

Resistant ammonia-oxidizing archaea endure, but adapting ammonia-oxidizing bacteria thrive in boreal lake sediments receiving nutrient-rich effluents

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Summary

Climate change along with anthropogenic activities changes biogeochemical conditions in lake ecosystems, modifying the sediment microbial communities. Wastewater effluents introduce nutrients and organic material but also novel microbes to lake ecosystems, simulating forthcoming increases in catchment loadings. In this work, we first used 16s rRNA gene sequencing to study how the overall sediment microbial community responds to wastewater in six boreal lakes. To examine forthcoming changes in the lake biogeochemistry, we focused on the ammonia-oxidizing archaea (AOA) and bacteria (AOB), and examined their functional and compositional community response to wastewater. Although we found the least diverse and least resistant prokaryotic communities from the most wastewater-influenced sediments, the community changed fast toward the natural composition with the diminishing influence of wastewater. Each lake hosted a unique resistant AOA community, while AOB communities were adapting, responding to environmental conditions as well as receiving new members from WWTPs. In general, AOB dominated in numbers in wastewater-influenced

sediments, while the ratio between AOA and AOB increased when moving toward pristine conditions. Our results suggest that although future climate-change-driven increases in nutrient loading and microbial migration might significantly disrupt lake sediment microbiomes, they can promote nitrification through adapting and abundant AOB communities.

Introduction

Microbes are important drivers of biogeochemical processes in lake ecosystems. However, climate change together with increasing anthropogenic activities in the lake watersheds is expected to significantly modify lake microbiomes. In the boreal area, increasing inorganic nitrogen and organic carbon loading from the agriculture and forest-dominated catchment areas (Erlandsson *et al.*, 2008; Mattsson *et al.*, 2017) will have a key role in shaping elemental cycling in lake environments, which are typically nutrient-poor. Biogeochemical conditions and microbial communities are generally tightly linked (Shade *et al.*, 2012; Kearns *et al.*, 2016; Reyes *et al.*, 2017), suggesting that future changes in the composition and function of microbial communities are expected in these ecosystems. However, microbial communities can be resistant and remain similar even under changing biogeochemical conditions (Bowen *et al.*, 2011).

Wastewater effluents introduce high amounts of inorganic nitrogen and organic material to aquatic ecosystems. They significantly alter the sediment microbial communities, decreasing diversity and population sizes (Drury *et al.*, 2013). In addition to changing microbial habitat characteristics, wastewater can introduce new microbes to the sediment community (Saarenheimo *et al.*, 2017), if wastewater-influenced and less-diverse communities show decreased resistance (Shade *et al.*, 2011). However, the influence of wastewater can be spatially limited, suggesting that significant changes in the sediment microbiology could be seen only in the near proximity of the effluent discharge. Indeed, we recently demonstrated that the sediment microbial community composition changed gradually toward the natural one when moving

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downstream from the wastewater discharge site in two boreal lake sites (Saarenheimo *et al.*, 2017). As both wastewater and catchment loadings alter habitat characteristics and may introduce novel microbes, these previous results suggest that future increases in catchment loadings could substantially modify the sediment microbiology.

Similar to catchment loading, wastewater may affect only certain taxonomic groups, which can then be reflected to biogeochemical processes. By bringing high amounts of inorganic nitrogen, the effect of wastewater, as well as of the loading from agricultural catchments, is targeting the nitrogen transforming microbial communities (Wakelin *et al.*, 2008). Denitrifying bacteria form a rather diverse group of bacteria, being less sensitive and functionally more redundant than nitrifying communities, which consist of only a few taxa and commonly respond to disturbances (Griffiths *et al.*, 2000; Wertz *et al.*, 2015). Indeed, nitrification has shown to be more dependent on the microbial community structure than denitrification (Cristina *et al.*, 2017), suggesting that the effect of wastewater could be targeted on nitrifying microbial community. In our recent study, we found that ammonia-oxidizing bacteria (AOB) dominated over ammonia-oxidizing archaea (AOA) at wastewater-influenced lake sediments (Saarenheimo *et al.*, 2017), but the relationship was reversed in the pristine sediments. Although a proportion of AOB was suggested to originate from wastewater treatment plant (see also Mußmann *et al.*, 2013; Pan *et al.*, 2018), the dynamics between AOA and AOB abundance were mainly directly driven by wastewater itself. Previous studies have reported that AOA thrive under stable and oligotrophic conditions, while dynamic and nitrogen-rich conditions favour AOB in aquatic environments (Laanbroek *et al.*, 2013; Bollman *et al.*, 2014; Reis *et al.*, 2015). From an ecological perspective, these results indicate that there might be some differences in the stability of AOA and AOB communities, seen as different resistance to changes in environmental conditions. Indeed, AOA have shown weaker resistance than AOB from drying-rewetting stress in soils (Thion *et al.*, 2014).

In this study, our first objective was to characterize the overall community stability and the effect of treated wastewater on lake sediment microbes, in order to understand how future higher catchment loadings could shape sediment microbiology. We hypothesized that wastewater-driven changes in sediment habitat characteristics would be directly reflected in microbial communities. We expected community diversity to decrease with increasing wastewater concentrations, by only certain groups being favoured by altered habitat characteristics. For example, *Bacteroidetes*, *Firmicutes*, *Nitrospira* and proteobacterial groups have been found to increase in wastewater-influenced sediments (Drury *et al.*, 2013; Lu and Lu 2014; Saarenheimo *et al.*, 2017). However, if there was a significant migration of

microbes from wastewater treatment plant, microbial community diversity could also increase or remain similar (Wakelin *et al.*, 2008). In both cases, we expected lower community resistance in wastewater-influenced sediments.

Our second objective was to specifically examine how AOA and AOB communities respond to wastewater. As AOA communities have been found to exhibit low diversity under disturbance (Newell *et al.*, 2014), and their abundance to be suppressed under nutrient-rich conditions (French *et al.*, 2012; Reis *et al.*, 2015; Soares *et al.*, 2016), we hypothesized that AOA would be more sensitive to nutrient-rich wastewater, showing decreasing diversity as well as decreasing overall abundance. We expected the abundance of AOB to increase in wastewater-influenced sediments, and hypothesized that it would be due to altered environmental conditions and/or introduction of novel AOB from wastewater treatment plant (WWTP). Furthermore, we hypothesized that we would observe a lower diversity in wastewater-influenced sediments caused by stronger selection, as there typically is environmental-driven niche differentiation between *Nitrosomonas* and *Nitrospira* groups (e.g., Peng *et al.*, 2013; Zhang *et al.*, 2015). However, we hypothesized that diversity could be promoted, if AOB groups coming from WWTP could successfully colonize the receiving sediments. For resistance, we expected AOA to exhibit weaker resistance to the wastewater disturbance than AOB, as has been shown for AOA communities facing physical disturbance (Newell *et al.*, 2014) or drying-rewetting (Thion *et al.*, 2014), suggesting that there might be a direct link between diversity and resistance, at least for AOA.

To investigate these objectives, we conducted a field sampling campaign in six boreal lakes in Central Finland in winter 2017. All lakes selected were large and humic, and received purified wastewater from nearby WWTPs. From each lake, reference samples were taken from a pristine sampling point, and wastewater-influenced samples along a wastewater gradient (downstream from wastewater effluent discharge site; Fig. 1). With the experimental design, we were able to relate changes in microbial community to the wastewater concentration, and to estimate whether higher catchment loadings in the future will shape nitrification, as AOB are considered to dominate ammonia oxidation in ammonium-rich environments (e.g., Di *et al.*, 2009; Pan *et al.*, 2018).

Results

Changes in overall sediment microbial community composition

Pristine microbial communities were generally similar, although Petäjävesi was slightly separated from the other

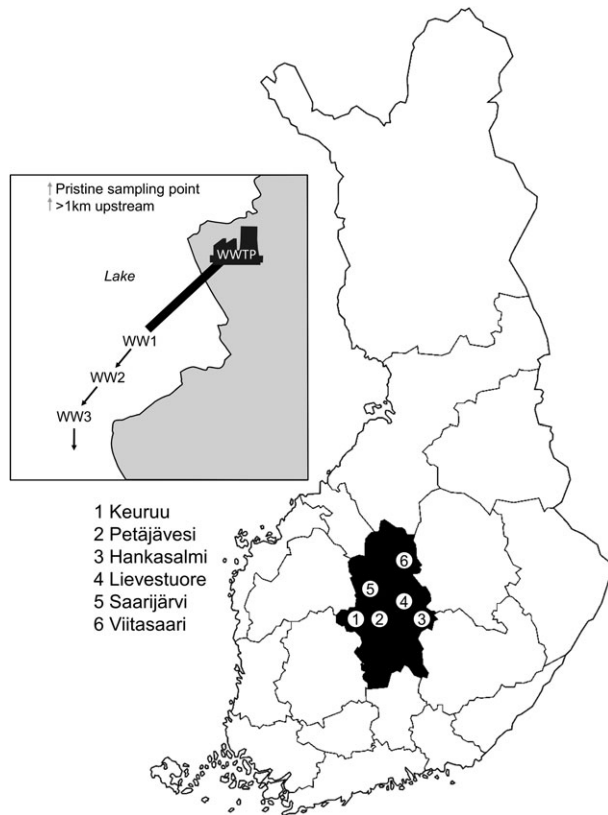


Fig. 1. Map of the location six study lake sites and the wastewater-influenced and pristine sampling points.

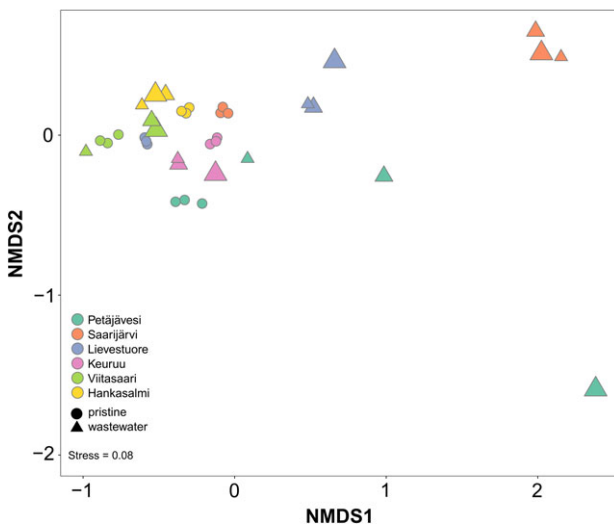


Fig. 2. Non-metric multidimensional scaling (axes NMDS1 vs. NMDS2) of prokaryotic sediment communities at the wastewater-influenced and pristine sampling points in six study lakes. Circles denote pristine sediment samples and triangles wastewater-influenced samples. The size of the triangle relates to the location of wastewater-influenced sampling point: WW1 being the largest triangles, WW2 the medium-sized triangles, and WW3 the smallest triangles.

sites, probably due to lower pH (Supporting Information Table S1). The composition of sediment microbial communities was different between pristine and wastewater-influenced sampling points (PERMANOVA, $F = 2.29$, $p = 0.003$; Fig. 2), the wastewater discharge point community being the most distinct as compared with the pristine communities. Based on the dotplot illustrating the differences in the standardized relative abundance of microbial taxa between the pristine sediments and wastewater discharge sampling point (Fig. 3), the abundance of several taxa was modified in the wastewater-influenced sediments. The effect of wastewater was highest in Petäjävеси and lowest in Hankasalmi (Fig. 3). The microbial communities were similar in all WWTPs sites, being dominated by certain beta-, epsilon- and gamma-proteobacterial, *Bacteroidetes*- and *Firmicutes*-type OTUs (Supporting Information Fig. S1). These groups increased at wastewater discharge points as compared with pristine sampling points, suggesting that they could originate from the WWTP (Fig. 3).

Wastewater-driven changes in sediment microbial community diversity and stability

In general, WWTP communities were less diverse than lake communities. The diversity and species richness of sediment microbes increased from the wastewater discharge site to the downstream sampling points (Fig. 4). In Saarijärvi, this was not seen due to different sampling strategy (see 'Materials and methods' section). The microbial community resistance decreased with wastewater influence, being lowest in Petäjävеси and Saarijärvi, and was positively related to diversity (Table 1; Supporting Information Fig. S2). In all lakes, the effect of wastewater was spatially restricted to the near proximity of the discharge point, as the similarity between lowest wastewater sampling point (WW3) and pristine samples was almost as high as between three pristine sample replicates (Table 1). This was again not seen in Saarijärvi. WWTP communities were the most similar between the six locations, while pristine communities exhibited 30% similarity and wastewater-influenced communities less than 20% similarity (Table 1).

Changes in nitrifying microbial communities under wastewater influence

In Petäjävеси, Lievestuore and Viitasaari, AOA dominated the pristine nitrifying community, but AOB were more abundant in Saarijärvi, Keuruu and Hankasalmi (Fig. 5). At the wastewater-influenced sampling points, AOB dominated in numbers in all lakes, except in Viitasaari and at the down-most point (WW3) in Hankasalmi. In Saarijärvi and Lievestuore, the abundance of both AOA and AOB

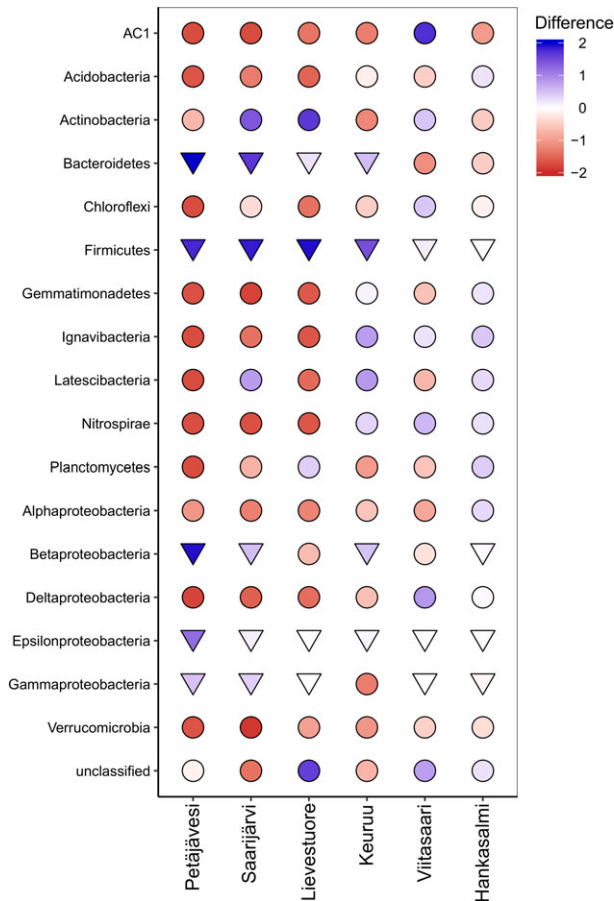


Fig. 3. Difference in the relative standardized abundance of most important microbial taxa (> 1% of all) between wastewater discharge sampling points and pristine sampling points in the study lakes. Red colour indicates a lower abundance and blue a higher abundance of certain taxonomic group at the wastewater discharge point than at the pristine reference site. Triangle denotes the possible WWTP-origin taxa. [Color figure can be viewed at [wileyonlinelibrary.com](#)]

was low at wastewater-influenced sampling points, AOA disappearing completely in Saarijärvi. The latter was also seen at the wastewater discharge point in Petäjavesi. The AOA:AOB increased with oxygen, and $\delta^{13}\text{C}$ and C:N of sediment, and decreased with increasing conductivity, temperature, ammonium concentration, and sediment C % and N% (Supporting Information Fig. S3).

The wastewater did not affect the AOA community composition (PERMANOVA, $F = 0.72$, $p > 0.05$; Fig. 6A), as each lake had their own unique AOA community ($F = 3.68$, $p = 0.001$). *Candidatus Nitrosotalea devanattera* dominated in Petäjavesi, Saarijärvi and Keuruu, while unclassified Nitrosopumilaceae was the main group in the other three lakes (Fig. 7A). Furthermore, there were clear differences in the AOA community composition between WWTPs, for example, unclassified Thaumarchaeota were abundant in Keuruu and Saarijärvi. The AOA community composition differed between WWTPs and wastewater-influenced sediments. For AOB, there

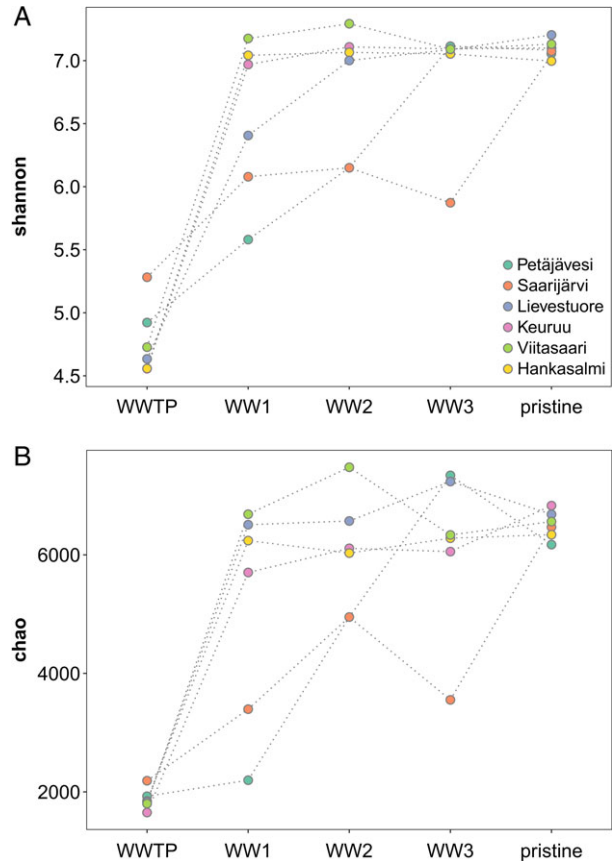


Fig. 4. A. Shannon diversity B. Chao species richness index for sediment microbiome in the six study lakes, based on the 16S rRNA gene sequencing. [Color figure can be viewed at [wileyonlinelibrary.com](#)]

was significant interaction between lake and sampling point affecting community composition (PERMANOVA, $F = 2.07$, $p = 0.001$; Fig. 6B), as in the most lakes, there were differences between pristine and wastewater-influenced sediments (Petäjavesi, Saarijärvi, Lievestuore, Keuruu, Viitasaari). Unclassified *Nitrosomonas* was the most abundant groups in WWTPs, and in the most wastewater-influenced sediments in Petäjavesi, Saarijärvi and Lievestuore, while *Nitrosospira briensis* was abundant in more pristine sediments. In Keuruu and Viitasaari, the abundance of *Nitrosomonas oligotropha* increased in wastewater-influenced sediments as compared with the pristine ones. If unclassified Nitrosomonadaceae (abundant in all samples) was excluded, *Nitrosomonas* dominated the wastewater-influenced sediments, while the abundance of *Nitrosospira* increased with decreasing wastewater influence (Fig. 7B).

Diversity and stability of AOA and AOB communities

AOA diversity and richness remained similar between wastewater-influenced and pristine sediments, but there

Table 1. Resistance, similarity between the lowest wastewater-influenced sampling point and pristine sampling points, and similarity between pristine sampling points of prokaryotic, AOA and AOB communities in six study lakes, and similarity between lakes at pristine and wastewater-influenced sampling points, and between the WWTPs.

	Resistance: community similarity between wastewater discharge point and pristine point			Community similarity between the lowest wastewater-influenced and pristine points			Similarity between pristine sampling points		
	16S	AOA	AOB	16S	AOA	AOB	16S	AOA	AOB
Petäjavesi	3.1%	na	0.9%	35.8%	38.8%	17.0%	45.4%	45.0%	*
Saarijärvi	11.5%	na	0.7%	11.9%	na	0.0%	43.5%	30.1%	38.7%
Lievestuore	19.5%	29.9%	12.1%	23.3%	34.6%	16.1%	46.2%	34.3%	36.0%
Keuruu	32.4%	35.0%	18.8%	35.3%	38.1%	29.2%	42.7%	39.4%	31.8%
Viitasaari	35.7%	33.7%	8.0%	44.0%	32.1%	11.8%	44.6%	39.6%	*
Hankasalmi	38.6%	na	26.5%	36.3%	na	26.2%	44.1%	na	26.8%
	Between-lakes similarity in pristine points			Between-lakes similarity under wastewater influence			similarity between WWTPs		
	16S	AOA	AOB	16S	AOA	AOB	16S	AOA	AOB
mean	30.4%	15.5%	21.4%	19.5%	16.6%	9.9%	32.4%	16.9%	22.5%
st.dev	1.9%	3.3%	4.0%	6.3%	4.3%	4.0%	3.7%	9.8%	7.9%

na, no sample, *, only one replicate from pristine point.

was substantial variation between the lakes. For AOB, we saw a decrease in the diversity and richness when moving downstream from the wastewater discharge

point, except in Saarijärvi and Hankasalmi where they increased (Supporting Information Fig. S4). Wastewater-influenced AOA communities were resistant, remaining

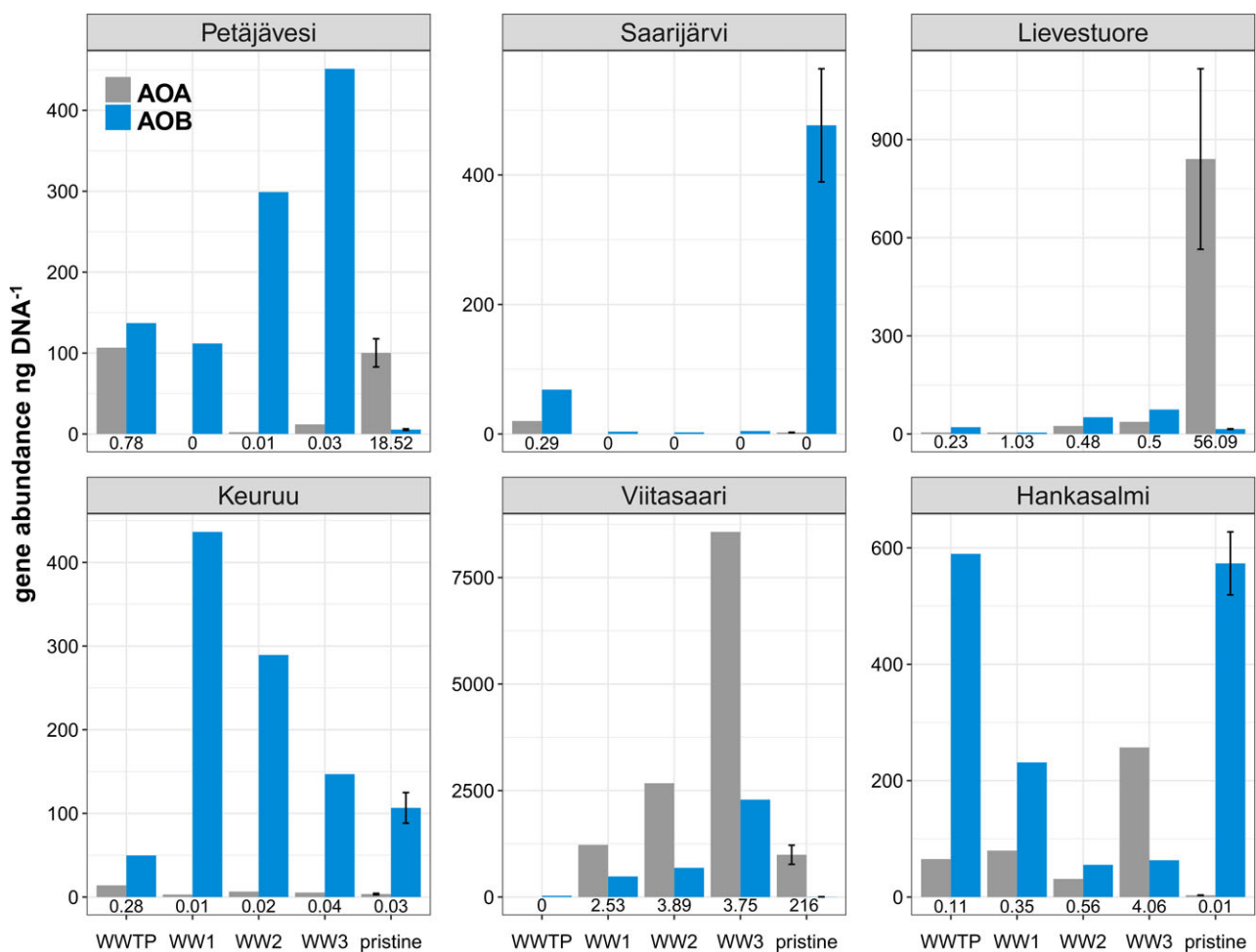


Fig. 5. Abundance of archaeal (AOA) and bacterial (AOB) *amoA* gene in relation to amount of DNA (gene abundance ng DNA⁻¹) in the six study lakes. Numbers below each bar denote for the AOA:AOB. [Color figure can be viewed at wileyonlinelibrary.com]

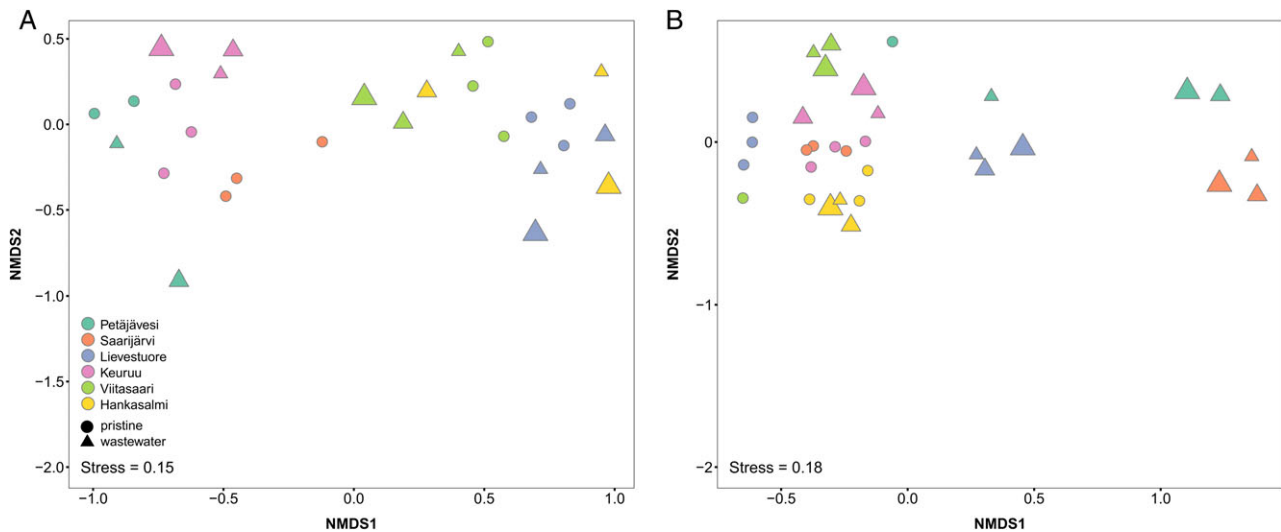


Fig. 6. Non-metric multidimensional scaling (axes NMDS1 vs. NMDS2) of A. AOA and B. AOB communities at the wastewater-influenced and pristine sampling points in six study lakes. Circles denote pristine sediment samples and triangles wastewater-influenced samples. The size of the triangle relates to the location of wastewater-influenced sampling point: WW1 being the largest triangles, WW2 the medium-sized triangles, and WW3 the smallest triangles.

similar with the pristine communities (Table 1). In contrast, AOB communities exhibited a low resistance to wastewater, and the similarity between the lowest wastewater-influenced sampling point (WW3) and pristine sampling points remained low. For AOA, between-lakes similarity was less than 17% for both pristine and wastewater-influenced sampling points, as also between-WWTPs similarity (Table 1). For AOB, the similarity between wastewater-influenced sites was less than 50% of the similarity between pristine sampling points, and the similarity between WWTPs was intermediate (Table 1). For both AOA and AOB, resistance was not related to community diversity (Supporting Information Fig. S5).

Discussion

We conducted a large-scale field campaign to examine the significance of wastewater as a disturbance for the sediment microbes, and to answer how ammonia-oxidizing bacterial and archaeal communities would respond to this disturbance. In general, wastewater-influenced communities were less diverse and less resistant, but the effect of wastewater was spatially limited. The AOA communities were resistant, but their abundance was generally low at the wastewater-influenced sediments. In contrast, AOB communities adapted fast to wastewater disturbance, increasing their abundance as compared with the pristine sediments.

Wastewater as a disturbance for sediment microbes

Our first objective was to examine the role of wastewater as a disturbance for sediment microbes. The differences

in the community composition between wastewater discharge site sample and pristine sediments showed a gradual decrease from Petäjälvesi to Hankasalmi. We used this overall microbial community response as an estimate of the biologically relevant influence of wastewater in further analysis. Agreeing with our hypothesis, the wastewater-influenced communities had lower diversity and richness than pristine sediment communities. This was especially pronounced in the three most wastewater-influenced lakes, Petäjälvesi, Saarijärvi and Lievestuore. Similar to general nitrogen additions (e.g., Kearns *et al.*, 2016), wastewater favoured the abundance of only certain specialized taxa, for example, *Bacteroidetes*, *Firmicutes*, *Beta*-, *Epsilon*- and/or *Gammaproteobacteria*. These groups are known to migrate from WWTPs to lake sediments (Saarenheimo *et al.*, 2017). In the three most influenced sites, we saw a significant increase in the abundance of these WWTP-originating groups, which being adapted to wastewater environment, could successfully colonize the receiving sediments and outcompete natural taxa. Furthermore, although high wastewater loading was discharged from some WWTPs (e.g., Viitasaari), it was rapidly diluted to the lake water, and less WWTP-origin microbes could establish than in the three most wastewater-influenced lake sites. This indicates that with the current water quality sampling, the functional effect of wastewater cannot be reliably estimated. Altogether, agreeing with Saarenheimo *et al.*, (2017), we demonstrated that wastewater acts as both physico-chemical and biological disturbance and that although wastewater effluents can introduce new members to the sediment microbial communities, they cannot promote diversity due to significant changes in habitat characteristics.

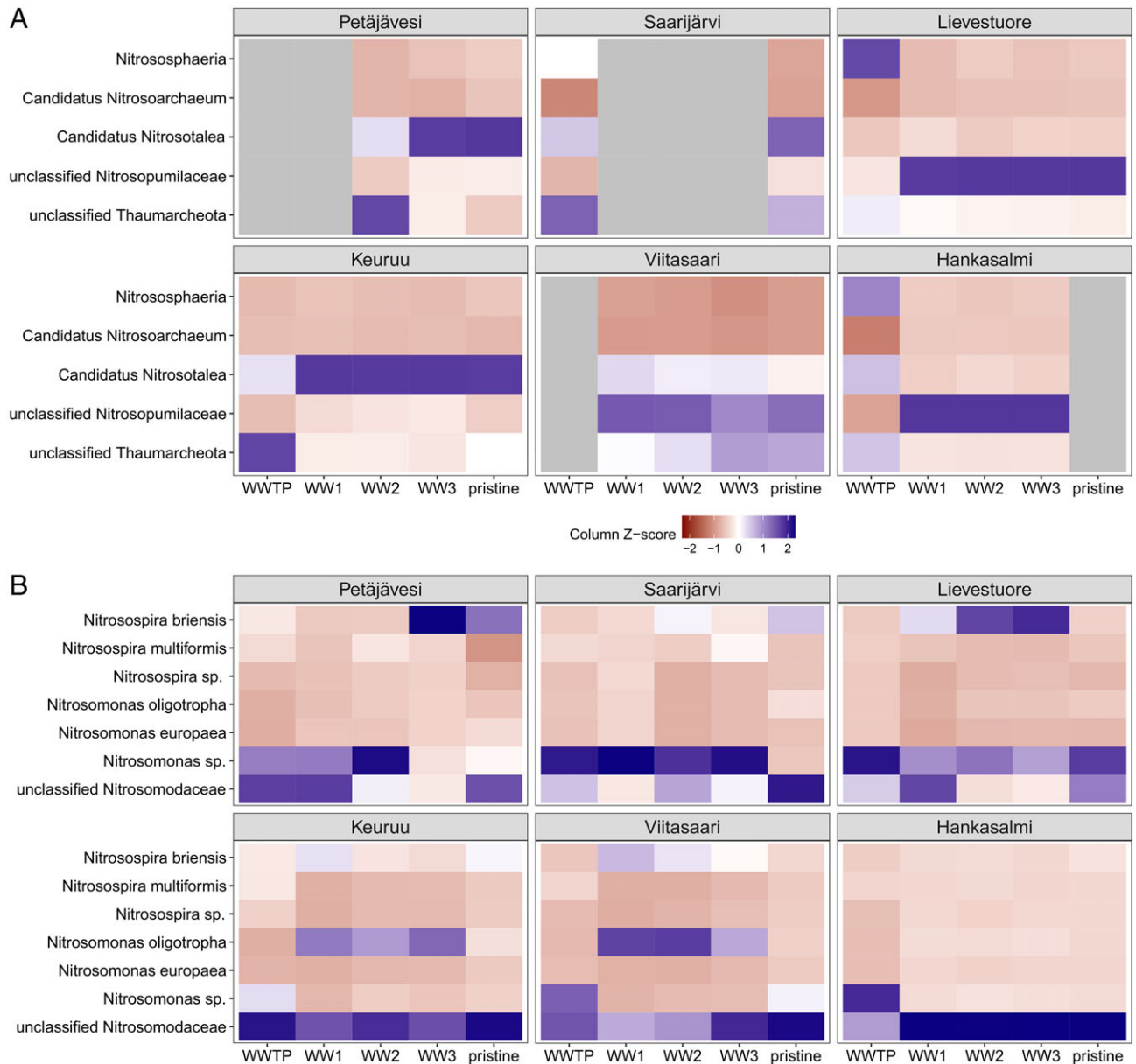


Fig. 7. Relative abundances of A. AOA and B. AOB taxonomic groups presented as z-scores (standardized values over samples). Red colour indicates a lower abundance, and blue a higher abundance of certain taxonomic group as compared with other taxonomic groups in one sample. Grey indicates that no AOA/AOB sequences were retrieved from the sample. [Color figure can be viewed at wileyonlinelibrary.com]

Although significant changes in the microbial communities were seen at the most wastewater-influenced sampling points, in all lakes except Saarijärvi, similarity between pristine communities and the communities at the down-most wastewater-influenced sampling point (WW3) approached the similarity within pristine communities already within our narrow sampling range. This was especially pronounced in Petäjavesi, where the resistance was the lowest (3% similarity), but the similarity between WW3 and pristine communities was only 10% lower than within pristine communities. These results suggest that although the future higher catchment

loadings can substantially modify sediment microbiology, their effect is expected to be spatially limited.

Response of AOA and AOB communities to wastewater

Recently, AOA were found to dominate pristine boreal lake sediments, while AOB were more abundant in the wastewater-influenced sediments (Saarenheimo *et al.*, 2017). Here, we saw no constant pattern between AOA and AOB abundance in pristine sediments, but we found wastewater significantly decreasing sediment AOA abundance, and AOA:AOB increasing when moving

downstream from the wastewater discharge site. In Viitasaari, AOA was found to dominate even at wastewater-influenced samples. One explanation for this could be that there, the highly abundant *amoA*-archaea were not true AOA, as WWTPs are known to host *amoA*-carrying non-nitrifying Thaumarchaeota (Mußmann *et al.*, 2011). Furthermore, the overall prokaryotic community analysis indicated wastewater influence being less significant in Viitasaari, so it is possible that the habitat conditions remained nutrient-poor enough to support high AOA abundance. However, the high AOA abundance in Viitasaari could also be explained with the presence of AOA groups being adapted to nutrient-rich conditions, as it was recently found in eutrophic Yellow River estuary sediments (Li *et al.*, 2018).

Previous studies have shown that the high ratio between AOA and AOB (AOA:AOB) could be an indicator of oligotrophic conditions in aquatic environments (Sims *et al.*, 2012), as AOA tolerate nutrient-poor conditions better than AOB. By combining the data from pristine and wastewater-influenced sediments, we demonstrated that the total abundance of AOA was suppressed in oxygen-depleted conditions, where concentration of ammonium was high, both being typical characteristics of wastewater-influenced sampling points. However, AOA:AOB was also connected to sediment characteristics, such as $\delta^{13}\text{C}$, which tended to be higher at wastewater-influenced sites. We also found a significant interaction between AOA:AOB and $\delta^{13}\text{C}$. The $\delta^{13}\text{C}$ values seem to be lake specific, and although all study lakes were humic, there were some differences in pH, which is known to control interactions between AOA and AOB (Gubry-Rangin *et al.*, 2011), suggesting that AOA:AOB was also partly driven by lake characteristics, for example, trophic state (Bollmann *et al.* 2014). Altogether, our results suggest that the factors governing the interactions between AOA and AOB are more diverse than previously anticipated.

Unlike we expected, we saw no significant decreases in AOA diversity or richness under wastewater influence. This agrees with the previous results from soil AOA communities under N fertilization (Daebeler *et al.*, 2017), but not the ones from physically disturbed estuarine sediments (Newell *et al.*, 2014), suggesting the wastewater was mainly changing nutrient environment and not significantly increasing turbulence in the sediment surface. Each lake hosted a unique AOA community and there was a clear niche specialization between AOA groups, as has been previously demonstrated in soils (Gubry-Rangin *et al.*, 2011). Different AOA taxa are known to be conservative toward environmental conditions, pH being the key factor (MacQueen and Gubry-Rangin 2016). In accordance with this, we found acidophilic *Candidatus Nitrosotalea devanaterrea* dominating the sediment

community in Petäjavesi, Saarijärvi and Keuruu with pH ranging between 6.1 and 6.7. Differences in the pH between WWTP and lake sediments could also explain why WWTP-AOA could not successfully colonize the lake communities. The resistance of AOA community probably explains why we saw significant decreases in their absolute abundance at the wastewater-influenced sampling points, as AOA communities could not adapt to changing habitat characteristics. In accordance, when comparing the wastewater discharge sites between lakes, AOA was found in high densities in Viitasaari, where the AOA community was most diverse and possibly also best adapted.

In opposite to AOA, wastewater-influenced AOB communities exhibited poor resistance, and remained distinct from the pristine ones when moving downstream from the wastewater discharge site. Interestingly, diversity and richness were highest at the wastewater discharge site in most lakes. In the most wastewater-influenced lakes, Petäjavesi and Saarijärvi, unclassified *Nitrosomonas* were highly abundant at both WWTPs and wastewater-influenced sampling points, suggesting that they have migrated from WWTP. In other lakes, there was no such connection between the composition of WWTP and sediment communities, so observed changes in the sediment AOB community composition were most likely due to the wastewater-driven habitat characteristics, promoting the abundance of *Nitrosomonas* groups, for example, *Nitrosomonas oligotropha*. Also previously, this group has been found to dominate in habitats affected by WWTP effluents (Mußmann *et al.*, 2013; references therein). When unclassified Nitrosomonadaceae was excluded, the abundance of *Nitrosomonas* was higher than that of *Nitrosospira* in wastewater-influenced sediments. These two AOB clades are physiologically distinct, *Nitrosospira*-like AOB being more abundant in low ammonium conditions, while *Nitrosomonas*-like AOB have been suggested to be adapted to grow in ammonium-rich environments, for example, in eutrophic sediments (Peng *et al.*, 2013; Reis *et al.*, 2015). Altogether, these results indicate that although wastewater can promote the abundance of certain AOB groups (*Nitrosomonas*) through habitat modification and migration from WWTP, other groups can still coexist. The higher diversity and the rapid response and capability to adapt to changes in habitat characteristics probably explains why we saw high AOB abundance in almost all wastewater-influenced sediments. However, even AOB could not grow under extreme wastewater influence in Saarijärvi and Lievestuore. As AOB abundance and nitrification rates are commonly tightly linked in ammonium-rich environments (e.g., Di *et al.*, 2009; Hampel *et al.*, 2018), the high abundance of AOB suggests also high nitrification activity at wastewater-influenced sediments.

Conclusions

In future, higher catchment loading is expected to shape microbial habitat characteristics by bringing high nutrient and organic matter concentrations to boreal lake ecosystems. Here, we simulated these forthcoming changes by comparing the microbiology of wastewater-influenced and pristine lake sediments. We demonstrated sediment microbial communities being generally poorly resistant, but exhibiting capability to approach the natural community composition with the diminishing impact of wastewater, suggesting that the effect of catchment loadings on the sediment microbiology is strong but spatially restricted. Furthermore, wastewater, as well as future increases in the catchment loadings, was seen to modify the interactions between AOA and AOB. AOA communities were lake-specific and resistant, and were thus strongly suppressed by wastewater, while AOB communities were dynamic, changing and diversifying and increasing in numbers. These results indicate that higher future catchment loading may promote more active nitrification through highly abundant AOB communities.

Experimental procedures

Sample and data collection

Sediment samples and environmental data were collected from six lakes located in Central Finland between 20 and 24 Feb 2017 (Fig. 1, Table 2, Supporting Information Table S1). All lakes receive purified wastewater from WWTPs (see Supporting Information Table S1 for more details) and were ice-covered during the sampling. Samples were taken from three wastewater-influenced sampling points (wastewater discharge point; WW1, and two points located 100 and 200 m downstream from the wastewater discharge point; WW2 and WW3). In Saarijärvi, the wastewater was discharged to a small pond Saarilampi, and samples taken from there were replicates rather than following any gradient. In addition to wastewater-influenced sediments, three replicate samples were taken from a pristine sampling point located upstream from the wastewater discharge point. Oxygen, temperature, conductivity and pH profiles were measured *in situ* using a portable field meter (YSI 6600V2-4 Multi-Parameter Water Quality Sonde, Yellow Springs Instruments, OH 45387, USA). For DNA-analysis and sediment characteristics, three replicate samples of the uppermost sediment (0–2 cm) were collected from each sampling point using Sandman sediment sampler, and stored at -20°C . Nitrate + nitrite (NO_x^-) and ammonium (NH_4^+) from water above the sediment surface (collected with Limnos sampler) were determined as in Rissanen and colleagues (2011). Wastewater discharge samples

(100 ml of effluent water) were collected from all WWTPs.

Microbial community composition

The sediment samples, as well as water samples from WWTPs, were freeze-dried (Alpha 1–4 LD plus, Christ) and DNA was extracted from 0.2 g of dried sediment using a PowerLyzer PowerSoil DNA extraction kit (MoBio Laboratories, Inc.). DNA was quantified with Quant-IT PicoGreen[®] dsDNA Assay Kit (Invitrogen). Changes in the community composition, richness, and diversity of organisms harbouring the 16S rRNA gene or archaeal or bacterial *amoA* genes were studied with next generation sequencing. V4 region of the prokaryotic 16S rRNA gene was PCR amplified with universal prokaryotic primers 515FB (GTGYCAGCMGCCGCGGTAA; Parada *et al.*, 2016) and 806R (GGACTACHVGGGTWTCTAAT; Caporaso *et al.*, 2011). PCR reaction of 25 μl consisted of Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Fisher Scientific), 5 ng of template DNA, 0.4 μM of each primer (Sigma Aldrich), and 0.02% Bovine Serum Albumin (Thermo Fisher Scientific). Thermal cycling consisted of 10 min initial denaturation at 95°C , followed by 24 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 60 s, followed by final elongation at 72°C for 10 min, was conducted on Bio-Rad CFX96 Real-Time System (Bio-Rad Laboratories). About 2 μl of the product was used as a template in a second PCR where Ion Torrent PGM sequencing adapters and barcodes were added to the ends using linker and fusion primers (0.04 μM of M13_515FB, 0.4 μM of IonA_IonXpressBarcode_M13 and P1_806R) in eight additional cycles with conditions otherwise identical to the first amplification (Mäki *et al.*, 2016). Products were purified with Agencourt AMPure XP purification system (Beckman Coulter Life Sciences, Indianapolis, IN, USA), quantified as earlier described, and pooled in equimolar quantities for sequencing on Ion Torrent PGM using Ion PGM Hi-Q View OT2 Kit for emulsion PCR, PGM Hi-Q View Sequencing Kit for the sequencing reaction and Ion 316 Chip v2 (all Life Sciences, Thermo Fisher Scientific).

To build archaeal and bacterial *amoA* PCR amplicon libraries, two PCR reactions were performed. In the first PCR, primer pairs ArchAmoAF/ArchAmoAR (Francis *et al.*, 2005) for archaeal *amoA*, and *amoA*-1F/*amoA*-2R (Rothauwe *et al.*, 1997) for bacterial *amoA* were used, with the M13 sequence (5'-TGTA AACGACG GCCAGT-3') linker attached to the 5' end of each forward primer (Mäki *et al.*, 2016). The first reaction mixture contained ~ 10 ng of DNA template, 0.5 μM of each primer for the selected target gene and 1 \times Maxima SYBR Green qPCR Master Mix to yield a total volume of 25 μl . The PCR procedure for archaeal *amoA* included an initial denaturation

Table 2. Brief description of the six study lakes and wastewater treatments plants (WWTP).

Sampling site	Lake name	Max depth	Mean depth	Lake area	WWTP	Mean discharge
		(m)	(m)	(km ²)	PE	m ³ /d
Keuruu	Keuruselkä	41	4.9	118.2	8200	2840
Petäjavesi	Jämsänvesi	27	4.2	8.8	1800	865
Hankasalmi	Kuuhankavesi	24	5.4	18.4	3100	760
Lievestuore	Lievestuoreenjärvi	70	9.9	40.2	2100	532
Saarijärvi	Saarijärvi	25	5.0	14.2	6700	2070
Viitasaari	Keitele	66	6.9	498.4	2800	1840

PE denotes for the population equivalent of the WWTP.

step at 95°C for 10 min and 40 cycles of amplification (95°C for 30 s, 53°C for 30 s, and 72°C for 40 s). The thermal cycling conditions for bacterial *amoA* were the same as for archaea, except that the annealing temperature was 56°C. In the second PCR, 2 µl of the first round PCR product was used as a template in a 25 µl PCR mixture, and Ion Torrent PGM forward sequencing adapter and barcodes were added to the 5' end of the product using IonA_IonXpressBarcode_M13 as a forward primer (see above) in 15 additional cycles with conditions otherwise identical to the first amplification. The PCR products were purified, DNA concentration measured, and the samples pooled in equal DNA amounts as described for 16S rRNA genes. In order to adjust the length of the archaeal and bacterial gene amplicons (~ 600 bp) for the Ion Torrent™ sequencing (recommended amplicon length ~ 400 bp), the sample pool was sheared, ligated to P1 adapter, size-selected and re-amplified as a single reaction as described in the study by Mäki and colleagues (2016). The sequencing protocol downstream followed that which was described for the 16S rRNA gene sequencing.

Analysis of gene sequences was done using Mothur (version 1.39.5; Schloss *et al.*, 2009). Sequences shorter than 150 bp, low-quality sequences with more than one mismatch in barcode/primer sequences, or with homopolymers longer than eight nucleotides, as well as barcodes, and primers were removed. Framebot (at FunGene website, <http://fungene.cme.msu.edu/FunGenePipeline>) (Fish *et al.*, 2013; Wang *et al.*, 2013) was used to correct frameshift errors in archaeal and bacterial *amoA* reads. Prokaryotic 16S rRNA gene sequences were aligned using Silva reference alignment (Release 128), while the alignment of the archaeal and bacterial *amoA* sequences was conducted using sets of aligned archaeal or bacterial *amoA* sequences retrieved from the FunGene website (http://fungene.cme.msu.edu/hmm_detail.spr?hmm_id=16). Chimeric sequences, denoted using Mothur's implementation of Uchime (Edgar *et al.*, 2011), were removed from each library. Sequences were divided into operational taxonomic units (OTUs) at 97% similarity levels for 16S rRNA gene and at 95% for archaeal and

bacterial *amoA*, and singleton OTUs (OTUs with only one sequence in the entire dataset) were removed.

16S rRNA gene sequences were assigned taxonomies with a naïve Bayesian classifier (bootstrap value cut off = 75%) (Wang *et al.*, 2007) using Silva database (Release 128) and sequences classified as chloroplast, mitochondria and eukaryota were removed. The taxonomic assignment of archaeal and bacterial *amoA* sequences was done similarly, but using in-house reference databases, which were compiled from reference *amoA* (either bacterial or archaeal) sequences and their taxonomies in the Nucleotide database (<https://www.ncbi.nlm.nih.gov/nucleotide/>). Finally, the data was normalized by subsampling to 11 099 sequences for 16S rRNA gene, 260 for archaeal *amoA*, and 356 for bacterial *amoA*. Sequence variation was adequately covered in these libraries as shown by Good's coverage, an estimate of the proportion of amplified gene amplicons represented by sequence libraries for each sample, that varied 0.82–0.95 for 16S rRNA gene, 0.95–1 for archaeal *amoA* and 0.95–0.99 for bacterial *amoA*. Sequences have been submitted to the NCBI's Sequence Read Archive under accession number SRP141667.

qPCR of archaeal and bacterial *amoA* genes

The abundance of archaeal and bacterial *amoA* genes was assessed using the Maxima SYBR Green/Fluorescein Master Mix for qPCR as in Saarenheimo *et al.*, (2017), except that we used DNA template amounts of 10 and 15 ng, and 55°C annealing temperature for archaeal *amoA*. Amplification efficiencies were 80% for AOA and 91% for AOB.

Statistical analysis

All statistical analyses were conducted using R version 3.4.3 (R Core Team, 2017). Non-metric multidimensional scaling (NMDS, conducted with metaMDS function in vegan package; Oksanen *et al.*, 2017) plots calculated based on Bray–Curtis distance matrix were used to visualize dynamics in the community structure of prokaryotes

in general, and of AOA and AOB (OTUs represented by at least two reads). Before NMDS, Wisconsin and square-root-transformations were applied to OTU abundance data. Differences in the prokaryotic/AOA/AOB community structure between lakes and sampling points were tested separately with permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001; McArdle and Anderson, 2001, function 'adonis' in vegan). Spearman Rank Order correlation was used to study interactions between environmental variables, AOA and AOB abundance, and AOA:AOB-abundance ratio. The resistance of the microbial community was defined as an average Bray–Curtis similarity between pristine sampling point and wastewater discharge sampling point (WW1) (Shade *et al.*, 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Relative abundances of taxonomic groups in the discharge of the six WWTPs studied.

Fig. S2. The relationship between resistance and Shannon diversity of the prokaryotic community at the wastewater-disturbed sampling points.

Fig. S3. The interactions between environmental variables, copy numbers of AOA and AOB and AOA:AOB. Red denotes for negative correlation and blue for positive correlation. Only statistically significant ($P < 0.05$) correlations are shown.

Fig. S4. Shannon diversity for A) AOA and B) AOB, and chao species richness index for C) AOA and D) AOB communities in six study lakes.

Fig. S5. The relationship between resistance and Shannon diversity of A) AOA and B) AOB community at the wastewater-disturbed sampling points.

Table S1. A detailed description on six study lakes. WW1: wastewater discharge sampling point, WW2: downstream wastewater-disturbed sampling point, WW3: the downmost wastewater-disturbed sampling point.