the nitrate removal, but a considerable part was accumulating in the system water and in the rinsing water of the foam fractionators.

Applied to the model, 94% of the nutrient balance of the system could be explained for nitrogen as well as 86% for phosphorus, so the model can be

considered as suitable for the simulation of a marine artificial recirculation system.

e) General considerations

Generally, it seems that the spatial dimensions of the different biological secondary units did not exactly match in the experiment. Therefore, system optimisation can include stocking density, harvesting processes or water flow rates. In order to calculate the right spatial dimensions for such a system it is necessary to define the criteria for optimisation: e.g. the financial benefit of the secondary steps or the nutrient retention within the different biological secondary elements. It is not possible to optimise all criteria simultaneously. Maximum biomass yield in all components at the same time is also impossible due to the different physiological requirements of the cultured organisms. Therefore, the model can be a powerful tool for optimising integrated recirculating systems.

f) Commercial applications

Although nutrient retention of the biological secondary units accounted for only one fifth of the total nutrient budget, the financial benefit of the additional biomass production can significantly improve the economical outcome. For example, the harvested worms may be directly sold as bait for fishers or as high quality aquaculture food (high proportions of polyunsaturated fatty acids). Preliminary calculations based on the MARE data and current market prices of €30/kg indicate a cost coverage of one year´s fish feed by selling the additional crop of worms.

The direct sale of macroalgae appears to be more difficult, but macroalgae can be also used in aquaculture as feed for more profitable organisms, like abalone (*Haliothis* spec.) or as dietary supplement for aquaculture fish species e.g. *Sparus aurata*. However, results from feeding experiments (this study, data not shown) did not show a significant difference in fish growth with a feed replacement of 5 %. Further options for the commercial usage of

cultured macroalgae are products from fermentation processes of algae (e.g. for cosmetics and pharmacy).

2.4.3 Modelling

In aquaculture a broad spectrum of models were developed for different species and various aquaculture productions forms (Roland and Brown, 1990; Piedrahita, 1990; Watten, 1992; Kochba *et al.*, 1994; Ellner *et al.* 1996, Cho and Bureau, 1998; Pagand *et al.*, 2000a; Lefebvre *et al.* 2001b; Gasca-Leyva *et al.*, 2002). It is obvious, that in models natural processes can only be described in a simplified way. During the development of this model 15 modules were identified and characterised within the particular system components (fish tank, macroalgae tank, detritivorous reactor, foam fractionation), although some were overlapping, e.g. nitrification.

For practical reasons different assumptions concerning the modelling process were made. The following paragraph lists and explains these assumptions:

It was assumed that the excretion of urea by fish is of minor importance. This was done despite the results presented by Dosdat and co-authors (1996) which showed that the excretion of urea represents about 15 % of total nitrogen excretion. Measurements of urea production during the experimental period were not performed and therefore modelling of urea production and remineralisation could not be achieved. It was further assumed that all settable solids are transferred to the detritivorous reactor. Another assumption was that *Nereis diversicolor* could not build up any lipid reserves. Lipids are well recognised as an energy source for marine invertebrates (Luis and Passos, 1995; Graeve and Wehrtmann, 2003; Sewell, 2005). Beyond that, lipids provide structural components for membranes in the form of phospholipids (Sargent and Whittle, 1981). This lack of reserves and essential components led to death of worms during the course of the experiment. Another pre-assumption was that no organic matter accumulates in the sediment of the detritivorous reactor. This could be proved by experimental results of sediment samples during the experiment and therefore this assumption was included into the model. Additionally, it

sumption was included into the model. Additionally, it was assumed that all the organic matter not being used by the worms ($B_{t,OM}$) will be used for denitrification processes. Conditions favouring denitrification or ANAMMOX (anaerobic ammonia oxidation) (suboxic areas, sufficient contents of organic matter and elevated nitrate concentrations) occurred during the experiment. The ANAMMOX process leads to the formation of N_2 assuming suboxic/anoxic conditions. Suboxic conditions are considered to develop at organic particle surfaces and in deeper sediment regions. However, elevated nitrite concentrations could not be detected being a prerequisite for the onset of the ANAMMOX process. Therefore, this process may be neglectable. Additionally, nitrate concentrations rose before integration of the macroalgae unit, indicating considerably higher nitrification rates compared to denitrification rates, although no quantifications were made. Although all these processes have to be taken into account, for practical reasons it was assumed that all available ammonia is converted to nitrate.

In contrast to many other static models, this dynamic model refers to the variable “production day” with the changing variable “fish growth” of *Sparus aurata.* Based on these data the influence of all presented modules was investigated. Within a numeric model iterative algorithms were developed for nutrient flows of each production day. It is based on functional correlations resulting from theoretical considerations, literature data and regression analyses of empirical and literature data.

The informational value of the model was evaluated by the modelled and experimental concentrations of nitrate and phosphate in the MARE system and showed good correlations (Fig. 14). However the model was not yet validated by an independent data set. The empirical data for fish growth were used for development and linear regression analyses for numeric modelling. So, transferability of the results to other recirculation system could not be statistically affirmed. Nevertheless, it can be assumed that the model represents a valuable tool to calculate the proper dimensions of future integrated recirculating system and to forecast nutrient flows within the different compo-

nents of such a system. It can be used as a reasonable basis for further investigations into this area.

Despite all achievements of the model systems like the presented MARE system still struggle with problems like the accumulation of dissolved inorganic nutrients. The excess of certain critical values can cause limitations in growth performance or even death of the cultured organisms. So far no recommendations concerning harvesting of the cultured organisms were made. In order to avoid production failures (due to e.g. reproduction events) worms for example should be harvested before they start reproduction.

2.5 Conclusions

MARE is an innovative concept for new marine aquaculture cultivation systems which allows not only water reprocessing but also nutrient recycling with the aim of enhancing economical profitability. The presented numeric modelling identified all essential biological processes and linked them to a complete tool, which is able to predict the growth of the cultured organisms as wells as concentrations of accumulating dissolved inorganic nutrients (nitrate and phosphate). The observed results enable the use of the model for future scientific questions, e.g. to integrate further biological steps for improving the nutrient recycling to replace technical solutions. The replacement of foam fractionators by filtering organisms (e.g. bivalves) and the development of a suitable microalgae photobioreactor system (Kube *et al.*, in prep., see Chapter 3 & 4) may be steps towards this direction. Although the first attempt of this new type of recirculation system was beyond optimal configuration, the experimental data and the developed model can help to calculate the nutrient fluxes and module sizes in virtual simulations to improve the biological and economical efficiency of future commercial recirculation systems (von Harlem, 2006).

2.6 Acknowledgements

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2.7 References

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Chapter 3

Cultivation of microalgae using a continuous photobioreactor system based on dissolved nutrients of a marine recirculation system

Kube N., Bischoff A.A., Wecker B., Waller U. (2006)

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Cultivation of microalgae using a continuous photobioreactor system based on dissolved nutrients of a marine recirculation system

Kube, N., Bischoff A.A., Wecker B., Waller U.

Abstract

In this study a photobioreactor system for continuous microalgae cultivation was developed and tested in an integrated recirculation system with a water renewal rate of less than 1% system volume (MARE = Marine Artificial Recirculating Ecosystem).

The photobioreactor system was equipped with a continuous water pretreatment unit and harvesting unit (foam fractionation) and tested for its applicability in aquaculture systems. *Nannochloropsis* was the target species and cultivated with a daily yield of 0.93 ± 0.5g dry weight m^{-2}. C/N ratio, energy content and organic amount was determined for the harvest. Growth performance and fatty acid profiles of *Nannochloropsis* were determined according to different nutrient concentrations at different irradiations (100, 250 and 400 µmol s^{-1} m^{-2}), and described by Michaelis-Menten-kinetics.

This study showed that a continuous cultivation without automatic regulation of light intensity and flow rates results in very unstable conditions regarding biomass concentration within the photobioreactors due to varying culture conditions. A semi-continuous system might be more feasible.

3.1 Introduction

Microalgae play an important role in mariculture. They are used for rearing fish larvae, bivalves, shrimps and crabs (Brown *et al.,* 1997; Moss, 1994) and for the nutrition of food organisms like rotifers and copepods (Støttrup and McEvoy, 2002; Pinto *et al.,* 2001). Different species are used for this purpose (e.g. *Nannochloropsis* spec., *Tetraselmis* spec., *Dunaniella* spec., *Isochrysis* spec., (Brown *et al.*, 1989, Rocha *et al.*, 2003). Especially microalgae characterized by an elevated content of polyunsaturated fatty acids are preferred (Volkman *et al.,* 1992; Renaud *et al.*, 1994; 1999). Considerable populations of microalgae are cultivated not only for aquaculture purposes with standard cultivation methods (Becker, 1994), e.g. large pond cultures and highly effective photobioreactor systems (Borowitzka, 1999; Pulz, 2001).

The usage of microalgae as a secondary integration step in marine recirculation systems for removing dissolved nutrients from the system water is a new goal in aquaculture (Hussenot, 2003; Schneider 2005). High nutrient concentrations are present in the discharge water of recirculation systems: nitrate and phosphate are the two major nutrients which accumulate in a recirculation system. In comparison with conventional microbial biofilters, microalgae have several advantages: nitrate and phosphate are consumed simultaneously and used for incorporation into biomass (primary production). Microalgae can also be regarded as a valuable "industrial" product with a variety of applications (food, cosmetics, human consumption, pharmacy etc.) (Wikfors and Ohno, 2001).

Aim of this study was the development of a continuous cultivation system for marine microalgae based on dissolved nutrients from a marine recirculation system. A maximal flowback of purified water (without algae) needs to be achieved, because in modern marine recirculation systems water discharge is less than 1% per day (Waller *et al.*, 2003).

New developments of systems for cultivation of microalgae in a secondary loop within a recirculation system are scarce because of the considerable technical effort for operation. Thus, these modules have been integrated into

fishfarms only occasionally. First approaches have been performed, but were mainly focused on benthic diatoms, although commercial value of diatoms appears to be limited (Tandler, 2003; Hussenot, 2003). Microalgae with commercial value or required nutrient composition for aquaculture purposes are mainly floating algae. Due to their floating way of living, harvesting of the algae is a technical problem. Standard methods like filtration, sedimentation, flocculation or centrifugation require high technical/financial investments and are labour intensive (Becker, 1994). Therefore, integration of filtrating organisms into the recirculation system like bivalves are cheaper and more valuable alternatives for removing microalgae from the system water. (Pfeiffer and Rusch, 2000; Hussenot *et al.*, 1998; Hussenot, 2003).

However, additional to the problems concerning the harvesting process of the algae, cultivation of free floating microalgae in fish effluent waters makes high demands compared to the cultivation using standard (sterile) media. Firstly, effluents from a fish tank are far from being sterile. Bacteria, fungi, viruses and particles may be transferred into the culture and may cause significant difficulties during operation. Additionally, denitrification processes within a recirculation system can cause an inappropriate N:P ratio of the system water (e.g. 4:1; see Chapter 2). This leads to limitation of nutrients (N limitation), resulting in poor growth and sudden crashes of cultures (Benemann 1992, own observations).

In summary, the conceptional design of a photobioreactor system in a secondary loop of a marine recirculation system needs to fulfill several requirements. Technical aspects are: (1) efficient water pretreatment to reduce microbial contamination, (2) sufficient nutrient flow and (3) simple and efficient harvesting. However, for practical reasons the photobioreactor must be easy to operate and to clean. Also adaptability to different algal species should be possible.

Nannochloropsis sp. was selected because of its profile of highly unsaturated fatty acids, its applicability as food and the wide range of temperatures and salinities tolerated by this species (Rocha *et al.,* 2003). *Nannnochloropsis* sp.

taxonomically belongs to the Eustigmatophyceae (Xanthophyceae, Chrysophyta). The flagellate cells (Fig. 1) are characterized by the possession of the xanthophylls heteroxanthine and vaucheriaxanthine and by a lack of chl b. Besides oil, chrysolaminarine and laminarine are accumulated as repository polymers.

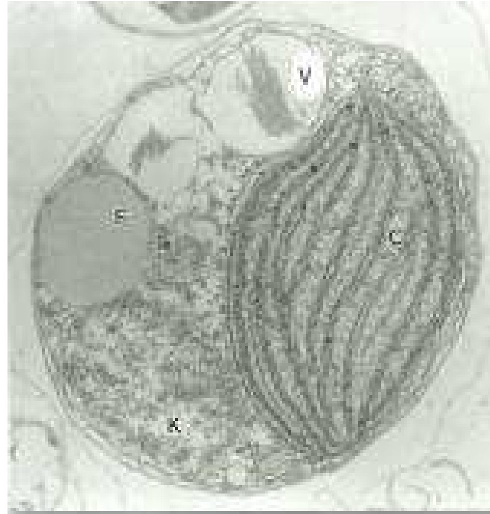

Fig. 1: *Nannochloropsis* sp. (image source: www.innovations-report.de)

3.2 Material and Methods

3.2.1 Design of the continuous photobioreactor system

The photobioreactor system used in this study consisted of three different units (Fig. 2): the "disinfection" unit, the microalgae cultivation unit and the harvesting unit. The system was operated as a bypass loop of the recirculation system for continuous treatment of the process water from the recirculation system.

The *„disinfection" unit* (Fig. 1) was an 8L foam fractionator (Sander, Uetze-Eltze, Germany) (1) combined with a water storage column. Water was permanently pumped (Eheim 1260) to the foam fractionator. Flow rate through the foam fractionator was manually adjusted to 32L/hour. The foam fractionation was operated with a compressed air/ozone mixture, produced by an ozone generator (Sander, Ozonizer A500). Redoxpotential (ORP) was measured in the outlet of the foam fractionator by a sensor and regulated to reach values between 500 and 600mV using a control module KM2000 (both Sensortechnik Meinsberg GmbH, Ziegra-Knobelsdorf, Germany).

Waste air from the foam fractionation and the waste collector was passed to an ozone decomposer filled with activated carbon. The pretreated water was transferred to the storage column where water got aerated to release residual ozone and to stabilize pH. Remaining water in the water storage column was discharged via an overflow pipe into the primary recirculation system.

The *microalgae cultivation unit* consisted of three parallel photobioreactors (Fig. 1, B). Each photobioreactor consisted of an acrylic column with a diameter of 20 cm and 1,50 m height, resulting in a total volume of 50 litres. The acrylic column was fixed with screws to a base plate of 50 x 40 cm. The top cover was designed to be removable. Y-shape air spargers were attached in the lower part of the reactor, producing large air bubbles in order to agitate/stir the culture. Four removable lamps (individually switched) with two fluorescent tubes each (daylight spectrum) were attached vertically around the bioreactor.

To ensure maximal return of water back to the primary recirculation system, an efficient *harvesting unit* needed to be included. Foam fractionation was considered to be the most efficient method to harvest the suspended algae from the outflow water of the photobioreactors. A second foam fractionator identical to the first one (see above) was installed (Fig. 2 and 3a, (4)). The foam fractionator was also operated with an ozone/air mixture. Harvested algae were collected in a separate container (box “harvest”, Fig. 2). Purified water was led back to the primary recirculation system (sampling point C). A redox potential sensor was installed to control the redox potential in the outflow water.

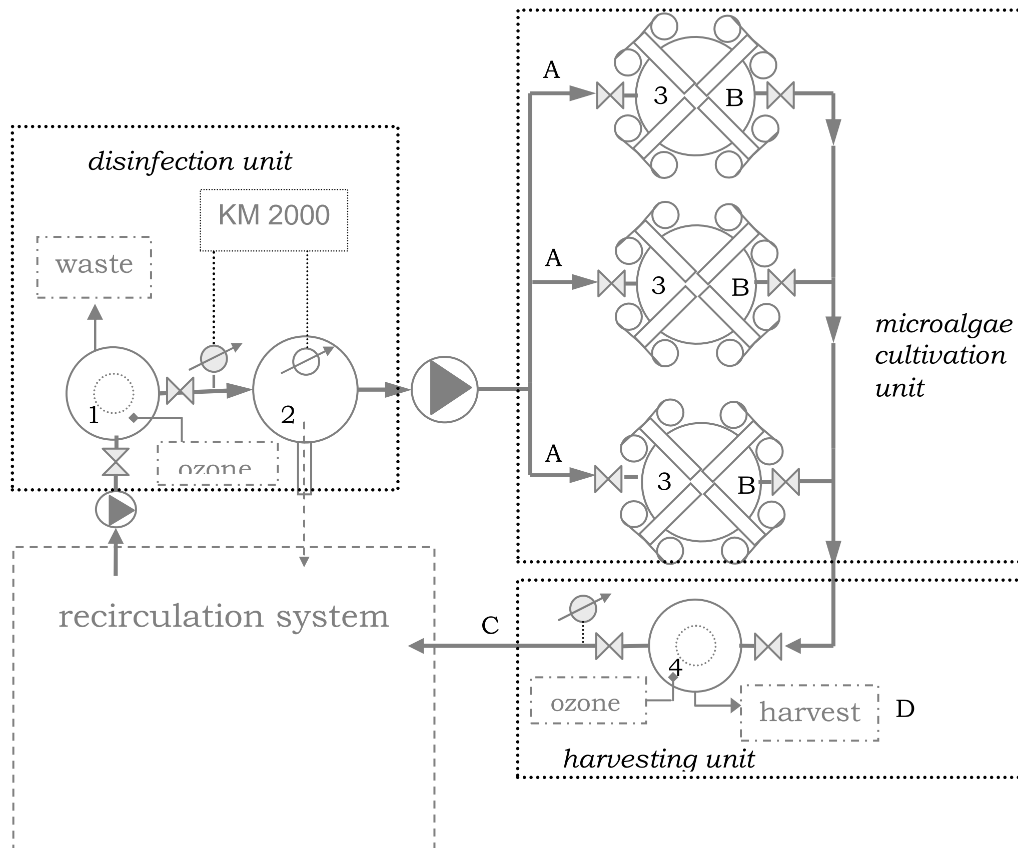

Fig. 2 Schematic drawing of the continuous photobioreactor system installed in a marine recirculation system: (1) = foam fractionator for water pretreatment supplied with ozone; (2) = storage column with aeration to remove residual ozone, broken arrow indicates overflow for dispensable water; (3) = photobioreactors with attached light tubes; (4) = harvesting foam fractionator with ozone; grey circle with arrow = electrode for redox potential; white circle with arrow = electrode for pH; technical symbols for pump (circle with triangle) and valves (double triangle), Arrows indicate flow of water. A,B,C,D = sampling points (Tab. 1).

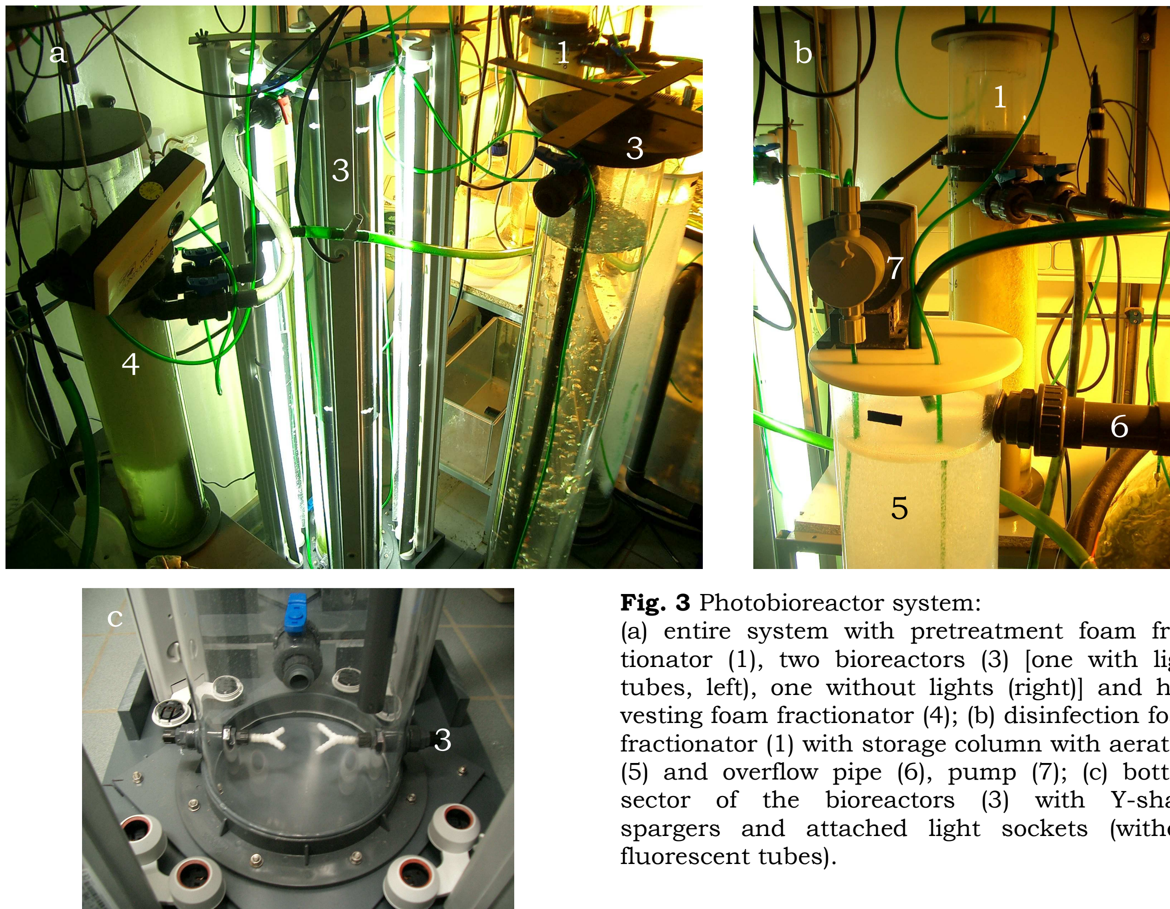

Fig. 3 Photobioreactor system:
(a) entire system with pretreatment foam fractionator (1), two bioreactors (3) [one with light tubes, left), one without lights (right)] and harvesting foam fractionator (4); (b) disinfection foam fractionator (1) with storage column with aeration (5) and overflow pipe (6), pump (7); (c) bottom sector of the bioreactors (3) with Y-shape spargers and attached light sockets (without fluorescent tubes).

3.2.2 Functional principle of the photobioreactors

The functional principle of the photobioreactor operation is analogous to a chemostat (Pirt, 1975, Fig. 4): medium is inserted into the reactor containing the culture at a constant flow rate (F). The total volume of the culture (V) remains constant by continuous removal following an overflow principle (Fig. 4). Thus, constant mixing processes are ensured in order to dispense the new media uniformly throughout the whole culture within a very short time.

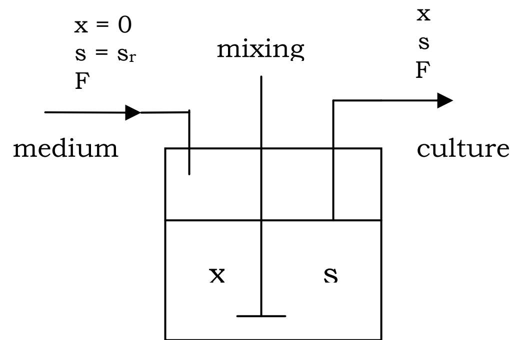

Fig. 4 Simplified principle of a chemostat (Pirt, 1975): x = biomass, s = growth limiting substrate, s_r = inflow concentration for growth limiting nutrient; F = flow rate; V = culture volume

If a growing batch culture is changed into continuous cultivation modus, there are different scenarios to be considered as a result of the cultivation efforts: (1) the rate of washout exceeds the growth rate (μ) and biomass accumulation in the photobioreactor will decrease and growth limiting substrate concentration will tend towards s_r; (2) the initial rate of washout will balance the growth rate, so microalgae will grow at maximum rates (μ_m) and (3) the rate of washout is minor to the maximum growth rate, the biomass will increase within the photobioreactor.

Therefore, growth of biomass can be described as follows for an infinitely small time interval *dt*:

$$\frac{dx}{dt} = \mu \times x - \frac{F \times x}{V} \quad \text{(Equ. 1)}$$

When the flow rate equalizes the specific growth in the photobioreactor, "steady state" is achieved. The steady state is a self-regulating process: biomass concentration and substrate concentration are acting following an oscillating relation: a decrease in biomass concentration will be associated with an increase in substrate concentration; this in turn will lead to an increase of the growth rate and thus, restore the steady state conditions. An increase in biomass concentration will have the reverse effects. Therefore, a certain time interval is required until this oscillation effect will tend to zero. So also

biomass output possibility (1) and (3) will also result in a steady state at constant nutrient inflows conditions after a certain time.

During the entire experimental period no real “steady state” can be expected to develop due to changing nutrient concentrations of the inflow process water. Thus, measurements of the critical dilution rates (rates where diluting washout of the culture takes place) were used to determine growth rate at different concentrations of the recirculation water. Flow rates of recirculation water were adjusted to a value, at which optical density (OD_{665}) within the bioreactor remained constant for several hours. “Steady state” conditions were assumed and specific growth was determined by measuring the proportion of microalgae removed from the photobioreactor within a certain time interval (1 hour):

$$\mu = \frac{\ln N_t - \ln N_0}{t} \qquad \text{(Equ. 2)}$$

where N_0 = cell number at t_0 in the bioreactor, N_t = cell number after t (calculated from cell numbers of bioreactor + cell numbers of outflow), t is time between two sampling points (1 hour).

Specific growth rates vary according to nutrient concentrations of the medium. The relationship between specific growth rate and growth-limiting nutrient can be described by a Michaelis-Menten-kinetic:

$$\mu = \frac{\mu_{\max} \cdot x}{K_s + x} \qquad \text{(Equ. 3)}$$

where μ_{max} = maximum growth rate, x = algae biomass, K_s = half saturation constant.

To calculate the nutrient concentrations (also called nutrient load) within the photobioreactor some additional aspects have to be taken into account. If a very small inflow volume is introduced into a larger volume (e.g. volume of the photobioreactor), dilution processes occur following an exponential equa-

tion: for example a dilution rate of 0.02 will not result in a 2% water exchange of the total photobioreactor volume. The percentage of exchanged water (R) has to be calculated as a function of the flow rate (F) and the volume of a single photobioreactor (V) for a definite time interval (t):

$$R = 1 - e^{\frac{-tF}{V}} \qquad \text{(Equ. 4)}$$

Considering this, the virtual effluent concentration ($c_{out,\ virtual}$) describes the hypothetic effluent concentration at the end of the sampling period, assuming that no algae are present. The function requires the measurement of the influent (c_{in}) and effluent (c_{out}) concentration at the beginning (t_1) and the end (t_2) of the sampling period.

$$c_{out,virtual} = R \cdot \frac{c_{in,t_1} + c_{in,t_2}}{2} + (1 - R) \cdot \frac{c_{out,t_1} + c_{out,t_2}}{2} \qquad \text{(Equ. 5)}$$

The virtual effluent concentration ($c_{out,\ virtual}$) was used to calculate the nutrient uptake rate (r_A) as the difference between the virtual and the measured effluent concentration (c_{out,t_2}) per time interval (t).

$$r_A = \frac{(c_{out,virtual} - c_{out,t_2})}{t} \qquad \text{(Equ. 6)}$$

This study was focused on the determination of specific growth rates of the microalgae with regard to different nutrient concentrations of the photobioreactor in order to define suitable dilution rates for the operation of the photobioreactors. This was tested at different radiation intensities (100µmol s^{-1} m^{-2}, 250µmol s^{-1} m^{-2}, 400µmol s^{-1} m^{-2}). The photobioreactor system was attached to a marine recirculation system for rearing sea bream (*Sparus aurata)* (see Chapter 5). It was operated during a 4 months experimental period. Each reactor was working with a different irradiation and was illuminated for 24 hours (Table 1).

Tab. 1 Number of fluorescent tubes for illumination and measured average irradiation

Photobioreactor	Number of fluorescent tubes	radiation (µmol s^{-1} m^{-2})
1	2	100
2	4	250
3	8	400

3.2.3 Algae

Nannochloropsis was obtained from a sterile stock culture strain, available at IFM-GEOMAR. Stock cultures served as backup and were cultured on f/2 medium (Guillard, 1975) applying sterile conditions.

Working cultures of 5 l volume for bioreactor inoculation were already grown on pretreated recirculation water.

3.2.4 Culture conditions

Water temperature of the recirculation system was kept at 20 ± 2.2°C and salinity at 23 ± 1.0 psu. Temperature in the bioreactors varied between 23 and 26°C due to different radiation intensities. pH values ranged from 7.5 to 8.5 and was not regulated automatically, but HCl was added when pH rose above values of 8.7. Compressed air was supplied to the photobioreactors in order to aerate the cultures.

Bioreactors were started as batch cultures until the exponential growth phase of microalgae was observed. When the exponential growth phase was reached, the photobioreactors were transferred to the continuous cultivation modus. Flow rates varied between 0.36L h^{-1} to 1.5L h^{-1}.

3.2.5 Sampling and analytical methods

The protocols of daily measurements are summarized in Table 2. Depending on the production mode (batch, continuous), samples were taken at different sampling points (A,B,C,D, Fig. 2).

Tab. 2 Daily measurements according to cultivation modus of the photobioreactor system included into a marine recirculation system. A,B,C,D are the sampling points (see Fig. 2). OD_{665} = optical density of microalgae cultures at 665nm wavelength.

Cultivation mode	**A** (inflow to bioreactors)	**B** (bioreactors)	**C** (outflow to MARE)	**D** (harvest)
Batch	-	OD_{665} pH dissolved nutrients	-	-
Continuous	pH dissolved nutrients	OD_{665} pH dissolved nutrients	OD_{665} pH dissolved nutrients	OD_{665} pH dry matter organic matter C/N energy

Optical density was measured at 665nm with a Hach spectrometer (10 ml sample). In order to allow rapid determination of cell numbers a linear relationship between cell counts and optical density was established. Cell numbers were counted using a light microscope and a haemocytometer (Fuchs-Rosenthal).

Water samples of approx. 20ml were centrifuged at 5000 rpm for 10min. To remove algae and then frozen at -20°C. Dissolved nutrients (PO_4-P, NO_3-N, NO_2-N, TAN) were analysed using an autoanalyzer AA3 (Bran-Lübbe GmbH, Norderstedt, Germany). pH was measured with a WTW multi 340 pH-meter.

Daily water volume in the harvest collection unit (Fig. 2, D) and cell numbers of harvested microalgae were recorded. 12 subsamples of microalgae harvest (10ml each) were centrifuged at 5000 rpm for 10min. The microalgae pellets formed by centrifugation were analysed as follows: content of dry matter was determined by drying the sample in a furnace at 60°C for 12 hours. Organic content was then measured by incineration of organic matter in a muffle furnace. C/N ratio was determined by gas chromatography (GC) in an element analyser (EURO EA elemental analyser, Milano, Italy). Calorific value was measured by combustion in an IKA calorimeter C4000. Weighing was performed using a Sartorius A 210 P (max. 200g).

Microalgae samples from the photobioreactors were filtered using 25mm glass-fibre filters (Whatman GF/F). The filters were pre-combusted for at least 12 hours at 550°C to remove traces of organic matter. POC and PON were measured applying gas chromatography. POP was converted to orthophosphate by cooking with potassium peroxydisulphate. The orthophosphate was then measured photometrically.

Fatty acids were extracted with dichloromethane: methanol (2:1, vol/vol) as described by Fink (Fink, 2006). PUFAs (polyunsaturated fatty acids) were quantified as fatty acid methyl esters (FAMEs) using a HP 5890 Series II GC (Agilent Technologies, Waldbronn, Germany) equipped with a DB 225 fused silica column (J&W Scientific, Folsom, USA) and a flame ionisation detector with heptadecanoic acid methyl ester and tricosanoic acid methyl ester as internal standards (Von Elert and Stampfl, 2000). Identification of the FAMEs was based on comparison of retention times to those of reference compounds.

3.3 Results

3.3.1 Feasibility of the photobioreactor system for algae cultivation

a) pretreatment of recirculation water and harvesting process

The photobioreactor system fulfilled the technical requirements concerning continuous water treatment and harvesting as well as space requirements. In general, the conceptional design of the photobioreactor system turned out to be feasible. It was possible to successfully cultivate *Nannochloropsis* in a continuous culture based on dissolved nutrients derived from the marine recirculation system.

Water from the detritivorous culture tank (harbouring *Nereis diversicolor*) was continuously pretreated with ozone in combination using foam fractionation. This treatment process was analyzed for its effectiveness in purifying highly loaded waste water from recirculation systems. As a result, disinfecting effect could be achieved due to the removal of particles and bacteria cells by the foam fractionation process. For details see Chapter 4.

The harvesting process could be successfully established. Harvest of all cultivated algae by the foam fractionation technique was achieved, which is represented by an average optical density at 665nm (OD_{665}) of 0.007 ± 0.007 (< 1 Mio. cells ml^{-1}). The daily amount of algae biomass yield averaged 2.49 ± 1.34g DW d^{-1}. Daily yields are shown in Tab. 3. At the beginning of the experiments the harvesting foam fractionator was operated without ozone. This turned out not to be effective enough, resulting in remaining algae in the outflow and an elevated harvesting volume (2.5 Litres) with relatively low cell densities. Addition of ozone improved the performance. At the end of the experimental period the harvest volume reached 0.184 ± 0.053 L d^{-1} at very high cell densities of 227.9 x 10^8 ± 6.7 x 10^8 cells ml^{-1} (Tab. 3). 99.68 ± 0.38 % of the water coming from the photobioreactors were transferred back to the main recirculation system.

b) nutritional value of the cultivated microalgae

The nutritional quality of the algae was increasing during the experimental period. The organic proportion increased from 40-50% to more than 80% of algae DW_{algae} (Tab. 3), coinciding with an elevated energy content of the microalgae. Measured energy values of 21.07 ± 4.6 KJ d^{-1} DW_{algae} indicate high nutritional quality of the microalgae. In initial samples a proportion of an unknown white substance could be observed, which was probably lime. This may explain the lower organic fraction, but not the high C:N ratio of the samples, because samples were pretreated with HCl in order to remove inorganic carbon before gaschromatographical determination.

Fatty acid profiles were determined at the end of the experiments to get another hint about the nutritional value of the cultivated microalgae (Tab. 4). Total amount of fatty acids varied from 16.72 ng fatty acids (FA)/µg POC_{algae} (100 µmol s^{-1} m^{-2}) to 14.01 ng FA/µg POC_{algae} (250 µmol s^{-1} m^{-2}). Values for full illumination was very low at 5.5 ng FA/µg POC_{algae}.

Highest values for Arachidonic acid (ARA) and Eicosapentaenoic acid (EPA) have been found in bioreactors at low irradiances at 1.12 ± 0.18 ng ARA/µg

POC_{algae} (100 µmol s^{-1} m^{-2}) and 1.16 ± 0.06 ng ARA/µg POC_{algae} (250µmol $s^{-1}m^{-2}$). Values for Eicosapentaenoic acid (EPA) were 2.54 ± 0.44 ng EPA/µg POC_{algae} and 2.66 ± 0.14 ng EPA/µg POC_{algae}, respectively. Values for irradiation at 400µmol s^{-1} m^{-2} were found to be dramatically low at 0.22 ± 0.04 ng ARA/µg POC_{algae} and 0.50 ng EPA/ µg POC_{algae} for EPA.

Relative amounts of highly unsaturated fatty acids compared to the total amount of fatty acids was high: relative amount of arachidonic acid (ARA, 20:4 n-4) of the total fatty acid content were at 6.7% (100µmol s^{-1} m^{-2}), 8.27% (250µmol s^{-1} m^{-2}) and 4.0% (400µmol s^{-1} m^{-2}), respectively. Values for eicosapentaenoic acid (EPA, 20:5 n-5) were at 15.2% (100µmol s^{-1} m^{-2}), 18.9% (250µmol s^{-1} m^{-2}) and 9.1% of total fatty acid amount (400µmol s^{-1} m^{-2}), respectively.

Tab. 3 Harvesting results by foam fractionation (total amount of all 3 photobioreactors with different irradiations at 100µmol s^{-1} m^{-2}, 250µmol s^{-1} m^{-2} and 400µmol s^{-1} m^{-2}, respectively). Flow rate per day = total flow through all three bioreactors, foam volume = harvested volume in the harvesting vessel, cell density, pH, yield (DW d^{-1}) in the harvest. Organic fraction, energy content and C/N ratio of the cultivated algae.

Date	Flow rate per day [L]	Foam volume [L]	Cell density [Mio ml^{-1} harvest]	pH Harvest	Yield DW (g d^{-1})	Org. fraction	Energy (KJ g^{-1})	N [mg g^{-1} DW]	C [mg g^{-1} DW]	C:N ratio
15.10.05	108.0	1.0	129.1	7.09	4.44	55.8	16.05	36.06	317.4	8.8
17.10.05	108.0	0.4	164.6	7.46	2.58	57.4	14.21	38.84	297.8	7.7
19.10.05	108.0	0.12	126.1	n.d.	0.62	50.2	13.85	29.62	263.8	8.9
20.10.05	108.0	0.58	133.8	7.09	3.01	53.6	13.22	24.90	213.1	8.6
27.10.05	73.4	2.5	68.8	7.15	n.d.	39.9	13.54	15.36	143.8	9.4
28.10.05	73.4	1.5	392.2	7.06	n.d.	53.9	9.16	31.16	298.2	9.6
29.10.05	73.4	0.98	115.6	7.3	2.69	83.4	14.27	45.01	386.2	8.6
31.10.05	73.4	1.75	65.3	7.42	3.07	78.9	23.53	52.46	458.1	8.7
1.11.05	73.4	0.35	207.4	7.06	1.69	87.9	24.63	58.8	499.3	8.5
2.11.05	73.4	0.4	214.2	7.15	3.44	82.9	22.88	57.2	473.3	8.3
16.11.05	260.8	0.8	25.0	7.03	-	82.5	21.39	49.8	408.2	8.2
18.11.05	108.0	0.4	31.80	n.d.	4.58	78.5	21.49	55.1	417.3	7.6
20.11.05	86.4	0.49	46.6	6.93	7.41	78.5	21.66	49.5	416.3	8.4
21.11.05	86.4	0.18	32.7	6.81	3.02	73.4	21.23	36.5	307.2	8.4
24.11.05	86.4	0.2	35.3	6.81	2.74	82.1	23.46	45.3	341.9	7.6
25.11.05	86.4	0.2	29.6	6.38	1.86	85.1	23.88	63.9	480.0	7.5
26.11.05	86.4	0.3	27.7	6.55	3.16	80.6	21.93	52.0	381.5	7.3
27.11.05	86.4	0.16	56.6	6.87	2.62	85.9	23.80	80.3	593.6	7.4
28.11.05	86.4	0.05	75.3	5.88	1.48	91.9	25.98	50.7	407.5	8.0
10.1.06	43.2	0.16	355.0	6.85	2.38	-	-	-	-	-
11.1.06	43.2	0.15	466.1	n.d.	1.45	85.1	23.61	53.8	463.0	8.6
16.1.06	64.8	0.18	704.1	6.81	1.58	84.0	22.82	62.8	506.6	8.1
17.1.06	43.2	0.22	674.5	6.94	1.88	84.8	24.19	65.6	472.8	7.2
18.1.06	60.5	0.19	667.9	7.43	1.56	85.1	23.78	60.6	448.6	7.4

Date		Foam volume [L]	Cell density [Mio ml^{-1} harvest]	PH Harvest	Yield DW (g d^{-1})	Org. fraction	Energy (KJ g^{-1})	N [mg g^{-1} DW]	C [mg g^{-1} DW]	C:N ratio
19.1.06	64.8	0.2	901.4	7.35	2.31	84.7	24.48	56.6	432.6	7.6
20.1.06	64.8	0.1	954.8	6.96	1.15	86.9	25.12	72.9	538.6	7.4
22.1.06	64.8	0.18	849.6	5.48	1.77	87.8	24.96	75.1	534.7	7.1
23.1.06	64.8	0.11	772.3	7.92	0.98	86.7	24.8	65.3	493.5	7.6
24.1.06	64.8	0.31	683.5	5.66	2.43	85.7	24.12	67.8	482.7	7.1
25.1.06	64.8	0.18	760.0	n.d.	2.10	84.9	22.97	64.3	468.6	7.3

Tab. 4 Fatty acid profiles of *Nannochloropsis* spec. from batch cultures at stationary phase at different irradiances (100µmol s^{-1} m^{-2}, 250 µmol s^{-1} m^{-2}, 400 µmol s^{-1} m^{-2})

Fatty acid: position of double bonds	nomenclature	100µmol $s^{-1}m^{-2}$		250 µmol s^{-1} m^{-2}		400 µmol s^{-1} m^{-2}	
		average ng FA/µg PO-C_{algae}	± SD	average ng FA/µg PO-C_{algae}	± SD	average ng FA/µg PO-C_{algae}	± SD
14:0	Myristic acid	0.23	± 0.32	0.15	± 0.34	-	-
16:0	Palmitic acid	5.75	± 0.79	5.12	± 0.33	2.01	± 0.46
16:1	Palmitoleic acid	3.44	± 0.34	2.76	± 0.17	0.68	± 0.16
18:1 (9)/(6)	Oleic acid	2.96	± 0.40	1.71	± 0.09	0.93	± 0.20
18:1 (11)	Vaccenic acid	0.14	± 0.08	0.10	± 0.01	0.37	± 0.07
18:3 (6,9,12)	y-Linolenic acid	0.03	± 0.04	0.09	± 0.00	-	-
18:3 (9,12,15)	a-Linolenic acid	0.51	± 0.06	0.26	± 0.01	0.79	± 0.37
20:4 (5,8,11,1)	Arachidonic acid (ARA)	1.12	± 0.18	1.16	± 0.06	0.22	± 0.04
20:5 (5,8,11,14,17)	Eicosapentaenoic acid (EPA)	2.54	± 0.44	2.66	± 0.14	0.50	± 0.10
	Total	16.72		14.01		5.5	

3.3.2 Specific growth rates and nutrient uptake rates of *Nannochloropsis* at different light intensities

The duty of a continuous photobioreactor system in a marine recirculation system is to maintain cultivation rates at constant rates with maximum yield rates. Therefore adequate flow rate according to the available nutrient concentration and hence specific growth rate needs to be adjusted: if the flow rate is higher than the specific growth rate of the microalgae, washout of biomass will set in and may result in a low performance of the photobioreactor.

Flow rates where washout happens are called “critical dilution rates” and were determined in this study at various nutrient concentrations within the photobioreactor by adjusting different flow rates during the four month experimental period at different irradiations (100µmol $s^{-1}m^{-2}$, 250µmol $s^{-1}m^{-2}$ and 400µmol $s^{-1}m^{-2}$) in order to obtain information about maximum specific growth rates within this type of photobioreactor.

Data are presented exemplary from the photobioreactor with the lowest irradiation (100µmol $s^{-1}m^{-2}$, Fig. 4, Tab. 3). Batch culture (black dots) was operated until a sufficient optical density was reached. During period A (Fig. 5) flow rate of 1.5 Litre per hour was adjusted at day 8 and resulted in a rapid decrease of biomass within 4 days. Therefore, continuous mode was changed to batch culture to increase cell numbers. At day 21 flow rate of 1 Litre/hour was adjusted (period B). For the initial cultivation days biomass remained constant, indicating that flow rate equalized specific growth rate of microalgae. From day 23 onwards a decrease of specific growth rates was observed. This is due to a slow but constant (until day 30) washout of algal biomass. Another continuous cultivation period was started at day 42 (period C) with a flow rate of 3.6 Litres per hour resulting in a rapid biomass decrease within a short time period. Flow rate was adjusted to 1.2 Litres/hour (period D). At this flow rate biomass values in the photobioreactor remained constant. Cultivation was stopped at day 58 due to a system failure. During that period water parameters (dissolved nutrients, pH etc., Tab. 4) remained constant.

In periods E,F,G flow rates needed to be adjusted to lower values, because of limited algae growth, because nitrate was limited in the recirculation system (see Chapter 5) (Tab. 4, period E,F,G). This resulted in lower growth rates of *Nannochloropsis* sp.

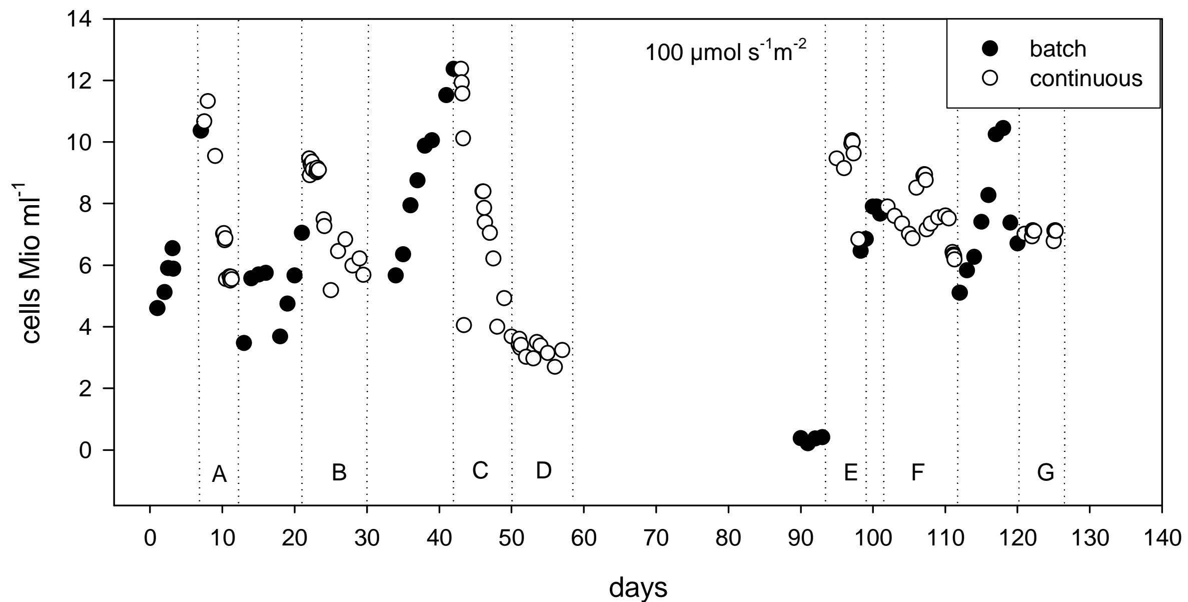

Fig. 5 Cell densities *of Nannochloropsis* per ml in the photobioreactor illuminated with 2 fluorescent tubes (100 µmol $s^{-1}m^{-2}$). Batch cultures (black dots) were used to reach sufficient densities, before operation mode was changed to continuous production (white dots). Letters indicate adjusted flow rates within the periods with continuous production: A = 1,5L/hour (dilution rate 0.03); B = 1L/hour (dilution rate 0.02); C = 3.6L/hour (dilution rate 0.08); D = 1.2L/hour (dilution rate = 0.027); E = 0.6L/hour (dilution rate 0.027); F = 0.9L/hour (dilution rate = 0.02); G = 0.36L/hour (dilution rate = 0.014) (for further details, see also Tab. 4).

Tab. 4 Water parameters during continuous production of *Nannochloropsis* spec. in the photobioreactor with low illumination (100µmol $s^{-1}m^{-2}$) during the 4 month experimental time. A,B,C,D,E,F,G are indicating different time intervals with different adjusted flow rates. D = dilution rate/h

Period	Flow rate L/h	D	pH	inflow concentration to photobioreactor				outflow concentration from photobioreactor			
				PO_4-P	NO_3-N	TAN	NO_2-N	PO_4-P	NO_3-N	TAN	NO_2-N
A	1.5	0.03	7.44 ±0.05	25.2 ± 2.2	60.5 ± 2.0	n.d.	n.d.	19.5 ± 19.5	56.2 ±3.0	n.d.	n.d.
B	1.0	0.02	8.01 ±0.21	30.6 ± 3.3	80.7 ± 8.4	0.93 ± 0.58	0.15 ± 0.08	20.5 ± 2.6	71.4 ± 8.4	0.17 ±0.08	0.19 ± 0.12
C	3.6	0.08	7.8 ± 0.1	32.2 ± 2.3	82.4 ±1.8	0.3 ± 0.2	0.5 ± 0.4	19.9 ± 2.4	72.6 ± 5.4	0.1 ± 0.03	1.4 ± 0.6
D	1.2	0.027	7.88 ± 0.2	37.3 ±1.28	92.1 ± 4.7	0.14 ± 0.06	0.07 ± 0.06	22.1 ± 4.3	87.1 ± 1.6	0.08 ± 0.05	0.18 ± 0.21
E	0.6	0.022	8.4 ± 0.4	28.9 ± 4.8	12.2 ± 1.6	2.6 ± 0.5	0.01 ± 0.01	11.5 ± 3.0	36.2 ± 3.0	0.1 ± 0.1	0.4 ± 0.1
F	0.9	0.02	7.51 ± 1.4	33.0 ± 6.3	8.9 ± 2.0	1.7 ± 0.97	0.04 ± 0.05	14.6 ± 7.2	5.5 ± 2.91	0.11 ± 0.11	0.06 ± 0.03
G	0.36	0.014	8.12 ± 0.1	41.2 ± 3.4	9.5 ± 0.9	3.7 ± 0.4	0.11 ± 0.07	25.9 ± 1.7	0.00 ± 0.2	0.06 ± 0.04	0.06 ± 0.03

Photobioreactors with irradiations of 250µmol $s^{-1}m^{-2}$ and 400µmol $s^{-1}m^{-2}$ showed similar performances, although washout rates differed. For determination of specific growth rates, time intervals with constant cell densities within the photobioreactor at a certain flow rate were chosen. Specific growth rate (µ) was determined for 1 hour intervals according to equation 2. These values were plotted against the nutrient concentrations within the photobioreactor according to equations 4 and 5.

Fig. 6 shows Michaelis-Menten-kinetics determined for each photobioreactor for nitrogen (sum of $N0_3$-N, TAN, NO_2-N). Kinetics for all three treatments turned out to be rather similar, maximum specific growth rates for all three radiation intensities were approx. $0.024h^{-1}$. Best growth and nutrient removal rates have been determined for the photobioreactor operated at 400µmol s^{-1} m^{-2} irradiation for short time periods. However, photobioreactor at high illumination was very unstable, wash out of biomass occurred very

often. Best long-term performance was observed for photobioreactor with 250µmol s^{-1} m^{-2} irradiation.

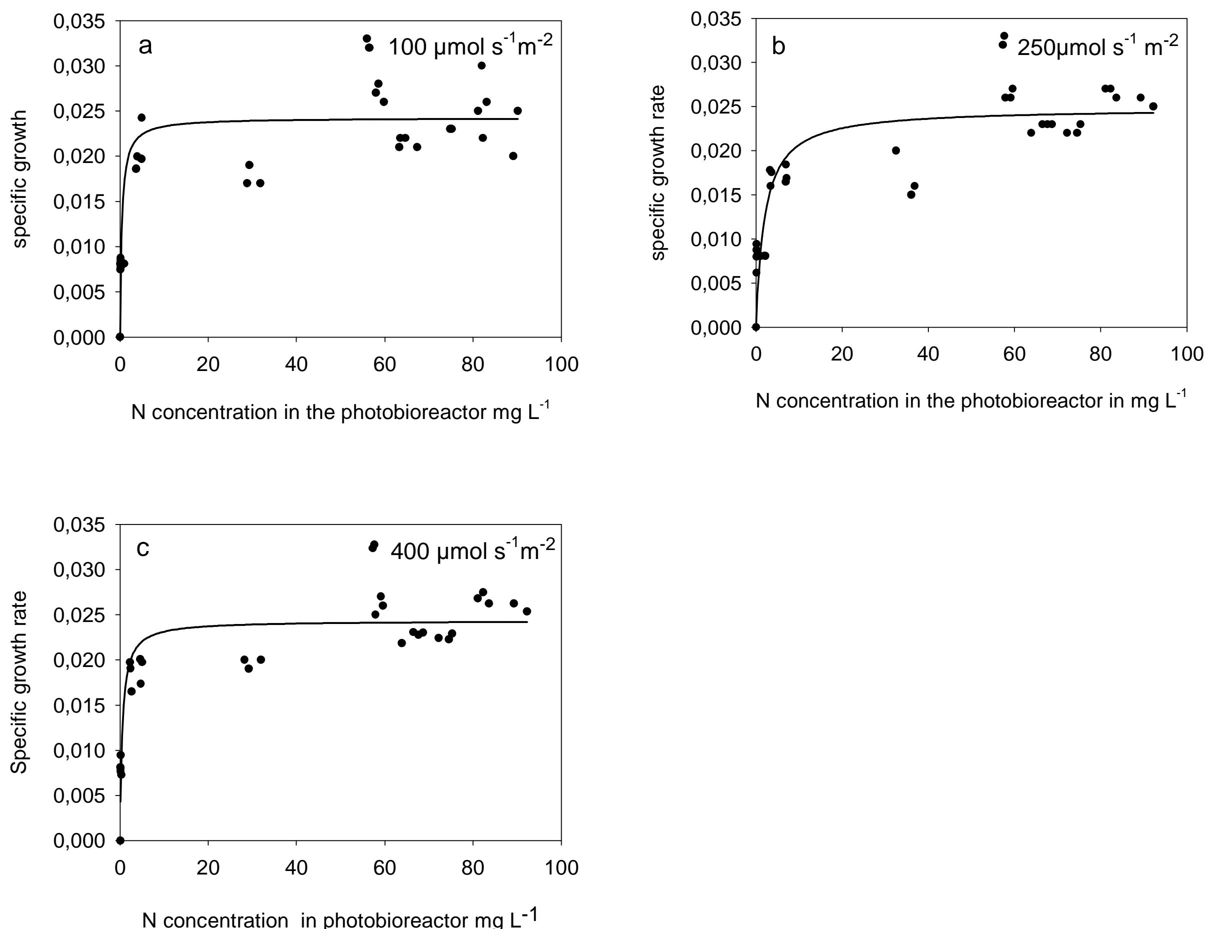

Fig. 6 Growth of *Nannochloropsis* spec. in continuous cultures of a photobioreactor system included in a marine recirculation system at different nutrient concentrations in the photobioreactor at different irradiations (100µmol s^{-1} m^{-2}, 250µmol s^{-1} m^{-2}, 400 µmol s^{-1} m^{-2}).

K_s values for all three kinetics indicate that nutrient limitation is normally not occurring within a recirculation system, as system water concentrations of nitrogen and phosphorus are normally above limiting values. However, very low nitrogen concentrations may occur, if an enhanced denitrification occurs within a recirculation system, as happened in the experimental trial within this study was accomplished (MARE II, see Chapter 5). Nitrogen concentrations tended toward zero, resulting in a lower growth performance of the microalgae.

Tab. 5 Growth performance of *Nannochloropsis* spec. in continuous cultures in photobioreactors included in a marine recirculation system at different irradiances (100µmol s^{-1} m^{-2}, 250µmol s^{-1} m^{-2}, 400µmol $s^{-1}m^{-2}$)
μ = specific growth rate, μ_{max} = maximum growth rate, K_s = half saturation constant (Michaelis-Menten-kinetic), SE = Standard Error; SD = Standard Deviation
a = mean of experimental data from continuous culture (n= 35), b = parameters from regression analysis (Sigma-Plot)

irradiance	100 Wm^{-2}			250 Wm^{-2}			400 Wm^{-2}		
	μ h^{-1} ± SD	μ_{max} h^{-1} ± SE	K_s ± SE	μ h^{-1} ± SD	μ_{max} h^{-1} ± SE	K_s ± SE	μ h^{-1} ± SD	μ_{max} h^{-1} ± SE	K_s ± SE
continuous	0.020 ± 0.008 [a]	0.024 ± 0.001 [b]	0.41 ± 0.17 [b]	0.020 ± 0.009 [a]	0.024 ± 0.001 [b]	2.01 ± 0.59 [b]	0.021 ± 0.008 [a]	0.024 ± 0.001 [b]	0.53 ± 0.15 [b]

Tab. 6 Calculation of nutrient uptake (r) per hour per litre of *Nannochloropsis* spec. in continuous cultures in photobioreactors included in a marine recirculation system at different irradiances (100µmol $s^{-1}m^{-2}$, 250mmol $s^{-1}m^{-2}$, 400µmol $s^{-1}m^{-2}$). r = uptake rate per hour, r_{max} = maximum growth rate; SD = Standard Deviation

a = mean of experimental data from continuous culture (n = 50); b = maximum observed value (n=1);

irradiance	100 Wm^{-2}				250 Wm^{-2}				400 Wm^{-2}			
	r	r_{max}	r	r_{max}	r	r_{max}	r	r_{max}	r	r_{max}	r	r_{max}
	PO_4-P	PO_4-P	NO_3-N	NO_3-N	PO_4-P	PO_4-P	NO_3-N	NO_3-N	PO_4-P	PO_4-P	NO_3-N	NO_3-N
	mg $L^{-1}h^{-1}$ ± SD	mg L^{-1} h^{-1}	mg L^{-1} h^{-1} ± SD	mg L^{-1} h^{-1}	mg L^{-1} h^{-1} ± SD	mg L^{-1} h^{-1}	mg L^{-1} h^{-1} ± SD	mg L^{-1} h^{-1}	mg L^{-1} h^{-1} ± SD	mg L^{-1} h^{-1}	mg L^{-1} h^{-1} ± SD	mg L^{-1} h^{-1}
continuous	0.190 ± 0.11 [a]	0.41 [b]	0.129 ± 0.09 [a]	0.29 [b]	0.219 ± 0.16 [a]	0.52 [b]	0.135 ± 0.14 [a]	0.48 [b]	0.294 ± 0.15 [a]	0.59 [b]	0.186 ± 0.15 [a]	0.51[b]

3.4 Discussion

3.4.1 Applicability of the photobioreactor design

In this study, *Nannochloropsis* sp. was cultivated for the first time based on dissolved nutrients from a marine recirculation system. The conceptional design of the photobioreactor system was feasible concerning the pretreatment of recirculation water for microalgae cultivation and the harvesting method of microalgae. For both processes, water pretreatment and harvesting, the foam fractionation technique was applied. Foam fractionation was also applied in previous studies for harvesting of algal cells (Csordas and Wang, 2004) and turned out to be a very effective harvesting method. Very dense algal suspensions were harvested at minimum volumes, the outflow water from the foam fractionators was clear. Thus, foam fractionation proved to be a very effective method for microalgae removal. However, there are other techniques to be considered within an integrated system. Filtrating organisms like bivalves can use microalgae directly, and might be an additional trophic level (Hussenot *et al.,* 1998, Pfeiffer and Rusch, 2000).

There are several types of bioreactors, most of them reported to have very high yield rates. These reactors are often operated at optimum light intensities (Richmond *et al.*, 2003; Pulz, 2001). Especially *Nannochloropsis* was efficiently cultivated in flat plate glass reactors (Cheng-Wu *et al.*, 2001). Reported cell densities for polyethylene bags or transparent fibreglass columns vary between 25-150 x 10^6 cells per ml^{-1} (0.1 to 0.6g ash-free dry mass L^{-1}), in flat plate reactors cell density can reach 5 to 6 x 10^8 cells ml^{-1} (12g dry cell mass m^{-2} day^{-1} or 650mg EPA m^{-2} day^{-1} (Cheng-Wu *et al.*, 2001).

These values could not be achieved during this study, in average 0.94g dry cell mass per m^2 and day could be harvested. This is considered to be mainly caused by the larger diameter of the photobioreactor columns, resulting in a comparably long distance for the light to reach all algal cells. Although the culture has been agitated by air bubbles, self shading of the algae induced photolimitation. In high-tech bioreactors the equal supply of all algal cells with light has to be guaranteed to ensure maximum growth rates of the mi-

croalgae (e.g. 1cm in flat plate reactors, Richmond *et al.*, 2003). However, cultivation in long tubular systems with a low diameter has also already been reported to show acceptable growth rates (Pulz, 1994; Borowitzka, 1996). These systems have high spatial requirements, which are often limited in aquaculture systems. In this study, it was not possible to install such photobioreactor systems due to spatial limitations of the laboratory.

A comparably large diameter of 20cm of the photobioreactor column was chosen in order to increase the culture volume. Hence a total volume of 50 Litres could be placed in a room providing an area of only 0.16m². The reduction of the optical path (equal supply with light for all algal cells) was compensated by agitating of the culture. Air bubbles were constantly directed through the tank to ensure an equal light supply of all algal cells. This "bubble column system" has also been applied during cultivation of microalgae and was considered to be suitable (Eriksen *et al.*, 1998). However, a strong agitation can cause cell damage and hence the intensity of the bubbling has to be carefully adjusted.

The culture has to be monitored cautiously. During cell division *Nannochloropsis* sp. new cell walls are generated and the "old" multilayered parent cell walls are released. The release of this parent cell wall coincides with the release of autoinhibitory substances (Rodolfi *et al.*, 2003). When cell densities reach a critical value, further growth can be inhibited by autoinhibiting substances released by the algae. Thus, both cell wall remains and autoinhibitors may negatively affect culture growth. Additionally, biofouling at the walls of the photobioreactor could be observed and may be another factor influencing continuous growth once maximum growth rates were reached. Because of this biofouling process, photobioreactors had to be cleaned every 2 to 3 weeks.

3.4.2 Nutritional value of microalgae

The harvested algae had a high energy content of up to 20kJ g^{-1} DW. This comparably high nutritional value makes the algae a suitable feed for other feed organisms like *Brachionus* or Copepods (Støttrup & Norsker, 1997). *Nannochloropsis* species are known for the high content of highly unsaturated fatty acids (HUFA): 20:4n-6 have a proportion of 1.9-3.9% (by weight of total fatty acids) and 20:5n-3 contents (12.1%-17.8% as weight percentage of total fatty acids) at a total lipid content of 10.3-16.1% (dry weight, Mourente *et al.,* 1990). Similar values have been observed in the cultivated algae during this study: the relative proportion of arachidonic acid (ARA, 20:4 n-4) of total fatty acid amount were at 6.7% (100µmol s^{-1} m^{-2}), 8.27% (250µmol s^{-1} m^{-2}) and 4.0% of total fatty acid amount (400µmol s^{-1} m^{-2}), respectively. Similar values were determined for eicosapentaenoic acid (EPA, 20:5 n-5) at 15.2% of total fatty acid amount (100µmol s^{-1} m^{-2}), 18.9% (250µmol s^{-1} m^{-2}) and 9.1% of total fatty acid amount (400µmol s^{-1} m^{-2}), respectively. The nutritional value of the cultivated microalgae was of special interest because of the applicability for successful fish larvae rearing (Støttrup and McEvoy, 2002).

The decreasing content of HUFA with increasing light intensities was already reported before (Fabregas *et al.*, 2003). Illumination exceeding light saturation (>220µmol s^{-1} m^{-2}) induces a dramatically decrease in the content of highly unsaturated fatty acids in the microalgal cells. Temperature also is reported to influence fatty acid synthesis in *Nannochloropsis*: with increasing temperatures up to 25°C an increasing trend in the content of HUFAs can be shown, but then a rapid decrease occurs when temperatures of 25°C are exceeded (James *et al.*, 1989). Also salinity has an influence on the content of HUFAs. Optimum salinities for *Nannochloropsis oculata* were reported to be 20-30ppt (Renaud and Parry, 1994). These salinities were therefore chosen for cultivation of Nannochloropsis sp. in the photobioreactors during this study.

3.4.3 Growth performance of Nannochloropsis spec. in continuous cultures

The specific growth rates determined from continuous cultures can only be regarded as approximate values characterizing a small time interval of several hours. The maximum specific growth rate of 0.025 h^{-1} can be reached at all irradiations, if the other cultivation conditions are suitable (e.g. nutrient supply, cell density, light intensity). But for long term cultivation periods, these values might be overestimated, because density in the photobioreactors was always decreasing after a certain time probably partly due to the release of autoinhibitory substances.

Another explanation is the absence of an automatic regulation of flow rate and illumination: it was not possible to reach a real "steady state" in the photobioreactors due to varying nutrient concentrations of the inflow water coming from the recirculation system. Generally, biomass and nutrient concentrations tend towards the steady state conditions with an oscillating process (Pirt, 1975). However, changes in cultivation conditions, especially nutrient concentrations, may disturb this process. Considering constant cultivation of microalgae at high biomass levels, a disturbance may result in an outwash of biomass. Steady state conditions are unlikely to develop in a recirculation system, where several conditions can change within a very short time (e.g. diurnal variation of ammonia, increasing temperatures (optimum 15-20°C, James *et al.*, 1989) etc.). These condition changes are followed by a decreased growth rate due to a *lag* phase needed by the algae to adapt to the new condition. This *lag* phase leads to an wash out of biomass in a continuous culture with a constant flow, if flow rate is constant in turn resulting in a reduced cell density in the photobioreactor. This cascade effect will continue: if the irradiation is not suitable for the "new" reduced cell density and is not adjusted, growth rate will continuously decrease (due to light limitation or photoinhibition, Richmond *et al.* 2003). Biomass will then be washed out even more rapidly. This process is considered to be the reason for the unstable performance of the photobioreactor at high irradiations (400µmol) during long-term experiments.

Algae growth in the photobioreactor at the lowest irradiation (100 µmol s^{-1} m^{-2}) was affected by light limitation, but this photobioreactor operated rather stable in long-term experiments compared to the bioreactors being treated with elevated radiation intensities. At this radiation intensity the algal growth rate may increase at a reduced velocity due to the lower illumination. The photobioreactor at 250µmol s^{-1} m^{-2} radiation intensity showed best long-term performance during the experiment, because light intensities fitted best to the varying cultivation conditions (temperature, nutrient availability), although biomass wash out also occurred.

These results fit to a laboratory study where light saturation for *Nannochloropsis* was achieved at 220µmol s^{-1} m^{-2} with no significant increase in steady state cell density or dry weight productivity at higher irradiations up to 480 µmol s^{-1} m^{-2} (Fabregas *et al.*, 2004). Similar results have been found in this study, although nutrient uptake rates can reach much higher values at irradiation of 400µmol $s^{-1}m^{-2}$.

In this study, growth of *Nannochloropsis* sp. was limited by nitrogen and could be described by a Michaelis-Menten-kinetic in relation to total nitrogen concentration, because in the photobioreactors different chemical forms of nitrogen (nitrate, ammonium and nitrite) were supplied to *Nannochloropsis.* It could not be proved that *Nannochloropsis* prefers one of these form, although according to the data hints are given for nitrate preference. In nature, summer and autumn phytoplankton blooms exhibits typical Michelis-Menten uptake kinetics for NO_3^-, NH_4^+ and urea, although also linear uptake rates may occur with increasing nitrogen concentrations due to eutrophication (Horner Rosser, 2004). Similar to the conditions in the presented photobioreactor, growth rates and nutrient uptake rates in natural systems are not in steady state but will fluctuate according to large and small-scale spatial and temporal variations in nutrient availability. Certain species (e.g. *Clamydomonas*) are able to maximise growth through short-term rapid utilisation. This is considered to be a mechanism for competitive success in a mixed population (Rhee *et al.,* 1988). *Nannochloropsis* was found to grow at

similar growth rates at nitrate, ammonia and urea (Lourenco *et al.,* 2002), indicating an acclimation of microalgae to the respective N sources (Levasseur *et al.*, 1993). In general, ammonium is the preferred chemical form of nitrogen and is readily taken up and assimilated by phytoplankton (Collos and Slawyk, 1981). In contrast to nitrate, microalgae do not need to reduce ammonia prior to assimilation (Syrett, 1981). However, some studies indicate that microalgae growth can be negatively influenced at high ammonia concentrations due to toxification effects (e.g. in media used in aquaculture or nutrient loaded effluents). Lourenco *et al.* (2002) showed that growth of *Nannochloropsis oculata* on urea resulted in a lower final biomass yield. However, supply of ammonia and urea was very low in the photobioreactor system, so toxification effects are considered to be unrealistic.

It has been shown in few studies that different nitrogen sources may result in effects on growth and biochemical composition (content of protein, lipids and carbohydrates, fatty acid profiles) (Lourenco et al., 2002; Fidalgo *et al.,* 1995). High nitrogen concentrations in the culture medium stimulates the accumulation of protein by microalgae (Fabregas *et al.*, 1989; Fidalgo *et al.*, 1995). This might be one reason of the high energy content of the microalgae.

Results from this study showed that uptake rates for phosphorus were higher than for nitrate. This was unexpected with regards to the Redfield ratio. However, it has been shown that in nutrient rich water phosphorus can be rapidly taken up, resulting in a depletion of external phosphorus concentration in the medium. Phosphorus can be stored inside the cells (Passarge *et al.*, 2006). Also, high intracellular concentrations of inorganic nitrogen can be built up if the nitrogen is available in large amounts in the medium (Lourenqo *et al.,* 1998), but nitrogen was limited at the end of the experimental period (see Chapter 5).

3.4.4 Filter efficiency of microalgae photobioreactors

In contrast to the macroalgae filter, microalgae are able to remove nitrate from the water due to longer retention times of nitrate within the culture. According to the specific growth dilution rates in the photobioreactors may not exceed 0.025 h^{-1}. For the presented photobioreactor system with a total volume of 150 Litres the flow rate per hour was appr. 3.75 Litres. This flow rate is far too low to be an effective biofilter for the removal of toxic ammonia and nitrite. However, daily removal rates of nitrate and phosphate have been determined at 0.87g PO_4-P d^{-1} and 0.57g NO_3-N d^{-1}, respectively, supporting the importance to control nitrate and phosphate concentrations within a recirculation system. Thus, simultaneous integration of microalgae photobioreactors and macroalgae filters into a recirculation system is recommended to achieve an optimal removal of all dissolved nutrients derived from fish cultivation (see also MARE II experiment, chapter 5).

3.5 Conclusion

The continuous culture mode turned out not to be suitable for a long term cultivation of microalgae within a recirculation system, if flow rate and light intensities are not automatically adjusted. It is technically possible to install an online flow-through-photometer and a computer controlled light regulation into a recirculation system. However, these installations are expensive and may exceed investment costs for aquaculture systems. Better results may be achieved with semicontinuous microalgae cultures, where a certain percentage of the total volume is replaced every day.

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Chapter 4

Ozonation and foam fractionation used for the removal of bacteria and particles in a marine recirculation system for microalgae cultivation

Kube N. and Rosenthal H.

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Abstract

The use of ozone in combination with foam fractionation was tested for its effectiveness in disinfecting highly loaded waste water in a bypass of a marine recirculation system used for microalgae cultivation. Water was treated with ozone at different levels as measured indirectly via different redox potentials (400mV, 500mV, 600mV). The redox levels were also applied at different retention times of the water in a foam fractionator (5min, 10min, 15min). A broad spectrum of water quality and microbial data for inflow and outflow samples was recorded: viable and total numbers of bacteria, qualitative analysis of living and dead cells (Live/Dead® staining), size distribution of particles and amount of attached living bacteria; pH, free ozone content and dissolved nutrients of the water.

The results showed clearly that ozone kills free floating bacteria under given operational conditions. However, almost all surviving bacteria were attached to mostly organic particles, where they seem to be sufficiently protected from ozone attack as revealed by the specific staining method. As anticipated by the operational criteria of the foam separation and contacting tower, much less bacteria and fine particles were detected in the outflow samples. It can be concluded the foam fractionation process contributes also substantially the mechanical removal of fines and aggregated bacteria cells.

4.1 Introduction

For several decades, interest in aquatic applications of ozone other than for drinking water sterilization (Legeron, 1984) has increased considerably (Rosenthal, 1981, Rosenthal and Wilson, 1987). Ozonation is now widely used and well-established in municipal waste-water treatment (Hoigne and Bader, 1980), in process-water polishing (Richard and Brener, 1984), in soft-drink manufacturing (Schneider and Rump, 1983), in pulp and paper waste-water treatment (Rice, 1984), in swimming pool water treatment (Eichelsdo-erfer and Jandik, 1985), and many other industrial applications including fish processing to extend the shelflife for fresh fish (Kötters *et al.*, 1997; Crappo *et al.*, 2004). Its application in aquaculture recirculation system has also been practiced for more than three decades (Rosenthal, 1981), mostly in experimental fish culture systems but also in a growing number of commercial production units, particularly in hatcheries. (Rosenthal and Wilson, 1987, Buchan *et al.*, 2004; Sander, 1998; Summerfelt *et al.*, 2002; Fraser, 2004).

This application gains importance (Brazil *et al.*, 1998; Waller *et al.*, 2002; Chen *et al.*, 2003) and has recently been increasingly studied in China (Gong *et al.*, 2002; Liu *et al.* 2003, 2004) while applying methodological principles previously developed by Rosenthal (1981).

While the argument for its use was often to disinfect the water, some of the studies clearly identified the benefits of ozone use in combination with foam fractionation and nitrite oxidation. (Rosenthal, 1981; Rosenthal and Wilson, 1987; Summerfelt, 2001). The need for disinfection or even „sterilisation“ (as in drinking water applications) is not even desired in aquaculture systems as these recycling systems work with living animals, and can be considered the as a „commercial ecosystem“ in which a balanced fauna and flora of organisms thrives (Rosenthal, 1981; Rosenthal and Black, 1993). Ozonation has, therefore, mostly been combined with foam fractionation to enhance the removal of particles.

It should be noted that the term „ozone“ is used here in a generic sense as ozone reacts with several seawater components to form secondary oxidants within seconds. Among ozone specialists the term TRO (total residual oxidants) is commonly used to reflect this fact while also circumventing the difficulty to exactly determine the ozone concentration, particularly in seawater (Buchan *et al.,* 2005), where some bromide radicals and hypobromous acid is formed (Tango and Gagnon, 2003). Further, the output of various mixtures of oxygen radicals in any electrical discharge ozonizer depends on system configuration and operational procedures (high frequency or low frequency ozonizer). It is also for this reason that the redox potential has been chosen as a convenient measure to characterize total radical oxidant (TRO) activity. Therefore, the common language usage of „ozone“ in this paper is meant to refer to TROs.

Although ozone cannot be considered a coagulant in the classical sense, many studies have evaluated its use as a coagulant aid. Dissolved organic matter has been identified as playing a primary role in particle stabilization and hence, ozonation – through electrostatic loading and polarization of the hydrophobic-hydrophilic ends - foster settling characteristics of suspended solids while also assisting in forming aggregates that attach to water-air interfaces, producing a stable foam which can be removed by counter-current stripping.

Depending on the organic and microbial load, there will certainly also be some reduction of bacterial counts, however, there have been only a few quantifying studies on the fate of bacteria in ozone contacting chambers (Wuhrmann and Meyrath, 1955), particularly with regard to virus inactivation (Akey and Walton, 1984; Liu *et al.*, 2004), however, there is very little information on the bactericidal effects of ozone application in conjunction with aquaculture recycling systems using foam fractionators. Certainly, the removal of unwanted fine particles which not only consume oxygen, but also are repeatedly in contact with respiratory tissues of the cultured species and – above all – potentially act as „carriers“ for facultative pathogens, is desirable and also reduces simultaneously the bacterial load.

The objectives of the present study were (a) to identify the „disinfection capacity“ of a foam fractionation system using ozone at three different levels and three different retention (contact) times in the contact chamber and (b) to investigate whether there are differences in the survival of free-floating bacteria in contrast to particle-attached microbial species.

4.2 Material and Methods

4.2.1 System configuration

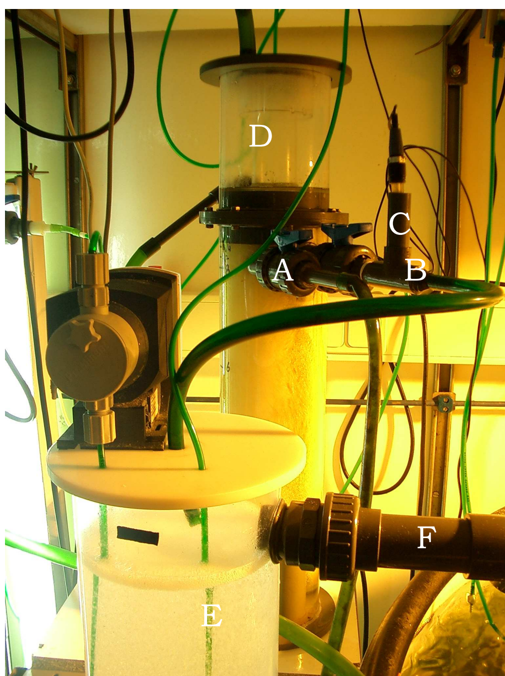
The examined foam fractionator was a small unit operated in an extra bypass (volume 8.4 L, Fig. 1). The purpose was mainly to remove fine particles while at the same time aiming to act as a disinfection unit. The effluent of this foam fractionator was supplied to a novel photobioreactor system, in which microalgae are produced from dissolved nutrients derived from a marine recirculation system. The system was stocked with *Sparus aurata* (Kube *et al.,* 2006a in prep., Chapter 3, Fig. 1). Considering the system configuration the foam fractionator acted as pretreatment of fish effluents in order to remove suspended particles and bacteria entering the microalgae production unit which consisted of three acrylic columns (50L each). For detailed information about the marine recirculation system and the microalgae production unit see Chapters 3 and 5, respectively.

Water from the main recirculation system was continuously pumped (Eheim pump type 1260) to feed the foam fractionator. The flow rate was manually adjusted by a ball valve and flow control unit (see Table 1). Ozone containing air was supplied to the foam fractionator at a rate of 200L/min (estimate from producer table). The feed line comes from the compressed air supply, which also supports the aeration of the entire recirculation system, including water storage column. The ozone generator was a product of the Sander Company (Uetze-Eltze, Germany; model S500, output characters: ozone production at 500mg h^{-1}, air flow rate 50 -1000 L h^{-1}). Air diffusers made of lime wood were used.

Redox potential was determined in the outlet of the foam fractionator by a electrode and regulated by a control module (model KM2000, Sensortechnik Meinsberg GmbH, Ziegra-Knobelsdorf, Germany) (Fig. 1, C). Foam condensate was collected in a separate container. Waste air from foam fractionation and waste container were treated with activated carbon. Pretreated water was transferred to a storage column (Fig. 1, E) with a constant aeration for outgassing residual ozone and to stabilize the pH. Spare water from the foam fractionator was discharged via an overflow into the main water circle (Fig. 1, F).

4.2.2 Sampling methods

Sampling was done at randomly chosen production days during the entire investigational period (April 2004 to March 2006). Different retention times were adjusted by a flow meter at levels according to Table 1 the day before the sampling started. This was to ensure that redox potential reached the desired level. However, we are aware that a certain flow rate in a given time does not necessarily result by definition in true retention times because of improper mixing and turbulences. However, for the purpose of this study the approximate time is considered to be adequate.



Tab. 1 Overview about the adjusted flow rates for the required retention time within the foam fractionator

Retention time	Flow rate/h
15 min	32 Litres
10 min	48 Litres
5 min	96 Litres

Fig. 1 Foam fractionator for the pretreatment of system water.
A = Inlet, B = Outflow, C = redoxpotential probe, D=Foam collector, E = water storage column with aeration, F=overflow back to the main system

Samples were taken at the inlet (Fig. 3, A) of the foam fractionator and at the outflow (B) after the calculated retention time.

Chemical analysis of water samples

A volume of 20ml was used to determine pH (model multi 340i, WTW, Germany). It was frozen at -20°C for later analysis of dissolved nutrients by an Autoanalyser AA3 (Bran-Lübbe, Norderstedt, Germany: Method no. G-016-91 for Nitrate-N, G-029-92 for Nitrite-N, G-102-93 for Ammonia-N, G-103-93 for Phosphate).

The content of TRO [mg L^{-1}] was measured in outflow samples using HACH-Permachem® Reagents (DPD Free Chlorine Reagent, 10ml). Extinction was measured with a spectrophotometer (Hach, Germany) and given in free chlorine concentrations. Values were multiplied with the factor 0.6769 to recalculate content of free total residual oxidants (from now on referred to as ozone).

Microbiological methods

1) CFU determination – viable cells

Bacterial abundances were determined using the pour-plate method by Koch (JAHR) with standard marine agar (TSB 3). Different dilutions were used for inflow and outflow samples (1ml to 1:1000 & 1:10.000 for inflow samples; 1:10 & 1:100 for outflow samples). Samples were incubated at room temperature for two weeks in darkness and colony forming units (CFU) were detected after incubation.

2) Epifluorescence Microscopy

a) TBN-total bacterial number

Different bacterial species have different growth requirements and therefore only a small proportion of the total bacterial number will be able to grow on standard agar. To determine the total number of bacteria, the Acridine Orange Direct Count (AODC) method was used. Acridine Orange binds to acidic cell components like DNA (orange stain, Jochem, 2001) and the stained cells

can be counted using epiflourescence microscopy. Therefore, immediately after sampling, 20 ml of the sample were transferred into a glass vial (Perkin Elmer, PLCAP/500), fixed with 0.5ml particle free formaline and stored at room temperature in darkness for later analysis.

For staining, samples were vacuum-filtrated (<200 mbar) through 0.2µm black polycarbonate filters preventing background fluorescence (ø25mm) (Whatman Nucleopore). Filtration volume for Acridin Orange samples ranged from 2 ml (inflow sample) to 6 ml (outflow sample). 1 ml of acridine orange was pipetted to the sample and the dye was incubated on the filter for 5min in darkness (STOCKING). Acridine Orange was then removed by filtration (200 mbar); filters were air-dried, embedded in oil (Cargille®) and kept in darkness before analysis using a Zeiss Axioplan epifluorescence microscope with a filter setting KP490,KP500; Teiler 510 & LT520 (450 – 490 nm emission).

b) Viable staining

Qualitative analysis of bacterial abundances distinguishing living and dead cells was performed with LIVE/DEAD® BacLight™ Bacterial Viability Kits for microscopy (Molecular Probes). This method includes two different nucleid acid stains differing in their spectral characteristics and in their ability to penetrate healthy bacterial cells. SYTO®9 (3.34 mM) is green fluorescent and labels all bacteria. In contrast, propidium iodide (20 mM) penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence, when both dyes are present (Molecular Probes, 2004). So, dead cells are stained in red, whereas living cells will be stained in green. For staining, 2 ml of sample water were transferred to a 2 ml Eppendorf cup and immediately stained with both dyes (3 µl). After 15min incubation at room temperature, bacterial samples were fixed with 0.07ml formaline and stored in darkness for later analysis.

For analysis, 2 ml of the samples (inflow and outflow samples) were filtered in order to enrich bacteria on the filter. Filters were mounted in oil (*Bac*Light

mounting oil) and stored in the darkness. Analysis was performed by epifluorescence microscopy analogue to the AODC method.

Calculation of TBN and viable staining

Total bacterial numbers and viable bacteria were determined by counting 30 grid fields (New Porton G12; Graticules Ltd., UK) with 1000x magnification. Total bacterial numbers were calculated using the following equation:

$$N = \left(\frac{F \cdot n_{grid}}{V} \right) \qquad \text{(Equation 1)}$$

where N is the total number of bacteria per ml, F is the conversion factor of 367.455 which reflects the area of the New-Porton G12 grid in relation to the total filter area (inner diameter of the filter), n_{grid} is the average number of bacteria per grid and V the filtrated volume. Total numbers were divided by 1.000.000 to express cell concentrations in Mio cells/ml.

Additionally, the size distribution of particles and the amount of bacteria living attached to particles were determined by viable staining. Therefore, 10 grid countings (New-Porton G12 grid, 0.44mm width, 0.74mm length each, 100x magnification) were summarized. Size classes were determined according to the grid: less than <10µm, 10-50µm, 50- 120µ, 120-240µm, >240µm.

Due to patchy distribution of the bacteria attached to particles, it was not always possible to count every single bacterium. Furthermore, only the top side of the particle was visible. Thus, bacteria attached to the back could therefore not be counted and integrated into further calculations. Nevertheless, the amount of visible bacteria attaced to particles was classified into 4 groups: 0 = none, + = 1 - 5, ++ = up to 20 ; +++ = >20.

4.3 Results

4.3.1 Viable counts

Viable plate counts were used as first indicator of disinfection efficiency. Table 1 shows that application of TRO´s at levels of 500mV and 600mV resulted in very low number of colony forming units (CFU) per ml, independent from the initial bacterial load of the inflow, which differs due to conditions in the recirculation system. For 600mV it was 0.600 ± 0.560 10^3 CFU/ml (15min retention time in foam fractionator); 0.144 ± 0.056 10^3 CFU/ml (10min retention) and 0.093 ± 0.025 10^3 CFU ml^{-1} (5min retention), respectively. Average numbers for 500mV were: 0.200 ± 0.149 10^3 CFU ml^{-1} (15min retention), 0.174 ± 0.187 10^3 CFU ml^{-1} (10min retention) and 0.039 ± 0.037 10^3 CFU ml^{-1} (5min retention), respectively. 15min retention time at 400mV gave similar results: 0.796 ± 0.321 10^3 CFU ml^{-1}. Less retention time resulted in a rapid increase to several thousand CFU ml^{-1}: 6.92 ± 5.34 10^3 CFU ml^{-1} (10 min retention time) and 23.91 ± 14.34 10^3 CFU ml^{-1} (5min retention time).

Tab. 2 Viable bacterial counts (pour-plate method) as determined from inflow and outflow samples of foam fractionator at different redoxpotential and water retention times. Data represent means of three parallel subsamples. Total sample number for 600mV = 8, taken over two operational days. All other operational redox levels (n = 5) are taken at the same day. % dead indicates percentage decrease from inflow samples.

	colony forming units (CFU) (10^3 per ml)								
retention time	15 min			10 min			5 min		
	Inflow	Outflow	% dead	Inflow	Outflow	% dead	Inflow	Outflow	% dead
600 mV	17.8	0.19	*98.93*	37.5	0.13	*99.65*	273.2	0.09	*99.97*
	13.6	1.72	*87.35*	26.8	0.14	*99.48*	475.8	0.12	*99.97*
	18.0	1.36	*92.44*	41.7	0.25	*99.40*	95.3	0.07	*99.93*
	1273.3	0.45	*99.96*	93.5	0.11	*99.88*	-	-	-
	14.2	0.38	*97.32*	27.0	0.09	*99.66*	-	-	-
	19.5	0.12	*99.38*	-	-	-	-	-	-
	29.0	0.25	*99.14*	-	-	-	-	-	-
	25.7	0.30	*98.83*	-	-	-	-	-	-
average		**0.6**	***96.6***		**0.14**	***99.6***		**0.28**	***99.9***
500 mV	983.3	0.49	*99.95*	630.7	0.51	*99.92*	246.7	0.03	*99.99*
	1246.7	0.13	*99.99*	696.7	0.23	*99.97*	368.3	0.005	*99.99*
	1333.3	0.08	*99.99*	480.0	0.11	*99.98*	201.7	0.02	*99.99*
	1180.0	0.19	*99.98*	666.7	0.01	*99.99*	220.0	0.03	*99.99*
	1046.7	0.11	*99.99*	583.3	0.01	*99.99*	171.7	0.11	*99.99*
average		**0.2**	***99.98***		**0.17**	***99.93***		**0.039**	***99.99***
400 mV	85.5	0.48	*99.44*	113.0	9.15	*91.90*	691.7	19.65	*97.16*
	283.2	1.37	*99.52*	534.5	1.91	*99.64*	619.0	43.44	*92.98*
	69.2	0.74	*98.93*	102.0	3.60	*96.47*	453.0	38.02	*91.61*
	90.1	0.87	*99.03*	700.2	3.54	*99.49*	372.2	10.12	*97.28*
	120.3	0.52	*99.57*	290.5	16.4	*94.35*	238.5	8.32	*96.51*
average		**0.796**	***99.3***		**6.92**	***96.4***		**23.91**	***95.11***

4.3.2 Quantitative and qualitative analysis

Not all bacteria grow on agarplates. Staining samples with acridin orange can provide total bacteria numbers, however this method does not differentiate between living and dead bacterial cells, so an overestimation of bacteria contamination may occur. Staining with LIVE/DEAD® allows a qualitative analysis to distinguish between living and dead bacteria cells.

The results clearly indicate that living bacteria were almost exclusively found attached to particles of different size (Fig. 3a). Unattached bacteria were almost exclusively stained red, and therefore identified as dead. Nevertheless after the ozone treatment living bacteria were still found while attached to particles (Fig. 3b), but in much smaller numbers. Both the amount of particles and free bacteria was much less than the inflow. Thus, most of them were removed by the foam fractionation process and were collected in the waste vessel (Fig. 3c).

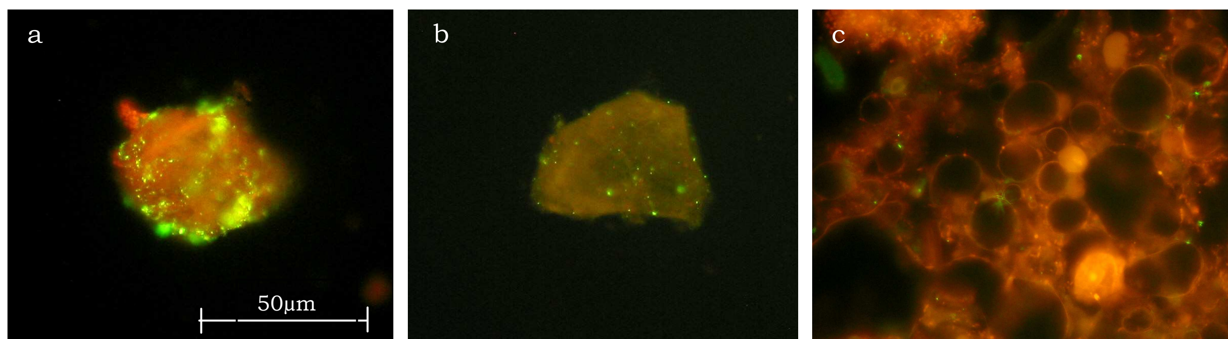


Fig. 3 Occurrence of living bacteria (green) attached to particles (red) (a) in the inflow and (b) in the outflow of the pretreated water. The particles are removed by the foam fractionation and can be found in the waste container (c).

The examination of inflow and outflow samples showed, that removal of unattached bacteria (Fig. 4a) and particles (Fig. 4b) was very effective at every treatment level tested, even at short retention times. Similar to the results on viable counts, outflow concentration of bacteria and particles (grey bars, Fig. 4a, b) are at constantly low levels, independent of the inflow concentration (black bars) (ca. 1000 particles per ml and 0.5 Mio. bacteria per ml).

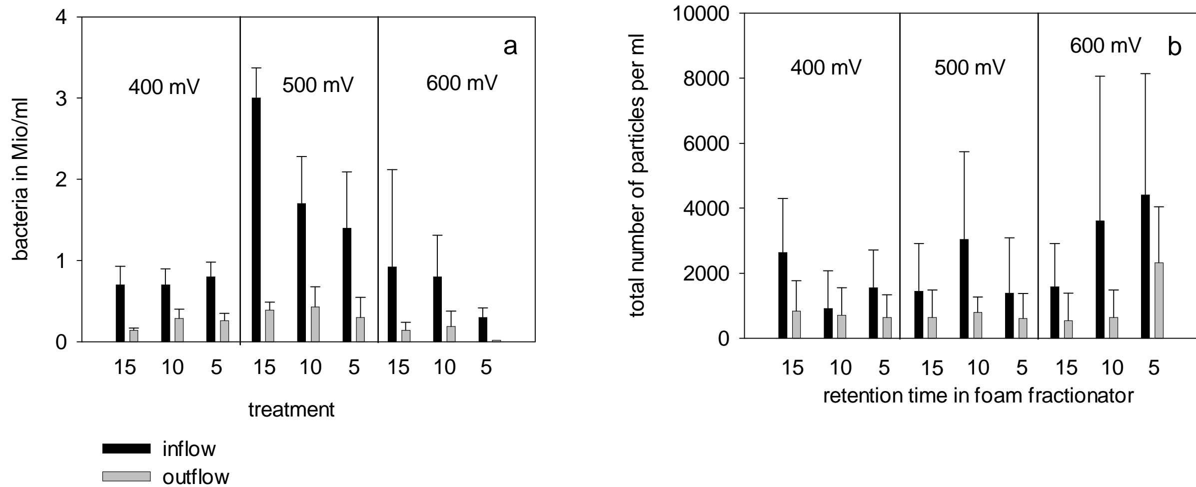


Fig. 4 Bacterial and particle counts in samples from the inflow and outflow of the foam fractionator. (a) bacteria (results from AO staining) and (b) total number of particles at different redox potentials (400mV, 500mV, 600mV) and retention times (15min, 10 min, 5 min). Results show low outflow concentration of both bacteria and particles (grey bars), independent from inflow concentrations (black bars).

If particles play the dominant role for bacteria contamination, we have to investigate the question: what sizes of particles can be found and is there a bacteria preference for attaching to certain particle sizes?

Size distribution of particles gave following results: At each treatment, size classes of less than 10µm and 10 - 50µm were the most frequent particle size. Both classes together represent at least 75%, sometimes almost 99% of the total number of the sample (Fig. 5a,b).

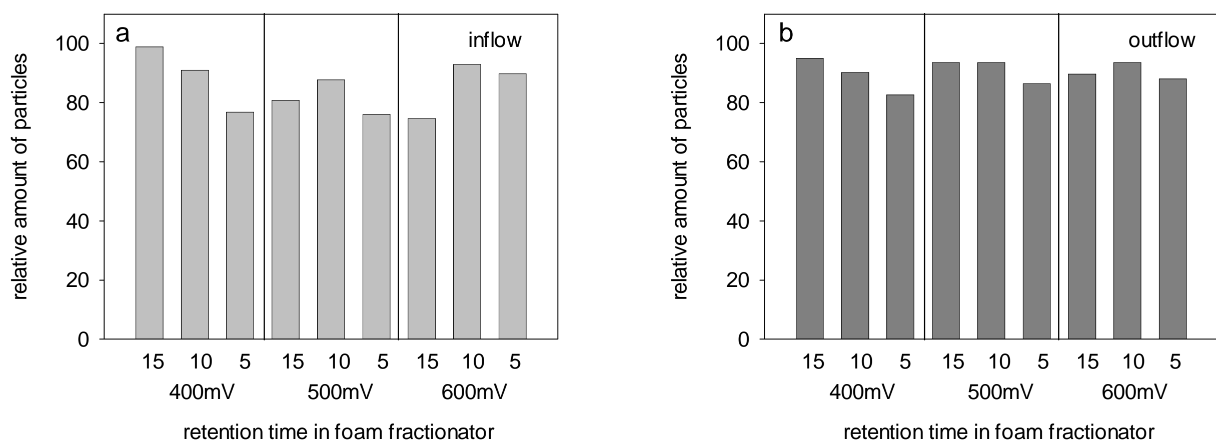


Fig. 5 Relative amount of particles of the size >10µm and 10-50µm in the (a) inflow samples and (b) outflow samples in relation to total number. Data represent average values of 5 replicates for each treatment at different redoxpotentials [mV]/retention time [min].

Regarding the abundance of particles with bacteria in relation to total particle numbers (Table 3, 4), the majority of particles <10µm was detected to be free of bacteria. This size class was dominated by dead bacteria forming agglomerations (2-10 bacteria) and were classified as particles without living bacteria.

In the size class 10 to 50µm, a variation between dominance of particles without bacteria and particles with attached bacteria was observed. In this size class very often eukaryotic microalgae were observed and declared as particles without bacteria (see also Tab. 4).

In particle size classes 50µm to 240µm the proportion of particles with attached bacteria increased, whereas the proportion of particles free of attached bacteria decreased (Tab. 3). Particles >240µm were dominated by *Sphaerotilus natans*, a sewage bacterium forming long chains. Particles identified as *Sphaerotilus natans* were assigned to the fraction “particle without bacteria” (Tab. 3, column >240µm “0”). *Sphaerotilus natans* chains were mainly found in inflow samples; in outflow samples they were only detected at an redox potential of 400mV (Tab. 3, >240µm “0”). In summary, increasing particle sizes can be correlated with increasing abundances of living bacteria attached to the particles.

According to the results, particles >50µm are of minor importance for particle contamination because of the low abundance of these size classes. Particles sizes of 10 to 50µm are of major relevance for the contamination on the one hand because of their high abundance and on the other hand because of the high abundances of particle-attached bacteria (Fig. 5).

Tab. 3 Summary of data for size distribution of particles and the amount of attached bacteria per ml sample for inflow (in) and outflow (out) samples for each treatment (redoxpotential [mV], retention time [min], left column). Particles were sorted according to size (first row of table) and bacteria amount: 0 = no living bacteria, + = 1-5 attached bacteria, ++ = up to 20 bacteria, +++ = more than 20 bacteria. Average & SD from 5 replicates for each treatment. Total = total numbers of particles

Redoxpotential [mV]/retention time[min] — Particle size	<10µm				10 - 50µm				50 - 120µm				120 - 240µm				>240µm				
att. bacteria	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++	0	+	++	+++	total
400/15 in	**2029** ±854	**153** ±205		**56** ±124	**181** ±188	**97** ±116	**28** ±62	**69** ±49	**14** ±31						**14** ±31						**2640** 1661
400/15 out	**389** ±349		**139** ±203	**14** ±31	**69** ±85	**83** ±76	**56** ±58	**42** ±38	**14** ±31							**14** ±31	**14** ±31				**834** 934
400/10 in	**208** **±139**	**69** **±85**	**69** **±155**		**56** **±31**	**153** **±151**	**181** **±293**	**97** **±144**	**14** **±31**	**28** **±38**	**28** **±62**		**14** **±31**								**917** **1161**
400/10 out	**125** ±91	**42** ±38			**111** ±105	**306** ±416	**56** ±58		**28** ±31	**14** ±31							**14** ±31		**14** ±31		**709** 839
400/5 in	**667** ±356	**97** ±79	**14** ±31	**28** ±38	**222** ±151	**111** ±38	**42** ±62	**14** ±31	**14** ±31	**42** ±38	**14** ±31	**28** ±38		**14** ±31	**14** ±31	**14** ±31	**181** ±79	**28** ±38		**14** ±31	**1556** 1166
400/5 out	**195** ±227	**28** ±38		**28** ±38	**167** ±105	**56** ±58	**42** ±38	**14** ±31	**42** ±62				**14** ±31	**14** ±31			**42** ±38				**639** 698
500/15 in	**903** ±681	**42** ±38		**14** ±31	**153** ±166	**42** ±93	**14** ±31		**97** ±79	**42** ±62	**14** ±31	**28** ±62	**28** ±62	**14** ±31		**28** ±62	**28** ±38				**1445** 1468
500/15 out	**514** ±642	**56** ±76			**28** ±38					**14** ±31		**14** ±31	**14** ±31								**639** 849
500/10 in	**1792** ±1471	**264** ±296			**459** ±305	**125** ±114	**28** ±62		**83** ±31	**167** ±135	**28** ±62		**28** ±62	**14** ±31			**42** ±93		**14** ±31		**3043** 2695
500/10 out	**452** ±216	**35** ±40			**226** ±67		**35** ±40		**35** ±69				**17** ±35								**799** 467
500/5 in	**264** ±257				**528** ±373	**139** ±163	**111** ±212	**14** ±31	**153** ±342	**56** ±91		**28** ±62	**14** ±31	**28** ±38		**14** ±31	**28** ±38	**14** ±31			**1389** 1700
500/5 out	**139** ±196				**361** ±345		**14** ±31	**14** ±31	**14** ±31	**14** ±31			**28** ±38	**14** ±31				**14** ±31			**611** 766
600/15 in	**361** ±134	**250** ±167		**69** ±69	**250** ±126	**83** ±91	**97** ±79	**69** ±98	**56** ±91		**14** ±31	**69** ±98	**14** ±31	**14** ±31	**28** ±62		**208** ±220				**1584** 1328
600/15 out	**153** ±193	**56** ±91	**83** ±186	**83** ±91		**14** ±31	**42** ±62	**56** ±91				**28** ±38	**14** ±31					**14** ±31			**542** 844
600/10 in	**1158** ±1300	**116** ±145	**69** ±69	**1297** ±2127	**208** ±69	**139** ±69	**69** ±69	**301** ±343			**23** ±40	**93** ±106			**23** ±40		**116** ±145				**3612** 4454
600/10 out	**514** ±642	**56** ±76			**28** ±38					**14** ±31		**14** ±31	**14** ±31								**639** 849
600/5 in	**1945** ±1965	**243** ±344	**556** ±491	**382** ±147	**139** ±0	**243** ±49	**139** ±196	**313** ±49		**69** ±98	**69** ±98	**69** ±0		**14** ±31		**35** ±49		**35** ±49	**69** ±98	**35** ±49	**4411** 3782
600/5 out	**1112** ±884	**104** ±49	**104** ±147		**382** ±147	**104** ±49	**208** ±98	**35** ±49		**139** ±98		**35** ±49						**69** ±98	**35** ±49		**2327** 1719

Tab. 4 Percentage of particles without (0) and with attached living bacteria (#) for each size class in inflow samples at different treatments (redoxpotential [mV]/ retention time [min], left column) in relation to total numbers (Table 5). Data present average values of 5 replicates at each treatment. Sum1 = sum of size class <10µm + 10-50µm; sum2 = sum of size classes 50-120µm + 120-240µm + >240µm.

	particle size classes											
INFLOW	<10µm		10-50µm			50-120µm		120-240µm		>240µm		
treatment	O	#	0	#	**Sum1**	0	#	0	#	0	#	**Sum2**
400/15	76,8	7,9	6,8	7,4	**98,3**	0,5	0	0	0,5	0	0	**1,1**
400/10	22,7	15,2	6,1	47,0	**90,9**	1,5	6,1	1,5	0	0	0	**9,1**
400/5	42,9	8,9	14,3	10,7	**76,8**	0,9	5,4	0	2,7	11,6	2,7	**23,2**
500/15	62,5	3,8	10,6	3,8	**80,8**	6,7	5,8	1,9	2,9	1,9	0	**19,2**
500/10	58,9	8,7	15,1	5,0	**87,7**	2,7	6,4	0,9	0,5	1,4	0,5	**12,3**
500/5	19,0	0,0	38,0	19,0	**76,0**	11,0	6,0	1,0	3,0	2,0	1,0	**24,0**
600/15	22,8	20,2	15,8	15,8	**74,6**	3,5	5,3	0,9	2,6	13,2	0	**25,4**
600/10	32,1	41,0	5,8	14,1	**92,9**	0	3,2	0	0,6	3,2	0	**7,1**
600/5	44,1	26,8	3,1	15,7	**89,9**	0	4,7	0	2,4	0	3,1	**10,2**

4.3.3 Influence of ozone on efficiency of foam fractionation

High values of free ozone are necessary for sterilization (Sander, 1998). Results from the present study clearly showed, that bacteria are able to survive treatments of 15min at 600mV redox potential when they live attached to particles. For decontamination of a recirculation system, it is therefore of major relevance to efficiently remove particles of 10 – 50 µm.

Two questions are implicated based on these results: i) does the content of free ozone have an impact on the removal efficiency and ii) which redox potential value is needed to efficiently remove particles? The solution of these problems is crucial for practical application, because technical ozone production is very cost-intensive.

Unfortunately, the results from this study could not be affirmed statistically due to the varying conditions during the microalgae cultivation period. When single data points are plotted against the content of free ozone, evidence was obtained for an redox potential higher than 400mV appearing to be crucial for efficient particle removal. Lower retention times (< 15min.) resulted in higher viable counts (Koch method, Fig. 6a), occurrence of *Spaerotilus natans* (Tab. 3, >240µm “0”, out) in the outflow sample and lower particle

removal efficiency (Fig. 6b). This effect could be alleviated by elevated retention times. Redox potential values > 500 mV did not yield a higher particle removal efficiency, even when retention times were varied. However, concerning this topic, further investigations using a comparable system are needed.

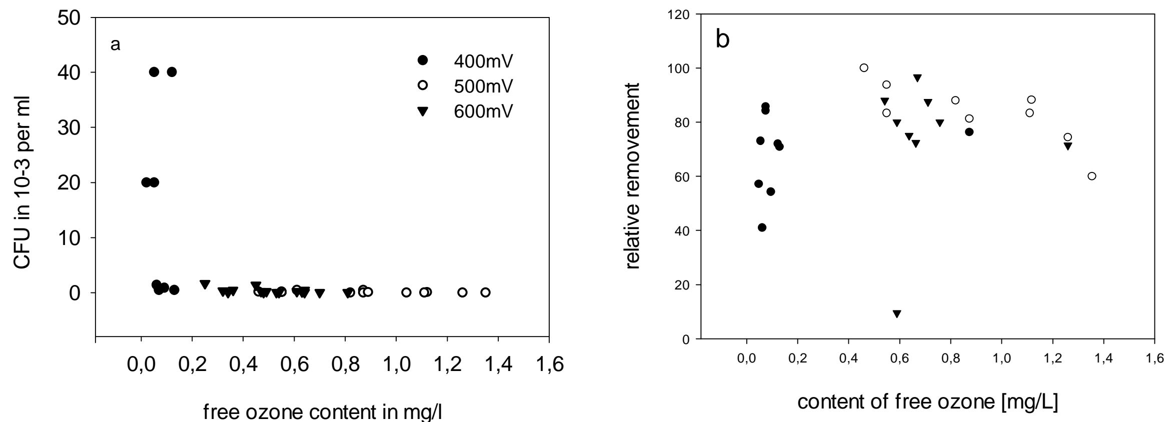


Fig. 6 Free ozone content for each treatment (5min, 10min and 15min. retention time) at total residual oxidant levels (TRO) in mgL^{-1}. (a) colony forming units, (b) relative amount of removed particles from inflow (data are shown for values with at least 2000 particles per ml inflow).

4.4 Discussion

Because of its known disinfection capacity, ozone is believed to be most effective to reduce the bacterial load of the water in aquatic applications (Sander, 1998). While this is true for drinking and process water sterilization where high dosages are employed, the use of ozone in aquaculture has never been exclusively targeted on this goal because of the risk to damage the cultured species at the same time (Brazil *et al.*, 1998). Therefore the application mostly aims at a multiple function with: (a) primarily enhanced removal of fine particles through aggregation (e.g. electro-static loading) and counter-current foam stripping, (b) simultaneous oxidation of organic compounds with multiple double bonds that are not easily degradable in biofilters (e.g. conversion into bio-degradable breakdown products to be returned to the biofilter) and finally (c) to reduce to some extent the overall microbial load (Rosenthal, 1981; Liltved, 2000; Summerfelt *et al.*, 2002).

The interest to understand the qualitative and quantitative effects of by-pass ozonation on overall reduction in pathogen counts has recently been revived in relation to recirculating aquaculture systems to combate fish diseases such as *Saprolegniasis* in freshwater systems (Forneris *et al.*, 2003) and marine halibut rearing facilities (Fraser, 2004) as well as shrimp hatcheries (Meunpol *et al.*, 2003). Bullock *et al.* (2002) clearly demonstrated that number of pathogen counts can be reduced in freshwater recirculation systems to reduce the risk of disease outbreaks such as bacterial gill diseases (BGD). While ozonation did reduce BKD mortality in fish, it failed in nearly all cases to produce even a one $\log^{10}$ reduction in numbers of heterotrophic bacteria in the system water or on gill tissue. Failure of the ozone to lower numbers of heterotrophic bacteria significantly or to prevent the causative BGD bacterium from occurring on gills was attributed to the short exposure time to ozone residual (35 s in contact chamber) and rapid loss of oxidation efficiency caused by levels of total suspended solids and nitrite. Our study clearly shows that retention time and thereby contact time, has certainly a major role in effectiveness to damage heterotrophic bacteria, due to increasing levels of TROs. The amount of total residual oxidants plays a major role for survival of bacterial fish pathogens. Sugita *et al.*, 1992, showed that bacterial counts of *Enterococcus seriolicida*, *Vibrio anguillarum* and *Pasteurella piscicida* decreased by more than 0.040 to 0.060 mg of total residual oxidants per litre, whereas no decrease in viable counts was observed at less than 0.018 to 0.028mg of TROs per litre. Similar results have been found in this study. Hence, the effectiveness of ozone treatment depends on TRO concentration, length of ozone exposure (contact time), pathogen loads and levels of organic matter.

However, it seems not advisable to apply ozone directly in the main stream of a fish culture recirculation system, because high levels of ozone can have a negative effect on fish health. Ozone appears to cause severe damage to all external tissues. This can result in disruption of respiration, osmoregulation and possibly excretion, resulting either in death or in the development of various sublethal secondary pathological effects (Paller and Heidinger, 1980).

But it is possible to introduce ozone in a by-pass stream which receives only a portion of the total flow. When this by-pass flow is returned to the main stream, the residual total radical oxidant concentration is drastically reduced, thereby greatly reducing the risk of toxic concentrations entering the fish culture tank. Further, residual ozone in the effluent of the counter current foam stripping unit will immediately react with organic particles and other reactants in the main stream, thereby quickly reducing the remaining TROs to safe levels.

Otherwise this study showed that use of foam fractionation supports the disinfecting process, as most of the living bacteria were determined to be attached to particles. Particles of the size less than 10µm and between 10 to 50µm turned out to be the major size class because of their major abundance in total numbers of suspended solids. This has been concluded also in other studies (Orellana *et al.*, 2005). One third of the solid load of a recirculation system can be found as suspended solids (Waller *et al.*, 2003a). High numbers of small particles provide a large surface area resulting in sufficient substrate for bacteria. This fraction needs to be removed, as to our knowledge this study documents for the first time by directly distinguishing between life and dead bacterial cells that bacteria can even survive treatments at high TROs at retention time of 15 minutes, when being attached to particles. Especially the surfaces of particles can provide microhabitats for bacteria (Curds, 1982; Acinas, 1999). The particles can serve as food source (i.e. in the marine realm, particle-attached bacteria are thought to play an important role in carbon cycling (Cammen and Walker, 1982; Irriberry *et al.*, 1990) but also provide protection from bacterivores (Curds, 1982). Even suboxic/anoxic conditions may develop and hence allow anaerobic bacteria to survive at these surfaces. In marine snow, several bacterial species are known to produce a capsular envelope in order to ascertain suitable conditions and to protect themselves against bacterivores (Heissenberger *et al.*, 1996).

There is one advantage of ozone application over UV-light use in recirculation systems. While UV-disinfection targets on DNA-destruction, ozone (total

radical oxidants) reacts with the surfaces of microbial membranes. While UV-light may only cause partial damage to bacterial cells and may enhance mutation (e.g. UV-resistant strains), ozone application is an all or none function. Either membrane double bonds are broken up by ozonlysis, or the membrane stays intact, partly being protected by surface contacts with particles, thereby permitting full survival of the cell.

For application purposes in aquaculture, a two step solid separation (sedimentation, foam fractionation) is needed to remove all size classes of solids and has been recommended before to achieve sufficient hygienic conditions in farming systems (Waller *et al.*, 2003a,b). Ozonation improves removal of total suspended solids in foam fractionators due to a decrease in particle stability (Rueter and Johnson, 1995).

4.5 Conclusion

The present study indicates that effective particle aggregation and removal can be achieved with relatively low ozone levels. At high suspended solid loads it seems not advisable to apply very high dosages as this will not achieve noticeable simultaneous disinfection as most bacteria will survive in attachment to individual particles and particle aggregates. However, even without killing bacteria, the overall bacterial counts will be reduced mainly as an effect of removal of large amounts of fine particles to which these bacteria adhere. The „disinfection“ is therefore indirect.

4.6 Acknowledgements

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Chapter 5

MARE – Marine Artificial Recirculated Ecosystem II: Influence of the nitrogen cycle in a marine recirculation system with low water discharge by cultivating detritivorous organisms and phototrophic microalgae

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(2006)

Chapter 5

MARE – Marine Artificial Recirculated Ecosystem II: Influence on the nitrogen cycle in a marine recirculation system with low water discharge by cultivating detritivorous organisms and phototrophic microalgae

Kube N., Bischoff A.A., Blümel M., Wecker B. and Waller U.

Abstract

Water as well as nutrient recycling gained more and more attention in recirculating aquaculture systems during the last few years. The newly developed recirculating system MARE (Marine Artificial Recirculating Ecosystem) showed great potential towards these requirements. The experiment MARE II focused on the integration of a detritus removing compartment using *Nereis diversicolor* (Polychaeta) and the development and integration of a microalgae bioreactor into the system.

Solids derived from the particulate fish waste were driving growth and reproduction of *Nereis diversicolor*. One complete life cycle of the polychaete was observed under artificial conditions within a period of 110 days. Continuous culture of the microalgae *Nannochloropsis* spec. (μ_{max} = 0.025 h^{-1}) was developed and integrated as secondary bypass system for the production of valuable microalgae nutrition.

The consumption of organics and the bioturbation effect of the worms kept the organic load of the sediment constant over a period of about 90 days. Due to the monotelic reproduction of the worm species larger individuals of *Nereis diversicolor* disappeared after spawning. The decreased food consumption led to an accumulation of organic material in the first section of the detritivorous reactor. This increased organic content created anoxic conditions leading to enhanced denitrification. Decreasing nitrate concentrations and increasing pH values in the system were the result of this development.

Even though bacterial activity at all nitrifying components (biofilter, sediment surface, system walls, etc.) could be measured, total ammonia-nitrogen (TAN) concentrations were increasing. These elevated NH_4 concentrations are assumed to be caused by another source than fish metabolism. Potential explanations for these elevated concentrations may be the reduced nitrification rate due to elevated denitrification rates or the assimilatory nitrate reduction processes both occurring when suboxic/anoxic conditions are present.

Keywords: marine recirculation system, integration, *Nereis diversicolor*, *Sparus aurata*, *Nannochloropsis* spec., artificial ecosystem

5.1 Introduction

The integration of secondary biological treatment components in marine recirculation systems is gaining more importance in order to reduce the environmental impact of aquaculture systems (e.g. eutrophication, Chopin et al., 2001). Cultivation of additional organisms within these systems may enhance their profitability (Chopin et al., 2003; von Harlem, 2006). However, the integration of additional organisms requires considerable financial investment during the setup.

Besides new developments in conventional production units (Neori et al., 2000), the first concept of a marine recirculation system with water and nutrient recycling was realised by integration of macroalgae filtration and marine ragworms (MARE, see Chapter 2). It could be shown that detritivorous organisms like *Nereis diversicolor* are suitable secondary organisms for utilization of suspended solid waste from fishtanks (Bischoff, 2003).

Macroalgael filters are the typical secondary treatment components for mariculture (Neori *et al.*, 2000; Chopin *et al.*, 2003). Nevertheless, integration of additional steps needs to be considered to reduce impacts of maricultures on the environment. Potential candidates are bivalves, shrimps, sea cucumbers and different microalgae, but only few attempts have been conducted to investigate the effectiveness of these organisms as additional steps in mariculture systems (Sphigel *et al.*, 1993; Neori et al., 2000).

The main task in marine recirculation aquaculture systems (RAS) is the removal of dissolved nutrients from the recirculated water. To date, there are almost no alternatives to bacterial or macroalgae filters for removal of dissolved nutrients. The use of microalgae filters was not tested intensively except for some studies mainly focused on diatoms (Hussenot, 2003; Tandler, 2003). However, suspended microalgae like *Nannochloropsis*, *Rhodomonas*, *Tetraselmis* etc. have a high value for aquaculture feed, e.g. for shrimps, herbivorous organisms (Rotatoria, Copepods) or larviculture (Sargent *et al.,* 1997; Brown *et al.*, 1997; Bessonart *et al.*, 1999). The present study was per-

formed in order to evaluate a novel continuous photobioreactor system with an automatic water pretreatment and harvesting process for the cultivation of microalgae as part of a recirculating aquaculture system.

5.2 Material and Methods

5.2.1 Modifications of the recirculation system

The system configuration of the investigated recirculation system is presented in Wecker *et al.*, 2006 (Chapter 2). Here, the first version of a low water discharge multi-loop recirculation system MARE (Marine Artificial Recirculation System, 4.5 m^3) is described. A modification of this system was also used in the present study (Fig. 1). Fish biomass was increased compared to MARE I; each fish tank (1) (700L each) was stocked with 35 Gilthead seabream (*Sparus aurata*) and 65 additional animals were cultivated in the former macroalgae tank (3, 1500L). Average size of fish in all tanks was 354.9 ± 48.8g, resulting in an initial stocking density of 9.8 kg per m^3 system volume at the start of the experiment.

The removal of dissolved nutrients was achieved by means of a photobioreactor system for the continuous cultivation of microalgae (for details see Chapter 3). This system consisted of a disinfection unit, a production unit and a harvesting unit (see also Chapter 3). In the disinfection unit water was pretreated in a foam fractionator at high redox potential values (500-600mV, 0.8-1.0mg L^{-1} total residual oxidants, TRO). Water was afterwards transferred to a degassing tower with compressed-air aeration to remove residual ozone (B). Further details of the “disinfection” and harvesting unit are outlined in Chapter 4 and Chapter 3, respectively.

The cultivation unit for microalgae consisted of three acrylic columns (20cm diameter, 1.50 m height) with Y-shape airdiffusers and an attached light tubes. *Nannochloropsis* sp. was cultivated and continuously harvested by a second foam fractionator. The foam condensate carrying the microalgae was collected in a separate tank. Water treated this way then flowed back into the main water circulation system at the outflow of tank 3.

In the modified system (MARE II) a trickling biofilter (7) was installed to enhance nitrification in order to maintain minimal concentrations of ammonia and nitrite. The bioactive surface of the biofilter was approx. 35m².

Several units were not modified for application in MARE II (Fig. 1): the detritivorous culture tank (2), two foam fractionators (5) and pump (4) were used analogous to MARE I. Surface area of the detritivorous culture tank was 2.08 m². Two PVC walls were integrated to enhance particle subsidence in the tank (Waller, 2000). The tank was filled with a 10cm thick layer of sand (grain size ≤ 2mm) providing the preferred sediment for the selected worm species. Foam fractionators (Erwin Sander Elektroapparate GmbH, Outside Skimmer III) were rinsed automatically with a secondary fresh water loop, which is not illustrated in Fig. 1. The water circulation was driven by a centrifugal pump (Argonaut G8) supplying fishtanks (700-800L h^{-1}), foam fractionators (1000L h^{-1}) and biofilters (500-1000 L h^{-1}), respectively. The detritivorous bioreactor was restocked with a second generation of *Nereis diversicolor,* at approx. 850 individuals per m².

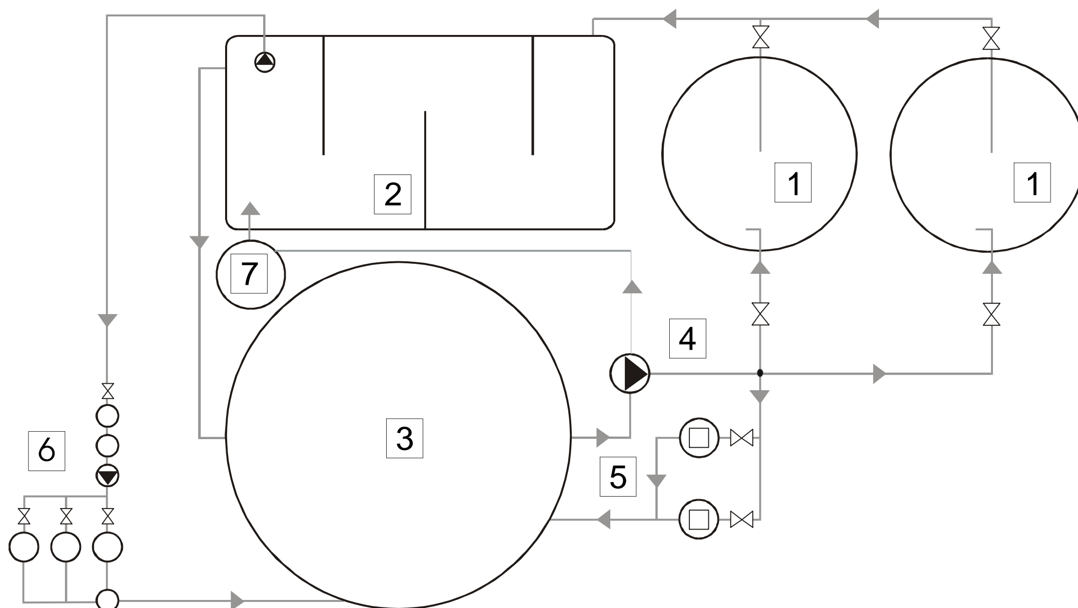

Fig. 1 Flow-chart of the MARE-system II (Marine Artificial Recirculating Ecosystem). Fishtanks = (1, 3), detritivorous culture tank = (2), pump = (4), foam fractionators = (5), microalgae cultivation system =(6) and trickling biofilter = (7). Double triangle = tap. Dimensions of the single modules are given in Chapter 2 and 3.

5.2.2 Measurements

The duration of the experiment was 5 months, lasting from 5th of September 2005 to 15th of February 2006.

Biomass determination

According to the average fish weight, fish were fed with pellets of 4.5 and 6 mm diameter, respectively (Biomar, Aqualife 17). The nutrition type was changed from trout feed to salmon feed after 3 months. Daily feeding rate (% fish body weight) was adjusted according to biomass determination of the fish. Feeding rate was changed from 1.0% to 0.8%, when the fish weight reached 500g.

Worm biomass was determined using four subsamples. Therefore, the detritivorous tank was divided into four areas and a sediment core of 9.5 cm diameter (800 cm^3) was sampled from each area. Sediment was removed using a sieve of 1 mm meh size collecting the worms. Measured average weight and stocking density of collected worms were used to calculate biomass parameters.

Optical density measurements were used to determine algal biomass in the bioreactors. Therefore, at the start of the experiment, a calibration was established in order to correlate algal biomass with measured optical density. Cells were counted using a light microscope and a haemocytometer (Fuchs-Rosenthal). Optical density of the sample was determined at 665 nm using a HACH SR2010 photometer.

During the experiment, algae were harvested by foam fractionation. The amount of algae in the harvesting unit was recorded using optical density measurements. 12 subsamples of the harvest were taken as replicates and centrifuged at 5000rpm for 10 minutes. Analysis of these samples is described in the subsection *Solid components* (see below).

Water chemistry

Water samples for chemical analysis of dissolved nutrients were taken daily at four definite points of the MARE-system: outlet fishtanks, outlet *Nereis*-tank, outlet fishtank 3 and outlet biofilter.

Water samples were stored at –20°C for later analysis. Concentration of PO_4-P, NO_3-N, NO_2-N and TAN were analysed using an Autoanalyzer 3 (Bran-Lübbe, Norderstedt, Germany).

Flow rates through fish tanks and foam fractionators were recorded and adjusted to 800L h^{-1} and 1000L h^{-1}, respectively. Flow rates of the microalgae reactors were recorded as well.

Online measurements of ozone reduction potential (ORP), pH and dissolved oxygen were recorded with an electronic control module (KM 2000, Meinsberg) and a portable measuring device (WTW multi 350).

Water level of the system was controlled daily and if necessary, adjusted with filtered brackish seawater. Salinity of the system water was 24.8±1.0 psu; artificial sea salt was used to increase salinity if necessary. The foam collectors were cleaned daily and the freshwater for rinsing the foam collectors was changed. The daily loss of water via the foam fractionators was recorded by the increased water volume in the secondary freshwater loop tank.

Solid components

Samples of rinsing water of the foam fractionator were taken weekly in order to determine the amount of suspended solids removed by the system. 12 tubes of 10ml sample volume were centrifuged and the supernatant was stored at -20°C for later water analysis of dissolved inorganic nutrient load. Dry matter and C/N ratio of the centrifuged pellets were determined as described below. Organic matter of the sediment was analysed weekly by the incineration technique using 5 subsamples.

For analysis of solid waste, harvested microalgae, tissues and sediment parameters the incineration technique was used as well: dry matter content was determined by dehydration of the sample in a drying furnace at 60°C overnight. Organic content was measured by incineration of organic matter in a muffle furnace. C/N ratio was determined by gas chromatography (GC) in an element analyser (EURO EA elemental analyser, Milano, Italy). Energy content was measured by complete sample combustion using an IKA calorimeter C4000. Weighing was performed using a Sartorius A 210 P (max. 200g) and a Sartorius U 4600 P (max. 4000g).

5.3 Results

The MARE experimental phase I showed a good performance of the system for the cultivation of *Sparus aurata* by integration of biological secondary steps (*Nereis diversicolor*, *Solieria chordalis*). A second experimental phase (MARE II) combining fish, ragworms and microalgae was started on 5th of September 2005 and was finished on 15th of February 2006. This period represents experimental days 480 to 643 of the whole MARE system (Chapter 2), but will here be referred to as "experimental days 1-163".

5.3.1 Module fish

Due to the increasing individual size of fish it was expected that fish growth switched from exponential to linear growth according to the Bertalanffy growth equation (Bone & Marshall, 1985). This hypothesis could be confirmed by experimental data; relevant data are presented in Fig. 3. Therefore, growth can be described by a linear regression:

$$f = y_0 + a \cdot x \qquad \text{(Equ. 1)}$$

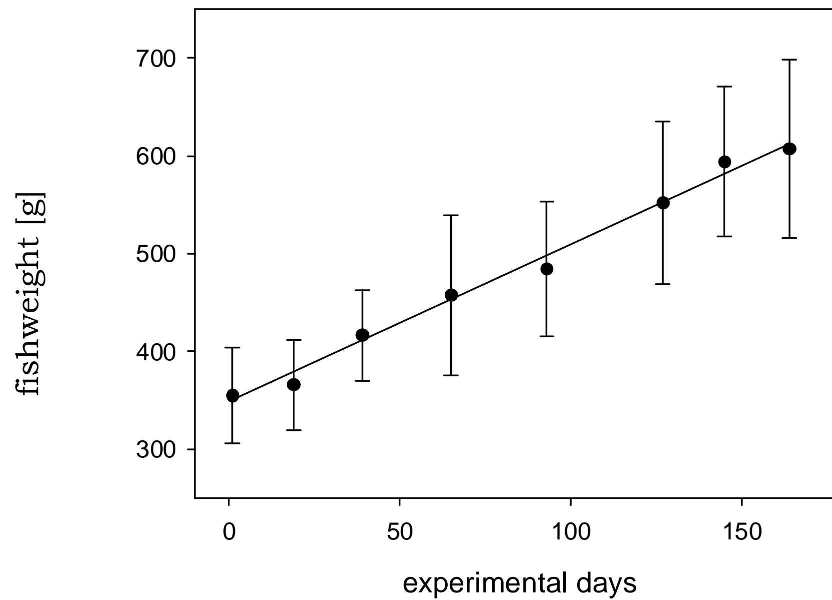

Fig. 3 Growth performance of *Sparus aurata* during the second MARE phase. Linear regression gave a= 1,60; y_0=-418, r^2=0.68.

5.3.2 Module detritivorous tank

Within 60 days after stocking, worms achieved a maximum weight of 0.62 ± 0.27g. Total worm biomass within the reactor was approx. 1.7 kg (Tab. 1, Fig. 5) with a standing stock density of approx. 1300-1400 individuals/m². Variations in stock density values are assumed to be caused by inaccuracies of the sampling method.

Tab. 1 Growth performance and biomass data of Nereis diversicolor during MARE II.

date	Experimental day	aver. worm weight ± SD [g]	stocking density ± SD [ind./m²]	total biomass [g]
10.10.2005	43	0.160 ± 0.259	895 ± 345	299
01.11.2005	65	0.243 ± 0.236	1011 ± 423	512
24.11.2005	88	0.369 ± 0.146	1371 ± 742	1057
15.12.2005	109	0.617 ± 0.273	1299 ± 120	1674
07.01.2006	132	0.584 ± 0.295	1112 ± 288	1356
26.01.2006	151	0.311 ± 0.329	953 ± 658	618
14.02.2006	170	0.240 ± 0.279	1631 ± 1696	818

The worm nutrition was solely based on the particulate matter from the fish tanks of the recirculating system. Tab. 2 gives an estimate of the food energy supplied to the worms at different time intervals during the course of the ex-

periment. The energy content ranged from 500 to 630 kJ d^{-1}. Assuming that 1400 worm individuals inhabit one square meter of sediment in the detritivorous reactor, approximately 162 J d^{-1} were available for an individual worm.

Tab. 2 Calculated total energy per day available for Nereis diversicolor in the bioreactor according to the fish feeding rate per day. Calculations were performed according to MARE I (Chapter 2).

Time interval	Fish feed per day [g]	daily solid waste per day ± SD [g]	Daily total organic load ± SD [g]	Daily total energy ± SD [kJ]
23.09. – 13.10.05	262.4	38.8 ± 9.2	25.0 ± 6.0	567.4 ± 135.1
13.10. – 08.11.05	292.0	43.1 ± 10.3	27.8 ± 6.6	631.4 ± 156.4
08.11. – 06.12.05	274.6	40.6 ± 9.7	26.2 ± 6.2	593.8 ± 141.6
06.12. – 09.01.06	232.6	34.6 ± 8.2	22.2 ± 5.3	503.0 ± 119.9
09.01. – 27.01.06	265.0	29.1 ± 9.3	25.3 ± 5.3	573.0 ± 136.4
27.01. – 15.02.06	285.2	42.1 ± 10.0	27.2 ± 6.5	616.6 ± 147.0

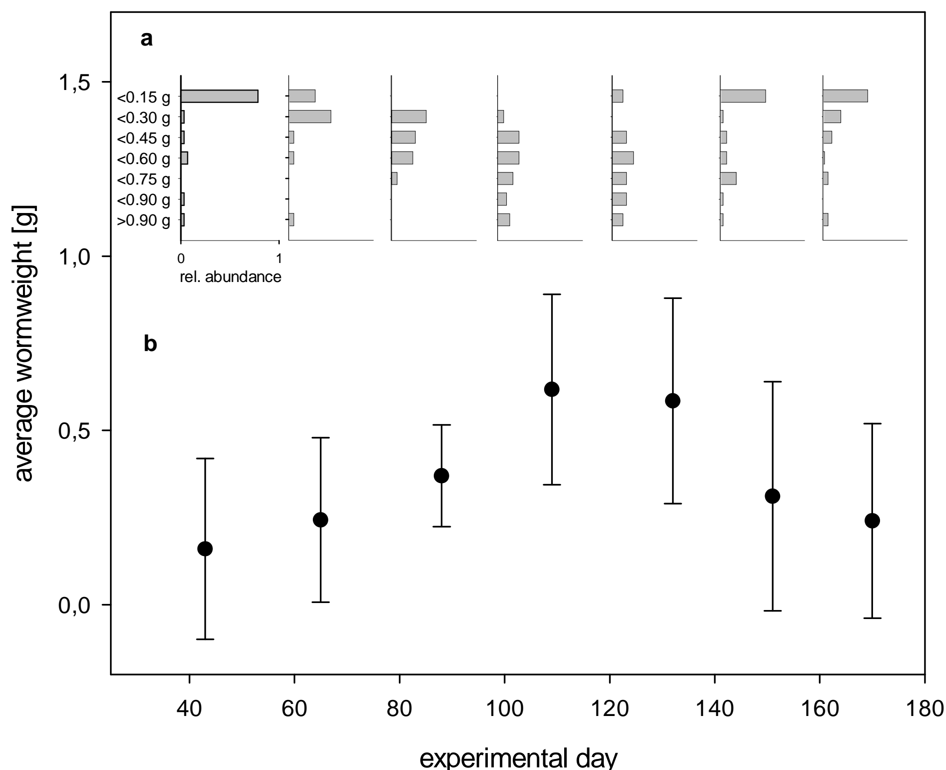

Fig. 5 Growth performance of *Nereis diversicolor* during MARE II (a) relative size distribution (weight classes) of every biomass determination. (b) average worm weight ± SD of each biomass determination (according to Tab. 1). The data indicate the generation cycle with reproduction around exp. day 109 and occurrence of new generation at day 132.

The growth performance of the worms is summarized in Fig. 5. The average individual weight at the beginning of the experiment was 0.160 ± 0.259 g.

Average individual worm weight increased until day 109 and slightly decreased at day 132 as shown in Fig 5 b). Maximum worm weight was between 0.617 ± 0.273 g and 0.584 ± 0.295 g. The average individual weight decreased substantially at day 151 and 170 to 0.311 ± 0.329 g and 0.240 ± 0.279 g, respectively. Fig. 5 a) shows the size distribution for the collected worms. A dramatical population decrease could be observed between day 120 and day 150.

Sexual maturity of the worms is indicated by a colour change from red to a greenish colour (Hartmann-Schröder, 1996), a colour change of the worms was observed for the first time on experimental day 60. Currently, no data are published concerning spawning weight of *Nereis diversicolor.* Data obtained during this study show, that worm body weight at time of maturation was approximately 0.8 g. Due to the monotelic reproduction mode (death after spawning), large individuals disappeared after spawning from the detritivorous culture tank. After the reproduction event, a biomass decrease could be observed within the system. Reproduction was observed for a period of approx. 20 days of the experiment. Individuals of the new generation were captured at day 100 for the first time, the start of the new "growing" generation can be assumed for day 120.

Reproduction could not be controlled but had an enormous influence on the system stability. Due to growth of fish larger amounts of suspended particles were transferred into the detritivorous tank. This increased particle load apparently was not completely used by the decreased worm biomass after day 132 leading to an increase of organic matter on the sediment in the first section of the wormtank at the water inlet. An increase of organic load was found at the first sampling point in the reactor. Further sampling points within the *Nereis*-tank did not show such an increase (Fig. 6).

The increased amount of organic matter in this reactor probably led to a cascade of effects within a short time interval from day 100 on: oxygen conditions in the first section of the detritivorous reactor changed from oxic to

suboxic/anoxic. Under these specific conditions, the process of denitrification is favoured, indicated by a rapid decrease of nitrate concentrations (Fig. 6b). Denitrification is a process releasing hydroxyl ions and consequently, pH increases. This could be experimentally confirmed by the pH measurements. Thus, to maintain system stability, a constant acid addition to regulate pH was required (Fig. 6d). Concurrently, TAN concentrations rapidly rose up to 10 mg L^{-1} (Fig. 6c). Flow rate through the biofilter was increased to 1000 L h^{-1} for the remaining experimental period and 1000 litres of water were exchanged in order to stabilize TAN concentrations. During MARE II, no loss of fish due to ammonia toxication could be observed.

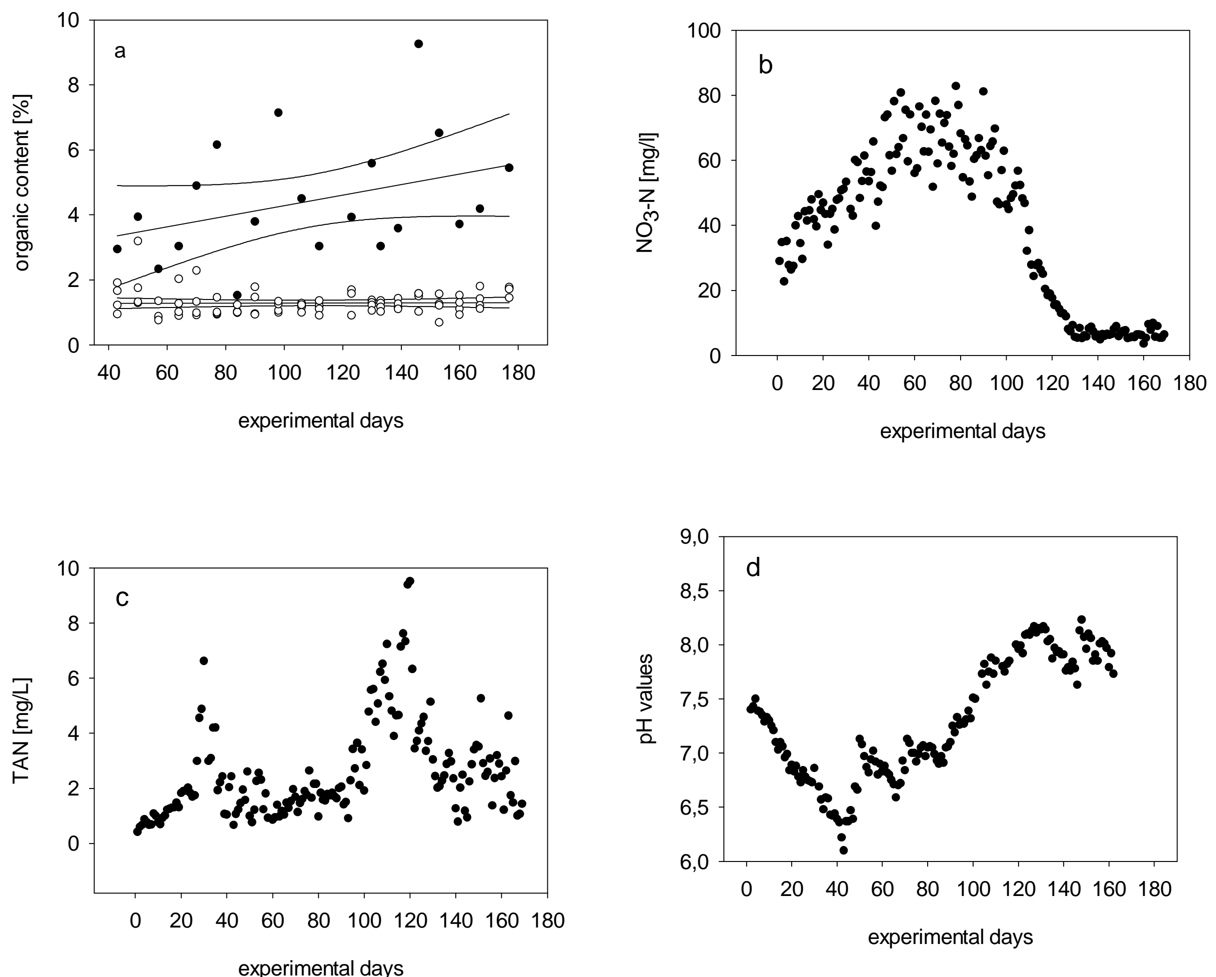

Fig. 6 (a) organic content of the sediment within the detritivorous culture tank: Amount of organic matter at sampling point 1 (closed circles) increased significantly during the experimental period (ANOVA, $p<0.001$). Open circles are all other sampling point in the detritivorous reactor. (b) NO_3-N concentrations in the system water during MARE II accumulation of nitrate occurred until day 100. Afterwards concentrations of NO_3-N decreased to less than 10mg/L^{-1} due to denitrification; (c) TAN concentrations of the system water. d) pH changes.

5.3.3 Module microalgae bioreactors

Photobioreactors for the continuous cultivation of *Nannochloropsis* sp. were included into the MARE-system. It was possible to cultivate Nannochloropsis in a continuous culture based on dissolved nutrients derived from the recirculation system. The daily harvested yield of algae biomass was 2.49 ± 1.34g DW d^{-1} in average. Harvest was very efficient: daily harvest volume was 0.184 ± 0.053 L d^{-1} at high cell densities of 227.9 x 10^8 ± 6.7 x 10^8 cells ml^{-1}. 99.68 ± 0.38% of the water coming from the photobioreactors were transferred back to the main recirculation system. Nutritional value of the algae was high: organic proportion reached more than 80% of the algae dry weight, measured energy value was 21.09 ± 4.6 KJ g^{-1} DW_{algae}. Total amount of fatty acids varied from 5.5ng fatty acids (FA)/µg POC_{algae} to 16.72ng FA/µg POC_{algae} according to irradiance of each photobioreactor. Relative amount of unsaturated fatty acids (arachidonic acid ARA, eicosapentaenoic acid EPA) to total amount of fatty acids ranged from 4.0 – 8.27% and 9.1 – 18.9%, respectively. For further details see Chapter 3.

The nutrient uptake rates have been determined in 1 hour growth experiments giving average values of 0.190 – 0.294 mg h^{-1} PO_4-P per litre culture volume and 0.129 – 0.186 mg h^{-1} NO_3-N per litre culture volume according to light intensity (see Chapter 3).

The biofilter efficiency of *Nannochloropsis* sp. were determined with two different methods: results from one hour growth experiments gave (depending on light intensity) average values for PO_4-P uptake of 0.190 – 0.294 mg h^{-1} per litre culture volume and for NO_3-N uptake 0.129 – 0.186 mg h^{-1} per litre culture volume, respectively (see Chapter 3, Tab. 6).

By using these values (average) amount of daily removed nutrients from the recirculation system can be calculated, assuming a total volume of photobioreactors of 3 x 50 litres and 24 hours cultivation time. According to this calculation the photobioreactor system of the presented dimension can remove a total amount of 0.87g of PO_4-P and 0.57g of NO_3-N per day.

The second method used to calculate the biofilter efficacy of the photobioreactors was the estimation of the daily removal of nutrients by water exchange due to continuous culture mode. According to the adjusted flow rate nutrient rich water from the recirculation system will be introduced at a certain volume to the photobioreactors. According to the laws of continuous culture (Pirt, 1975) an equal volume of treated water will flow out of the photobioreactors and back into the main recirculation system at the same time. The difference between the measured dissolved nutrient concentration of the inflow (nutrient rich water) and dissolved nutrient concentration of the outflow (treated water) were determined for each experimental day. Data are shown in Tab. 3. Values vary from 2.1 mg to 28.1 mg PO_4-P per litres discharge and 0.6 mg to 20.4 mg NO_3-N, because of the variation of the dissolved nutrient concentrations within the photobioreactors during the entire experimental time. However, daily values were multiplied with the total volume of recirculation water, passing the photobioreactor system at each experimental day according to the adjusted flow rate. Values for total amount of removed PO_4-P and NO_3-N per day vary due to changing conditions within the recirculation system. Surprisingly, taking the average values of all data over the entire experimental period, similar values in comparison to the first calculation were determined: in average 0.88g PO_4-P and 0.57g NO_3-N per day were removed from the recirculation system by the photobioreactor system.

Tab. 3 Differences of the inflow and outflow concentration of the photobioreactor system and daily amount of removed nutrients and flow rates during the experimental period. Optical density (OD_{665}) of the outflow from the harvesting unit.

Date	Flow rate per day [L]	OD_{665} after harvest	Δ inflow – outflow conc. [mg L^{-1}]				amount of removed nutrients per day [g d-1]			
			PO_4-P	NO_3-N	TAN	NO_2-N	PO_4-P	NO_3-N	TAN	NO_2-N
14.10.05	108.0	-	2.1	3.8	-	-	0.23	0.41		
15.10.05	108.0	0.062	3.6	5.1	-	-	0.39	0.55		
19.10.05	108.0	0.138	8.2	18.0	1.14	0.00	0.89	1.94	0.12	0.00
20.10.05	108.0	0.026	4.8	12.5	0.58	0.16	0.52	1.35	0.06	0.02
27.10.05	73.4	0.136	14.5	20.4	1.04	0.02	1.06	1.50	0.08	0.00
28.10.05	73.4	0.102	11.0	26.0	1.38	0.17	0.81	1.9	0.1	0.01
29.10.05	73.4	0.020	14.3	19.5	1.7	0.08	1.05	1.43	0.13	0.01
31.10.05	73.4	0.172	13.8	13.6	0.68	0.08	1.01	1.0	0.05	0.01
01.11.05	73.4	0.002	14.1	5.3	0.1	0.3	1.03	0.39	0.01	0.02
02.11.05	73.4	0.000	15.7	14.0	0.2	0.2	1.15	1.03	0.02	0.02
18.11.05	108.0	0.001	13.9	2.5	0.0	0.1	1.5	0.27	0.00	0.01
23.11.05	86.4	0.001	5.6	1.6	0.1	0.04	0.48	0.14	0.01	0.00
24.11.05	86.4	0.000	14.6	2.8	0.0	0.03	1.26	0.24	0.00	0.00
25.11.05	86.4	0.003	19.7	9.6	0.7	0.01	1.7	0.83	0.06	0.00
27.11.05	86.4	0.002	25.2	2.3	0.0	0.0	2.19	0.20	0.00	0.00
28.11.05	86.4	0.000	25.4	2.2	0.0	0.0	2.19	0.19	0.00	0.00
18.1.06	60.5	0.009	28.1	0.6	1.31	0.02	1.7	0.04	0.08	0.00
19.1.06	14.4	0.013	23.6	2.9	3.19	0.00	0.34	0.04	0.05	0.00
20.1.06	21.6	0.013	23.9	2.9	3.2	0.00	0.52	0.06	0.07	0.00
21.1.06	21.6	0.011	17.6	3.7	2.13	0.06	0.38	0.08	0.05	0.00
22.1.06	21.6	0.012	11.3	3.8	1.94	0.03	0.24	0.08	0.04	0.00
23.1.06	21.6	0.017	12.0	1.7	1.62	0.00	0.26	0.04	0.04	0.00
24.1.06	21.6	0.008	11.0	3.5	0.22	0.07	0.24	0.08	0.01	0.00
4.2.06	14.4	0.025	3.9	0.6	3.8	0.02	0.06	0.01	0.06	0.00
Average							**0.88**	**0.57**	**0.05**	**0.00**
SD							*0.63*	*0.63*	*0.04*	*0.01*

5.4 Discussion

5.4.1 Module fish tank

The laboratory conditions in MARE II were similar to MARE. However, fish tanks 1 and 2 became too small for the constantly growing fish. In order to analyze the system stability with respect to high total fish biomass, tank 3 (macroalgae tank in MARE) was stocked with additional fish. The load of dissolved nutrients was enhanced for tests of microalgae performance. Due to the changes in the stocking of the tanks suspended solids were transferred mainly to the foam fractionators. At the end of the experimental period the

functionality of foam fractionation was restricted due to large quantities of suspended solids caused by the incomplete first step for removal of suspended solids.

5.4.2 Module detritivorous tank

The performance of the detritivorous reactor as a sink for solid particles produced by the fish was sufficient during the growth period of the worms. But, as soon as worm reproduction (monotelic, *Nereis diversicolor* dies after spawning) started, organic matter was accumulating in the detritivorous tank, resulting in anoxic conditions in turn endangering the successful cultivation of the fish. The natural life cycle of an organism of critical importance for system stability therefore may lead to the breakdown of the system. These results necessarily led to the question of the suitability of *Nereis diversicolor* for integration in aquaculture systems and the search for possible alternatives. The applicability of sea cucumbers (*Parastichopus californicus, Stychopus japonicus,* Ahlgren 1998, Bregman 1994) is discussed as a possible alternative organism for *Nereis diversicolor* in integrated aquacultural systems. The performance of sea cucumbers for removal of settable solids appeared to be good (Ahlgren 1998, Bregman 1994), but the commercial value of these organisms is questionable. Parallel to MARE, the performance of shrimps (*Crangon crangon*) for the removal of settable solids was examined (data not shown). This experiment showed that the performance of *Nereis diversicolor* can be considered as superior to the shrimps concerning their capacity of settable solid removal. Therefore, currently there seems to be no alternative to *Nereis diversicolor* as a secondary organism in integrated aquaculture systems. Another possibility of avoiding the problems observed in MARE II is the integration of several detritivorous tanks equipped with *Nereis diversicolor* in different life cycle stages and their controlled connection to the main system, depending on the stages of life.

5.4.3 Module microalgae bioreactors

Although ammonia is the preferred chemical form of nitrogen and readily taken up by phytoplankton (Collos and Slawyk 1981, Levasseur *et al.*, 1993),

cultivated *Nannochloropsis* sp. mainly took up nitrate due to the lack of sufficient ammonia concentrations in the inflow.

Nannochloropsis was not sufficiently provided by this nutrient due to the low flow rates through the photobioreactor system. Flow rates are limited by the maximum specific growth rate and may not exceed 0.025 h^{-1} dilution rate to avoid biomass washout (see Chapter 3). Maximum total water flow per day through the photobioreactors was recorded at 108 L per day, which is still a negligible volume regarding total nutrient budget of the MARE-system.

Hence, the photobioreactor system can not fulfil the requirements of a biofilter for removal of toxic ammonia and nitrite from recirculation water. However, hints are given that the simultaneous integration with macroalgae filter may be possible, because macroalgae filter are not competing for nitrate due to their preference for ammonia (see Chapter 2).

The conceptional design of the photobioreactor system was feasible regarding pre-treatment of the water and harvesting process. Cultivated microalgae can be used as valuable feed for feeding organisms (*Brachionus* and copepods), bivalves, *Nereis diversicolor* or fish larvae (Støttrup and McEvoy, 2002).

5.4.4 Nitrogen cycle

Although not primarily intended, this experiment showed substantial insights into the nitrogen cycle within the system:

Over three months, TAN and nitrite concentrations were at low levels. Nitrate concentration was accumulating or remained stable till day 100 indicating nitrification processes.

The system started to show instabilities when the onset of worm reproduction was observed. The high amount of organic load produced by the fish could not be efficiently degraded by the worms due to their decreased biomass. Consequently, anaerobic conditions developed in the first sector of the

Nereis-tank, favouring denitrification (Rheinheimer, 1988). Indications for anaerobic conditions were approved during sampling (dark colour of sediment, no worms, increased occurrence of gas bubbles). Increasing phosphate values can also be an indication for anaerobic conditions, but in the system, this increase can also be due to the subsequent addition of phosphate via the feed. This addition of phosphate can be considered as the major contribution to the increasing phosphate values. Evidence for an enhanced denitrification activity was obtained by i) the rapid decrease of nitrate and ii) the subsequent increase of pH (Rheinheimer, 1988). Additionally, iii) bacterial mats identified as *Beggiatoa* sp. were observed. *Beggiatoa* sp. is known as a sulphur-oxidising bacterium (Madigan, 2001). The observed black colour of the sediment indicates the presence of H_2S, explaining the presence of *Beggiatoa* sp. as an H_2S-oxidising bacterium. Furthermore, the ability for denitrification is also described for *Beggiatoa* sp. on freshwater sediments (Sweerts et al., 1990) and indications were found for denitrification ability of this species in marine habitats as well (McHatton et al., 1996).

There is another nitrate-consuming process of possible relevance within the system. The reverse course of the nitrification process is called assimilatory nitrate reduction. Many bacterial species are able to reduce nitrate in order to obtain ammonia for biomass synthesis (Madigan, 2001). This process would also lead to a nitrate consumption and the two processes can be considered as major processes contributing to the observed decreasing nitrate values. Additionally, nitrate ammonification is performed by several bacteria in order to obtain reduction equivalents for fermentation. However, this process is inhibited by elevated concentrations of ammonia and may therefore be of minor relevance for the explanation of the observed effects.

Another process was observed to coincide with decreasing nitrate concentrations. The TAN concentrations showed strongly increasing values (Fig. 6b and c). Measured TAN values (measured daily) exceeded the TAN concentrations explicable by fish metabolism (3.35 ± 0.33 mg L^{-1}) only. There are two microbial processes removing ammonia: nitrification and ANAMMOX (Madigan, 2001). Nitrification is an oxic process and ammonia is thereby con-

verted to nitrate. A special trickling biofilter was integrated into the MARE II experiment in order to ensure a constant nitrification process. All surfaces (sediment, tank walls and tubes) are also considered to provide habitats for nitrifying bacteria. Losordo and Wethers (1997) estimated the TAN removal by nitrification outside the biofilter in the range of 30 to 50 %. This could not be proved by Schneider (2000) who showed little contribution of other system components (water, pipes, tanks, sedimentation unit) compared to the aerobic biofilter unit. However during the experiments of Schneider (2000) high values for FCR for African catfish (*Clarias gariepinus*) occurred (experimental FCR = 2.5). This resulted in high organic waste loads, which Bovendeur (1989) showed will inhibit nitrification processes. Rydl (2005) observed during her analysis of bacterial activities of a recirculating aquaculture system that turnover rates of bacteria on wall material and biofilter filling material are comparable. Turnover rates of water samples from the system were lower compared to the rates of the wall material and the biofilter filling material.

A part of the previously “nitrifying surface” was lost (considering the total nitrification budget) when a part of the sediment surface within the worm-tank became anoxic. In this part of the system, ammonia was no longer removed by nitrification, but nitrate was removed due to denitrification and possible assimilatory nitrate reduction.

The increasing nitrite concentrations may also be explained by this process. Nitrite concentrations are supposed to increase, when nitrification is partially inhibited due to unfavourable environmental conditions (e.g. suboxic/anoxic).

ANAMMOX is an anaerobic process converting ammonia and nitrite resulting in the formation of elementary nitrogen. Anoxic conditions were present in the *Nereis*-tank but ammonia as well as nitrite concentrations increased indicating that the ANAMMOX process was not of major importance in the system.

All these processes are strongly depending on the prevailing oxygen concentrations. Unfortunately, no oxygen concentrations were measured within the different system units, therefore the processes assumed to have happened within the system after the onset of worm reproduction only can rely on measured concentration changes of TAN, NO_2, NO_3.

Thus, the cross-linking and interactions of these nitrogen-converting processes, especially nitrification and denitrification strongly depending on each other may explain the observed changes in the system concerning the different nitrogen compounds.

The different interactions within the nitrogen cycle had different impacts on the other system modules: i) due to the increased pH, the fraction of dissociated ammonia (NH_3) was close to toxic levels (observed = 0.035 mg L^{-1}, literature data = 0.05 mg L^{-1}, Losordo et al., 1998). Despite these unfavourable conditions, no mortality and unusual behaviour among the fish could be detected. Fish even tolerated 10 mg L^{-1} TAN for a short while at pH 7.96., although reduced feeding activity occasionally occured. It can therefore be assumed, that fish is able to adapt to higher ammonia levels, when the water quality is good.

ii) the anoxic conditions in the sediment of the worm tank may have led to elevated worm mobility towards the oxic areas within the sediment, in turn increasing the organic load in the first section of the tank and therefore further enhancing denitrification.

iii) the foam fractionating process was not efficient enough to successfully remove bacteria attached to particles and microalgae did not show a comparable performance to the macroalgae for the removal of nitrate also favouring denitrification.

5.4.5 General recommendations

The experiments in this study confirmed, that the accurate adaptation of all components of a system is crucial (Losordo *et al.*, 1999; Waller *et al.*, 2003). In the first three months of the experiment, MARE II proved to be a stable system, but at the onset of worm reproduction the system started to become instable.

Based on the results from this study, the simultaneous use of several bioreactors with different worm generations is recommended to allow year round production without any disturbances possibly due to the worm life cycle. It is also recommended, that sediment removed by the worm harvesting process is brought back to the system (assuming aerobic conditions of the *Nereis* tank). Early life stages of *Nereis diversicolor* are rather small (< 1mm diameter) and cannot be easily detected using macroscopic methods. Therefore they are easily lost using the sieving method for harvesting. A mesh size reduction of the sieve would solve this problem, but an efficient sediment removal is not guaranteed by using smaller mesh sizes, also leading to incorrect worm biomass estimates.

The importance of a cautious monitoring of the different nitrogen-converting processes is also strongly recommended as a result of this study. Therefore, in possible future experiments it is also crucial to obtain oxygen concentrations in different compartments of the system and in different regions of the compartments (water, different sediment layers) in order to be able to react before anoxic conditions can develop.

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Kiel, den

____

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