Evolutionary ecology of natural comammox *Nitrospira* populations

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Microbes commonly exists in diverse and complex communities where species interact, and their genomic repertoires evolve over time. Our understanding of species interactions and evolution has increased in the last decades, but most studies of evolutionary dynamics are based on single species in isolation or in experimental systems composed of few interacting species. Here, we use the microbial ecosystem found in groundwater-fed sand filters as a model to avoid this limitation. In these open systems, diverse microbial communities experience relatively stable conditions, and the coupling between chemical and biological processes is generally well defined. Metagenomic analysis of 12 sand filters revealed systematic co-occurrence of at least five comammox *Nitrospira* species, likely promoted by low ammonium concentrations. These *Nitrospira* species showed intra-population sequence diversity, although possible clonal expansion was detected in few abundant local comammox populations. They showed low homologous recombination and strong purifying selection, the latter process being especially strong in genes essential in energy metabolism. Positive selection was detected on genes related to resistance to foreign DNA and phages. We found that, compared to other habitats, groundwater-fed sand filters impose strong purifying selection and low recombination on comammox *Nitrospira* populations. These results suggest that evolutionary processes are more affected by habitat type than by species identity. Together, this study improves our understanding of species interactions and evolution in complex microbial communities, and sheds light on the environmental dependency of evolutionary processes.

41 **Introduction**

42

43 Microorganisms dominate the tree of life based on species diversity, and they play an essential
44 role in all global biogeochemical cycles. Microbial species interact with each other and with the
45 environment (ecological processes), and also undergo changes in their genomic repertoire over time
46 (evolutionary processes). Yet, the interaction between ecological and evolutionary processes is
47 largely unknown, especially for complex open communities. For many years, most studies of
48 microbial communities in open, complex environments have focused on ecological aspects as it was
49 believed that evolutionary changes happen at much larger timescale (1). However, in recent years,
50 with the development of population-genomics analysis, researchers have started to investigate jointly
51 the ecological and evolutionary processes. Yet, most studies of evolutionary dynamics remain based
52 on single species in isolation (2) or on experimental systems composed of only a few interacting
53 species (3). While these analyses have helped to better understand some aspects of evolutionary
54 patterns, they have limitations because they lack many characteristics of true natural populations (e.g.,
55 spatial structure, existence of microdiversity, predation, immigration). On the other hand, observing
56 populations in the wild also has limitations because the conditions vary with little control (hence,
57 uncontrolled variation in population size, selection regime) and because the typically unknown
58 ecophysiology of retrieved genomes makes it difficult to interpret the observed patterns. Therefore,
59 studying well-defined model microbial ecosystems can help to understand ecological and
60 evolutionary processes in microbial communities (4).

61 Rapid sand filters (RSF), widely used to produce drinking water from surface- or
62 groundwater, are useful model systems as they are characterized by stable conditions; active growth,
63 primarily driven by the oxidation of ammonia, methane, and other inorganic compounds present at
64 low concentration in the influent water; large populations (10^9 – 10^{10} cells/g); significant mixing;
65 continuous but limited immigration from prokaryotes in the influent water; no dispersal between
66 separate sand filters (resulting in allopatric populations); and relatively well defined coupling between
67 chemical and biological processes (5–8). In addition, the microbial communities inhabiting these
68 systems, which are usually stable across time (9), have been broadly described, showing a general
69 dominance of complete ammonia oxidizers (comammox) (6, 10, 11). These recently discovered
70 microorganisms are expected to have a relatively simple ecology (due to their chemolithoautotrophic
71 metabolism) (12), yet are poorly studied in terms of what drives their diversity, distribution and
72 evolution. Furthermore, as comammox bacteria occur in RSF as coexisting populations (10, 13), RSF
73 offer an opportunity for resolving fine-scale genomic heterogeneity within closely related strains, and
74 investigate if they show similar patterns in evolutionary processes (such as selection or
75 recombination).

76 Of particular interest is to what extent the evolutionary processes that drive the diversification
77 of comammox *Nitrospira* are dependent on their environment, as opposed to intrinsic properties of
78 the species. Environmental dependency of microbial evolution has been investigated from different
79 perspectives. Several studies have focused on genome signatures variations (GC, tetranucleotide
80 signatures, codon usage, purine-pyrimidine ratio) associated with different environments (reviewed
81 in Dutta and Paul (2012) (14)). Others have studied bacterial adaptation to shifting environments (15),
82 or have targeted a specific evolutionary process across several lifestyles (e.g.: homologous

recombination (16) or selection (17)). Most of these studies, however, considered different species living in different environments, or closely related species with a different lifestyle (i.e. free-living organisms vs pathogens). Yet, little is known about ongoing evolutionary processes of species belonging to the same lineage inhabiting different open environments. In this study, taking advantage of the multiple comammox species present in several groundwater-fed RSF, we thoroughly investigated evolutionary processes in this environment, and compared these observations with those in comammox species inhabiting other environments.

Results and Discussion

We examined ecological and evolutionary patterns within comammox dominated-bacterial communities inhabiting groundwater-fed rapid sand filters. To that end, we retrieved metagenome-assembled genomes (MAGs) from 12 similarly operated waterworks in Denmark, using a combination of automatic and manual binning, followed by several refinement steps to improve the bin quality (Fig. S1). To remove redundancy, a single representative was selected for each set of genomes that shared an average nucleotide identity (ANI) greater than 99%. This resulted in a total of 189 MAGs (genome completeness = 83.9% ± 13.6, contamination = 1.9% ± 1.4; Table S1), 18 of them classified as *Nitrospira* spp. (completeness = 89.1% ± 9.2, contamination = 2.5% ± 1.1; Table S2). These *Nitrospira* MAGs spanned 16 putative species (further on simply referred to as 'species') using a threshold average nucleotide identity (ANI) of ≥ 95% (18–20). The phylogenomic analysis placed one *Nitrospira* species into lineage I, 14 into lineage II, and one into other lineages (Fig. 1). Of the 16 *Nitrospira* species, 12 were classified as comammox *Nitrospira* (5 clade A and 7 clade B) (Fig. 1). As expected, the genomes classified as comammox *Nitrospira* also contained genes essential for complete ammonia oxidation, such as those of the ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO) operons. Further description of the *Nitrospira* MAGs can be found elsewhere (21).

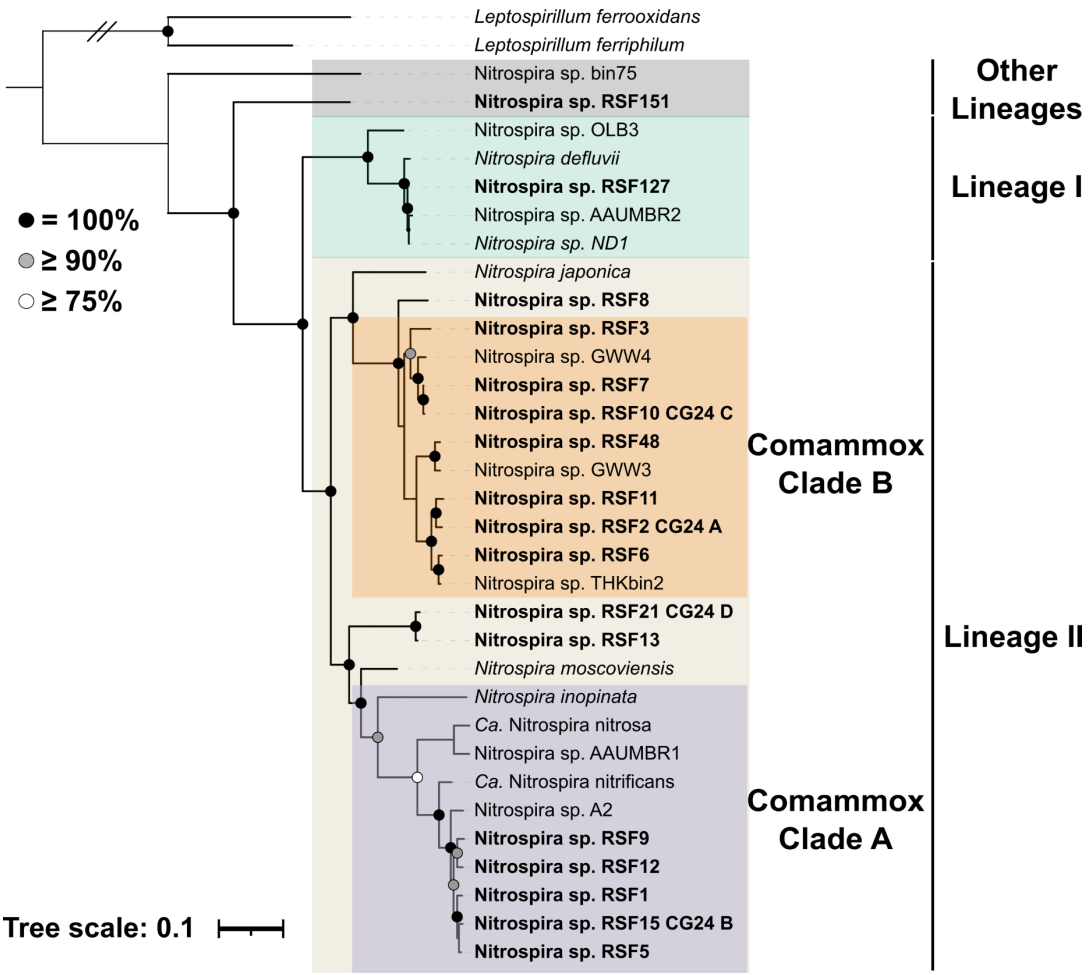

Fig. 1. Phylogenomic affiliation of *Nitrospira* MAGs retrieved from 12 waterworks.

A phylogenetic tree was built based on the concatenation of 120 proteins. *Nitrospira* MAGs retrieved in this study are highlighted in boldface. Lineages and sublineages are shown with colours (lineage I, green; lineage II, light brown; Other lineages, grey; comammox clade A purple; comammox clade B, orange). *Leptospirillum* was used to root the tree. The strength of support for internal nodes as assessed by bootstrap replicates is indicated as coloured circles (top left legend).

Nitrospira species comprised a large proportion of the microbial communities of the waterworks (27 - 70%), and comammox represented a large fraction of *Nitrospira* spp. (76 - 98%) (Fig. 2 and Table S3). For most of the RSFs, the variation among the abundance of the different *Nitrospira* MAGs between duplicate samples was low (Fig. S2), suggesting a high spatial homogeneity in the top layer of the filters, probably due to the frequent mixing caused by backwash, and hence supporting the representativity of our sampling. Multiple *Nitrospira* species (at least 5; 10 on average) co-occurred in all the waterworks (Fig. 2). However, there was no consistent dominance pattern. In some cases, a single species, but not always the same, dominated (WW4 and WW5), while, in other, two (WW9 and WW12) or more species dominated (Fig. 2). The chemical characteristics of the water explained 57% of the variance in *Nitrospira* composition (permutation test: $p < 0.001$; Table S4), suggesting that water chemistry is a strong filter for the assembly of these nitrifying communities. Among the measured water constituents, the influent ammonium concentration best explained *Nitrospira* distribution (explained 18%; permutation test: padjust = 0.02) (Table S4). Higher comammox species richness was detected in waterworks treating lower ammonium concentration (Fig. S3, $R^2 = 0.54$, $p < 0.01$). Canonical *Nitrospira* and canonical ammonia oxidizers were more abundant in waterworks receiving influent with higher ammonium (Fig. S4). These observations are in line with the prediction that higher ammonium concentration favours the emergence of division of labour between canonical ammonia and nitrite oxidiser (22). Nevertheless, we observed that a comammox species (RSF3) seemed to cope with slightly higher ammonium concentrations as well (Fig. S4). Interesting, besides the mentioned RSF3, the other comammox *Nitrospira* spp. tended to cluster with those ones sharing clade affiliation (Fig. S4), which is based on the phylogeny of ammonia monooxygenase subunit. On another note, the distribution pattern of *Nitrospira* species across the waterworks was not related to their geographic distance (Mantel test: r statistics = 0.08 and significance > 0.05).

Significant correlation among the abundances of the comammox species was rare; only few strong positive correlations were detected (RSF5 and RSF15_CG24_B (ρ=0.84), RSF6 and RSF11 (ρ=0.84)), while no significant negative correlations were observed (Fig. S5). This absence of obvious competitive exclusion pattern, together with the aforementioned description of co-existence of multiple comammox species, suggest that these microorganisms seem to exploit slightly different niches. A possible explanation might be that the co-occurrent comammox *Nitrospira* species had different ammonia affinities, and they would occupy different sites in the highly porous filter material (23). So far, little is known about the variability of ammonia affinity in comammox *Nitrospira* as this feature has only been reported for two comammox species (24, 25), however, a wide range of ammonia affinities have been observed in other ammonia oxidizers (26). Another reason could lie in the abundance of unique genes in each comammox *Nitrospira* species (> 250 in average (21)). Although the function of most of them is still unknown, these unique genes might promote ecological

variation. Traits such as chemotactic strategies, attachment to particles strategies, secondary metabolism or defence against predation have been proposed to explain this phenomenon in other coexisting microorganisms (Reviewed in Louca *et al.*, (2018) (27)).

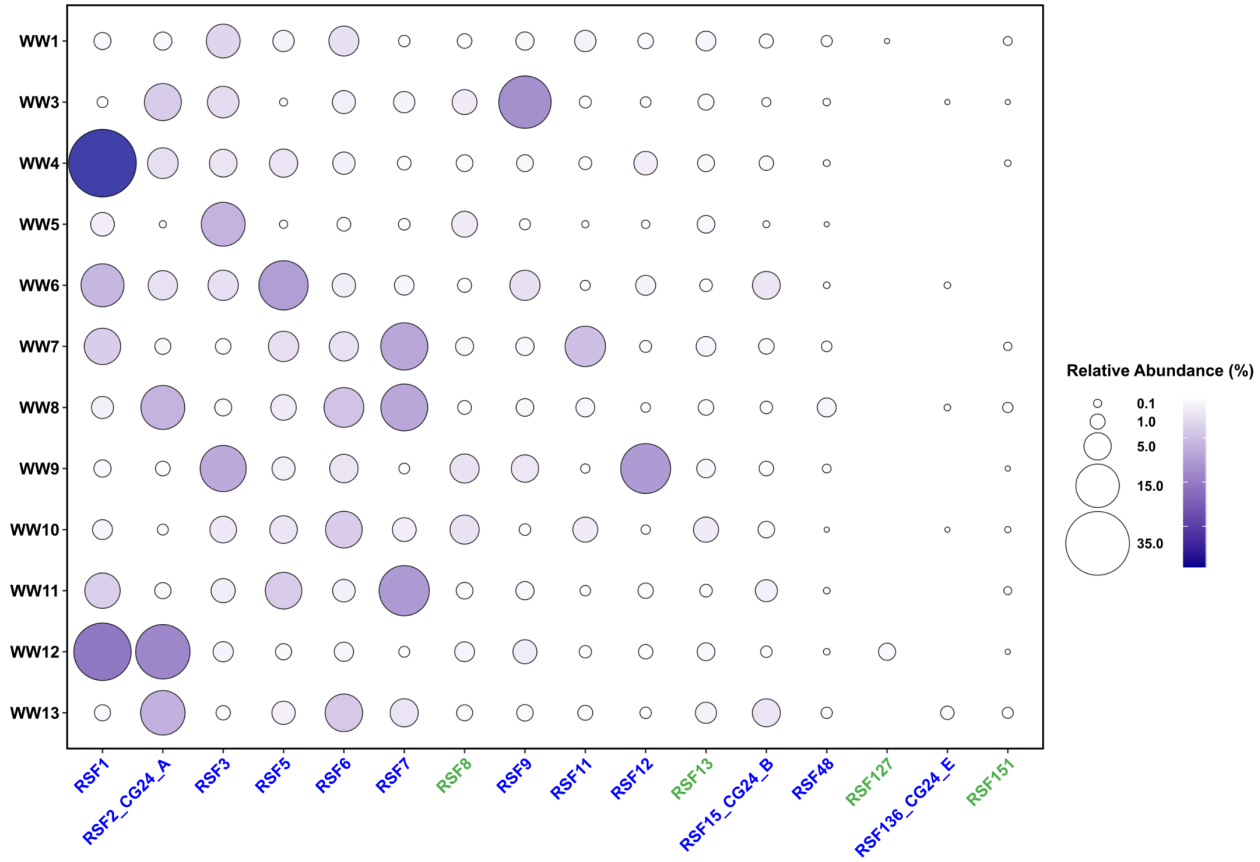

Fig. 2. Abundance of *Nitrospira* species across 12 waterworks.
Relative abundance of 16 *Nitrospira* species in 12 waterworks. Comammox and canonical *Nitrospira* species are denoted in blue and green, respectively.

Microdiversity within *Nitrospira* species

Strain-level analysis across the waterworks revealed that the *Nitrospira* populations contained intra-population sequence diversity. We exploited the shotgun metagenomic data to perform strain-level analyses on the 12 most abundant *Nitrospira* species (genome completeness = 92.4% ± 5.5, contamination = 2.7% ± 0.8) based on single nucleotide polymorphisms (SNPs). The number of SNPs/Mbp in the populations across the waterworks ranged from 14,437 to 45,664 (Table S5). Looking into the populations at local scale (species within waterworks), the number of SNPs/Mbp ranged from 249 to 37,663 (Table S6).

We observed a wide range of microdiversity (measured as nucleotide diversity (π)) among populations (Fig. 3A): canonical *Nitrospira* RSF8 was the most diverse species, with three times more nucleotide diversity than the less diverse *Nitrospira* species of our study (RSF1 and RSF12) (Fig. 3A). Depending on the *Nitrospira* population, we detected instances of both homogeneous microdiversity across the waterworks (e.g., RSF5 and RSF8), as well as a high microdiversity

202 variation depending on the waterworks (e.g., RSF1, RSF9 and RSF11) (Fig. S6). Based on our
203 observations of high species-level comammox *Nitrospira* diversity at low ammonium concentration,
204 we hypothesised that such conditions also promote high microdiversity. However, this was not the
205 case, as the correlations of microdiversity with ammonium concentration or with comammox species
206 richness were not significant for any species ($p > 0.05$). This suggests different drivers for inter-
207 species vs within-species diversities.

208 While all species showed significant microdiversity across waterworks, we detected a few
209 highly abundant comammox populations with almost no microdiversity at the local scale (e.g.,
210 comammox *Nitrospira* RSF1 and RSF12 in in WW4 and WW9, respectively; Fig. S7), which
211 suggests local clonal expansions of these comammox populations. Along the same line, the analysis
212 of major allele frequencies of common SNPs revealed that only a fraction of the within-species
213 variants diversity is found locally. Thus, for specific species, we detected different within-species
214 variants among the waterworks, in some cases, distinct single variants being dominants (e.g.:
215 comammox RSF3 in WW10B vs WW5; Fig. S8). These results contrast with species level
216 observations, where all the diversity was represented in each waterworks (as they all contain most of
217 the *Nitrospira* spp.; Fig. 2).

218 Similar to what we observed at species level, there was no significant correlation between
219 similarity in subspecies composition and the geographic distance of the waterworks, with the
220 exceptions of *Nitrospira* sp. RSF2 ($p < 0.01$) and *Nitrospira* sp. RSF8 ($p < 0.05$) (Fig. S9). However,
221 we observed an inter-waterworks organisation of the genetic structure at most loci across the studied
222 genomes, indicating that the *Nitrospira* populations were more similar within than between
223 waterworks. For each gene, we calculated pairwise fixation indexes (F_{ST}) to measure differences in
224 allele frequencies between populations of the same species found in two distinct waterworks. The
225 mean gene F_{ST} values were ≥ 15% for all *Nitrospira* species (Fig. S10), the most extreme case being
226 RSF5 ($F_{ST} > 40\%$) (Fig. S10). In few species (RSF2, RSF7 and RSF8), a higher dispersal ($F_{ST} < 20\%$)
227 of most alleles between waterworks was observed (Fig. S10). These observations differ from those
228 on soil bacterial populations across a meadow obtained using the same approach, where most of the
229 populations had mean gene F_{ST} values < 5% (28). These contrasting results support the notion that
230 populations in the waterworks are much more allopatric than the ones in other environments such as
231 the mentioned meadow.

232 We also investigated local regions of the *Nitrospira* genomes with significantly higher
233 F_{ST} values, as this is characteristic of local population-specific (here, in each waterworks) selective
234 pressures (28). A few loci with unusually high F_{ST} were found in some of the *Nitrospira* populations
235 (Fig. S11 and Table S7), one of them containing genes involved in nitrogen assimilation (*Nitrospira*
236 sp. RSF2) (Table S7). However, only two of these loci with unusually high site-specific
237 differentiation of alleles (high F_{ST}) also had few recombinant events, and low nucleotide diversity
238 (Table S7), which can be considered as a signal of recent selective sweep (28). These results suggest
239 that, contrary to what has been observed in several natural populations (28–31), gene-specific sweeps
240 seem to play a minor role in the evolution of *Nitrospira* spp. inhabiting the waterworks. A possible
241 explanation could be the low recombination rate that characterised the waterworks *Nitrospira*
242 populations (Fig. 3B, discussed below), as opposed to genome-wide sweeps that are associated to low
243 recombination rates, gene-specific sweeps are expected to occur with high recombination rates (32).

Overall, across the 12 waterworks, all species presented significant genomic microdiversity but this diversity was not always represented locally, with a few occurrences of patterns consistent with clonal expansion. The reason for the difference of within-species diversity across waterworks is unknown but the allopatric nature of the communities likely contributes to their persistence.

Evolutionary processes at whole-genome level

The *Nitrospira* populations were characterised by a low degree of homologous recombination. We investigated this evolutionary process based on linkage disequilibrium (D' is only < 1 if all possible combinations of a pair of biallelic sites are observed (33); lower D' values indicate higher levels of homologous recombination; Fig. 3B). A similar low degree of homologous recombination was observed for other non-*Nitrospira* abundant populations inhabiting the waterworks (n=12; genome completeness = 92.6% ± 5.5, contamination = 2.1% ± 2.0) (Fig. 3B and Table S5). In general, this evolutionary process was lower in the waterworks populations than in populations inhabiting a grassland meadow (Fig. 3B), where a similar strain-level analysis was conducted (28). To further examine the relative effect of homologous recombination on the genetic diversification of the populations, we measured the rates at which nucleotides become substituted as a result of recombination versus mutation using the *r/m* ratio. Most of the *Nitrospira* populations had a relatively low *r/m* (*r/m* < 2) compared to recombinogenic species reported in literature (*r/m* > 4) (34) (Fig. S12), although in one case (RSF15_CG24_B) the rate was similar to the value reported for a *S. flavogriseus* population (*r/m* = 28) considered to be approaching panmixia (35). Overall, these results suggest a low effect of recombination in the populations inhabiting the waterworks. Increasing recombination rate has been associated with fluctuating environments as a source of variation which can accelerate adaptation favouring survival in this type of environments (36, 37). On the other hand, constant environments - as the waterworks studied here - tend to reduce the recombination rate of their residents (36).

The *Nitrospira* populations were characterised by strong purifying selection. We used the relation between non-synonymous and synonymous polymorphisms (pN/pS) to investigate this evolutionary process. We detected pN/pS < 1, indicating purifying selection, for all *Nitrospira* species (Fig. 3C). Similar results were observed for other abundant populations of the waterworks (Fig. 3C). Purifying selection has frequently been observed in wild populations, and it was the case for populations inhabiting a grassland meadow (28) (pN/pS = 0.56 ± 0.17, n = 19), but this process seems to be especially strong in the waterworks populations (pN/pS = 0.34 ± 0.05, n = 24) (Two-Sample t-

test, $p < 0.0001$) (Fig. 3C). This suggests that their genomes could have reached an adaptive optimum for this stable environment, which is maintained by purging non-synonymous mutations.

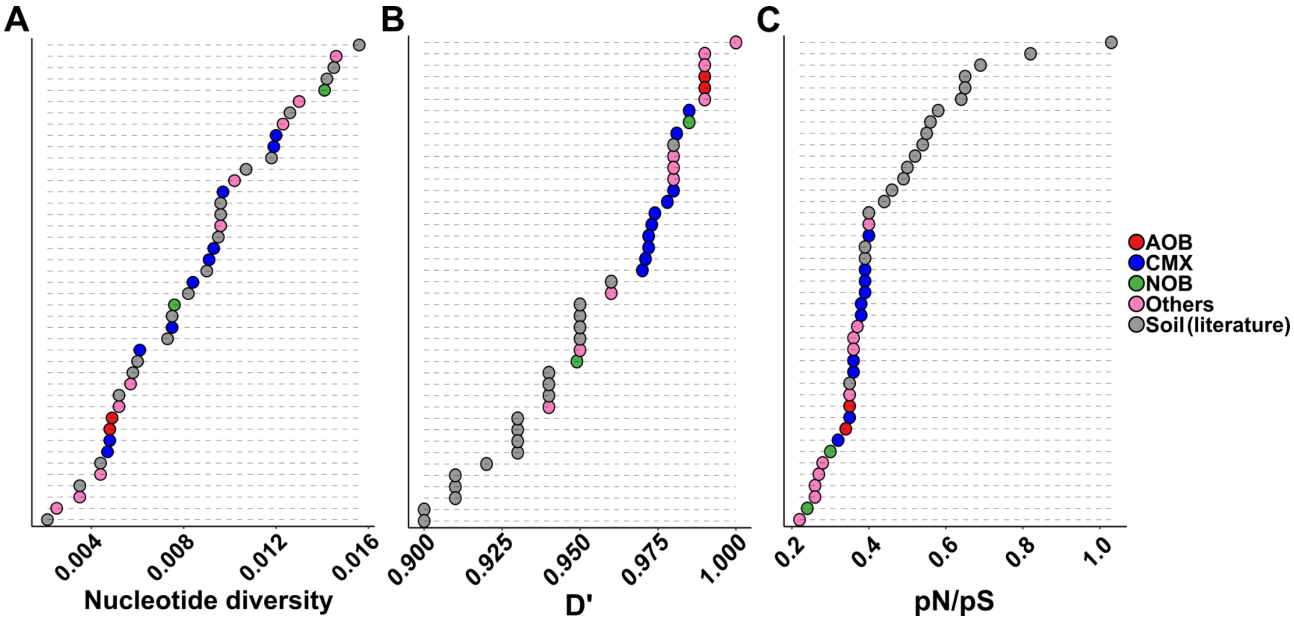

Fig. 3. Evolutionary metrics of *Nitrospira* populations across 12 waterworks compared to other organisms and locations.

A) Nucleotide diversity (π) of the most abundant bacterial populations across 12 waterworks, and the most abundant bacterial populations across a grassland meadow (denoted as 'Soil') [38]. B) Homologous recombination (D') of most abundant bacterial populations across 12 waterworks, and most abundant bacterial populations across grassland meadow. C) Selection (pN/pS ratio) of most abundant bacterial populations across 12 waterworks, and most abundant bacterial populations across grassland meadow. AOB: ammonia oxidizing bacteria, CMX: comammox, NOB: nitrite oxidizing bacteria, Others: non-nitrifying abundant bacteria present in the waterworks. The data reported in this table (including the MAGs ID) can be found in Table S5.

Interestingly, the degree of recombination and diversity across different *Nitrospira* populations varied substantially with habitat (Fig. 4). High variability of recombination in closely related bacterial species has occasionally been reported (38), and lifestyle appears as one of the most relevant factors to explain this variability (16, 38). Our analysis in *Nitrospira* populations from different habitats (drinking water treatment plants (DWTP), freshwaters and soils) suggests that the environment also influences ongoing evolutionary processes: different bacterial types in the same environment tended to share similar features (Fig. 4), while the evolutionary characteristics of comammox *Nitrospira* populations differed depending on the environment where they were retrieved (Fig. 4). Comammox species in the studied waterworks and in other DWTP were characterised by low recombination, strong purifying selection and moderate microdiversity (Fig. 4). On the other hand, comammox present in freshwater and in soils had higher microdiversity and, especially, recombination rate (Fig. 4 and Table S5). Intriguingly, we consistently observed that, in drinking water treatment systems, canonical *Nitrospira* species showed features similar to those of comammox *Nitrospira* but with even stronger purifying selection (Fig. 4). This feature, together with the much lower richness observed in canonical *Nitrospira* compared to comammox bacteria (Fig. 2), suggests

305 that competition can play a more intense role in canonical *Nitrospira*, which might select for few
306 species optimally adapted to this type of stable environment. However, a broader analysis is required
307 to confirm this hypothesis.
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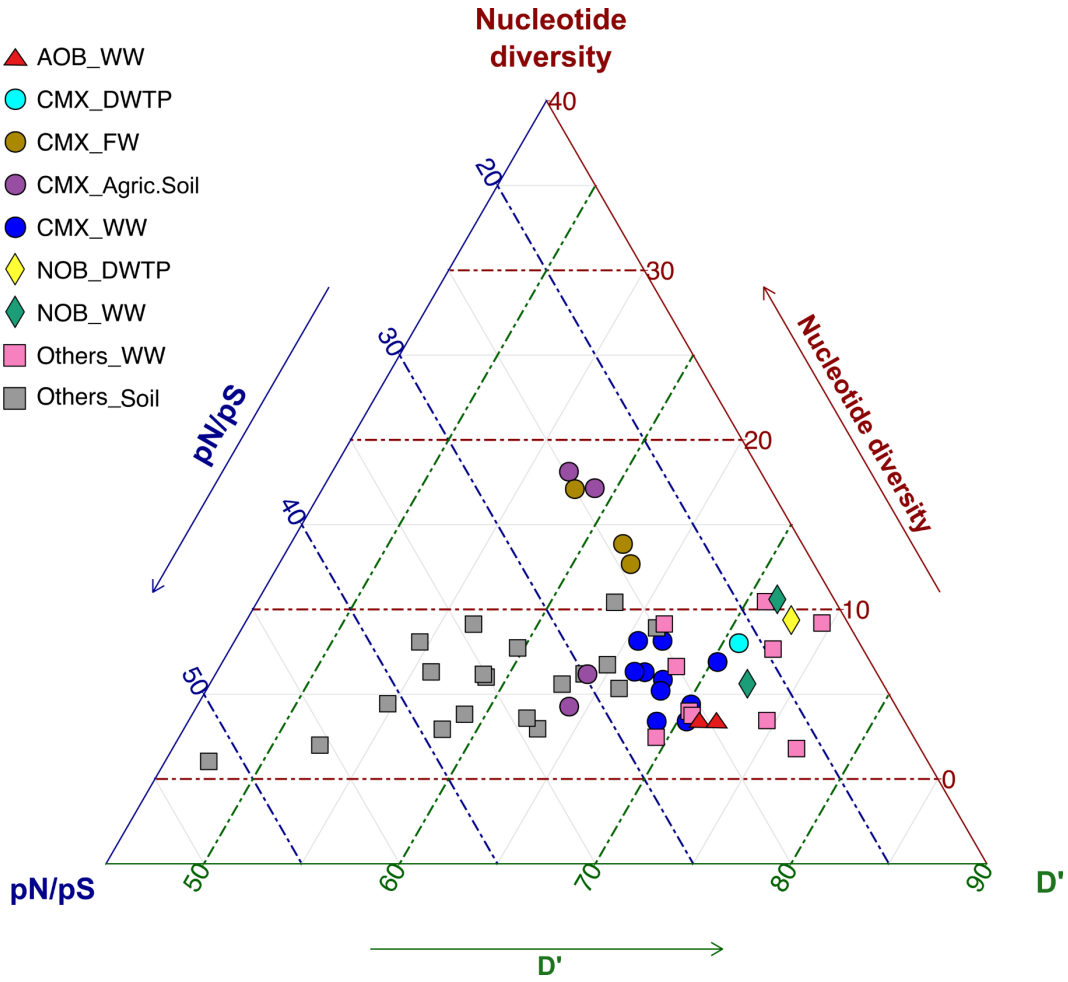

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311 **Fig. 4. Impact of environment and microbial type in evolutionary metrics**
312 Triplot composed of the nucleotide diversity, pN/pS ratio and D' values for the bacterial populations of this
313 study (WW), the most abundant bacterial populations across a grassland meadow [38] (Soil), and other
314 abundant *Nitrospira* populations recovered from other systems (Table S5; CMX_FW, CMX_DWTP,
315 NOB_DWTP, and CMX_Agric.Soil). The data reported in this table (including the MAGs ID) can be found
316 in Table S5.
317
318 **Evolutionary processes at the gene level**
319 In addition to a genome-wide analysis, we investigated the evolutionary processes at the gene
320 level. In the studied *Nitrospira* populations, genes involved in nitrification (ammonia
321 monooxygenase: *amoA* and *amoB*; hydroxylamine dehydrogenase: *haoA* and *haoB*; nitrite
322 oxidoreductase: *nxrA* and *nxrB*) generally had a similar nucleotide diversity (π) (Fig. 5A) and
323 homologous recombination rate (D') (Fig. 5B) compared to the rest of the genome, but with higher
324 levels of purifying selection (pN/pS) (Fig. 5C). The nucleotide diversities of genes related to

nitrification were very similar with the exception of *amoB*, which had a significantly lower nucleotide diversity than *nxrB* ($p < 0.05$) (Fig. 5A). A similar pattern was detected for the recombination, but in this case *amoA*, as well as *amoB*, had significantly lower recombination than *nxrB* ($p < 0.05$) (Fig. 5B). We observed a very strong purifying selection for most of the nitrifying genes, especially for *amoA*, *nxrA*, and *nxrB* ($p < 0.01$) (Fig. 5C). In the case of *nxrB*, not a single non-synonymous mutation was found in most of the *Nitrospira* species (0-1 non-synonymous sites vs 17-66 synonymous sites), even though this gene had a higher nucleotide diversity and homologous recombination (Fig. 5A and Fig. 5B). Our observations on selection are in line with previous studies, as generally, essential genes and enzymes catalysing reactions that are difficult to by-pass through alternative pathways are subject to higher purifying selection compared to nonessential ones (39–42).

Even though the average pN/pS values were below 1 in all *Nitrospira* species (Fig. 3), indicating purifying selection, genes with pN/pS values above 1 and significantly higher than the genomic average were detected in each species (Fig. S13). Many of those genes under positive selection were related to defence mechanism against phages (e.g. genes putatively involved in phage entry into cells, ribonucleases, genes coding proteins associated to restriction-modification systems, and genes related to toxin-antitoxin systems (Table S8)). Comparable findings were made in other abundant species from the waterworks (Table S9), in the additional *Nitrospira* populations retrieved from other environments (Table S9), as well as in *E. coli* and *Vibrio* sp. strains, respectively (43, 44). These observations suggest that positive selection in phage-related genes is widespread across bacteria, and highlights the evolutionary arms race occurring between phages and bacteria as an important driver in bacterial ecology and evolution (32, 45, 46). Additionally, we found nondefense, mobile genetic elements, such as transposons and integrases, with significantly higher pN/pS values than the genome average in the *Nitrospira* spp. (Table S10).

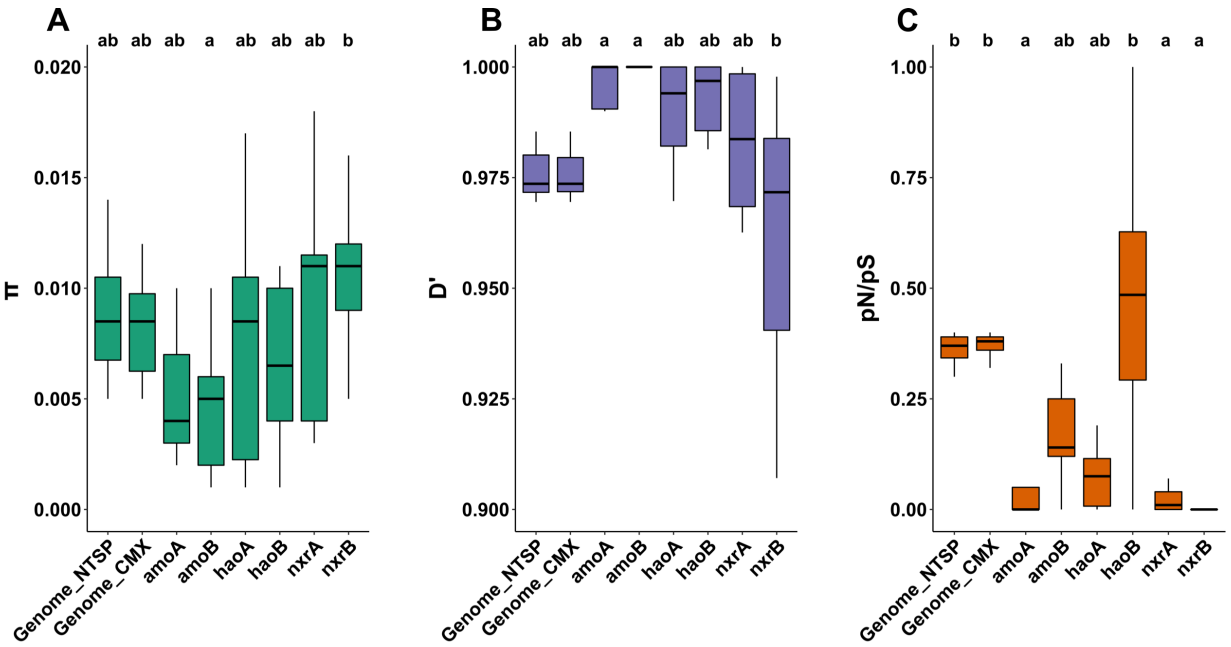

Fig. 5. Evolutionary metrics of nitrification genes in *Nitrospira* populations across 12 waterworks.

Left) Boxplot of nucleotide diversity of *Nitrospira* bacterial populations for whole genome (all *Nitrospira* and comammox *Nitrospira*) and nitrification genes. Differences between the mean nucleotide diversities were assessed by a Dunn's test; same letter means no significant difference ($p < 0.05$). Middle) Boxplot of linkage disequilibrium of *Nitrospira* bacterial populations for whole genome (all *Nitrospira* and comammox *Nitrospira*) and nitrification genes. Differences between the mean linkage disequilibriums were assessed by a Dunn's test; same letter means no significant difference ($p < 0.05$). Right) Boxplot of pN/pS ratios of *Nitrospira* bacterial populations for whole genome (all *Nitrospira* and comammox *Nitrospira*) and nitrification genes. Differences between the mean pN/pS ratios were assessed by a Dunn's test; same letter means no significant difference ($p < 0.01$).

Conclusions

A major unresolved question is how the relationship between ecology and evolution shapes complex communities in the environments. Here we use a model microbial system with relatively stable conditions to examine this question. By analysing the microbial communities in 12 groundwater-fed rapid sand filters, we observed that co-occurring comammox *Nitrospira* spp. dominate all sites, but with local differences in species composition. This suggest that the abundant comammox *Nitrospira* spp. exploit slightly different niches, which could partially be explained by the inlet water chemistry composition and the different genetic repertory. At subspecies level, comammox *Nitrospira* spp. are characterised by strong purifying selection and low recombination. These features, together with the occasional genome sweeps we detected, suggest that the subspecies possibly occupy a narrow niche to which they are optimally adapted. In contrast, we also showed that the relative magnitude of these evolutionary processes was different in comammox *Nitrospira* in habitats where environmental conditions are less stable and immigration more intense. Thus, we conclude that the evolutionary processes that drive the diversification of *Nitrospira* are dependent on the environment, as opposed to intrinsic properties of the species.

Materials and methods

Sampling, sequencing, and metagenomic assembled genomes recovery

The sampling description, DNA extraction and sequencing have been previously described (21). Briefly, filter material was collected from two locations at the top of the filters of 12 Danish waterworks. DNA was extracted from 0.5 g of sand material using the MP FastDNA Spin Kit (MP Biomedicals LLC, Solon, USA). DNA libraries were generated using the 24 extracted DNA with the Nextera XT DNA library preparation kit (Illumina Inc.) according to the manufacturer's instructions. Samples were sequenced in one lane, with 2×150 paired read sequencing on the Illumina HiSeq4000 at BGI's facility in Copenhagen. Generated reads were trimmed and filtered, including adapters removal, using Trimmomatic v0.22 (threshold quality=15; minimum length=40) (47). FastQC (Babraham Bioinformatics (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)) was used to evaluate the quality of the obtained reads. Co-assembly of high-quality reads was conducted using IDBA-UD (48) with the options --pre_correction --min_contig 1000. Additionally, 24 single-sample assemblies were performed following the same procedure. As it has been shown that the combination of multiple binning algorithms outperforms the usage of one single algorithm (49), here we performed metagenomic binning using MetaBAT (50), MaxBin2 (51), CONCOCT (52), MyCC (53), Binsanity

394 (54) and COCACOLA (55) (Fig. S1). The quality of the resulted genomes was improved using
395 Binning refiner (56). Simultaneously, we ran the software metaWRAP (57), which also take
396 advantage of multiple binning tools to recover genomes from the co-assembly. The generated bins
397 were improved using the refinement module of metaWRAP. Selection of the best genomes recovered
398 with the different binning algorithms was done with DAS Tool (58). dRep (59) was applied to
399 dereplicate all the selected best bins with the secondary clustering threshold set at 99% genome-wide
400 Average Nucleotide Identity (gANI). On the other hand, mmgenome (60) was applied to the single-
401 sample assemblies to recover *Nitrospira* genomes following the strategy described elsewhere (13).
402 The generated *Nitrospira* genomes were combined with the dereplicated bins, and subjected to the
403 reassembly module of metaWRAP with the aim of improving the quality of the bins. dRep at 99%
404 gANI was used again for dereplication. RefineM (--cov_corr = 0.8) (61) was used to refine the
405 resulting dereplicated bins by removing scaffolds with divergent GC-content or tetranucleotide
406 frequencies. Furthermore, the binning and refinement modules from metaWRAP were applied to the
407 co-assembly of the six RSF samples used in Palomo et al. (2018) (5). Obtained bins together with the
408 reported ones in Palomo et al. (2016 and 2018) (5, 13) were dereplicated using dRep at 99% gANI.
409 A refinement step with RefineM was applied on the resulting dereplicated bins. These refined bins,
410 together with the bins recovered from the 12 waterworks of this study were dereplicated as described
411 above. In addition, the assembly quality of the *Nitrospira* bins were improved by alignment against
412 related complete or draft genomes using the Multi-Draft based Scaffolder (MeDuSa) (62). The overall
413 procedure here described can be visualised in the Fig. S1. The completeness and contamination of
414 the bins were evaluated using CheckM (63).
415
416 **Species abundance estimation**
417 A 95% average nucleotide identity (ANI) cut-off was used to define species as proposed by
418 Klappenbach *et al*. (2007) (18). The retrieved MAGs were dereplicated using dRep with the
419 secondary clustering threshold set at 95% gANI. Among the genomes classified as belonging to the
420 same species, the one with higher quality was chosen as representative genome. The species
421 abundance and coverage of each representative genome across the metagenomes were assessed using
422 MIDAS (64). Briefly, MIDAS uses reads mapped to 15 universal single-copy gene families (with
423 ability to accurately recruit metagenomic reads to the correct species (64)) to estimate the abundance
424 and coverage of bacterial species from a shotgun metagenome. We used the species retrieved in this
425 study to build the database of universal-single-copy genes.
426
427 **Genome classification and annotation**
428 MAGs were classified using the classify workflow of the GTDB-Tk v.0.1.3 tool (65). Open reading
429 frames were predicted using Prodigal v. 2.63 (66), and annotated using blastp (67) against NCBI nr
430 (68), UniProt (69), KEGG (70), PFAM (71) and eggNOG (72). Genes were assigned to antiphage
431 defense systems using the strategy described in Doron *et al.* (2018) (73).
432
433 **Phylogenetic analysis**
434 Phylogenetic analyses of *Nitrospira* genomes were conducted with the GTDB-Tk v.0.1.3 tool (65)
435 [21] using the *de novo* workflow with a set of 120 single copy marker proteins and the genome

436 taxonomy database (GTDB) (74). Concatenated alignments were used to construct a maximum
437 likelihood tree using RAxML v. 8.2.11 (75) with 200 rapid bootstraps (determined using the
438 autoMRE option) and the LG likelihood model of amino acid substitution with gamma distributed
439 rates and fraction of invariant sites (-m PROTGAMMAILGF; best model determined using ProtTest
440 v. 3.4.2 (76)). The tree was rooted using two *Leptospirillum* species as outgroup. The rooted tree was
441 visualized using the online web tool from the Interactive Tree of Life (iTol) (77).
442
443 **Read mapping, SNP calling, and population genomic analysis**
444 The population genomic analysis was done following the approach described in Crits-Christoph et al
445 (2020) (28). High-quality reads were mapped to an indexed database of the 176 species MAGs
446 recovered from the waterworks using BWA-MEM (78). The resulting alignments were filtered using
447 samtools (79) view -q30 to remove reads with mapping quality less than 30, and also with the script
448 filter_reads.py (28) (with the options: -m 96 to retain reads with a percent identity of at least 96% to
449 the reference; and -q 2 to assure uniquely best mapping read pairs in the index). Downstream
450 population genomic analysis was performed on the 24 most abundant species genomes (12 *Nitrospira*
451 ones, and 12 other abundant species genomes). For each of these species genomes, we analysed data
452 in samples that passed a cutoff of at least 50% of the genome being covered with at least 5× coverage.
453 149 out of 576 sample genome comparisons (24 genomes × 24 samples) passed this minimum
454 requirement. Sample read mappings were pooled by each waterworks and by all samples across the
455 waterworks. Nucleotide diversity (π), linkage disequilibrium (D') and pN/pS ratio were calculated
456 for each sample, each waterworks and across all the waterworks as described elsewhere (28) using
457 the scripts provided by the authors. F_{ST} was calculated following the same procedure but on sites
458 segregating across two waterworks being compared (for all the possible waterworks comparisons).
459 As Crits-Christoph *et al*. (2020) (28) recommended, only sites with a coverage of at least 20× in each
460 waterworks was used to calculate F_{ST}. In addition, genes with coverage in a waterworks outside of
461 the range of two standard deviations were excluded from the analysis. As previously suggested (28),
462 a two-sample Wilcoxon test was conducted to find out if average linkage of highly differentiated loci
463 differed from the genomic average for each species. Similarly, a two-sample *t*-tests was used to
464 conclude if average nucleotide diversity of highly differentiated loci differed from the genomic
465 average. Both sets of tests were corrected for multiple hypotheses using the Benjamini–Hochberg
466 method. Recombination to mutation ratios were inferred using mcorr (34).
467 Same strain-level analysis as the one described above was conducted in other *Nitrospira* MAGs
468 previously recovered (21) that passed a cutoff of at least 50% of the genome being covered with at
469 least 5× coverage in any of the metagenomes were *Nitrospira* MAGs were found to be present (21).
470
471 **Statistical Analyses.**
472 All statistical tests were performed using R v3.5.2 (80). Due to the compositional nature of
473 sequencing data (81), for all statistical analyses, species abundances were analysed as followed: zeros
474 were replaced with an estimate value using the Count Zero Multiplicative approach with the
475 zCompositions R package (82), and data were further centred log-ratio transformed. *Nitrospira*
476 community dissimilarities were calculated using the Jaccard index. The correlation between the
477 *Nitrospira* community dissimilarities and geographic distances was calculated using the Mantel test

478 (significance obtained after 100,000 permutations). Same analysis was used to assess the correlation
479 between the *Nitrospira* community dissimilarities and the water composition dissimilarity, as well as
480 the correlation between major allele dissimilarities and geographic distances.
481 Proportionality between abundances of the species across the 24 metagenomes were calculated using
482 the propr R package (83) (with the options metric = "rho", ivar = "clr") and visualised using the
483 corrplot R package (84). For the network analysis, the function getNetwork from propr R package
484 was used to retain proportionalities > 0.56 (FDR < 5%). The network was visualised using the igraph
485 R package (85).
486 Redundancy analysis (RDA) was performed in a stepwise way using the *ordiR2step* function of the
487 *vegan* package (86). The analysis was conducted using centred log-ratio transformed *Nitrospira*
488 species abundances and chemical data of influent water. The constrained ordination model and the
489 variable significance were determined by permutation tests (1000 permutations) with anova.cca in
490 vegan. Before this analysis, collinearity among explanatory variables was evaluated using variance
491 inflation factor (VIF) in the *fmsb* package (87). The function *vif_func* was used to perform a stepwise
492 approach until all variables above a VIF of 5 were excluded (88). Ternary plot was performed using
493 the R package ggtern (89) using the nucleotide diversity, pN/pS ratio and D' values for the bacterial
494 populations retrieved from the waterworks, as well as most abundant bacterial populations across
495 grassland meadow (28), and other *Nitrospira* populations abundant in other systems (Table S5).
496 Differences between the mean nucleotide diversities of the nitrifying genes, whole *Nitrospira*
497 genomes, and whole comammox *Nitrospira* genomes were assessed using Kruskal–Wallis ANOVA
498 followed by Dunn's test with the Holm-Bonferroni correction. Same analysis was performed for
499 linkage disequilibrium and pN/pS ratios.
500
501 **Chemical analysis of influent water**
502 Ammonium was measured using a standard colorimetric salicylate and hypochlorite method (90),
503 while nitrite was analysed using a standard method adapted from Grasshoff *et al*. (1983) (91). Nitrate
504 and sulfate were measured by ion chromatography according to AWWA-WEF method 4110 (92).
505 Iron, manganese and copper were determined by ICP-MS (7700x, Agilent Technologies), while
506 calcium by ICP-OES (Varian, Vista-MPX CCD Simultaneous ICP-OES). Dissolved oxygen and pH
507 were measured with a handheld meter (WTW, Multi 3430, with FDO® 925 and SenTix® 940
508 probes).
509
510 **Data availability**
511 All raw sequence data and *Nitrospira* genomes retrieved from the Danish rapid sand filters have been
512 deposited at the NCBI BioProject database under accession number PRJNA384587. The rest of the
513 retrieved draft genomes from the Danish rapid sand filters are available on figshare
514 (https://doi.org/10.6084/m9.figshare.12962075). Data produced from the strain-level analysis
515 (nucleotide diversity, SPNs, linkage statistics and F_{ST} metrics) are available on figshare
516 (https://figshare.com/projects/Evolutionary_ecology_of_natural_comammox_Nitrospira_population
517 s/91217).
518

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Authors contributions

A.P conceived the study and performed the bioinformatic analyses. A.P and A.D led interpretation of the results supported by O.X.C and B.F.S. A.P drafted the manuscript with input from A.D, O.X.C and B.F.S. All authors contributed to manuscript revision, and approved the final version of the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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