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Innovations for nitrate removal in recirculating aquaculture systems

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THE FUTURE INTERESTS ME

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I'M GOING TO SPEND THE REST OF MY LIFE THERE.

Mark Twain

FÜR MEINE ELTERN

GALINA UND JAKOB

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GENERAL INTRODUCTION

Aquaculture production systems

Aquaculture production systems can be categorized based on production intensity per water volume into four categories: extensive, semi-intensive, intensive and super-intensive (Lekang, 2013; Bregnballe, 2015).

Extensive production systems, for instance ponds, are characterized by low production per water volume. Produced fish are kept at low densities and the human interference is limited to the maintenance of the aquatic habitat and the stocking of the fish. These production systems typically have low levels of technology. In a semi-intensive system the natural productivity of a pond is complemented by fertilization of the water and with supplementary natural or artificial feeds. This allows higher stocking densities and hence higher production per volume of water. The technological effort is higher compared to extensive systems. In intensive aquaculture farming, such as flow-through systems, the production per volume of water is usually much higher compared to the latter production forms. To achieve this, complex management and high technological effort for water treatment are necessary. Intensive aquaculture involves the control of most environmental conditions, high stocking densities, and artificial feeding to increase the productivity and yields. In super-intensive systems the water remains in a closed circuit and is reused after processing in water treatment units. These closed aquaculture recirculating systems (RAS) are isolated from the external environment and hence allow the full control of environmental conditions under highest technological and financial effort. Due to the efficient recycling of water highest production output per volume of water is achieved.

Each aquaculture production type bears advantages and disadvantages. Super-intensive systems usually have high costs, due to the investment and high energy requirement of the used technology. In contrast extensive systems can be managed without any energy costs. Apart from that, an extensively used pond will produce 0.2 kg fish per m^2, consuming 45 m^3 of water per produced kg fish. Super-intensive systems have a productivity in the range of 10 to 100 kg fish per m^2 and the water consumption is less than 1 m^3 per kg fish production (Browdy et al., 2012; Bregnballe, 2015). Hence, super-intensive systems use the available space and water resources much more efficient.

Due to highly efficient use of water in super-intensive systems, metabolic end products of organisms, inherent in the system, tend to accumulate. In fish, digestion and metabolism of nitrogen containing molecules such as proteins and other essential dietary components, results in nitrogen excretion (Wright and Anderson, 2001). These nitrogen containing excretion products can accumulate in the system and pose a danger to the cultured fish species at certain concentrations (Camargo et al., 2005; van Bussel et al., 2012; Schram et al., 2014; Good et al., 2017).

Nitrogen in aquaculture systems

The primary end product of the metabolic degradation of the nitrogen containing feed compounds by fish is mainly ammonium (NH_4^+), which is released via the gills (Wright and Anderson, 2001). Ammonium is metabolized by bacteria in the first step of nitrification (*Figure GI - 1*) to nitrite (NO_2^-) and in the second step to nitrate (NO_3^-). Usually in natural aquatic habitats, no excessive nitrogen enrichment in form of nitrate occurs. A large number of organisms are capable of biological nitrate removal by either assimilatory or dissimilatory pathways. One dissimilatory pathway is the denitrification (*Figure GI - 1*). Denitrification, a catabolic pathway, is defined as the microbial process of reducing nitrate (NO_3^-) and/or nitrite (NO_2^-) stepwise to nitric oxide (NO), nitrous oxide (N_2O), and nitrogen (N_2). Only under anoxic conditions and if an organic carbon source is sufficiently available, facultative anaerobic bacteria switch from aerobic to anaerobic respiration using the carbon source as electron donor and nitrate as electron acceptor.

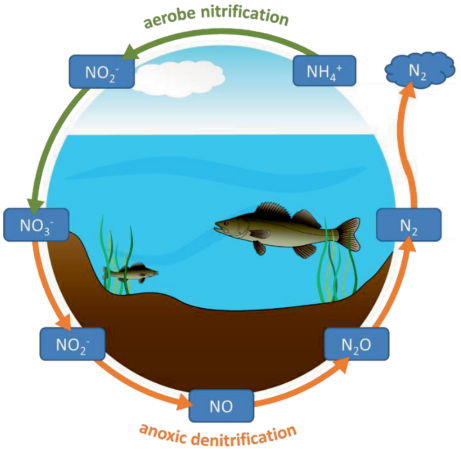

Figure GI - 1. *Simplified scheme of nitrification and denitrification in aquatic habitats.*

A large number of microorganisms are capable of denitrification, hence it is a ubiquitous process and occurs in terrestrial and aquatic ecosystems. Denitrification is present in tropical and temperate soils, in natural and intensively managed ecosystems, in marine and freshwater environments, in wastewater treatment plants, manure stores, and aquifers (Skiba, 2008). During denitrification, a net removal of nitrogen is realized since gaseous nitrogen compounds (N_2O and N_2) can leave the system and emit into the atmosphere.

In intensive RAS denitrifying processes do usually not take place, since the required environmental conditions are typically not present (anoxic conditions and a sufficiently available organic carbon source). RAS involve high stocking densities and artificial feeding to increase productivity and yields of the system. This is only possible with full control of environmental conditions, ensuring high oxygen saturation and low organic load in the water, both preventing denitrification. As a result of inhibited denitrification and limited water replacement, nitrate accumulates in RAS.

Influence of nitrate on fish

Nitrate has been proven to have a negative impact on health and growth of fish at species-specific concentrations (Scott and Crunkilton, 2000; Shimura et al., 2004; McGurk et al., 2006; van Bussel et al., 2012; Schram et al., 2014). Some of the negative effects described in these studies are methemoglobinemia, endocrine disruption, and general histological damages. Also, nitrate causes damages in gills, intestinal ampulla, liver, and kidney. Furthermore, nitrate impairs the feed conversion ratio, condition factor and the spleen-somatic index.

In this thesis, pike perch (*Sander lucioperca*) and European sea bass (*Dicentrarchus labrax*) were used as model organisms. The influence of nitrate on pike perch, reared in RAS, was already investigated (Schram et al., 2014). Until now, only little is known about the influence of nitrate on performance parameters and the health status of sea bass reared in RAS. Sea bass are usually farmed in seawater ponds, lagoons, and sea cages (Bagni, 2005). Nevertheless, sea bass is, next to turbot (*Psetta maxima*), one marine species which is intensively produced in RAS (Blancheton, 2000; Martins et al., 2010). It can be expected that the production of marine species in RAS will further increase, as already practiced for freshwater species, such as pike perch and salmonids. To meet appropriate water quality parameters in production cycles, it is necessary to estimate species specific requirements concerning the water quality.

Within ***Chapter 1*** of this thesis the influence of nitrate on performance parameters and health status of on-growing sea bass was clarified. The results allow to estimate the effects of nitrate on sea bass production in RAS.

Chapter 1 discusses following research questions:

- Does nitrate have a significant impact on performance parameters of sea bass?
- Does nitrate affect health status and mortality of sea bass?
- What are threshold values for production of this species in RAS?
- How sensitive are sea bass towards nitrate in relation to literature data given for other species?

Nitrate removal in RAS

As nitrate is proven to have negative impacts on fish and naturally occurring nitrate removal is usually not present in RAS, an artificial nitrate removal is crucial for fish production in closed RAS. Several denitrification units were already introduced to RAS (van Rijn et al., 2006), but bear various challenges. Difficulties arising in activated sludge systems are for instance sludge separation problems described by Tandoi et al. (2017). Upflow Anaerobic Sludge Blanket Reactors can require a comparably long incubation phase for start-up (Liu et al., 2003; Yang et al., 2003). Fixed/packed bed reactors tent to silt and need to be washed every few days, resulting in additional work and in irregular denitrification rates (Sauthier et al., 1998). Fluidized bed biofilter must be operated permanently in a narrow water flow range in order to lift and maintain proper bed expansion, resulting in high energy costs for water pumping (Summerfelt, 2006). Autotrophic sulphur reactors can decrease the pH and increase sulphate concentration in the rearing water (Oh et al., 2001), both affecting fish health negatively. Furthermore, existing conventional heterotroph denitrification processes are often accompanied by challenges, such as the accurate dosage of a carbon source (e.g. methanol), mandatory for denitrification but potentially hazardous.

The need of novel denitrification systems ensuring safe, efficient and easy to run denitrification processes at low maintenance costs are stringently required to guarantee a trendsetting aquaculture production form. One novel denitrification system, the low-maintenance **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor; *Figure GI - 2*; patented by Müller-Belecke and Spranger, 2014) was introduced for the first time by Müller-Belecke et al. (2013).

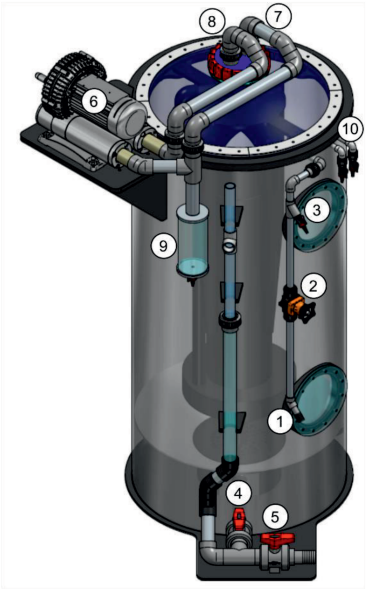

Figure GI - 2. *The **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor, 0.75 m³). Water inlet (1), flow rate valve (2), methanol inlet (3), water outlet (4), sludge release (5), side channel vacuum pump (6) with inlet (7) and outlet (8), condensate trap (9), and pressure relief opening (10).*

The design of the SID-Reactor is based on a combined function principle of a fix bed as well as moving bed biofilm reactor (Müller-Belecke et al., 2013). The SID-Reactor is usually filled with floating biocarriers making up 60% of the reactors water volume. The biocarriers serve as substrate available to population by bacteria. Heterotrophic denitrifying bacteria colonize the substrate and transform nitrate into inert nitrogen gas (dissimilative nitrate reduction). The reactor chamber itself is closed with a gas tight top cover to prevent oxygen influx by ambient air, enabling anoxic conditions. Additionally, the nitrogen gas produced during denitrification will accumulate at the top of the reactor chamber forming a gas bubble. Within a defined time interval, the fixed biocarriers are swirled up. A side channel vacuum pump forces the inherent oxygen-poor gas from the top to the bottom of the SID-Reactor and sets the biocarriers into motion. The aim is to prevent the biocarriers from clogging due to bacterial growth (self-cleaning effect of the SID-Reactor). A small opening on the top of the reactor allows excessive nitrogen gas to leak and furthermore allows pressure compensation at times when the side channel vacuum pump is running. Further components of the SID-Reactor are a container for a

carbon source and a peristaltic dosage pump constantly adding the carbon source to the influent water of the SID-Reactor.

Promising first results of Müller-Belecke et al. (2013), working with the SID-Reactor in freshwater, revealed a high potential of an easy to use, safe and efficient denitrification system. Within the scope of this thesis the further technological enhancement of the SID-Reactor was striven for. Additionally, specifying the effects of basal operating settings of the SID-Reactor would clarify whether the SID-Reactor is a promising denitrification system for nitrate removal also in marine RAS applications. In ***Chapter 2*** of this thesis the influence of specific operating settings of the SID-Reactor on water quality parameters and denitrification performance were evaluated. During a long-term experimental trial the effects of varying hydraulic retention time, backflushing intervals, and carbon to nitrogen ratios were monitored in order to derive recommendations for a safe and efficient denitrification process.

Chapter 2 discusses following research questions:

- Is the SID-Reactor a suitable denitrification unit for nitrate removal in marine RAS?
- How does the hydraulic retention time influence water quality and denitrification performance?
- Do varying backflushing intervals, preventing the SID-Reactor from clogging, also have a negative impact on water quality and denitrification performance?
- What is an optimum carbon to nitrogen ratio to achieve best denitrification performance?
- Does an imprecise dosing of the carbon source (methanol) have an effect on water quality and denitrification?

Chapter 3 of this thesis documents the possible replacement of methanol by biodegradable polyhydroxyalkanoate (PHA) plastics in the SID-Reactor used in freshwater RAS. The replacement of methanol as a hazardous carbon source by a non-hazardous carbon source would increase safety not only for fish but also for RAS staff. In contrast to conventional denitrification systems the operating principle of the SID-Reactor is potentially not restricted to use either a liquid carbon source or a solid carbon source. The identification of basal operating settings and the use of a safe carbon source will reduce denitrification related problems in RAS, enhance denitrification processes, and will result in a safer and easier to control SID-Reactor.

Chapter 3 discusses following research questions:

- Is it possible to replace methanol by a biodegradable PHA granulate?
- What are the effects of a PHA fuelled SID-Reactor compared to a methanol fuelled SID-Reactor and a control RAS without a denitrification unit on water quality?
- Does the denitrification performance differ using PHA or methanol as a carbon source?
- Is the application of PHA as a carbon source economically viable?

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CHAPTER 1

Nitrate has a low impact on performance parameters and health status of on-growing European sea bass (*Dicentrarchus labrax*) reared in RAS

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Abstract

Coming along with the intensification of fish production in modern recirculating aquaculture systems (RAS) the water re-use in RAS is increasing. Accompanied by the water re-use, metabolic end products of fish and bacteria continuously accumulate in RAS process water. Nitrate, which is an end-product of biological nitrification processes, accumulates to high concentrations potentially influencing various physiological parameters of fish. The present study focusses on the effects of different nitrate concentrations on performance and health status of on-growing European sea bass (*Dicentrarchus labrax*). On-growing sea bass were exposed to four different nitrate-N (NO_3^--N) concentrations (control (C): 0 mg L^{-1}; low nitrate-N (LN): 125 mg L^{-1}; medium nitrate-N (MN): 250 mg L^{-1}; high nitrate-N (HN): 500 mg L^{-1}) in a triplicate experimental design in twelve small-scaled RAS for 10 weeks. The nitrate concentrations were adjusted with a sodium nitrate and potassium chloride solution (Na^+/ K^+ weight-ratio of 1:27). Rising nitrate levels during the exposure period, caused by the accumulation of metabolic nitrate, were avoided by batch water exchange twice a week. Final biomass, final individual weight, condition factor (CF), feed conversion ratio (FCR) and spleen-somatic index (SSI) were not significantly affected by nitrate exposure within the concentration range tested. Specific growth rate (SGR), and total mortalities did not significantly differ between the treatment groups either, although a trend towards decreasing SGR and increasing mortalities at high nitrate levels was obvious. This study revealed, that the hepato-somatic index (HSI) and daily feed intake (DFI) were significantly negative correlated to with increasing nitrate levels. However, the sensitivity of on-growing sea bass towards nitrate toxicity seems to be low compared to other aquaculture fish species recently tested.

Keywords: marine aquaculture; RAS; metabolic end products; nitrate; sea bass; toxicity; life stage

1 Introduction

Due to the rising demand for fish products and concurrent stagnating fisheries production, new and independent production methods are necessary to meet the demand. The lack of space for expansion, the limited fresh water availability and the tightened wastewater regulations are considered as the main impediments for further expansion of conventional systems (Badiola et al., 2012; Dalsgaard et al., 2013). Hence, an intensified fish production in recirculating aquaculture systems (RAS) is of growing interest (Bregnballe, 2015; Dalsgaard et al., 2013). The technological progress of RAS can lead to a lower environmental impact due to the reduction of wastewater emissions (Deviller et al., 2004). Simultaneously, an increasing number of new species is introduced to aquaculture (www.diversifyfish.eu; Robles and Mylonas, 2017; Tacken et al., 2015).

European sea bass (*Dicentrarchus labrax*) and its general production as well as the improvement of husbandry conditions and intensification of production is in the focus of recent studies (Buscaino et al., 2010; Conides and Glamuzina, 2006; Dülger et al., 2012; Eroldoğan et al., 2004; Pichavant et al., 2001; Sammouth et al., 2009; Vectesi et al., 2012; Waller et al., 2015). Yet, the successful production of new species in RAS depends on the identification of biological requirements of the specific species of interest (Dalsgaard et al., 2013). On the one hand, the isolation of RAS from the environment comprises many advantages like wide independence of location, improved hygiene management, good monitoring of most husbandry parameters and low water demand (Martins et al., 2010). On the other hand, the low water replacement rates may lead to the accumulation of metabolic end products of fish and bacteria in the rearing water (Deviller et al., 2004; Schram et al., 2014). Specifically, within the nitrification process, primarily ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) convert ammonia/ammonium (NH_3/NH_4^+) to nitrite (NO_2^-) and further to nitrate (NO_3^-), which can accumulate and reach harmful concentrations (Hrubec et al., 1996; Kincheloe et al., 1979; McGurk et al., 2006; Pierce et al., 1993; Scott and Crunkilton, 2000; Shimura et al., 2004, 2002; van Bussel et al., 2012; Westin, 1974).

The not yet fully clarified pathways of nitrate uptake by fish are discussed to be passive diffusion across the gills and/or the gastro intestinal tract after ingestion of water (Bath and Eddy, 1980; Grosell and Jensen, 1999; Jensen, 1995; Learmonth and Carvalho, 2015; Schram et al., 2012; Stormer et al., 1996; van Bussel et al., 2012). However, the effects of nitrate are various and highly dependent on biotic factors like fish species, life stage, or fish size. Also, abiotic factors such as the duration of exposure e.g. short-term (acute toxicity) or long-term exposure (chronic toxicity) are influential

(McGurk et al., 2006; Scott and Crunkilton, 2000). Furthermore, cross-serial dependencies to other stressors (toxicants, water chemical parameters, initial health, etc.) can amplify negative effects (Wendelaar Bonga, 1997). In general nitrate impacts a various number of physiological parameters and impairs survival of various fish species (Kincheloe et al., 1979; McGurk et al., 2006; Pierce et al., 1993; Scott and Crunkilton, 2000; Shimura et al., 2002; van Bussel et al., 2012; Westin, 1974). A widely described physiological effect is the methemoglobinemia, causing a reduced carrying capacity of oxygen by blood cells (Davidson et al., 2017; Learmonth and Carvalho, 2015; Pereira et al., 2017; Wang and Chu, 2016; Wuertz et al., 2013). Methemoglobinemia is induced by endogenous conversion of nitrate to nitrite and the subsequent oxidation of haemoglobin to methaemoglobin. Furthermore, previous studies associated endocrine disruption in fish to nitrate exposure (Freitag et al., 2015; Hamlin et al., 2008). Another negative effect of nitrate was reported by van Bussel et al. (2012), who observed a negative influence of nitrate on feed conversion ratio (FCR), condition factor (CF) and the spleen-somatic index (SSI). Pereira et al. (2017) reported general histological damages caused by increasing nitrate levels, while Shimura et al. (2004) specify nitrate related damages in gills, intestinal ampulla, liver, and kidney causing symptoms similar to nutritional deficiency.

Consequently, it is essential to evaluate the influence of nitrate on health and production, and state nitrate threshold values for every species reared in RAS. Hence, European sea bass was used as one model organism for marine warm water species to evaluate related effects of four different nitrate concentrations on production performance and health status.

2 Material & methods

2.1 Experimental animals

Approximately 1700 European sea bass were obtained from neomar GmbH (Voelklingen, Germany) and kept as a fish stock in a large scaled RAS (40 m^3) at the marine aquaculture facility (Gesellschaft für Marine Aquakultur Aquakultur (GMA) mbH, Büsum, Germany). For the experimental trials 408 sea bass (34 per RAS) with an initial body weight of approximately 108 g were randomly distributed among twelve separate experimental RAS and adapted to the experimental conditions for ten days. The average initial biomass was 3.69 ± 0.02 kg per tank, resulting in a stocking density of 13.17 ± 0.07 kg per m^3. Fish that died during the adaptation phase were replaced. Individuals that died during the experimental trial were replaced by fish with an equal

weight to keep the nominal stocking density. The newly stocked fish were marked after sedation by clipping the gill cover. All marked fish were excluded from final individual-based analysis (hepato-somatic index, spleen-somatic index, individual length, individual weight and condition factor) and the determination of the survival rate in order to guarantee that only fish exposed to the respective nitrate concentrations over the whole experimental trial were considered for final analysis of the mentioned parameters. For group based parameters (specific growth rate, feed conversion ratio and daily feed intake) marked fish were included for calculations.

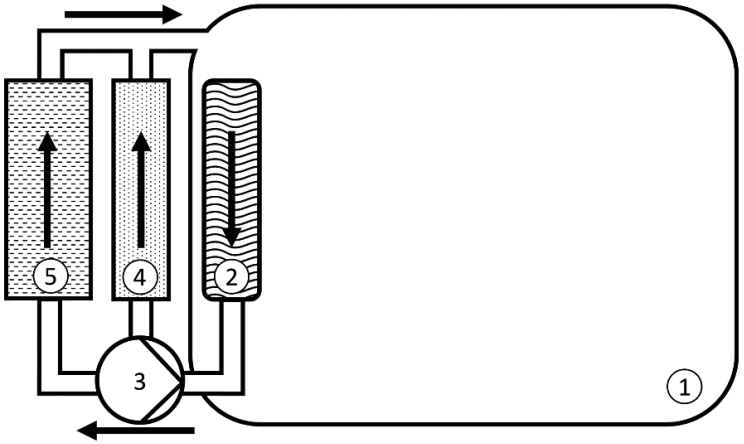

***Figure 1 - 1**. Set-up of one of the 12 used experimental recirculating aquaculture systems (RAS). 320 L total volume, 280 L tank volume, arrows indicate water flow. Water from the rearing tank (1) passes a particle filter (2) and is distributed by a water pump (3) to a protein skimmer (4) and a moving bed biofilm reactor (MBBR) (5) with a total flow rate of 500 L h^{-1} (100 L h^{-1} from the protein skimmer and 400 L h^{-1} from the MBBR).*

2.2 Experimental RAS

As shown in *Figure 1 - 1* each RAS (320 L total volume) consisted of a rearing tank (280 L; 0.7 m^2 footprint, Kunststoff-Spranger GmbH, Plauen, Germany), a Hamburger mat particle filter (HMF), a moving bed biofilm reactor (MBBR, Kunststoff-Spranger GmbH, Plauen, Germany) and a protein skimmer (Model 1AH 1100, Erwin Sander, Uetze-Eltze, Germany). Total flow rate was 500 L h^{-1} (400 L h^{-1} through the MBBR and 100 L h^{-1} through the protein skimmer) and equal to an exchange rate of 1.8 times the tank volume per hour. The temperature was adjusted to 25°C by a temperature-controlled 500 W heater (Aqua Medic, Bissendorf, Germany). The pH was

kept constant at 7.5 during the whole experimental period, by using sodium hydrogen carbonate powder ($NaHCO_3$). Technical oxygen was used to ensure oxygen saturations of >100%. The photoperiod was set to 14 hours light and 10 hours dark. Before the onset of the experiment, the RAS was filled with sand filtered, UV and ozone treated North Sea water (practical salinity (S): 25 - 27). Two-thirds (210 L) of the system water were exchanged twice a week. Fish were fed a commercial feed (Aller Green, Emsland Aller Aqua GmbH, Gloßen, Germany) until apparent satiation six days per week, three times a day. Uneaten pellets were re-collected from the system and counted for the determination of daily feed intake.

2.3 Installation of the nitrate treatments and experimental conditions

Based on the experimental design of van Bussel et al. (2012) the experimental RAS were setup with nominal nitrate concentrations of 125 mg L^{-1} NO_3^--N (low nitrate, LN), 250 mg L^{-1} NO_3^--N (medium nitrate, MN), 500 mg L^{-1} NO_3^--N (high nitrate, HN), and a positive control group (control, C) with the lowest possible concentration, determined by the nitrate production in the system and the water replacement. The nitrate concentration between the three replicates of each treatment was statistically equal ($p > 0.05$), in contrast to the treatment groups, which were statistically different ($p < 0.05$), validating the experimental setup. After adjusting the RAS to the respective nitrate concentration sea bass were distributed among the treatments. Each experimental group was kept in triplicates within the ten weeks trial. The nitrate concentrations were adjusted with a sodium nitrate ($NaNO_3$, Art.-Nr.8601.5, Roth GmbH+Co. KG, Karlsruhe, Germany) and potassium chloride (KCl, Art.-Nr. 6781.3, Roth GmbH+Co. KG, Karlsruhe, Germany) solution. The utilization of both salts provided a Na^+/K^+ weight-ratio of 1:27, which matches the concentration found in natural seawater and avoids imbalances of cellular homeostasis as recommended by van Bussel et al. (2012) and Romano and Zeng (2009, 2007). To avoid rising nitrate levels during the experiment, 210 L water per RAS were exchanged twice a week in batches, resulting in actual nitrate-N concentrations over the experimental trial shown in *Figure 1 - 2*. Prior to the water exchange, the prevailing nitrate-N concentration was measured. Based on this value the required amount of sodium nitrate and potassium chloride was added to the 210 L of exchange water (sand filtered, UV treated and aerated North Sea water from a storage tank) to keep nominal nitrate concentrations. No sodium nitrate and potassium chloride was added to the exchange water of the control treatment.

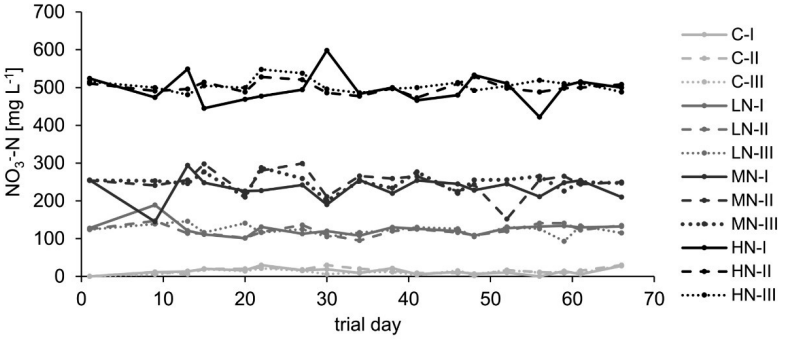

Figure 1 - 2. *Nitrate-N concentration of the twelve experimental RAS over the ten weeks trial. Each treatment, control (C), low nitrate (LN), medium nitrate (MN) and high nitrate (HN) is arranged in triplicate design (I to III).*

Throughout the experimental period, all water quality parameters (except salinity and nitrate) shown in *Table 1 - 2* were in a safe range for sea bass according to references given in *Table 1 - 1*. Significant differences between treatment groups (C, LN, MN, and HN) were observed for salinity, nitrite, nitrate, and pH ($p < 0.05$, *Table 1 - 2*, indicated by small letters a, b, c, d). All other water quality parameters revealed no significant differences between treatment groups.

Although salinity was slightly lower than the recommended minimum of 28, it can be stated that the prevailing values had no negative impact on the sea bass as a euryhaline species (Algers et al., 2008; Eroldoğan and Kumlu, 2002; Jensen et al., 1998; Marino et al., 1994). Significant differences for salinity between the treatment groups correlated with nitrate concentrations. Nitrate concentrations were adjusted with a sodium nitrate solution, which in turn contributes to salinity. Similar to salinity, nitrite also correlates positively with nitrate levels. The used measurement method of nitrite is based on the Griess reaction (Griess, 1879) where the chemical reaction can be influenced at very high levels of nitrate to slightly higher nitrite values. Nevertheless, referring to Blancheton (2000), nitrite values of all treatments were always below the harmful concentration of <2 mg L^{-1} NO_2^--N and therefore, a negative impact on the sea bass can be excluded.

Altogether, the prevalent water quality parameters listed in *Table 1 - 2* (except NO_3^--N) can be excluded as factors negatively affecting production and health performance of on-growing sea bass.

***Table 1 - 1**. Safe range of water quality parameters for rearing European sea bass* (*Dicentrarchus labrax*).

Parameter	Range	Unit	Reference
Temperature	22 - 26	°C	(Algers et al., 2008; Blancheton, 2000; Claireaux et al., 2006; Dülger et al., 2012; Person-Le Ruyet et al., 2004)
pH	6.5 - 8.5		(Algers et al., 2008; Blancheton, 2000)
Practical Salinity (S)	Euryhaline (>28)		(Algers et al., 2008; Conides and Glamuzina, 2006)
Oxygen saturation	>86	%	(Algers et al., 2008; Blancheton, 2000; Thetmeyer et al., 1999)
TAN	< 2 - 6 (6 mg L^{-1} at a pH of 8)	mg L^{-1}	(Blancheton, 2000; Dosdat et al., 2003; Lemarié et al., 2004)
NO_2^--N	< 2	mg L^{-1}	(Blancheton, 2000)

TAN, Total ammonia nitrogen; NO_2^--N, nitrite nitrogen

Table 1 - 2. *Mean values (± SD) of water quality parameters, including n, respectively. Values for water quality were obtained during the experimental trial of ten weeks for each RAS of each treatment.*

Treatment	NO_3^--N [mg L^{-1}]	Temperature [°C]	pH	DO [mg L^{-1}]	DO [%]	TAN [mg L^{-1}]	NO_2^--N [mg L^{-1}]	Practical Salinity
C	13.4 ^{a} ± 7.8	25.0 ± 0.4	7.3^{a} ± 0.2	9.9 ± 0.9	121.5 ± 11.1	0.10 ± 0.09	0.07 ^{a} ± 0.03	25 ^{a} ± 1
LN	123.4 ^{b} ± 15.3	24.9 ± 0.2	7.3 ^{a} ± 0.2	9.9 ± 0.9	122.5 ± 11.0	0.11 ± 0.10	0.11 ^{ab} ± 0.05	25 ^{a} ± 1
MN	243.4 ^{c} ± 30.0	24.9 ± 0.3	7.3 ^{a} ± 0.1	9.8 ± 0.9	120.9 ± 11.2	0.11 ± 0.08	0.13 ^{ab} ± 0.05	26 ^{b} ± 1
HN	501.1 ^{d} ± 26.2	24.9 ± 0.3	7.4 ^{b} ± 0.2	10.0 ± 0.9	123.2 ± 10.5	0.11 ± 0.08	0.15 ^{c} ± 0.07	27 ^{c} ± 1
n	54	183	189	159	159	90	90	82

Superscript letters within one column indicate significant differences (ANOVA with Bonferroni or Kruskal-Wallis-test with Dunn-Bonferroni post hoc test. $p < 0.05$). C, control; LN, low nitrate; MN, medium nitrate; HN, high nitrate; DO, dissolved oxygen; TAN, total ammonia nitrogen; NO_2^--N, nitrite nitrogen; NO_3^--N, nitrate nitrogen.

Table 1 - 3. *Mean values (± SD) of production and health parameters of on-growing European sea bass, including n respectively. Values are given for sea bass at the start of the experiment and for treatments at the end of the ten weeks trial, respectively.*

	n per treatment	C 0 mg L^{-1} NO_3^--N	LN 125 mg L^{-1} NO_3^--N	MN 250 mg L^{-1} NO_3^--N	HN 500 mg L^{-1} NO_3^--N
Initial biomass [kg]	3	3.69 ± 0.01	3.68 ± 0.01	3.69 ± 0.02	3.69 ± 0.02
Final biomass [kg]	3	5.77 ± 0.10	5.61 ± 0.21	5.64 ± 0.24	5.52 ± 0.07
Initial individual weight [g]	15	108.4 ± 0.3	108.3 ± 0.4	108.5 ± 0.6	108.6 ± 0.7
Final individual weight [g]	15	169.7 ± 3.0	165.1 ± 6.3	165.8 ± 7.1	162.4 ± 2.2
CF[1]	15	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
SGR [% d^{-1}][2]	3	0.95 ± 0.03	0.90 ± 0.08	0.90 ± 0.10	0.86 ± 0.04
FCR [g g^{-1}][3]	3	1.66 ± 0.04	1.67 ± 0.12	1.66 ± 0.14	1.66 ± 0.02
DFI [% d^{-1}][4]	3	1.58 ± 0.03	1.48 ± 0.04	1.49 ± 0.07	1.42 ± 0.09
HSI [%][5]	15	2.0 [a] ± 0.4	1.8 [a] ± 0.2	1.8 [a,b] ± 0.3	1.5 [b] ± 0.2
SSI [%][6]	15	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Total mortality [%]	3	0.98 ± 1.39	1.96 ± 2.77	2.94 ± 4.16	6.86 ± 3.67

Superscript letters within one row indicate significant difference (Kruskal-Wallis-test with Dunn-Bonferroni post hoc test. $p < 0.05$). n=20 for start values, n=15 for C, LN, MN and HN at the end of the experimental trial. CF, condition factor; SGR, specific growth rate; FCR, feed conversion ratio; DFI, daily feed intake; HSI, hepato-somatic index; SSI, spleen-somatic index.

[1] $\boldsymbol{CF} = initial\ individual\ weight\ [g] / initial\ individual\ length\ [cm^3] * 100$
[2] $\boldsymbol{SGR}\ [\%\ day^{-1}] = (\ln(initial\ biomass[kg]) - (\ln(final\ biomass[kg])) / feeding\ days * 100$
[3] $\boldsymbol{FCR}\ [kg\ kg^{-1}] = total\ feed\ intake\ [kg] / (final\ biomass\ [kg] - initial\ biomass[kg])$
[4] $\boldsymbol{DFI}\ [\%\ d^{-1}] = SGR * FCR$
[5] $\boldsymbol{HSI}\ [\%] = liver\ weight\ [g] / final\ individual\ weight\ [g]$
[6] $\boldsymbol{SSI}\ [\%] = spleen\ weight\ [g] / final\ individual\ weight\ [g]$

2.4 Water quality analysis

Physic-chemical parameters (*Table 1 - 2*) were monitored regularly during the experimental trial of ten weeks to ensure optimum rearing conditions. Temperature, oxygen concentration and saturation (Handy Polaris, OxyGuard International A/S, Farum, Denmark), and pH (multi 350iWTW, Weilheim, Germany) were measured daily. Practical salinity (S) (Refractometer HI 96822, HANNA Instruments, Woonsocket, USA), total ammonia nitrogen (TAN) and nitrite (NO_2^--N) (MColortestTM, Merck KGaA, Darmstadt, Germany), as well as nitrate (NO_3^--N) (cadmium reduction method 8171, DR 2800 photometer, Hach-Lange GmbH, Berlin, Germany) were measured twice per week.

2.5 Growth and health parameters

Prior and after the exposure experiment, fish of each RAS were group weighed to determine initial and final biomass in each RAS. Furthermore, the individual weight and length of five randomly selected sea bass per RAS (in total 15 fish per treatment) was determined prior and after the experiment. Subsequently, the sea bass were anesthetized, killed and dissected to measure liver and spleen weight. Additionally, total feed intake and daily mortalities were documented. From the collected data specific growth rate (SGR), feed conversion ratio (FCR), daily feed intake (DFI), condition factor (CF), hepato-somatic index (HSI) and spleen-somatic index (SSI) were determined according to formulas in *Table 1 - 3*.

2.6 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics Version 20 (IBM Corporation, New York, USA). Data was tested for normal distribution based on graphical residual analysis and for homoscedasticity using the Levene-Test ($p < 0.05$).

For normal distributed homoscedastic data (oxygen contend and saturation, temperature, pH, nitrate, practical salinity, individual weight, condition factor and spleen-somatic index), the ANOVA, based on triplicates of the four treatments, was followed by Bonferroni post-hoc test ($p < 0.05$) in order to evaluate the differences between individual treatment groups (C, LN, MN, and HN).

For non-normally distributed heteroscedastic data (total ammonia nitrogen, nitrite, initial biomass, final biomass, specific growth rate, feed conversion ratio, daily feed intake, hepato-somatic index and total mortality), Kruskal-Wallis-Test followed by Dunn-Bonferroni post-hoc test ($p < 0.05$) was performed in order to evaluate the differences between individual treatment groups (C, LN, MN, and HN).

The correlation of nitrate between health and performance parameters was tested by bivariate Spearman rank correlation analysis ($p < 0.05$)

3 Results and discussion

Data for initial and final weight showed that sea bass increased their average weight within the ten weeks trial by 30 - 40% to 169.7 g, 165.1 g, 165.8 g and 162.4 g for C, LN, MN, and HN, respectively. Consequently the growth resulted in specific growth rates of 0.86 to 0.95% d^{-1} (*Table 1 - 3*). In general, fish showed no hampered growth performance within the trial compared to sea bass growth evaluated in other studies within a similar size class. Lemarié and Toften (2002), and Sammouth et al. (2009) reported SGRs of 0.71 to 0.81% d^{-1} for on- growing sea bass (100 - 150 g). Also, the DFI of 1.42 to 1.58% d^{-1} and FCR of approximately 1.66 g g^{-1} for C, LN, MN, and HN, were in an appropriate range for on-growing sea bass (Pichavant et al., 2001; Sammouth et al., 2009).

At the end of the ten weeks trial, comparison of production and health parameters between treatment groups (C, LN, MN, and HN) revealed no significant differences for all parameters listed in *Table 1 - 3* except for HSI ($p < 0.05$). Additionally, HSI ($rs=-0.486$, $p=0.000$, $n=12$) and DFI ($rs=-0.605$, $p=0.037$, $n=12$) significantly decreased with increasing nitrate levels according to the rank correlation analysis *(Figure 1 - 3*). For pikeperch and African catfish nitrate-mediated reduction of feed intake and growth was documented by Schram et al. (2012, 2014), stating that especially reduced feed intake is a strong and sensitive indicator for deleterious nitrate levels. Hence, nitrate is most likely the reason for the reduced DFI observed in the present study. Adams et al. (1992) reported a relationship between unspecified toxicant exposure and physiological effects in redbreast sunfish (*Lepomis auritus*). In that study elevated levels of detoxification enzymes were associated with low lipid levels, histopathological damage, and reduced growth. It is obvious that first signs of intoxication on histo-pathological changes occurred, while liver functions include detoxification and energy consuming protein synthesis (e.g. detoxification enzymes). In the presented study, reduced HSI could be the first indicator for nitrate related intoxication of sea bass. The intoxication leads to increased energy consuming detoxification causing reduced lipid levels and consequently results in lower HSI values (Adams et al., 1992). It can be assumed that an intoxication leads to more intense effects on the health and the growth performance at longer exposure time. Pereira et al. (2017) documented general and histological damages of juvenile zebrafish (*Danio rerio*) with increasing nitrate levels.

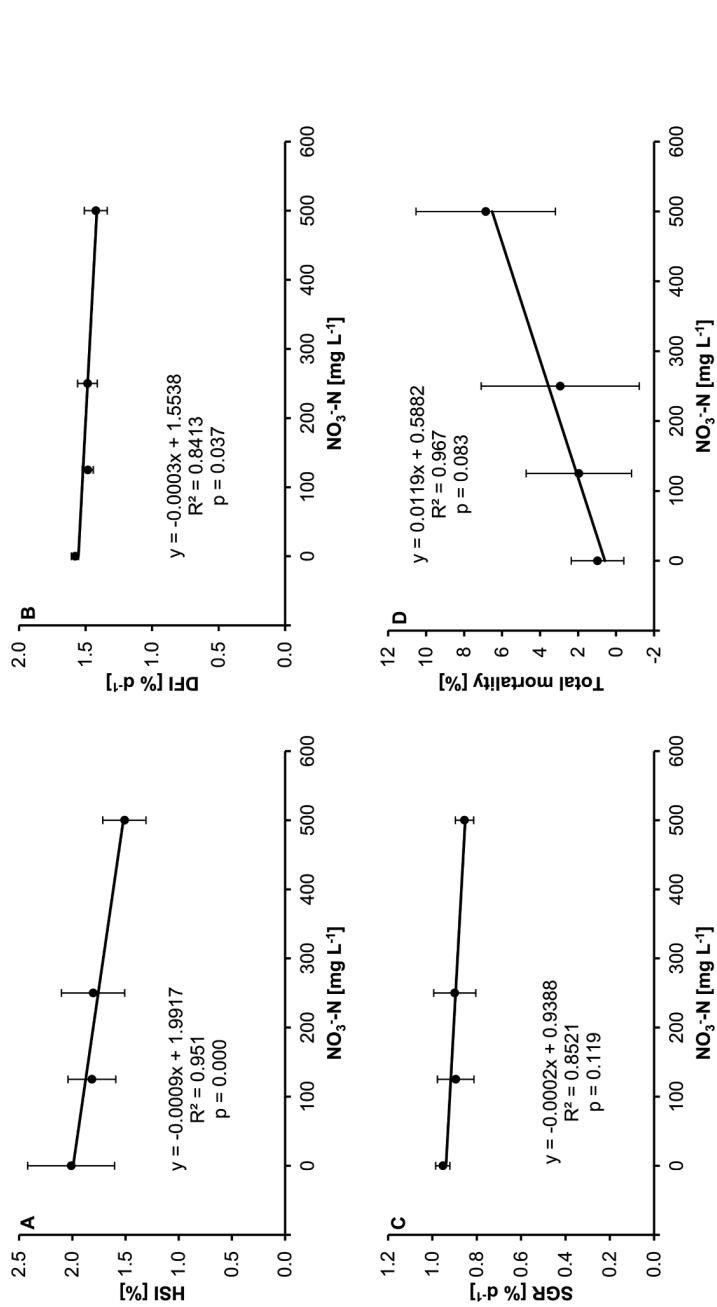

Figure 1 - 3. *(A) Hepato-somatic index (HSI), (B) Daily feed intake (DFI), (C) Specific growth rate (SGR) and (D) Total Mortality of sea bass at corresponding nitrate (NO_3-N) levels.*

Within the study histopathological changes were observed for gills (oedema, hyperaemia, haemorrhages, and hyperplasia and necrosis of epithelial cells), skin (epidermis desquamation and dermis desquamation), kidney (vacuolar degeneration of epithelial cells of renal tubules, hyperaemia, haemorrhage, and others), liver (hepatocyte vacuolization, hypertrophy and necrosis, and depletion of lipid reserves), and intestine (goblet cells hyperplasia in the anterior intestine, vacuolization and hypertrophy of the enterocytes of the posterior intestine, and others). Coincident, toxic effects of nitrate in Medaka fish (*Oryzias latipes*) were observed in the gills, the intestinal ampulla, the liver, and the kidney (Shimura et al., 2004). Especially the chronic nitrate exposure can impair hepatic function and consequently lead to symptoms similar to those of nutritional deficiency (Shimura et al., 2004). Results of the present work match these statements since the significant negative correlated HSI and DFI ($p < 0.05$, *Figure 1 - 3*) towards higher nitrate levels can lead to reduced growth and production performance, even though not statistically significant in this study. The statistical analyses of variances for SGR and total mortalities revealed no significant differences ($p > 0.05$), possibly caused by high standard deviation. Nevertheless, the rank correlation analysis revealed that SGR values ($rs=-0.475$, $p=0.119$, $n=12$) tended to show a significant negative correlation and mortalities ($rs=0.521$, $p=0.083$, $n=12$) tended to show a significant positive correlation with higher nitrate levels (*Figure 1 - 3*). Van Bussel et al. (2012) reported a negatively linear correlated effect of nitrate on SGR, final weight, final length, and biomass yield starting at concentrations of 125 mg L^{-1} NO_3^--N for juvenile turbot (*Psetta maxima*) exposed to nitrate concentrations equal to the nitrate concentration used in the presented study. Additionally van Bussel et al. (2012) revealed, that mortality, FCR, CF, and SSI were significantly higher in MN and HN compared to C and LN groups. Comparing results of the present study to results of van Bussel et al. (2012) it can be suggested that on-growing sea bass are more tolerant to elevated nitrate levels compared to juvenile turbot. Similar, Schram et al. (2014) observed no effects of nitrate on physiology and growth of juvenile pikeperch at nitrate-N levels as high as 358 mg L^{-1}, concluding that nitrate is relatively harmless to fish. The impact of toxicants on physiological parameters is likely attributed to a large number of environmental factors in combination with the fish species. As stated by Kroupova et al. (2005), Levit (2010) and Schram et al. (2014) the toxicity of ammonia, nitrite and nitrate depends on the fish species, the life stage and the fish size, among others. Furthermore, the type of rearing water (freshwater, brackish, or seawater) and the coherent relation between salinity of the water and nitrate uptake can possibly influence nitrate toxicity. However, the impact of salinity on nitrate toxicity has not yet been adequately evaluated, as mentioned by

Davidson et al. (2017) and van Bussel et al. (2012). As long as the exact pathways of nitrate uptake remain unclear, the influence of salinity on nitrate toxicity is questionable. Supporting this, the listing of "No Observed Effect Concentrations" (NOEC) in *Table 1 - 4* demonstrates how diversely nitrate affects different freshwater and marine fish species. Additionally, it should be taken into account that in the majority of studies, rather young fish were tested. European sea bass of the present study with an average initial weight of approximately 108 g were older and/or bigger in comparison to fish tested in the toxicity studies listed in *Table 1 - 4*. The rather high tolerance of on-growing sea bass towards nitrate might demonstrate an increased robustness compared to other species shown in *Table 1 - 4* (interspecific differences). Supporting Kroupova et al. (2005) and Levit (2010) it seems natural, that tolerance towards nitrate differs not only interspecific but also intraspecific. In general, older life stages are less sensitive to stressors such as water pollutants than earlier life stages (intraspecific differences) (Schram et al., 2014; Wendelaar Bonga, 1997). Comparative studies for the intraspecific influence of nitrate on health and performance parameters across various life stages and fish sizes are still insufficient (Camargo et al., 2005; Hickey and Martin, 2009).

Besides biotic factors also abiotic factors, such as nitrate exposure duration (acute toxicity or chronic toxicity), affect health and performance parameters of fish. Several studies revealed that fish can tolerate high nitrate concentrations ranging from 116 to >3000 mg L^{-1} NO_3^--N in a short time period (acute toxicity), while threshold concentrations of long-term exposure (chronic toxicity) were far lower with 1.6 to 358 mg L^{-1} NO_3^--N *Table 1 - 4*. For sea bass, Vectesi et al. (2012) observed no statistically significant changes in immunological and haematological parameters within a short-term 48 h exposure to 100 and 700 mg L^{-1} nitrate (23 and 158 mg L^{-1} nitrate-N). However, fish produced in RAS are exposed to increasing nitrate concentrations, resulting from the system, over months and years. To rely on LC50 values as nitrate threshold concentrations would be inaccurate for a RAS owner. To state threshold concentrations for fish produced in RAS long-term studies require further research (Davidson et al., 2014; Good et al., 2017). Additionally, as stated by Schram et al. (2014), threshold concentrations derived from experimental studies should be applied cautiously outside the size range of tested fish species. Therefore further studies are mandatory to determine nitrate threshold concentrations for different fish species and life stages produced on long-term production cycles in RAS.

Table 1 - 4. *Lethal concentration (LC50) of nitrate (NO_3^--N) and/or "No Observed Effect Concentration" (NOEC) for different fish species at different life stages comparing different studies.*

Species	Common name	Life Stage	LC 50		NOEC		Author
			Duration	mg L^{-1} NO_3^--N	Duration	mg L^{-1} NO_3^--N	
Carias gariepinus	African catfish	Juvenile	-	-	42 d	140	(Schram et al., 2012)
Centropristis striata	Gulf black sea bass	Juvenile	4 d	2400	-	-	(Pierce et al., 1993)
Coregonus clupeaformis	Lake whitefish	Fry	4 d	1903	126 d	25	(McGurk et al., 2006)
Monacanthus hispidus	Planehead filefish	Juvenile	4 d	573	-	-	(Pierce et al., 1993)
Oncorhynchus kisutch	Coho Salmon	Fry	-	-	30 d	> 4.5	(Kincheloe et al., 1979)
Oncorhynchus mykiss	Rainbow trout	Fingerlings, Fry	7 d	1061	30 d	2.3	(Kincheloe et al., 1979; Westin, 1974)
Oncorhynchus mykiss	Rainbow trout	Fry	-	-	30 d	> 4.5	(Kincheloe et al., 1979)
Oncorhynchus mykiss	Rainbow trout	Juvenile	-	-	3 m	< 80	(Davidson et al., 2014)
Oncorhynchus tshawytscha	Chinook salmon	Fingerlings, Fry	7 d	1084	30 d	4.5	(Kincheloe et al., 1979; Westin, 1974)
Oryzias latipes	Medaka	Fry	4 d	116 - 166	160 d	25	(Shimura et al., 2002)
Pimephales promelas	Fathead minnow	Larvae	4 d	1341	-	-	(Scott and Crunkilton, 2000)
Pomacentrus leucostritus	Beaugregory	Juvenile	4 d	> 3000	-	-	(Pierce et al., 1993)
Psetta maxima	Turbot	Juvenile	-	-	42 d	< 125	(van Bussel et al., 2012)
Raja eglanteria	Clearnose skate	Juvenile	4 d	> 960	-	-	(Pierce et al., 1993)
Salmo clarki	Cutthroat trout	Fry	-	-	30 d	7.6	(Kincheloe et al., 1979)
Salmo salar	Atlantic salmon	Post-smolt	-	-	8 m	100	(Davidson et al., 2017; Good et al., 2017)
Salvelinus namaycush	Lake trout	Fry	4 d	1121	146 d	1.6	(McGurk et al., 2006)
Sander lucioperca	Pikeperch	Juvenile	-	-	42 d	358	(Schram et al., 2014)
Trachinotus carolinus	Florida pompano	Juvenile	4 d	1000	-	-	(Pierce et al., 1993)

LC50, lethal concentration which causes the death of 50% of a group of test animals within a certain time period; NOEC, "No Observed Effect Concentration", the concentration of a pollutant that will not harm the species exposed within a certain time period; d, days; m, months; NO_3^--N, nitrate nitrogen.

4 Conclusion

In the present study, a significant impact of elevated nitrate levels of up to 500 mg L^{-1} NO_3^--N HSI and DFI of on-growing sea bass was documented. Our data revealed a significant influence of nitrate on HSI at concentrations >250 mg L^{-1} NO_3^--N. An overall negative correlation of HSI and DFI with increasing nitrate values was significant. Furthermore, a trend towards lower SGR and higher mortality at higher nitrate levels indicates a potentially negative impact of nitrate on further health and performance parameters. Thus, for a long-term production cycle negative effects of increased nitrate levels on SGR, growth, and mortality can be expected. Therefore, long-term research covering a complete production cycle in RAS is necessary to determine the entire effects of nitrate. However, compared to other aquaculture fish species recently tested, the sensitivity of on-growing sea bass towards nitrate toxicity seems to be relatively low.

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CHAPTER 2

Impact of hydraulic retention time, backflushing intervals, and C/N ratio on the SID-Reactor denitrification performance in marine RAS

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Abstract

In recirculating aquaculture systems (RAS) the high water re-use in combination with insufficient treatment of the process water can lead to the accumulation of nitrate, amongst other metabolic end products. For the efficient removal of nitrate in a marine RAS, a **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor) was investigated in this study. Within three consecutive experimental trials the effect of varying hydraulic retention time (HRT), backflushing intervals (BFI), and carbon to nitrogen (C/N) ratios on water quality parameters and denitrification performance (rate and efficiency) were monitored. Different HRTs of 2, 4, and 6 hours and additionally BFIs of 10, 30, 60, and 90 minutes were evaluated. The tested C/N ratios, realized using methanol (MeOH) as a carbon source, were 1.1, 1.5, 1.9, 2.1, 2.3, 2.7, 3.1, and 3.5 (mg MeOH per mg NO_3^--N). The experiments revealed that a HRT of 2 hours resulted in the highest denitrification rate (497 g d^{-1} m^3 biocarriers) but a lower denitrification efficiency of 64%. A HRT of 6 hours had highest denitrification efficiency of 81% but a lower denitrification rate (253 g d^{-1} m^3 biocarriers). Furthermore, it was evident that backflushing intervals every 10 minutes resulted in a decreased denitrification efficiency of 29%, while intervals every 90 minutes increased the maintenance effort. Overall, backflushing intervals every 30 and 60 minutes showed the best results. A C/N ratio of 2.3 seemed to be sufficient to ensure an optimal denitrification performance, incorporating all single tested water quality parameters. The results of this study allow an easy, efficient and safe application of a SID-Reactor with the purpose of nitrate removal in marine RAS.

Key words: process water treatment; anoxic; nutrient excretion; ammonia; nitrite; nitrate; methanol; metabolic end product; filter clogging; oxygen; oxidation-reduction potential; self-cleaning inherent gas denitrification reactor; backflushing intervals

1 Introduction

Production of fish in closed recirculating aquaculture systems (RAS) gained increasing interest over the last decades. This trend led to an intensification of production, simultaneously minimizing the environmental impact (Badiola et al., 2012; Bregnballe, 2015; Dalsgaard et al., 2013). Contemporaneous, environmental regulations have tighten and especially waste water regulations do affect RAS design and operation in terms of waste management (European Commission, 2009). New and existing production facilities will have to consider technical adaptations to legal frames and improved RAS designs to fulfil regulations.

Bregnballe (2015) postulated that "super" intensive RAS with a 99.6% degree of recirculation are trendsetting. This high degree of re-use of water is only possible if solid waste treatment and denitrification systems are ensured. Depending on the production site and the cultivated fish species, high re-use of water can lead to problems concerning water quality. Accumulating metabolic end products of fish and bacteria can reach toxic concentrations for fish, if water consumption is limited and the cultivated species is rather sensitive. For instance nitrate, as the metabolic end product of nitrification, could have a negative impact on health and growth performance of fish at species-specific concentrations (Davidson et al., 2017, 2014; Good et al., 2017; Hrubec et al., 1996; Kincheloe et al., 1979; McGurk et al., 2006; Pierce et al., 1993; Schram et al., 2014, 2012; Scott and Crunkilton, 2000; Shimura et al., 2004, 2002; Torno et al., 2018; van Bussel et al., 2012; Westin, 1974).

A denitrification unit included in RAS can reduce and/or eliminate nitrate and its effects on fish and environment. Though uncommon 10 years ago (van Rijn et al., 2006), nowadays an increasing amount of denitrification systems is applied to RAS. Especially, when high stocking densities and low water replacement are followed by high nitrate concentrations. In general, research on enhanced denitrification practices has accelerated within the last decades (Addy et al., 2016). However, conventional denitrification systems are an enormous challenge for RAS staff since maintenance and operation of these systems are challenging. Therefore, the need for a safe, efficient, and easy to operate denitrification system is necessary. Badiola et al. (2012) reported the major constraints on management and future challenges concerning RAS in a survey, involving RAS based production companies, researchers, system suppliers, and consultants. Main limitations were identified as: poor designs of the systems and poor management due to an absence of skilled people. As Lekang (2013) states, the challenge in RAS is "[…] to bring together both technological and biological knowledge within the aquaculture field".

Research and development in the domain of water treatment technology produces sophisticated systems showing smart solutions for existing problems. For instance, Müller-Belecke et al. (2013) introduced the low-maintenance **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor; patented by Müller-Belecke and Spranger, 2014), which is also the subject of the current study. They tested the performance of the SID-Reactor with different carbon sources (denatured ethanol, methanol, acetic acid, and glycerin) and changing operational modes (low energy demand, low carbon demand, and high performance). It has been demonstrated that the daily routine of operation and maintenance of the SID-Reactor was simple and done within a few minutes a day. Hence, the SID-Reactor is a promising denitrification unit for RAS. However, to make the SID-Reactor as safe and user-friendly as possible, it is still necessary to evaluate the influence of the most important operating settings to guarantee an optimized and safe denitrification process.

In denitrification systems, the adjustment of hydraulic retention time (HRT) shows a high impact on the nitrate removal rate and efficiency. While relatively long HRTs result in high denitrification efficiency (percent of removed NO_3^--N) and low denitrification rates (total amount of removed NO_3^--N per time unit), short HRTs result in the opposite effect (Addy et al., 2016; Lepine et al., 2016; Oh et al., 2001; Timmermans and van Haute, 1983; Wang and Chu, 2016).

When changing the HRT, generally two opposing scenarios can be expected. (I) At low HRTs a relatively high amount of oxygen (hindering denitrification performance) and nitrate (increasing denitrification performance) enters the reactor via the inlet water. (II) At increased HRTs, a relatively low amount of oxygen (promoting denitrification performance) and nitrate (lowering denitrification performance) enters the reactor via the inlet water. It is necessary to find a balanced setting where as much nitrate as possible can be treated without disturbing denitrification processes by increased oxygen influx.

Another major problem reported for biological filter systems is the clogging through microbial growth and particulate organic matter, thus limiting filter performance (Alonso et al., 1997; Eding et al., 2006; Hozalski and Bouwer, 1998; Kamstra et al., 1998; Lepine et al., 2016; Mara et al., 2003; McMillan et al., 2003; Moretti et al., 1999a; Paller and Lewis, 1982; Rakocy et al., 2006; Sastry et al., 1999). Conventional denitrification units based on the moving bed biofilm design or fixed bed design require a backflushing of the biocarriers to prevent clogging and breakdown of the denitrification performance. Backflushing of the denitrification unit is often accompanied by a severe

change in environmental conditions for bacteria, causing a temporary breakdown of the denitrification performance.

Safe denitrification relies on the accurate dosage (C/N ratio) of an external carbon source (in this study methanol), which is mandatory to fuel denitrification. An accurate methanol dosage is able to improve water quality by reducing the nitrate and rising the alkalinity. Under dosage will lower the denitrification rate and stimulate nitrite formation (Hamlin et al., 2008; Sauthier et al., 1998; Timmermans and van Haute, 1983; Yang et al., 2012) as well as hydrogen sulphide production (Bregnballe, 2015), which are both toxic for aquatic species cultured in RAS. Based on stoichiometric calculations, anticipated 1.9 g of methanol is required to reduce 1.0 g of NO_3^--N (Cheremisinoff, 1995). Since the C/N ratio depends on several factors (e.g. type of carbon source and cell synthesis of bacteria) the determination of an accurate dosage is mandatory for safe denitrification processes in specific technical setups.

The aim of this study was to evaluate the effect of (1) different HRTs, (2) varying BFIs, and (3) altered C/N ratios on denitrification performance and water quality parameters in a marine RAS equipped with a SID-Reactor. Three consecutive trials were performed within one large scale RAS stocked with European sea bass (*Dicentrarchus labrax*) to evaluate the operational settings and safe use of the SID-Reactor.

2 Materials & Methods

2.1 Experimental Setup

2.1.1 Recirculation Aquaculture System (RAS)

The utilized RAS (*Figure 2 - 1*, 40 m^3 in total, Kunststoff-Spranger GmbH, Plauen, Germany), was filled with sand-filtered, UV- and ozone-treated North Sea water (practical salinity: 24 - 30). Light regime was adjusted to 16 h light and 8 h darkness. Water from the ten rearing tanks (each 2.5 m^3) was drained to a drum filter (60 µm mesh size) and further to a pump sump. To ensure oxygen saturation in the rearing tanks, an oxygen cone was included into the RAS. A Moving-Bed-Biofilm-Reactor (MBBR, 4.5 m^3 total volume, 25 $m^3\ h^{-1}$ water flow) was filled with 1.5 m^3 of biocarrier (HEL-X®, diameter: 12 mm, surface: 859 $m^2\ m^{-3}$, specific surface area (SSA): 704 $m^2\ m^{-3}$, density: 0.95 g, Christian Stöhr GmbH & Co. Elektro- und Kunststoffwaren KG, Marktrodach, Germany) for aerobe nitrification. Furthermore, the RAS was equipped with a skimmer (Helgoland 500, 11 $m^3\ h^{-1}$ water flow, Erwin Sander Elektroapparatebau GmbH, Uetze-Eltze, Germany) supported by ozone (C-Lasky DSI/DTI, 10 g h^{-1} ozone, AirTree Europe GmbH, Baunatal, Germany). Water temperature in RAS was kept constant at 25°C by a heat exchanger. The pH was kept between 7.3 and 7.5, by adding sodium hydrogen carbonate powder ($NaHCO_3$) to the rearing water. The oxygen saturation of the rearing water was maintained on average >100% (>8 mg L^{-1}), guaranteeing a sufficient oxygen supply for the fish as well as the aerobe biofilter systems. Further water parameters (salinity, total ammonia nitrogen (TAN) and NO_2^--N) were monitored and kept in a safe range in accordance to sea bass requirements summarized in Torno et al. (2018). To keep the intended basal nitrate concentration stable at 40 mg L^{-1} throughout the experimental trials, nitrogen in form of a 20% urea and 80% ammonia solution (CH_4N_2O as powder, 25% NH_3, Carl Roth GmbH & Co.KG, Karlsruhe, Germany) was added to the system when necessary. The nitrogen was added directly into the MBBR with the help of an automatic dosage pump (RAININ, Dynamax®, Model RP-1, Rainin Instrument, Oakland, CA, USA).

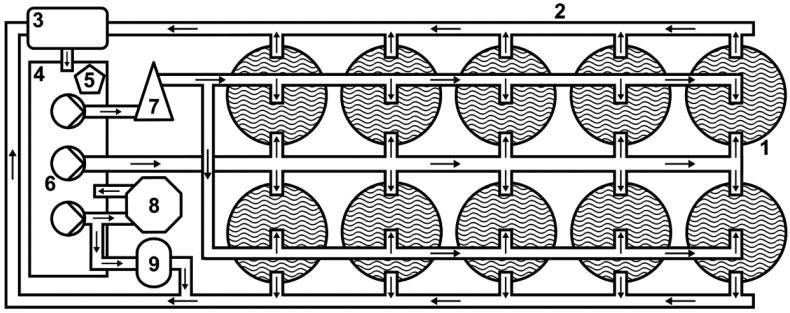

Figure 2 - 1. *The 40 m³ experimental recirculating aquaculture system (RAS). The RAS includes ten 2.5 m³ rearing tanks (1), an outlet drain (2), a drum filter (3), a pump sump (4), a protein skimmer (5), three circulation pumps (6), an oxygen cone (7), an aerobe MBBR nitrification filter (8), and the anoxic SID-Reactor (9). The water flow is indicated by arrows.*

2.1.2 Experimental Animals

Approximately 1700 European sea bass were obtained from neomar GmbH (Voelklingen, Germany) and acclimatized to the conditions in the experimental RAS in the facilities of the Gesellschaft für Marine Aquakultur (GMA) mbH (Büsum, Germany). Sea bass were distributed among nine out of ten rearing tanks at an average stocking density of 40 kg per m³. Fish were fed manually until apparent satiation with a commercial feed (Aller Green, Emsland Aller Aqua GmbH, Gloßen, Germany) twice per day, resulting in a basal nitrogen load into the RAS. Feeding rate was adjusted regularly to 1% of the total biomass per tank.

2.1.3 Denitrification System

The same Self-cleaning Inherent gas Denitrification-Reactor (SID-Reactor, Kunststoff-Spranger GmbH, Plauen, Germany) was used for the whole experimental duration for the purpose of nitrate removal. The design of the SID-Reactor is based on a combined function principle of a fix bed as well as moving bed biofilm reactor (Müller-Belecke et al., 2013). The reactor (*Figure 2 - 2*) had a total volume of 0.85 m³, whereas the water level in the reactor was adjusted to 0.75 m³. The SID-Reactor was filled with 0.45 m³ floating biocarriers (originating from the aerobe MBBR). The biocarriers resulted in a specific surface area of 386.5 m² available to population by bacteria. Denitrifying bacteria transform nitrate into inert nitrogen gas (dissimilative nitrate reduction) due to following simplified chemical reaction chain:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

The reactor chamber itself is closed with a gas tight top cover to prevent oxygen influx by ambient air, enabling anoxic conditions. Additionally, the nitrogen gas produced during denitrification will accumulate at the top of the reactor chamber. Within a defined time interval, the fixed carriers were swirled up. A side channel vacuum pump (0.55 kW, 120 m^3 h^{-1} gas flow, 100 mbar, type: SV 8.130/1-01, Gebr. Becker GmbH, Wuppertal, Germany) forced the inherent oxygen-poor gas from the top to the bottom of the SID-Reactor and set the biocarriers into motion. Aim was to prevent the biocarriers from clogging due to bacterial growth (self-cleaning effect of the SID-Reactor). A small opening (Ø = 0.5 cm) on the top of the reactor allowed excessive nitrogen gas to leak and furthermore allowed pressure compensation at times when the side channel vacuum pump was running.

Further components of the SID-Reactor were a container (20 L) for a carbon source and a peristaltic dosage pump (Kronos 50, Seko Deutschland GmbH, Mainz-Kastel, Germany) constantly adding the carbon source to the influent water of the SID-Reactor. The flow rate was adjusted by a diaphragm valve with a digital flow meter (Signet 2551, Georg Fischer AG, Schaffhausen, Switzerland).

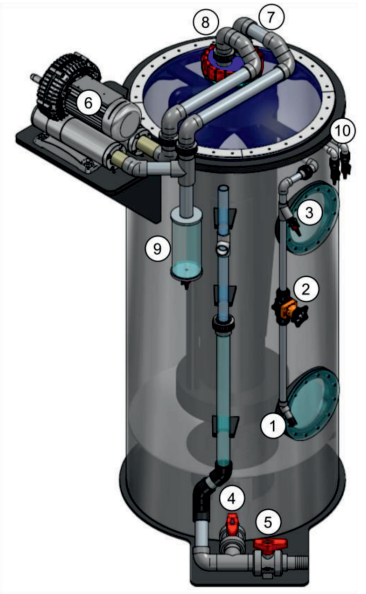

Figure 2 - 2. *The **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor). Inlet (1), flow rate valve (2), methanol inlet (3), outlet (4), sludge release (5), side channel vacuum pump (6) with inlet (7) and outlet (8), condensate trap (9), and pressure relief opening (10).*

2.2 Experimental Design

2.2.1 Pre-Trial Phase

After 60 days of adaptation of the sea bass to the RAS conditions denitrification processes were started within a start-up process divided into three consecutive steps.

Within the first step, the SID-Reactor was operated without biocarriers and without methanol dosage to adjust basal hydraulic settings orientated on Müller-Belecke et al. (2013) for a period of two weeks. At this point, the flow-through was set to 750 L h^{-1}, equal to a HRT of 1 hour. In a second step, biocarriers from the aerobe MBBR were added to the SID-Reactor chamber and the unit was operated for further three weeks allowing bacteria to adapt. During this period the flow-through was reduced stepwise from 750 to 125 L h^{-1}, decreasing the oxygen load into the SID-Reactor. In a third step, flow through was kept at 125 L h^{-1} and methanol was applied to the system based on the nitrate concentration of the rearing water, finally initiating the denitrification process. Subsequently, the experiment with three consecutive trials was performed. Each experimental trial was started only under the premise that a stable denitrification process was present.

2.2.2 Consecutive experimental trials

Trial I: Hydraulic Retention Time (HRT)

Within the first experimental trial the aim was to evaluate the effects of varying HRTs on denitrification efficiency and rate of the SID-Reactor. Three different HRTs of 2, 4 and 6 hours (HRT-2, HRT-4 and HRT-6) resulting in nominal hydraulic loads of 375, 188 and 125 L h^{-1}, respectively, were tested. Water quality parameters were monitored (daily: temperature, oxygen saturation and content, ORP, and pH; every three days: ANC, TAN, NO_2^--N, and NO_3^--N). Based on recommendations of Müller-Belecke et al. (2013) biofilter media was back flushed every 30 minutes for 30 seconds. During the first trial, methanol dosage was set to a C/N ratio of 2.7.

Trial II: Backflushing Intervals (BFI)

The SID-Reactor used in this study allowed to adjust the time intervals of automated backflushing of the biocarriers from constant to every 99 minutes. Four different BFIs were tested in order to assess the SID-Reactor's denitrification performance. Water quality parameters were monitored (every two days: temperature, oxygen saturation and content, ORP, pH, and turbidity; every four days: ANC, TAN, NO_2^--N, and NO_3^--N). Within the scope of this work a BFI consist of three phases:

Phase I: Initiation of biocarrier movement. To overcome inertia of biocarriers in the water column, the side channel vacuum pump automatically runs for 10 seconds at full load (100 mbar pressure and 120 $m^3\ h^{-1}$).

Phase II: Biocarriers are kept in movement for 30 seconds during an energy-saving, reduced operating level of the side channel vacuum pump.

Phase III: The side channel vacuum pump automatically turns off for a certain time period.

Within the four tested BFIs, phases I and II were kept unchanged. The duration of phase III was altered to result in backflushing every 10, 30, 60, and 90 minutes (treatments BFI-10, BFI-30, BFI-60, and BFI-90). Based on the results of the first experimental trial a HRT of 2 hours was chosen. During the second trial, methanol dosage was kept unchanged at a C/N ratio of 2.7.

Trial III: C/N ratio

For the heterotrophic denitrification processes a 50% methanol (CH_3OH, BÜFA Chemikalien GmbH & Co. KG, Oldenburg, Germany) solution was used. In previous trials it was observed that the dosage of pure methanol led to clogging (not examined in closer detail) of the inlet valve. Therefore, methanol was diluted with deionized water in a weight ratio of 1:1 resulting in a 50% methanol solution, avoiding the problem.

Seven different C/N ratios, starting from an assumed over dosage (C/N ratio of 3.5) to an under dosage (C/N ratio of 1.1), were tested in following order within a third experimental trial as shown in *Figure 2 - 3*. Water quality parameters (oxygen concentration, ORP, TAN, NO_2^--N, NO_3^--N, and TN reduction) were monitored daily. Methanol addition was adjusted to the actual nitrate level every 24 hours. Every 48 hours the overall methanol dosage was reduced by 0.4, resulting in the seven tested C/N ratios. The HRT was kept stable at 2 hours (375 L h^{-1}).

At the end of the first test series, the denitrification performance of the SID-Reactor was stabilized with a C/N ratio of 2.3. Hereafter, a second test series with the same ratios was conducted to prove the repeatability of the observations.

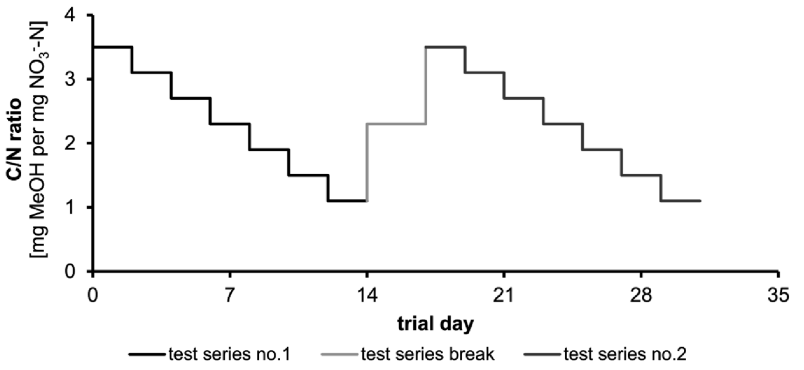

Figure 2 - 3. *Experimental procedure for the determination of optimum carbon to nitrogen (C/N) ratio.*

2.3 Data collection

2.3.1 Water quality analysis

Data for following water quality parameter assessment were collected for both, inlet and outlet of the SID-Reactor. Hereby, the inlet water was equal to the rearing water in the RAS. Total ammonia nitrogen (TAN, salicylate method, method 8155, Hach Lange GmbH, Düsseldorf, Germany), nitrite (NO_2^--N, diazotization method, method 8507, Hach Lange GmbH, Düsseldorf, Germany), and nitrate (NO_3^--N, cadmium reduction method, method 8171, Hach Lange GmbH, Düsseldorf, Germany) were measured photometrically (DR 2800, Hach Lange GmbH, Düsseldorf, Germany). Furthermore, turbidity (NTU, PCE-TUM 20, PCE Deutschland GmbH, Meschede, Germany), acid neutralizing capacity (ANC) by titration of 0.1 mmol L^{-1} hydrochloric acid, and salinity (HI 96822, Hanna Instruments Deutschland GmbH, Vöhringen, Germany) were measured. For automatic data logging, three sensors were installed in the outlet of the SID-Reactor. Data were logged for oxygen saturation (%), oxygen concentration (mg L^{-1}), and water temperature (°C) (Handy Polaris, OxyGuard International A/S, Farum, Denmark). Likewise, the pH-value (SenTix®41, WTW pH 3310, Xylem Analytics Germany Sales GmbH & Co.KG, Weilheim, Germany), and the ORP (GMH 5550, GHM Messtechnik GmbH, Regenstauf, Germany) were logged. Due to a malfunction of the ORP sensor in the SID-Reactor inlet, no data of the inlet water could be recorded. Additionally, particle characteristics were documented photographically in the BFI trial and used as further assessment criteria.

2.3.2 SID-Reactor denitrification performance

The overall denitrification efficiency is described as NO_3^--N reduction in percent (*Table 2 - 2* and *Table 2 - 4*). For comparison reasons with other studies the denitrification rate is described by the NO_3^--N reduction in milligram NO_3^--N per litre of treated water and hour (mg L^{-1} h^{-1}), in milligram NO_3^--N per litre of treated water, hour and cubic meter of biocarriers (mg L^{-1} h^{-1} m^3 biocarriers), and in gram NO_3^--N per day and cubic meter of biocarriers (g d^{-1} m^3 biocarriers) (*Table 2 - 2* and *Table 2 - 4*). The denitrification rate was calculated based on the difference between the NO_3^--N concentration of the inlet and the outlet water of the SID-Reactor. Results for total nitrogen (TN) reduction are expressed in the same way. TN reduction was calculated based on the differences in concentration between inlet and outlet water, taking only TAN, NO_2^--N and NO_3^--N into account. Results for denitrification efficiency were discussed in percent and for denitrification rate in g d^{-1} m^3 biocarriers.

2.4 Statistical Analysis

Since the present survey was performed in a unique technical scale production RAS all experiments are based on a consecutive experimental design. Mean values and standard deviation presented in the tables are based on repeated measurements (=n) over the trial phase.

To evaluate the optimum C/N ratio, a segmented linear regression with one dependent variable (oxygen concentration, ORP, TAN, NO_2^--N, NO_3^--N, and TN reduction) and the independent variable C/N ratio was performed. Statistical analyses were performed using SegReg (R.J. Oosterbaan, www.waterlog.info). The segmentation was done by presenting a break point, resulting in a broken line graph. The calculation of the optimum break point was based on maximizing the statistical coefficient of explanation, and performing tests of significance. Statistical significance of the segmented linear regression with break point was done by analysis of variance (ANOVA) and F-tests ($p < 0.05$).

3 Results

3.1 Hydraulic Retention Time (HRT) influences water quality

3.1.1 SID-Reactor inlet water parameters

All measured water quality parameters (*Table 2 - 1*) for the inlet water of the SID-Reactor, also representing the rearing water, were close to equal for all three HRTs and showed only slight fluctuations. During the experimental trial, HRT-2, HRT-4, and HRT-6 resulted in average hydraulic loads of 348, 181 and 116 L h^{-1}, respectively, and were comparable to the nominal settings (*Table 2 - 1*). The water temperature for all three settings fluctuated between 23 and 25°C. The oxygen saturation in the rearing water was on average >100%. The pH was between 7.3 and 7.5, while ANC ranged between 2.4 and 2.9 mmol HCl L^{-1}. Average TAN concentrations (*Table 2 - 1*) varied depending on HRT. The highest TAN was at HRT-2 (0.7 mg L^{-1}), while lower values for the HRT-4 and HRT-6 treatment (0.2 and 0.3 mg L^{-1}, respectively) were measured. Similar, average NO_2^--N concentration (*Table 2 - 1*) was higher at the HRT-2 (0.4 mg L^{-1}) compared to the HRT-4 and HRT-6 (both 0.1 mg L^{-1}). The average NO_3^--N concentrations were 42, 33, and 49 mg L^{-1} for HRT-2, HRT-4, and HRT-6, respectively.

3.1.2 SID-Reactor outlet water parameters

For all three settings, oxygen saturation in the outlet was below 4% (0.3 mg L^{-1}, *Table 2 - 1*). Furthermore, pH (7.6 to 7.9) and ANC (4.9 to 5.6 mmol HCl L^{-1}) were higher in the outlet of the SID-Reactor compared to the inlet for all HRT settings. Negative ORP values (-125 to -145 mV) were measured in the outlet throughout all HRTs. Average TAN concentrations for HRT-2 (0.2 mg L^{-1}) and HRT-6 (0.1 mg L^{-1}) were slightly lower in the outlet compared to the inlet, while for HRT-4 the TAN concentration (0.3 mg L^{-1}) was slightly higher. Differences were more evident in average NO_2^--N values. For all three HRT settings, NO_2^--N values were higher in the outlet compared to the inlet. An average NO_2^--N value of 3.9 mg L^{-1} was observed in HRT-2 compared to 0.8 and 1.7 mg L^{-1} for HRT-4 and HRT-6, respectively. However, lower average NO_3^--N concentrations in the reactor effluent compared to the inlet were an indicator for denitrification processes for all three tested HRTs. The lowest average NO_3^--N outlet concentration was measured for HRT-6 (10 mg L^{-1}), followed by a HRT-4 (12 mg L^{-1}). The HRT-2 resulted in the highest average NO_3^--N concentrations (15 mg L^{-1}) at the SID-Reactor outlet.

Table 2 - 1. *Mean values (± SD) of water quality parameters for the SID-Reactor inlet and outlet depending on hydraulic retention times (HRT) of 2, 4 and 6 hours.*

hydraulic retention time (HRT)		[h]	2		4		6	
nominal hydraulic load		[L h^{-1}]	375		188		125	
trial days		[d]	29		26		36	
actual hydraulic load		[L h^{-1}]	348 ± 38	(n=29)	181 ± 35	(n=26)	116 ± 10	(n=36)
	temperature	[°C]	23.5 ± 1.2	(n=29)	24.7 ± 0.3	(n=26)	25.2 ± 0.2	(n=36)
	oxygen	[mg L^{-1}]	8.9 ± 0.5	(n=29)	8.7 ± 0.0	(n=26)	8.4 ± 0.0	(n=36)
	oxygen	[%]	109 ± 7	(n=29)	107 ± 1	(n=26)	103 ± 0	(n=36)
	ORP	[mV]	-	-	-	-	-	-
inlet	pH		7.3 ± 0.1	(n=29)	7.5 ± 0.1	(n=26)	7.5 ± 0.1	(n=36)
	ANC	[mmol HCl L^{-1}]	2.6 ± 0.3	(n=8)	2.9 ± 0.3	(n=10)	2.4 ± 0.5	(n=13)
	TAN	[mg L^{-1}]	0.7 ± 0.6	(n=9)	0.2 ± 0.1	(n=10)	0.3 ± 0.1	(n=12)
	NO_2^--N	[mg L^{-1}]	0.4 ± 0.2	(n=9)	0.1 ± 0.0	(n=10)	0.1 ± 0.0	(n=12)
	NO_3^--N	[mg L^{-1}]	42 ± 8	(n=9)	33 ± 7	(n=10)	49 ± 8	(n=11)
	temperature	[°C]	24.7 ± 0.8	(n=29)	24.8 ± 0.3	(n=26)	24.3 ± 0.3	(n=36)
	oxygen	[mg L^{-1}]	0.3 ± 0.2	(n=29)	0.2 ± 0.2	(n=26)	0.2 ± 0.1	(n=36)
	oxygen	[%]	4 ± 3	(n=29)	3 ± 2	(n=26)	3 ± 1	(n=36)
	ORP	[mV]	-126 ± 44	(n=28)	-145 ± 96	(n=26)	-125 ± 45	(n=36)
outlet	pH		7.9 ± 0.2	(n=29)	7.6 ± 0.0	(n=26)	7.9 ± 0.1	(n=35)
	ANC	[mmol HCl L^{-1}]	4.9 ± 0.8	(n=8)	5.0 ± 0.8	(n=10)	5.6 ± 0.7	(n=13)
	TAN	[mg L^{-1}]	0.2 ± 0.1	(n=9)	0.3 ± 0.4	(n=10)	0.1 ± 0.1	(n=12)
	NO_2^--N	[mg L^{-1}]	3.9 ± 2.8	(n=9)	0.8 ± 0.8	(n=10)	1.7 ± 1.2	(n=12)
	NO_3^--N	[mg L^{-1}]	15 ± 5	(n=9)	12 ± 6	(n=10)	10 ± 10	(n=9)

SID-Reactor, **S**elf-cleaning **I**nherent gas **D**enitrification Reactor; ORP, oxidation-reduction potential; ANC, acid neutralization capacity; TAN, total ammonia nitrogen; NO_2^--N, nitrite; NO_3^--N, nitrate; -, no data available.

3.1.3 Denitrification performance

With 81% the overall NO_3^--N reduction efficiency (*Table 2 - 2*) was highest at HRT-6, whereas both other HRT settings showed a NO_3^--N reduction efficiency of approximately 64%. Taking into account the average NO_3^--N reduction rate, rates of 497 g NO_3^--N d^{-1} m^3 biocarriers were measured for HRT-2 and minor denitrification rates of 198 and 253 g NO_3^--N d^{-1} m^3 biocarriers were measured for HRT-4 and HRT-6, respectively.

Similar, the overall TN reduction efficiency (*Table 2 - 2*) was highest for HRT-6 (78%). The TN reduction for HRT-4 (61%) was lower, and HRT-2 had the lowest values (56%). The TN reduction rate was highest with 443 g N d^{-1} m^3 biocarriers again for HRT-2, while the reduction rates for HRT-4 and HRT-6 were 190 and 245 g N d^{-1} m^3 biocarriers respectively.

Table 2 - 2. *Mean values (± SD) of denitrification efficiency and rate depending on hydraulic retention times (HRT) of 2, 4 and 6 hours.*

hydraulic retention time (HRT)		[h]	2	4	6
nominal hydraulic load		[L h^{-1}]	375	188	125
trial days		[d]	29	26	36
NO_3^--N reduction	efficiency	[%]	64 ± 13	64 ± 39	81 ± 15
	rate	[mg L^{-1} h^{-1}]	27 ± 9	21 ± 9	41 ± 8
		[mg L^{-1} h^{-1} m^3 biocarriers]	59 ± 20	47 ± 19	90 ± 18
		[g d^{-1} m^3 biocarriers]	497 ± 176	198 ± 83	253 ± 56
TN reduction	efficiency	[%]	56 ± 14	61 ± 18	78 ± 17
	rate	[mg L^{-1} h^{-1}]	24 ± 9	20 ± 8	39 ± 9
		[mg L^{-1} h^{-1} m^3 biocarriers]	53 ± 19	45 ± 18	87 ± 21
		[g d^{-1} m^3 biocarriers]	443 ± 178	190 ± 81	245 ± 61

Number of data points (n) per treatment: HRT of 2: n=9; HRT of 4: n=10; HRT of 6: n=9.
NO_3^--N, nitrate; TN, total nitrogen.

Table 2 - 3. *Mean values (± SD) of water quality parameters for the SID-Reactor inlet and outlet depending on backflushing interval (BFI) of every 10, 30, 60, and 90 minutes.*

backflushing intervals (BFI)		[every x minutes]	10		30		60		90	
hydraulic retention time (HRT)		[h]	2		2		2		2	
nominal hydraulic load		[L h^{-1}]	375		375		375		375	
trial days		[d]	6		19		21		21	
actual hydraulic load		[L h^{-1}]	347 ± 26	(n=6)	365 ± 25	(n=10)	336 ± 12	(n=9)	343 ± 20	(n=13)
inlet	temperature	[°C]	24.9 ± 0.2	(n=6)	25.0 ± 0.2	(n=10)	25.3 ± 0.1	(n=9)	25.2 ± 0.4	(n=13)
	oxygen	[mg L^{-1}]	8.8 ± 0.1	(n=6)	9.0 ± 0.1	(n=10)	8.7 ± 0.0	(n=9)	8.6 ± 0.1	(n=13)
	oxygen	[%]	108 ± 1	(n=6)	111 ± 1	(n=10)	108 ± 0	(n=9)	107 ± 1	(n=13)
	ORP	[mV]	-	-	-	-	-	-	-	-
	pH		7.3 ± 0.15	(n=6)	7.3 ± 0.1	(n=10)	7.3 ± 0.0	(n=9)	7.3 ± 0.1	(n=13)
	ANC	[mmol HCl L^{-1}]	3.7 ± 0.2	(n=3)	3.0 ± 0.3	(n=5)	3.8 ± 0.4	(n=4)	3.1 ± 0.3	(n=6)
	TAN	[mg L^{-1}]	0.5 ± 0.1	(n=2)	0.4 ± 0.2	(n=5)	0.4 ± 0.1	(n=4)	0.3 ± 0.1	(n=6)
	NO_2^--N	[mg L^{-1}]	0.5 ± 0.0	(n=2)	0.2 ± 0.1	(n=5)	0.2 ± 0.0	(n=4)	0.1 ± 0.1	(n=6)
	NO_3^--N	[mg L^{-1}]	51 ± 6	(n=2)	26 ± 4	(n=5)	49 ± 2	(n=4)	47 ± 3	(n=6)
	turbidity	[NTU]	0.6 ± 0.6	(n=4)	0.1 ± 0.3	(n=8)	1.2 ± 0.9	(n=6)	0.5 ± 0.5	(n=8)
outlet	temperature	[°C]	25.4 ± 0.3	(n=6)	25.6 ± 0.2	(n=10)	25.7 ± 0.1	(n=9)	25.5 ± 0.4	(n=13)
	oxygen	[mg L^{-1}]	0.4 ± 0.1	(n=6)	0.1 ± 0.0	(n=10)	0.2 ± 0.0	(n=9)	0.2 ± 0.0	(n=13)
	oxygen	[%]	5 ± 1	(n=6)	2 ± 0	(n=10)	2 ± 0	(n=9)	3 ± 0	(n=13)
	ORP	[mV]	-67 ± 12	(n=6)	-217 ± 51	(n=10)	-152 ± 15	(n=9)	-194 ± 14	(n=13)
	pH		7.5 ± 0.1	(n=6)	7.9 ± 0.2	(n=10)	8.1 ± 0.1	(n=9)	8.2 ± 0.1	(n=13)
	ANC	[mmol HCl L^{-1}]	5.8 ± 0.3	(n=3)	4.6 ± 0.2	(n=5)	7.1 ± 0.2	(n=4)	6.3 ± 0.4	(n=6)
	TAN	[mg L^{-1}]	0.2 ± 0.1	(n=2)	0.5 ± 0.7	(n=5)	0.7 ± 0.2	(n=4)	0.8 ± 0.3	(n=6)
	NO_2^--N	[mg L^{-1}]	16.4 ± 0.1	(n=2)	1.6 ± 0.8	(n=5)	3.1 ± 1.3	(n=4)	4.3 ± 2.8	(n=6)
	NO_3^--N	[mg L^{-1}]	36 ± 6	(n=2)	6 ± 2	(n=5)	8 ± 2	(n=4)	12 ± 5	(n=6)
	turbidity	[NTU]	7.4 ± 5.3	(n=4)	2.8 ± 1.0	(n=8)	2.0 ± 0.9	(n=6)	1.5 ± 1.3	(n=9)

SID-Reactor, **S**elf-cleaning **I**nherent gas **D**enitrification Reactor; ORP, oxidation-reduction potential; ANC, acid neutralization capacity; TAN, total ammonia nitrogen; NO_2^--N, nitrite; NO_3^--N, nitrate; -, no data available.

3.2 Backflushing interval (BFI) influences water quality

3.2.1 SID-Reactor inlet water parameters

For all backflushing intervals (BFI-10, BFI-30, BFI-60 and BFI-90) the inlet water quality parameters were comparable to each other (*Table 2 - 3*). No differences could be observed for temperature (ranging at 25°C) and oxygen saturation (107 to 111%). Furthermore, pH (ranging at 7.3) and ANC (3.0 to 3.8 mmol HCl L^{-1}) remained unaffected through the course of the experimental trial. The highest TAN was measured for BFI-10 (0.5 mg L^{-1}), while TAN was slightly lower (0.3 to 0.4 mg L^{-1}) for the other three intervals. Similar, NO_2^--N was slightly elevated (0.5 mg L^{-1}) in BCI-10 compared to the other intervals (0.1 to 0.2 mg L^{-1}). For the NO_3^--N concentration, average values of 51, 26, 49 and 47 mg L^{-1} were measured for BFI-10, BFI-30, BFI-60, and BFI-90, respectively. Turbidity was low for all four intervals (0.1 to 1.2 NTU).

3.2.2 SID-Reactor outlet water parameters

Outlet water quality parameters of the SID-Reactor (*Table 2 - 3*) revealed several differences between the different BFIs. Oxygen saturation was highest for BFI-10 (5%) compared to the other three intervals (<3%). For all four intervals, the oxygen saturation was lower in the outlet water compared to the inlet water (>100%). ORP ranged from -152 to -217 mV for BFI-30, BFI-60 and BFI-90, whereas ORP for BFI-10 was higher with -67 mV. However, for all BFIs ORP was negative in the outlet water. For all four intervals, pH increased throughout the reactor passage, showing lowest values for BFI-10 (pH 7.5) and highest for BFI 90 (pH 8.2). ANC (4.6 to 7.1 mmol HCl L^{-1}) was likewise higher for all BFIs compared to the inlet water. TAN was low for all BFIs, showing the lowest values for BFI-10 (0.2 mg L^{-1}) and highest for BFI-90 (0.8 mg L^{-1}). For all four BFIs the NO_2^--N concentrations in the outlet water were higher compared to inlet water. The highest average NO_2^--N values of 16.4 mg L^{-1} were measured for BFI-10, whereas for BFI-30, BFI-60, and BFI-90 low NO_2^--N values of 1.6, 3.1, and 4.3 mg L^{-1} were measured, respectively. Turbidity of the outlet water was highest at BFI-10 (7.4 NTU) followed by BFI-30 (2.8 NTU), BFI-60 (2.0 NTU), and BFI-90 (1.5 NTU).

3.2.3 Denitrification performance

The overall NO_3^--N reduction efficiency (*Table 2 - 4*) was highest for BFI-30 and BFI-60 (78 and 84%, respectively), followed by BFI-90 (75%), while BFI-10 had the lowest observed NO_3^--N reduction efficiency (29%). The overall NO_3^--N reduction rate was highest for BFI-60 and BFI-90 (733 and 629 g d^{-1} m^3 biocarriers, respectively). BFI-30 showed a reduced NO_3^--N reduction rate (390 g d^{-1} m^3 biocarriers) and BFI-10 had the lowest reduction rate (280 g d^{-1} m^3 biocarriers). With regard to the TN reduction, BFI-10 showed negative values for denitrification efficiency (-2%) and denitrification rate (-15 g d^{-1} m^3 biocarriers), consequently indicating an increase of TN in the outlet water compared to the inlet water. Apart from this, BFI-30 and BFI-60 resulted in the highest TN reduction efficiency (71 and 76%), followed by BFI-90 (65%). The TN reduction rate was highest for BFI-60 and BFI-90 (676 and 547 g d^{-1} m^3 biocarriers), and lowest for BFI-30 (362 g d^{-1} m^3 biocarriers).

Table 2 - 4. *Mean values (± SD) of denitrification efficiency and rate depending on backflushing interval (BFI) of every 10, 30, 60, and 90 minutes.*

backflush Interval (BFI)		[every x minutes]	10	30	60	90
hydraulic retention time (HRT)		[h]	2	2	2	2
nominal hydraulic load		[L h^{-1}]	375	375	375	375
trial days		[d]	6	19	21	20
NO_3^--N reduction	efficiency	[%]	29 ± 3	78 ± 5	84 ± 5	75 ± 11
	rate	[mg L^{-1} h^{-1}]	15 ± 0	21 ± 3	41 ± 4	35 ± 5
		[mg L^{-1} h^{-1} m^3 biocarriers]	33 ± 0	46 ± 6	91 ± 8	78 ± 12
		[g d^{-1} m^3 biocarriers]	280 ± 32	390 ± 68	733 ± 85	629 ± 117
TN reduction	efficiency	[%]	-2 ± 0	71 ± 7	76 ± 8	65 ± 17
	rate	[mg L^{-1} h^{-1}]	-1 ± 0	19 ± 3	38 ± 5	30 ± 8
		[mg L^{-1} h^{-1} m^3 biocarriers]	-2 ± 0	42 ± 8	84 ± 11	67 ± 18
		[g d^{-1} m^3 biocarriers]	-15 ± 4	362 ± 64	676 ± 105	547 ± 155

Number of data points (n) per treatment: BFI of 10: n=2; BFI of 30: n=5; BFI of 60: n=4; BFI of 90: n=6
NO_3^--N, nitrate; TN, total nitrogen

3.2.4 Particle characteristics of the outlet water

Apart from the influence of the BFI on water quality parameters, it was observed that particle characteristics (not examined in closer detail) in the outlet were influenced by the BFI. The longer the BFI, the bigger and denser the particles released directly after backflushing (*Figure 2 - 4*). Particles for BFI-10 are not shown, since no clear denitrification was observed. BFI-30 lead to particles of a few millimetres, and a more homogeneous particle distribution in the outlet water. BFI-60 and BFI-90 resulted in an increased particle size with an upper particle size range of centimetres. For BFI-90 clogging of the SID-Reactor's drain tube occurred several times throughout the day and consequently resulted in higher maintenance work (not further quantified).

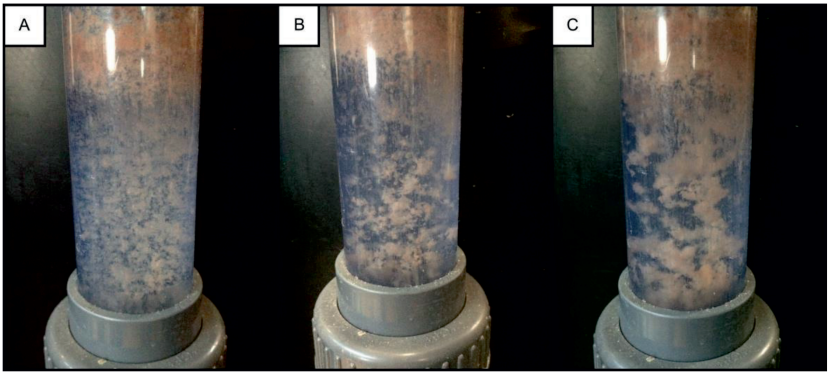

Figure 2 - 4. *Particle characteristics in the SID-Reactor outlet. Small, medium and large particles are formed for backflushing intervals every 30 (A), 60 (B), and 90 (C) minutes, respectively.*

3.3 C/N ratio influences water quality

The breakpoints of the segmented linear regressions are shown in *Figure 2 - 5* and are summarized in *Table 2 - 5*. A 95% confidence belt and a 95% confidence block of the break point are shown. The break point for oxygen saturation was determined at a C/N ratio of 2.1 (*Figure 2 - 5A*) and an oxygen saturation of <4% (<0.3 mg L^{-1}). Reducing the C/N ratio resulted in higher oxygen saturations of up to 8% (0.5 mg L^{-1}). For ORP, a break point at a C/N ratio of 1.7 (*Figure 2 - 5B*) was calculated (approximately -150 mV). Lowering the C/N ratio further increased the ORP to -75 mV, whereas during increased C/N ratio the ORP remained at approximately -150 mV. Taking into account TAN and NO_2^--N concentrations of the outlet of the SID-Reactor,

an optimum break point was determined at a C/N ratio of 2.3 (*Figure 2 - 5C&D*) with concentrations of 0.1 mg L^{-1} TAN and 2.5 mg L^{-1} NO_2^--N. At a C/N ratio below 2.3, TAN and NO_2^--N increased up to 1.0 and 14 mg L^{-1}, respectively. In contrast, TAN and NO_2^--N outlet concentrations were approximately 0.1 and 2.5 mg L^{-1}, respectively, for C/N ratios above 2.3. The break point for NO_3^--N was present at a C/N ratio of 2.1 (*Figure 2 - 5E*) and a nitrate concentration of 8 mg L^{-1} in the reactor outlet. Increasing the C/N ratio resulted in stable NO_3^--N concentrations in the outlet of the SID-Reactor of 8 mg L^{-1}. Reducing the C/N ratio resulted in increased average NO_3^--N concentrations of up to 35 mg L^{-1} in the outlet. Considering the TN reduction efficiency, the break point was determined for a ratio of 2.2 with an average TN reduction of 75% (*Figure 2 - 5F*). Reducing the C/N ratio resulted in a reduced TN reduction efficiency of up to -16% (equal to a nitrogen increase), whereas an increase of the C/N ratio resulted in a stable TN reduction of approximately 75%.

Table 2 - 5. *C/N ratio at break point for the dependent variables oxygen saturation, ORP, TAN, NO_2^--N, NO_3^--N, and TN reduction, respectively.*

dependent variable	C/N ratio at break point
oxygen [%]	2.1
ORP [mV]	1.7
TAN [mg L^{-1}]	2.3
NO_2^--N [mg L^{-1}]	2.3
NO_3^--N [mg L^{-1}]	2.1
TN reduction [%]	2.2

C/N ratio, carbon to nitrogen ratio; ORP, oxidation-reduction potential; TAN, total ammonia nitrogen; NO_2^--N, nitrite; NO_3^--N, nitrate; TN, total nitrogen.

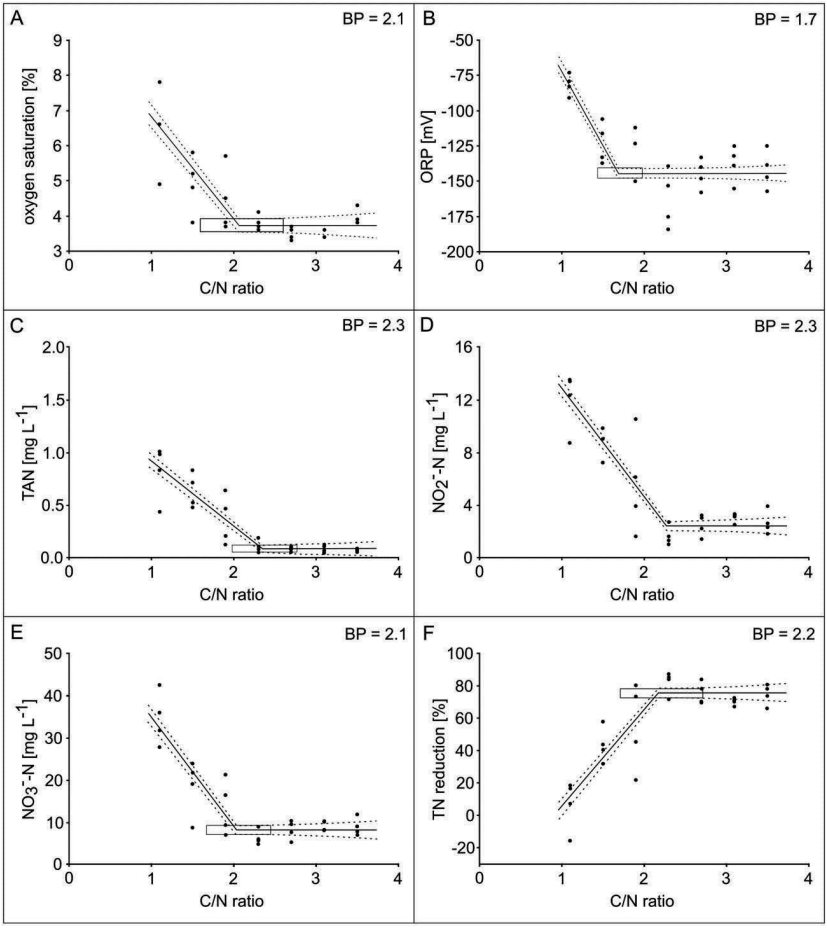

Figure 2 - 5. *Segmented linear regressions with one dependent variable and the independent variable C/N ratio [mg mg^{-1}]. A, Oxygen saturation [%]; B, ORP [mV]; C, TAN [mg L^{-1}]; D, NO_2^--N [mg L^{-1}]; E, NO_3^--N [mg L^{-1}], and F, TN reduction [%]. For the tested variables a 95% confidence belt and a 95% confidence block of the break point (BP) is shown. For each variable, each C/N ratio has 4 data points.*

4 Discussion

4.1 Hydraulic Retention Time (HRT)

Hydraulic retention time is one of the most important operational parameters influencing biofilters, both nitrification (Eding et al., 2006; Summerfelt, 2006) and denitrification filter systems (Addy et al., 2016; Christianson et al., 2015; Christianson and Schipper, 2016; Chu and Wang, 2011; Guo et al., 2017; Healy et al., 2015; Lampe and Zhang, 1996; Lepine et al., 2016; Oh et al., 2001; Plósz et al., 2003; Suhr et al., 2013; Timmermans and van Haute, 1983; van Rijn and Rivera, 1990; Wang and Chu, 2016). Several studies showed a high impact of HRT on diverse parameters of biofilter systems, such as TAN removal efficiency (Summerfelt, 2006), sulfate production (Christianson et al., 2015; Oh et al., 2001), alkalinity production (Lepine et al., 2016), and the effect of C/N ratio on denitrification processes (Suhr et al., 2013), amongst others. The effects of HRT on denitrification performance are unavoidable. A general recommendation for an optimal HRT range cannot be given. The HRT range is highly dependent on the denitrification principles (autotroph / heterotroph / mixotroph), the reactor type and size, temperature, and others. The influence of HRT on denitrification processes should be evaluated for every denitrification type separately. This was done in the first part of the experiment for the SID-Reactor.

As most denitrifying organisms are facultative anaerobe heterotrophs they have the ability to use nitrate as electron acceptor instead of oxygen. In the presence of organic electron donors, and as long as oxygen is sufficiently available, bacteria will use molecular oxygen as electron acceptor instead of nitrate. Under aerobe conditions, oxygen is energetically favoured over nitrate by these bacteria (Hamlin et al., 2008; Plósz et al., 2003; van Rijn et al., 2006). Under anoxic conditions, nitrate is metabolically favoured as electron acceptor, thus reducing each nitrate ion to one nitrite ion. Hence, oxygen is an important kinetic inhibitor of denitrification processes and possibly results in the production of intermediate products such as NH_4^+ and NO_2^- (Guo et al., 2017; Hamlin et al., 2008; Oh and Silverstein, 1999; Plósz et al., 2003; Yang et al., 2012). To guarantee stable denitrification processes, it is essential to achieve anoxic conditions within a denitrification unit. The SID-Reactor is designed to allow oxygen to solely enter via the influent. At a low HRT more oxygen is transported into the denitrification unit, compared to a higher HRT. Consequently, a low HRT could negatively affect denitrification. Within the HRT trial, oxygen concentration in the outflow of the SID-Reactor reached values of ≤ 0.3 mg L^{-1} for all three HRT settings. Therefore, it can be concluded that the wide spectrum of heterotrophic bacteria was

capable to reduce the influx of molecular oxygen of the inlet water, causing anoxic conditions in the SID-Reactor. However, within the first part of the experiment, the denitrification efficiency was decreased for low HRT-2 compared to higher HRT-6, thus indicating the negative effect of low HRT on denitrification efficiency.

In anoxic conditions, a negative ORP is concomitant with efficient denitrification. Lee et al. (2000) observed that nitrate is converted to nitrite when ORP drops below 0 mV. Hereby, nitrite accumulated through the range of 0 to -225 mV. From -225 to -400 mV nitrite was converted to N_2. However, optimum ORP value for denitrification seems to be dependent on the carbon source and the inlet water used (Drtil et al., 1999; Müller-Belecke et al., 2013). When ethanol, methanol and glycerine were used, the ORP sets in at below -120 mV. When acetic acid was used instead, ORP sets in at -70 mV (Müller-Belecke et al., 2013) at a similar NO_3^--N reduction. In a different study, Hamlin et al. (2008) also used methanol as a carbon source and the ORP value was between -20 to -150 mV. In the present trial part, the average ORP values for the tested HRTs were within the recommendations for denitrification and ranged between -125 and -145 mV. The ORP values were not actively regulated, but a result of the SID-Reactor in combination with the experimental design.

In RAS, the nitrification in aerobe biofilters leads to reduced pH and a loss of alkalinity in the rearing water. To counteract this, alkalinity supplements such as sodium hydrogen carbonate ($NaHCO_3$) are commonly used. During denitrification processes, pH and ANC values tend to increase. This results in reduced use of alkalinity supplements (van Rijn et al., 2006). Within the present study, no differences of pH and ANC increase were measured between the tested HRTs of 2, 4 and 6 hours.

As a first conclusion, all three tested HRTs resulted in low oxygen saturations and a negative ORP in the outlet water of the reactor after adding methanol as a carbon source, enabling denitrification processes. Finally, lowered NO_3^--N values, increased pH and alkalinity in the outlet water, compared to the inlet, were proved for all three tested HRTs. Hence, all three HRTs can be recommended for successful denitrification processes in a SID-Reactor. The HRT-2 and HRT-6 revealed lower TAN and only slightly elevated NO_2^--N values in the outlet compared to the inlet water. Slightly higher TAN values in the outlet compared to the inlet were measured for HRT-4. No coherence to HRT or other parameters can explain this finding. A possible explanation could be a side effect, as a result of degradation of organic matter, occurring simultaneously in the anoxic environment. This could result in the observed increase of TAN concentration in HRT-4 (Guo et al., 2017).

In general, elevated NO_2^--N values are common in denitrification processes. They potentially indicate a limitation of carbon or an inadequate HRT, resulting in incomplete denitrification, and an associated increase in nitrite (Guo et al., 2017; Hamlin et al., 2008; van Rijn and Rivera, 1990). As a consequence, denitrification units used in bypass in RAS, are able to affect the overall nitrite load of the rearing water. In the present study, elevated nitrite values in the outlet water (*Table 2 - 1*) did not affect the rearing water. A slightly elevated nitrite concentration in the SID-Reactor outlet did not affect the rearing water, possibly because this compound was easily oxidized in the on-farm aerobe nitrifying filter. At no time during the operation time of the SID, other measured water parameters reached harmful concentrations for sea bass in the rearing water. Thus, the three tested HRTs were suitable for the SID-Reactor operation.

Since the tested HRTs resulted in predominantly equal water quality parameters, distinct differences between HRT-2, HRT-4, and HRT-6 hours were evaluated in terms of denitrification efficiency and denitrification rate (*Table 2 - 2*). A relatively long HRT of 6 hours resulted in highest relative nitrate reduction in percent (denitrification efficiency). On the contrary, a long HRT of 6 hours resulted in the lowest absolute nitrate reduction in g d^{-1} m^3 biocarriers (denitrification rate). At a HRT of 2 hours the opposite was found (highest denitrification rate and lowest denitrification efficiency). As a consequence, an optimized HRT is not the same based on nitrate removal rate versus nitrate removal efficiency. Thus, an optimized HRT depends mainly on the pursued objective.

As a result of the HRT trial the optimal operation of the SID-Reactor with regard to HRT reveals two feasible options. Targeting to reduce as much NO_3^--N as possible in the whole system, low HRT of 2 hours (high hydraulic loads) should be favoured. Targeting lowest NO_3^--N values in the outlet water of the denitrification unit, high HRT of 6 hours (low hydraulic loads) should be favoured.

4.2 Backflushing Intervals (BFI)

A general problem for most biological filters caused by clogging is the channelization of water through narrow areas of the biofilter units, possibly leading to a completely blocked water flow (Paller and Lewis, 1982; Sastry et al., 1999). Shedding of biofilm caused by clogging can occur, causing high levels of fine suspended solids in the water column (Eding et al., 2006; Kamstra et al., 1998). With regard to aerobe biofilter systems, clogging may reduce the nitrification capacity (Eding et al., 2006; Michaud et al., 2006). For example, anaerobe decaying organic matter can cause an ammonia release, thus hampering the overall nitrification process (Rakocy et al., 2006). With regard to denitrifying biofilters, clogging may reduce the denitrification capacity. For example, Sauthier et al. (1998) reported increased nitrite production and eventually increased nitrite concentration in the outlet water in proportion to the amount of clogged biocarriers. Hence, the prevention of clogging of biofilter media is crucial to ensure a consistent and efficient denitrification performance. The self-cleaning characteristics of the SID-Reactor allow to adjust automated backwashing of biofilter media, resulting in reduced clogging, good denitrification performance, and low maintenance.

One result from the BFI trial was, that the BFI-10 had lower NO_3^--N and TN reduction compared to the other intervals (BFI-30, BFI-60, and BFI-90). Furthermore, an enormous increase of the outlet NO_2^--N concentration was noticeable, forcing a termination of the BFI-10 setting after 6 trial days. The observations made during the BFI-10 setting can be explained by the following facts. On the one hand, physical stress on bacteria could be the cause for decreased denitrification rates after backflushing. On the other hand, an increased oxygen influx can change anaerobic conditions of a denitrification device to aerobic conditions, thus affecting the metabolism of bacteria. Physical stress on bacteria generally results in removal of biofilm by fluid shear or abrasion of biomass by the collision of biocarriers (Camargo et al., 2005; Chaudhary et al., 2003; Hozalski and Bouwer, 1998). If the BFI is too frequent, physical stress possibly could have resulted in an excessive removal of bacterial mass, maybe even greater than bacterial growth, thus reducing the total number of bacteria. The elevated turbidity in the SID-Reactor outlet (*Table 2 - 3*) at BFI-10 indicates the mentioned increased removal of bacterial mass. As a consequence of the decreased bacterial population, the oxygen content and subsequently the ORP increased in the reactor chamber. This might have resulted in the observed formation of nitrite (Guo et al., 2017; Oh and Silverstein, 1999; Plósz et al., 2003; Yang et al., 2012). This eventually resulted in lower denitrification efficiency and rates, as it was observed for BFI-10 (*Table 2 - 4*). Furthermore, small amounts of

oxygen can possibly enter the reactor during pressure compensation at times when the side channel vacuum pump runs in the course of backflushing. If the backflushing is too frequent, the amount of oxygen entering the SID-Reactor might increase. As a result, the rearing water in the denitrification unit is enriched with oxygen and consequently a higher ORP is present. This may have led to the production of denitrification intermediates (e.g. nitrite) and could explain the decreased denitrification performance of the SID-Reactor.

Low denitrification efficiency of 29% NO_3^--N reduction (*Table 2 - 4*) and a four times higher NO_2^--N concentration (16.4 mg L^{-1}, *Table 2 - 3*) was observed for BFI-10 compared to the other BFI settings. The increase in TN (negative TN reduction, *Table 2 - 4*) for the BFI-10 setting, which can possibly explained by decay processes, is even more severe. Due to the lowered denitrification performance the positive effect of the denitrification on pH (van Rijn et al., 2006) is also hampered. This can be concluded from the lowered pH for BFI-10 (*Table 2 - 3*) in comparison to the other treatments. Hence, backflushing the biofilter media every 10 minutes is a too frequent interval.

In contrast to results for BFI-10, backflushing every 30, 60, and 90 minutes resulted in appropriate low oxygen of ≤0.2 mg L^{-1} and low ORP of <-150 mV (*Table 2 - 3*). Furthermore, nearly no intermediate nitrite production was observed for BFI-30, BFI-60, and BFI-90, indicating a good denitrification performance. Hence, denitrification efficiency of ≥75% NO_3^--N and ≥65% TN reduction were measured for BFI-30, BFI-60, and BFI-90 (*Table 2 - 4*), at a similar range. However, the denitrification rate for BFI-30 treatment revealed a lowered NO_3^--N reduction rate of 390 g d^{-1} m^3 biocarriers compared to BFI-60 and BFI-90 treatments with 733 and 629 g d^{-1} m^3 biocarriers, respectively (*Table 2 - 4*). Attributed to the lower initial NO_3^--N concentration in the inlet for BFI-30 (*Table 2 - 3*) a direct comparison with the other BFI settings is hampered. Ideally, equal initial NO_3^--N concentrations in the inlet water for all BFI settings would have resulted in more homogeneous denitrification rates, since the denitrification efficiency in percent is comparable between the treatments (*Table 2 - 4*).

The problem of clogging is described in numerous studies as well as the need of a filter design preventing clogging and/or a method for removing dead biofilm with the aim of low maintenance effort (Alonso et al., 1997; Chu and Wang, 2011; Eding et al., 2006; Hozalski and Bouwer, 1998; McMillan et al., 2003; Moretti et al., 1999b; Müller-Belecke et al., 2013; Paller and Lewis, 1982; Rakocy et al., 2006; Sastry et al., 1999; Wang and Chu, 2016). It was noticeable within this present study, that by increasing the BFI, bigger and denser particles were released from the SID-Reactor (*Table 2 - 4*). Bigger

particles led to increased clogging of the outlet tube, increasing the overall maintenance effort. A BFI every 90 minutes resulted in clogging of the biofilter bed as well as outlet tubing. For the evaluation of the backflushing intervals, the change in particle characteristics should not be underestimated and considered in future studies.

As a conclusion from the BFI trial, it can be stated that clogging of the SID-Reactor can effectively be prevented by regular backflushing of the biofilter media. However, a frequent backflushing (every 10 minutes) resulted in a collapse of denitrification possibly conditioned by shear force and increased oxygen content. In contrast, a rare backflushing (every 90 minutes) favours clogging and increased the maintenance effort. Thus, a BFI of every 30 to 60 minutes can be recommended for the SID-Reactor resulting in both, good denitrification efficiency and denitrification rates. A solution allowing an adequate backflushing technique and frequency should be taken into consideration throughout the process of designing of biofilter systems (aerobe and anaerobe).

4.3 C/N ratio

The performance of heterotroph denitrification reactors depends on organic carbon sources as an electron donor. Insufficient endogenous organic carbon supply may limit the application of in situ heterotrophic denitrification. The addition of external carbon sources is essential in particular in RAS aquaculture (Lampe and Zhang, 1996). The correct choice of carbon source is a consistent subject of several aquaculture related studies. The most commonly used carbon sources are acetate (acetic acid), ethanol, glucose, glycerol, methanol, and molasses (Bregnballe, 2015; Clifford and Liu, 1993; Hamlin et al., 2008; Klas et al., 2006; Lampe and Zhang, 1996; Lekang, 2013; Müller-Belecke et al., 2013; Oh et al., 2001; Sauthier et al., 1998; Timmermans and van Haute, 1983; van Rijn et al., 2006, 1996; van Rijn and Rivera, 1990; Yang et al., 2012). However, methanol is often preferred because it is relatively inexpensive and it produces less sludge than other carbon sources (Hamlin et al., 2008). Methanol was also chosen as the carbon source in the present study even though it remains objectionable in terms of toxicity (Kaviraj et al., 2004), especially at over dosage. During an over dosage non-metabolized methanol could pollute the rearing water and potentially harm the cultivated fish. Despite this, non-metabolized methanol would also increase the operating costs of a RAS. Therefore, it is highly important to avoid an inadequate methanol dosage when operating a denitrification device.

According to stoichiometry, the methanol demand to fuel denitrification is 1.9 g of methanol for each 1.0 g of nitrate-N, which is equal to a C/N ratio of 1.9 (Cheremisinoff, 1995). Since nitrogen and carbon are also used by denitrifying bacteria for cell synthesis, Kadlec and Wallace (2009) suggest an additional demand of 0.57 g methanol, summing up to 2.47 g methanol for 1.0 g nitrate-N. Timmermans and van Haute (1983) report a C/N ratio of 2.55 while Clifford and Liu (1993) state an optimum C/N ratio of 2.7 resulting in >95% denitrification. Higher demand of 2.9 g methanol for 1.0 g nitrate-N have been reported by Theis and Hicks (2012).

From the results obtained in this study, it is obvious that relating a C/N ratio solely to one single water parameter is misleading (*Table 2 - 5*). Hence, it is necessary to rank the water parameters (depending on C/N ratio) according to their importance. The break point analysis for oxygen saturation and ORP revealed a C/N ratio of 2.1 and 1.7, respectively, to achieve lowest oxygen saturation and ORP (*Figure 2 - 5A&B*). A C/N ratio of 2.1 would also match with the break point for low NO_3^--N concentration in the outlet of the SID-Reactor (*Figure 2 - 5E*). However, a C/N ratio of 2.1 or 1.7 would result in the production of intermediate products, since the optimum C/N ratio for TAN and NO_2^--N is 2.3 (*Figure 2 - 5C&D*). Incomplete denitrification is associated with an increase of intermediate TAN and NO_2^--N production (Guo et al., 2017; Hamlin et al., 2008; Oh and Silverstein, 1999; Plósz et al., 2003; van Rijn and Rivera, 1990; Yang et al., 2012). At high concentrations, both intermediate products have the potential to harm the cultivated fish species. Therefore, a C/N ratio keeping all of the critical parameters in optimum ranges should be chosen.

In conclusion from the C/N trial, it can be stated that in order to determine a sufficient C/N ratio for denitrification processes it is necessary to take all relevant and denitrification dependent water quality parameters into account. The optimum carbon dosage should be determined under the guiding principle: as much as necessary, as little as possible. With a C/N ratio of 2.3 the oxygen saturation and ORP would be kept as low as possible and at the same time TAN, NO_2^--N and NO_3^--N concentrations in the outlet would show the lowest values. Additionally a C/N ratio of 2.3 would also be sufficient to result in an overall TN reduction as high as 75% (*Figure 2 - 5F*).

5 Conclusion

The determination of basal operating parameters and a deeper understanding of their effects on denitrification are necessary for one of the newest generation of denitrification devices in order to allow stable and efficient nitrate removal in RAS. The present work contributes to a better understanding of the effects of varying HRT, BFI, and C/N ratios on denitrification processes and water quality when using a SID-Reactor. The main results of this study can be summarized as:

- A HRT of 6 hours resulted in a denitrification efficiency of up to 81% nitrate reduction, but an overall denitrification rate of 253 g d^{-1} m^3 biocarriers. A HRT of 2 hours resulted in a reduced denitrification efficiency of 64%, but an overall denitrification rate of 497 g d^{-1} m^3 biocarriers.
- A BFI of 10 minutes resulted in an increase of oxygen in the denitrification unit and an inhibition of denitrification processes. Furthermore, the increased physical stress resulted in an excessive removal of bacteria, and concomitantly increased turbidity. Both, increased oxygen content and removal of bacteria, resulted in reduced denitrification processes that might even show signs of total breakdown. In contrast, a BFI of 90 minutes favoured clogging of biofilter media resulting in elevated maintenance effort. Hence, a BFI of 30 to 60 minutes is recommended when running a SID-Reactor.
- A C/N ratio of 2.3 is sufficient to keep all relevant water quality parameters for denitrification processes in optimum ranges with low production of intermediates. In general, one parameter solitarily gives a misleading recommendation and might result in non-optimal dosage of carbon source (C/N ratio).

The results of this study allow an easy, efficient and safe application of a SID-Reactor with the purpose of nitrate removal in RAS.

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CHAPTER 3

Replacement of methanol by biodegradable polyhydroxyalkanoate (PHA) plastics in the SID-Reactor for an efficient and safe use in RAS

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Abstract

An efficient and safe nitrate elimination by biological denitrification is still one of the most promising but challenging tasks in the development of recirculating aquaculture systems (RAS). Solid-phase denitrification has proved to be a promising technique to remove nitrate from water and hence gained interest over the last years.

The aim of this study was to evaluate the replacement of methanol as a liquid carbon source by biodegradable plastic granulate made of polyhydroxyalkanoate (PHA). In this study the PHA granulate was used as biofilm carrier and as solid carbon source in the novel **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor). An experiment was conducted over 110 days in three 1 m^3 RAS. Each RAS was stocked with pikeperch (*Sander lucioperca*) at a density of 24 kg m^{-3}. The first RAS was operated as control without a denitrification system. The second RAS was operated with a SID-Reactor fuelled with methanol as external carbon source and the third RAS was operated with a SID-Reactor fuelled with PHA granulate functioning as biofilm carrier and carbon source at the same time. Using PHA as a solid carbon source in the SID-Reactor resulted in a maximum denitrification efficiency of 40% nitrate removal, achieved after 6 days. Using methanol as a carbon source resulted in a maximum denitrification efficiency of up to 98% nitrate removal, achieved after 21 days. No significant differences were observed between the denitrification rate for the PHA (1245 g d^{-1} m^3 substrate) and the methanol (1042 g d^{-1} m^3 substrate) fuelled SID-Reactors. Values for turbidity, total ammonia, nitrite, and total organic carbon were lower in the rearing water, without any extreme values, using PHA compared to using methanol. Furthermore, both denitrification units caused an increase in alkalinity and pH, resulting in an overall 50% saving of alkalinity supplements in contrast to RAS without denitrification. The results demonstrate that the functional principle of the SID-Reactor, usually fuelled with a liquid carbon source, is also suitable for the application with a solid carbon source.

Key words: RAS; process water treatment; ammonia; nitrite; nitrate; *Sander lucioperca*; PHA; methanol; biodegradable plastics; filter clogging; self-cleaning inherent gas denitrification reactor; solid-phase denitrification

1 Introduction

The accumulation of metabolic end products of fish and bacteria gained an increasing importance in recirculating aquaculture systems (RAS). When water consumption is limited and an appropriate filter system is absent nitrate accumulates as a potentially toxic metabolic end product of bacterial nitrification in the rearing water of RAS (Davidson et al., 2017; Hrubec et al., 1996; Kincheloe et al., 1979; McGurk et al., 2006; Pierce et al., 1993; Schram et al., 2014, 2012; Scott and Crunkilton, 2000; Shimura et al., 2004, 2002; Torno et al., 2018; van Bussel et al., 2012; Westin, 1974). Recent studies demonstrated the negative impact of nitrate on cultivated fish species if maximum tolerance values are exceeded (Davidson et al., 2017; Schram et al., 2014, 2012; Torno et al., 2018; van Bussel et al., 2012). A possible way to minimize nitrate concentrations in RAS is an appropriate denitrification system. However, as reviewed by van Rijn et al. (2006) challenges for conventional denitrification systems remain, even though these systems are established since decades. Novel denitrification systems such as the **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor) contribute to easy, safe and efficient denitrification in RAS (Müller-Belecke et al., 2013). The performance of denitrifying bacteria depends on organic carbon sources as an electron donor. One of the biggest obstacles to efficient and safe denitrification is the accurate dosage of a mandatory carbon source. Furthermore, numerous carbon sources are potentially hazardous, highly flammable, and cause along security risks during storage, transportation and operation. Additionally, a complex control and monitoring of denitrification processes and water quality parameters is necessary to avoid negative impacts of the denitrification unit on RAS water quality. For instance, an insufficient dosage of the carbon source entails the risk of intermediate denitrification product formation such as ammonium/ammonia and nitrite. Whereas an overdosing can pollute RAS water with increased organic carbon loads. Hence, insufficient doses and overdosing can result in deterioration of the RAS water quality.

Solid-phase denitrification in aquaculture has been shown to be a promising alternative and has gained interest over the past years (Boley et al., 2000; Chu and Wang, 2011a; Gutierrez-Wing et al., 2012; P. Li et al., 2016; Shen et al., 2013b; Wang and Chu, 2016). In contrast to conventional denitrification systems, solid-phase denitrification is characterized by the use of solid water-insoluble carbon sources. It is potentially able to resolve dosage problems, provides a stable water quality, and potentially decreases the nitrate-related negative impact on fish (Boley et al., 2000; Gutierrez-Wing et al., 2012; Wang and Chu, 2016). During denitrification the biodegradable solid carbon source is available for bacteria only by decomposition and functions as biocarrier at the

same time. The amount of organic carbon decomposed by bacteria correlates with nitrate concentrations in the water column (Wang and Chu, 2016). Consequently, the control of the process is simple and the risks of inaccurate dosage are negligible (Boley et al., 2000; Chu and Wang, 2011a). Solid water-insoluble, and biodegradable substrates can be made from natural plant-like materials or synthetic polymers. As microbial carbon and energy storage materials polyhydroxyalkanoates (PHAs) are expected to be easily metabolized by a broad variety of microorganisms under denitrifying conditions. Hence, PHA is discussed as being the most suitable solid carbon substrates for denitrification in water treatment (Gutierrez-Wing et al., 2012; Hiraishi and Khan, 2003).

The aim of this study was to evaluate the replacement of methanol by biodegradable PHA plastics in the SID-Reactor. The effects on water quality and denitrification performance were compared between two SID-Reactors fuelled either with PHA or methanol. The replacement of methanol as a hazardous carbon source by a non-hazardous carbon source would increase safety not only for fish but also for RAS staff. Furthermore, the possibility of free selection of either a liquid carbon source or a solid carbon source would prove that the operating principle of the SID-Reactor is highly versatile.

2 Materials & Methods

2.1 Experimental recirculating aquaculture systems (RAS)

Three experimental RAS of identical construction were used for the experiment. The first RAS (RAS-C) functioned as a control and was not equipped with a denitrification unit. The second RAS (RAS-M) was equipped with a methanol fuelled denitrification unit. The third RAS (RAS-P) was equipped with a PHA granulate fuelled denitrification unit. The three used RAS *(Figure 3 - 1*, 1 m^3 in total, Kunststoff-Spranger GmbH, Plauen, Germany), were filled with tap water. Each rearing unit was equipped with an aerobe biofilter system. The system consisted of a Hamburg mat filter (HMF), a Moving-Bed-Biofilm-Reactor (MBBR, 0.12 m^3 total volume) filled with 0.06 m^3 of biocarrier (HEL-X®, diameter: 12 mm, surface: 859 m^2 m^{-3}, specific surface area (SSA): 704 m^2 m^{-3}, density: 0.95g, Christian Stöhr GmbH & Co. Elektro- und Kunststoffwaren KG, Marktrodach, Germany), and a protein skimmer (Aqua Medic Turboflotor Blue 3000, AB Aqua Medic GmbH, Bissendorf, Germany) supported by ozone (Ozone 300, 300 mg h^{-1} ozone, AB Aqua Medic GmbH, Bissendorf, Germany). Furthermore, a

UV-light disinfection (Aqua Medic Helix Max UV 55W, AB Aqua Medic GmbH, Bissendorf, Germany) was connected to the outlet of the aerobe biofilter system. To adjust the required oxygen saturation in the rearing unit, an oxygen diffuser was included into the RAS.

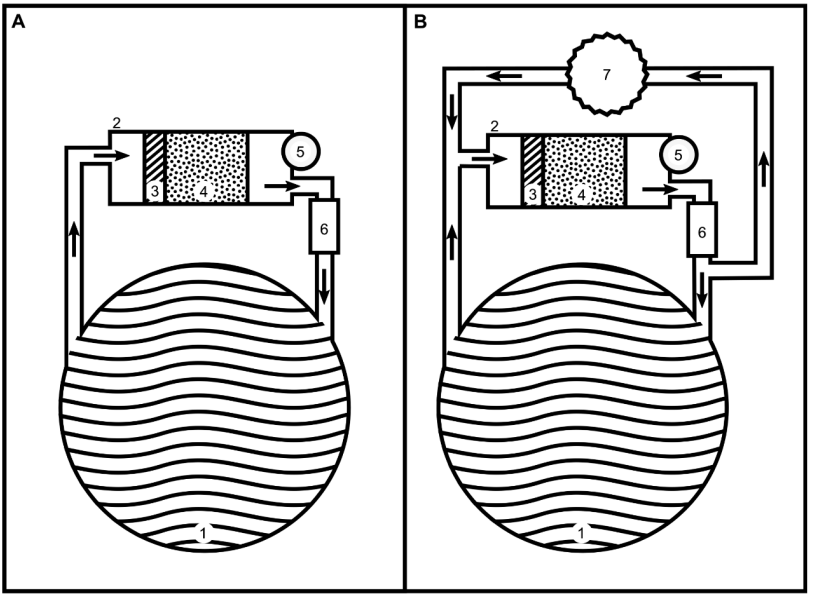

Figure 3 - 1. *Set-up of the (A) experimental control RAS and the (B) two RAS equipped with a* ***S****elf-cleaning* ***I****nherent gas* ***D****enitrification Reactor (SID-Reactor). Each RAS was equipped with a rearing tank (1), a biofilter system (2) consisting of a hamburger mat filter (3), a moving bed biofilm reactor (4), and a protein skimmer (5). The outflow from the biofilter system passed through a UV-disinfection unit (6). Furthermore, two out of the three RAS were equipped with a SID-Reactor (7). The water flow is indicated by arrows.*

2.2 Biodegradable polyhydroxyalkanoate (PHA) granulate

In this study a polyhydroxyalkanoate polymer granulate was applied both as biodegradable carbon source and as substrate for microbial attachment. The PHA (VVK Vertrieb Veredelter Kunststoffe GmbH, Siegburg, Germany) granulate was produced from renewable vegetal resources according to ASTM D6866 standard. This quality grade is according to the compostation and biodegradation norm ASTM D6400. Furthermore, the PHA granulate grade was suitable for use in food contact applications in the European Union as a raw material with 1935/2004/EEC and Plastics Regulation 10/2011. Additionally, the PHA granulate was produced by using ingredients listed in Table 1 of Annex 1 of Plastics Regulations 10/2011. No specific migration limits (SML) are listed for the ingredients.

2.3 Denitrification System

The design of the **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor, Kunststoff-Spranger GmbH, Plauen, Germany) is based on a combined function principle of a fixed bed and a moving bed biofilm reactor (Müller-Belecke et al., 2013). The SID-Reactors used in this study were small scaled versions of the SID-Reactor used by Müller-Belecke et al. (2013) (*Figure 3 - 2*) and had a total volume of 27 litres each. The water level in the reactors was adjusted to 22 litres. The SID-Reactor of RAS-M was filled with 11 litres of floating biocarriers (HEL-X®, diameter: 12 mm, surface: 859 $m^2\ m^{-3}$, specific surface area (SSA): 704 $m^2\ m^{-3}$, density: 0.95g, Christian Stöhr GmbH & Co. Elektro- und Kunststoffwaren KG, Marktrodach, Germany) shared equally by the aerobe MBBR of the three experimental RAS. The SID-Reactor for RAS-P was filled with 11 litres (7.5 kg) of biodegradable PHA granulate, which were soaked in a separate water tank during a start-up phase, filled with tempered (25°C) tap water. The biocarriers provided a specific surface area of 9.5 m^2, the PHA granulate of 13.8 m^2 to be populated by bacteria. Each reactor chamber itself was closed with a gas tight top cover to prevent oxygen influx by ambient air, enabling anoxic conditions. Additionally, the nitrogen gas produced during denitrification accumulated at the top of the reactor chamber. Within a defined time interval, the fixed biocarriers and the PHA granulate were swirled up. A side channel vacuum pump (0.145 kW, 4.2 $m^3\ h^{-1}$, 1 bar, type: DTE3, Gardner Denver Thomas GmbH, Germany) forced the inherent oxygen-poor gas from the top to the bottom of the SID-Reactor and set the biocarriers and the PHA granulate into motion. This was done to prevent biocarriers and granulate from clogging due to bacterial growth (self-cleaning effect of the SID- Reactor). A mesh was installed in the upper third of the SID-Reactor, to prevent biocarriers and PHA granulate from flushing out. A small opening (Ø = 0.5 cm)

on the top of the reactor allowed excessive nitrogen gas to leak and furthermore allowed pressure compensation while the side channel vacuum pump was running. According to results of ***Chapter 2*** the hydraulic retention time (HRT) was set to 2 hours and the biocarrier circulation interval to 30 minutes for both SID-Reactors.

The methanol fuelled SID-Reactor (RAS-M) was equipped with a container (20 L) for methanol and a peristaltic dosage pump (Dynamik Series, Seko Deutschland GmbH, Mainz-Kastel, Germany) constantly adding methanol to the influent water of the SID-Reactor. The flow rate of both SID-Reactors was adjusted by a diaphragm valve with a variable area flow meter (Georg Fischer AG, Schaffhausen, Switzerland).

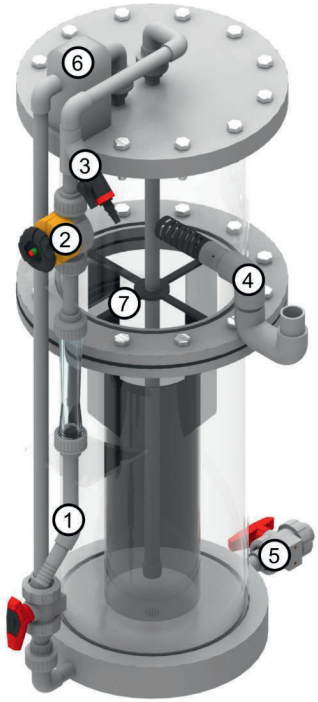

Figure 3 - 2. *Drawing of a small scaled* ***S****elf-cleaning* ***I****nherent gas* ***D****enitrification Reactor (SID-Reactor). Inlet (1), flow rate valve (2), methanol inlet (3), outlet (4), residual water outlet (5), side channel vacuum pump (6), and gaze (7).*

2.4 Experimental setup and duration

For the experimental trial pikeperch (*Sander lucioperca*) were obtained from Aqua Pri (Vejen, Denmark) and kept in a large scaled RAS (40 m³) prior to the experiments at the experimental facility Gesellschaft für Marine Aquakultur (GMA) mbH (Büsum, Germany). For the experimental trial 385 pikeperch with an initial body weight of approximately 190 g were randomly distributed among the three RAS resulting in an average initial biomass of 24.3 kg per RAS. The pikeperch acclimatized to the experimental conditions for two weeks. Light regime was adjusted to 14 h light and 10 h darkness. Beginning with the second day after stocking, pikeperch were daily fed 243 g of a commercial feed (Aller Metabolica, Emsland Aller Aqua GmbH, Gloßen, Germany) by an automatic feeder. Feeding rate was adjusted at the beginning of the experimental trial to 1% of the total biomass per tank. The experiment lasted 110 days (excluding acclimatization) and was divided into three consecutive steps. Within the first step (start-up phase) all three RAS were maintained without a denitrification unit for 53 days to allow nitrate accumulation in the systems. During the start-up phase attention was paid on differences between the three treatment groups concerning the monitored water quality parameters (*Table 3 - 1*). A higher nitrite concentration in RAS-M was traced back to a reduced water flow in the MBBR, due to a malfunction of the water pump. Hence, the water pump was replaced. After ensuring the same starting conditions concerning the monitored water quality parameters for the three experimental RAS, the second step (experimental phase I) was initiated. In the experimental phase I (lasting 10 days) the SID-Reactors were installed to RAS-M and RAS-P, respectively, and basal settings were adjusted. As a third step the experimental phase II (47 days) was started by adding methanol to the inlet water of SID-Reactor of RAS-M. In both experimental phases water quality parameters (*Table 3 - 2* and Table 3 - 3) and denitrification performance (*Table 3 - 4*) were monitored.

Table 3 - 1. *Mean values (±SD) of water quality parameters of the RAS rearing water including number of data points (=n) respectively. Values for water quality were obtained during the start-up phase of 53 days (trial days 1 to 53) for each RAS.*

		RAS-C	RAS-M	RAS-P	n
Temperature	°C	24.9 ± 0.3	24.9 ± 0.4	25.0 ± 0.6	39
ORP	mV	186.5 ± 17.3	189.0 ± 17.5	186.9 ± 17.2	28
Oxygen	%	110.5 ± 7.2	112.9 ± 8.1	113.5 ± 7.4	41
Oxygen	mg L^{-1}	9.0 ± 0.5	9.2 ± 0.7	9.3 ± 0.6	39
pH		7.5 ± 0.1	7.5 ± 0.1	7.4 ± 0.1	40
Turbidity	NTU	0.6 ± 0.7	0.5 ± 0.6	0.8 ± 1.0	50
TAN	mg L^{-1}	0.2 ± 0.1	0.3 ± 0.4	0.8 ± 0.1	51
NO_2^--N	mg L^{-1}	1.5^a ± 1.8	2.9^b ± 2.8	1.5^a ± 1.8	51
TOC	mg L^{-1}	7.1 ± 2.2	7.4 ± 2.9	7.0 ± 1.8	28

Superscript letters within one row indicate significant differences between RAS (Kruskal-Wallis-test with Dunn-Bonferroni post hoc test. $p < 0.05$). RAS, recirculating aquaculture system; ORP, oxidation-reduction potential; TAN, total ammonia nitrogen; NO_2^--N, nitrite-nitrogen; TOC, total organic carbon; RAS-C, control RAS without a denitrification unit; RAS-M, RAS with a methanol fuelled denitrification unit; RAS-P, RAS with a polyhydroxyalkanoate fuelled denitrification unit.

2.5 Data collection

2.5.1 Water quality analysis

For all three RAS data of water quality parameters analysis were collected for the rearing water equalling the inlet water of the SID-Reactors. Additionally for RAS-M and RAS-P data for the SID-Reactor outlet water were collected. Total ammonia nitrogen (TAN, salicylate method, method 8155, Hach Lange GmbH, Düsseldorf, Germany), nitrite (NO_2^--N, diazotization method, method 8507, Hach Lange GmbH, Düsseldorf, Germany), and nitrate (NO_3^--N, cadmium reduction method, method 8171, Hach Lange GmbH, Düsseldorf, Germany) were measured photometrically (DR 2800, Hach Lange GmbH, Düsseldorf, Germany). Furthermore, turbidity (NTU, PCE-TUM 20, PCE Deutschland GmbH, Meschede, Germany), acid neutralizing capacity (ANC) by titration of 0.1 mmol L^{-1} hydrochloric acid, and salinity (HI 96822, Hanna Instruments Deutschland GmbH, Vöhringen, Germany) were measured. Oxygen saturation (%), oxygen concentration (mg L^{-1}), and water temperature (°C) (Handy Polaris, OxyGuard Internaional A/S, Farum, Denmark) were measured by sensors. Likewise, the pH-value (SenTix®41, WTW pH 3310, Xylem Analytics Germany Sales GmbH & Co.KG, Weilheim, Germany), and the oxidation-reduction potential (ORP) (GMH 5550, GHM Messtechnik GmbH, Regenstauf, Germany) were measured.

2.5.2 SID-Reactor denitrification performance

The overall denitrification efficiency is described as NO_3^--N reduction in percent. For comparison with other studies the denitrification rate is additionally described by the NO_3^--N reduction in milligram NO_3^--N per liter of treated water and hour (mg L^{-1} h^{-1}), in milligram NO_3^--N per liter of treated water, hour and cubic meter of substrate (mg L^{-1} h^{-1} m^3 substrate), and in gram NO_3^--N per day and cubic meter of substrate (g d^{-1} m^3 substrate) (*Table 3 - 4*). The denitrification rate was calculated based on the difference between the NO_3^--N concentration of the inlet and the outlet of the SID-Reactor. The price (€ kg^{-1}) for both used carbon sources (methanol and PHA), the consumption of carbon source (kg kg^{-1} NO_3^--N) and the costs of denitrification (€ kg^{-1} NO_3^--N) are given in *Table 3 - 5*. The costs of denitrification was calculated according to following formula:

(1) RSC = TSC / TNR

with RSC=Relative substrate consumption (kg kg^{-1} NO_3^--N), TSC=total substrate consumption (kg), and TNR=total NO_3^--N reduction (kg NO_3^--N).

(2) DC = RSC * SP

with DC=denitrification costs (€ kg^{-1} NO_3^--N) and SP=substrate price (€ kg^{-1}).

2.6 Statistical Analysis

The present study was performed in unique experimental RAS and thus, all experiments are based on a consecutive experimental design. Mean values and standard deviation presented in tables are based on repeated measurements over the trial phase. Statistical analyses were performed using IBM SPSS Statistics Version 20 (IBM Corporation, New York, USA). Data was tested for normal distribution based on graphical residual analysis and for homoscedasticity using the Levene-Test ($p < 0.05$).

With regard to water quality analysis, for normally distributed homoscedastic data, the ANOVA, based on the three treatments, was followed by Bonferroni post-hoc test ($p < 0.05$) in order to evaluate the differences between individual treatment groups (RAS-C, RAS-M, and RAS-P). For non-normally distributed heteroscedastic data, Kruskal-Wallis-Test followed by Dunn-Bonferroni post-hoc test ($p < 0.05$) was performed in order to evaluate the differences between individual treatment groups.

With regard to the denitrification performance, t-tests ($p < 0.05$) for normally distributed data were performed to investigate differences in denitrification rate (in g d^{-1} m^3 substrate) between the treatment groups.

For the nitrate concentrations in all three experimental RAS and for the denitrification efficiency for both SID-Reactors regression lines and turning points were determined according to best fit using SigmaPlot 14.0 (Systat Software Inc., London, United Kindom).

3 Results

3.1 Water quality parameters of the experimental RAS

3.1.1 Experimental phase I

During experimental phase I all monitored water quality parameters of the rearing water (*Table 3 - 2*) showed no significant differences between the three tested RAS. Average temperature for all three RAS was 25°C and the oxygen saturation was >100% (> 8.5 mg L^{-1}). Average ORP ranged from 152 to 155 mV, average pH ranged from 7.4 to 7.5 and ANC from 2.5 to 3.4 mmol HCl L^{-1} for the three RAS. Turbidity was low (0.4 to 0.9 NTU) for all three RAS during the experimental phase I. Furthermore, low TAN (0.2 mg L^{-1}) and NO_2^--N (0.5 mg L^{-1}) values were measured. The TOC concentration in the tested RAS ranged between 8.7 and 9.7 mg L^{-1}.

Table 3 - 2. *Mean values (±SD) of water quality parameters of the RAS rearing water including number of data points (=n) respectively. Values for water quality were obtained during the experimental phase I of 10 days (trial days 54 to 63) for each RAS.*

		RAS-C	RAS-M	RAS-P	n
Temperature	°C	24.8 ± 0.1	25.1 ± 0.4	25.0 ± 0.3	9
ORP	mV	155 ± 24	154 ± 26	152 ± 28	9
Oxygen	%	106 ± 14	104 ± 14	104 ± 13	9
Oxygen	mg L^{-1}	8.6 ± 1.2	8.5 ± 1.1	8.5 ± 1.1	9
pH		7.4 ± 0.1	7.4 ± 0.1	7.5 ± 0.1	9
ANC	mmol HCl L^{-1}	2.5 ± 0.3	3.0 ± 0.1	3.4 ± 0.8	4
Turbidity	NTU	0.5 ± 0.7	0.4 ± 0.5	0.9 ± 0.9	10
TAN	mg L^{-1}	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	6
NO_2^--N	mg L^{-1}	0.5 ± 0.6	0.5 ± 0.6	0.5 ± 0.6	6
TOC	mg L^{-1}	8.7 ± 1.3	9.7 ± 1.6	9.7 ± 0.8	5

RAS, recirculating aquaculture system; ORP, oxidation-reduction potential; ANC, acid neutralization capacity; TAN, total ammonia nitrogen; NO_2^--N, nitrite-nitrogen; TOC, total organic carbon; RAS-C, control RAS without a denitrification unit; RAS-M, RAS with a methanol fuelled denitrification unit; RAS-P, RAS with a polyhydroxyalkanoate fuelled denitrification unit.

3.1.2 Experimental phase II

During experimental phase II (*Table 3 - 3*) no significant differences for temperature, ORP, oxygen saturation and concentration were observed in the rearing water of the three RAS. Significant differences ($p < 0.05$) were observed for pH, ANC and turbidity of the rearing water. The significantly highest pH of 7.7 was measured for RAS-M. In contrast, the lowest average pH of 7.4 was measured for RAS-C, which was not significantly different from the average pH of 7.5 measured for RAS-P. Similarly, average ANC of 3.0 mmol HCl L^{-1} for RAS-C was significantly lower compared to an average ANC of 7.1 mmol HCl L^{-1} for RAS-M. ANC for RAS-P was 4.1 mmol HCl L^{-1} and not significantly different compared to the other two RAS. Average turbidity of 2.6 NTU was significantly higher in RAS-M compared to 0.8 NTU in RAS-C. In RAS-P turbidity was 1.8 NTU and did not significantly differ compared to the other two RAS. With regard to average TAN, NO_2^--N, and TOC no significant differences between the three experimental RAS were observed, possibly due to the high standard deviations. In addition to average water quality parameters in the rearing water of the three RAS, *Figure 3 - 3* shows the measured TAN, NO_2^--N and TOC concentration in the outlet water of the two SID-Reactors over the duration of experimental phase I and II. Several measuring peaks were observed for TAN, NO_2^--N and TOC in the outlet water of the SID-Reactor of RAS-M. In contrast, no peaks were observed in the outlet water of the SID-Reactor of RAS-P.

Table 3 - 3. *Mean values (±SD) of water quality parameters of the RAS rearing water including number of data points (=n) respectively. Values for water quality were obtained during the experimental phase II of 47 days (trial days 64 to 110) for each RAS.*

		RAS-C	RAS-M	RAS-P	n
Temperature	°C	24.6 ± 0.5	24.7 ± 0.5	24.8 ± 0.5	38
ORP	mV	126 ± 18	112 ± 21	121 ± 18	34
Oxygen	%	113 ± 11	113 ± 19	114 ± 12	38
Oxygen	mg L^{-1}	9.4 ± 1.0	9.3 ± 1.6	9.3 ± 1.0	38
pH		7.4^{a} ± 1.0	7.7^{b} ± 0.2	7.5^{a} ± 0.1	38
ANC	mmol HCl L^{-1}	3.0^{a} ± 0.4	7.1^{b} ± 2.6	4.1^{ab} ± 0.6	19
Turbidity	NTU	0.8^{a} ± 1.0	2.6^{b} ± 2.2	1.8^{ab} ± 1.7	31
TAN	mg L^{-1}	0.4 ± 0.1	1.1 ± 2.0	0.4 ± 0.1	19
NO_2^--N	mg L^{-1}	0.8 ± 0.5	0.8 ± 0.6	0.5 ± 0.3	19
TOC	mg L^{-1}	17.5 ± 6.1	24.4 ± 10.4	19.2 ± 5.1	18

Superscript letters within one row indicate significant differences between RAS (Kruskal-Wallis-test with Dunn-Bonferroni post hoc test. $p < 0.05$). RAS, recirculating aquaculture system; ORP, oxidation-reduction potential; TAN, total ammonia nitrogen; NO_2^--N, nitrite-nitrogen; TOC, total organic carbon; RAS-C, control RAS without a denitrification unit; RAS-M, RAS with a methanol fuelled denitrification unit; RAS-P, RAS with a polyhydroxyalkanoate fuelled denitrification unit.

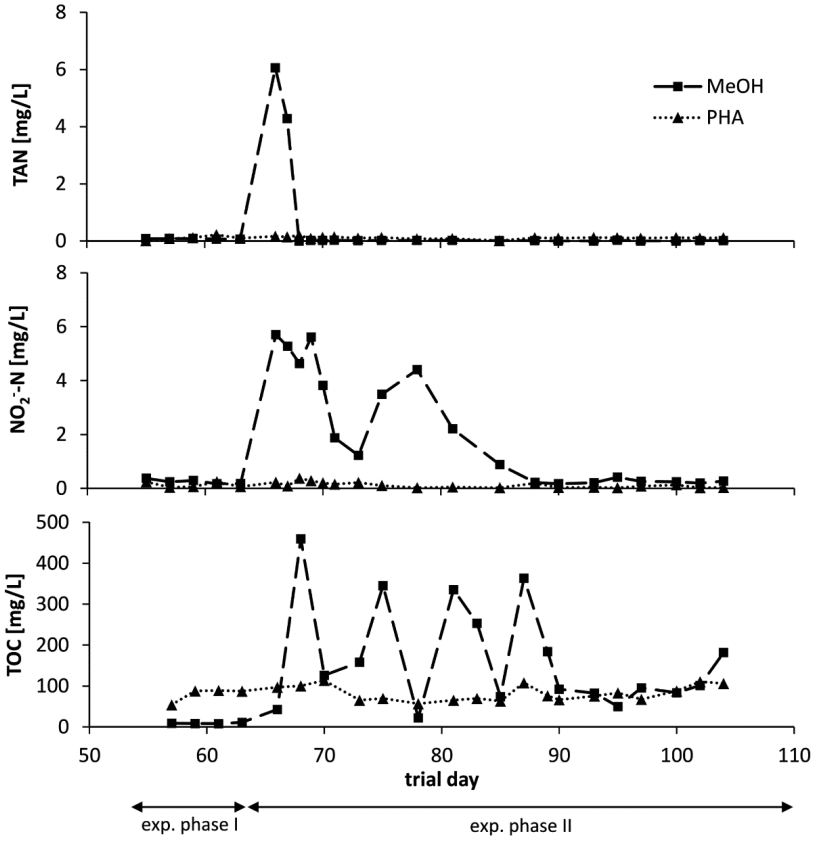

Figure 3 - 3. *Course of total ammonium nitrogen (TAN), nitrite (NO_2^--N) and total organic carbon (TOC) in the outlet water of the MeOH and PHA fuelled SID-Reactors.*

3.2 NO_3^--N concentration and reduction in the experimental RAS

During the experimental phases I and II the course of NO_3^--N concentration was monitored in the rearing water of the three experimental RAS (*Figure 3 - 4*). At the beginning of experimental phase I for all three RAS an initial NO_3^--N concentration of approximately 130 mg L^{-1} was measured. At the end of experimental phase II, the NO_3^--N concentrations for RAS-C, RAS-M and RAS-P were 450, 50 and 160 mg L^{-1}, respectively. For RAS-C a constant increase in NO_3^--N concentration with some fluctuations was observed during experimental phase I and II (trial day 54 to 110). The regression analysis revealed a positive linear correlation of NO_3^--N concentration and trial day. No plateau phase was observed for nitrate in RAS-C during the entire experimental phase. For RAS-M an increase in NO_3^--N concentration was observed during phase I (trial day 54 to 63), whereby no methanol was dosed to the SID-Reactor. At the start of experimental phase II (trial day 64) methanol dosage was initiated. However, NO_3^--N concentration in RAS-M continued to increase to approximately 190 mg L^{-1} – the first turning point (T1) at day 67 (*Figure 3 - 4*). In the further course of the experiment, the NO_3^--N concentration decreased to approximately 50 mg L^{-1} - the second turning point (T2) at day 91. From trial day 92 until day 110, the end of experimental phase II, the NO_3^--N concentration remained on a plateau at approximately 50 mg L^{-1}. The regression analysis revealed a 3-segmented piecewise linear correlation of NO_3^--N concentration and trial day. In RAS-P a constant increase of the nitrate concentration up to 160 mg L^{-1} was observed during experimental phase I and II (trial day 54 to 110). The regression analysis revealed a positive linear correlation of NO_3^--N concentration and trial day. No plateau phase was reached during the entire experimental phase, even though the slope of NO_3^--N increase was obviously lower compared to RAS-C.

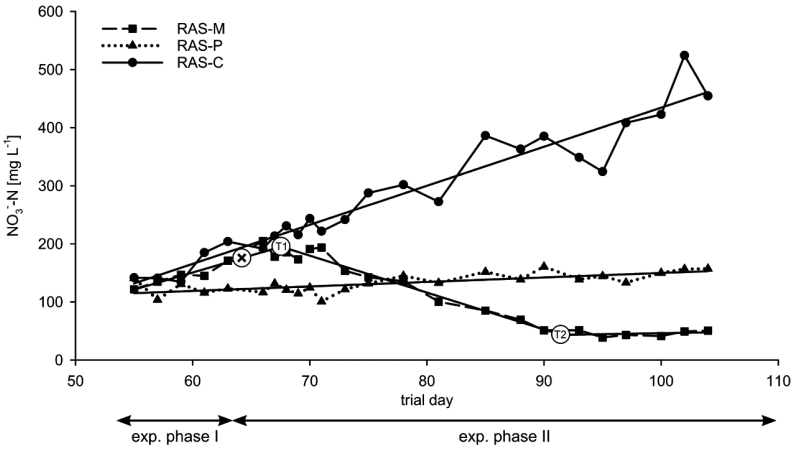

Figure 3 - 4. *Course of nitrate (NO_3^--N) concentration in the rearing water of RAS-C, RAS-M, and RAS-P. RAS-C, control RAS without a denitrification unit; RAS-M, RAS with a methanol fuelled denitrification unit; RAS-P, RAS with a polyhydroxyalkanoate fuelled denitrification unit. The x marks the start of the methanol dosage into the SID-Reactor of RAS-M. T1 and T2 mark turning points for nitrate concentration in RAS-M. Regression lines and turning points were determined according to best fit using SigmaPlot 14.0 (Systat Software Inc., London, United Kindom).*

With regard to the denitrification efficiency (in percent), several differences between the methanol and PHA fuelled SID-Reactors were observed (*Figure 3 - 5*). During experimental phase I no denitrification in the methanol fuelled SID-Reactor was detected. At the beginning of experimental phase II a denitrification efficiency of approximately 6% was observed. The denitrification efficiency increased up to approximately 98% at trial day 85, a turning point (T_M) in the denitrification course. From trial day 85 to 110 the denitrification efficiency remained on a plateau at approximately 98%. Hence, 21 days had passed from start of the denitrification process by methanol dosage, until denitrification efficiency of 98% was reached. The regression analysis revealed a 2-segmented piecewise linear correlation of denitrification efficiency and trial day. In contrast to the methanol fuelled SID-Reactor, the PHA fuelled SID-Reactor revealed a denitrification efficiency of approximately 6% already after connection to the RAS at the beginning of experimental phase I. At trial day 60, 6 days after connecting the SID-Reactor to RAS-P, the denitrification efficiency increased up to approximately 40%, a turning point (T_P) in the denitrification course (*Figure 3 - 5*). During the further progress of the experiment the denitrification efficiency remained at approximately 40% with some fluctuations. No clear increase or decrease of the denitrification efficiency was obvious for the PHA fuelled SID-Reactor. The regression analysis revealed a 2-segmented piecewise linear correlation of denitrification efficiency and trial day.

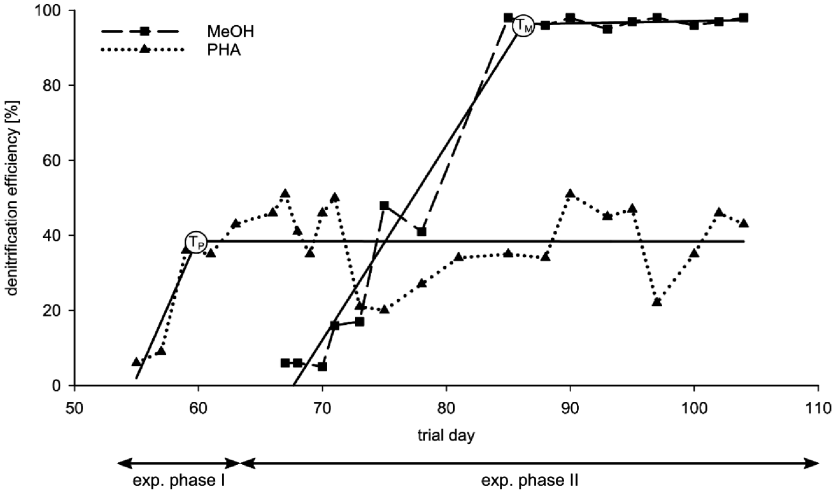

Figure 3 - 5. *Course of denitrification efficiency for the methanol (MeOH) and polyhydroxyalkanoate (PHA) fueled SID-Reactor during experimental phase II. TM, turning point for the denitrification efficiency of the methanol fueled SID-Reactor; TP, turning point for the denitrification efficiency of the PHA fueled SID-Reactor. Regression lines and turning points were determined according to best fit using SigmaPlot 14.0 (Systat Software Inc., London, United Kindom).*

Table 3 - 4. *Mean values (± SD) of denitrification rate for the SID-Reactors of RAS-M and RAS-P during experimental phase II.*

		Experimental phase II	
		RAS-M	RAS-P
Trial days	[d]	47	47
Data points	[n]	17	19
NO_3^--N reduction rate	[mg L^{-1} h^{-1}]	43 ± 20	52 ± 15
	[g L^{-1} h^{-1} m^3 substrate]	3.95 ± 1.85	4.60 ± 1.34
	[g d^{-1} m^3 substrate]	1042 ± 489	1245 ± 339

Number of data points (n) per treatment: RAS-M: n=17; RAS-P: n=19. NO_3^--N, nitrate; TN, total nitrogen; RAS-M, RAS with a methanol fuelled denitrification unit; RAS-P, RAS with a polyhydroxyalkanoate fuelled denitrification unit.

With regard to the denitrification rate (in g d^{-1} m^3 substrate) of the SID-Reactor of RAS-M and RAS-P (*Table 3 - 4*) no significant differences were observed. The denitrification rate was 1042 g d^{-1} m^3 substrate and 1245 g d^{-1} m^3 substrate for the methanol and the PHA fuelled SID-Reactor, respectively.

Comparing the costs for denitrification (*Table 3 - 5*) under the given experimental conditions it is evident that reducing 1 kg NO_3^--N is 3.4 times less expensive using methanol than using PHA granulate. To reduce 1 kg NO_3^--N 6.86 kg of methanol and 3.36 kg of PHA were required in the current setup. The price for one kg MeOH was 1.77€ and for one kg PHA granulate 12.30€. Consequently, the reduction of 1 kg NO_3^--N costed 12.10€ using methanol and 41.30€ using PHA.

Table 3 - 5. *Substrate consumption and costs for methanol (MeOH) and polyhydroxyalkanoate (PHA).*

		MeOH	PHA
Substrate price (SP)	[€ kg^{-1}]	1.77	12.30
Total substrate consumption (TSC)	[kg]	2.77	1
Total NO_3^--N reduction (TNR)	[kg NO_3^--N]	0.404	0.298
Relative substrate consumption (RSC)[1]	[kg kg^{-1} NO_3^--N]	6.86	3.36
Denitrification costs (DC)[2]	[€ kg^{-1} NO_3^--N]	12.10	41.30

1RSC = TSC / TNR
2DC = RSC * SP

4 Discussion

With regard to water quality parameters during experimental phase I (trial day 54 to 63) no significant differences between the three tested RAS were observed. Therefore, it can be assumed that connecting the SID-Reactors and setting them into operation had no negative influence on water quality and functionality of RAS components like the nitrifying MBBR. During experimental phase II (trial day 64 to 110) a significant influence of both denitrification systems on the pH and ANC was observed. In RAS, the nitrification in aerobe biofilters leads to reduced pH and a loss of alkalinity in the rearing water. To counteract this, alkalinity supplements such as sodium hydrogen carbonate ($NaHCO_3$) are commonly used. During denitrification processes, pH and ANC values tend to increase. This results in reduced use of alkalinity supplements (van Rijn et al., 2006). According to stoichiometry, alkalinity decreases by 7.14 mg $CaCO_3$ per mg NH_4^+-N during nitrification. During heterotrophic denitrification alkalinity increases by 3.57 mg $CaCO_3$ per mg NH_4^+-N (van Rijn et al., 2006). Due to the stoichiometric calculation a saving of alkalinity supplements of 50% can be expected. To keep the pH in RAS-C stable at 7.4 it was necessary to add in total 3.95 kg of $NaHCO_3$ to the system. Nearly twice as much as the $NaHCO_3$ addition of 2.0 kg for RAS-M and 2.2 kg for RAS-P. Hence, a saving of approximately 50% in alkalinity supplements was documented for both RAS equipped with a SID-Reactor. Accompanied with pH, values

for ANC were significantly higher in RAS-M compared to the control, hence again demonstrating the positive influence of SID-Reactor denitrification on pH and alkalinity. Thus, both SID-Reactors showed a positive influence on pH and alkalinity stability.

A possible negative effect of the methanol fuelled SID-Reactor on water quality was observed for the water turbidity in the RAS. Compared to RAS-C, turbidity in RAS-M was significantly higher. Turbidity measurements can be used to estimate the growth parameters of bacteria (Métris et al., 2003). Therefore, the increase in turbidity in RAS-M could be a result of increased bacterial growth conditioned by increased organic carbon load in the system due to a methanol over-dosage. An indicator for imprecise methanol dosage is the total organic carbon (TOC) value in the SID-Reactor outlet water of RAS-M. During experimental phase II, several peaks in TOC concentration in the SID-Reactor outlet water were measured. If methanol that has not completely been consumed by denitrifying bacteria, is flushed out of the SID-Reactor it can enhance bacterial growth in the rearing water of RAS. Connected to bacterial growth turbidity can also increase. As biodegradable solid carbon sources are only available to bacteria by enzymatic decomposition, the risks of inaccurate dosage can be avoided (Chu and Wang, 2011a; Gutierrez-Wing et al., 2012; Shen et al., 2013b). Bacteria react to nitrate levels in the water column and hence use only the required amount of organic carbon necessary to reduce the available amount of nitrate (Wang and Chu, 2016).

An increase of intermediate ammonium and nitrite production in denitrification units is a result of incomplete denitrification caused for example by imprecise carbon dosage (Guo et al., 2017; Hamlin et al., 2008; Oh and Silverstein, 1999; Plósz et al., 2003; van Rijn and Rivera, 1990; Yang et al., 2012). At high concentrations, both intermediate products have the potential to harm the cultivated fish species. Even though the higher TAN and NO_2^--N values in the rearing water of RAS-M were not significantly different in comparison to the other two RAS, the influence of the methanol fuelled SID-Reactor on TAN and NO_2^--N was obvious. Taken together, observed peaks for TAN, NO_2^--N, and TOC in the outlet water of the methanol fuelled SID-Reactor demonstrate the importance of accurate carbon dosage. Deterioration of the RAS rearing water could be a consequence, if dosage is not accurate. For RAS-P with the PHA fuelled SID-Reactor none of these earlier mentioned negative effects were observed. This matches the conclusion, that PHA based granulates do not have a potential risk of releasing dissolved organic carbon and causing deterioration of effluent water (Hiraishi and Khan, 2003).

With regard to the NO_3^--N concentration different concentration profiles were observed in the three RAS during the experimental trial. In a closed RAS without a denitrification unit, nitrate accumulates over time, due to the nitrogen load originating from the fish feed, as observed for RAS-C. In a RAS equipped with a denitrification unit with a net nitrate removal lower than the net nitrate production, nitrate also accumulates over time, but at a comparable lower rate. This was observed for RAS-P. In a RAS with a denitrification unit having a net nitrate removal higher than the net nitrate production, nitrate concentration decreases over time. If the net nitrate removal is equal to the net nitrate production a plateau phase is reached. In RAS-M the last two scenarios were observed. At the beginning of the experimental phase II the net nitrate removal in RAS-M was higher than the net nitrate production, resulting in declining nitrate concentrations in the RAS water. At the end of experimental phase II a plateau of the nitrate concentration in RAS-M was observed. Hence, at the end of experimental phase II in RAS-M the net nitrate removal was equal to the net nitrate production. In all three experimental RAS feeding regimes were equal, resulting in equal net nitrate production. Additionally due to identical flow through, the same amount of water was treated by both SID-Reactors. Furthermore, no statistical differences were observed for the denitrification rates of both SID-Reactors. Hence, the varying nitrate concentrations in the RAS at the end of the experiment are most likely attributed to varying denitrification efficiencies of both SID-Reactors.

In RAS-P nitrate concentration increased from approximately 130 mg L^{-1} up to 160 mg L^{-1}. The PHA fuelled SID-Reactor reached relatively quickly a denitrification efficiency of 40% nitrate removal after 6 trial days. Hence, the PHA fuelled SID-Reactor removed approximately 52 to 64 mg nitrate per treated litre rearing water during the experiment. In RAS-M denitrification started at day 67 at a concentration of 190 mg L^{-1} at a low denitrification efficiency of approximately 6%. Hence, the methanol fuelled SID-Reactor removed approximately 11 mg nitrate per treated rearing water at the beginning. During the course of the experiment denitrification efficiency increased up to 98% at trial day 85. At this point the nitrate concentration in RAS-M was 85 mg L^{-1}, resulting in a nitrate removal of 83 mg per treated litre rearing water. Towards the end of the experiment nitrate concentration in RAS-M decreased to approximately 50 mg L^{-1}, resulting in a nitrate removal of 49 mg per treated litre rearing water. Based on this, determining the average denitrification rates for both SID-Reactors results in a similar nitrate removal in g d^{-1} m^3 substrate.

Using PHA granulate as substrate and carbon source at the same time, resulted in an immediate, though low, denitrification efficiency of 6% after connecting the PHA fuelled SID-Reactor to RAS-P. During installation of the SID-Reactors to the RAS (experimental phase I) the SID-Reactor of RAS-M was not fuelled with a carbon source. If the endogenous amount of organic carbon is insufficient to achieve stable denitrification processes in a denitrification unit, an external carbon source is mandatory (van Rijn et al., 2006). Based on stoichiometric calculations, anticipated 1.9 g of methanol is required to reduce 1.0 of NO_3^--N (Cheremisinoff, 1995). Since nitrogen and carbon are also used by denitrifying bacteria for cell syntheses methanol requirement is above the C/N ratio of 1.9 (Kadlec and Wallace, 2008). As reported in ***Chapter 2*** a sufficient C/N ratio of 2.3 for stable and efficient denitrification performance of a SID-Reactor. TOC of approximately 10 mg L^{-1}, representing endogenous organic carbon in RAS-M, did not meet the required C/N ratio for denitrifying 130 mg L^{-1} NO_3^--N. Hence, the present endogenous organic carbon concentration of the system was not sufficient to initiate denitrification processes in the SID-Reactor in RAS-M. As consequence, the nitrate concentration in RAS-M increased comparable to RAS-C during experimental phase I. Three days after the beginning of experimental phase II a measurable decrease of the nitrate concentration in RAS-M was observed. For the methanol fuelled SID-Reactor the maximum denitrification efficiency was achieved 21 days after the start of methanol dosage.

Several authors also report a lag-time until full denitrification performance is achieved when using biodegradable granulate as a carbon source (Boley et al., 2000; Shen et al., 2013b). However, in other studies the start of denitrification process showed no lag-time (Gutierrez-Wing et al., 2012). In the present study a lag-time was observed for both SID-Reactors. It is obvious, that a colonization of the substrate by bacteria and the adaptation of the bacterial community to the environmental conditions takes time. However, denitrification efficiency of approximately 40% for the PHA fuelled SID-Reactor seemed to be low compared with 98% of the methanol fuelled SID-Reactor and compared to the denitrification efficiency reported in other studies. As summarized by Wang and Chu (2016) denitrification efficiency is commonly >90%, using different biodegradable polymers as a carbon source. Clogging and short-circuiting of a denitrification reactor due to excess biomass production can cause a decline in the denitrification efficiency and are reported using denitrification units with biodegradable granulate as a carbon source (Boley et al., 2000; P. Li et al., 2016). Due to the self-cleaning characteristics of the SID-Reactor clogging and short-circuiting is prevented (Müller-Belecke et al., 2013; ***Chapter 2***). With the SID-Reactor as

denitrification unit no clogging or short-circuiting was observed for both SID-Reactors during the experimental trial, allowing a proper assessment of the denitrification performance.

Although denitrification performance is often reported, a direct comparison of data is not accurate, due to the lack of information for environmental situations and the exact chemical compounds used. Generally, there is a various number of different PHA types (Kessler et al., 2001; Raza et al., 2018; Steinbüchel, 1992; Zhang et al., 2006). It can be assumed that they differ in physical and chemical properties due to structural variations (Raza et al., 2018). Furthermore, in the biosynthesis of PHAs no pure PHAs of a single type are produced but rather mixtures of more than one monomer (Raza et al., 2018). Hence, it is conceivable that the PHA available in this study had a composition resulting in comparable low denitrification efficiency. However, the differences of the diverse substrate groups can also be exploited by compounding. Compounding can result in improved physical and chemical properties and reduce costs (Hiraishi and Khan, 2003; Shen et al., 2013a; Shen and Wang, 2011; Wu et al., 2012). Next to compounding the optimization of the surface-to-volume ratio could enhance the overall denitrification performance (Chu and Wang, 2011b; Gutierrez-Wing et al., 2012; Hiraishi and Khan, 2003). Increasing the surface-to-volume ratio could have a high impact on the denitrification performance of the substrate, hence making the denitrification more efficient by allowing a larger quantity of bacteria to populate the substrate.

However, using plastics in aquaculture, even though of biological origin, could raise concerns associated with the intake of microplastics by fish and potential contamination of the human food supply (Lusher et al., 2017). Uptake of microplastics by environmentally exposed organisms has been reported in a wide range of habitats, including the sea surface, water column, benthos, estuaries, beaches, aquaculture, and the deep sea (Bergmann et al., 2015; GESAMP, 2015; Lusher et al., 2017; Taylor et al., 2016). As reviewed by Lusher et al. (2017) uptake, movement and adverse effects of microplastic particles were observed in whole organisms and tissues, such as gills, intestinal tract and liver.

For PHA likewise the hazard potential was observed in several studies in different areas of research. These studies revealed that PHA and its degradation products have been proven to be non-toxic and are without indication of carcinogenic effects (Ali and Jamil, 2016; Z. Li et al., 2016; Peng et al., 2011). Furthermore, PHAs were already being used in the medical field as sutures, cardiovascular patches, orthopaedic pins, adhesion barriers, stents, nerve guides, bone marrow scaffolds, wound dressings,

cellular growth support, and further applications (Ali and Jamil, 2016; Chen and Wu, 2005). Hence, PHA reveals best prerequisites for the application in aquacultural fish production. Yet, in the present study the effluent water from each SID-Reactor was processed in a biofilter system, removing particles from the water column, minimizing the risk of PHA particle ingestion by fish. Nevertheless, future research projects should clarify if PHA poses a risk for fish and whether the release and accumulation of substances originating from PHA have negative long-term effects on fish.

Concerning the economic costs in this study only the cost of substrates were taken into consideration. Due to the purchase of minimum quantities the price of 1.77€ for methanol and 12.30€ for PHA was not representative for economically working RAS production systems. In the case of larger purchase quantities lower prices can be expected. As reported in other studies the price of biodegradable plastics can start at just a few Euros (Boley et al., 2000; Wang and Chu, 2016).

Furthermore, methanol dosage was probably not always precise with regard to fluctuating TOC values in the outlet water of the methanol fuelled SID-Reactor. Hence, comparing the consumption of methanol and substrate costs difficult. At the end of the experimental trial a substrate consumption of 3.36 kg PHA per reduced kg of NO_3^--N was calculated. In this study the consumption of substrate is higher compared to other studies, but still in a similar range. Substrate consumption reported for PHB were 2.9 kg kg^{-1} NO_3^--N (Gutierrez-Wing et al., 2012), for PHBV 1.49 to 1.65 kg kg^{-1} NO_3^--N (Chu and Wang, 2016), and for PCL ranging from 1.27 to 3.7 kg kg^{-1} NO_3^--N (Boley et al., 2000; Chu and Wang, 2013, 2011a). These differences could be attributed first of all to the different group of substances, but also to differences in physical properties (e.g. density, surface quality, etc.) and chemical composition within a group of substances. Nevertheless, substrate costs for denitrification processes with biodegradable polymers are more expensive compared to conventional liquid carbon sources (Wang and Chu, 2016). The future reduction of production costs and compounding of biodegradable plastics with cheap organic substances will make the solid-phase denitrification more economic (Wang and Chu, 2016). Beyond that, to compare the operating costs of a methanol fuelled SID-Reactor with a PHA fuelled SID-Reactor not only the price of substrates has to be taken into account, but also the costs for process control, labour costs for maintenance, costs for safety precautions, and others.

5 Conclusion

The results of this study reveal a great potential using a SID-Reactor in combination with biodegradable polymers as a solid carbon source with the purpose of nitrate elimination in RAS. In contrast to conventional denitrification systems the operating principle of the SID-Reactor is not restricted to use either a liquid carbon source or a solid carbon source. Using PHA in combination with a SID-Reactor revealed several advantages compared to a methanol fuelled SID-Reactor. Under the given experimental conditions start of denitrification performance of the PHA fuelled SID-Reactor showed a shorter lag-time in contrast to the methanol fuelled SID-Reactor. However denitrification efficiency was lower compared to the methanol fuelled SID-Reactor. Furthermore, turbidity, TAN, NO_2^--N, and TOC values were lower in the rearing water without any extreme values using PHA compared to using methanol. Future research should focus on further reduction of production costs of biodegradable plastics, compounding of different groups of substances creating new substrates with advanced physical and chemical properties, and the evaluation of this new substrates in potential in RAS applications.

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GENERAL DISCUSSION

Super-intensive recirculating aquaculture systems (RAS) offer a great potential for a sustainable and species-appropriate aquaculture, due to their highly efficient use of available space and water resources, and the control of most husbandry parameters. Nevertheless, intensive fish production is followed by high accumulation rates of nitrate - a possibly toxic substance. Thus, this thesis aimed to improve nitrate removal in RAS by using the novel **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor). Furthermore, knowledge gaps concerning nitrate toxicity to European sea bass (*Dicentrarchus labrax*) were filled. The results of the performed experiments are reported within three main chapters of this thesis.

As a first step the influence of nitrate on production parameters and health of on growing European sea bass reared in marine RAS was evaluated (***Chapter 1***). Subsequently, SID-Reactor denitrification performance was investigated with regard to hydraulic retention time, backflushing intervals and the carbon to nitrogen (C/N) ratio (***Chapter 2***). Finally, the possible replacement of methanol by biodegradable polyhydroxyalkanoate (PHA) plastics in the SID-Reactor was evaluated (***Chapter 3***).

Increasing fish demand

Fish consumption increased from 10 kg per person per year in 1960 to 20 kg per person per year in 2013, ensuring approximately 17% of the global population's animal protein intake (FAO Fisheries and Aquaculture Department, 2016). A further increase in per capita consumption of fish is estimated, as it provides a key source of proteins, essential amino-acids and minerals, important for human nutrition. Hence, fish production has to be enhanced and intensified to satisfy the future demand for fish. However, world capture fisheries stagnate since the 1990s. The increasing demand for fishery products has mainly to be ensured by the increasing aquaculture fish production (FAO Fisheries and Aquaculture Department, 2016). A growth and intensification of production in turn will increase the pressure on present water resources and the environment. The increasing demand for fish cannot be realized using extensive and semi-intensive production systems. The lack of space for expansion, the limited fresh water availability and the tightened wastewater regulations are considered as the main impediments for further expansion of conventional systems (Badiola et al., 2012; Browdy et al., 2012; Dalsgaard et al., 2013) Nevertheless, the growth of the aquaculture sector in a sustainable manner is a key factor for the sustainability of

global fish production (Merino et al., 2012). As a consequence, sustainable aquaculture has to utilize natural resources, such as space and water, in the most optimal way.

Aquaculture contains the potential to enhance resilience of the global food system with the application of super-intensive RAS. These systems have a high degree of recirculation of up to 99.6% and use as little as 300 litres or less of water per kilo of fish produced, involving sludge treatment and denitrification systems (Bregnballe, 2015). Using these super-intensive systems allows the greatest benefit of the available space and water resources. The diversification of aquaculture systems by farming a wide range of aquatic species contributes important elements of stability to the food production sector (Troell et al., 2014). A total of 580 aquatic species, including 362 finfish species, were farmed worldwide in 2014 (FAO Fisheries and Aquaculture Department, 2016). All in all, aquaculture can contribute to a sustainable food production system, with the effective resource use, diversification of suitable farmed species, well adapted fish feeds, and species-appropriate aquaculture production systems (Troell et al., 2014).

New fish species in recirculating aquaculture systems

Efforts are made to explore the biological and socio-economic potential of new fish species for the expansion of the aquaculture industry (www.diversifyfish.eu; Robles and Mylonas, 2017). To ensure the best environmental conditions during the production of a respective fish species, RAS provide several advantages. The wide independence of location, improved hygiene management, good monitoring of most husbandry parameters, reduced water usage, nutrient recycling, and controlled waste management allow species-appropriate fish farming. Furthermore, the separation of RAS from the environment allows for a sustainable and environmentally friendly production of fish. With the aim to benefit from the mentioned advantages marine fish species, usually farmed in sea cages, are also farmed in RAS (Blancheton, 2000; Martins et al., 2010). One of the relevant marine species farmed in RAS is the European sea bass and was therefore chosen as a model organisms in the experiments of ***Chapter 1*** and ***Chapter 2***.

However, production of fish in RAS bears several challenges. For instance, the low water replacement rates may lead to the accumulation of metabolic products of fish and bacteria in the rearing water (Deviller et al., 2004; Schram et al., 2014). Amongst others, steroid hormones (e.g. cortisol and testosterone), carbon dioxide, nitrate, phosphate, and heavy metals (e.g. arsenic and copper) are proven to accumulate in water (Martins et al., 2010; Mota et al., 2017, 2014; Steinberg et al., 2017). These and

other metabolic products can impair fish growth and health at species specific concentrations. For nitrate, a metabolic end product of the microbial nitrification, the negative impact at species related concentrations was already proven for several fish species (Davidson et al., 2014; Good et al., 2017; Schram et al., 2014, 2012; van Bussel et al., 2012). Still, the pathways of nitrate uptake of fish is still unclear and should be clarified as mentioned by Davidson et al. (2014) and van Bussel et al. (2012). However, until now only little is known about the influence of nitrate on performance parameters and the health status of sea bass. Obvious, if European sea bass are produced intensively in RAS, they are exposed to elevated nitrate levels compared to conventional production systems such as sea cages. Therefore, it is necessary to determine how and at which concentration nitrate impacts sea bass. Consequently, ***Chapter 1*** focuses on the impact of four tested nitrate levels on production and health parameters of on-growing European sea bass. The results of the experiment have shown a significant negative correlation of hepato-somatic index and daily feed intake with increasing nitrate levels. Furthermore, for specific growth rate, and total mortalities a trend towards decreasing specific growth rate and increasing mortalities was obvious at high nitrate levels. However, compared to other species nitrate has a low impact on European sea bass production performance and health. Therefore it could be concluded that on-growing sea bass are rather insensitive to nitrate and thus suitable for RAS production.

Additionally to the nitrate exposure experiment in ***Chapter 1*** an extensive comparison of current literature demonstrated that the negative effect of nitrate is dependent on abiotic factors like duration of exposure. For fish production in RAS the long term toxicity is of high relevance, since the cultivated fish species is exposed to potential contaminants over the long term production cycle. Moreover, contaminant substances can accumulate in RAS over multiple production cycles over years and reach not naturally occurring concentrations. Hence, the introduction of new fish species should be accompanied by the evaluation of threshold values for the most important water bound chemical compounds based on data from long term experiments rather than LC50 values. Consequently, the experiment of ***Chapter 1*** was performed in a long term experimental trial over 110 days.

As discussed in ***Chapter 1*** the negative effect of nitrate is also dependent on biotic factors like fish species, life stage, and fish size. Hence, further research is necessary to determine threshold values for different life stages of the cultivated species separately. Additionally, cross-serial dependencies to other parameters (toxicants, water quality, initial health, etc.) can amplify or weaken negative effects

(Wendelaar Bonga, 1997). In this context it would be highly interesting to clarify the impact of salinity on nitrate tolerance of fish, as reliable data is missing. All in all, to clarify the impact of nitrate on sea bass and other fish species in RAS, experiments under production conditions covering a full production cycle would be necessary.

However, research should not only determine at which concentration a chemical compound impairs fish health and growth, but research should also contribute to a solution for these problems.

Denitrification in next generation recirculating aquaculture systems

During the production of fish in RAS, several chemical compounds are well-known to impair fish health. However, not only animal welfare concerns, but also the economic loss are important for commercial production. Growth reduction and increased mortalities of the cultivated fish species are equal to an economic loss. Furthermore, tightened wastewater regulations restrict the production capacity of conventional systems (Dalsgaard et al., 2013). An appropriate technical solution should be readily provided by the system designer or should be available as a technical upgrade. Research groups contribute to knowledge gain for removal of phosphate/phosphorous (Barak et al., 2003; Barak and Van Rijn, 2000), carbon dioxide (Moran, 2010; Summerfelt et al., 2000), heavy metals (Jung and Lovitt, 2011), total organic carbon (Mook et al., 2012), humic acids (Kourdali et al., 2014), therapeutics (Aitcheson et al., 2000), sludge and solids in general (Cripps and Bergheim, 2000; Summerfelt et al., 1999), and others.

As discussed in ***Chapter 1*** nitrate removal is crucial, in the case of nitrate sensitive fish species or life-stages. With this objective ***Chapter 2*** deals with nitrate removal of the novel **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor), developed and patented by Müller-Belecke and Spranger (2014). Based on the results of ***Chapter 1*** sea bass were chosen as a model organisms for the experiments. Particularly because sea bass demonstrated to be less sensitive towards nitrate compared to other species, sea bass was a qualified fish species for the experiments of ***Chapter 2***. Using sea bass it was assumed that animal welfare aspects during the trials were affected as little as possible regarding nitrate exposure. Prove of the SID-Reactor denitrification concept was already reported by Müller-Belecke et al. (2013). In this thesis experiments were performed to evaluate the influence of changing basal operating parameters on water quality and denitrification performance of the SID-Reactor. The overall performance of

biofilters in RAS is affected amongst other by the hydraulic retention time (HRT) (Addy et al., 2016; Lepine et al., 2016; Oh et al., 2001; Timmermans and van Haute, 1983; Wang and Chu, 2016) and backflushing of the filter substrate against clogging (Alonso et al., 1997; Eding et al., 2006; Lepine et al., 2016; Mara et al., 2003; McMillan et al., 2003; Moretti et al., 1999; Rakocy et al., 2006; Sastry et al., 1999). Results of ***Chapter 2*** demonstrate that ideal hydraulic retention time differed among optimized denitrification efficiency (HRT of 6 hours) and denitrification rate (HRT of 2 hours). Backflushing intervals (BFI) every 30 to 60 minutes prevented biofilter clogging and resulted in denitrification efficiency up to 84%.

Next to HRT and BFI settings the addition of external carbon sources is essential for heterotrophic denitrification in RAS aquaculture (Lampe and Zhang, 1996). However, inaccurate dosage results in deterioration of RAS water (Hamlin et al., 2008; Kaviraj et al., 2004; Sauthier et al., 1998; Timmermans and van Haute, 1983; Yang et al., 2012). The correct dosage of the carbon source determines whether a denitrification unit influences fish production positive or negative. As described in ***Chapter 2*** the C/N ratio was determined by segmented linear regression and revealed a methanol requirement of 2.3 mg MeOH per 1.0 mg NO_3^--N, when considering common water quality parameters in RAS.

By adjustment of the basal operating settings and technical improvements the nitrate removal of the SID-Reactor was nearly doubled from 451 g NO_3^--N per m^3 moving bed volume and day reported by (Müller-Belecke et al., 2013) up to 870 g NO_3^--N per m^3 moving bed volume achieved within this thesis. Furthermore, the evaluation of basal operating parameters helped to guarantee stable, efficient, and safe application of the SID-Reactor in RAS. Moreover, the results are also valuable for the operation of other denitrification units.

Polyhydroxyalkanoate in the SID-Reactor

As reported in ***Chapter 2***, one of the biggest obstacles identified by researchers and confirmed by producers, is the negative impact of inaccurate dosage of the mandatory carbon source (Hamlin et al., 2008; Kaviraj et al., 2004; Sauthier et al., 1998; Timmermans and van Haute, 1983; Yang et al., 2012). Therefore a complex control and monitoring of denitrification processes and water quality parameters is indispensable to ensure accurate dosage of the carbon source. In addition, numerous carbon sources are potentially hazardous to fish and RAS staff, highly flammable, and can cause security risks during storage, transportation, and operation. Solid-phase denitrification has proved to be a promising alternative to remove nitrate from water,

excluding the above mentioned obstacles (Hiraishi and Khan, 2003; Horiba et al., 2005; Wang and Chu, 2016). In solid phase denitrification, biodegradable polymers are used as carbon source and biofilm carrier for denitrifying microorganisms at the same time (Wang and Chu, 2016). The design and the operation principle of denitrification systems is usually restricted to a certain type of carbon source, liquid or solid. ***Chapter 3*** demonstrates that the operation principle of the SID-Reactor is, in contrast to conventional denitrification systems, not restricted to either carbon source. During the experimental trials, the removal of nitrate was achieved by using polyhydroxyalkanoate (PHA) in the SID-Reactor. PHA was proven to be a safe and easy to use carbon source, while the of the SID-Reactor shows advantages compared to conventional systems. The combination of PHA and the SID-Reactor could make denitrification a natural part of future RAS. Concerning solid-phase denitrification in the SID-Reactor, future research could enhance the application of biodegradable plastics. Results of ***Chapter 3*** show that three topics need to be improved using PHA granulate as a carbon source.

First of all, costs of the PHA granulate have to be reduced in order to make it economically more attractive. Substrate costs for PHA were 3.4 times higher compared to methanol within this study. The costs of the carbon sources used are not fully representative for economically working RAS, since bulk purchase in commercial RAS is more profitable. Nevertheless, raw material prices are significantly higher for biodegradable plastics compared to conventional carbon sources. Although as stated in ***Chapter 3***, to compare the actual costs of methanol fuelled SID-Reactor with a PHA fuelled SID-Reactor not only the price of substrates has to be taken into account, but also the costs for process control, labour costs for maintenance, costs for safety precautions, and others. Reducing production cost for biodegradable plastics would consequently also reduce operation costs. Nevertheless, an assessment of all factors of costs would show the real expenses for denitrification using biodegradable plastics.

Second, the denitrification efficiency while using PHA as a carbon source could be improved by compounding different substances. Results of ***Chapter 3*** revealed a denitrification efficiency of approximately 40% for the PHA fuelled SID-Reactor. This is low compared to the denitrification efficiency of 98% observed for the methanol fuelled SID-Reactor and compared to the denitrification efficiencies reported in other studies summarized by Wang and Chu (2016). The low denitrification efficiency could be the result of a slow biodegradation by bacteria. Chemical structure, molecular weight, glass transition temperature, melting temperature, elasticity, crystallinity, surface area, hydrophilic, and hydrophobic properties are chemical and physical properties

influencing biodegradation (Tokiwa et al., 2009). Compounding different groups of substances can result in new physical and chemical properties increasing biodegradation (Tokiwa et al., 2009). Targeting particularly denitrification processes in RAS compounding of biodegradable plastics can contribute to customized solutions.

Beside substrate costs and compounding, the third topic should deal with the material surface, which also influences the biodegradation rate. Biodegradation of solid materials occurs on the surface. The biodegradation rate increases with increasing available surface area of the substrate (Chinaglia et al., 2018). Biofilter media, used in RAS applications, is characterized by a high specific surface area (surface area per unit volume, m^2 m^{-3}) where biofilm can be established. Especially the protected surface, where the bacterial film is protected from abrasion, is important in this context. Conventional biofilter media presents a very complex structure (*Figure GD - 1A*). In contrast, the used PHA granulate has no protected surface area, due to its cylindrical structure (*Figure GD - 1B*). Hence, colonization by bacteria is not optimal. Processing of PHA to a complex structure could increase denitrification by biodegradation, due to increased protected specific surface area.

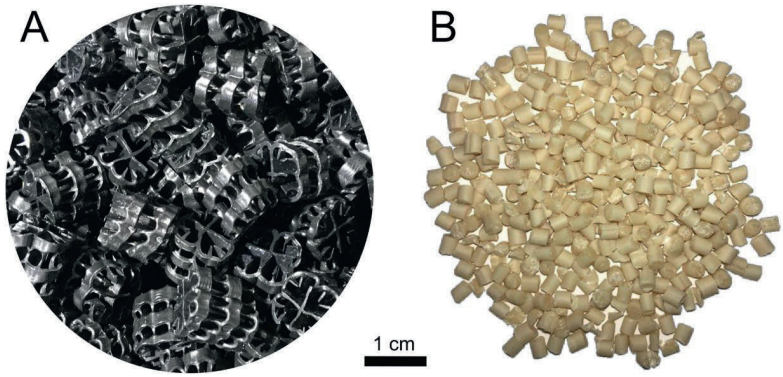

Figure GD - 1. *Biofilter media used in this thesis. A, Conventional biocarriers (HEL-X®, diameter: 12 mm, Christian Stöhr GmbH & Co. Elektro- und Kunststoffwaren KG, Marktrodach, Germany). B, polyhydroxyalkanoat (PHA) granulate (VVK Vertrieb Veredelter Kunststoffe GmbH, Siegburg, Germany).*

However, not only the available surface area has an influence on bacterial attachment, but also the surface structure. New PHA granulate has a rather smooth surface with small unevenness (*Figure GD - 2A*), whereas used PHA granulate has cavities caused by bacterial degradation (*Figure GD - 2B*). These cavities possibly contribute to increased bacterial colonization providing protection from abrasion. Hence, processing of PHA granulate resulting in a rough surface should be favoured. As reported in ***Chapter 3*** final denitrification efficiency of the PHA fuelled SID-Reactor was reached after a lag-time. This delay was explained by the natural course of bacterial growth. However, the formation of cavities could possibly promote bacterial growth and should be investigated in more detail in the future.

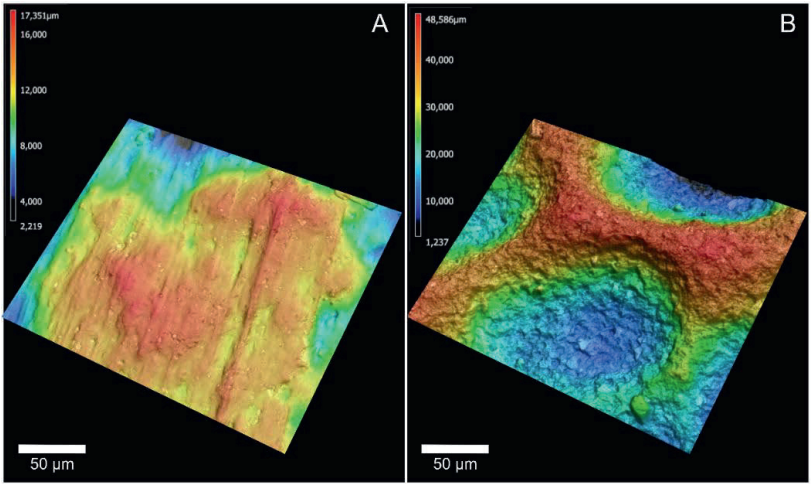

Figure GD - 2. *Surface structure of new (A) and used (B) polyhydroxyalkanoate (PHA) granulate.*

Key factors contributing to a future application of biodegradable plastics in RAS denitrification systems will be cost reduction of the substrate, improvement of chemical properties (compounding), and physical properties (protected surface area and structure). Once those obstacles have been overcome, the SID-Reactor in combination with biodegradable plastics as biofilm carrier and solid carbon source will set a new standard in terms of RAS denitrification.

Knowledge transfer

Most research studies are usually conducted in laboratory systems to generate reliable data. Laboratory systems (short-term, small-scale, and single-factor) can only partially simulate conditions in commercial RAS (long-term, large scale, and multi-factor), thus up-scaling of results is difficult (Colt, 2006; van Rijn et al., 2006; Zhu and Chen, 1999). In this context, a close cooperation between research and industry, involving producers, would be highly recommendable and could accelerate knowledge gain (Badiola et al., 2012).

During this thesis a high degree of knowledge transfer was striven for. Through published journal articles, presentations at conferences, and information events results of this thesis were made accessible to specialists, the interested public and customers of the SID-Reactor. Next to the scientific output the close contact to producers, research facilities, commercial sea-life centres, and the Spranger-Kunststoffe GmbH (project partner and manufacturer of the SID-Reactor) guaranteed a practice-oriented processing of the whole project. Resulting from this close contact and onsite instruction of clients, a SID-Reactor manual was written. The manual consist of different chapters, dealing with general information on SID-Reactor technique and biological background, explaining the start-up process, the influence of operating parameters, maintenance, and troubleshooting. Continuous knowledge gain by experiments and feedback of the clients contribute to a constant improvement of the manual.

Further research

During the experiments a shift in the bacterial community with operation time and in comparison to the aerobe MBBR was observed (unpublished data). A detailed analysis of the bacterial community would be a highly valuable knowledge gain. Having knowledge about the bacterial community and the needed environmental conditions would allow a targeted improvement of removal processes.

Within this thesis, the main focus was the use of the SID-Reactor for nitrate removal. Obviously, bacteria are also capable of metabolic pathways processing other substances from their environment. There are some indications, that the SID-Reactor potentially removes substances (2-methylisoborneol and geosmin) responsible for off-flavour (unpublished data). Furthermore, it would be highly interesting to investigate whether and which other undesirable substances in RAS could be removed by bacteria under anoxic conditions in the SID-Reactor. Some other undesirable substances could be phosphorous, humic acids, and therapeutics.

Another research subject could be the simultaneous nitrification and denitrification (SND) in the SID-Reactor. Walters et al. (2009) demonstrated SND processes in a biological reactor with biodegradable carrier material. An efficient SND process in the SID-Reactor would make an aerobe biofilter system in the RAS superfluous. Simultaneous nitrification and denitrification in one biofilter unit would reduce acquisition and operation cost of a RAS, increasing its profitability.

Conclusion

With the help of the results gained in this thesis knowledge gaps were filled concerning the nitrate toxicity to European sea bass, showing that sea bass is a promising species for RAS production. Furthermore, the SID-Reactor contributes to the advancement of RAS and helps to keep up with the intensification of fish production. In addition, further research could open up new application fields for the SID-Reactor. All in all, the development of state-of-the-art water treatment systems will result in future-oriented RAS, crucial to achieve sustainable growth of aquaculture.

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SUMMARY

The demand for fishery products is increasing constantly since decades. Hence, the future challenge for the fish production industry will be to satisfy this need for fish, a key source of protein, essential amino-acids, and minerals. Because, world capture fisheries stagnate since the 90s, the increasing demand for fishery products has to be ensured predominantly by the aquaculture production.

Present land based aquaculture production systems can be categorized into extensive, semi-intensive, intensive, and super-intensive systems. The latter have the highest production of fish per used water volume and thus use the available space and water resources most efficient. Furthermore, the independence of recirculating aquaculture systems (RAS) from the environment keeps their environmental impact as low as possible and allows the control of all husbandry conditions during fish production. However, production of fish in RAS bears also several obstacles. Modern super-intensive RAS are characterized by a high water re-use coming along with intensification of fish production. The recycling of rearing water results in the accumulation of metabolic end products of fish and bacteria in the water column. One metabolic end product is nitrate, which is formed during biological nitrification process. Nitrate influences various physiological parameters of fish at species related concentrations.

This thesis first evaluates possible effects of nitrate on a marine fish species (***Chapter 1***). Furthermore, nitrate removal using the **S**elf-cleaning **I**nherent gas **D**enitrification Reactor (SID-Reactor) was investigated. To allow stable and efficient nitrate removal using the SID-Reactor, basal operation parameters were evaluated and a deeper understanding of their effects on denitrification and water quality was striven for (***Chapter 2***). Additionally, to exclude the risk of incorrect dosing of potentially hazardous carbon sources, mandatory for heterotrophic denitrification, it was evaluated whether solid biodegradable plastics were suitable as carbon source for the application in the SID-Reactor (***Chapter 3***).

In ***Chapter 1***, the impact of nitrate on performance parameters and health status of on growing European sea bass (*Dicentrarchus labrax*), used as a marine model fish species, reared in RAS was evaluated. On-growing sea bass were exposed to four different nitrate concentrations between 0 and 500 mg L^{-1} in a triplicate experimental design in twelve small-scaled experimental RAS for 10 weeks. At the end of the experiment, final biomass, final individual weight, condition factor, feed conversion ratio, and spleen-somatic index were not significantly affected by nitrate exposure within the concentration range tested. Furthermore, specific growth rate, and total mortalities did not significantly differ between the treatment groups either, although a trend towards decreasing specific growth rate and increasing mortalities at high nitrate levels was visible. The results demonstrate the significant negative correlation of hepato-somatic index and daily feed intake with increasing nitrate levels.

All in all, nitrate significantly impaired only for a few parameters monitored in this study. Hence, the sensitivity of on-growing sea bass towards nitrate toxicity seems to be low compared to other aquaculture fish species. However, in the case of fish species or life-stages more sensitive towards nitrate, a nitrate removal via denitrification is essential.

In ***Chapter 2***, nitrate removal in a marine RAS using the SID-Reactor was investigated. Within a consecutive experimental approach, effects of varying hydraulic retention time (HRT), backflushing intervals (BFI), and carbon to nitrogen (C/N) ratios on water quality parameters and denitrification performance were monitored. During the first test series three different HRTs were tested. The experiments revealed that a HRT of 2 hours resulted in the highest denitrification rate (497 g d^{-1} m^3 biocarriers) but a lower denitrification efficiency of 64%. A HRT of 6 hours had highest denitrification efficiency of 81% but a lower denitrification rate (253 g d^{-1} m^3 biocarriers). During the second test series, four BFIs were evaluated. It was evident that backflushing intervals every 10 minutes resulted in a decreased denitrification efficiency of 29%, while intervals every 90 minutes increased the maintenance effort. Overall, backflushing intervals every 30 and 60 minutes showed the best results. During the third test series, seven carbon to nitrogen ratios were evaluated using methanol as a carbon source. The results indicated that a C/N ratio of 2.3 was sufficient to ensure an optimal denitrification performance, incorporating all single tested water quality parameters. However, incorrect carbon dosing resulted in deterioration of water quality.

In ***Chapter 3***, the replacement of methanol as a liquid carbon source by biodegradable plastic granulate made of polyhydroxyalkanoate (PHA) used as biofilm carrier and as solid carbon source was investigated. In the experiment, the SID-Reactor was used in freshwater RAS stocked with pikeperch (*Sander lucioperca*). Three RAS (1 m^3) were used for the trial. The first RAS was operated as control without a denitrification system. The second RAS was operated with a SID-Reactor fuelled with methanol as external carbon source and the third RAS was operated with a SID-Reactor fuelled with PHA granulate functioning as biofilm carrier and carbon source at the same time. For all three RAS, water quality was documented and compared to each other. Furthermore, the denitrification performance of the PHA and methanol fuelled SID-Reactors were compared to each other. Values for turbidity, total ammonia, nitrite, and total organic carbon were lower in the rearing water when PHA was used in the SID-Reactor compared to methanol. Furthermore, both denitrification units caused an increase in alkalinity and pH, resulting in an overall 50% saving of alkalinity supplements in contrast to RAS without denitrification. Using PHA as a solid carbon source in the SID-Reactor resulted in a maximum denitrification efficiency of 40% nitrate removal. Using methanol as a carbon source resulted in a maximum denitrification efficiency of up to 98% nitrate removal. However, no significant differences were observed between the denitrification rate for the PHA and the methanol fuelled SID-Reactors. The results demonstrate that the functional principle of the SID-Reactor, usually fuelled with a liquid carbon source, is also suitable for the application with a solid carbon source.

This study revealed a low impact of nitrate on production performance and health of on-growing sea bass. For production of more nitrate sensitive fish species, the SID-Reactor is an appropriate denitrification unit. Safe, efficient and easy to handle denitrification was demonstrated using the SID-Reactor. Based on the results of this thesis and the accompanying general technical improvements, denitrification performance of the SID-Reactor was enhanced. Additionally, general maintenance effort can be reduced if the results of this thesis are taken into account. Taking the application of the SID-Reactor to the next step, biodegradable plastics were proven as a suitable carbon source and biofilm carrier at the same time. The lower denitrification efficiency with PHA is negligible since the operation of the SID-Reactor is predominant. The use of biodegradable plastics in the SID-Reactor is a forward-looking and state-of-the-art approach for water treatment in RAS.

ZUSAMMENFASSUNG

Seit vielen Jahren ist ein steigender Bedarf an Fischereiprodukten zu verzeichnen. Die zukünftige Aufgabe der Fischindustrie wird es sein, diesen Bedarf an Fisch, eine wichtige Proteinquelle, reich an essenziellen Aminosäuren und Mineralstoffen, zu decken. Die weltweiten Erträge der Fangfischerei stagnieren jedoch seit den 90iger Jahren. Somit muss der wachsende Bedarf an Fischereiprodukten durch Fisch aus der Aquakultur gedeckt werden.

Aktuelle landbasierte Aquakultur-Produktionssysteme können in extensive, semi-intensive, intensive und super-intensive Systeme kategorisiert werden. Die letzteren zeichnen sich durch den höchsten Fischertrag pro genutztem Wasservolumen aus. Somit nutzen super-intensive Kreislaufanlagen (KLA) die verfügbaren Flächen und Wasserressourcen am effizientesten. Des Weiteren können durch die Isolation der KLA von der Umwelt, alle relevanten Haltungsparameter während der Fischproduktion kontrolliert und beeinflusst werden. Durch die Isolation wird der Einfluss von KLA auf die Umwelt außerdem auf ein Mindestmaß reduziert. Der Einsatz von KLA weist jedoch auch einige Hindernisse auf. Moderne super-intensive KLA sind durch eine intensive Fischproduktion und hohe Wasserwiederverwertung gekennzeichnet. Durch das Wiederverwenden von Anlagenwasser können sich jedoch Stoffwechselendprodukte von Fischen und Bakterien im Haltungswasser anreichern. Ein Stoffwechselendprodukt, das während der biologischen Nitrifikation gebildet wird, ist Nitrat. Sobald die Nitratkonzentration Art-spezifische Grenzwerte übersteigt, können physiologische Parameter von Fischen negativ beeinflusst werden.

In der vorliegenden Arbeit wurde daher zu Beginn untersucht, in welchem Ausmaß Nitrat eine marine Fischart beeinflussen kann (***Kapitel 1***). Des Weiteren wurde die Nitrat Entfernung durch den **S**elbstreinigenden **I**nertgas **D**enitrifikations Reaktor (SID-Reaktor) untersucht. Um einen stabilen und effizienten Nitratabbau zu gewährleisten wurden dabei grundlegende Betriebsparameter untersucht und beurteilt (***Kapitel 2***). Zudem wurde ein umfassendes Verständnis der Betriebsparametereffekte auf Denitrifikationsleitung und Wasserqualität angestrebt. Bei der heterotrophen Denitrifikation ist die Zudosierung einer Kohlenstoffquelle unerlässlich. Um das Risiko einer Fehldosierung dieser teilweise toxischen Kohlenstoffquellen auszuschließen, wurde der mögliche Einsatz eines biologisch abbaubaren Kunststoffes untersucht (***Kapitel 3***).

In ***Kapitel 1*** wurden die Auswirkungen von Nitrat auf Leistungs- und Gesundheitsparameter von Europäischen Wolfsbarschen (*Dicentrarchus labrax*) untersucht. Die Wolfsbarsche dienten dabei als eine Aquakultur-relevante, marine Modelfischart, die in KLA produziert wird. In 12 klein-skalierten, experimentellen KLA wurden die Wolfsbarsche in einem triplikaten Versuchsansatz für 10 Wochen vier unterschiedlichen Nitratkonzentrationen zwischen 0 und 500 mg L^{-1} ausgesetzt. Am Ende des Versuches waren Endgewicht, Endlänge, Konditionsfaktor, Futterverwertung und der spleen-somatic Index durch die getesteten Nitratkonzentrationen weitestgehend unbeeinflusst. Des Weiteren waren spezifische Wachstumsraten und Mortalität ebenfalls nicht signifikant unterschiedlich zwischen den Versuchsgruppen. Jedoch war bei hohen Nitratkonzentrationen ein Trend zu geringeren spezifischen Wachstumsraten und steigender Mortalität zu erkennen. Die Ergebnisse in ***Kapitel 1*** zeigen eine signifikant negative Korrelation von hepato-somatic Index und Futteraufnahme in Zusammenhang mit steigenden Nitratkonzentrationen.

In dieser Studie konnte ein negativer Effekt von Nitrat für wenige der untersuchten Parameter dokumentiert werden. Somit kann die Empfindlichkeit von Wolfsbarschen gegenüber Nitrat, im Vergleich zu anderen Fischarten, als gering eingeschätzt werden. Dennoch kann im Fall von empfindlicheren Entwicklungsstadien oder anderen Fischspezies, eine Nitrat Entfernung durch Denitrifikationsreaktoren unabdingbar sein.

In ***Kapitel 2*** wurde die Nitrat Entfernung durch den SID-Reaktor in einer marinen KLA untersucht. Die Effekte von unterschiedlichen hydraulischen Retentionszeiten (eng.: hydraulic retention time; HRT), Rückspül-Intervallen (eng.: backflushing intervals; BFI) und Kohlenstoff- zu Stickstoffverhältnissen (C/N Verhältnis) wurden in einem konsekutiven experimentellen Ansatz im Hinblick auf Wasserqualität und Denitrifikationsleistung untersucht. Während der ersten Testserie wurden drei unterschiedliche HRTs geprüft. Es konnte gezeigt werden, dass eine HRT von 2 Stunden in höchster absoluter Denitrifikationsrate (497 g d^{-1} m^3 Aufwuchskörper), aber in geringer relativer Denitrifikationseffizienz von 64% resultierte. Eine HRT von 6 Stunden hatte hingegen die höchste relative Denitrifikationseffizienz von 81%, aber eine niedrigere absolute Denitrifikationsrate (253 g d^{-1} m^3 Aufwuchskörper) zur Folge. Während der zweiten Testserie wurden vier unterschiedliche BFIs untersucht. Es war ersichtlich, dass ein BFI alle 10 Minuten in einer geringen Denitrifikationseffizienz von 29% resultierte, wobei ein BFI alle 90 Minuten in einem gestiegenen Wartungsaufwand resultierte. Insgesamt zeigten BFIs alle 30 bis 60 Minuten die besten Ergebnisse. Während der dritten Testserie wurden sieben C/N Verhältnisse getestet, wobei Methanol als Kohlenstoffquelle genutzt wurde. Die Ergebnisse zeigen, dass ein C/N

Verhältnis von 2.3 ausreichend war, um eine optimale Denitrifikationsleistung zu erzielen. Eine Über- und Unterdosierung führte hingegen zu einer Verschlechterung der Wasserqualität.

In ***Kapitel 3*** wurde untersucht, ob Methanol als flüssige Kohlenstoffquelle durch einen biologisch abbaubaren Kunststoff aus Polyhydroxyalkanoat (PHA) ersetzt werden kann. Das genutzte PHA Granulat fungierte dabei gleichzeitig als Aufwuchskörper und feste Kohlenstoffquelle. Der Versuch wurde mit drei Süßwasser-KLA (1 m^3), besetzt mit Zandern (*Sander lucioperca*), durchgeführt. Die erste KLA fungierte als Kontrolle und wurde ohne ein Denitrifikationssystem betrieben. Die zweite KLA wurde mit einem Methanol betriebenen SID-Reaktor ausgestattet. An die dritte KLA wurde ein PHA betriebener SID-Reaktor angeschlossen. Während des Versuches wurden Wasserqualitätsparameter von allen drei KLA dokumentiert und verglichen. Des Weiteren wurde die Denitrifikationsleistung der zwei SID-Reaktoren verglichen. Wenn PHA als Kohlenstoffquelle genutzt wurde, waren Werte für Trübung, Gesamtammonium, Nitrit und für gesamt organischen Kohlenstoff geringer. Außerdem hatte der Einsatz von beiden SID-Reaktoren im Vergleich zur Kontrolle einen Anstieg der Alkalinität und des pH-Wertes zur Folge, sodass eine 50%ige Einsparung von Alkalinitätssupplementen möglich war. Beim Gebrauch von PHA als Kohlenstoffquelle wurde eine relative Denitrifikationseffizienz von 40% Nitratentfernung erzielt. Beim Einsatz von Methanol als Kohlenstoffquelle wurde eine relative Denitrifikationseffizienz von 98% Nitratentfernung erzielt. Im Hinblick auf die absolute Denitrifikationsrate wurden jedoch keine signifikanten Unterschiede zwischen dem PHA und dem Methanol betriebenen SID-Reaktor festgestellt. Grundsätzlich zeigen die Ergebnisse aus ***Kapitel 3***, dass das Funktionsprinzip des SID-Reaktors, der üblicherweise mit einer flüssigen Kohlenstoffquelle betreiben wird, auch für den Einsatz mit einer festen Kohlenstoffquelle geeignet ist.

Zusammenfassend zeigt diese Arbeit, dass der Europäische Wolfsbarsch eine eher Nitrat unempfindliche Spezies ist. Bei der Produktion von empfindlicheren Fischarten ist der Einsatz eines SID-Reaktors als angemessen zu beurteilen. Der Reaktor ist sicher und leicht zu bedienen, wobei eine effiziente Nitrat Elimination erzielt werden kann. Des Weiteren konnte die Denitrifikationsleistung durch die Ergebnisse dieser Arbeit und durch technische Anpassungen verbessert werden. Wenn die Ergebnisse dieser Arbeit berücksichtigt werden, kann der allgemeine Wartungsaufwand des SID-Reaktors zusätzlich reduziert werden. Um den Einsatz des SID-Reaktors auf den letzten Stand der Forschung zu bringen, wurde ein biologisch abbaubarer Kunststoff als geeignete Kohlenstoffquelle und als Aufwuchskörper eingesetzt. Auch wenn die

Denitrifikationseffizienz beim PHA im Vergleich zum Methanol betriebenen SID-Reaktor geringer war, zeigte der Einsatz von PHA erhebliche Vorteile, die als richtungsweisend für die Zukunft zu bewerten sind.

APPENDIX - METHODOLOGY OF WATER ANALYTICS

1 Spectrophotometrically measurements

Determination of the nitrogen compounds ammonia nitrogen, nitrite nitrogen, and nitrate nitrogen were performed spectrophotometrically using the DR 2800 photometer (Hach-Lange GmbH, Berlin, Germany)

1.1 Ammonium nitrogen

Ammonia nitrogen (NH_3-N) was analysed according to the powder pillow procedure by (Hach Company, 2015a) based on the Salicylate Method. During the reaction ammonia compounds form, combined with chlorine, monochloramine. Monochloramine in turn reacts with salicylate and forms 5-aminosalicylat, which is oxidized with the help of sodium nitroprusside catalyst and forms a blue-coloured compound. The blue colour results in combination with the yellow colour from the excess reagent in a final green-coloured solution. The spectrophotometrically measurement takes place at a wavelength of 655 nm.

1.2 Nitrite nitrogen

Nitrite nitrogen (NO_2^--N) was analysed according to the powder pillow procedure by (Hach Company, 2015b) based on the USEPA Diazotization Method. During the reaction nitrite reacts with sulfanilic acid and forms an intermediate diazonium salt. This salt reacts with chromotropic acid resulting in a pink coloured complex, proportional to the amount of nitrite present in the water sample. The spectrophotometrically measurement takes place at a wavelength of 520 nm.

1.3 Nitrate nitrogen

Nitrate nitrogen (NO_3^--N) was analysed according to the powder pillow procedure by (Hach Company, 2015c) based on the Cadmium Reduction Method. During the reaction cadmium metal reduces nitrate in the water sample to nitrite. The nitrite reacts with sulfanilic acid in an acidic medium and forms an intermediate diazonium salt. This salt reacts with gentisic acid resulting in an amber coloured complex. The spectrophotometrically measurement takes place at a wavelength of 400 nm.

2 Determination of carbon and nitrogen compounds

A TOC-Analyser equipped with an auto sampler (TOC-LCPH/CPN PC-Controlled Model, Shimadzu Deutschland GmbH, Duisburg, Germany) was used to determine carbon and nitrogen containing compounds in water samples. With the TOC-Analyser following carbon compounds can be determined: total carbon (TC), inorganic carbon (IC), total organic carbon (TOC), dissolved carbon (DC), dissolved inorganic carbon (DIC), dissolved organic carbon (DOC), purgeable organic carbon (POC), and non-purgeable organic carbon (NPOC). Furthermore nitrogenous compounds can be determined in form of total nitrogen (TN). The TOC-Analyser applies the combustion catalytic oxidation method at 720°C with a detection range of 4 µg L^{-1} to 30,000 mg L^{-1}. The catalytic oxidation method ensures the efficient oxidation of not only easily decomposable, low-molecular-weight organic compounds, but also hard to decompose insoluble and macromolecular organic compounds.

The following section (2.1 PRINZIPLES OF ANALYSIS) is quoted from the TOC-Analyser user's manual (Shimadzu Corporation, 2010).

2.1 Principles of analysis

Two types of carbon are present in water: organic carbon and inorganic carbon. Organic carbon (TOC) bonds with hydrogen or oxygen to form organic compounds. Inorganic carbon (IC or TIC) is the structural basis for inorganic compounds such as gas carbonates and carbonate ions. Collectively the two forms of carbon are referred to as total carbon (TC) and the relationship between them is expressed TOC=TC-IC. Nitrogen is also present in water in two types: organic and inorganic. The sum of these is referred to as total nitrogen (TN). The principles underlying TC and TN analysis are explained in the following sections.

2.1.1 Principles of total carbon analysis

Sample is introduced into the total carbon (TC) combustion tube, which is filled with an oxidation catalyst and heated to 680°C. The sample is burned in the combustion tube and, as a result, the TC components in the sample are converted to carbon dioxide. Carrier gas, which flows at a rate of 150 mL min-1 to the combustion tube, carries the sample combustion products from the combustion tube to an electronic dehumidifier, where the gas is cooled and dehydrated. The gas then carries the sample combustion products through a halogen scrubber to remove chlorine and other halogens. Finally, the carrier gas delivers the sample combustion products to the cell of a non-dispersive infrared (NDIR) gas analyser, where the carbon dioxide is detected. The NDIR outputs

an analogue detection signal that forms a peak; the peak area is measured by the TOC-Control L software.

The peak area is proportional to the TC concentration of the sample. A calibration curve equation that mathematically expresses the relationship between peak area and TC concentration can be generated by analysing various concentrations of a TC standard solution. The TC concentration in a sample can be determined by analysing the sample to obtain the peak area and then using the peak area in the calibration curve equation.

2.1.2 Principles of inorganic carbon analysis

Two methods for measuring inorganic carbon (IC) using the TOC-L are available: analysis within the injection syringe and analysis using the optional IC reactor. In both methods, the measured IC consists of carbon derived from carbonates, hydrogen carbonates and dissolved carbon dioxide.

Defining IC

The IC measured by TOC analysis consists of the carbon contained in carbonates and in carbon dioxide dissolved in water. By acidifying the sample with a small amount of hydrochloric acid to obtain a pH less than 3, all carbonates are converted to carbon dioxide (CO_2) by the following reactions:

$Me_2CO_3 + 2HCl \rightarrow CO_2 + 2MeCl + H_2O$

$MeHCO_3 + HCl \rightarrow CO_2 + MeCl + H_2O$

Carbon dioxide and dissolved carbon dioxide in the sample are volatilized by bubbling (sparging) air or nitrogen gas that does not contain carbon dioxide through the sample.

2.1.3 Analysis using the IC reaction vessel (H type instrument)

The TOC-L IC reactor kit is used to sparge the IC reaction solution (acidified reaction liquid) with carrier gas. Sample is injected into the IC reaction vessel and the IC in the sample is converted to carbon dioxide, which is volatilized by the sparging process and detected by the NDIR.

2.1.4 Analysis within the syringe (N type instrument)

The sample is acidified to pH 3 or lower in the syringe, using hydrochloric acid. The sample is sparged with carrier gas and the IC in the sample is converted to carbon dioxide and detected by the NDIR.

2.1.5 Principles of Non-Purgable Organic Carbon analysis

After acidifying the sample to pH 2 to 3, sparge gas is bubbled through the sample to eliminate the IC component. The remaining TC is measured to determine total organic carbon, and the result is generally referred to as TOC. However, in the TOC-L, this analysis value is referred to Non-Purgeable Organic Carbon (NPOC) to distinguish it from the TOC value obtained by calculating the difference between TC and IC. NPOC stands for non-purgeable organic carbon and refers to organic carbon that is present in a sample in a non-volatile form.

NPOC and TOC (obtained by IC elimination) described in the TOC-related standard methods and referred to in water quality-related test methods (JIS, ASTM, EPA, EN) are identical. Purgeable organic substances in the sample can be lost during the sparging process. Consequently, when the sample contains purgeable organic substances, TOC should not be measured by the NPOC method. If the dissolved purgeable organic component in the water sample is large, the amount volatilized during sparging is relatively small. Generally, the amount of purgeable organic substances in natural environmental, public and purified water is small; as a result, NPOC can be referred to as TOC.

2.1.6 Principles of Purgeable Organic Carbon

Purgeable Organic Carbon (POC) analysis is used to measure the volatilized component of TOC, which is produced during the NPOC sample sparging process. As a result, NPOC+POC is equivalent to TOC. POC analysis is performed as follows.

Sparge gas containing the volatilized CO_2 and POC components of the sample is carried to the lithium hydroxide-filled CO_2 absorber to eliminate the CO_2 that was converted from the IC in the sample. The gas, which now contains only the POC component of the sample, then passes through the combustion tube to be oxidized. The POC component is convertedto CO_2 during oxidation and the CO_2 is detected by the NDIR. Data processing is conducted in the same manner as for TC.

POC is not precisely defined. Factors that determine whether or not, or to what degree, a volatile organic carbon component is volatilized during sparging include the type of organic compound, the gas/liquid contact with the sparge gas, and the ambient temperature during sparging. The target of POC analysis is the organic component present in the aqueous phase. As a result, organic compounds that are highly soluble in water (such as methanol or ethanol) and not easily volatilized by sparging produce almost no peaks in POC analysis.

Organic compounds with low solubility in water (such as methylene chloride or chloroform) produce sharp peaks in POC analysis. Over a long period of time, compounds such as acetone and methyl isobutyl ketone generate extremely broad, tailing peaks with no specific end time.

The lithium hydroxide-filled CO_2 absorber eliminates the carbon dioxide that was generated along with the POC substances. Therefore, POC components that are easily caught in the CO_2 absorber (such as esters) produce low values in POC analysis. POC results obtained using this technique are not absolute. The user should take all the above factors into consideration when measuring POC using this instrument.

2.1.7 Principles of measuring Total Organic Carbon

Total Organic Carbon (TOC) can be measured using the following 3 methods:

- TC-IC Method
- NPOC Method
- POC+NPOC Method

TC-IC Method

In the TC-IC method, TOC is measured as the difference between the TC and IC analysis values. The TC-IC method is not recommended for samples that contain more IC than TOC (samples where TC consists almost entirely of IC); the NPOC method is recommended for such samples. The TOC value determined using the TC-IC method includes errors associated with each of the individual TC and IC measurements, and can therefore result in a large error in the TOC value.

Because detection accuracy decreases with increasing IC concentration, the NPOC method is also recommended for samples containing IC concentrations exceeding 10ppm for TOC/TN catalyst and 5ppm for high sensitivity catalyst.

NPOC Method

The NPOC method is the most widely used TOC analysis method. The NPOC method is not recommended for samples that foam during sparging. Samples that become foamy during sparging tend to form bubbles that flow out of the syringe, which removes the concentrated TOC sample components and leaves a small amount of TOC in the syringe. The TC-IC method is recommend in this situation.

Moreover, since TOC cannot be correctly measured in samples that coagulate or precipitate when acidified, use the TC-IC method.

POC+NPOC Method

The POC+NPOC method should be used when the amount of POC present in the sample cannot be disregarded.

2.1.8 Principles of measuring Total Nitrogen

When a sample is introduced into the combustion tube (furnace temperature 720°C), the Total Nitrogen (TN) in the sample decomposes to nitrogen monoxide. Nitrogen gas does not become nitrogen monoxide under these circumstances. The carrier gas, which contains the nitrogen monoxide, is cooled and dehumidified by the electronic dehumidifier. The gas then enters a chemiluminescence gas analyser, where the nitrogen monoxide is detected. The detection signal from the chemiluminescence gas analyser generates a peak and the TN concentration in the sample can then be measured.

3 Other water chemical parameters

3.1 Oxygen

For dissolved oxygen measurement a hand-held oxygen meter was used (Handy Polaris, OxyGuard International A/S, Farum, Denmark). The probe is of the Mackareth cell type and consists of a two-electrode electrochemical cell with a thin gas-permeable membrane separating the electrodes and the electrolyte solution from the analyzed media. During measurement oxygen diffuses through the membrane. The electrochemical reduction of oxygen generates a potential of the working electrode. The sensor current is proportional to the oxygen concentration in the water and is processed by the hand-held unit to an output value.

3.2 Oxidation-Reduction Potential

For the measurement of oxidation-reduction potential (ORP) a hand-held ORP meter was used (GMH 5550, GHM Messtechnik GmbH, Regenstauf, Germany). The ORP indicates that the sample being measured has an oxidizing or reducing effect relative to the hydrogen normal electrode. The measurement is carried out with the widespread silver / silver chloride electrodes, a reference system with 3 molar potassium chloride solution.

3.3 Salinity

Practical salinity was measured by a refractometer (Refractometer HI 96822, HANNA Instruments, Woonsocket, USA). The salinity is determined by measuring the refractive index of seawater. The refractive index is an optical property of a substance and the number of particles dissolved therein. The refractive index is defined as the ratio of the speed of light in empty space to the speed of light in the substance. In HI 96822, the light from an LED passes through a prism that is in contact with the sample. An image sensor determines the critical angle at which the light no longer breaks through the sample. The refractometer converts the refractive index of the sample into one of the common salinity units.

3.4 pH

The pH was measured by a hand-held pH meter (SenTix®41, WTW pH 3310, Xylem Analytics Germany Sales GmbH & Co.KG, Weilheim, Germany). The determination of the pH was done by the potentiometric method. This method uses the electrical potential of pH-sensitive electrodes (hydrogen-, metal- and glass- electrodes) as a

measurement signal. The sensor current is processed by the hand-held unit to an output value.

3.5 Acid Neutralizing Capacity

The acid neutralising capacity (ANC) of the water was determined by the method of titration according to (Radtke et al., 1998). ANC is defined as the equivalent of all bases or base producing substances in the water sample that can be titrated by a strong acid to equivalence point (pH 4.3). For titration a colour indicator (methyl orange) was added to 100 mL of a water sample. By adding 0.1 molar hydrochloric acid the colour of the liquid changes from yellow to orange/red at the point when a pH-value of 4.3 is reached.

3.6 Turbidity

Water turbidity was measured with the help of a turbidity meter (Turbidity Meter, PCE-TUM 20). The method is used to determine the concentration of suspended particles in a grab sample by measuring rectangular emitted light of 850 nm wavelength. A photodiode that captures the light produces an electronic signal which is converted to a specific turbidity value. The turbidity meter works in accordance to ISO 7027 and gives the turbidity of the water sample as a value expressed in nephelometric turbidity units (NTU). The meter measures in a range from 0 to 1000 NTU.

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