Relevance and Diversity of *Nitrospira* Populations in Biofilters of Brackish RAS

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**Abstract**

Lithoautotrophic nitrite-oxidizing bacterial populations from moving-bed biofilters of brackish recirculation aquaculture systems (RAS; shrimp and barramundi) were tested for their metabolic activity and phylogenetic diversity. Samples from the biofilters were labeled with ¹³C-bicarbonate and supplemented with nitrite at concentrations of 0.3, 3 and 10 mM, and incubated at 17 and 28 °C, respectively. The biofilm material was analyzed by fatty acid methyl ester - stable isotope probing (FAME-SIP). High portions of up to 45% of *Nitrospira*-related labeled lipid markers were found confirming that *Nitrospira* is the major autotrophic nitrite oxidizer in these brackish systems with high nitrogen loads. Other nitrite-oxidizing bacteria such as *Nitrobacter* or *Nitrodoca* were functionally not relevant in the investigated biofilters. *Nitrospira*-related 16S rRNA gene sequences were obtained from the samples with 10 mM nitrite and analyzed by a cloning approach. Sequence studies revealed four different phylogenetic clusters within the marine sublineage IV of *Nitrospira*, though most sequences clustered with the type strain of *Nitrospira marina* and with a strain isolated from a marine RAS. Three lipids dominated the whole fatty acid profiles of nitrite-oxidizing marine and brackish enrichments of *Nitrospira* sublineage IV organisms. The membranes included two marker lipids (16:1 cis 7 and 16:1 cis 11) combined with the non-specific acid 16:0 as major compounds and confirmed these marker lipids as characteristic for sublineage IV species. The predominant labeling of these characteristic fatty acids and the phylogenetic sequence analyses of the marine *Nitrospira* sublineage IV identified organisms of this sublineage as main autotrophic nitrite-oxidizers in the investigated brackish biofilter systems.

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**Introduction**

In biofilters of recirculation aquaculture systems (RAS), nitrite oxidation by lithoautotrophic bacteria (NOB) is the most important process to prevent the cultivated organisms from intoxication with nitrite [1]. Nitrite is formed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaeca (AOA) during the first process of aerobic nitrification. Together with heterotrophic bacteria, the nitrifying communities form biofilms on carrier elements in biofilter systems [2–3]. Although recirculation aquaculture systems are increasing worldwide [4], we still lack a detailed understanding of the process of bacterial nitrification, and especially nitrite oxidation, in marine RAS [5].

The guild of NOB is phylogenetically heterogeneous and comprises bacteria of at least five different genera: *Nitrobacter*, *Nitrospina*, *Nitrosoccus*, *Nitrospira* and the recently discovered *Nitrotoga*. Co-existence of two [6–10], or three [11–12] genera of NOB in the same habitat has previously been reported. NOB grow slowly due to the low energy yield during oxidation of nitrite [13]. Thus the successful cultivation of NOB is a challenging and time consuming task. Consequently, NOB cultures have only rarely been established in the laboratory [14].

Hovanec et al. [15], and Juretschko et al. [16], were first to identify *Nitrospira* as the dominant NOB in freshwater aquaria and wastewater treatment plants. Later this genus was also detected in marine aquaculture and aquaria systems [2–3,17]. However, only two studies so far have found *Nitrospira* as the dominant nitrite oxidizer in marine aquacultural biofilters [5,18]. Here, we aimed to expand the metabolic activity data from Keuter et al. [5] of marine RAS by analyzes of the metabolic activity of NOB in brackish RAS.

Metabolic activity of nitrite-oxidizing populations can be assessed with DNA-based or chemotaxonomic methods. A DNA-based method of assimilation of labeled substrates performed by Radajewski et al. [19] provided the characterization of the ecology and function of methanol-utilizing organisms from forest soil. The incorporation of labeled substrates followed by gas chromatography and combustion isotope ratio spectrometry (GC-C-IRMS) was first used by Boschker et al. [20] for acetate assimilation of sulphate-reducing bacteria. More recently, quadrupole GC-MS (gas chromatography coupled with mass spectrometry) analyses of labeled lipids with ¹³C-acetate have been conducted [21] to study soil associated bacteria and fungi. The GC-MS analysis is generally less sensitive compared to GC-C-
IRMS, but GC-MS systems are readily available to most laboratories [22]. A method based on quadrupole GC-MS for the labeling with 13C-bicarbonate of autotrophic populations in complex environments was described by Knief et al. [23]. Lipid analyses with fatty acid profiling in combination with stable-isotope probing (FAME-SIP) are very sensitive, making this a suitable technique for the quantification of autotrophic 13C-assimilation in nitrite supplemented biofilter material. For the analyses of the nitrite-oxidizing genera Nitrobacter, Nitrococcus, Nitrospira, Candidatus Nitrotooga and Nitrospina [14] by FAME-SIP, reference data sets for marker lipids are available [10–11,24–25]. Especially the genus Nitrospira can be differentiated from the other genera of NOB based on characteristic lipid markers. These NOB are characterized by varying combinations of specific major compounds, which are the palmitoleic acid isomers cis-7 and cis-11 [24]. The different content of the characteristic lipids has been proved to be a good indicator for the presence of Nitrospira populations.

To determine the relevant NOB in two moving-bed biofilters of a brackish (17–21 psu (practical salinity units)) recirculation aquaculture system (shrimp and barramundi), we used FAME-SIP after incubation of the nitrifying community with different nitrite concentrations and temperatures. The chemotaxonomic analyses were supplemented by a cloning approach to get in-depth information about the diversity and the taxonomic positions of Nitrospira populations. Further, the fatty acid profiles of enrichment cultures obtained from the biofilter of the shrimp RAS and from marine sediments of the Laptev Sea were determined in order to expand the marker lipids data set of marine Nitrospira species.

**Methods**

**Samples and enrichments**

The samples were collected from two brackish recirculation aquaculture systems (RAS) in Strande (Germany (Marifarm; EAP AquaCulture AG)).

One system comprised shrimp (Penaeus vannamei) tanks with a total volume of 189 m³. The moving-bed biofilter of this system has a volume of 11.3 m³ and was started in early 2007 with approx. 3 m³ black and white high density polyethylene (HDPE) biocarriers (type HX09KL, Stöhr, Marktrodach, Germany) with a total surface area of 836 m²/m³ and a protected surface area of 494 m²/m³. The white biocarriers were made of new HDPE material, and the black were made of recycled HDPE material with the addition of 3% carbon black (Figure S1).

In the second, smaller experimental system, barramundi (Lates calcalifer) was reared in four tanks of a total volume of 10.8 m³. This system was started in 2006. Here, the biofilter comprised of 10 tanks containing 160 l water each, of which two were filled with 96 l black HX09KL (recycled material) and two with white HX09KL (new material) biocarriers.

Both systems ran at 26 to 29°C, the oxygen concentration was between 6.5 and 7 mg/l and pH was kept between 6.8 and 7. Make up water was taken from the Baltic Sea nearby, filtered and ozonized.

For the whole cell fatty acids profiles, enrichment cultures were started with biocarriers from the biofilter of the shrimp system as inoculum. The carriers were separated in white and black ones; cells were removed by shaking vigorously with glass beads (1.7–2 mm) and incubated for several months in marine mineral salt medium [26] with 70% brackish sea water at 28°C. Consumption of nitrite (1 mM) was regularly monitored with the Griess–Ilosvay spot test [27] and supplemented with NaNO2 (2.5 M stock solution) if necessary. In addition, the marine Nitrospira-enrichment culture, “S11”, which originated from marine sediments of the Laptev Sea in the permafrost region of Siberia [29], was incubated in marine mineral salt medium supplemented with 0.3 mM NaNO2 as described above. This culture was grown at 10°C and 17°C, respectively. Whole cell fatty acid profiles were analyzed from biomass from the two enrichments (see below).

For labeling experiments and subsequent cloning of Nitrospira-specific DNA fragments, biocarriers from the shrimp biofilter and from both biofilter components of the barramundi system (white and black biocarriers, respectively) were taken in October 2008. Nitrite and nitrate concentrations in the shrimp aquaculture system were 97 and 300 μM respectively, as determined by ion pair chromatography (Elite LaChrom System, Hitachi, Krefeld, Germany [29]). We measured the potential nitrite oxidation rate following the protocol by Spieck and Lipski [14]. The maximum nitrification potential, calculated as the average of two parallels, was 400 nmol NO2 *h⁻¹ per biocarrier of the shrimp biofilter. In the barramundi system 1000 μM nitrate was measured, while nitrite could not be detected. The potential nitrite oxidation rates at sampling time were 1000 (white) and 1400 (black) nmol NO2 *h⁻¹ per biocarrier.

**Lipid analyses**

Whole cell fatty acid profiles. Cells were harvested by centrifugation (10,000 × g; 20 min) when dense flocs had developed. Fatty acid methyl esters (FAMEs) were prepared according to Sasser [30]. The analyses of the FAMEs by GC-MS were performed as described previously [24].

Labeling experiments. In 500 ml gastight flasks filled with 100 ml marine mineral medium [26] prepared with 70% seawater (Baltic Sea; Fischland/Darß, Germany), 100 biocarriers from the two barramundi biofilters (black [Bb] or white [Bw] biocarriers) and the biofilter of the shrimp system (mixed black and white biocarriers [Sh]) were incubated at temperatures of 17°C and 28°C with 20 mM NaH13CO3 and NaNO2 concentrations of 0.3, 3 and 10 mM, resulting in a total of 18 different incubations. Nitrite consumption was tested with the Griess–Ilosvay spot test [27] twice a week and initial concentrations were readjusted if necessary. The oxygen content of the gas phase above the suspension was measured regularly by subjecting 10 μl of gas phase samples to GC-MS. The fatty acids from the sample biomass were extracted when one of samples from the same biofilter, incubated with the same temperature, contained less than 5% oxygen in the gas phase above the medium. Then all three flasks with the different nitrite concentrations (0.3 mM, 3 mM and 10 mM) were harvested. This resulted in incubation times of 41 up to 55 days (17°C) and 27 up to 41 days (28°C). We obtained biomass by shaking the HDPE carriers with glass beads (2 mm) in a horizontal shaker at 400 rpm overnight. The suspended biomass was concentrated by centrifugation (10,000 × g; 20 min).

Lipids were extracted from biomass and converted to fatty acid methyl esters (FAMEs) as described by Knief et al. [23]. The resulting FAMEs were analyzed by GC-MS, and the degree of labeling was quantified using the SIM-mode (single ion monitoring) as described previously [23]. The natural concentration of 13C in biofilm samples was analyzed from samples without added NaH13CO3 and these values were subtracted from those of 13C-labeled samples.

Significance of labeled amounts of fatty acids was calculated based on a one-tailed Student’s t-test. The 13C-content (L) was calculated for unlabeled references from fatty acids of original biofilm samples. Based on these data the t-test was used for the calculation of threshold values (T). Labeling was significant with amounts L>T (p<0.1). The threshold value was calculated for the
fatty acids of the biofilm samples with percentage of 3.7% (p<0.1).
Therefore, all fatty acids of the biofilters samples with degree of
labeling ≥4% were deemed to be labeled.

16S rRNA gene analyses

Primer design. *Nitrospira*-specific primer NS1036R (5’-
GCCGACCTGAGCTGCT-3’) was designed by using the
PROBE-DESIGN Tool from ARB ([31]; version ssu_ian04.cor-
r_optarb [http://www.arb-home.de]). Specificity of the primer
was checked with the online databases RDP II with the Probe
Match function (Ribosomal Database Project; [32]) and NCBI with the BLAST function (Basic local alignment search tool; [33]).
Furthermore, the sequences were checked for chimera and sequence anomalies using the Pintail program [34]. The sequences were named according to their origin (Bw: baramundi white biocarrier, Bb: baramundi black biocarrier, Sh: shrimp biocarrier) and the incubation temperature (17 or 28°C). Sequences derived from the gas tight flask incubations were labeled with the appendix OS (acc. nos. HE793388 – HE793423).

In addition to the incubation with NaH13CO3 in gas tight flask, a further set of incubations of the biocarrier samples was prepared in Erlenmeyer flasks with normal caps and without supplemented 13C. Biofilm from the carriers was harvested in a horizontal shaker at 400 rpm overnight. DNA was extracted from the 10 mM nitrite samples and the 16S rRNA genes were cloned and sequenced according to Keuter et al. [5]. The “Sh” DNA extracts were amplified with the primer pair Jur8F/1036R [16], and “Bb” and “Bw” DNA extracts were amplified with the primer pair Jur8F/ Ns1036R [16,35]. Sequences resulting from this cloning approach are labeled with the appendix HH (acc. nos. JQ900181-JQ900201; JX028301).

Results

Whole cell fatty acids of enrichments

The enrichment cultures inoculated with black and white biocarriers from the shrimp system (Sh) consisted of 19–26% of the fatty acid 16:1 cis7 and 14–19% of the lipid 16:1 cis11. The major fatty acid composition of the enrichment culture from the Laptev Sea (S11) was similar (16:1 cis7: 32%; 16:1 cis11: 40%–44%).

Table 1. Whole fatty acid profiles of marine enrichments and *Nitrospira marina* 295 (fatty acids in %).

| Cultivation temperature | Ecomares 2.1b | Sh (black carrier) | Sh (white carrier) | S11 |
|-------------------------|-------------|-------------------|-------------------|-----|
|                         | 28°C | 17°C | 28°C | 28°C | 28°C | 10°C | 17°C |
| 120                     | 0.6  | -    | -    | -    | -    | -    | -    |
| 140                     | 1.4  | 1.0  | -    | 1.9  | 3.3  | 1.0  | 0.8  |
| 15.0 iso                | 0.8  | -    | -    | 7.3  | 6.1  | -    | -    |
| 15.0 antieisco          | -    | -    | -    | 2.4  | 4.1  | -    | 1.3  |
| 15.0                    | -    | -    | -    | -    | 2.4  | -    | -    |
| 16.0 iso                | -    | -    | -    | 2.6  | -    | 0.7  | 1.7  |
| 161 cis7                | 30.4 | 40.7 | 37.1 | 25.8 | 19.0 | 32.4 | 31.8 |
| 161 cis9                | -    | -    | -    | 2.1  | -    | 2.4  | 1.0  |
| 161 cis10               | -    | 1.9  | 2.8  | -    | -    | -    | 1.1  |
| 161 cis11               | 15.5 | 30.8 | 19.8 | 18.9 | 13.8 | 44.2 | 40.3 |
| 160                     | 36.5 | 24.4 | 35.3 | 29.9 | 39.3 | 14.0 | 15.7 |
| 160 11methyl            | 0.8  | -    | 2.3  | -    | -    | -    | -    |
| 170 iso                 | 0.8  | -    | -    | 2.3  | -    | -    | 0.3  |
| 181 cis9                | 1.8  | -    | -    | 1.8  | -    | 0.8  | 0.7  |
| 181 cis11               | 0.6  | -    | -    | 2.6  | 5.4  | 3.1  | 1.3  |
| 180                     | 8.7  | 2.2  | 2.8  | 2.5  | 3.4  | 1.3  | 1.5  |
| 190 cyclo 9–10          | -    | -    | -    | -    | 3.1  | -    | 0.6  |

*a* data from Lipski et al. [24].

*b* data from Keuter et al. [5].

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Additionally, all enrichments showed the lipid 16:0 as major compound with percentages from 14 up to 39% (Table 1). Data of two previously analyzed *Nitrospira* cultures, strains *Nitrospira marina* 295 [24] and Ecomares 2.1 [5], which contained high percentages of the lipid 16:1 cis7 (30–41%) in combination with 16:1 cis11 (16–31%), are also presented in Table 1.

**Table 2.** Labeled amounts of major compounds from nitrite-oxidizing bacteria.

|                | 16:1 cis7 | 16:1 cis9 | 16:1 cis11 | 18:1 cis11 | 16:0 |
|----------------|-----------|-----------|------------|------------|------|
| Labeled amounts (%) | 6–13 | 30–44 | 6–13 | 19–45 | 6–10 | 11–29 |
| Significance (p) | <0.1 | <0.005 | <0.025 | <0.005 | <0.05 | <0.01 |
| Dominating lipids | | | | | | |
| *Nitrospira IV* | ++ | ++(+) | ++(+) | | | |
| *Nitrobacter* | ++ | ++ | | | | |
| *Nitrooccus* | ++ | ++ | ++ | | | |
| *Nitrospina* | ++ | ++ | | | | |
| *Nitrota* | ++ | ++ | | | | |

aRange of the labeled amounts from the 3 moving bed biofilters (incubations with 10 mM nitrite and both used temperatures), see also Figure 2 for detailed information of Nitrospira marker lipids.

bMarked lipids of Nitrospira sublineage IV, detailed information see Table 3.

As can be seen in Table 2, the major labeled fatty acids for the respective taxon were 16:0 and 16:1 cis9. These compounds showed the highest percentage of labeled fatty acids in all samples, with percentages ranging from 32–45% for fatty acid 16:1 cis9 and 26–45% for fatty acid 16:1 cis11.

**Figure 1.** Whole fatty acid profile of the three original biofilter samples. Samples from the moving-bed biofilters were analyzed in parallels (error bars represent the standard deviation of n = 2 samples of each moving-bed system). Data from the barramundi biofilters are shown by white and black bars (white and black biocarriers), and those from the shrimp biofilter by grey bars.

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**Figure 3.** Whole fatty acid profile of the three original biofilter samples. Samples from the moving-bed biofilters were analyzed in parallels (error bars represent the standard deviation of n = 2 samples of each moving-bed system). Data from the barramundi biofilters are shown by white and black bars (white and black biocarriers), and those from the shrimp biofilter by grey bars.

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|----------------|-----------|-----------|------------|------------|------|
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| Significance (p) | <0.1 | <0.005 | <0.025 | <0.005 | <0.05 | <0.01 |
| Dominating lipids | | | | | | |
| *Nitrospira IV* | ++ | ++(+) | ++(+) | | | |
| *Nitrobacter* | ++ | ++ | | | | |
| *Nitrooccus* | ++ | ++ | ++ | | | |
| *Nitrospina* | ++ | ++ | | | | |
| *Nitrota* | ++ | ++ | | | | |

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**Discussion**

**Metabolically active NOB in the biofilters**

Members of the genus *Nitrospira* often co-exist with other NOB, or even dominate the nitrite oxidation in many engineered ecosystems such as wastewater treatment plants, aquaria and aquaculture biofilters, as indicated by several nucleic acid-based studies [2–3,15–18,37]. Fluorescence in situ hybridization (FISH) with rRNA-targeted probes is well practical for analyses of the
The low degree of labeled and ria of the deep-branching phylum Nitrobacter oxidizing autotrophic organisms, 7 and conditions we used [23].

Cells, show a considerable incorporation of label under the assumed that only active autotrophic cells, but not heterotrophic degree of labeling never exceeded 3.8% (data not shown). It is assimilation of 13C in the course of the labeling experiments. The compound of different heterotrophic cells, showed no significant occurrence mainly in heterotrophic cells. The lipid 18:1 cis-11 isomer of hexadecenoic acid, reflects the major role of fatty acids, polyunsaturated and iso-/anteiso-branched fatty acids, were detected in lower percentages for all samples. All these lipids occur mainly in heterotrophic cells. The lipid 18:1 cis-9, a compound of different heterotrophic cells, showed no significant assimilation of 13C in the course of the labeling experiments. The degree of labeling never exceeded 3.8% (data not shown). It is assumed that only active autotrophic cells, but not heterotrophic cells, show a considerable incorporation of label under the conditions we used [23].

The high degree of labeled Nitrospira-specific compounds, the cis-7 and cis-11 isomer of hexadecenoic acid, reflects the major role of Nitrospira cells in the biofilters. Marker lipids of the other nitrite-oxidizing autotrophic organisms, Nitrobacter, Nitrospina, Nitrooccus and Candidatus Nitrotoga were only moderately labeled (Table 2). The low degree of labeled Nitrobacter marker fatty acids at 10 mM nitrite contrasts the expectation that this NOB is often found as dominant NOB in habitats with high substrate availability [39–41], outcompeting Nitrospira which has high substrate affinity at low nitrite concentration [42]. However, Nitrobacter- and Nitrosga-like bacteria were detected by specific PCR in samples from the shrimp (both) and the barramundi (only Nitrospira) filters (data not shown), but no relevant activity could be assigned to these genera by the labeling approach.

In contrast to Nitrobacter (Alphaproteobacteria), Nitrospira-like bacteria of the deep-branching phylum Nitrospirae [43] grow slowly, and seem to be better adapted to low oxygen concentrations [44]. Their high nitrite affinities led to the assumption, that Nitrospira are K-strategists, which thrive at low nitrite concentrations [44–45]. The high metabolic activity of Nitrospira populations, especially at a concentration of 10 mM nitrite (Figure 2) is remarkable. In line with our finding, Maixner et al. [35] suggested a broader differentiation of ecotypes within the genus Nitrospira on an imaginary scale reaching from K-strategist to r-strategist. The Nitrospira in the studied brackish RAS hence might have been r-strategists, or/and bear currently unidentified features that are advantageous compared to other NOB.

Nitrobacter has rarely been reported to be a major NOB in marine habitats [8,46]. Rather the marine genera Nitrooccus or Nitrospira would be theoretically more suitable as putative co-inhabitants or competitors in brackish or marine RAS. Both genera are tolerant to high nitrite concentrations [47] and Nitrospira is thought to be the most abundant nitrite oxidizer in the oceans [48], though sound data on the composition and distribution of NOB in the oceans are still lacking [49]. In contrast to the theory, no specific fatty acids of either of these NOB were labeled during our experiments and there was no hint for their relevance in the analyzed biofilters.

The similar pattern of labeled fatty acids at different nitrite concentrations suggests that only one dominant NOB was active under the different conditions. The amounts of the specific labeled acids of Nitrospira correlated positively with the nitrite concentrations. Both moving-bed-systems ran at 26 to 29°C, but the metabolic activity was not influenced by the incubation temperatures of 17 and 28°C. We detected similar activities at the original temperature of the system and the lower temperature conditions suggesting the autotrophic organisms are very resistant to a decrease in temperature. While the temperature difference of 9°C did not seem to have any influence on the activity of the NOB the type of the biocarriers did affect the activity of the NOB: In almost all incubations, the bacterial community on the white biocarriers of the barramundi biofilter incorporated more 13C-bicarbonate than the black carriers of the second barramundi biofilter, even though both biofilters were supplied by the same effluent water from the barramundi tanks. This is in accordance with results from a range of activity tests on black and white biofilter from the barramundi biofilter between June 2008 and June 2009; the nitrite biofilter by grey bars.
Figure 3. Phylogenetic tree based on 16S rRNA gene sequences. The tree was constructed by neighbor-joining algorithm showing four different clusters (1–4). Nodes ≥65% supported by bootstrap values (based on 1000 iterations). The tree shows sequences of the marine Nitrospira sublineage IV with clones of the brackish RAS biofilters. The sequence of Nitrospira moscoviensis from sublineage II was defined as outgroup. The sequences obtained from analyzed biofilters were named after their origin (Bw: barramundi white biocarrier; Bb: barramundi black biocarrier; Sh: shrimp biocarrier), the incubation temperature (17 or 28 °C), as well as the appendix OS (acc. nos. HE793388 – HE793423) and HH (acc. nos. JQ900181-JQ900201; JX028301) for different cloning approaches. Sequences ≥800 bp was marked with ‘***’. Scale bar = 1% sequence divergence.

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In the current study we found only sequences of Nitrospira moscoviensis using clone libraries of the barramundi and shrimp biocarriers. Higher abundances of Nitrospira marina biofilters of a shrimp RAS. Quantification by qPCR showed Nitrospira moscoviensis sequences related to gene sequences related to temperature [35,51–52]. Brown authors associate population shifts or differences by physical or plants, while species of the sublineage VI (N. calida-lineage) seem to be restricted to hot springs. Sequences of sublineage IV have habitat specific [25,37]. For instance, species of sublineage I containing only one described strain, each of which might be Nitrospira in Biofilters of Brackish RAS

### Table 3. Marker lipids for Nitrospira species from whole fatty acid profiles.

| sublineage    | Species                  | 16:1 cis7 | 16:1 cis11 | 16:0 | 16:0 11methyl |
|---------------|--------------------------|-----------|-----------|------|--------------|
| sublineage I  | Candidatus Nitrospira defluvii<sup>a</sup> | ++        |           | +    |              |
| sublineage II | Nitrospira moscoviensis<sup>b</sup>     | ++        | +         | ++   | ++           |
| sublineage IV | Nitrospira marina<sup>c</sup>           | ++        |           | +    | ++           |
|               | Enrichment Ecomares 2.1<sup>d</sup>     | ++        | ++        | +    |              |
|               | Enrichment S11            | ++        | ++        | +    |              |
|               | Enrichment Sh black       | ++        | +         | ++   |              |
|               | Enrichment Sh white       | +         | +         | ++   |              |
| sublineage V  | Candidatus Nitrospira bockiana<sup>e</sup> | +         | ++        | ++   |              |
| sublineage VI | Nitrospira calida<sup>f</sup>         | +         | ++        | ++   |              |
|               | Enrichment Ga II<sup>g</sup>    | ++        | ++        |      |              |

<sup>a</sup>data from Spieck et al. [10].
<sup>b</sup>data from Lipski et al. [24].
<sup>c</sup>data from Keuter et al. [5].
<sup>d</sup>data from Lebedeva et al. [15].
<sup>e</sup>: marker lipids with percentage ≤20%; ++: marker lipids with >20% of whole fatty acids.

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These results are interesting, since there is no difference in shape, structure or size of the white and black biocarriers. The only difference is that white biocarrier consisted of new HDPE material while the black HDPE biocarriers were made of recycled material pigmented with black carbon. Reasons for that are unclear and would require further studies.

**Phylogenetic relationship in sublineage IV**

We assessed micro-diversity of Nitrospira-strains in the biofilters of the brackish RAS, by constructing a clone library using semi-specific Nitrospira primer pairs with DNA from the 10 mM nitrite incubations at both temperatures. We assumed that the bacteria in the biofilters would be halotolerant or halophilic, since the systems were run with water from the Kiel Bight, a part of the Baltic Sea which can reach salinities of up to 30 psu. Our phylogenetic analyses showed that sequences from the analyzed biofilters belong to the marine Nitrospira sublineage IV, and some were highly similar to the few known marine cultures. So far six sublineages of Nitrospira-strains are known, mostly containing only one described strain, each of which might be habitat specific [25,37]. For instance, species of sublineage I (Candidatus N. defluviil-lineage), are found in wastewater treatment plants, while species of the sublineage VI (N. calida-lineage) seem to be restricted to hot springs. Sequences of sublineage IV have mainly been derived from marine habitats.

In various engineered ecosystems Nitrospira-related strains, from either different or the same sublineages, co-exist [18,30]. Some authors associate population shifts or differences by physical or chemical factors like nitrite concentration, oxygen content or temperature [35,51–52]. Brown et al. [53] discovered 16S rRNA gene sequences related to Nitrospira marine-like organisms and also sequences related to Nitrospira moscoviensis-like organisms in biofilters of a shrimp RAS. Quantification by qPCR showed higher abundances of Nitrospira marine-like organisms in biofilters from sublineage IV than Nitrospira moscoviensis-like organisms from sublineage II. In the current study we found only sequences of Nitrospira from sublineage IV grouped into four different clusters using clone libraries of the barramundi and shrimp biocarriers.

Cluster 1 comprised the largest number of sequences originating from all incubations and included the strains Nitrospira marine and Ecomares 2.1. The latter was isolated from the moving-bed biofilter of a marine RAS in Büsum, Germany (North Sea water) [5]. However, a range of sequences confirmed three further clusters within the marine Nitrospira sublineage. Sequences from the colder incubation of the shrimp biocarriers were found clustering together with culture S11 (cluster 2). The culture S11 was observed to convert moderate nitrite concentrations (34–36 µM/day) irrespectively of the temperature (range between: 10–28°C) [28]. Population shift experiments with varying nitrite concentrations [33] revealed shifts within a few days, which is a relatively short time for nitrifiers. Therefore, the incubation at 17°C for approx. 55 days might have favored growth of bacteria of cluster 2.

Except for the colder incubation of biocarriers from the shrimps filter, cluster 4 comprised sequences from all incubation variants together with the sequence of enrichment culture M1-marine, a Nitrospira coexisting with the Ecomares 2.1 in the marine RAS run with North Sea water [5]. No representative of cluster III has been cultivated so far.

The genotypic information of marine and brackish habitats revealed high similarities of the sequences within the marine Nitrospira sublineage. This indicates that there are indeed different ecotypes in Nitrospira sublineage IV, although no grouping into marine and brackish strains could be observed. The marine sublineage IV is the only sublineage of Nitrospira with several isolated or highly enriched and physiologically studied representatives. Experiments on the strains S11 [28], Ecomares 2.1 [5], and N. marina 295 [26] revealed that members of this sublineage, even very close relatives such as N. marina 295 and Ecomares 2.1, can differ immensely in their substrate tolerances, temperature optima or substrate conversion rates. Such physiological diversity, a prerequisite for niche differentiation, might therefore explain the high phylogenetic micro-diversity. Micro-scale conditions in biofilms of biofilter systems can vary extremely over time and/or space [35,44], enabling the co-existence of strains with differing physiological preferences for resources.
New data sets of fatty acid profiles in sublineage IV

*Nitrospira*-typical fatty acids are used as biomarker molecules for the *in situ* detection of this NOB in natural environments. Moreover, different sublineages can be identified by certain combination of these acids. For instance, two cultures in the same sublineage, *N. calida* and GaII (sublineage VI), exhibited considerable differences in their fatty acids profile [23].

Beside *N. marina* and its close relative Ecomares 2.1, the culture S11 from the Laptev Sea is the third culture of the marine *Nitrospira*-sublineage IV, of which a fatty acid profile has been generated. This profile and profiles of the two enrichment cultures from the shrimp biofilter (black and white biocarriers) were similar to the profiles of the other two *Nitrospira* strains originating from marine environments. The specific fatty acids of *Nitrospira* sublineage IV consisted of the two marker lipids 16:1 cis7 and 16:0 cis11 in combination with the non-specific fatty acid 16:0 as major compounds in the membranes (Table 1). This characteristic pattern (Table 3) is distinguishable from the lipid profiles of the sublineages I, II, V and VI [10,24–25]. The lipid pattern of sublineage III is not yet determined due to the lack of enrichment cultures for this group. The marker lipid analyses confirmed the possibility of differentiation of marine *Nitrospira* strains of sublineage IV from other *Nitrospira* sublineages using this method.

Conclusion

The labeling approach with $^{13}$C as substrate indicated the presence and the metabolic activity of a *Nitrospira*-related organism as the dominant nitrite-oxidizer in analyzed brackish moving-bed filter systems. The abundance of *Nitrospira*-like organisms was shown under various incubation parameters. The sequencing approach of the 16S rRNA genes revealed four *Nitrospira* phyotypes, but only the second cluster was restricted to the specific incubation conditions of low temperature. Our study could also confirm that whole fatty acid profiles of currently known *Nitrospira* sublineage IV organisms from marine habitats always consist of the two marker lipids 16:1 cis7 and 16:1 cis11 combined with the non-specific acid 16:0 as major compounds in the membranes.

Supporting Information

Figure S1 New high density polyethylene biocarriers. Biocarriers of the type HX09KL (Stohr, Marktrodach, Germany) made of new material (white) and of recycled material (black color, due to the addition of 3% carbon black). Scale of the ruler in centimeter (cm).

(TIF)

Figure S2 Nitrite oxidizing potentials of black and white biocarriers from the shrimp biofilter. 10 biocarriers were shaken in 50 ml mineral medium spiked with 1 mM nitrite. Bars right axis: nitrite-oxidizing potentials (in nmol substrate per hour) of NOB on 1 recycled (stripes) or new (dots) HDPE biocarrier. Left axis: nitrate concentrations (black line) of the biofilter water indicating the *N* load of the system over the sampling period of one year.

(TIF)

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Author Contributions

Conceived and designed the experiments: MK SK ES AL. Performed the experiments: MK SK. Analyzed the data: MK SK TE. Contributed reagents/materials/analysis tools: ES EB. Wrote the paper: MK SK AL. Critically revised the manuscript: EB ES TE.

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