Supporting Information for

Growth of *Nitrosococcus*-Related Ammonia Oxidizing Bacteria Coincides with Extremely low pH Values in Wastewater with High Ammonia Content

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SUPPLEMENTARY MATERIALS & METHODS

pH measurement. The pH was monitored on-line with pH sensors (Sentix 41, WTW, Weilheim, Germany and 405-DXX-S8/225, Mettler-Toledo, Greifensee, Switzerland) connected to amplifiers (pH-meter 605, Metrohm, Herisau, Switzerland and pH 340, WTW, Weilheim, Germany). The pH sensors were calibrated with pH standard solutions 7 and 4 on a bi-weekly basis. The on-line pH measurements were recorded with one minute intervals using a data logger (Memograph S, RSG40, Endress + Hauser, Reinach, Switzerland).

Characteristics of the inoculum. AOB from the inoculum were adapted to total ammonia concentrations of around 900 mgNH_4-N·L^{-1}, a pH of around 6 and total salt concentrations of around 250 mmol·L^{-1}. Additionally, 20 mL of activated sludge from the Eawag pilot-scale wastewater treatment plant operated with municipal wastewater were added to each reactor in order to diversify the initial AOB populations in the reactors. The amount of biomass added from the municipal wastewater treatment plant was very small compared to the amount of biomass added with the carriers from the urine reactor (< 10%).

Trace elements. A volume of 2 mL of the trace element solution (Table S2) was added to each liter of influent solution for the urine reactors, and 1 mL per liter of influent solution for the wastewater reactors. To compensate for water losses due to evaporation, 180 and 30 mL of deionized water were added to each liter of urine and wastewater influent.

Liquid phase sampling and analysis. Concentrations of total inorganic carbon (TIC) and total ammonia were measured biweekly in the influent. Total ammonia, total nitrite, and nitrate in the reactor were measured weekly, total and soluble chemical oxygen demand (COD) biweekly. The concentrations of phosphate (PO_4-P), chloride (Cl), sodium (Na) and potassium (K) in the influent and the reactor were measured in at least 8 samples during the first 90 days of reactor operation. Liquid phase samples were filtered (0.45 µm, MN GF-5, Macherey–Nagel, Düren, Germany) prior to analysis. Samples for total COD were homogenized (DIAX 600, Heidolph Instruments, Schwabach, Germany) prior to analysis.

The cations sodium and potassium and the anions nitrate, chloride, and phosphate were analyzed with ion chromatography (IC 881 Compact IC pro, Metrohm, Herisau, Switzerland). Total ammonia (NH_4^+ and NH_3) and total nitrite (NO_2^- and HNO_2) were either measured with IC or photometrically with cuvette tests (LCK 303, LCK 341, LCK 342, Hach-Lange, Berlin, Germany). Soluble and total COD were quantified photometrically with cuvette tests (LCK 614). TIC was measured using a total inorganic/total organic carbon analyzer (TOC-L, Shimadzu, Kyoto, Japan) according to manufacturers' protocol.[1] Nitrification rates were calculated based on the input and output load of total ammonia. The output flow rate was corrected for a constant humidity loss through evaporation of 7.5 mL·d^{-1} caused by aeration of the reactors.

Batch experiments for NOB activity. The activity of NOB in urine reactor UR-Na and in wastewater reactor WWR-Na was checked on days 171 and 172, respectively, which was clearly after the pH shift in all reactors. For this purpose, 100 mL of Kaldnes® biofilm carriers were removed from UR-Na and WWR-Na, respectively, and were placed in a batch reactor with a volume of 250 mL. The temperature in the reactor was controlled with a water jacket (Colora Messtechnik GmbH, Lorch, Germany) at 24.3 ± 0.4°C and pH was monitored. The solution was stirred at 350 rpm using magnetic stirrers (RCT classic, IKA®-Werke GmbH & Co. KG, Staufen, Germany). The solution in the batch reactor contained KH_2PO_4 at 0.8 g·L^{-1} and $NaNO_2$ at 0.05 g·L^{-1}. To prevent chemical degradation of HNO_2 at low pH values,[2] the pH of the solution was adjusted to 6 by adding NaOH once in the beginning of the experiment. The

pH was not controlled actively, but remained stable during the experiment (pH 6.0 ± 0.03 with UR-Na, pH 6.1 ± 0.03 with WWR-Na). The temporal trends in total nitrite and nitrate concentrations were analyzed by taking aliquots in an hourly interval. The experiments lasted 10 hours (WWR-Na) and 24 hours (UR-Na).

Biomass sampling and conditioning. The biomass of all four reactors was sampled every week. For each sampling point, four biofilm carriers were removed and replaced by new, unused carriers. The total number of removed biofilm carriers of around 90 was small compared to the estimated 900 carriers in the reactor. The carriers were cut in pieces using a sterile scalpel in preparation for direct use in DNA extraction kits and then stored at -20°C prior to molecular analysis.

Extraction of genomic DNA. Genomic DNA was extracted from two biofilm carriers from days -6, 7, 20, 27, 34, 41, 48, 55, 61, 69, 77, 90, 111, 131, and 154 using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA), with adaptations to manufacturer's protocol. In short, the bead-beating step was performed under conditions close to the MIDAS field guide[3] in series of 4×20 s at 6 m s^{-1} separated by 2 min on ice. The purified DNA extracts were eluted in a final volume of 60 µL of nuclease-free water provided with the kit. The quality and rough concentration of the DNA extracts were assessed using a NanoDrop-1000™ UV/VIS spectrophotometer (Thermo Fischer Scientific Inc., Wilmington, DE, USA). DNA extracts were characterized by a median concentration of 18 ng·uL^{-1} and an absorbance ratio (260 to 280 nm) of 1.8. DNA concentrations per carrier (gDNA·$carrier^{-1}$) were calculated from the measured DNA concentrations and by assuming a liquid volume of 60 µL per carrier.

***In silico* analysis and sequencing with primers targeting archaea.** *In silico* testing of the primers set 341F (5'-CCTACGGGNGGCWGCAG-3') / 785R (5'-GACTACHVGGGTATCTAATCC-3') was conducted against the SILVA database of 16S rRNA gene reference sequences[4] by following Klindworth, et al. [5] with one mismatch allowed, and indicated that this primer pair can provide a theoretical coverage of 94% of the more than 400'000 reference sequences related to the kingdom of bacteria as well as of 95 to 100% of the reference sequences related to known AOB and NOB.[6]

The samples from day -6 and 61 were analyzed with the primer pair 340wF (5'-CCCTAYGGGGYGCASCAG-3') / 958R (5'-YCCGGCGTTGAMTCCAATT-3') (v3-v6) for archaea diversity, as well as 926F (5'-AAACTYAAAKGAATTGRCGG-3') / 1392R (5'-ACGGGCGGTGTGTRC-3') (v6-v8) for bacterial and archaea diversity.

PCR for AOA. PCR for the detection of the *amoA* gene of ammonium oxidizing archaea was carried out using the primer-set (Arch-amoAF: STAATGGTCTGGCTTAGACG and Arch-amoAR: GCGGCCATCCATCTGTATGT) and PCR method of Francis, et al. [7]: PCR cycling conditions were as reported previously, except using 40 cycles and the Promega GoTaq G2 Flexi DNA Polymerase and buffer with 2 mM $MgCl_2$ (Promega, Madison, WI, USA). Reaction products were visualized by gel electrophoresis on 1.4% agarose gels stained with EtBr. Positive controls consisting of a plasmid containing a cloned *amoA* PCR amplicon derived from activated sludge and previously sequenced to verify identity[8] were run alongside samples, and samples from reactor 1 in 10 to 1000-fold dilutions were spiked with positive control DNA to test for inhibition problems.

qPCR for archaea and *Nitrosococcus* OTU 1. All qPCR reactions were performed on a LightCycler 480-II (Roche, Rotkreuz, Switzerland) and analyzed using the LightCycler 480 ver. 1.5.1 software (Roche, Rotkreuz, Switzerland). Total reaction volumes were 10 µl with a sample volume of 2 µl. DNA extracts with DNA concentrations ranging from 9 to 166 ng $µl^{-1}$

were diluted 1000 times (bacteria) or 100 times (archaea, *Nitrosococcus* OTU assay) and repeated at 100- or 10-fold dilutions if results were below or close to the limit of detection. 10- to 1000-fold dilutions of selected samples were also run in parallel to test for inhibitory effects.

Real-time PCR protocols for the quantification of bacterial 16S rRNA genes were carried out using the primer and probe set (Bact349F/Bact806R, Probe Bac516F) of Takai and Horikoshi [9]. Reaction conditions were adapted as follows: For bacterial 16S: 95°C 10 min initial denaturation and 45 cycles of: 95°C for 40 sec, 53°C for 40 sec, and 72°C for 1 min, using LightCycler 480 Probes Master hot start reaction mix (Roche, Rotkreuz, Switzerland) and each primer at a concentration of 0.9 $\mu mol \cdot L^{-1}$ and the probe at 0.3 $\mu mol \cdot L^{-1}$. Archaeal 16S rRNA genes were quantified using the primer and probe set (Arch349fF and Arch806R, Probe Arch516F) of Takai and Horikoshi [9]. Reactions were set up using the LightCycler 480 Probes Master hot start reaction mix (Roche, Rotkreuz, Switzerland) with each primer at a concentration of 1 $\mu mol \cdot L^{-1}$ and the probe at 0.15 $\mu mol \cdot L^{-1}$ and the following cycling conditions: 95°C for 10 min initial denaturation and 45 cycles of 95°C for 20sec, 60°C for 2400 sec. Results were evaluated using the point-fit method for absolute quantification.

To confirm the dynamics of a *Nitrosococcus*-related OTU 1 obtained from sequencing, a TaqMan qPCR assay was developed. Target and reference sequences were assembled from the reference sequence obtained by our own Illumina sequencing and closely related reference sequences obtained from public databases and aligned. A primer and TaqMan probe set specific for the target OTU sequence and discriminating against all other sequences was designed using AlleleID (ver. 7.82; PREMIER Biosoft, Palo Alto, CA): Forward primer: Nc. acid. F-1: CGCTACCTACAGAAGAAG; reverse primer: Nc. acid. R-1: GGGATTTCACACCTAACTTA; Probe: Nc. acid. FAM-AAACCGCCTACATGCCCTTT-TAMRA. Reaction chemistry was the same as described for the bacterial 16S assay and cycling conditions were: initial denaturation of 95°C for 5 min and 45 cycles of 95°C for 10 sec, 60°C for 20 sec.

Thermodynamic calculations with PHREEQC. The computer program PHREEQC Interactive (Version 2.15.0)[10] was used to calculate the minimal pH values that can be reached, if the total ammonia in synthetic urine or synthetic wastewater is completely converted to nitrate. We used the database "wateq4f.dat" included in the PHREEQC package. For the initial solution, we used the measured concentrations in the reactors and the observed reactor pH values (Table S4). The chloride ion was used for charge balance. The proton release from the oxidation of ammonium to nitrate was simulated by the addition of chloride ions and a charge balance of pH. We assumed that two moles of protons are released per mole of ammonium that is oxidized to nitrate.

SUPPLEMENTARY RESULTS

Nitrogen mass balance

Table S3 shows the mass balance of the nitrogen compounds in both urine and wastewater reactors. High nitrogen emissions of 53 and 50% were observed in UR-K and UR-Na, respectively, while nitrogen emissions from the wastewater reactors were low. The nitrogen loss from the liquid phase is balanced by emissions of NO, NO_2, and N_2O in the off-gas. In addition, at low pH values, HNO_2 might be stripped from the reactors. HNO_2 was not analyzed in the off-gas, but maximal emissions were estimated to be 1.2 and 1.8 $mgN \cdot L^{-1} \cdot d^{-1}$ for UR-K and UR-Na assuming equilibrium between liquid and air, a Henry coefficient for HNO_2 of 0.00083 $mol(g) \cdot mol^{-1}(aq)$ [11] and average HNO_2 concentration in the liquid of 3.4 and 5.3 $mgN \cdot L^{-1}$. HNO_2 emissions are therefore far lower than the observed NO emissions.

Sequencing with primers targeting archaea

Very few sequences of AOA from affiliates of the family of *Nitrososphaeraceae* were found in UR-K at day -6, but none of the sequencing results revealed AOA at the later data point after 61 days.

Batch experiment for NOB

Figure S4 displays the total nitrite and nitrate concentrations in two batch experiments with biofilm carriers from urine reactor UR-Na and wastewater reactor WWR-Na, respectively. In the batch experiment with sludge from UR-Na the total nitrite as well as the nitrate concentrations remained constant, whereas with sludge from WWR-Na the total nitrite was converted to nitrate. Hence, NOB were active in the wastewater but not in the urine reactors. This results support the molecular analysis where NOB remained below the detection limit in the urine reactors, but were still present in the wastewater reactors.

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SUPPLEMENTARY TABLES

Table S1: Recipes for the synthetic influent solutions to the urine and wastewater reactors. Urine and wastewater reactors contained different total ammonia and total salts concentrations. The urine reactors (UR-K and UR-Na) as well as the wastewater reactors (WWR-K and WWR-Na) differed in their potassium and sodium concentrations.

		UR-K	UR-Na	WWR-K	WWR-Na
KCl	$g \cdot L^{-1}$	4.4	0	0	0
NaCl	$g \cdot L^{-1}$	0	3.6	0	0
NH_4Cl	$g \cdot L^{-1}$	0	0	0.6	0.6
NH_4OH (25% NH_3)	mL	5.5	5.5	0	0
NH_4HCO_3	$g \cdot L^{-1}$	6	6	0	0
$NaHCO_3$	$g \cdot L^{-1}$	0	0	0	1.7
$KHCO_3$	$g \cdot L^{-1}$	0	0	2	0
KH_2PO_4	$g \cdot L^{-1}$	0.8	0	0.05	0
$NaH_2PO_4 \cdot 2H_2O$	$g \cdot L^{-1}$	0	0.95	0	0.06

Table S2: Recipe for the trace element solution added to the synthetic influent solutions. A volume of 2 mL of the trace element solution was added to each liter of influent solution in the urine reactors, and 1 mL per liter of influent solution in the wastewater reactors.

$FeSO_4 \cdot 7H_2O$	$mg \cdot L^{-1}$	172
$ZnCl_2$	$mg \cdot L^{-1}$	20
$MnCl_2 \cdot 4H_2O$	$mg \cdot L^{-1}$	47
H_3BO_3	$mg \cdot L^{-1}$	6
$CuSO_4 \cdot 5H_2O$	$mg \cdot L^{-1}$	3
$Na_2MoO_4 \cdot 2H_2O$	$mg \cdot L^{-1}$	2
NaCl	$mg \cdot L^{-1}$	584
KCl	$mg \cdot L^{-1}$	746
$MgSO_4 \cdot 7H_2O$	$mg \cdot L^{-1}$	2465
$CaCl_2 \cdot 2H_2O$	$mg \cdot L^{-1}$	1470

Table S3: Nitrogen mass balances of the urine reactors (UR-K and UR-Na) and wastewater reactors (WWR-K and WWR-Na) on day 246. On this day, the gaseous emissions for NO, NO2 and N2O were measured). Mass balances for the liquid were calculated from the total ammonia input and the total nitrogen output (sum of total ammonia, nitrate and total nitrite). Nitrogen losses from the liquid phase are balanced by gaseous nitrogen emissions (sum of NO, NO_2, N_2O). HNO_2 emissions were not analyzed but estimated using Henry's Law to be below 1.2 and 1.8 $mgN \cdot L^{-1} \cdot d^{-1}$ for UR-K and UR-Na. Indicated precisions were calculated by error propagation of the standard deviations for liquid and gas phase measurements.

		UR-K	**UR-Na**	**WWR-K**	**WWR-Na**	
Liquid phase:						
Input	Total ammonia	17.4 ± 0.7	18.7 ± 0.7	8.4 ± 0.3	8.2 ± 0.3	$mgN \cdot L^{-1} \cdot d^{-1}$
Output	Total ammonia	3.6 ± 0.1	4.2 ± 0.2	0.9 ± 0.0	0.6 ± 0.0	$mgN \cdot L^{-1} \cdot d^{-1}$
Output	Nitrate	4.5 ± 0.2	5.0 ± 0.2	8.0 ± 0.3	7.2 ± 0.3	$mgN \cdot L^{-1} \cdot d^{-1}$
Output	Total nitrite	0.03 ± 0.00	0.04 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	$mgN \cdot L^{-1} \cdot d^{-1}$
Nitrogen mass balance (Input – Output)		**9.2 ± 0.7**	**9.4 ± 0.8**	**-0.5 ± 0.5**	**0.4 ± 0.4**	$mgN \cdot L^{-1} \cdot d^{-1}$
		53	50	-6	5	%
Gas phase:						
Output	NO	8.7 ± 1.3	7.1 ± 1.1	< 0.5	< 0.5	$mgN \cdot L^{-1} \cdot d^{-1}$
Output	NO_2	1.3 ± 0.3	1.6 ± 0.4	< 0.3	< 0.3	$mgN \cdot L^{-1} \cdot d^{-1}$
Output	N_2O	0.4 ± 0.1	0.2 ± 0.0	< 0.1	<0.1	$mgN \cdot L^{-1} \cdot d^{-1}$
Total nitrogen emissions		**10.4 ± 1.3**	**8.9 ± 1.1**	**< 0.9**	**< 0.9**	$mgN \cdot L^{-1} \cdot d^{-1}$

Table S4: Measured concentrations and standard deviations in all four reactors. The urine reactors (UR-K and UR-Na) contained higher total ammonia and total salt concentrations than the wastewater reactors (WWR-K and WWR-Na). UR-K and WWR-K contained high potassium, but low sodium concentrations, whereas UR-Na and WWR-Na contained high sodium, but low potassium concentrations.

		UR-K	UR-Na	WWR-K	WWR-Na
NH_4-N	$mg \cdot L^{-1}$	**875 ± 231**	**842 ± 176**	**15.6 ± 3.2**	**12.3 ± 3.1**
NO_3-N	$mg \cdot L^{-1}$	959 ± 148	916 ± 103	152 ± 8.2	150 ± 8.0
PO4-P	$mg \cdot L^{-1}$	192 ± 19.0	194 ± 12.6	12.1 ± 2.5	13.0 ± 3.2
Cl	$mg \cdot L^{-1}$	2490 ± 234	2250 ± 66.2	459 ± 66.4	459 ± 91.9
Na	$mg \cdot L^{-1}$	**13.2 ± 4.6**	1620 ± 86.5	**6.1 ± 1.4**	524 ±41.9
K	$mg \cdot L^{-1}$	2920 ± 457	**25.8 ±21.3**	887 ±70.1	**13.2 ± 4.4**
COD dissolved	$mg \cdot L^{-1}$	123 ± 39.1	103 ± 34.7	12.2 ± 4.1	11.8 ± 4.2
COD particulate	$mg \cdot L^{-1}$	201 ± 97.6	124 ± 40.5	26.9 ± 28.6	22.2 ± 9.1
Total salts	$mmol \cdot L^{-1}$	**283 ± 46.2**	**266 ± 26.5**	**48.3 ± 4.6**	**48.1 ± 5.4**

SUPPLEMENTARY FIGURES

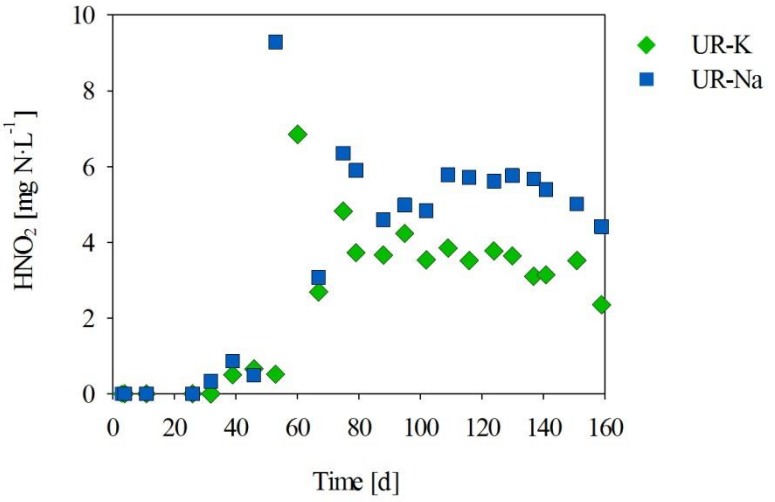

Figure S1: HNO_2 concentrations in both urine reactors (UR-K and UR-Na) calculated from nitrite and pH.

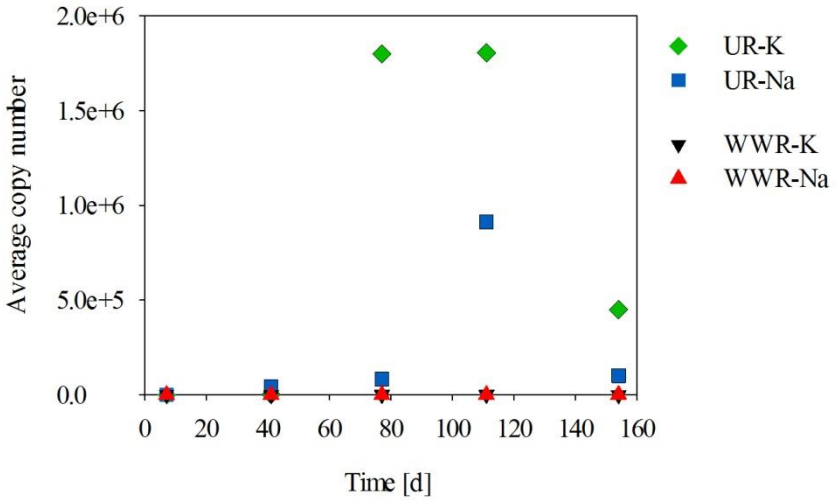

Figure S2: Average copy number of *Nitrosococcus* OTU 1 in the urine reactors (UR-K and UR-Na) and in the wastewater reactors. Copy numbers were below a value of 1000 in all samples of the wastewater reactors (WWR-K and WWR-Na).

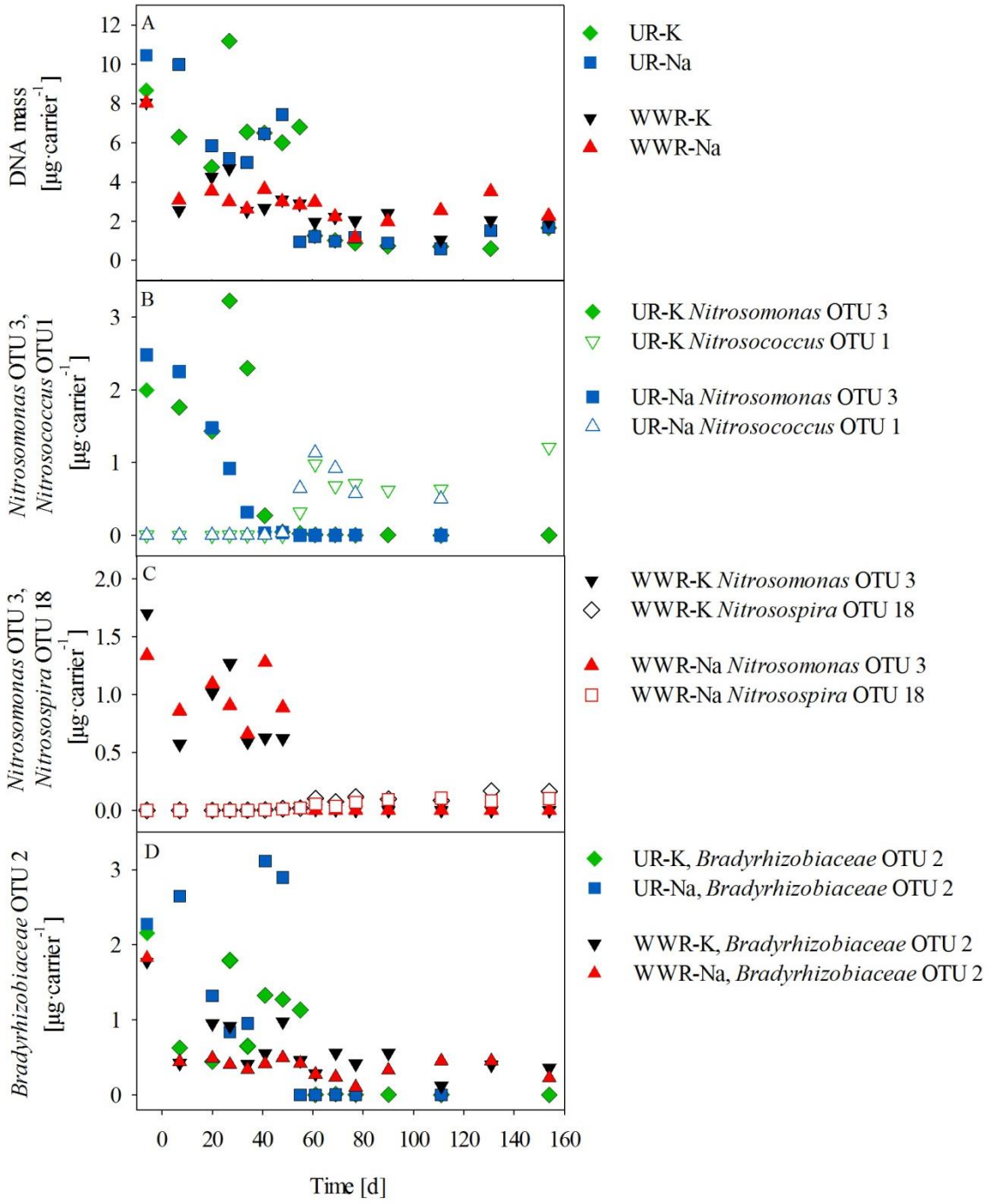

Figure S3: (A) Measured DNA concentrations in all reactors, (B) specific DNA concentrations of *Nitrosomonas* OTU 3 and *Nitrosococcus* OTU 1 in the urine reactors (UR-K and UR-Na), (C) specific DNA concentrations of *Nitrosomonas* OTU 3 and *Nitrosospira* OTU 18 in the wastewater reactors (WWR-K and WWR-Na), as well as (D) specific DNA concentrations of *Bradyrhizobiaceae* OTU 2 comprising the genus *Nitrobacter*.

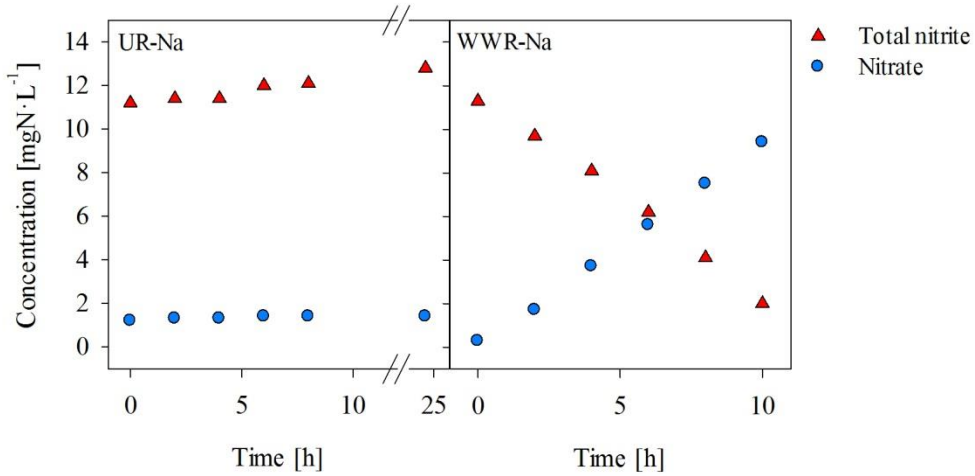

Figure S4: Concentrations of total nitrite and nitrate in two batch experiment performed with biofilm carriers from urine reactor UR-Na (left) and wastewater reactor WWR-Na (right), respectively.

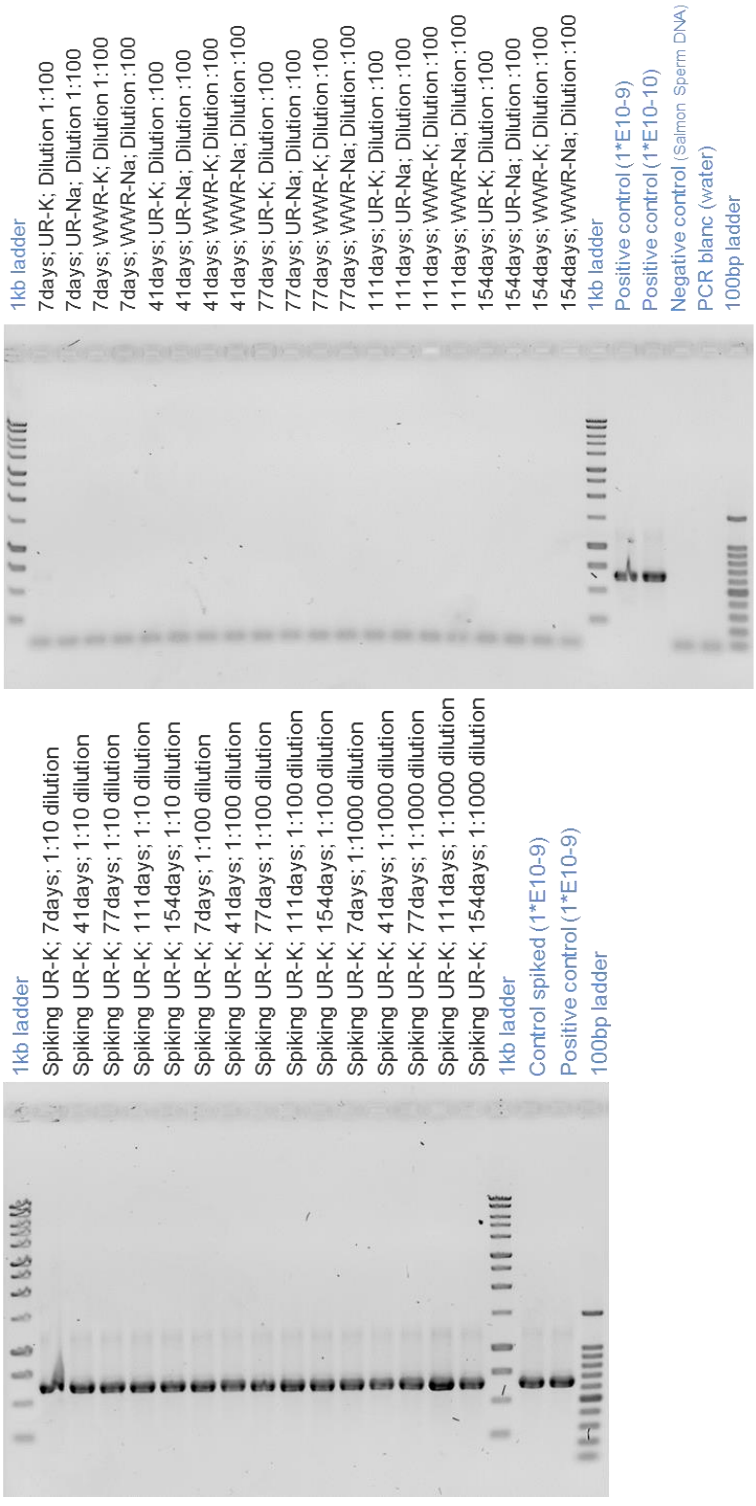

Figure S5: PCR for ammonia oxidizing archaea. 5 samples of each reactor and a positive control were tested. Furthermore samples of urine reactor UR-K at different time points and dilutions were spiked with the positive control. Positive results in the spiked samples show that negative results were not due to PCR inhibition problems.

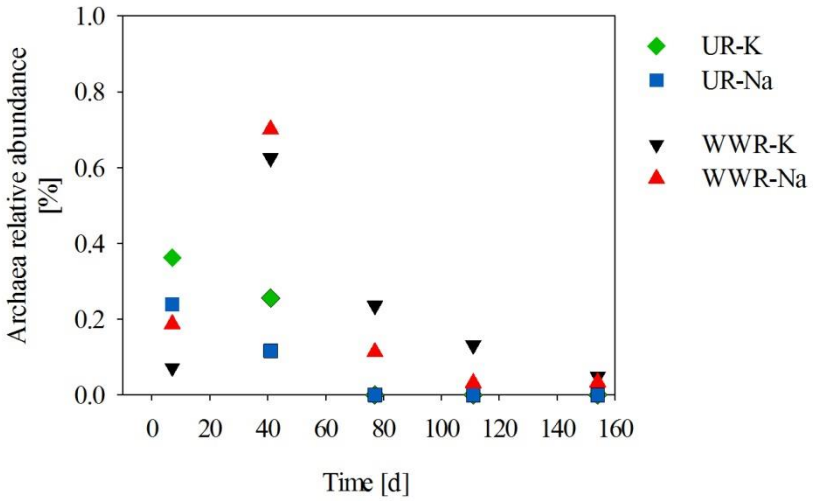

Figure S6: Relative abundance of archaea compared to the total sum of bacteria and archaea in the urine reactors (UR-K and UR-Na) as well as in the wastewater reactors (WWR-K and WWR-Na). Archaea were below the detection limit of 50 gene copies / reaction in the later samples of the urine reactors (days 77, 111 and 154).

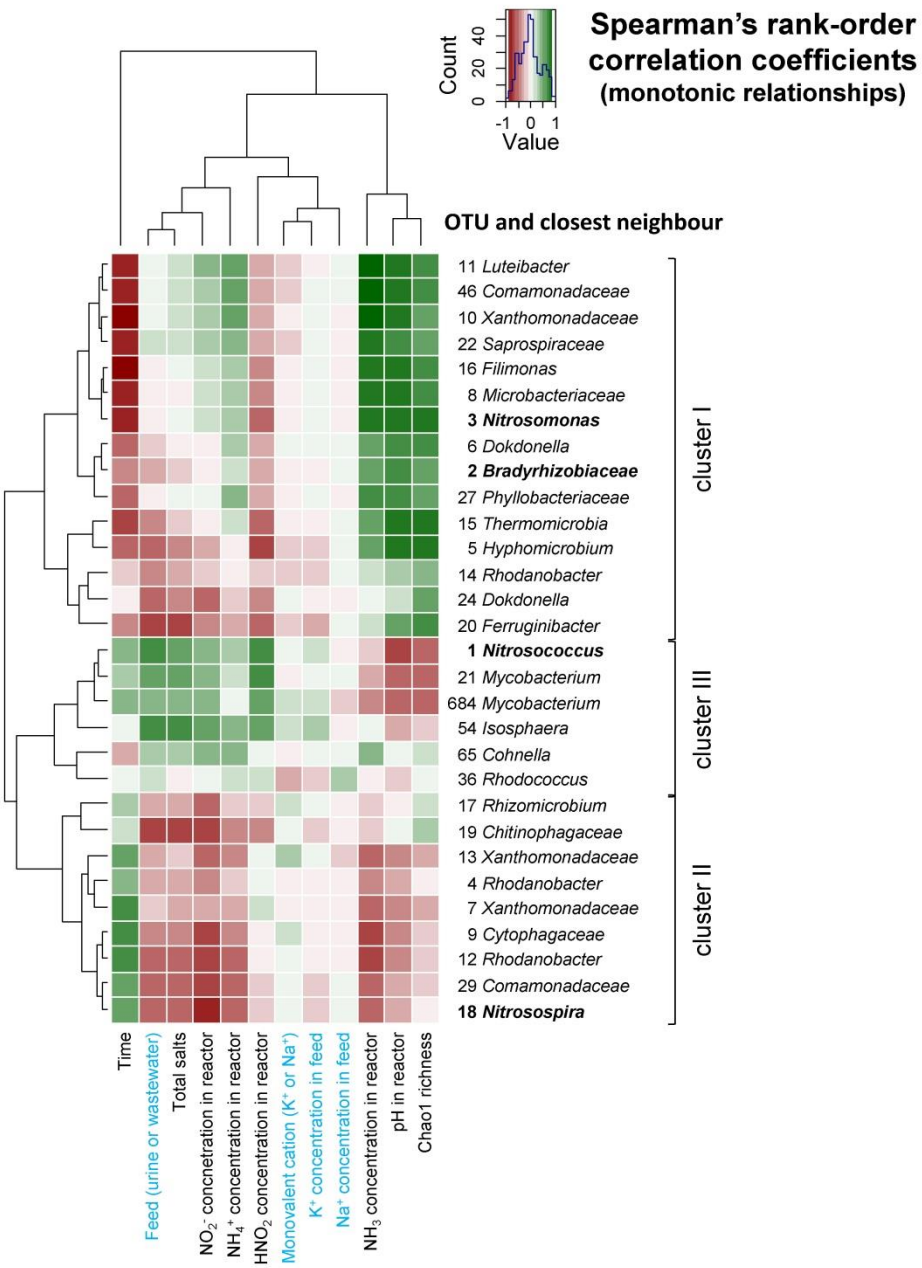

Figure S7: Heatmaps of Spearman's rank-order correlations computed to assess the monotonic relationships between predominant phylotypes (vertical axis) that displayed relative abundance above 5% and that shared similar dynamics in function of environmental conditions (horizontal axis) prevailing in the urine and wastewater reactors. The fixed operational conditions are provided in blue font, while the environmental variables that evolved over the experimental period are provided in black font. Three main clusters of phylotypes were identified in function of positive (green gradient) and inverse (red gradient) correlations. Cluster I is notably composed of *Nitrosomonas* OTU 3 which relative abundance positively correlated with the higher pH conditions that prevailed in the first experimental period. Cluster II notably comprises *Nitrosospira* OTU 3 that was mainly selected by the wastewater matrix under lower pH condition. Cluster III is mainly represented by *Nitrosococcus* OTU 1 and *Mycobacterium* OTUs 21 and 684 that dominated the bacterial community in the urine-based reactors at low pH. Only low correlations were obtained between the dynamics of OTUs and the type of monovalent cation.