

# Nitrate removal from aquaculture effluents using woodchip bioreactors improved by adding sulfur granules and crushed seashells

Mathis von Ahnen, Per Bovbjerg Pedersen and Johanne Dalsgaard

## ABSTRACT

This study examined the effects on nitrate removal when adding sulfur granules and crushed seashells to a woodchip bioreactor treating aquaculture effluents. Using a central composite design, the two components were added at three levels (0.000, 0.125 and 0.250 m<sup>3</sup>/m<sup>3</sup> bioreactor volume) to 13 laboratory-scale woodchip bioreactors, and a response surface method was applied to find and model the optimal mixture ratios with respect to reactor performance. Adding 0.125 m<sup>3</sup>/m<sup>3</sup> sulfur granules improved the total N removal rate from 3.27 ± 0.38 to 8.12 ± 0.49 g N/m<sup>3</sup>/d compared to pure woodchips. Furthermore, the inclusion of crushed seashells together with sulfur granules helped to maintain the pH above 7.4 and prevent a production (i.e., release) of nitrite. According to the modeled response surfaces, a sulfur granule:crushed seashell:woodchip mixture ratio containing about 0.2 m<sup>3</sup> sulfur granules and 0.1 m<sup>3</sup> crushed seashells per m<sup>3</sup> reactor volume would give the best results with respect to high N removal and minimal nitrite release. In conclusion, the study showed that N removal in woodchip bioreactors may be improved by adding sulfur granules and seashells, contributing to the optimization of woodchip performance in treating aquaculture effluents.

**Key words** | denitrification, nitrogen, seashells, sulfur, woodchips

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## INTRODUCTION

Practical solutions for removing nitrate (NO<sub>3</sub><sup>-</sup>-N) from aquaculture effluents are needed to reduce the environmental impact of the aquaculture industry. Existing NO<sub>3</sub><sup>-</sup>-N removal techniques in aquaculture, such as heterotrophic denitrification reactors, usually rely on the costly input of external carbon sources and are often difficult to operate (van Rijn *et al.* 2006). Denitrifying woodchip bioreactors have proven to be a simple, low-maintenance technology for removing nitrogen (N) from ground- and surface waters (Schipper *et al.* 2010; Christianson *et al.* 2012), and recent studies have demonstrated that they may also be applied for removing NO<sub>3</sub><sup>-</sup>-N from aquaculture effluents (Lepine *et al.* 2016; von Ahnen *et al.* 2016a, 2016b). Nitrogen removal rates in denitrifying woodchip bioreactors are, however, rather low (2–22 g NO<sub>3</sub><sup>-</sup>-N/m<sup>3</sup>/d; Schipper *et al.* 2010) compared to those reported for other types of denitrification reactors applied in aquaculture (24–3,984 g NO<sub>3</sub><sup>-</sup>-N /m<sup>3</sup>/d; van Rijn *et al.* 2006). Hence, despite simple installation and presumably sustained treatment for several years with low maintenance costs, the low N removal rates may limit

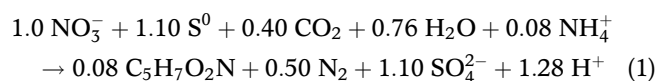
the implementation of woodchip bioreactors in aquaculture since large reactors would be needed to completely remove the incoming NO<sub>3</sub><sup>-</sup>-N loads (von Ahnen *et al.* 2016b).

The low removal rates in woodchip bioreactors are probably linked to limited carbon availability, i.e., it appears that the complex polysaccharide structures in the wood cannot be readily utilized by heterotrophic denitrifying bacteria. This has been corroborated by several studies showing that N removal rates are not improved by reducing the size of the woodchips, i.e., assuming that this should increase carbon availability (Robertson *et al.* 2000; Greenan *et al.* 2006; van Driel *et al.* 2006; Cameron & Schipper 2010).

Robertson (2010) speculated that the removal of N is not restricted to the surface of woodchips but that it also takes place a few millimeters into the wood. This was deduced from dark-colored rims observed in the outer regions of woodchip cross sections. Consistent with this, other studies have found high activity of sulfur-denitrifying and sulfate (SO<sub>4</sub><sup>2-</sup>) reducing bacteria in the deep-layer biofilm inside the wood, while heterotrophic denitrifiers seem to dominate

in the biofilm on woodchip surfaces (Yamashita & Yamamoto-Ikemoto 2006, 2008; Yamashita *et al.* 2011).

The potential contribution of sulfur-based denitrification to the overall N removal in denitrifying woodchip bioreactors is, however, not well studied. Sulfur-based denitrification is an autotrophic process performed by a number of common soil bacteria that use inorganic carbon sources ( $\text{CO}_2$  or  $\text{HCO}_3^-$ ) together with elemental sulfur (S) or reduced sulfur compounds (e.g.  $\text{S}_2\text{O}_3^{2-}$ ,  $\text{H}_2\text{S}$ ,  $\text{SO}_3^{2-}$ ) to gain energy from denitrification (Korom 1992). With elemental sulfur as an electron donor, the following stoichiometric equation for sulfur-based autotrophic denitrification has been established (Batchelor & Lawrence 1978):



Autotrophic denitrification produces less sludge compared to heterotrophic denitrification (Oh *et al.* 2001), whereas it consumes alkalinity and produces sulfate (Sahinkaya & Kilic 2014).

Previous studies have shown that adding soluble organics (methanol, ethanol, acetate, and glucose) to laboratory-scale batch reactors packed with granular elemental sulfur stimulated mixotrophic denitrification (Lee *et al.* 2001; Oh *et al.* 2001, 2003). Furthermore, Christianson & Summerfelt (2014) and Christianson *et al.* (2015) showed that sulfur granules (~99.9% elemental sulfur) may be used for autotrophic denitrification in aquaculture wastewater. Consistent with this, we speculated whether sulfur-based autotrophic denitrification could be stimulated in woodchip bioreactors operated under mixotrophic conditions.

To examine this, a pre-trial was run at DTU Aqua, Hirtshals, Denmark to test the effect on the treatment performance of laboratory-scale woodchip bioreactors treating aquaculture effluent when adding increasing concentrations of sodium thiosulfate (0.00, 0.05, 0.10, 0.15, 0.20, 0.25 g  $\text{NaS}_2\text{O}_3/\text{l}$ ). The reactors were all operated at an equal empty bed contact time (EBCT) of 10 hours. The pre-trial showed that higher doses of  $\text{NaS}_2\text{O}_3$  resulted in increased N removal while effluent alkalinity and pH, as expected when sulfur-based autotrophic denitrification occurs, decreased. The trial, however, also showed that higher concentrations of  $\text{NaS}_2\text{O}_3$  led to unwanted, elevated  $\text{NO}_2^-$ -N effluent concentrations.

Sulfur-based denitrification reactors commonly contain alkaline buffer material to counteract alkalinity consumption (Zhang & Lampe 1999). Sengupta *et al.* (2006) and

Sengupta *et al.* (2007) investigated the buffering capacity of three different solid-phase buffers (marble chips, limestone and oyster shells) when applied to sulfur-based denitrification and concluded that oyster shells were the most suitable based on: (i) the rate of dissolution of the buffer and the buffering agent released (carbonate, bicarbonate, or hydroxide); (ii) the ability of the buffer material surface to act as a host for microbial attachment; (iii) the turbidity of the solution upon release of the buffering agent; and (iv) economics.

Based on these findings, we hypothesized that N removal rates in woodchip bioreactors might be improved by simultaneously adding sulfur granules and crushed seashells. We anticipated that this would have positive effects on potential mixotrophic denitrification processes by mitigating alkalinity consumption and reducing sulfate production as shown for drinking water treatment (Sahinkaya & Dursun 2012). The purpose of the current study was thus to investigate the removal rates of N, and additional inorganic and organic nutrients, in laboratory-scale woodchip bioreactors treating aquaculture effluents when containing different ratios of sulfur granules and crushed seashells. Process performance was specifically evaluated based on N removal rates, accumulation of nitrite ( $\text{NO}_2^-$ -N), alkalinity consumption, and sulfate production.

## MATERIALS AND METHODS

### Experimental design

A central composite design (CCD), using the Design-Expert version 9 software (Stat-Ease, Inc., Minneapolis, USA), was applied to explore the interactions and find the optimal combination of adding sulfur granules and crushed seashells to woodchip bioreactors with respect to nutrient removal rates. A review of the CCD as part of the response surface methodology can be found in Bezerra *et al.* (2008). Consistent with a face-centered CCD, the two variables were set at three coded levels each:  $-1$  (low),  $0$  (central) and  $+1$  (high), corresponding to actual contents of each component mixed into the woodchip beds of 0.000, 0.125 and 0.250  $\text{m}^3/\text{m}^3$ . Hence, at the highest addition of both components only half of the reactor volume was occupied by woodchips. The center point combination of the design matrix (i.e., 0.125/0.125  $\text{m}^3/\text{m}^3$ ) was replicated five times (i.e., in five separate reactors), while all other combinations consisted of single determinations giving a total of 13 separate reactors in the experimental set-up.

## Laboratory-scale woodchip bioreactors

The 13 horizontal-flow woodchip bioreactors (400 × 89 × 150 mm) were constructed in polyvinyl chloride (PVC) as described in von Ahnen *et al.* (2016a). Each filter consisted of a packed-bed zone containing a mixture of woodchips, sulfur granules, and crushed seashells in the ratios specified by the CCD (Table 1). The packed-bed was separated from an inlet and an outlet zone by vertically inserted PVC grids to promote an even plug flow, and water exited the bioreactor outlet zone via an overflow.

Prior to the experiment, crushed seashells (2.0–5.0 mm, OSTREA, Van der Endt-Louwerse c.v., Yerseke, The Netherlands) and sulfur granules (2.5–5.5 mm, Sulfur Pearls, Aqua Medic GmbH, Bissendorf, Germany) were washed separately in water from an associated recirculating aquaculture system (RAS; see the ‘Wastewater’ section) to remove any fines and remnants from the manufacturing process. The coarse-textured woodchips used (*Salix viminalis*;  $4.3 \pm 0.8 \times 0.8 \pm 0.4$  cm,  $n = 30$ ; Ny Vraa Bioenergy I/S, Tylstrup, Denmark) had been used in the reactors for approximately one year treating aquaculture effluent under different operating conditions. Prior to the current experiment they were therefore pooled and thoroughly mixed before being randomly redistributed back into the reactors. Sulfur granules and crushed seashells were evenly mixed into the woodchip packing during this process in the ratios specified in Table 1. A layer of pure woodchips was added on top of all reactors extending approximately 40 mm above the water surface.

To determine the total porosity of the different fill media mixtures, water was drained from the reactors at the end of the trial and the media was left to air dry at room temperature for 4 weeks. The reactors were subsequently filled with water, topping up with water after 24 hours to account for any absorption by the media. Total porosity (%) was subsequently determined from the total volume of water added (excluding water in the bioreactor inlet and outlet zones) relative to the volume of the packed-bed chamber.

## Wastewater

The water to be treated in the laboratory-scale woodchip bioreactors originated from a low-intensity freshwater RAS comprising a 720 L rearing tank, a drum filter, a moving bed biofilter, and a trickling filter. The rearing tank was stocked with 40 kg juvenile rainbow trout (*Oncorhynchus mykiss*). The fish were fed 400 g feed/day of a commercial diet (3 mm EFICO Enviro 920, BioMar, Denmark), and the system was operated with a daily amount of make-up water of 1.15 m<sup>3</sup>.

Each day 100 l of system water was collected at 15:00 and successively filtered through a 100 and 60 μm mesh into a stand-alone holding tank. From here, the water was transferred manually into a smaller reservoir feeding directly into the experimental bioreactors (see ‘Laboratory-scale woodchip bioreactors’ section) after the addition of 30 mg/L NaNO<sub>3</sub>-N. This was done to ensure that the filter processes at no time became nitrate limited. Hence, water in the reservoir was replaced and NaNO<sub>3</sub>-N was added on a daily basis, ensuring continuous feeding of water into the experimental woodchip bioreactors. Water from the reservoir was pumped into each reactor at a flow rate of 0.2 L/h using peristaltic pumps (BT 100-2 J and BT 300-2 J, pump heads YZ1515X, Langer Instruments Corp., New Jersey, USA), corresponding to EBCTs of 15 hours.

Dosing with NaNO<sub>3</sub>-N changed the original C:N ratio in the water but did not measurably affect any other water quality parameters except nitrate as intended. The composition of the inlet water to the bioreactors is shown in Table 1. The temperature in the reservoir feeding into the bioreactors averaged  $17.6 \pm 0.2$  °C ( $n = 9$ ), while oxygen averaged  $5.68 \pm 0.25$  mg O<sub>2</sub>/L ( $n = 9$ ).

## Sample collection and analyses

The experimental woodchip bioreactors were operated for 25 days under the conditions specified in the ‘Wastewater’ section prior to the actual sampling campaign. During sampling, which took place on three consecutive days, all water from the overflow of each reactor was collected continuously for 24 hours in plastic containers kept on ice. In addition, a grab sample was taken from the reservoir each day. After collection, the samples were kept in suspension using a magnetic stirrer while subsamples were withdrawn and filtered through 0.2 μm sterile syringe filters (Filtropur S 0.2 μm, Sarstedt, Germany) prior to analysis of total ammonia nitrogen (TAN; DS 224 1975), NO<sub>2</sub><sup>-</sup>-N (DS 223 1991), NO<sub>3</sub><sup>-</sup>-N (ISO 7890-1 1986), N<sub>total</sub> (ISO 7890-1 1986; ISO 11905-1 1997), and SO<sub>4</sub><sup>2-</sup> (cuvette test LCK153, Hach Lange GmbH, Düsseldorf, Germany).

Other subsamples were filtered through 0.45 μm mixed cellulose ester filters (Whatman, GE Healthcare, UK) and analysed for dissolved five-day biochemical oxygen demand (BOD<sub>5-diss</sub>; first sampling day only; ISO 5815-2 2003), dissolved chemical oxygen demand (COD<sub>diss</sub>; first sampling day only; 4–40 mg/L test kits, Spectroquant®, Merck Millipore, Darmstadt, Germany) (ISO 15705 2002) and orthophosphate (PO<sub>4</sub><sup>3-</sup>-P).

Finally, unfiltered subsamples were analysed for total BOD<sub>5</sub> (BOD<sub>5-total</sub>; first sampling day only; ISO 5815-2

**Table 1** | Composition of reservoir (woodchip bioreactor inlet) water and removal rates of organic and inorganic nutrients obtained in the experimental runs with reactors containing different amounts of sulfur granules and crushed seashells

Concentrations in reservoir (mg/L)													
	$\text{NO}_3^- \text{-N}$	$\text{N}_{\text{total}}$	TAN	$\text{NO}_2^- \text{-N}$	$\text{SO}_4^{2-} \text{-S}$	$\text{BOD}_{5\text{-total}}$	$\text{BOD}_{5\text{-diss}}$	$\text{COD}_{\text{total}}$	$\text{COD}_{\text{diss}}$	$\text{PO}_4 \text{-P}$	$\text{CaCO}_3$	pH	
	$42.40 \pm 0.33$	$43.29 \pm 0.07$	$0.42 \pm 0.01$	$0.18 \pm 0.01$	$71.90 \pm 0.83$	3.36	1.76	15.3	13.1	$0.62 \pm 0.02$	$330 \pm 1.54$	$7.96 \pm 0.03$	
Removal rate ( $\text{g}/\text{m}^3/\text{d}$ ) <sup>b</sup>													
Exp. runs <sup>a</sup>	$\text{NO}_3^- \text{-N}$	$\text{N}_{\text{total}}$	TAN	$\text{NO}_2^- \text{-N}$	$\text{SO}_4^{2-} \text{-S}$	$\text{BOD}_{\text{total}}$	$\text{BOD}_{\text{diss}}$	$\text{COD}_{\text{total}}$	$\text{COD}_{\text{diss}}$	$\text{PO}_4^{3-} \text{-P}$	$\text{CaCO}_3$	Outlet pH	Porosity (%)
Sulfur/seashell													
0 (-1)/0 (-1)	$3.60 \pm 0.16$	$3.27 \pm 0.38$	$0.20 \pm 0.01$	$0.00 \pm 0.01$	$3.50 \pm 0.65$	0.66	0.15	0.80	0.59	$-0.03 \pm 0.01$	$-12.07 \pm 1.07$	$7.75 \pm 0.01$	66
0 (-1)/0.125 (0)	$3.17 \pm 0.50$	$2.82 \pm 0.45$	$0.21 \pm 0.01$	$0.03 \pm 0.01$	$5.30 \pm 0.96$	0.57	0.17	1.28	0.80	$0.00 \pm 0.01$	$-30.12 \pm 1.51$	$7.80 \pm 0.01$	53
0 (-1)/0.25 (+1)	$3.02 \pm 0.12$	$2.56 \pm 0.16$	$0.21 \pm 0.01$	$0.02 \pm 0.02$	$4.68 \pm 0.97$	0.52	0.06	0.75	0.16	$0.01 \pm 0.01$	$-29.81 \pm 0.37$	$7.82 \pm 0.01$	49
0.125 (0)/0 (-1)	$9.53 \pm 0.57$	$8.12 \pm 0.49$	$0.21 \pm 0.01$	$-0.50 \pm 0.02$	$-25.12 \pm 1.63$	0.58	0.03	0.59	-0.05	$-0.01 \pm 0.01$	$30.01 \pm 1.35$	$7.16 \pm 0.01$	52
0.125 (0)/0.25 (+1)	$6.35 \pm 0.76$	$6.08 \pm 0.68$	$0.20 \pm 0.01$	$0.01 \pm 0.02$	$-18.54 \pm 2.29$	0.47	0.06	0.64	0.11	$0.01 \pm 0.01$	$-18.17 \pm 1.92$	$7.51 \pm 0.02$	44
0.25 (+1)/0 (-1)	$8.91 \pm 1.02$	$7.60 \pm 0.94$	$0.20 \pm 0.01$	$-0.61 \pm 0.03$	$-24.59 \pm 1.55$	0.58	0.05	0.32	-0.05	$-0.01 \pm 0.01$	$31.82 \pm 1.87$	$7.15 \pm 0.01$	50
0.25 (+1)/0.125 (0)	$7.87 \pm 0.78$	$7.67 \pm 0.69$	$0.20 \pm 0.01$	$-0.07 \pm 0.02$	$-23.34 \pm 1.54$	0.44	-0.02	0.8	0.27	$0.00 \pm 0.01$	$-17.03 \pm 1.91$	$7.52 \pm 0.03$	47
0.25 (+1)/0.25(+1)	$6.52 \pm 0.75$	$6.33 \pm 0.70$	$0.19 \pm 0.01$	$0.00 \pm 0.02$	$-18.01 \pm 1.32$	0.45	-0.06	0.85	0.16	$0.03 \pm 0.01$	$-18.67 \pm 1.39$	$7.51 \pm 0.01$	46
0.125 (0)/0.125(0)	$7.47 \pm 0.57$	$7.19 \pm 0.50$	$0.21 \pm 0.01$	$0.06 \pm 0.02$	$-19.79 \pm 2.74$	0.58	0.09	1.17	0.69	$0.02 \pm 0.01$	$-15.04 \pm 1.49$	$7.49 \pm 0.00$	52
0.125 (0)/0.125(0)	$7.76 \pm 0.14$	$7.52 \pm 0.22$	$0.20 \pm 0.01$	$0.03 \pm 0.01$	$-21.03 \pm 2.10$	0.47	-0.02	1.01	0.27	$0.00 \pm 0.01$	$-19.61 \pm 1.90$	$7.46 \pm 0.01$	51
0.125 (0)/0.125(0)	$7.77 \pm 0.41$	$7.61 \pm 0.36$	$0.19 \pm 0.01$	$-0.01 \pm 0.02$	$-21.21 \pm 2.46$	0.46	-0.02	0.96	0.32	$0.02 \pm 0.01$	$-18.20 \pm 2.27$	$7.46 \pm 0.00$	51
0.125 (0)/0.125(0)	$6.66 \pm 0.26$	$6.49 \pm 0.08$	$0.21 \pm 0.01$	$0.05 \pm 0.01$	$-19.79 \pm 2.24$	0.39	-0.06	1.33	0.69	$-0.02 \pm 0.01$	$-22.54 \pm 0.57$	$7.50 \pm 0.01$	48
0.125 (0)/0.125(0)	$7.43 \pm 0.41$	$7.16 \pm 0.53$	$0.20 \pm 0.01$	$0.00 \pm 0.02$	$-20.50 \pm 1.09$	0.53	-0.03	0.69	0.21	$0.00 \pm 0.01$	$-20.27 \pm 2.68$	$7.49 \pm 0.01$	51

<sup>a</sup>Experimental runs are shown as combinations of sulfur granule and seashell contents where values with the original unit ( $\text{m}^3/\text{m}^3$ ) are followed by the coded central composite design (CCD) values (see the 'Experimental design' section) in parentheses.

<sup>b</sup>Removal rates are expressed as g removed per  $\text{m}^3$  of reactor volume (i.e. volume of immersed media) per day. Removal rates of  $\text{NO}_3^- \text{-N}$ ,  $\text{N}_{\text{total}}$ , TAN,  $\text{NO}_2^- \text{-N}$ ,  $\text{SO}_4^{2-} \text{-S}$ ,  $\text{PO}_4^{3-} \text{-P}$ ,  $\text{CaCO}_3$  are shown as the averages of three consecutive sampling days with standard deviation. Removal rates of  $\text{BOD}_{5\text{-total}}$ ,  $\text{BOD}_{5\text{-diss}}$ ,  $\text{COD}_{\text{total}}$  and  $\text{COD}_{\text{diss}}$  represent single measurements obtained during the first sampling day.

2003), total COD ( $\text{COD}_{\text{total}}$ ; first sampling day only; 4–40 mg/L test kits, Spectroquant<sup>®</sup>, Merck Millipore, Darmstadt, Germany), (ISO 15705 2002) and alkalinity (T50 Titrator, Mettler-Toledo GmbH, Switzerland).

Samples were stored in darkness at 3 °C and analysed within the two weeks after collection. Subsamples for  $\text{COD}_{\text{total}}$  and  $\text{COD}_{\text{diss}}$  analyses were preserved until then by adding 1% of sulfuric acid ( $\text{H}_2\text{SO}_4$ ; 21.4%, Merck KGaA, Darmstadt, Germany). All analyses were carried out in double determination.

Results are reported as averages of the three sampling days except those for organic matter ( $\text{BOD}_{5\text{-total}}$ ,  $\text{BOD}_{5\text{-diss}}$ ,  $\text{COD}_{\text{total}}$  and  $\text{COD}_{\text{diss}}$ ), which are reported only for samples obtained on the first day of sampling.

Oxygen, pH, and temperature were measured three times per day in the reservoir (bioreactor inlet water) and in the centre of the outlet zones of all filters using Hach Lange HQ40 multimeters (Düsseldorf, Germany).

## Data analyses

Volumetric removal rates ( $\text{g}/\text{m}^3/\text{d}$ ) of the different inorganic and organic nutrients were calculated based on the differences between woodchip bioreactor inlet and outlet concentrations, the flow rate, and the effective filter volume (i.e., the volume of the submerged media packing). To compare overall reactor performances with the stoichiometry of heterotrophic and autotrophic denitrification processes, respectively, the ratios of  $\Delta\text{SO}_4^{2-}/\Delta\text{N}_{\text{total}}$  as well as  $\Delta\text{CaCO}_3/\Delta\text{N}_{\text{total}}$  were calculated, with  $\Delta$  referring to the change in concentration from bioreactor inlet to bioreactor outlet.

Second order polynomial models of the form:

$$y = \beta_0 + \beta_1 * A + \beta_2 * B + \beta_3 * A * B + \beta_4 * A^2 + \beta_5 * B^2 \quad (2)$$

where  $y$  refers to a measured response (e.g. volumetric removal rate),  $\beta_0$ – $\beta_5$  are constants,  $A$  refers to the sulfur granule content ( $\text{m}^3/\text{m}^3$ ), and  $B$  refers to the crushed seashell content ( $\text{m}^3/\text{m}^3$ ) were fitted to the investigated responses using the Design-Expert version 9 software to determine the relationship between the two factors and generate response surface plots.

Coefficients of determination ( $R^2$ ) were used to evaluate the adequacy of the model fits after insignificant ( $P > 0.05$ ) terms had been excluded from the model equations.

Spearman correlation analyses were performed using SigmaPlot version 13.0 (Systat Software Inc., CA, USA) considering  $P \leq 0.05$  as significant.

## RESULTS

Table 1 summarizes the calculated removal rates in the woodchip bioreactors, and Table 2 summarizes the second order polynomial models fitted to the removal rates, outlet pH, and total bioreactor media porosity.

### Total nitrogen and nitrate removal

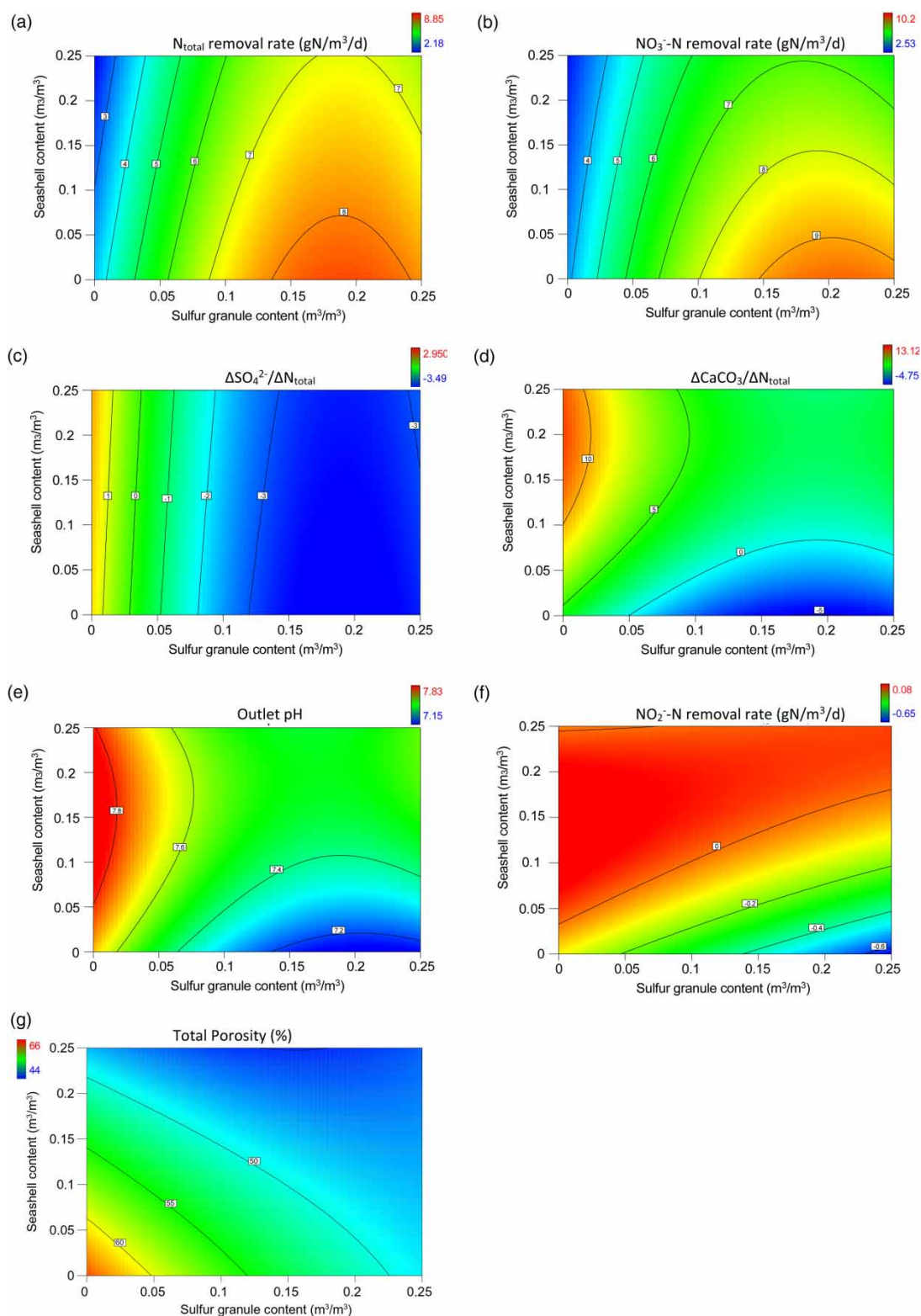
Total nitrogen was removed in all woodchip bioreactors due mainly to the removal of  $\text{NO}_3^-$ -N. Removal rates of  $\text{N}_{\text{total}}$  and  $\text{NO}_3^-$ -N ranged from  $2.56 \pm 0.16$  to  $8.12 \pm 0.49$   $\text{g N}/\text{m}^3/\text{d}$  and from  $3.02 \pm 0.12$  to  $9.53 \pm 0.57$   $\text{g NO}_3^-$ -N/ $\text{m}^3/\text{d}$ , respectively (Table 1).

Nitrogen removal increased when more sulfur granules were included in the bioreactors (Figure 1(a) and 1(b)). Consistent with this, the linear term representing the sulfur granule content in the second order polynomial models (term A) fitted to the removal rates of  $\text{N}_{\text{total}}$  and  $\text{NO}_3^-$ -N, respectively (Table 2) was significant ( $P < 0.001$ ). Compared to the  $\text{N}_{\text{total}}$

**Table 2** | Model equations (including  $R^2$ ) describing removal rates ( $\text{g}/\text{m}^3/\text{d}$ ) of  $\text{N}_{\text{total}}$ ,  $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N as well as ratios of  $\Delta\text{CaCO}_3/\Delta\text{N}_{\text{total}}$  and  $\Delta\text{SO}_4^{2-}/\Delta\text{N}_{\text{total}}$  and total media porosity (%)

Equation #	Response	Model equation	$R^2$
3	$\text{N}_{\text{total}}$ removal rate ( $\text{g}/\text{m}^3/\text{d}$ )	$\text{N}_{\text{total}}$ removal rate ( $\text{g}/\text{m}^3/\text{d}$ ) = $3.55 + 51.27 * A - 5.35 * B - 136.01 * A^2$	0.90
4	$\text{NO}_3^-$ -N removal rate ( $\text{g}/\text{m}^3/\text{d}$ )	$\text{NO}_3^-$ -N removal rate ( $\text{g}/\text{m}^3/\text{d}$ ) = $3.84 + 54.50 * A - 4.60 * B - 28.82 * A * B - 131.48 * A^2$	0.89
5	$\text{NO}_2^-$ -N removal rate ( $\text{g}/\text{m}^3/\text{d}$ )	$\text{NO}_2^-$ -N removal rate ( $\text{g}/\text{m}^3/\text{d}$ ) = $-0.10 - 2.18 * A + 3.41 * B + 9.51 * A * B - 12.28 * B^2$	0.91
6	$\Delta\text{SO}_4^{2-}/\Delta\text{N}_{\text{total}}$	$\Delta\text{SO}_4^{2-}/\Delta\text{N}_{\text{total}} = -4.14 + 96.10 * A - 77.58 * B - 248.97 * A^2 + 193.60 * B^2$	0.97
7	$\Delta\text{CaCO}_3/\Delta\text{N}_{\text{total}}$	$\Delta\text{CaCO}_3/\Delta\text{N}_{\text{total}} = 4.14 - 96.10 * A + 77.58 * B + 248.97 * A^2 - 193.60 * B^2$	0.97
8	Outlet pH	Outlet pH = $7.69 - 5.39 * A + 2.42 * B + 4.56 * A * B + 13.00 * A^2 - 7.87 * B^2$	0.96
9	Total porosity (%)	Total porosity (%) = $64.08 - 90.95 * A - 64.67 * B + 208 * A * B + 126.48 * A^2$	0.90

Factor A represents the sulfur granule content ( $\text{m}^3/\text{m}^3$ ) while factor B represents the crushed seashell content ( $\text{m}^3/\text{m}^3$ ) in the fill media mixture within the investigated range of 0–0.25 ( $\text{m}^3/\text{m}^3$ ) for both A and B.



**Figure 1** | Contour plots of the effects of different sulfur granules and crushed seashells mixtures on the volumetric removal rates of  $N_{\text{total}}$ ,  $\text{NO}_3^-$ -N and  $\text{NO}_2^-$ -N (a, b and f, respectively); ratios of  $\Delta\text{SO}_4^{2-}/\Delta N_{\text{total}}$  and  $\Delta\text{CaCO}_3/\Delta N_{\text{total}}$  (c and d, respectively); and outlet pH (e) and total porosity (g).

removal rate in the pure woodchip bioreactor, an increase of up to 148% was achieved by adding sulfur granules (Table 1).

In contrast, increasing the content of crushed seashells slightly reduced the removal rates of  $N_{\text{total}}$  and  $\text{NO}_3^-$ -N independently of adding sulfur granules (Figure 1(a) and 1(b)). Hence, the multiplier term B was negative in both models and roughly 10 times smaller than A (Table 2).

### Sulfate

Removal rates of sulfate ranged between  $-25.12 \pm 1.63$  and  $5.30 \pm 0.96$  g  $\text{SO}_4^{2-}/\text{m}^3/\text{d}$  (Table 1), i.e., there was a production (negative removal rates) of sulfate in several reactors. Sulfate removal rates correlated negatively with the removal rates of  $N_{\text{total}}$  (correlation coefficient,  $r = -0.96$ ,  $P < 0.001$ ,  $n = 13$ ),  $\text{NO}_3^-$ -N ( $r = -0.98$ ,  $P < 0.001$ ,  $n = 13$ ), and  $\text{CaCO}_3$  ( $r = -0.59$ ,  $P < 0.001$ ,  $n = 13$ ), while they correlated positively with outlet pH ( $r = 0.83$ ,  $P < 0.001$ ,  $n = 13$ ). Hence, the concentration of sulfate in the reactor outlets as well as the amount of sulfate produced per unit of  $N_{\text{total}}$  removed increased with the sulfur granule content in the media mixtures (Tables 1 and 2, Equation (6), and Figure 1(c)).

### Alkalinity and pH

pH in the reactor outlets varied between  $7.15 \pm 0.01$  and  $7.82 \pm 0.01$ , and removal rates of  $\text{CaCO}_3$  ranged between  $-30.12 \pm 1.51$  and  $31.82 \pm 1.87$  g  $\text{CaCO}_3/\text{m}^3/\text{d}$  (Table 1), i.e., both consumption (in reactors containing woodchips and sulfur granules only) and production of alkalinity was observed. Fill media mixtures composed of woodchips and crushed seashells resulted in the highest outlet  $\text{CaCO}_3$  concentrations and pH, while the opposite was true for fill media mixtures containing woodchips and sulfur granules only (Figure 1(e), Table 1). Consistent with this, the interaction term (AB) in the model predicting outlet pH was significant (Table 2, Equation (8)). Production of alkalinity simultaneous with the removal of N was observed in the reactor containing woodchips only as well as in all reactors containing mixtures of crushed seashells and woodchips (Figure 1(d)). In contrast, consumption of alkalinity accompanied by N removal was observed in the reactors containing mixtures of woodchips and sulfur granules (Tables 1 and 2, Equation (7), Figure 1(d)).

### Nitrite

Removal rates of  $\text{NO}_2^-$ -N ranged between  $-0.61 \pm 0.03$  and  $0.06 \pm 0.02$  g  $\text{NO}_2^-$ -N/ $\text{m}^3/\text{d}$  (Tables 1 and 2, Equation (5),

Figure 1(f)). They correlated negatively, although not very strongly, with removal rates of  $N_{\text{total}}$  ( $r = -0.54$ ,  $P = 0.043$ ,  $n = 13$ ),  $\text{NO}_3^-$ -N ( $r = -0.56$ ,  $P = 0.044$ ,  $n = 13$ ), and  $\text{CaCO}_3$  ( $r = -0.58$ ,  $P < 0.035$ ,  $n = 13$ ), and positively with sulfate removal rates ( $r = 0.60$ ,  $P = 0.029$ ,  $n = 13$ ).

### Organic matter and orthophosphate

There was a small removal of  $\text{COD}_{\text{total}}$  and  $\text{BOD}_{5\text{-total}}$  in all the reactors, while removal rates of  $\text{COD}_{\text{diss}}$ ,  $\text{BOD}_{5\text{-diss}}$ , and  $\text{PO}_4^{3-}$ -P were insignificant (Table 1) with no obvious dependency on the content of either sulfur granules or crushed seashells (Table 1).

### Media porosity

Total porosity of the different fill media mixtures ranged between 44 and 66% and decreased with the contents of crushed seashells and/or sulfur granules (Table 1, Figure 1(g)). Consistent with this, both linear terms (A and B) in the fitted model describing total porosity were negative (Table 2, Equation (9)).

## DISCUSSION

### Nitrogen removal, sulfate and alkalinity production and consumption

The results of the current study sustained that both sulfur-based autotrophic denitrification and heterotrophic denitrification took place in the woodchip bioreactors depending on the woodchip:sulfur granule:crushed seashell configuration. Hence, the observed correlation between nitrogen removal and sulfate production (Figure 1(a)–1(c)), combined with a concurrent reduction in alkalinity and pH (deduced from the two runs containing woodchips and sulfur granules only; Figure 1(e), Table 1) supports that N was removed in these reactors due (partly) to sulfur-based autotrophic denitrification fueled by the sulfur granules.

The  $\text{NO}_3^-$ -N removal rate in the reactor containing only woodchips (i.e.,  $3.60$  g/ $\text{m}^3/\text{d}$ ) fits within the lower range of  $\text{NO}_3^-$ -N removal rates observed in typical denitrifying woodchip beds treating ground- and surface water ( $2$ – $22$  g  $\text{NO}_3^-$ -N/ $\text{m}^3/\text{d}$ ; Schipper *et al.* 2010). The removal rate is, however, somewhat lower than that observed during the first half year in a pilot-scale woodchip bioreactor operated at a commercial outdoor fish farm ( $7.06 \pm 0.81$  g  $\text{NO}_3^-$ -N/ $\text{m}^3/\text{d}$ ; von Ahnen *et al.* 2016b). The woodchips used in the

current study had already been applied for one year and may have lost some of their 'reactivity'. Hence, Robertson (2010) investigated woodchip media of different ages and concluded that woodchips lose about 50% of their reactivity during the first year of operation, but that removal rates then remain relatively stable for a number of years thereafter.

According to stoichiometry, sulfur-based autotrophic denitrification with elemental sulfur as an electron donor produces  $2.5 \text{ g SO}_4^{2-}$  for each  $\text{g NO}_3^-$ -N that is reduced (Equation (1)). In all reactors containing sulfur granules, sulfate production was higher than anticipated based on this stoichiometry. The additional sulfate may potentially have derived from aerobic bacterial oxidation of sulfur granules near the bioreactor inlets (Suzuki *et al.* 1992; Zhang & Lampe 1999; Beller *et al.* 2006). Sulfate production is thus higher under aerobic than under anoxic conditions (Zhang & Lampe 1999). Furthermore, the additional sulfate production may, in the current study, have masked any sulfate reduction taking place in the bioreactors. Sulfate reduction in denitrifying bioreactors usually only occurs when nitrate concentrations are very low (Robertson *et al.* 2009; Robertson 2010; Elgood *et al.* 2010) because sulfate-reducing bacteria are typically outcompeted by denitrifying bacteria for available organic carbon (Appelo & Postma 1994). However, some sulfate reduction may still occur at elevated nitrate concentrations in the more reduced micro niches within the wood (Yamashita & Yamamoto-Ikemoto 2006, 2008; Yamashita *et al.* 2011). Consistent with this, a small removal of sulfate was observed in the three reactors with no sulfur granules added (Table 1, Figure 1(c)).

Aside from the production of sulfate, autotrophic denitrification with elemental sulfur consumes  $4.57 \text{ g}$  alkalinity as  $\text{CaCO}_3$  per  $\text{g NO}_3^-$ -N reduced (Equation (1)). Alkalinity consumption was a little less than this theoretical alkalinity requirement in the two experimental runs containing woodchips and sulfur granules only. Alkalinity may have been produced simultaneously to autotrophic denitrification in these bioreactors by heterotrophic denitrification taking place on the woodchip surfaces (Lepine *et al.* 2016). Furthermore, alkalinity may have been produced via sulfate reduction (van Rijn 2007) inside the wood as there was net production of alkalinity observed in the reactor containing pure woodchips (Table 1).

In reactors containing both sulfur granules and crushed seashells, the crushed seashells seemed able to compensate for the alkalinity loss resulting from the autotrophic denitrification process, as also observed by Sengupta *et al.* (2007). This alkalizing effect of crushed seashells was further

sustained by an increase in alkalinity and outlet pH in the two reactors containing woodchips and crushed seashells compared to the reactor with woodchips only (Table 1, Figure 1(e) and 1(d)). Hence, in these two reactors production of alkalinity per unit N removed was much larger ( $9.50$ – $9.87 \text{ g CaCO}_3$  produced per  $\text{g NO}_3^-$ -N reduced) than what could be explained by alkalinity production due to heterotrophic denitrification only ( $3.57 \text{ g CaCO}_3$  produced per  $\text{g NO}_3^-$ -N reduced to  $\text{N}_2$ ; van Rijn *et al.* 2006) (Figure 1(d), Table 1).

Differences between the two amounts of crushed seashells on outlet pH and alkalinity production were very small, indicating that there was no linear effect of the content of seashell on alkalinity. It thus appears that seashells provide alkalinity 'on demand', and that there is no risk of overdosing alkalinity in the form of seashells because the dissolution rate depends on pH, with higher dissolution rates at lower pH (Waldbusser *et al.* 2011).

Koenig & Liu (2002) reported a range of optimal sulfur:limestone ratios (1:0–1:1) depending on the ratios of incoming to theoretically required alkalinity ( $>1.7$  –  $<0.5$ ). The fairly high alkalinity ( $330 \pm 1.54 \text{ mg CaCO}_3/\text{L}$ ) and pH ( $7.96 \pm 0.03$ ) in the incoming water in this study may thus have reduced the importance of crushed seashells as a buffering media, being more relevant in settings where influent alkalinity and pH are lower.

## Nitrite

The buffering effect of the crushed seashells seems to have had an impact on outlet nitrite concentrations. Hence, the highest net production rates of  $\text{NO}_2^-$ -N ( $-0.50$  and  $-0.61 \text{ g NO}_2^-$ -N/ $\text{m}^3/\text{d}$ , respectively) were observed in the two reactors with no seashells but only woodchips and sulfur granules (Table 1). In addition, these two reactors had the lowest outlet pH values measured (i.e.,  $7.15$ – $7.16$  versus  $7.46$ – $7.82$  in the other reactors). Consistent with this, Furumai *et al.* (1996) investigated the effects of pH and alkalinity on the sulfur-denitrification process, and found a strong relationship between pH and nitrite accumulation at pH below 7.4. Hence, it appears that the pH within the reactor should be maintained above 7.4 to avoid the production of nitrite, which in the current study was achieved by adding crushed seashells.

## Organic matter and orthophosphate

The small removal of organic matter observed was most likely due to particle entrapment within the media mixtures.



Hence, there was no leakage of dissolved organic matter or  $\text{PO}_4^{3-}\text{-P}$  consistent with the fact that the woodchips applied in the study had been used for a long period of time (Schipper *et al.* 2010; von Ahnen *et al.* 2016b).

### Media porosity

Adding more crushed seashells while maintaining the sulfur granule content constant slightly reduced the N removal rates. In addition, increasing the sulfur granule content lead to a non-linear (diminishing) increase in N removal rates (supported by significant  $A^2$  terms in the models describing the removal rates of  $N_{\text{total}}$  and  $\text{NO}_3^- \text{-N}$ ; Table 2 Equations (3) and (4)). These observations may likely be explained by the reduced media porosity (Table 1, Figure 1(g)). Hence, the relatively small sulfur granules (2.8–5.6 mm) and crushed seashells (2–5 mm) may have occupied the voids in between the woodchips (up to 5 cm in length), thereby reducing porosity and the hydraulic retention time. Furthermore, the heterogeneous size distributions of woodchips versus crushed seashells and sulfur granules may have altered the hydraulic conductivity in some parts of the reactors and favored channeling of the water.

### Optimal process condition

According to the fitted models, a woodchip bioreactor containing about  $0.2 \text{ m}^3/\text{m}^3$  sulfur granules and  $0.1 \text{ m}^3/\text{m}^3$  crushed seashells would lead to both high N removal and only little nitrite production (Table 2, Figure 1(a), 1(b) and 1(f)). This optimal sulfur granule:crushed seashell volume ratio of 2:1 comes close to the optimal sulfur:limestone volume ratios reported for purely autotrophic processes (van der Hoek *et al.* 1992; Zhang & Lampe 1999).

## CONCLUSION

Woodchip bioreactors are a potential new method for removing nitrate from the effluents of commercial fish farms. This study confirms that the overall volumetric nitrate removal rates in woodchip bioreactors can be improved by adding sulfur granules and crushed seashells. The alkalizing effect of seashells may be particularly important to the overall reactor performance when the typically low-alkaline effluents from commercial sites are treated.

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