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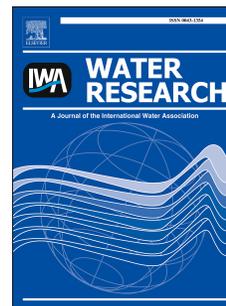
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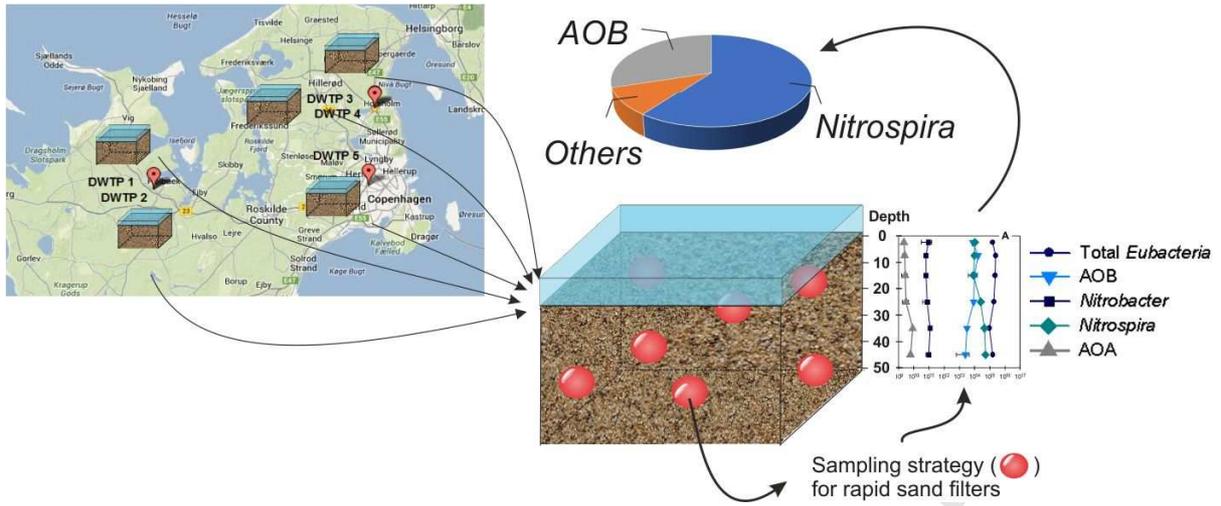
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Density and distribution of nitrifying guilds in rapid sand filters for drinking water production: dominance of *Nitrospira* spp.

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1 Abstract

2 We investigated the density and distribution of total bacteria, canonical Ammonia Oxidizing Bacteria (AOB)
3 (*Nitrosomonas* plus *Nitrospira*), Ammonia Oxidizing Archaea (AOA), as well as *Nitrobacter* and
4 *Nitrospira* in rapid sand filters used for groundwater treatment. To investigate the spatial distribution of these
5 guilds, filter material was sampled at four drinking water treatment plants (DWTPs) in parallel filters of the
6 pre- and after-filtration stages at different locations and depths. The target guilds were quantified by qPCR
7 targeting 16S rRNA and *amoA* genes. Total bacterial densities (ignoring 16S rRNA gene copy number
8 variation) were high and ranged from 10^9 to 10^{10} per gram (10^{15} to 10^{16} per m^3) of filter material. All
9 examined guilds, except AOA, were stratified at only one of the four DWTPs. Densities varied spatially
10 within filter (intra-filter variation) at two of the DWTPs and in parallel filters (inter-filter variation) at one of
11 the DWTPs. Variation analysis revealed random sampling as the most efficient strategy to yield accurate
12 mean density estimates, with collection of at least 7 samples suggested to obtain an acceptable (below half
13 order of magnitude) density precision. *Nitrospira* was consistently the most dominant guild (5 to 10% of

14 total community), and was generally up to 4 orders of magnitude more abundant than *Nitrobacter* and up to 2
15 orders of magnitude more abundant than canonical AOBs. These results, supplemented with further analysis
16 of the previously reported diversity of *Nitrospira* in the studied DWTPs based on 16S rRNA and *nxrB* gene
17 phylogeny (Gülay et al., 2016; Palomo et al., 2016), indicate that the high *Nitrospira* abundance is due to
18 their comammox (complete ammonia oxidation) physiology. AOA densities were lower than AOB densities,
19 except in the highly stratified filters, where they were of similar abundance. In conclusion, rapid sand filters
20 are microbially dense, with varying degrees of spatial heterogeneity, which requires replicate sampling for a
21 sufficiently precise determination of total microbial community and specific population densities. A
22 consistently high *Nitrospira* to bacterial and archaeal AOB density ratio suggests that non-canonical
23 pathways for nitrification may dominate the examined RSFs.

24

25 Keywords

26 RSF, nitrifying guilds, AOB, NOB, AOA, comammox, *Nitrospira*

27

28 1. Introduction

29 Rapid sand filters (RSFs) are widely used in groundwater treatment to remove compounds like NH_4^+ , Fe^{2+}
30 and Mn^{2+} to below drinking water quality standards; removal occurs by a combination of physico-chemical
31 and biochemical means. The maximum allowed NH_4^+ (and NO_2^-) concentration in the effluent from the
32 drinking water treatment plants (DWTPs) in Europe is 0.5 mg/L (and 0.1 mg/L) (Council Directive 98/93/EC
33 1998), unless stricter limits are applied by the individual member states (e.g. 0.05 mg/l (and 0.01 mg/L) in
34 DK). NH_4^+ residuals in non-chlorinated systems can cause O_2 consumption in the distribution network
35 (Zhang et al. 2009), as well as accumulation of toxic NO_2^- due to incomplete nitrification (Lytle et al. 2007).
36 In chlorinated distribution systems, NH_4^+ removal is important to control the chlorine residuals and avoid the
37 formation of disinfection byproducts (Lytle et al. 2013, Rittmann et al. 2012).

38 NH_4^+ removal in RSFs has been assumed to be a two-step biological oxidation process, first to NO_2^- and then
39 to NO_3^- . Oxidation to NO_2^- has typically been attributed to canonical Ammonia Oxidizing Bacteria (AOB)
40 such as *Nitrosomonas* and *Nitrospira* and Ammonia Oxidizing Archaea (AOA); both AO types have been
41 identified in biological filters (Bai et al. 2013, de Vet et al. 2011, Tatari et al. 2016), although their relative
42 contribution to NH_4^+ removal in these engineered systems has not yet been examined. AOB predominance
43 over AOA seems to link with the NH_4^+ concentration, with lower concentrations favoring the predominance
44 of AOA, potentially due to their higher affinity for NH_4^+ (Martens-Habbenha et al., 2009; Niu et al., 2013;
45 Sauder et al., 2012). NO_2^- oxidation in RSFs has been attributed to *Nitrospira* and *Nitrobacter*, although
46 *Nitrospira* has been detected at much higher densities compared to *Nitrobacter* (Albers et al. 2015, Tatari et
47 al. 2016). Additionally, *Nitrospira* but not *Nitrobacter* were detected in a trickling filter treating groundwater
48 (de Vet et al. 2009).

49 Few studies have reported on densities of different nitrifying guilds, yet such information might provide
50 clues on the importance of different guilds in the overall RSF performance (i.e. ammonia removal) under
51 certain operational conditions. In trickling filters treating groundwater, mean filter densities of AOB (10^7 - 10^9
52 AOB cells /g filter material) vastly exceeded those of AOA (10^5 cells /g filter material) as quantified by
53 *amoA*-based qPCR (de Vet et al. 2009). AOB mean densities of 10^6 - 10^9 copies/g filter material were
54 reported (quantified by *amoA*-based qPCR) for granular activated carbon filters treating surface water (Niu
55 et al. 2013). The large range in reported densities (for single systems) would seem worrying, yet
56 quantification of total bacteria, AOB and AOA in biological sand filters has occasionally revealed substantial
57 depth stratification and spatial variation (Bai et al. 2013, Lee et al. 2014, Tatari et al. 2016, Gülay et al.,
58 2016). Hence, appropriate sampling may be essential to ensure representative density estimates of microbial
59 guilds in RSFs.

60 Relative densities of different microbial guilds can also be compared to predicted ratios based on assumed
61 physiology and stoichiometry; large deviations would then challenge the underlying assumptions. Recently
62 we discovered anomalous relative densities *Nitrospira* to AOB in RSFs, with *Nitrospira* comprising up to
63 45% of all community 16S rRNA gene amplicons with canonical AOB attaining on average 2.5% (Gülay et

64 al., 2016). These unexpectedly high *Nitrospira* densities do not agree with stoichiometry assuming
65 *Nitrospira* to have physiology of a nitrite oxidizing bacterium (NOB), which would predict ratios of AOB to
66 NOB of 2 to 3 (Hagopian and Riley 1998, Winkler et al. 2012). Recently, some *Nitrospira* types have been
67 found to carry a full set of *amo* and *hao* genes, which may enable them to oxidize also NH_4^+ and therefore
68 carry out complete NH_4^+ (to NO_3^-) oxidation; all *amo* containing *Nitrospira* described to date belong to
69 *Nitrospira* sublineage II (Daims et al. 2015, Palomo et al. 2016, van Kessel et al. 2015, Pinto et al., 2015).
70 Nevertheless, direct proof for NH_4^+ oxidizing activity by *Nitrospira* in RSFs has not yet been provided.

71 This work addressed the following questions. First, is there significant spatial variation in the distribution of
72 total bacteria and nitrifying guilds (AOB, AOA, *Nitrobacter* and *Nitrospira*) in RSFs? Second, do the
73 relative densities of the nitrifying guilds agree with their expected roles and physiologies in RSFs? Hence,
74 we conducted an extensive and spatially explicit survey of nitrifying guilds in replicate filters at 4 DWTPs.
75 The data were used to examine spatial variation, and identify optimal sampling strategies. Relative density
76 ratios were examined and related to the operating conditions to infer roles of the different guild members.
77 Finally, given the consistently high *Nitrospira* presence, we assessed the relative abundance of *Nitrospira*
78 sublineages based on *nxB* and 16S rRNA gene analysis.

79 2. Materials & Methods

80 2.1. Overview of the investigated DWTPs

81 The investigations included four Danish DWTPs treating groundwater that have been in operation for 6 to 40
82 years. Groundwater at the investigated DWTPs is abstracted from deep limestone anoxic aquifers and the
83 treatment train consists of an aeration step and a double filtration step. Pre-filters have a bed of coarse sand
84 material and are intended to retain the Fe-hydroxides, formed by the oxidation of Fe^{2+} . Pre-filter effluent is
85 supplied to the after-filters, which consists of a 0.4 to 0.7 m deep bed of fine sand on top of gravel. Design
86 parameters and selected water quality characteristics at the investigated DWTPs are summarized in Table 1.
87 Pre- and after-filters at the investigated DWTPs are backwashed at regular times by air scouring followed by
88 high water flowrates (Table 1).

89 DWTP 1 and 2 are located at Sjælsø (Nordvand A/S), in the greater Copenhagen area. The two DWTPs are
90 operated independently and receive raw water with significantly different CH₄ content (Table 1). DWTP 3
91 (Langerød) is located in Holbæk (Fors A/S) and consists of two parallel lines, West and East (W, E) which
92 were operated nearly identically, but displayed different performance with regards to NH₄⁺ removal (effluent
93 NH₄⁺ concentration 0.05 and 0.15 mg/L from E and W, respectively). DWTP 4 is Islevbro waterworks
94 (HOFOR A/S) located in the greater Copenhagen area, and has very low NH₄⁺ and CH₄ concentrations in the
95 raw water (Table 1).

96 2.2. Filter material sampling

97 Microbial density was quantified for filter material collected from the pre- and after-filters at the investigated
98 DWTPs. The sampling strategy was designed to investigate depth stratification, spatial variation within a
99 filter (intra-filter variation) and variation between parallel filters (inter-filter variation). Filter material was
100 core-sampled using a Plexiglas cylinder (1 m height and 5 cm inner diameter), closed on the one end with a
101 rubber stopper. The sampler was pushed into the filter and gently pulled retaining a 40-60 cm filter material
102 core. One pre-filter was sampled at 1-3 random locations at DWTPs 1-3. The pre-filters at DWTP 4 could
103 not be sampled because of the coarse filter material (Table 1) that could not be collected with the core
104 sampler. 2-3 parallel after-filters were sampled at all DWTPs, and a filter material core was collected from 1-
105 3 randomly selected locations in each filter. All cores were sampled at approximately 2/3 of a filter run cycle
106 between two consecutive backwashing events. The filter material cores were divided into the following depth
107 segments: 0-5, 5-10, 10-20, 20-30, 30-40, 40-50 cm that were transferred to the lab on ice and frozen until
108 further analysis.

109 2.3. Quantification of the selected microbial guilds by qPCR

110 The density of total bacteria (*Eubacteria*), β -proteobacterial AOB (*Nitrosomonas* spp and *Nitrosospira* spp),
111 *Nitrobacter* spp, and *Nitrospira* spp was quantified by 16S rRNA gene targeted qPCR in each filter material
112 segment. DNA was extracted from 0.5 g drained filter material using MP FastDNA SPIN Kit (MP
113 Biomedicals LLC) previously shown effective for DNA extraction from iron oxide rich environments (Kato
114 et al. 2013). Earlier efforts at removing/solubilizing metal oxides by pretreatment with oxalic acid did not

115 enhance DNA recovery, and neither did pretreatment by sonication. The extracted DNA was eluted in 100 μ l
116 Tris-EDTA buffer and its concentration and purity estimated by measuring absorbance at 260 and 280 nm
117 (NanoDrop). A group specific region on the 16S ribosomal-RNA encoding the *rrs* gene (16S rRNA) was
118 targeted by the primer sets 1055f and 1392R for total bacteria (Ferris et al. 1996, Lane 1991), CTO189A/B/C
119 and RT1 for β -proteobacterial AOB genera *Nitrosomonas* and *Nitrospira* (Hermansson and Lindgren
120 2001), Nspra675f and Nspra746r for the *Nitrospira* genus (Graham et al. 2007) and FGPS872f and
121 FGPS1269r for the *Nitrobacter* genus (Degrange and Bardin 1995). AOB and AOA were also quantified by
122 *amoA* targeted qPCR, using the *amoA*1f and *amoA*2r primers for β -proteobacterial AOB (Rotthauwe et al.
123 1997) and the *amoA*F and *amoA*R primers for AOA (Francis et al. 2005). Specificity of the 16S rRNA and
124 *amoA* targeted primers for beta-proteobacterial AOB was confirmed and published separately (Dechesne et
125 al., 2016); specificity of the *amoA* targeted primers for AOA was confirmed here by clone library analysis;
126 the 16S rRNA targeted primers for *Nitrospira* were confirmed *post hoc* by *in silico* comparison with the 16S
127 rRNA amplicon libraries of the same samples (Gülay et al. 2016) to be specific and cover 96% cover of all
128 *Nitrospira* diversity.

129 The qPCR analyses were run in duplicate in a Chromo4 thermocycler using the Opticon Monitor 3 software
130 (Bio-Rad Laboratories). Each qPCR reaction contained 12.5 μ l of 2 \times iQ SYBR Green Supermix (Bio-Rad
131 Laboratories), 500 nM of each primer, DNA template (10 ng) and DNA/RNA-free water (Mol. Bio.) to 25
132 μ l. The thermal cycling conditions consisted of an initial 5 min denaturation at 95°C, followed by 40 cycles
133 of 30 s at 94°C, primer annealing for 30 s at 55°C for total bacteria, 30 s at 60/56°C for AOB (16S-rRNA),
134 60 s at 50°C for *Nitrobacter*, 30 s at 64°C for *Nitrospira*, 60 s at 60°C for AOA (*amoA*), 40 s at 55°C for
135 AOB (*amoA*), and 1 min extension at 72°C. After the 40th cycle, a final DNA extension at 72°C for 10 min
136 was performed followed by cooling at 4°C. The melting curve analysis (gradient 0.2°C/s, range 70-95°C)
137 (Ririe et al. 1997) showed single peaks for all qPCR reactions. Reported qPCR reactions had amplification
138 efficiencies between 97 and 100%; addition of BSA (to address potential PCR inhibition) had no further
139 positive effect. The gene copy number was obtained by comparing the cycle threshold values of the sample

140 against a standard curve for each qPCR target. To express microbial densities per unit volume of filter
141 material, bulk densities at each depth were calculated by weighing 10-20 mL of drained filter material.

142 2.4. Statistical analysis of microbial densities

143 The measured densities in pre- and after-filters were statistically analyzed to assess their spatial variation at
144 three levels: depth stratification, intra-filter variation and inter-filter variation. Spearman's rank correlation
145 tests ($\alpha=0.05$) were performed to examine the correlation between density of a specific guild and filter depth.
146 To investigate intra-filter variation, the depth profiles from distinct locations in a filter were tested for
147 differences by paired t-tests ($\alpha=0.05$) in all possible combinations. Lastly, inter-filter variation was
148 investigated by paired t-tests ($\alpha=0.05$) on mean depth profiles in parallel filters. The mean depth profile in a
149 filter was calculated as the mean density at each depth for all sampling locations.

150 To assess how the sampling effort relates to the measured average density of a specific guild, we examined
151 the distribution of the total bacterial mean density in the after-filters. This analysis examined three sampling
152 strategies: random sampling, sampling at different depths and sampling at different locations. Detailed
153 information about statistical analyses is provided in the Supplemental Information (SI).

154 2.5. Bioinformatic analysis

155 We evaluated the lineages that constitute the comammox *Nitrospira* population genome CG24 earlier
156 identified in DWTP 4 (Palomo et al., 2016) and the *Nitrospira* distribution across its lineages at all DWTPs
157 based on earlier published 16S rRNA amplicon libraries (Gülay et al., 2016). Details on bioinformatics
158 analysis are in the SI.

159

160 3. Results & Discussion

161 3.1. Density of total bacteria, AOB, *Nitrobacter*, *Nitrospira* and AOA in RSFs

162 The targeted microbial guilds were quantified by qPCR in the filter material collected from the pre- and
163 after-filters. Filter material density and microbial density values are reported (Table 2) as means of the

164 densities at all depths, all sampled locations and all parallel filters for each filtration stage at each DWTP.
165 The mean filter material densities were in the range of $1.0\text{-}1.7\times 10^6$ g/m³ (Table 2), with variation caused by
166 the different filter material types and sizes (Table 1) and degrees of mineral coating (Gülay et al. 2014).

167 Microbial densities, measured per unit filter material mass, are readily converted into volume using the
168 estimated filter material densities. While both density expressions are presented (Table 2) for the examined
169 guilds, the following discussion considers volumetric densities.

170 Total bacterial (*Eubacteria*) 16S rRNA gene densities ranged from 1.1 to 16×10^{15} copies/m³ filter material
171 (Table 2). The density was highest in the pre-filters at DWTP 2, and was substantially higher than the density
172 in the after-filters at the same DWTP (Table 2). At the other investigated DWTPs, bacterial density was
173 similar in pre- and after-filters (Table 2). The high density observed in the pre-filters is surprising, as pre-
174 filters were historically thought to remove primarily Fe²⁺ and mainly through chemical oxidation and
175 precipitation (Sharma et al., 2005). It appears that the resulting oxy-hydroxide precipitates provide a highly-
176 porous mineral coating on the filter grains, which can support the high microbial density (Gülay et al., 2014).

177 Canonical AOB (*Nitrosomonas* spp. and *Nitrospira* spp.) were identified in all investigated filters at 16S
178 rRNA gene densities ranging from 0.5 to 35×10^{13} copies/m³ filter material. Densities in the pre-filters were
179 higher than in the after-filters at all DWTPs, with one exception (DWTP 3-E, Table 2). High AOB densities
180 in the pre-filters indicate that NH₄⁺ removal takes place in both filtration steps. For example, at DWTP 3 the
181 NH₄⁺ concentrations after aeration were 0.89-0.94 mg/L and decreased to 0.54-0.75 after the prefilters and
182 further to 0.007-0.1 mg/L after the after filters. This waterworks (DWTP 3) had different nitrification
183 behaviors in the parallel lines: DWTP 3-W consistently removed NH₄⁺-N more efficiently and to a lower
184 level (e.g. 0.007-0.01 mg/L) than DWTP 3-E (e.g. 0.077-0.11 mg/L), which is reflected by a higher AOB
185 density (particularly in the prefilters) in DWTP 3-W than in DWTP 3-E (Table 2).

186 *Nitrobacter* 16S rRNA gene densities ranged across two orders of magnitude: from 3.6 to 500×10^{10}
187 copies/m³ in the investigated filters (Table 2). *Nitrobacter* pre-filter densities were higher than the respective
188 after-filter densities at all DWTPs. *Nitrospira* were present in all investigated filters at 16S rRNA gene

189 densities ranging from 0.97 to 33×10^{14} copies/m³ filter material. *Nitrospira* densities at two waterworks
190 (DWTPs 1 and 2) were slightly (4 to 6 fold) higher in pre-filter versus after-filters, with the opposite trend in
191 a third waterworks (DWTP 3, Table 2). Consistently, *Nitrospira* densities were at least 2 orders of magnitude
192 higher than *Nitrobacter* densities, suggesting *Nitrospira* as dominant NO₂⁻ oxidizer at all DWTPs. Another
193 study on Danish DWTPs reports that *Nitrospira* accounted for up to 8% of all prokaryotic amplicon
194 sequences and detected no *Nitrobacter* sequences in RSFs (Albers et al. 2015); however the lack of
195 replication and the exceedingly small amplicon library size (558 sequences/samples) challenge the
196 robustness of those observations. Similarly *Nitrospira*, but not *Nitrobacter*, were detected by DGGE analysis
197 of 16S rRNA gene fragments from a groundwater fed trickling filter (de Vet et al. 2009). *Nitrospira*
198 sequences also accounted for up to 51% of the clone library in samples from an anthracite/sand dual media
199 filter fed with groundwater (White et al. 2012). Finally, in our microbial community diversity analysis on the
200 same DWTPs, we similarly observed *Nitrospira* sp. as the most abundant taxon in the 16S rRNA amplicon
201 libraries (from 19% to 71% in all DWTPs (Gülay et al., 2016)).

202 AOA *amoA* gene densities were below detection limits in one of the after-filters (DWTP 3), while at the
203 other waterworks their densities ranged from 4.0 and 20×10^9 copies/m³ filter material. AOA were typically 4
204 orders of magnitude less abundant than AOBs, except in the DWTP 4 after-filters, where highest AOA
205 densities were observed (Table 2).

206 Overall, RSF are microbially dense, and the targeted guilds are often at least as heavily present in the pre-
207 filters as in the after-filters, revealing an important role of pre-filters in biological processes.

208 3.2. Spatial distribution of the investigated microbial guilds

209 In the pre-filters, limited stratification was observed (except in DWTP 2, where AOB and *Nitrospira*
210 densities decreased with depth and in DWTP 1 where *Nitrospira* densities increased with depth (Fig 1, Table
211 SI 1)). Stratification in the after-filters was noted for some microbial guilds at DWTP 2 and 3, and for all
212 microbial guilds (except AOA) at DWTP 4 (Fig. 1, Table SI 2), with densities decreasing with depth. No
213 stratification was observed in the after-filters at DWTP 1 (Fig. 1).

214 Backwashing frequency and strategy are crucial to control the mixing and redistribution of the filter material,
215 eventually preventing or allowing permanent stratification in the filter. Stratification at DWTP 3 and 4 is
216 maintained after backwashing due to the presence of substantial mineral coatings on the filter material
217 grains, which forces larger – and less dense grains – to remain at the top of the filter, and smaller – denser
218 grains – to settle in deeper filter regions, thus maintaining a permanent distribution (Gülay et al. 2014). In
219 line with these observations, sand grains in DWTP 1 and 2 have a very low degree of mineral coating at all
220 depth levels and show no evidence of biomass stratification (Gülay et al. 2014). Depth stratification in
221 groundwater-fed periodically backwashed RSFs has been observed before (Bai et al. 2013).

222 In individual pre-filters, replicate profiles at different sampling locations yielded essentially similar density
223 distributions for all microbial populations (except for *Nitrobacter* (Table SI 3)) and the same was observed in
224 the after-filters (Table SI 4). This stands in contrast to previous studies (at DWTP 1) which documented local
225 hydraulic heterogeneity in both pre- and after-filters, and suggested a strong effect on local NH_4^+ removal
226 rate (Lopato et al. 2011, Lopato et al. 2013). Clearly, the hydraulic heterogeneity does not result in
227 observable differences in horizontal densities of microbial groups.

228 However, parallel after-filters at a DWTP often had different profiles: densities of total bacteria, AOB and
229 *Nitrospira* varied across the filters of both lines at DWTP 3, whereas 50% of the compared profiles were
230 significantly different at DWTP 4 and no significant variation was observed at DWTP 1 and 2 (Tables SI 5-
231 7). Inter-filter variation at both lines of DWTP 3 was much larger than the intra-filter variation, and the
232 opposite was observed at DWTPs 1 and 2. Hence, spatial variation of the targeted guilds does not follow a
233 predictable pattern at the investigated DWTPs.

234 3.3. Investigation of mean density precision and required sampling effort

235 The increase in precision of the estimated mean density was evaluated as a function of sampling effort and
236 sampling strategy ((i) random sampling of s samples across locations, parallel filters, and depth; (ii) sampling
237 all locations for d randomly chosen depths; (iii) and sampling full profiles at l randomly chosen locations).
238 Setting half-an-order of magnitude as an acceptable precision, the mean density and associated precision

239 were computed at the investigated DWTPs: clearly precision increased with increasing sampling effort (Fig.
240 2, Fig. SI 1). With DWTP 4 as an example, sampling at 7 random combinations of locations and depths was
241 required to meet the demanded precision (Fig. 2). Sampling at different depths would require 14 samples (2
242 randomly chosen depths at all investigated filter locations and parallel filters), sampling at different locations
243 and filters would require 17 samples (at least 3 locations, each location sampled at 5-6 depths). Clearly
244 random sampling yielded an acceptably precise density estimate with lowest effort, and there was no
245 advantage of exhaustively sampling depths profiles or horizontal profiles.

246 The mean bacterial density estimates were more precise at DWTPs 1-3, where collection of a singular
247 sample would already give an estimate within half-an-order of magnitude precision (Fig. SI 1). For almost
248 all DWTPs, random sampling yields the highest precision compared to sampling at different depths or
249 locations (Fig. SI 1). The only exception was at DWTP 3-W, where sampling of 1 filter location at all depths
250 (7 samples) yields a higher precision than collecting 7 random samples (Fig. SI 1). However, fewer than 7
251 samples are required to obtain a mean precision above the acceptable limit of half-an-order of magnitude.

252 Overall, the mean density precision can vary significantly at different DWTPs. In all cases random sampling
253 across filters, filter locations and depths yielded the highest precision for a given number of samples.
254 According to the precision required by each study, the number of samples needed may vary significantly. In
255 this study, each sample had a mass of 100-333 g drained filter material, depending on the depth interval
256 (segmented every 5 or 10 cm as described in sec. 2.2) and the bulk density. Ultimately, 7 samples randomly
257 collected should generally be sufficient to provide a precision of the mean of half order of magnitude, even at
258 DWTPs with heterogeneous microbial spatial distributions. Our recommendations apply to density, but may
259 not apply to community diversity or composition. When the same samples were subject to community
260 analysis via 16S rRNA amplicon sequencing, triplicate samples within a RSF were compositionally different
261 from each other at the whole community level (Gülay et al., 2016); yet were identical in terms of dominant
262 taxa (>1%) (Gülay and Smets, 2015).

263 3.4. Relative abundance of microbial guilds

264 The fraction of canonical AOB (abundance vs total bacteria) ranged from 0.004 and 0.07 in the pre-filters,
265 and from 0.002 and 0.12 in the after-filters (Fig. 3, Panels A and D). Abundances in the pre- and after-filters
266 were similar at most DWTPs except at DWTP 1, where abundance in the pre-filters was approximately one
267 order of magnitude higher than in the after-filters. No patterns were observed with depth.

268 The ratio of canonical AOB to *Nitrospira* ranged from 0.02 and 1.9 in the pre-filters, and from 0.04 and 0.6
269 in the after-filters (Fig. 3, Panels B and D). Ratios were consistently below 1 in all RSFs except in one set of
270 pre-filters (DWTP 1), where AOB were more dominant than *Nitrospira* in the filter top (ratio 1.9). Even
271 here, an increase in *Nitrospira* density with depth (Table SI 1) made AOB less dominant than *Nitrospira* at
272 the bottom of the filter (ratio 0.05). Overall, while the proportions varied, canonical AOB (i.e. *Nitrosomonas*
273 plus *Nitrospira* genus) were consistently less abundant than *Nitrospira* genus.

274 Similar anomalous *Nitrospira* to *Nitrosomonas* (or more correctly *Nitrosomonas* + *Nitrospira*) abundance
275 ratios have been reported by others. These reports were based on relative sequence abundances in clone or
276 amplicon libraries in both groundwater (e.g. 3.7% vs 33.4% (Nitzsche et al. 2015); 3.2% vs 16.9% (White et
277 al. 2012) *Nitrosomonas* and *Nitrospira* abundance, respectively) and surface water fed drinking water
278 treatment gravity filters (0.8% vs 17.3% (Feng et al. 2012), 0.07% vs 17 *Nitrosomonas* and *Nitrospira*
279 abundance, respectively (LaPara et al. 2015)). Proposed, yet unproven, explanations for these observations
280 were PCR primer bias, differences in 16S rRNA gene copy numbers (Nitzsche et al. 2015), presence of
281 unidentified NH_4^+ oxidizers (LaPara et al. 2015) and presence of dormant *Nitrospira* cells (Martiny et al.
282 2005). Winkler et al. (2012) proposed that unexpectedly high NO_2^- oxidizer (e.g. *Nitrospira*) abundance in a
283 community may also be caused by a NO_2^- oxidation/ NO_3^- reduction loop, which would be driven by
284 incomplete heterotrophic denitrification. While bulk phase dissolved oxygen concentrations in both pre- and
285 after-filters are near saturation, strictly heterotrophic anaerobes (*Xanthomonadales* and *Anaerolineales*) have
286 been reported in amplicon libraries of the after-filters (Gülay et al., 2016), and existence of the proposed
287 $\text{NO}_2^-/\text{NO}_3^-$ loop, and its contribution to the observed *Nitrospira* abundance should be examined.

288 Recent discoveries have revealed that the physiological abilities of *Nitrospira* may extend far beyond NO_2^-
289 oxidation. Genomic and physiological evidence has shown that certain *Nitrospira* strains can oxidize
290 hydrogen, cyanate and simple organic compounds such as formate (Koch et al., 2014) (Palatinszky et al.,
291 2015). The chemical nature of the groundwater and the initial aeration/stripping makes cyanate or hydrogen
292 unlikely candidates to support *Nitrospira* growth, especially in the downflow units. Similarly, because
293 assimilable organic carbon in the feed groundwater is low, only decay products from the RSF microbiome
294 would be available to support heterotrophic *Nitrospira* growth. The qPCR results, and our previous
295 survey (Gülay et al., 2016), indicate that *Nitrospira* is the most abundant taxon: such abundance is not likely
296 supported by metabolic decay products of a microbial community. Of specific interest is the recent discovery
297 that some *Nitrospira* genomes harbor *amo* genes and have the potential for NH_4^+ oxidation – in addition to
298 NO_2^- oxidation – endowing them the physiology of complete ammonia oxidation (comammox) (Daims et al.,
299 2015; Palomo et al., 2016; Pinto et al., 2015; van Kessel et al., 2015). The described enriched and isolated
300 comammox *Nitrospira* strains - *Candidatus Nitrospira inopinata*, *Cand. N. nitrosa* and *Cand. N. nitrificans*
301 (Daims et al., 2015)(van Kessel et al., 2015) – all belong to *Nitrospira* lineage II, with *N. moscoviensis* as
302 best known canonical NOB as reference strain (Pester et al., 2014).

303 Given the striking dominance of the *Nitrospira* genus in all DWTPs (Fig. 1, Table 2), we analyzed its
304 composition across *Nitrospira* lineages from the 16S rRNA amplicon libraries at the same DWTPs (Gülay et
305 al., 2016): *Nitrospira* lineage II dominated at all DWTPs, representing consistently over 95%, with not-yet-
306 named lineages accounting for $3.5\% \pm 0.03$, of all *Nitrospira* reads (Fig. 4 Fig SI 2, Table SI 8).

307 Earlier, we identified, from the DWTP 4 metagenome, a *Nitrospira* population genome with comammox
308 capability (CG_24) (Palomo et al., 2016). This population genome consisted of several sub-genomes, but no
309 efforts were made to separate the individual genomes and examine their phylogeny. Here – based on
310 phylogenetic analysis of CG24's *nxB* content - we show that the five different constituent *nxB* sequence
311 types are lineage II (Fig. 4). As *nxB* and 16S rRNA genes yield congruent *Nitrospira* phylogenies (Pester et
312 al., 2014), and given the high abundance of CG24 in the community metagenome (up to 30 % of all mapped
313 reads (Palomo et al., 2016)); *Nitrospira* in DWTP 4 are likely primarily comammox *Nitrospira*. By

314 extension, these results indicates that the high *Nitrospira* abundances (compared to canonical AOBs) in all
315 examined DWTPs are, to a large extent, the result of the NH_4^+ oxidation capability of *Nitrospira*.

316 AOA were consistently less abundant than AOB (based on *amoA* targeted qPCR) in both pre- and after-
317 filters at DWTPs 1, 2, and 3, (Fig. 3, Panels C and F) and occasionally at or below detection limit. AOA have
318 been identified in other drinking water treatment plants – where occasionally they are abundant (Kasuga et
319 al., 2010; van der Wielen et al., 2009). When detected in this study, the AOA/AOB ratios ranged between
320 3×10^{-6} and 8×10^{-3} in the pre-filters, and between 2×10^{-5} and 0.2 in the after-filters. While the AOA/AOB
321 ratios were calculated based on archaeal and bacterial *amoA* targeted qPCR, quantification based on the
322 chosen *amoA* PCR primers (Rotthauwe et al. 1997) underestimates AOB densities (based on 16S rRNA
323 targeted qPCR) at some DWTPs (DWTPs 1, 2 and 4 but not at DWTP 3 (Fig. SI 3)). This underestimation
324 was recently identified as caused by the preferential amplification of *amoA* Cluster 7 vs. Cluster 6A AOBs
325 (Dechesne et al. 2016). In addition, the employed *amoA* primers would not quantify comammox *amoA*
326 (Daims et al. 2015). Considering this bias, the AOA to AOB ratios at DWTPs 1 and 2 would be even lower.
327 No consistent trend of the AOA/AOB ratio with depth was observed at any of these DWTPs. AOAs were at
328 significant abundance compared to AOB in only one of the waterworks (DWTP 4) (AOA/AOB from 0.26 in
329 the top 0-5 cm to 20 at the bottom 40-50 cm (Fig. 3, Panel F)). The increased ratio (by roughly 2 orders of
330 magnitude) was mainly due a reduction in AOB abundance (Fig. 1, Panel C) and may suggest a significant
331 role of AOA at the bottom of the filter. Yet, as *amoA* based qPCR also underestimates AOB densities at
332 DWTP 4 (Fig SI 3), the true ratios are higher and AOA may have a secondary role even at the bottom of the
333 filter at DWTP 4. The relative enrichment of AOA over AOB at the filter base (with lower NH_4^+ supply) is,
334 nevertheless, consistent with the notion that AOA are competitive over AOB in conditions of reduced energy
335 supply, such as in soils (Leininger et al. 2006, Verhamme et al. 2011) and ocean (Wuchter et al. 2006), most
336 likely due to their ability to cope with energy stress (Martens-Habbena et al. 2009, Valentine 2007). At the
337 system level, similar correlations between AOA and AOB predominance and NH_4^+ loading are apparent,
338 with AOB densities (in both pre- and after-filters) increasing with loadings, and AOA densities decreasing

339 with loading (Fig. 5) consistent with observations that AOA densities increase inversely with ammonium
340 gradients (Sauder et al., 2012).

341 In sum, notwithstanding the relatively low influent ammonium concentrations (max. 1 mg/L) at all DWTPs
342 canonical AOB are numerically dominant over AOA, and both are vastly inferior to *Nitrospira*.

343

344 4. Conclusions

345 This work presents the first comprehensive investigation of the density and spatial distribution of total
346 bacteria, AOB, *Nitrobacter*, *Nitrospira* and AOA by qPCR at a number of full scale DWTPs. The main
347 conclusions are:

- 348 • Microbial density can vary spatially in a filter and in parallel filters at a DWTP. Specifically, we
349 observed strong stratification of nitrifying guilds in one DWTP, but not in others. Horizontally, intra-
350 or/and inter filter variation were significant at the investigated DWTPs, indicating the need of proper
351 experimental design to obtain representative results.
- 352 • Variation analysis for total bacteria estimated that 7 random filter material samples need to be
353 collected at a DWTP with high spatial variation to obtain an acceptable precision for the mean (half
354 an order of magnitude). At the other investigated DWTPs, one sample collected from a random
355 location can already provide a density estimate within an acceptable (half an order of magnitude)
356 precision interval. Higher sample replication will typically be required if the goal is to describe mean
357 microbial community composition (Gulay et al, 2016)
- 358 • *Nitrospira* were roughly 4 orders of magnitude more abundant than *Nitrobacter* in all investigated
359 filters, suggesting that they should be the predominant NO_2^- oxidizers. Yet, *Nitrospira* were also up
360 to almost 2 orders of magnitude more abundant than AOBs, and we suggest that the *Nitrospira*
361 abundance is primarily caused by their NH_4^+ oxidation capability (comammox).

- 362 • Among the canonical ammonia oxidizers, AOB exceed AOAs in RSFs, except in RSFs with strong
363 stratification; and AOB are favored at DWTPs operating at higher loading rates.

364

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- 508

509

510 **Table 1.** Selected water quality parameters and characteristics at the 4 investigated DWTPs.

		DWTP 1 (Sjælsø 1)	DWTP 2 (Sjælsø 2)	DWTP 3 (Langerød)	DWTP 4 (Islevbro)
Raw water ⁱ	NH ₄ ⁺ (mg/L)	0.51 ± 0.21	1.08 ± 0.44	1.09 ± 0.17	0.41 ± 0.20
	CH ₄ (mg/L)	0.12 ± 0.17	3.73 ± 2.67	0.07 ± 0.04	0.02 ± 0.03
	Fe ²⁺ (mg/L)	2.9 ± 1.7	1.9 ± 1.1	3.0 ± 0.2	1.8 ± 1.1
	HCO ₃ ⁻ (mg/L)	N/A ⁱⁱ	N/A	360 ± 8	350 ± 48
	NVOC (mg/L)	2.7 ± 0.4	2.3 ± 0.3	2.6 ± 0.2	2.3 ± 0.3
	Dissolved O ₂ (mg/L)	0.65 ± 0.3	0.87 ± 0.4	0.31 ± 0.1	1.1 ± 0.6
	pH	N/A	N/A	7.4 ± 0.1	7.4 ± 0.3
Aeration	Aerator type	Cascade	Intensive tray (INKA)	Cascade	Corrugated plate (Coplator)
Pre-filters	Filter flow (×10 ³ L/h)	80	60	100	288
	Active layer depth (m)	0.4 + 0.4	0.3	0.7	2.0
	Material ⁱⁱⁱ	1.6-2.5 mm anthracite + 3.0- 5.0 mm sand	3.0-5.0 mm sand	2.0-3.0 mm sand	40-50 mm gravel
	Backwashing frequency (d) ^{iv}	13	10	1	30
	Backwashing procedure	5 m air scouring followed by 5 min water upwash at 1000 m ³ /h	3 min air and water flushing followed by 15 min water upwash at 1550 m ³ /h	15 min air scouring followed by 4 min water upwash at 920 m ³ /h	N/A
After-filters	Filter flow (×10 ³ L/h)	52	60	50	72
	Active layer depth (m)	0.4	0.4	0.65	0.7
	Material	1.5-2.0 mm calcined flint	0.8-1.4 mm sand	0.8-1.4 mm sand	0.8-1.2 mm sand
	Backwashing frequency (d) ⁱⁱⁱ	12	14	10	14
	Backwashing procedure	5 min air scouring followed by 5 min water upwash at 1000 m ³ /h	5 min air scouring followed by 8 min water upwash at 1550 m ³ /h	5 min air scouring followed by 5 min water upwash at 600 m ³ /h	3 min air scouring at 90 m/h followed by water 10 min upwash at 450 m ³ /h
Treated water ⁱ	NH ₄ ⁺ (mg/L)	<0.05	<0.05	0.11 ± 0.11	<0.05
	CH ₄ (mg/L)	N/A	N/A	<0.01	<0.01
	Fe ²⁺ (mg/L)	<0.1	<0.1	<0.1	<0.1
	HCO ₃ ⁻ (mg/L)	N/A	N/A	360 ± 8	350 ± 13
	NVOC (mg/L)	2.4 ± 0.5	3.3 ± 0.6	2.5 ± 0.3	2.2 ± 0.1
	Dissolved O ₂ (mg/L)	N/A	N/A	9.7 ± 0.5	8.7 ± 0.4
	pH	N/A	N/A	7.7 ± 0.1	7.4 ± 0.1

511 i. Water quality data are means provided by the DWTPs

512 ii. N/A: Not Available

513 iii. Nominal size range before filter start-up. Actual filter material size may be different due to mineral deposition on the filter material

514 iv. Backwashing frequency is regulated from the total filter flow. Calculation of average backwashing interval here is based on the

515 average filter flow

516 **Table 2.** Filter material bulk densities and microbial densities per mass and per volume of filter material.
517 Reported values are means calculated from equal weighing of measured values at all depths, sampling
518 locations in a filter and parallel filters at each DWTP. Mean values are reported with their standard deviation.

519

ACCEPTED MANUSCRIPT

Filter material bulk density						
Pre-filters			After-filters			
	($\times 10^6$ g/m ³ filter material)	N ⁱ	($\times 10^6$ g/m ³ filter material)		N ⁱ	
DWTP 1	1.0 \pm 0.25	6	1.2 \pm 0.06		12	
DWTP 2	1.4 \pm 0.04	5	1.5 \pm 0.03		11	
DWTP 3-E	1.7 \pm 0.05	6	1.6 \pm 0.05		13	
DWTP 3-W	1.5 \pm 0.07	5	1.6 \pm 0.05		14	
DWTP 4	N/S	-	1.5 \pm 0.28		12	
Total bacteria (<i>Eubacteria</i>) ⁱⁱⁱ						
	Mass density	Volumetric density ⁱⁱ	N	Mass density	Volumetric density	N
	($\times 10^9$ copies/g)	($\times 10^{15}$ copies/m ³)		($\times 10^9$ copies/g)	($\times 10^{15}$ copies/m ³)	
DWTP 1	2.0 \pm 1.0	1.8 \pm 0.56	6	0.9 \pm 2.2	1.1 \pm 0.27	29
DWTP 2	12 \pm 4.6	16 \pm 6.1	14	1.6 \pm 6.3	2.5 \pm 0.97	30
DWTP 3-E	3.1 \pm 1.2	5.1 \pm 2.0	11	3.5 \pm 17	5.7 \pm 2.9	26
DWTP 3-W	6.6 \pm 1.7	9.8 \pm 2.7	5	4.0 \pm 17	6.5 \pm 2.8	32
DWTP 4	N/S ^v	N/S	-	8.8 \pm 13	11 \pm 16	40
AOB (<i>Nitrosomonas</i> spp and <i>Nitrosospira</i> spp) ⁱⁱⁱ						
	($\times 10^7$ copies/g)	($\times 10^{13}$ copies/m ³)	N	($\times 10^7$ copies/g)	($\times 10^{13}$ copies/m ³)	N
DWTP 1	19 \pm 16	16 \pm 12	11	0.41 \pm 17	0.49 \pm 0.21	30
DWTP 2	13 \pm 6.5	17 \pm 8.7	14	2.0 \pm 0.88	3.0 \pm 1.4	30
DWTP 3-E	5.8 \pm 4.2	9.6 \pm 6.8	11	15 \pm 14	24 \pm 22	26
DWTP 3-W	23 \pm 7.8	35 \pm 12	5	12 \pm 12	20 \pm 19	25
DWTP 4	N/S	N/S	-	1.2 \pm 2.3	1.3 \pm 2.5	35
<i>Nitrobacter</i> ⁱⁱⁱ						
	($\times 10^4$ copies/g)	($\times 10^{10}$ copies/m ³)	N	($\times 10^4$ copies/g)	($\times 10^{10}$ copies/m ³)	N
DWTP 1	7.8 \pm 1.5	7.6 \pm 2.0	12	3.0 \pm 1.8	3.6 \pm 2.1	12
DWTP 2	370 \pm 230	500 \pm 310	14	19 \pm 7.9	29 \pm 12	10
DWTP 3-E	8.7 \pm 3.0	15 \pm 4.9	17	6.1 \pm 0.91	10 \pm 15	7
DWTP 3-W	23 \pm 16	34 \pm 23	10	5.4 \pm 4.0	8.6 \pm 6.3	11
DWTP 4	N/S	N/S	-	9.7 \pm 16	11 \pm 16	36
<i>Nitrospira</i> ⁱⁱⁱ						
	($\times 10^8$ copies/g)	($\times 10^{14}$ copies/m ³)	N	($\times 10^8$ copies/g)	($\times 10^{14}$ copies/m ³)	N
DWTP 1	3.4 \pm 2.3	3.8 \pm 3.4	17	0.81 \pm 0.21	0.97 \pm 0.26	17
DWTP 2	24 \pm 13	33 \pm 17	15	3.3 \pm 1.1	5.1 \pm 1.7	25
DWTP 3-E	1.7 \pm 0.88	2.8 \pm 1.4	10	5.2 \pm 4.1	8.6 \pm 6.9	23
DWTP 3-W	5.9 \pm 0.85	8.8 \pm 1.4	5	8.9 \pm 5.4	14 \pm 8.8	23
DWTP 4	N/S	N/S	-	8.4 \pm 13	9.7 \pm 14	46
AOA ^{iv}						
	($\times 10^3$ copies/g)	($\times 10^9$ copies/m ³)	N	($\times 10^3$ copies/g)	($\times 10^9$ copies/m ³)	N
DWTP 1	6.0 \pm 3.9	5.7 \pm 3.3	12	18 \pm 14	21 \pm 17	18
DWTP 2	15 \pm 11	20 \pm 15	14	3.8 \pm 1.7	5.7 \pm 2.6	15
DWTP 3-E	2.3 \pm 1.1	3.9 \pm 1.9	17	< 1 ^{vi}	< 1.6 ^{vi}	-
DWTP 3-W	2.6 \pm 2.5	4.0 \pm 3.8	5	< 1 ^{vi}	< 1.6 ^{vi}	-
DWTP 4	N/S	N/S	-	320 \pm 120	460 \pm 260	6

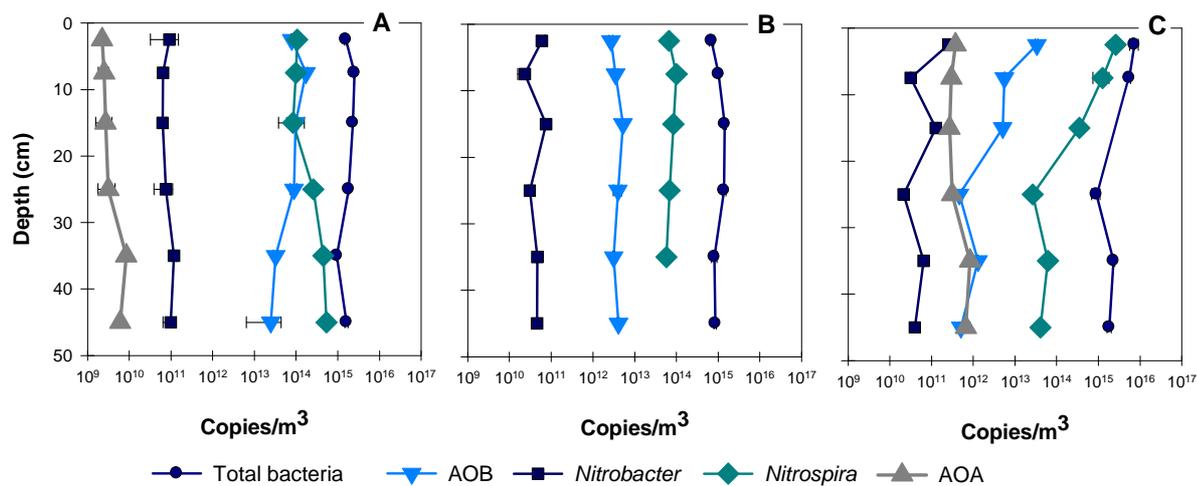
i. Number of samples used to calculate mean density values

ii. Conversion from filter material mass to volume units was done by multiplying with the mean bulk density

iii. Quantified by 16S rRNA targeted qPCR, iv. Quantified by *amoA* targeted qPCR

v. N/S: Not sampled due to coarse filter material in the pre-filter at DWTP 4

vi. Densities below detection limit

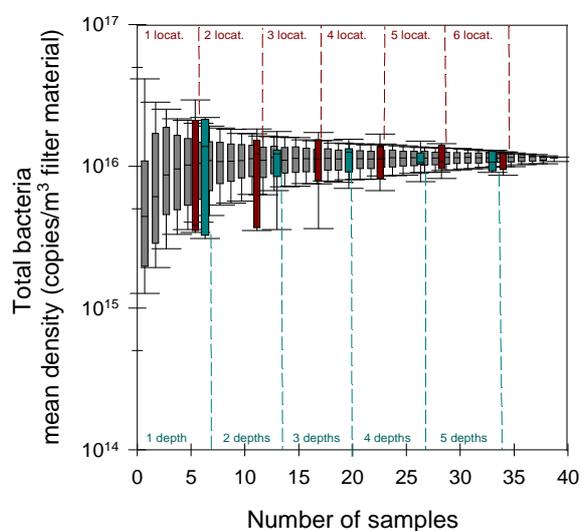


521

522 **Figure 1.** Depth profiles of the volumetric densities at one location of: one pre-filter at DWTP 1 (Panel A),
 523 one after-filter at DWTP 1 (Panel B) and one after-filter at DWTP 4 (Panel C). Densities were quantified by
 524 qPCR and error bars represent the standard deviation of the analytical method.

525

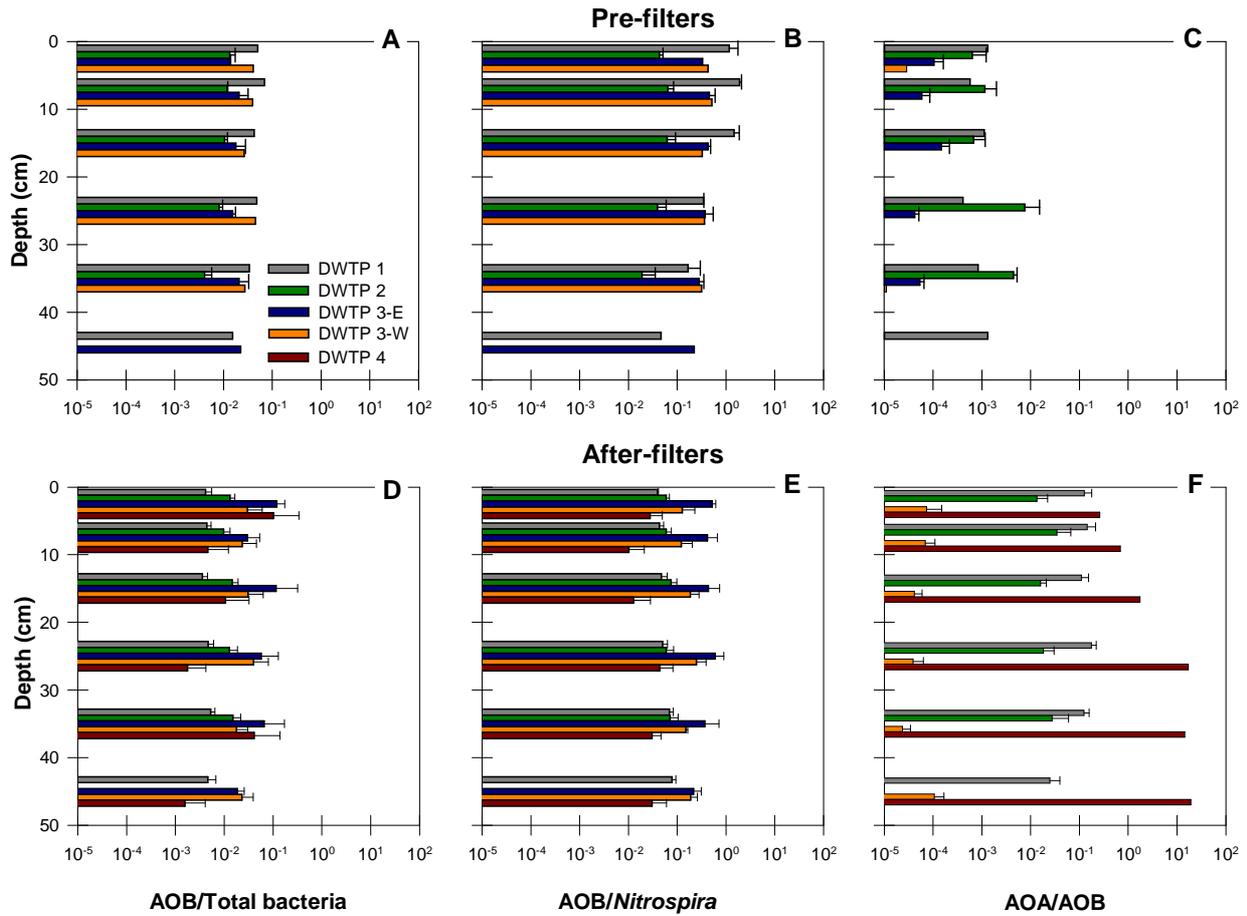
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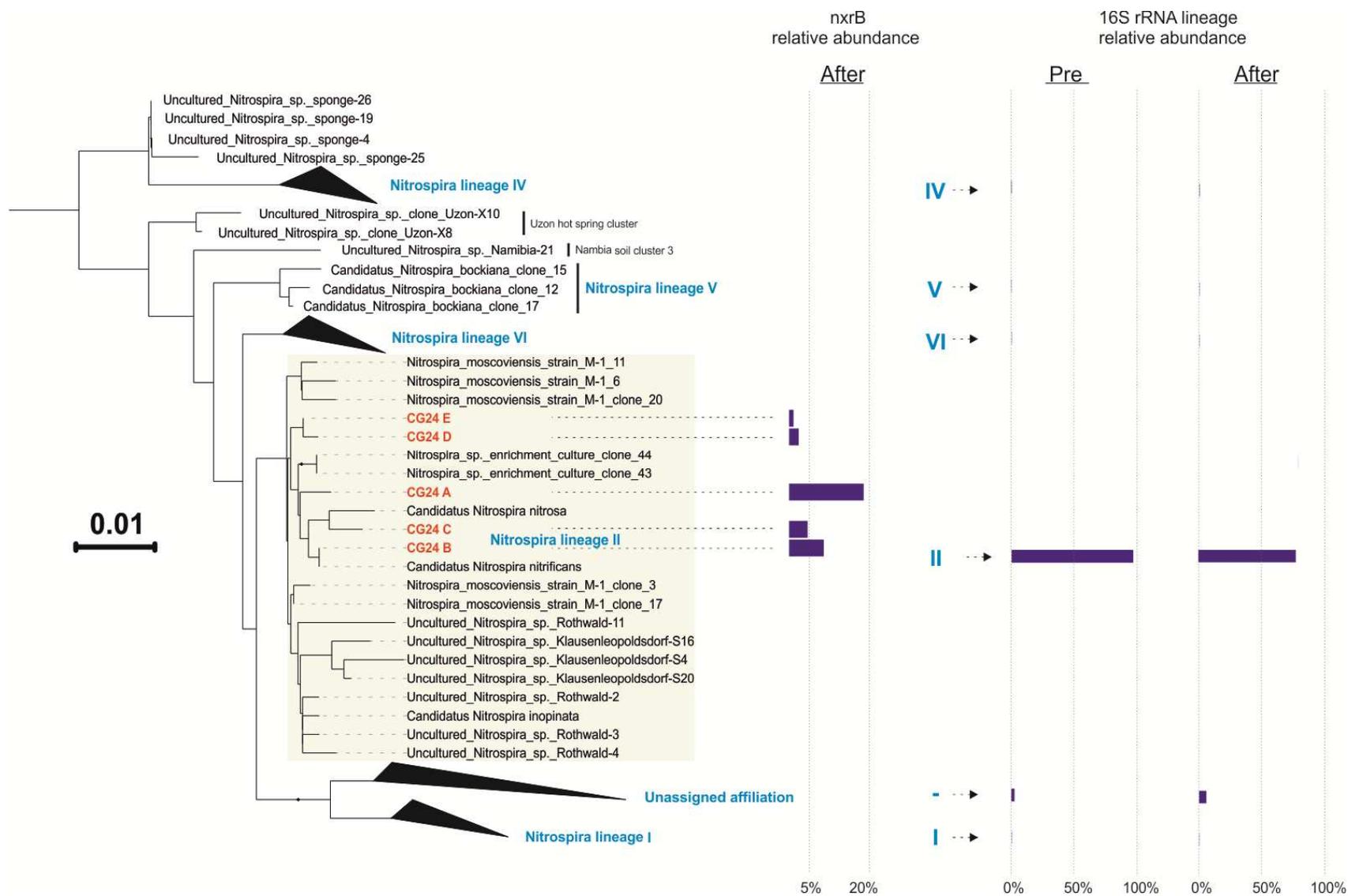


528 **Figure 2.** Distribution of total bacterial mean density at DWTP 4 based on sample number and sampling
529 strategy. Random sampling includes all random combinations of samples at different depths, locations and
530 parallel after-filters. Sampling at different depths includes all locations at these specific depths and sampling
531 at different locations includes all depths at these specific locations.



532

533 **Figure 3.** Density ratios in the pre-filters (Panels A-C) and after-filters (Panels D-F) at all investigated
 534 DWTPs. Panels A and D: ratio of AOB to total bacteria quantified by 16S rRNA-specific qPCR; Panels B
 535 and E: ratio of AOB to *Nitrospira* quantified by 16S rRNA-specific qPCR; Panels C and F: ratio of AOA to
 536 AOB quantified by the *amoA*-specific qPCR. Bars are means of all the sampled locations in all parallel
 537 filters and error bars are the standard deviation of the mean.

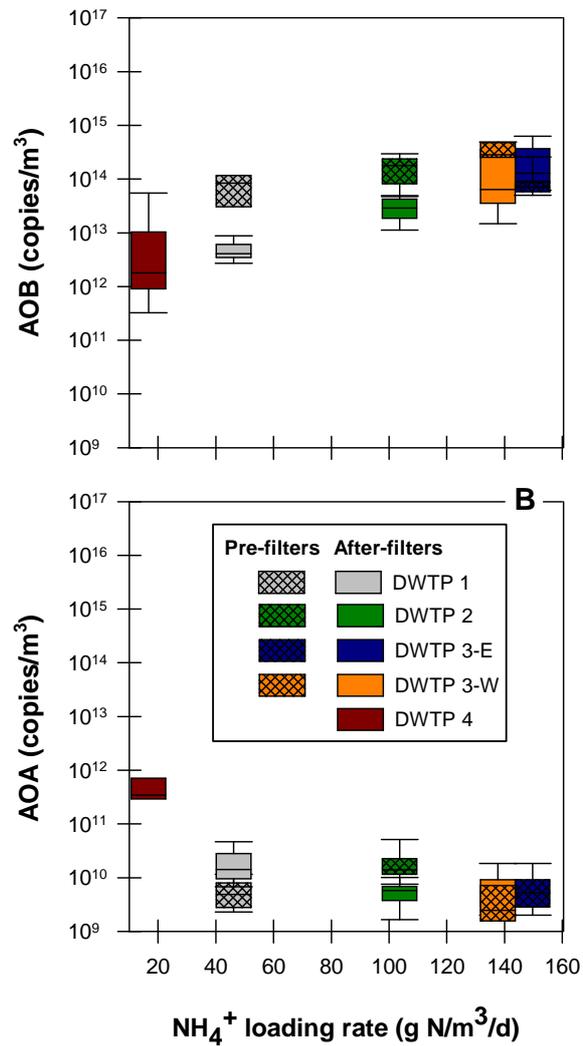


538

539 **Figure 4.** Phylogenetic analysis of *nxB* genes retrieved from the CG24 *Nitrospira* population genome
540 (Palomo et al., 2016) with NCBI database recorded sequences as reference (Pester et al., 2014). The scale bar
541 indicates 1% estimated sequence divergence. Relative abundance of CG24 *nxB* genes (compared to total
542 *nxB* gene in the community metagenome) and relative abundance of *Nitrospira* 16S rRNA sublineages
543 (compared to all *Nitrospira* sequences) sequences are reported.

544

545



546

547 **Figure 5.** Volumetric densities of AOB (Panel A) and AOA (Panel B) in the investigated pre- and after-
 548 filters as a function of the NH_4^+ loading rate to the DWTP.

549

Highlights

- Microbial density in RSFs are high (upto 10^9 to 10^{10} per gram (10^{15} to 10^{16} per m^3) but vary spatially.
- Acceptable mean precision of density estimates requires 7 random samples.
- *Nitrospira* were more abundant than *Nitrobacter* and AOBs.
- *Nitrospira* abundance is likely caused by their NH_4^+ oxidation capability (comammox).
- AOB consistently exceed AOBs in RSFs, except in RSFs with strong stratification.