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Copper-induced stimulation of nitrification in biological rapid sand filters for drinking water production by proliferation of *Nitrosomonas* spp.

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14 ABSTRACT

15 Copper is a co-factor of the ammonia monooxygenase, an essential enzyme for the activity of ammonia
16 oxidizing prokaryotes (AOP). Copper dosing at less than 1 μg/L stimulated ammonium removal in the
17 poorly-nitrifying biological filters of three full-scale drinking water treatment plants. Upon copper
18 dosing, the ammonium concentration in the effluent decreased from up to 0.18 to less than 0.01 mg NH_4^+-
19 N/L. To investigate how copper dosing affected the filter microbial community, we applied amplicon
20 sequencing and qPCR targeting key nitrifying groups, including complete ammonia oxidizing
21 (comammox) *Nitrospira*. Copper dosing increased the abundance of different nitrifiers. Multiple
22 *Nitrosomonas* variants (betaproteobacterial ammonia oxidizers), which initially collectively represented
23 1% or less of the total community, increased almost 10 fold. Comammox *Nitrospira* were abundant and
24 increased too, but their relative abundance within the AOP decreased because of *Nitrosomonas*
25 proliferation. No other consistent change in the filter communities was detected, as well as no adverse
26 effect of copper on the filters functionality. Our results show that copper dosing in three independent
27 plants was associated with consistent growth of AOP and that efficient nitrification was achieved through
28 the joint contribution of comammox *Nitrospira* and an increasing fraction of betaproteobacterial
29 ammonia oxidizers.

30

GRAPHICAL ABSTRACT

- *Please see separate file* -

INTRODUCTION

When anaerobic groundwater is treated for drinking water production, biological granular media filters are commonly used to remove ammonium through nitrification after aeration. In this aerobic microbial process, ammonia oxidizing bacteria (AOB)[1] or archaea (AOA)[2] oxidize ammonia to nitrite, which is further oxidized by nitrite oxidizing bacteria (NOB)[1] to nitrate. Besides canonical ammonia and nitrite oxidizing microorganisms, members of the genus *Nitrospira* have been reported recently to catalyze the complete oxidation pathway from ammonia to nitrate (comammox)[3–5]. These different groups of nitrifying microorganisms often co-occur in biological filters treating groundwater for drinking water production[6–9],where they grow attached on the surface or in the porous mineral coatings of the filter media[10], and co-exist with other microbial taxa with diverse physiologies[8,11,12]. Ammonium removal is sometimes incomplete during biofiltration, causing excessive ammonium concentrations in the finished drinking water. This is problematic, because in systems without disinfection, nitrification during water distribution can result in microbial regrowth (possible growth of pathogens, aesthetic problems) and material corrosion[13].

Poor nitrification performance can sometimes be resolved by dosing the trace metal copper[14]. Copper is a cofactor for the enzyme ammonia monooxygenase[15], which catalyzes ammonia oxidation to hydroxylamine. When copper was added at µg/L level to a biological rapid sand filter that displayed incomplete ammonium removal, nitrification increased and ammonium was removed to <0.01 mg NH_4^+-N/L within less than 3 weeks[14]. A further, more comprehensive investigation at 10 groundwater treatment plants showed a consistent effect of copper dosing to remediate poorly nitrifying biofilters[16]. The progressive but rapid increase in nitrification efficiency suggests nitrifier growth stimulated by copper addition. Yet, this has not been examined. If correct, it is possible that such growth would modify the

composition of nitrifiers. Therefore, the present study aimed to answer the following: is copper-induced stimulation of nitrification in biological rapid sand filters associated with an increase in nitrifier density and/or with a change of composition within the nitrifying genera? This question was investigated at three full-scale drinking water treatment plants.

MATERIAL AND METHODS

Drinking water treatment plants

The three investigated drinking water treatment plants (DWTPs) are located in Denmark (Holmehave DWTP (VCS Denmark): 55°17'24.9"N, 10°11'19.8"E, Nærum DWTP (Novafos, formerly Forsyningen Allerød Rudersdal): 55°49'04.5"N, 12°31'24.2"E, and Glostrup DWTP (Glostrup Forsyning): 55°40'02.3"N 12°23'00.1"E). Groundwater from anaerobic aquifers is treated through aeration (plate aeration at Holmehave, stairs aeration at Nærum and Glostrup), followed by filtration with single stage, gravity driven, rapid sand filters (0.8-1.4 mm quartz sand at all plants and active depths of 0.6 m at Nærum, 0.75 m at Holmehave, and 0.6 m at Glostrup), and the treated water is distributed without disinfection. Influent water to the filters (Table 1) is relatively high in alkalinity. These treatment plants have experienced incomplete ammonium removal for several years (filter effluent concentrations > 0.01 mg NH_4-N/L; Table 1 and [16]) at relatively low hydraulic loading rates of 1.6, 1.9, and 2.5 m/h at Nærum, Holmehave and Glostrup DWTP, respectively.

79 **Table 1. Water quality characteristics at the investigated drinking water treatment plants**

	Holmehave			Nærum			Glostrup		
	influent	effluent		influent	effluent		influent	effluent	
		Before dosing	After dosing		Before dosing	After dosing		Before dosing	After dosing
Temp [ºC]	9.7	9.8	9.6	9.6	9.7	9.4	9.8	10.0	10.0
pH [-]	7.4	7.4	7.4	7.7	7.6	7.7	7.4	7.3	7.3
Dissolved oxygen [mg L^{-1}]	8.9	8.8	8.6	9.8	8.9	8.4	9.2	8.7	8.2
NH_4^+ [mg N L^{-1}]	0.36	0.14	<0.01	0.34	0.18	<0.01	0.42	0.17	<0.01
NO_2^- [mg N L^{-1}]	0.006	<0.002	<0.002	0.006	0.009	<0.002	0.003	<0.002	<0.002
NO_3^- [mg N L^{-1}]	<0.1	0.22	0.40	0.33	0.51	0.69	<0.1	0.23	0.40
total Fe [mg L^{-1}]	1.34	0.008	0.005	2.07	0.008	0.010	1.78	0.014	0.010
Mn [mg L^{-1}]	0.308	0.034	0.001	0.09	0.009	0.003	0.06	0.008	0.002
H_2S [mg L^{-1}]	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
CH_4 [mg L^{-1}]	<0.02*	n.d.	n.d.	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
PO_4^{3-} [mg P L^{-1}]	0.066	<0.01	<0.01	0.018	<0.01	<0.01	<0.01	<0.01	<0.01
Alkalinity [mg HCO_3^- L^{-1}]	329	331	322	348	340	341	442	436	434
Ca [mg L^{-1}]	103	102	103	n.d.	106	105	113	n.d.	n.d.
Cl^- [mg L^{-1}]	25	33	36	n.d.	66	65	57	n.d.	n.d.
SO_4^{2-} [mg L^{-1}]	7	8	9	n.d.	43	46	15	n.d.	n.d.
NVOC [mg L^{-1}]	1.5	1.4	1.4	n.d.	1.7	1.9	1.8	1.7	1.8

n.d.: not determined, * value from raw water

80

81 Water and filter media were sampled from a filter at each DWTP before and after copper dosing, and
82 water samples were also collected during the dosing. At the three plants, copper was dosed through

electrolysis, employing rod-shaped 4 mm diameter copper electrodes[17]. Nitrification activity of these test filters was evaluated based on the influent and effluent concentrations of ammonium, nitrite and nitrate, and volumetric ammonium removal rates (ARR) were calculated[16]. Additionally, nitrification activity of reference filters, which did not receive dosing, was monitored at all DWTPs.

Water and media sampling

Influent water was collected from the top of the filters and effluent water from sampling taps. Water from the filters was abstracted from 10 cm below the surface of the filter by a peristaltic pump (Ole Dich, 101 ACR) at 25 mL/min through PTFE tubing inside a stainless-steel probe inserted into the filter at a 45º angle. Detailed sampling procedure and sample handling and storage are described elsewhere[14].

Filter media samples were collected from the top 10 cm of the filters with a stainless steel grab sampler and homogenized. Sub-samples were transferred into sterile 50 mL PE vials, and transported on ice to the laboratory for processing. All filter media samples were collected at the same time within a filtration cycle (one day after backwash). At Holmehave DWTP, media samples were collected from the test (Cu dosed) filter at six random locations (biological replicates) and from the reference (without Cu dosing) filter at three locations, just before the start of the copper dosing to the test filter (day 0) and after 67 days of dosing. At Glostrup DWTP, samples were collected from the test and the reference filter at six random locations (biological replicates). The test filter (Cu dosed) was sampled 171 days before dosing onset (day -171), just before the start of the copper dosing (day 0) and after 43 days of dosing, and the reference filter (without Cu dosing) was sampled 171 days before dosing onset (day -171) and just before the start of the copper dosing to the test filter (day 0). Due to operational reasons, approx. 20 cm of top layer sand were removed from both the test and the reference filter, 90 days before the start of the dosing (day –

90). At Nærum DWTP, samples were collected from the test filter at two locations, just before dosing began (day 0) and after 116 days of dosing.

Chemical parameters

Ammonium,nitrite, phosphate, and hydrogen sulfide were analyzed with colorimetric methods (APHA 4500-NH_3 F, 4500-NO_2^- B, 4500-P F, and 4500-S^{2-} D [18]) with quantification limits of 0.01 for ammonium and 0.002 mg N/L for nitrite. Concentrations of total iron, manganese, calcium, and copper (detection limit of 0.01 µg Cu/L) in water samples were determined by ICP-MS - inductively coupled plasma mass spectrometry (Agilent Technologies, 7700 Series ICP-MS), analogous to EPA method 6020A[19]. Nitrate, chloride, and sulfate were analyzed with ion chromatography (APHA 4110-B [18]). Non-volatile organic carbon (NVOC) was determined by wet-oxidation method, according to APHA 5310 D [18]. Methane concentrations were determined in the headspace by gas chromatography (GC). Water phase concentrations under equilibrium conditions were calculated based on the Henry's Law constant. Dissolved oxygen, temperature and pH were monitored with a hand held meter (WTW, Multi 3430, with FDO® 925 and SenTix® 940 probes). Total alkalinity (as HCO_3^-) was determined with titrimetric method (APHA 2330 [18]).

DNA extraction

DNA was extracted from 0.5 g of wet drained sand using FastDNA™ spin kit for soil (MP Biomedicals, Solon, OH, USA) according to manufacturer's instructions. DNA was extracted in duplicates from each of the biological replicate sand samples. The DNA concentration was determined using NanoDrop ND-1000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and the extracts were stored at -20 °C until further analysis.

125 ***Quantitative PCR, PCR amplification and Illumina Sequencing***

126 Quantitative PCR (qPCR) was used to quantify total *Bacteria*, ammonia oxidizing bacteria, ammonia
127 oxidizing archaea, nitrite oxidizing bacteria (*Nitrospira* and *Nitrobacter*) and comammox *Nitrospira*
128 (both clades in a single assay, specificity illustrated Figure S8). This was performed with a Chromo4
129 thermocycler using Opticon Monitor version 3 software (BioRad). Each qPCR reaction contained 12.5
130 µL of 2X iQ SYBR green Supermix (catalog no. 170-8880, Bio-Rad Laboratories), 500 mM primer,
131 DNA template (10 ng), and DNA/RNA free water (Mo Bio Laboratories) to 25 µL (ref[11]). Primer details,
132 PCR and qPCR conditions are given in Table S1, S2 and S3. For each sample, 50 µL total extracted DNA
133 was sent for 16S rRNA gene PCR amplification, purification and amplicon sequencing using the Illumina
134 MiSeq platform at the DTU Multi Assay Core Center (Kgs. Lyngby, DK). Gene copy numbers from
135 qPCR were converted to cell densities, assuming one 16S rRNA gene per cell of AOB[11], one *amoA* gene
136 per cell of comammox *Nitrospira*[20], and two *nxrB* genes per cell of *Nitrospira*[20]. Copy numbers of 16S
137 rRNA genes for total bacteria were corrected based on the average ribosomal operon number per
138 organism in each sample determined by comparing 16S rRNA gene amplicon data to the rrnDB
139 database[21] and calculating the average copy number per sample in R (script available at
140 https://github.com/ardagulay). Abundance changes caused by copper addition are reported as fold
141 change, calculated as the ratio of the value measured after copper supplementation divided by the value
142 before copper supplementation.

143 ***Bioinformatics analysis***

144 Sequences generated as paired FASTQ files were processed using DADA2 (Version - 1.4)[22]. DADA2
145 was used for quality filtering, trimming and de-replicating the reads, inferring sequence variation through

default error model parameters, merging paired reads, removing chimeras, and assigning taxonomy using Silva reference database v123 and custom taxonomy files for nitrifiers. Further analysis on the sequence and taxonomy table generated using DADA2 was performed using Phyloseq R Package (Version - 1.7.12)[23]. The Phyloseq extension DESeq2 (Version – 1.16.2)[24] was used to detect differential relative abundances among the sequence variants or taxa between the two sampling points. This method uses negative binomial generalized linear models and allows adjusting the significance threshold of the Wald test (here 0.05) after adjusting the P values for false discovery rate. Reference 16S rRNA gene sequences for comammox *Nitrospira* were obtained from GenBank and from re-analysis of amplicon sequencing data from a sand filter for which metagenomic data had shown dominance of comammox[8,25]; see Supplementary Methods, Fig S1 and Table S4. These sequences were then aligned with sequences from this study using ClustalW in MEGAX [26,27] to generate a maximum of likelihood tree. Sequence variants were putatively assigned to a comammox clade if they were more than 98.5% similar to one of the comammox *Nitrospira* reference sequences.

Nucleic Acid Sequences

Raw sequence files were deposited into the Sequence Read Archive at GenBank under the study accession number SRP129820.

RESULTS AND DISCUSSION

Copper dosing stimulates nitrification

Prior to copper dosing, nitrification had been insufficient for several years in the biological filters of the three DWTPs, resulting in excessive NH_4^+ concentration in the effluent[16]. Just before dosing, the effluent concentrations were 0.14 mg NH_4^+-N/L at Holmehave, 0.18 mg NH_4^+-N/L at Nærum, and 0.17 mg NH_4^+-

N/L at Glostrup (Table 1). The DWTPs therefore violated the national water quality standard of 0.039 mg NH_4^+-N/L [28] by a factor of 3.6 - 4.6. At Holmehave, the filter removed 0.60 g NH_4^+-N/m^3 filter material/h at an ammonium loading rate of 0.95 g NH_4^+-N/m^3 filter material/h (Figure 1), corresponding to 63% removal. At Nærum DWTP, only 44% of the influent ammonium was removed, where the filter only achieved a removal rate of 0.22 g NH_4^+-N/m^3/h when loaded with 0.50 g NH_4^+-N/m^3/h. At Glostrup DWTP, ammonium loading and removal rates varied slightly (Figure 1), but when loaded with 0.90 g NH_4^+-N/m^3/h the day before dosing onset, the filter achieved a removal rate of 0.53 g NH_4^+-N/m^3/h, corresponding to a 59% removal. These removal rates were low, compared with 3.4 and 5 g NH_4^+-N/m^3/h achieved by similar systems[6,7], albeit operated under higher ammonium loading rates. Nitrite filter effluent concentrations were <0.002 mg NO_2^--N/L at Holmehave and Glostrup DWTPs, but were elevated at 0.009 mg NO_2^--N/L at Nærum (Table 1 and[16]) documenting that nitrite breakthrough is sometimes a problem, with a low regulatory standard of 0.003 mg NO_2^--N/L at the effluent of DWTPs[28].

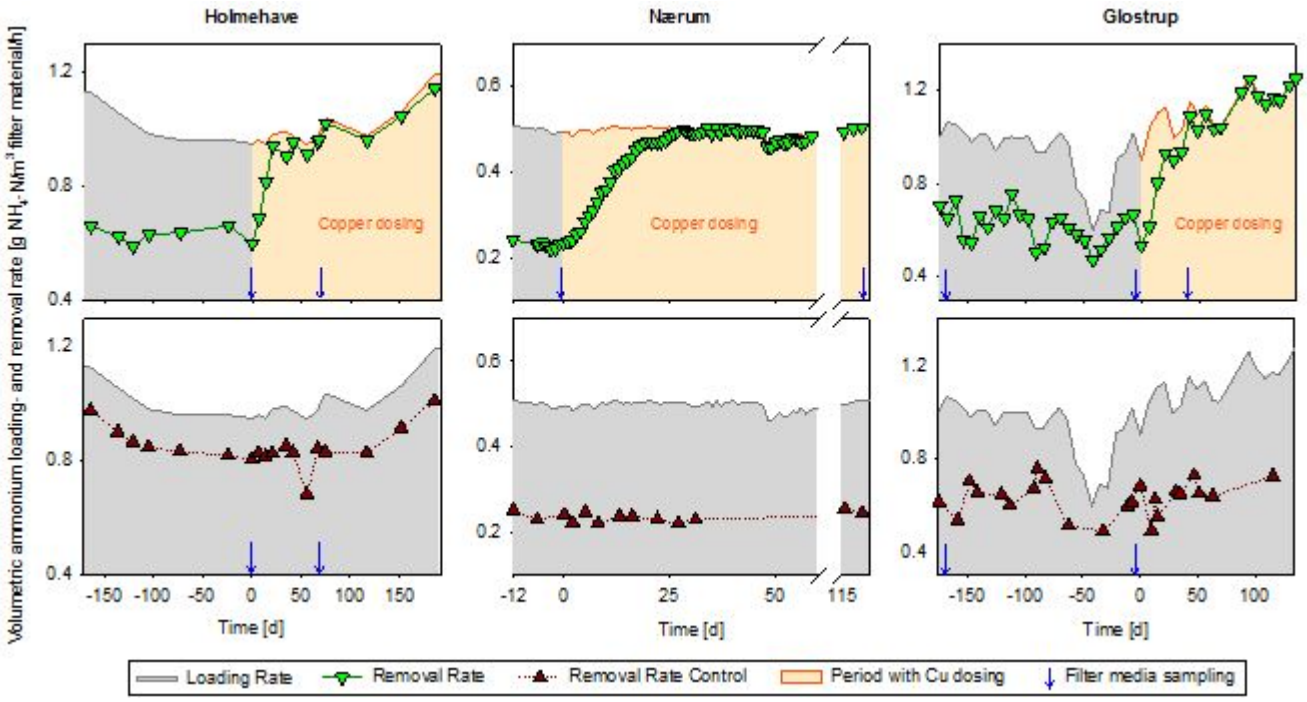


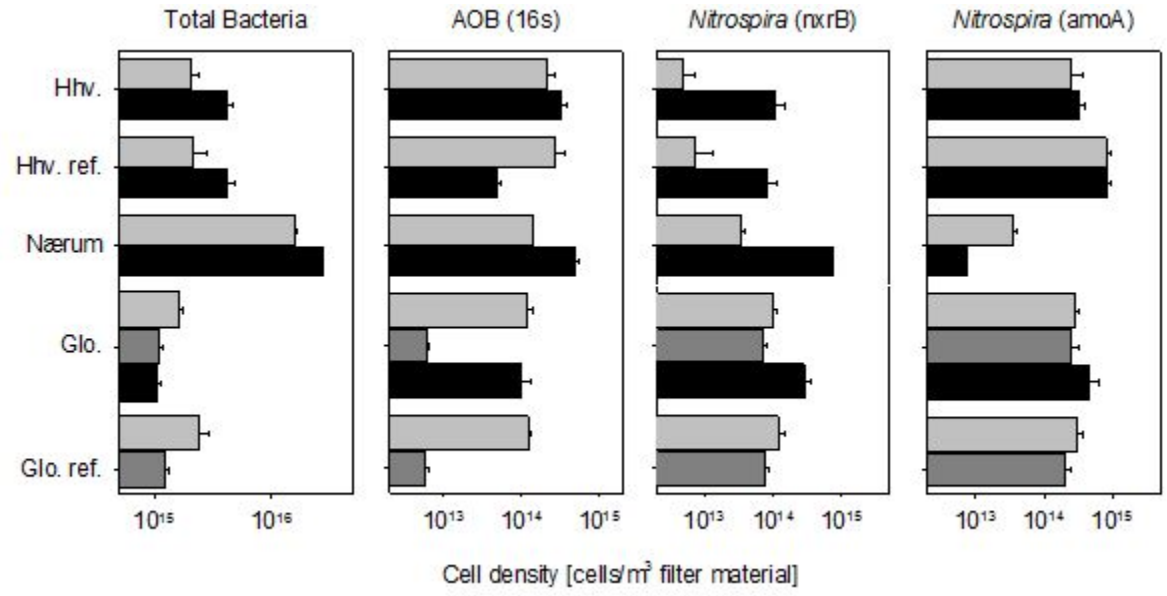
Figure 1. Top: Stimulation of ammonium removal rate by copper dosing (start on day 0) in the test filters at Holmehave, Nærum, and Glostrup DWTPs, at given ammonium loading rate. Bottom: Absence of improvement in the ammonium removal performance of the reference filters, which did not receive copper. Blue arrows mark filter media sampling. Partly redrawn from[16].

Ammonium removal responded promptly to copper dosing, with increased rates within only a few days for all plants (Figure 1). Effluent concentrations of <0.01 mg NH_4^+-N/L were achieved after 22, 21 and 30 days of dosing for Nærum[14], Holmehave, and Glostrup[16], respectively. During dosing, the test filters were exposed to average influent dosing concentrations of 0.34 ± 0.22 µg Cu/L (n=11) for Nærum, 0.85 ± 0.24 µg Cu/L (n=8) for Holmehave, and 0.70 ± 0.19 µg Cu/L (n=9) for Glostrup. The ammonium removal of the reference filters, which were not subjected to copper dosing, did not change during the investigation at any of the sites (Figure 1). At Holmehave DWTP, approximately 80% of the ammonium was removed in the reference filter regardless of the ammonium loading rates (Figure 1) while this fraction increased from 63 to approx. 97% in the test filter in only 3 weeks of dosing (Figure 1). At Nærum and Glostrup DWTPs, the ammonium removal rates achieved by the reference filters were and remained comparable to those achieved by the test filters before dosing (Figure 1). Hence, the increased nitrification in the test filters was a result of the copper dosing. Importantly, additional filter functions such as the removal of iron and manganese were not impaired by the dosing at any plant (Table 1).

Effect of dosing on nitrifier abundance

Microbial densities within the top 10 cm were quantified by qPCR targeting the 16S rRNA gene for total bacteria and AOB, *nxrB* for *Nitrospira* and *Nitrobacter*, and *amoA* for AOB, AOA, and comammox

200 *Nitrospira* (Figure 2 & Figure S2). The quantified AOA, AOB and comammox are collectively referred
201 to as ammonia oxidizing prokaryotes (AOP).



202

203 **Figure 2.** Cell densities measured by qPCR before dosing of copper (light grey bars) and 43, 67 or 116
204 days after start of dosing (black bars) to the test filters at Holmehave, Nærum and Glostrup DWTPs. For
205 Glostrup, a second sampling of both filters, right before dosing of the test filter, was conducted (dark
206 grey bars), because the top 20 cm of the filter material had been removed since the first sampling. The
207 reference filters at Holmehave (Hhv. ref.) and Glostrup (Glo. ref.) did not receive copper dosing. Error
208 bars represent the standard error of the mean from replicate samples.

209 After dosing began, AOB absolute densities estimated from qPCR of the 16S rRNA gene increased in
210 all filters receiving copper, albeit to different degrees (Figure 2). We observed fold changes of 1.5 in
211 Holmehave, of 3.4 in Nærum, and of more than 15 in Glostrup. This large increase in Glostrup is partly
212 due to the fact that AOB abundance measured just before dosing was low (Figure 2, dark grey bars),
213 most likely because sand from the top of the filter had been removed between the first and second

sampling. The decrease of AOB density after the sand removal suggests some degree of biomass stratification, which has been described previously in such systems[9]. Compared to the abundances of AOB derived from 16S rRNA gene-based qPCR, those measured by the *amoA* targeted qPCR were between 2 and 3 orders of magnitude lower (Figure S2), which is likely due to the poor coverage of the *amoA* primers, as reported previously[7,29]. Yet, the two qPCR methods yielded results that matched in their temporal trends (increase vs. decrease in AOB abundance) for both test and reference filters at Holmehave and at Glostrup DWTPs. This was, however, not verified for Nærum DWTP, where AOB abundance increased when measured with 16S rRNA gene-based qPCR, but showed no significant change when quantified with *amoA* targeted qPCR (Figure S2). AOA densities were close to or below the qPCR quantification limit (10 gene copies per reaction) and were thus under 8×10^{10} cells/m^3 filter material (Figure S2), 2 to 4 orders of magnitude lower than previously reported in similar full scale filters with good nitrification peformance[9]. In relative terms, this amounted to only 0.0008-0.007% of total AOP, and dosing did not change their abundance significantly. Considering that cell specific ammonia oxidation rates of AOA are generally 1 to 2 orders of magnitude lower than for AOB[30], the contribution of AOA to ammonium removal in the present study is likely negligible. Some AOA have been suggested to have a higher requirement for copper than AOB[31,32] which may contribute to their low relative abundance in these copper limited systems.

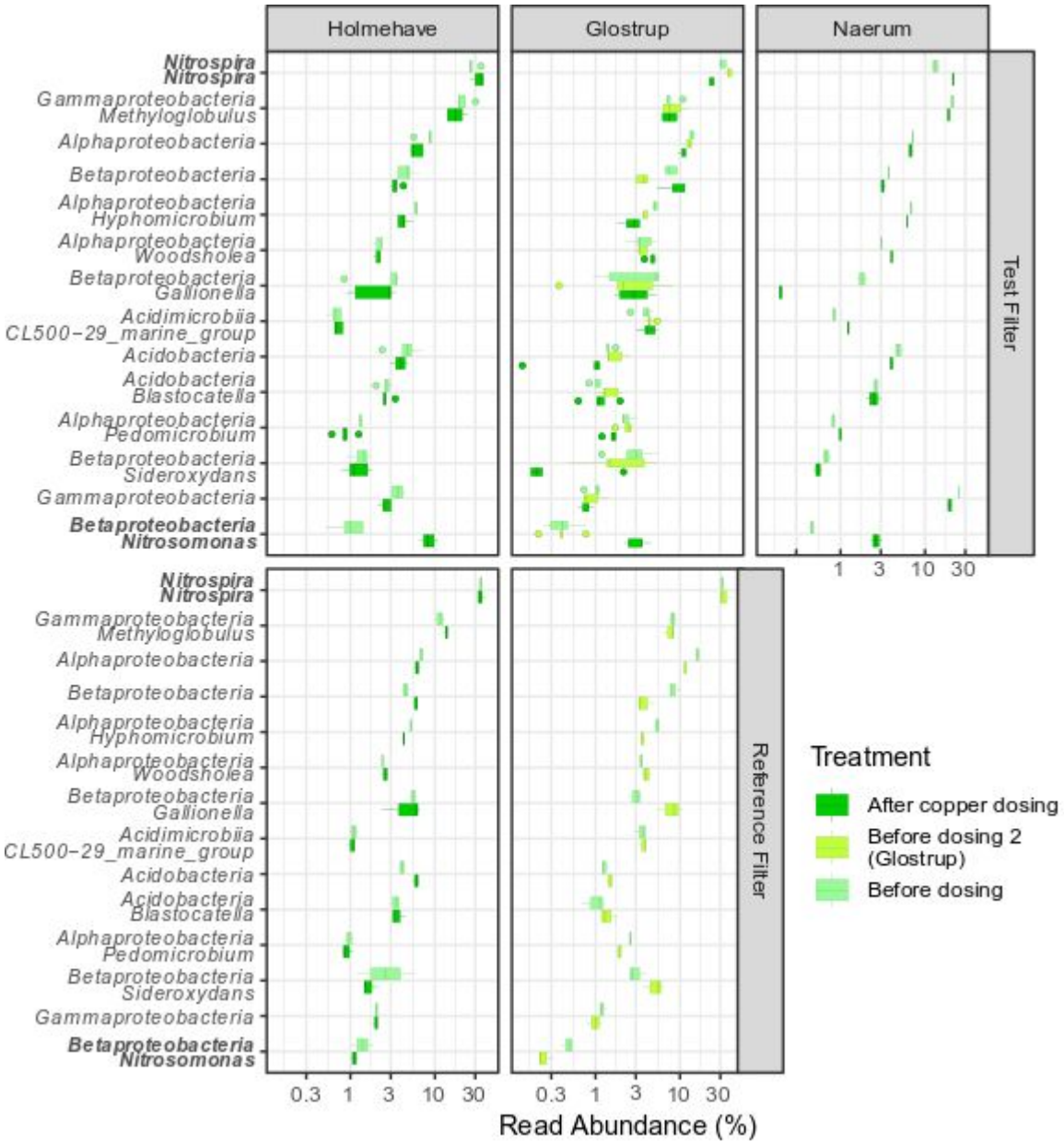
Application of newly developed qPCR primers targeting *amoA* of *Nitrospira*[25] revealed the presence of comammox *Nitrospira* at both DWTPs. Before dosing, and compared to AOB (16S rRNA gene), comammox *Nitrospira* densities were slightly higher (16%) at Holmehave, and lower (75%) at Nærum DWTP. Interestingly, compared to before dosing, comammox *Nitrospira* increased slightly (fold change: 1.3) in the filter at Holmehave, and decreased distinctly (fold change: 0.2) at Nærum DWTP (Figure 2).

At Glostrup DWTP, comammox Nitrospira were more abundant than AOB (16S rRNA gene) just before copper dosing was started (Figure 2, dark grey bars), but their increase (1.8 fold) was smaller than that of AOB. *Nitrospira* (*nxrB*) densities increased at all three plants after the dosing; however, this was also observed in the reference filter at Holmehave (Figure 2). The melting curve for *Nitrobacter nxrB* qPCR had multiple peaks and amplicon sequencing yielded no *Nitrobacter*-related sequences (*Nitrobacter* data is thus not shown). Therefore, *Nitrospira* spp. must have been the dominant nitrite oxidizers. Total bacterial density increased significantly in filters dosed with copper at Holmehave and Nærum (p-value of 0.003 for both plants). However, it is difficult to ascribe the increase to copper dosing, as total bacteria also increased in the reference filter at Holmehave, and no significant change was observed in the test filter at Glostrup (Figure 2). Overall, qPCR analyses showed that copper dosing increased the abundance of canonical, betaproteobacterial AOB at all three investigated sites, in stark contrast to the Holmehave reference filter without copper dosing. For comammox *Nitrospira,* a less pronounced growth was observed at Holmehave and Glostrup and even a decrease in abundance at Nærum DWTP (Figure 2). These differences may originate from the fact that sampling occurred later relative to the beginning of dosing at Nærum than at the other sites.

Effect on relative abundance of nitrifiers assessed at the genus and sequence variant levels

To obtain a deeper and broader insight in the microbial communities, they were subjected to amplicon sequencing of the 16S rRNA gene. After quality filtering, 2,415,214 sequences were distributed among 2279 sequence variants. Of the total number of sequences, 702,639 (~29%) belonged to 27 sequence variants assigned to the genus *Nitrospira* and 34,282 (~1.4%) to 23 sequence variants assigned to the genus *Nitrosomonas*.

First, we used the amplicon sequencing information to confirm the effect of copper dosing on the groups targeted by our qPCR assays. The copper dosing did not provoke a drastic change in the microbial composition of the filters (Figure 3). *Nitrospira* was and remained the dominant genus in all plants (Figure 3, top), which is consistent with previous studies of microbial composition in groundwater-fed rapid sand filters[8,25]. In the reference filters, the relative abundance of both *Nitrosomonas* and *Nitrospira* genera remained stable, except for a slight decrease for *Nitrosomonas* between the two pre-copper dosing samplings in Glostrup (Figure 3, lower panels). In the test filters, however, the relative abundance of *Nitrosomonas* increased almost 10-fold after copper dosing started (Figure 3, top panels). The relative abundance of *Nitrospira*, which was initially more than 20 times that of *Nitrosomonas*, did not show such an increase; in fact, an increase or a decrease was detected depending on the site: modest increase Nærum and Holmehave (p-value <0.001), but decrease for Glostrup (p<0.001) (Tables S5, S6, and S7). The only other nitrifiers detected were *Nitrotoga* (<1% of the total reads), which did not significantly respond to copper addition.



272

273 **Figure 3.** Taxonomic distribution of 16S rRNA gene sequences in the copper-dosed and the reference
274 filters. The relative abundances of the 14 most abundant genera are presented. The sequences are grouped
275 according to their assigned genus (labeled as "*Class Genus*", NA indicates unidentified genera),
276 nitrifying genera are highlight in bold. Box plots are computed from 5 replicate samples for Holmehave
277 and Glostrup test filters, 3 for the reference filters, and 2 for Nærum test filter. Dots represent data points

that lie at least 1.5 interquartile ranges below the first quartile or above the third quartile. For Glostrup, "Before dosing 2" denotes the sample taken just before the onset of dosing, but after sand was removed from the filter.

Furthermore, we investigated if copper dosing affected taxa other than nitrifiers. In Holmehave test filter, sampled just two months after initiating dosing, no genera had changed in relative abundance (Figure S3 and Table S5), and only a few did in Nærum*: Ca. Odyssella* and unclassified *Acidobacteria* increased significantly (fold change ~6 and 2, respectively) and *Gallionella* decreased significantly (fold change ~0.1); Figure S4 and Table S6). In contrast, the community composition at Glostrup after the onset of copper dosing differed more largely from its previous states, described 214 and 43 days earlier, with respectively 45 and 54 non-nitrifying genera detected as having varied in relative abundance (Figure S5 and Table S7). Many of these have unknown function but several of the genera which decreased in relative abundance were likely involved in the metabolism of sulfur (*Sulfurovum*, *Sulfuricella, Sulfuritalea, Desulfobulbus)* or in manganese oxidation (*Hyphomicrobium*, *Pedomicrobium*). We could not identify toxicity thresholds for these negatively affected genera in the literature. However, for *Escherichia coli*, copper concentrations of at least 3.8×10^5 µg Cu/L has been reported as inhibiting growth[33], which is more than five orders of magnitude above the range measured in these filters (<1 µg Cu/L). Therefore, it is highly unlikely that the observed decreases in relative abundance were caused by copper toxicity. These were thus either temporal fluctuations unrelated to the dosing or, more likely, were the result of intensified competition for other nutrients with organisms that benefited from copper dosing. Even if some of these genera could be implicated in important functions such as removal of iron (a likely function of *Gallionella*[8,34]) or of manganese, no deterioration of the effluent water quality was

observed (Table 1), possibly because their activity was compensated by other microbes (via functional redundancy) and/or by abiotic oxidation.

To investigate how the microbial composition within the dominant nitrifying genera was affected by copper dosing, we performed a sequence variant-level analysis of the test filters. Eleven sequence variants of *Nitrosomonas* were detected in more than a single sample, of which two were very abundant at all sites (~90% of all *Nitrosomonas* reads) (Figure 4). These two sequence variants belong to different branches of *Nitrosomonas* Cluster 6, with 16S_3168 closely related to *Nitrosomonas oligotropha* strain Nm45 (97% identity) and thus putatively assigned to Cluster 6A[35], while 16S_3167 could not be placed in a subcluster (closest type strain: *Nitrosomonas marina* strain Nm22 with 95% identity) (Figure S5). *Nitrosomonas* 6A members (*N. oligotropha* and *N. ureae*) are commonly adapted to very low ammonium concentrations (1-5 mM ammonium) with a maximum tolerance to ammonium of 50-200 mM [36]. They are usually considered as the betaproteobacterial AOB with the highest affinity for ammonium (Ks in the 1.9-4.2 μM range)[37]. The ability of Cluster 6 members to produce ample amount of extracellular polymeric substances (EPS) also make them well adapted to growth at the surface of the filter material[38]. The prevalence of *Nitrosomonas* cluster 6 observed here is consistent with previous findings in similar biological sand filters for drinking water production, where members of *Nitrosomonas* cluster 6A (*Nitrosomonas* sp. Is79A3) and cluster 7 were dominant among the AOB[8]. The heatmap of *Nitrosomonas* sequence variants (Figure 4) indicated that copper addition did not markedly modify the composition within this genus, even though only three or four most abundant sequence variants were statistically significantly positively affected in terms of relative abundance within the total community (2.2-3.8 fold across all plants; all adjusted p-values $<5 \times 10^{-5}$). This demonstrates that the increase of *Nitrosomonas*

was not restricted to a specific type, possibly because of the relative homogeneity of the initial composition (only cluster 6 representatives).

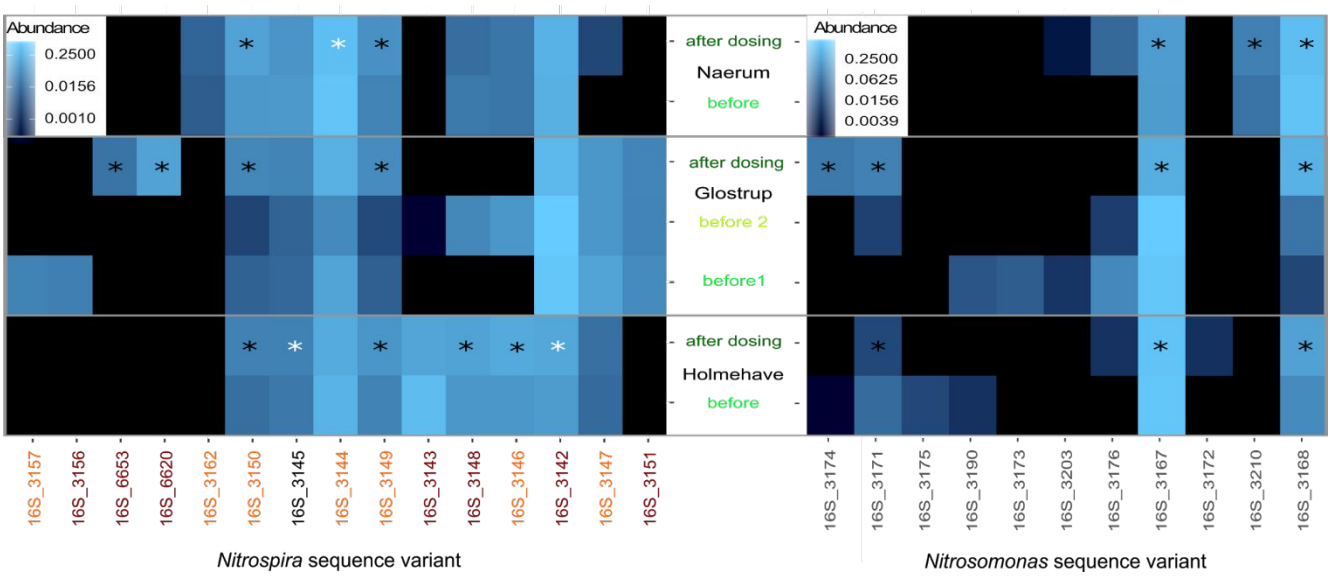


Figure 4. Abundance of sequence variants assigned to *Nitrospira* (left) and *Nitrosomonas* (right) relative to the total number of sequence variants of each genus for the test filters of three DWTP before and after copper addition, averaged over 2 to 5 replicate samples. Stars denote significant increase in the sequence variant relative abundance upon copper addition (white and back symbols for increase lower or larger than one log2, respectively). The color of the *Nitrospira* sequence names indicate their putative classification as canonical NOB (black) or comammox clade A (dark red) or clade B (orange).

Considering *Nitrospira*, 15 sequence variants were detected in more than a single sample (Figure 4), which is relatively low considering the high abundance of this genus. All these variants were assigned to *Nitrospira* lineage II (Figure S6), a broadly distributed lineage often found in freshwater and in drinking

water distribution systems, known to contain comammox representatives. Comammox *Nitrospira* do not form a monophyletic group within lineage II, but are instead interspersed with nitrite-oxidizing *Nitrospira*[39,40]. Therefore, uncertainties are associated with using the 16S rRNA gene as a marker to distinguish comammox from non-comammox (strict NOB) *Nitrospira*. Nevertheless, based on their close similarity with previously described 16S rRNA gene sequences of comammox (always >98.8%, Figure S6), we propose that eleven of the variants are likely comammox bacteria. These include the two most abundant variants (~45% of total *Nitrospira*), which we tentatively assigned to comammox Clade A (16S_3142) and B (16S_3144). Such a high prevalence of comammox was recently demonstrated or suggested in similar rapid sand filters[8,9,25] as well as the joint presence of clades A and B[25,40]. Like for *Nitrosomonas*, the general picture within the genus upon copper supplementation is one of compositional stability (Figure 4), although our statistical analysis detected proliferation at the community level for only some of the dominant variants, most of them putative comammox (six in Holmehave, three in Nærum, and four in Glostrup, all adjusted $p < 0.003$). Only two (16S_3149, 16S_3150, both putative comammox clade B) significantly increased in the three plants, with fold increase in the 2.6-6.1 range.

Therefore, the copper induced stimulation of nitrification did not markedly modify the composition within the two most implicated genera, suggesting that this did not constitute a strong differential selective pressure at the sub-genus level. The composition in the three plants for these two genera was very similar down to the sequence variant level, with most of the dominant variants being shared. This probably contributes to their consistent response to copper supplementation.

Contribution of betaproteobacterial AOB and comammox to total AOP

353 To assess the relative contribution of betaproteobacterial AOB and comammox *Nitrospira* to total
354 ammonia oxidizing prokaryotes (AOP) before and after dosing, estimates of AOB and comammox
355 *Nitrospira* abundances from targeted qPCR were summed up and their fractions calculated (Figure 5,
356 left). Based on qPCR estimates, the fraction of betaproteobacterial AOB increased from 44% to 51% at
357 Holmehave, from 80 to 98% at Nærum, and from 2 to 18% at Glostrup, comparing just before ("be2")
358 and 43 days after dosing onset (Figure 5, left). Based on amplicon sequencing, the initial fraction of
359 comammox *Nitrospira* was significantly larger at all plants (Figure 5, right). The discrepancy between
360 the results of the two methods may have been caused by an overestimation of AOB abundance by the
361 qPCR targeting the 16S rRNA gene[29]. An in-depth analysis of biases associated with the *amoA Nitrospira*
362 primers has not been performed, but it is known that the primers present mismatches to *Nitrospira amoA*
363 genes, thus biases in amplification resulting in over- and/or under-estimation of different comammox
364 *Nitrospira* likely exist. However, the trend of an increasing fraction of betaproteobacterial AOB with
365 copper dosing was consistent (Figure 5), which means that comammox *Nitrospira* became relatively less
366 -and canonical ammonia oxidizers relatively more- prevalent within total AOP as a result of the copper
367 dosing. This indicates that once copper limitation was lifted, *Nitrosomonas* had higher fitness than
368 comammox *Nitrospira,* at least transitorily. This higher fitness of AOB may be explained by their
369 comparatively higher maximal specific growth rate [41–43], which should also translate into comparatively
370 higher cell specific ammonia utilization rate. The increased activity of *Nitrosomomas* was indeed
371 evidenced in Holmehave by the nitritite accumulation which transiently occurred after copper dosing was
372 initiated[16]. By contrast, comammox *Nitrospira* outcompetition of AOB in copper deficient conditions
373 demonstrates their ability to efficiently compete for copper uptake at low concentration, as proposed
374 based on comparative genomics[20]. In addition, comammox *Nitrospira* certainly have other traits that
375 make them well adapted to conditions in these rapid sand filters (very high affinity to ammonia, high

yield) as they dominate over *Nitrosomonas* in the vast majority of the DWTP filters without copper deficiency we surveyed[25]. This is why we do not expect copper dosing to result in a complete change of dominance within the AOP.

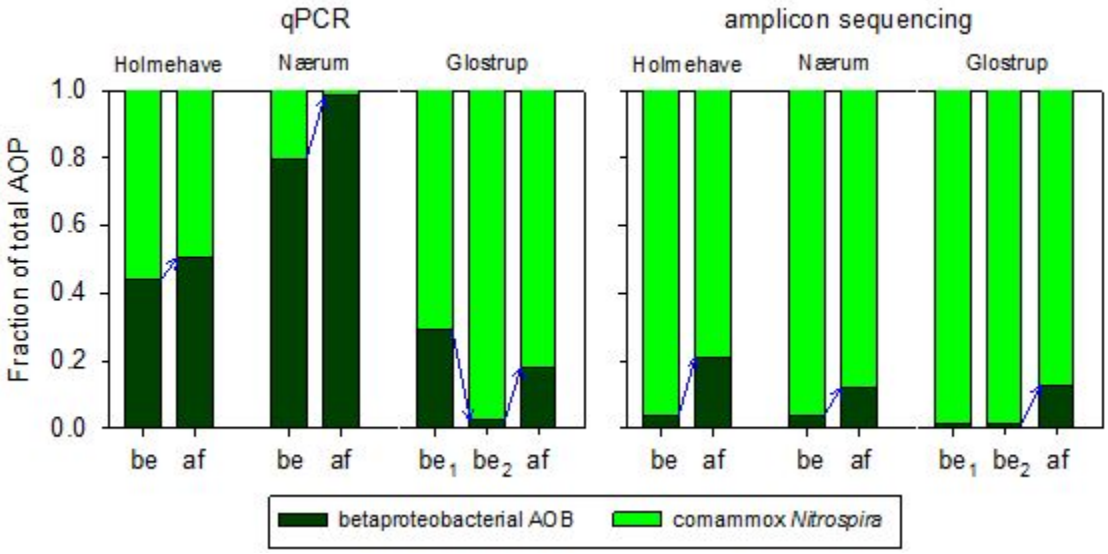


Figure 5 – Effect of copper dosing on the contribution of betaproteobacterial AOB and comammox *Nitrospira* to total AOP abundance, as estimated with two types of molecular approaches. The qPCR based quantification was performed using 16S rRNA gene (betaproteobacterial AOB) and *amoA* (comammox *Nitrospira*). For Glostrup, "be_2" is the second sample before the onset of dosing, but after sand was removed from the filter.

Overall, our study showed that copper-induced stimulation of nitrification in biological rapid sand filters involved changes in nitrifiers abundance. The genus *Nitrospira* was and remained the most abundant; however, the copper-induced change of *Nitrosomonas* relative abundance was much higher than for *Nitrospira*. This proliferation of betaproteobacterial AOB certainly contributed to the rapid increase in ammonium removal. Our finding that copper dosing did not consistently markedly affect the relative abundance of other microbial guilds in the filters is of practical relevance. Taken together, our results

391 clarify the consequences of copper dosing on copper-deficient rapid sand filters: it consistently stimulates
392 nitrifiers growth and increases the fraction of *Nitrosomonas* within weeks, but with lasting effect
393 (detected after four months of dosing in Nærum DWTP). This strengthens the notion that the joint
394 contribution of AOB and comammox *Nitrospira* ensures efficient nitrification in groundwater-fed rapid
395 sand filter for drinking water production.

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404 AUTHOR CONTRIBUTIONS

405 [+]Florian B. Wagner and Vaibhav Diwan contributed equally as first authors.

406 NOTES

407 The authors declare no competing financial interest.

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559

560 SUPPORTING INFORMATION

561 **Table S1.** Primer and targeted gene used for qPCR and PCR amplification for sequencing.

562 **Table S2.** qPCR and PCR conditions.

563 **Table S3.** Reagent concentrations and volumes for a single PCR reaction.

564 **Table S4**. Result of the matching of *Nitrospira* comammox MAG to their putative 16Sr RNA gene partial sequence
565 for Islevbro rapid sand filter.

566 **Table S5.** List of the genera that significantly responded to copper dosing at the Holmehave test filter.

567 **Table S6.** List of the genera that significantly responded to copper dosing at the Nærum test filter.

568 **Table S7.** List of the genera that significantly changed abundance between initial and the final sampling at the
569 Glostrup test filter

570 **Figure S1.** Correlation of the relative of abundance *Nitrospira* phylotypes as estimated by amplicon sequencing
571 and by metagenomic sequencing and MAG reconstruction in Islevbro rapid sand filter.

572 **Figure S2.** Cell density measured by qPCR before and after dosing of copper in the test filters at Holmehave
573 Nærum, and Glostrup DWTPs.

574 **Figure S3.** Taxonomic distribution of 16S rRNA gene sequences for the 50 most abundant genera before and after
575 copper dosing in the test filter at Holmehave DWTP.

576 **Figure S4.** Taxonomic distribution of 16S rRNA gene sequences for the 50 most abundant genera before and
577 after copper in the test filter at Naerum DWTP.

578 **Figure S5.** Taxonomic distribution of 16S rRNA gene sequences for the 50 most abundant genera before and
579 after copper dosing in the test filter at Glostrup DWTPs.

580 **Figure S6.** Evolutionary relationships of AOB sequence variants from this study to reference Cluster 6 AOB.

581 **Figure S7.** Evolutionary relationships of *Nitrospira* sequence variants from this study to reference *Nitrospira*.

582 **Figure S8.** Gel electrophoresis of PCR products from the PCR targeting comammox *Nitrospira amoA* from
583 DNA extracted from DWTPs

Stimulation of NH_4^+ removal by Cu dosing in drinking water biofilters