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RESEARCH ARTICLE

The activity of nitrifying microorganisms in a high-altitude Andean wetland

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One sentence summary: Andean wetlands have functionally diverse nitrogen cycling microbes displaying different activities at sites with contrasting ammonium concentrations and salinities.

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ABSTRACT

High-altitude wetland holds freshwater springs, evaporitic ponds and lagoon with variable salinity and nutrients, potentially influencing the ecology of nitrifying communities. In this study, nitrifying microorganisms in Salar de Huasco (Chile) were surveyed to determine bacterial and archaeal contribution to ammonium (AO), nitrite oxidation (NO), ammonium uptake (AU) during wet and dry seasons. The activity signals from these groups were assessed by specific *amoA*-qPCR transcription, ¹⁵N tracer studies and addition of group specific inhibitor experiments for nitrifying microorganisms (N1-guanyl-1, 7-diaminoheptane [GC7]-archaeal specific and allylthiourea [ATU]-bacterial specific). Nitrifying communities, i.e. *Nitrosopumilus*, *Nitrosospira*, *Nitrosomonas*, *Kuenenia* and *Nitrospira*, were more frequent (~0.25% of 16S rRNA sequences) at low salinity sites. Bacterial *amoA*-qPCR transcripts also increased at low salinity and along in situ ammonium increase observed between wet/dry seasons. Nutrient changes through time and ¹⁵N tracer experiments results showed that AO and NO were detected and peaked mainly at low salinity-high ammonium sites (<37 000 μS cm⁻¹ and >0.3 μM), whereas AU was predominant at evaporitic sites. Our results indicate that salinity and ammonium affect the nitrifying communities that are potentially more active at low-salinity sites but persistent at saltier evaporitic areas of the wetland when ammonium is available.

Keywords: ammonia-oxidation; nitrite-oxidation; AOB; AOA; nitrifier diversity; salinity

INTRODUCTION

The high-altitude plateau (>3800 m above sea level) of the Southern American Andes Altiplano are characterized by athalasoaline (different from seawater) salt flats from former paleolakes and active wetlands, like Salar de Huasco (Risacher, Alonso and Salazar 2003; Risacher and Fritz 2009). High daily temperature fluctuations between -15°C and 20°C and irradiance up to 1000 W m⁻² are typical of this extreme ecosystem (Aceituno 1997). The wetland is comprised of diverse aquatic sites such as, springs, ponds and the main evaporitic lagoon, characterized by variable salt concentrations associated with differential proportions of chloride, sulfate, calcium, sodium, potassium and magnesium (Risacher, Alonso and Salazar 2003; de la Fuente and Niño 2010). The aquatic sites of the wetland from the spring towards the main lagoon and surrounding ponds have a salinity gradient from moderate towards saline, which is characterized by a range in conductivity of less than 650 to greater than 63 100 μS cm⁻¹ (Dorador et al. 2010). In addition to salinity, other chemical and physical variables including total nitrogen, temperature and pH have been reported to influence the macro and microorganism community structure of the Altiplano wetlands (Dorador, Pardo and Vila 2003; Dorador et al. 2008; Márquez-García et al. 2009; Demergasso et al. 2010).

Nitrification, the sequential oxidation of ammonia to nitrite and then to nitrate, is carried out by two functional guilds, archaeal and bacterial ammonia-oxidizers (AOA and AOB) and aerobic nitrite-oxidizing bacteria (NOB), or by complete ammonia oxidizers (comammox) by a single microorganism recently reported in the *Nitrospira* phylum (Daims et al. 2015; van Kessel et al. 2015). Nitrification has been shown to be influenced by salinity along with other variables such as light, low oxygen and substrate concentration, see reviews by e.g. (Oren 2001; Horz et al. 2004; Erguder et al. 2009; Hatzenpichler 2012). For example, AOB abundances have been shown to be negatively correlated with salinity in the Chesapeake Bay and Charente river (Bouskill et al. 2012; Hugoni et al. 2015). Whereas, in other estuaries (San Francisco Bay and subterranean estuary), AOB are more abundant than AOA at high-salinity sites, in ecosystems also influenced by variable C/N ratios and dissolved oxygen concentrations (Mosier and Francis 2008; Santoro et al. 2008). Moreover, species or ecotypes associated with lower or higher salinity areas have been detected in 16S rRNA genes and functional gene (*amoA*) surveys (Coci et al. 2005; Francis et al. 2005; Freitag, Chang and Prosser 2006; Caffrey et al. 2007; Sahan and Muyzer

2008). Furthermore, groups adapted to low and high salinity have been isolated, i.e. *Nitrosoarchaeum limnia* BG20 and *Nitrosopumilus salaria* BD31 (Blainey et al. 2011; Mosier et al. 2012; Mosier et al. 2012b).

In Salar de Huasco, 16S rRNA and *amoA* gene studies indicated the presence of nitrifying communities (Dorador et al. 2008; Dorador et al. 2010). Additional high-altitude ecosystems harboring nitrifiers include living stromatolites from Socompa lake at the Argentinian Altiplano (Farias et al. 2013) and in Tibetan lakes (Jiang et al. 2009; Hu et al. 2010). Nitrification rates at high-altitude aquatic ecosystems are scarce, recently, a report from different altitude lake ecosystems (2289–3160 m) indicate that nitrifying communities (AOB and AOA) are active at low rates but only AOA presented a negative influence of lake elevation (Hayden and Beman 2014).

Our study examines the hypotheses that salinity and ammonium were the main environmental factor shaping the potentially active nitrifying microbial communities and determine the contribution of the bacterial and archaeal guilds to an ammonia oxidation, nitrite oxidation and ammonium uptake in a high-altitude wetland plateau setting.

MATERIALS AND METHODS

Salar de Huasco, located at 20° 18'S, 68°, 50'W, was visited during three periods: August 2011, February and July 2012, as part of the field campaigns MOSE 1, 2 and 3 (Microbial Observatory of Salar Experiments), a series of field studies that have been running since 2011. Different methods were considered to evaluate our hypotheses during the fieldtrips. First, during 2011, 16S rRNA subunit pyrosequencing was used to identify potentially active nitrifiers within the aquatic microbial community from contrasting salinity sites *in situ* and from ¹⁵N tracer and inhibitor experiments incubation and select targets for functional gene assays (qPCR) used in all fieldtrips.

The field campaigns also considered different climatic seasons; August and July were associated with dry season (austral winter), but with variable annual average precipitation. Monthly average precipitation (<http://snia.dga.cl/BNAConsultas/reportes> from DGA, Chile) from meteorological stations situated at Altos del Huasco hills limiting Salar de Huasco basin (4044 013 m.a.s.l., Latitude 20° 10'13", Longitude 68° 46'13", only for 2012) and further north at Coyacagua (4013 m.a.s.l., Latitude 20° 03'11", Longitude 68° 48'27" for 2011 and 2012), indicates that the annual precipitation was higher in 2012 than in 2011. August 2011 and July

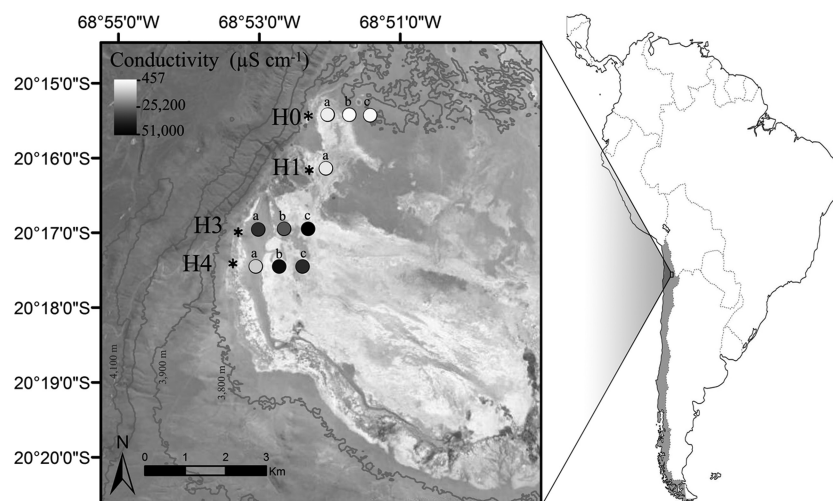


Figure 1. Map of the study area, Salar de Huasco, showing the sampling sites and the variability in conductivity observed between different field trips (August 2011 (a), February (b) and July 2012 (c)). Sampling sites: spring (H0), Pool (H1), Pond (H4) and lagoon (H3). Contour plots represent meters above sea level.

2012 registered no precipitation, as typical for the dry season, whereas February 2012 (austral summer), presented between 75.2 and 125 mm monthly precipitation, reported from Altos del Huasco and Coyacagua, respectively. The sites were selected considering due to their variability in conductivity and included; spring (H0), pool (H1), main lagoon (H3) and pond (H4) (see map in Fig. 1). The sites were GPS marked to ensure reproducible sampling at the same location, however, shifts in the water levels of ~ 1 m altered where sampled could be collected at the lagoon and pond mainly associated with the second dry season (austral winter). *In situ* determinations and water sampling for nutrients and experiments incubations were carried out in water samples collected from the edge of different sites avoiding sediment resuspension using a jar and a pre-filtration funnel with a 200 mm mesh to fill up a carboy (10 L), since the wetland is characterized by very shallow depths (20–30 cm depth). Time between sampling and beginning of the experiment incubation was ~ 1 –2 h depending on the distance between sampling site and the field laboratory. During transport and experiment setup in the lab, the water was kept in the dark.

Conductivity, temperature, pH and oxygen were determined *in situ* using a portable Thermo Scientific Orion Star Multiparameter (model A329) device. Water samples for ammonium concentration determination were collected directly into Schott Glass Bottles (DURAN). Ammonium concentrations were determined according to (Holmes et al. 1999) and fluorescence was measured using a Turner design® fluorometer in the field. The detection limit of this technique was $0.01 \mu\text{M}$. Water for nitrate, nitrite and phosphate determinations was filtered through a GF/F filter (Whatman), and stored frozen until analysis (-20°C). These analyses were carried out in a Seal AutoAnalyzer AA3 segmented flow (Laboratorio de Biogeoquímica, Universidad de Concepción), following standard colorimetric methods for nitrate and nitrite (Strickland and Parsons 1972) and for phosphate (Murphy and Riley 1962). The detection limit for nitrate, nitrite and phosphate were, respectively, 0.015, 0.011 and 0.051. Water for RNA (60 mL) extractions was filtered onto $0.22 \mu\text{m}$ hydrophilic PVDF filters, (GVWP02500, Millipore) in the field using sterile syringes and filter holders of 25 mm diameter. The filters were preserved with RNAlater® solution (Ambion, Austin, Texas) and stored in liquid nitrogen in the field and transferred to a -20°C freezer until extraction.

Tracer experiments for ammonia oxidation and nitrite oxidation

To determine ammonia and nitrite oxidation rates, a tracer experiment addition using $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_2^-$ was used in combination with known inhibitors for both process (Fig. S1, Supporting Information). Polycarbonate bottles (1 L for ammonia oxidation and 0.5 L for nitrite oxidation) were incubated with an addition of $^{15}\text{NH}_4\text{Cl}$ or $\text{Na}^{15}\text{NO}_2$ (both 99% at $0.5 \mu\text{mol mL}^{-1}$), to a final concentration of 10% ambient ammonium or nitrite concentration, respectively. Five treatments in duplicate were done as follows: (i) $^{15}\text{NH}_4^+$ (as a positive net nitrification control); (ii) $^{15}\text{NH}_4^+$ with allylthiourea ($84 \mu\text{M}$ -to inhibit bacterial nitrifiers); (iii) $^{15}\text{NH}_4^+$ with N1-guanyl-1, 7-diaminoheptane (0.4 mM -to inhibit archaeal nitrifiers); (iv) $^{15}\text{NO}_2^-$ (as a nitrite oxidation control), (v) $^{15}\text{NO}_2^-$ with sodium azide ($34 \mu\text{M}$ - as an inhibitor of nitrite oxidation and denitrification (Ginestet et al. 1998; Hatzenpichler et al. 2008; Jansson, Malandrin and Johansson 2000; Löscher et al. 2012; Berg et al. 2015)). In all cases, an extra control bottle was spiked with tracer and immediately sampled, to evaluate initial conditions. Remaining bottles were incubated for 6–7 h in the dark and in a water bath (c.a. 10°C) in the field habilitated laboratory. At all-time points, bottles were subsampled for dissolved inorganic nitrogen concentrations ($\text{DIN} = \text{NH}_4^+, \text{NO}_2^-, \text{NO}_3^-$), RNA and isotopic analysis. Samples for isotope analyses were filtered (GF/F; $0.7 \mu\text{m}$ nominal pore size) and frozen until analysis.

Ammonia and nitrite oxidation tracer analyses and rates determinations

Nitrite oxidation rates were determined based on $^{15}\text{NO}_3^-$ production in incubations with $^{15}\text{NO}_2^-$. After removal of any unused $^{15}\text{NO}_2^-$ from the initial amendment using sulfamic acid, $^{15}\text{NO}_3^-$ was converted to $^{15}\text{NO}_2^-$ with cadmium and then to N_2 with sulfamic acid (McIlvin and Altabet 2005; Fuessel et al. 2012). For experiments with $^{15}\text{NH}_4^+$ additions, the same analysis procedure was followed, except no initial sulfamic acid addition was made for combined analysis of $^{15}\text{NO}_2^- + ^{15}\text{NO}_3^-$, which we further refer to as ammonia oxidation. The N_2 produced ($^{14}\text{N}^{15}\text{N}$ and $^{15}\text{N}^{15}\text{N}$) was analyzed on a gas-chromatography isotope ratio mass spectrometer (GC-IRMS) as in (Dalsgaard et al.

2012). Rates for both processes were inferred from the slope of the linear regression of ^{15}N production with time and corrected for initial labeling percentage of the substrate pool. A t-test was applied to determine if rates were significantly different from zero ($P < 0.05$). Detection limits were estimated from the median of the SE of the slope, multiplied by the t value for $P = 0.05$. Thus, detection limits were 12.9 nM d^{-1} for ammonia oxidation and 23.8 nM d^{-1} for nitrite oxidation. Rates were only determined for August 2011 and July 2012 experiments, as the samples from experiments performed during February 2012 defrosted during transport.

DIN rates of change through time and determination of ammonium oxidation potential

To determine the contribution of ammonia oxidizers in the remineralization of this ecosystem, including bacteria and archaea contribution, changes in DIN concentration from ^{15}N tracer experiments over time ($\mu\text{M d}^{-1}$) were also used to estimate rates and assess the bacteria and archaea contribution.

The DIN rates were estimated by subtracting the mean final concentrations from the mean initial concentrations; then dividing the difference by the incubation time. An error was calculated using the standard deviations of the averages. Positive rates indicated a production of DIN, whereas negative values indicated their consumption. Ammonium oxidation potential was assumed to occur, when a significant inhibition of AOB and AOA occurs; resulting in a higher accumulation of ammonium in ATU and GC7 amended experiments (Lam et al. 2004; Molina et al. 2005; Farías et al. 2009). A total ammonium oxidation potential was calculated from ATU incubations, since both AOB and AOA (T_{AOB}) are expected to be inhibited by this treatment (see reference above). This rate was obtained by the following equation: $T_{\text{AOB}} = R_{[^{15}\text{NH}_4^+ \text{ ATU}]} - R_{[^{15}\text{NH}_4^+]}$. In addition, considering that mainly AOB are expected to be active in GC7 incubations (see references above), the following equations were used to evaluate the potential contribution of both AOB and AOA: $R_{\text{AOB}} = R_{[^{15}\text{NH}_4^+ \text{ GC7}]}$ and $R_{\text{AOA}} = R_{[^{15}\text{NH}_4^+]} - R_{[^{15}\text{NH}_4^+ \text{ GC7}]}$.

Ammonium uptake rates

A parallel experiment to determine ammonium uptake was carried out *in situ* during February and July 2012. 500 mL of water was collected at each sampling site and amended with $^{15}\text{NH}_4\text{Cl}$ (99% at $0.5 \mu\text{mol mL}^{-1}$), to a final concentration of 10% ambient ammonium concentration in order to prevent stimulation of uptake activity. Bottles were incubated *in situ* and incubations were terminated by the filtration of biomass with a gentle vacuum ($<100 \text{ mm Hg}$) onto pre-combusted GF/F filters (450°C for 12 h). Filters were then dried at 60°C for 24 h and stored at constant temperature until laboratory analysis by IRMS (Finnigan Delta Plus). The net DIN uptake rate was estimated according to previous studies (Dugdale and Wilkerson 1986), and the following equation:

$$\rho_{\text{DIN}} = (\text{RPON}/\text{RDIN} * t) * [\text{PON}]$$

where RPON and RDIN represent the ^{15}N atom % excess enrichment in the PON and DIN pools compared to natural abundance, and [PON] represents the final PON concentration; t represents the incubation time in hours (h).

RNA extraction and molecular methods

RNA was extracted using Ambion[®] RNA extraction kit (AM1560) following the manufacturer's specifications with the addition of a mechanical disruption step with 200- μm -diameter zirconium beads (Low Binding Zirconium Beads, OPS Diagnostics) for two rounds of 30 s ($\sim 3000 \text{ rpm}$) using a Mini-Beadbeater-8 (Biospec Products). RNA concentration and quality (A_{260}/A_{280} ratio) was determined spectrophotometrically (Synergy Mx Microplate Reader, BioTek Instruments). DNA was removed using the TURBO DNA-free kit (Applied Biosystems) and RNA was tested for residual DNA by bacterial 16S rRNA gene PCR amplification following (Stevens and Ulloa 2008).

Active nitrifying assemblages and accompanying microbial community composition and structure analyses

The active microbial community composition was studied by 16S rRNA subunit sequence analysis to determine changes within sites, the experimental approach (response to addition and inhibitors) and to identify main known nitrifiers. RNA was retrotranscribed with random primers provided by the ImProm-II Reverse Transcription System (Promega Corp. Madison, Wisconsin, United States), using August 2011 samples taken *in situ* (H1, H0, H3 and H4) and samples from ^{15}N tracer incubation experiments for ammonia and nitrite oxidation.

Bacterial and archaeal 16S rRNA gene pyrolibraries from V1-V3 and V3-V5, were generated, respectively using primers 28F (5'-GAGTTTGATCNTGGCTCAG) and 519R (5'-GTNTTACNGCGGCKGCTG) and Arch340F (5'-CCCTAYGGGGYGCASCAG) and Arch958R (5'-YCCGGCGTTGAMTCCAATT) at the Research and Testing Laboratory (RTL, Texas, USA). The 16S rRNA gene sequences retrieved were curated following the Ribosomal Data Project pipeline by removing primers and barcodes, filtering low quality and length reads, including sequences with ambiguity codes (Cole et al. 2014). The trimmed sequences were then taxonomically classified using the non-redundant M5RNA database (integrating SILVA, Greengenes, and RDP databases) available from the metagenomics RAST server (MG-RAST, v3.3.6) pipeline. We only considered sequences with a minimum e-value $1e^{-20}$, $>70\%$ identity cutoff and $>50\%$ alignment. In order to determine the presence of new candidate phyla, the microbial composition *in situ* were taxonomically reclassified using the automatic software pipeline SILVAngs available from <https://www.arb-silva.de/> (Quast et al. 2013).

The 16S RNA pyrolibraries were deposited in the European Nucleotide Archive under accession Nr. PRJEB8781 with the following run access numbers: ERR789831-ERR789866.

Microbial community-specific analyses

Phylogenetic analyses were carried out for aerobic ammonia and nitrite oxidizer groups using 16S RNA subunit sequences retrieved from pyrosequencing ($\sim 400 \text{ bp}$) and highly similar blast hits from environmental sequences. Maximum likelihood algorithms were run using the FastTree 2.0 method and 1000 bootstrap resampling available in BOSQUE software (Ramírez-Flandez and Ulloa 2008).

Clustering analyses were carried out using Operational Taxonomic Units (OTUs) considering the species level classification to evaluate shifts in microbial community structure associated with environmental factors such as salinity. The data was

first normalized using the fourth-root-transformation and Manhattan distance and UPGMA linkage for clustering was determined using R 2.15.3. Uncertainty of the cluster analyses was estimated through bootstrap analyses (1000 times resampling) using *puclust* (Suzuki and Shimodaira 2006).

AOB and *Nitrosopumilus ammonia monooxygenase* transcripts in situ and within the experiments

The presence of AOB and AOA was studied first by PCR detection of *amoA* transcript. Reverse transcriptions were carried out using 20 ng of total RNA with Reverse Transcription System Improm-II (Promega Corp.) and specific reverse primers for bacteria and archaea (see below). A specific assay for *Nitrosopumilus maritimus* was used (see below) based on the detection of this group in pyrosequencing result during the first sampling combined with a negative amplification using general AOA and ecotypes A and B primers (Beman, Popp and Francis 2008).

The qPCR reactions were analyzed using MX3000P (Stratagene) for 2011 and StepOnePlus™ Real-Time PCR System (Applied Biosystems) for 2012 fieldtrips. A 20 μL reaction mixture with 1 μL of specific cDNA as template, 0.4 μM of primers and the Fast EvaGreen master mix (Biotium) for MX3000P and Power SYBR® Green PCR Master Mix (Applied Biosystems) for StepOnePlus™.

The primers *amoA1F*–*amoA2R* (Rotthauwe et al. 1997) were used to estimate the relative contribution of AOB. For *Nitrosopumilus*, an internal forward primer, *Nmar423F* (3' AAC CTG ATA ACR GTW GCA GAC C 5') was used in combination with the reverse primer *Arch-amoAR* (Francis et al. 2005) following (Elizondo-Patrone et al. 2015). The amplification was carried out in replicates as follows: 3 min at 95°C, and then 40 cycles consisting of 30 s at 95°C, 30 s at 56°C and 45 s min at 72°C with a detection step at the end of each cycle. Negative controls were also included. A melting curve was run at the end of all the qPCR assays. The CT cutoff value ~ 30 . The specificity of qPCR assays was verified by checking melting curves and by running the PCR products in agarose gels.

Plasmids containing *amoA* gene fragments were used as qPCR standards. The plasmids were extracted using Wizard Plus SV Minipreps (Promega) and quantified using the Quant-iT Broad Range DNA assay (Qubit Fluorometer, Invitrogen). For AOB and *Nitrosopumilus maritimus*, the clones ST080550.F7 (GU066855) and clone 05 (KJ555107) were used as standards. Standard curves were prepared using 10 fold dilutions series from 4×10^7 – 10^1 . The PCR efficiencies and correlation coefficients for the standard curves were as follows: AOB ($E = 92.2$ – 95.3 , $r^2 = 0.984$ – 0.999) and *Nitrosopumilus* ($E = 95.5\%$ – 106.4% , $r^2 = 0.986$ – 0.998) assays. The data were analyzed by using the MX-3000 software and StepOne software package (v.2.2.2). The gene copy numbers were then multiplied by a factor associated with the RNA retro-transcribed and divided by total RNA extracts obtain for each sample as a standardization procedure to compare the different sites and samples, since RNA yields varied between samples (Table S1, Supporting Information).

Picoplankton abundance was estimated by flow cytometry for 2012 in the different experiments. For this purpose, 1.35 mL of water was fixed with glutaraldehyde (0.1% final concentration) and stored in liquid nitrogen during transport and at -80°C until analysis. Subsamples of 150 μL were processed on a FACSCalibur flow cytometer (Becton Dickinson) equipped with an ion-argon laser of 488 nm of 15 mW at the Universidad de Concepción.

Spearman rank correlations were calculated to determine the association between the different variables studied, ammonia and nitrite oxidation rates and, qPCR results with conductivity and substrate availability. Bray Curtis similarity matrix of square-root transformed data (qPCR counts) were used to determine potential amendments effects. This analyses were done using PRIMER software (Anderson, Gorley and Clarke 2008).

RESULTS

Physical-chemical conditions of the wetland and nitrogen recycling rates

The wetland of Salar de Huasco was characterized by a large variability in physical-chemical parameters among the spring (H0), pond (H4) and lagoon (H3) sites and between the different samplings (Fig. 1 and Table 1). Conductivity was variable between the different sampling dates; the spring was always characterized as freshwater ($<610 \mu\text{S cm}^{-1}$), the lagoon varied between hyposaline and mesosaline ($26\,800$ – $51\,000 \mu\text{S cm}^{-1}$), whereas the pond changed from subsaline ($3050 \mu\text{S cm}^{-1}$) towards mesosaline ($37\,000$ – $49\,700 \mu\text{S cm}^{-1}$) (Table 1). Referential PSU values based in seawater ranged between 0.3 and 48.4 (Table 1). Oxygen was also variable and lower concentrations were observed at the spring and lagoon sites ($\sim 4.6 \text{ mg L}^{-1}$) compared with the pond site ($<8.5 \text{ mg L}^{-1}$). In general, ammonium, nitrite, nitrate and phosphate presented higher concentrations during 2012 compared with 2011 sampling (Table 1). Phosphate concentrations were higher at the lagoon (18.8 – $102 \mu\text{mol L}^{-1}$) and at the pond site during 2012 samplings. Phosphate was the only chemical variable significantly correlated with conductivity (Spearman Rank, $r = 0.89$; $P < 0.001$). Nitrate concentrations were $\geq 8.9 \mu\text{mol L}^{-1}$ at the spring site and at all sites during February 2012. Nitrite concentrations were comparable at the spring, pond and lagoon during August 2011 and July 2012 ($<0.32 \mu\text{mol L}^{-1}$), whereas higher nitrite concentrations (0.38 – $3.5 \mu\text{mol L}^{-1}$) were found at the pond and spring sites during February and July 2012, respectively. Ammonium concentrations were low during August 2011 ($\sim 0.11 \mu\text{mol L}^{-1}$) and increased to a maximum of $0.83 \mu\text{mol L}^{-1}$ at the spring site during July 2012. Associated with the physical and chemical variability in the aquatic sites sampled, nitrifying activities (ammonia and nitrite oxidation) were variable during our sampling, showing similar trends but contrasting magnitudes depending also on the method used (Table 2 and Figs 2 and 3). Based on the ^{15}N tracer experiments, ammonia oxidation were only determined at the pond during August 2011 (55 nM d^{-1}), showing a 34% decrease in ATU amendments (Table 2 and Fig. 2A). Unfortunately, February 2012 samples for these analyses were lost due to unfreezing during transport; thus only potential rates were determined during that period (Fig. 3A). During the second dry period studied (July 2012) ammonia oxidation were detected at all the aquatic sites ranging between 40.2 and 84.8 nM d^{-1} . These rates were highest at the spring and pond during July 2012 potentially associated with the higher ammonium concentration (Fig. 2C; Fig. S2, Supporting Information). In contrast, the mesosaline lagoon has the lowest rates despite the high ammonium concentration (Fig. 2C; Fig. S2, Supporting Information).

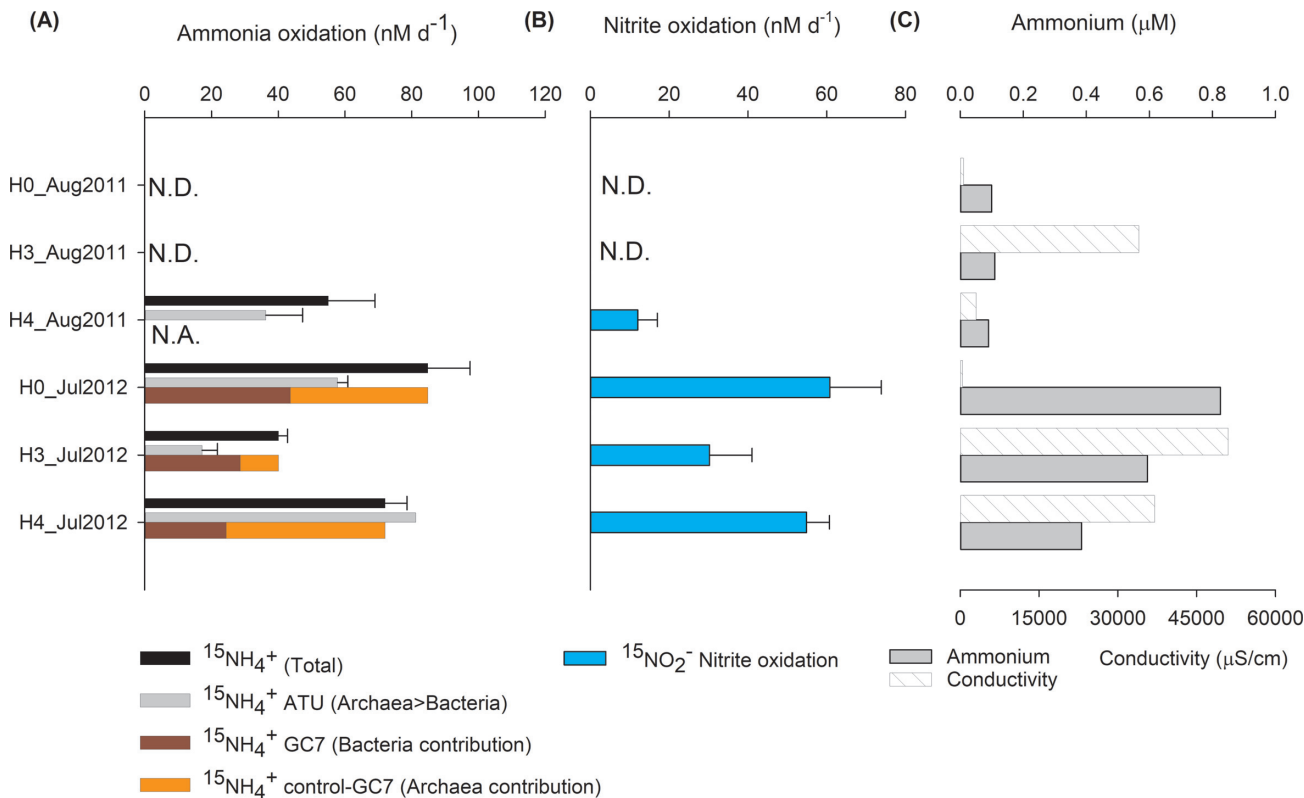
The relative contribution of archaeal versus bacteria was determined based on a reduction of ammonia oxidation between 29% and 67% and 31% and 42% in the GC7 and ATU treatments compared to the control, respectively (Table 2). The bacterial ammonia oxidation rate decreased at mesosaline sites

Table 1. In situ physical-chemical variables determined during the different fieldtrips in Salar de Huasco. Nutrients standard deviations are given in parenthesis. Not detected (n.d.).

Date	Site	Temperature (°C)	Conductivity ($\mu\text{S cm}^{-1}$ /Salinity (PSU))		Oxygen (mg L ⁻¹)	Ammonium ($\mu\text{mol L}^{-1}$)	Nitrite ($\mu\text{mol L}^{-1}$)	Nitrate ($\mu\text{mol L}^{-1}$)	Phosphate ($\mu\text{mol L}^{-1}$)
Aug2011	H0 Spring	15.5	610/0.36		6.3	0.11 (0.05)	0.18 (0.03)	15.8 (2.29)	1.64 (0.22)
	H1 Pool	11.6	697/0.46		5.5	0.09 (0.02)	0.26 (0.07)	0.97 (0.03)	0.39 (0.014)
	H3 Lagoon	18.2	34,000/24.9		7.3	0.11(0.01)	0.23 (0.07)	6.83 (0.70)	18.8 (0.52)
	H4 Pond	14.6	3,050/2.02		7.1	0.09 (0.005)	0.31 (0.03)	1.23 (0.05)	0.93 (0.04)
Feb2012	H0 Spring	14.5	457/0.28		7.7	0.30 (0.05)	0.38 (0.01)	11.1 (0.04)	0.90 (0.07)
	H3 Lagoon	10.1	26,800/23.7		4.7	0.32 (0.06)	3.2 (0.37)	10.9 (0.08)	78.1 (5.16)
	H4 Pond	9.1	49,700/48.4		6.5	0.24 (0.03)	3.5 (0.32)	8.16 (0.01)	220 (12.81)
Jul2012	H0 Spring	14.6	476/0.29		5.8	0.83 (0.06)	0.32 (0.06)	19.9 (0.24)	5.16 (0.59)
	H3 Lagoon	14.1	51,000/43.5		5.9	0.59 (0.05)	n.d.	6.21 (0.28)	102.4 (0.81)
	H4 Pond	5.1	37,000/39.03		8.5	0.38 (0.06)	n.d.	9.81 (0.014)	55.08 (0.72)

Table 2. Ammonium and nitrite oxidation rates (nM d^{-1}) determined experimentally during August 2011 and July 2012. The standard error is presented between parentheses. *Single value. Not analyzed (N.A.). Not Detected (N.D.) at 12.9 and 23.8 nM d^{-1} , respectively, for $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_2^-$.

Date	Site	$^{15}\text{NH}_4^+$	$^{15}\text{NH}_4^+ + \text{ATU}$	$^{15}\text{NH}_4^+ + \text{GC7}$	$^{15}\text{NO}_2^-$	$^{15}\text{NO}_2^- + \text{Azide}$
Aug2011	H0 Spring	N.D.	N.D.	N.D.	N.D.	N.D.
	H3 Lagoon	N.D.	N.D.	N.D.	N.D.	N.D.
	H4 Pond	55.0 (14.0)	36.3 (11.0)	N.A.	12.0 (5.0)	N.D.
Jul2012	H0 Spring	84.83 (12.63)	57.78 (3.05)	43.61 (2.68)	60.77 (13.07)	N.D.
	H3 Lagoon	40.07 (2.68)	17.19 (4.57)	28.6 (5.44)	30.23 (10.8)	25.51 (9.42)
	H4 Pond	72.01 (6.55)	81.2 (*)	24.38 (5.53)	54.87 (5.81)	33.03 (10.9)

**Figure 2.** Ammonia oxidation rates and the relative contribution of bacteria and archaea ammonia oxidizers (only during July 2012) (A) and nitrite oxidation rates (B) measured at the different sites sampled. Ammonium concentration and conductivity determined in situ (C).

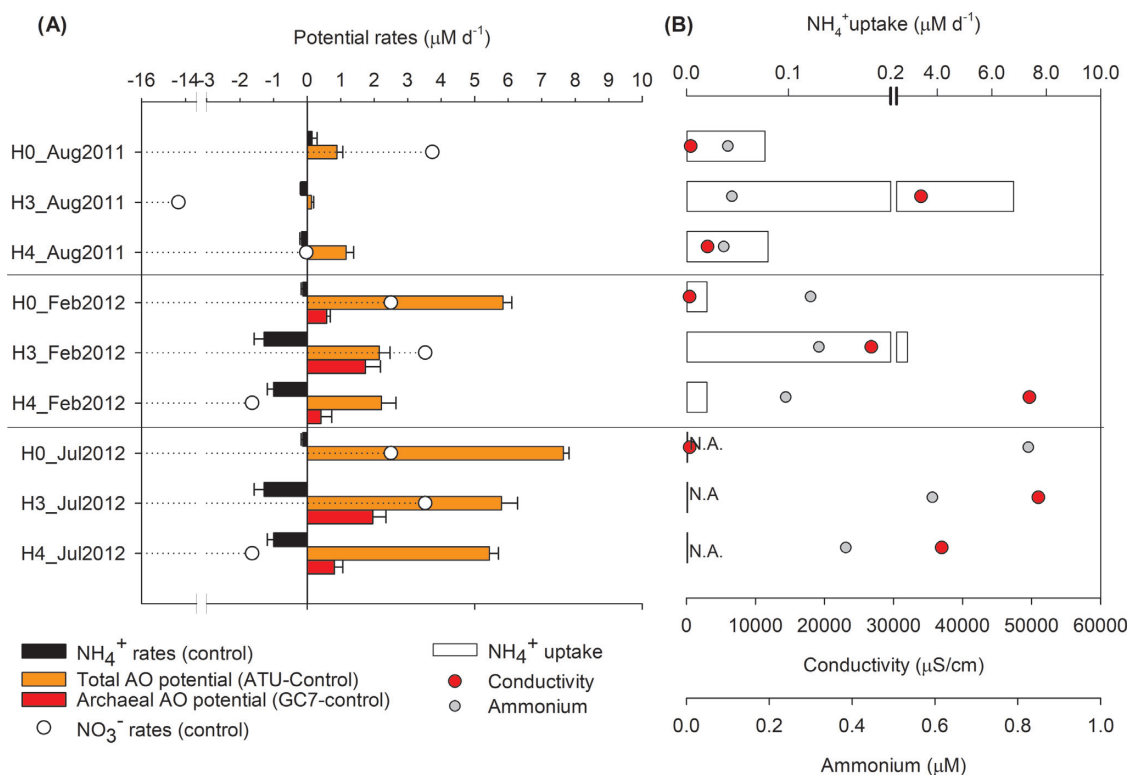


Figure 3. Nutrient rates of change through time (ammonium, nitrate) and ammonia oxidation (total and Archaeal potential) determined within endpoint tracer experiments bottles (A). Ammonium uptake rates determined at the different sites during August 2011 and February 2012 and in situ ammonium concentration and conductivity (B).

($\sim 24.4 \text{ nM d}^{-1}$ at pond and lagoon) compared to the freshwater spring site (43.6 nM d^{-1} , Table 2 and Fig. 2A) and increased with in situ ammonium concentration (Table 1). However, bacterial ammonia oxidation represented a significant fraction of the total ammonia oxidation determined at the lagoon (71%), followed by the spring and pond sites (51% and 33%, respectively). On the other hand, the archaeal ammonia oxidation (Table 2) ranged between 11.5 and 47.6 nM d^{-1} showing the opposite trend as bacteria (Fig. 2A), reaching a higher contribution at the spring. A similar trend was supported by the rates determined at $^{15}\text{NH}_4^+$ ATU experiments (Fig. 2A).

In contrast, total ammonia oxidation (AO) based on NH_4^+ net changes in concentration was 1–2 orders of magnitude higher than ammonia oxidation rates determined from ^{15}N tracer additions (Fig. 3A). These rates were associated with a high NO_3^- accumulation at the spring during all sampling periods, the lagoon only during February 2012 and the pond site during July 2012. AO potential decreased at sites with high conductivity and increased with high in situ ammonium concentrations at almost all sites particularly during the last sampling (Fig. 3B). During 2012 sampling, the difference between GC7 and control NH_4^+ rates of change suggest an archaeal AO contribution of ca. 50% of the total AO potential. These rates also showed a decline at sites with high conductivity and low ammonium concentration (Fig. 3B).

Nitrite oxidation also followed ammonia oxidation rates pattern were in the limits of detection during 2011 (Fig. 2B), but higher (30.2 – 60.8 nM d^{-1}) at all sites during July 2012 (Table 2). These rates showed a reduction in treatments with azide mainly at the spring, followed by the lagoon and pond, by 100%, 40% and 15%, respectively (Table 2).

Spearman rank correlation indicates negative but non-significant correlation between ammonia and nitrite oxidation rates using tracers (July 2012) and potential AO rates (total and archaeal) with conductivity (Fig. S2, Supporting Information). Total and archaeal AO potential were significantly correlated with in situ ammonium concentrations (r 0.74–0.90; p 0.01–0.04).

In general, ammonium rates of change were negative at the different sampling sites, except for the spring site during August 2011 and July 2012, and the pond in July 2012. These rates were significantly correlated with conductivity (r -0.81 ; p 0.008). This result was coincident with ammonium uptake evaluated during 2011 and February 2012 (Fig. 3). At the lagoon (high conductivity site), ammonium was in high demand compared to the other sites 2.9 – 6.8 versus $<0.08 \mu\text{M d}^{-1}$, respectively (Fig. 3).

Composition of nitrifying microorganisms compared with total community based on 16S rRNA pyrolibraries and *amoA*-qPCR counts

Nitrifying microorganisms (ammonia/nitrite oxidizers, anammox) represented $<0.25\%$ of the pyrosequencing reads from in situ samples and our rate experiments (Table S2, Supporting Information; Fig. 4). Nitrifying bacteria affiliated with *Nitrosospira* and *Nitrosomonas* were found in the spring, pond and pool sites ($<0.08\%$ of total bacterial sequences). These sequences had a low similarity with cultured representatives (87%–92%) but were closely related with uncultured bacteria sequences derived from soil, including CO_2 exposed (Dunbar et al. 2012), rice field (Qiu et al. 2008) and arid environments such as saline-alkaline lakes (available from GenBank; Fig. S3, Supporting Information). Other

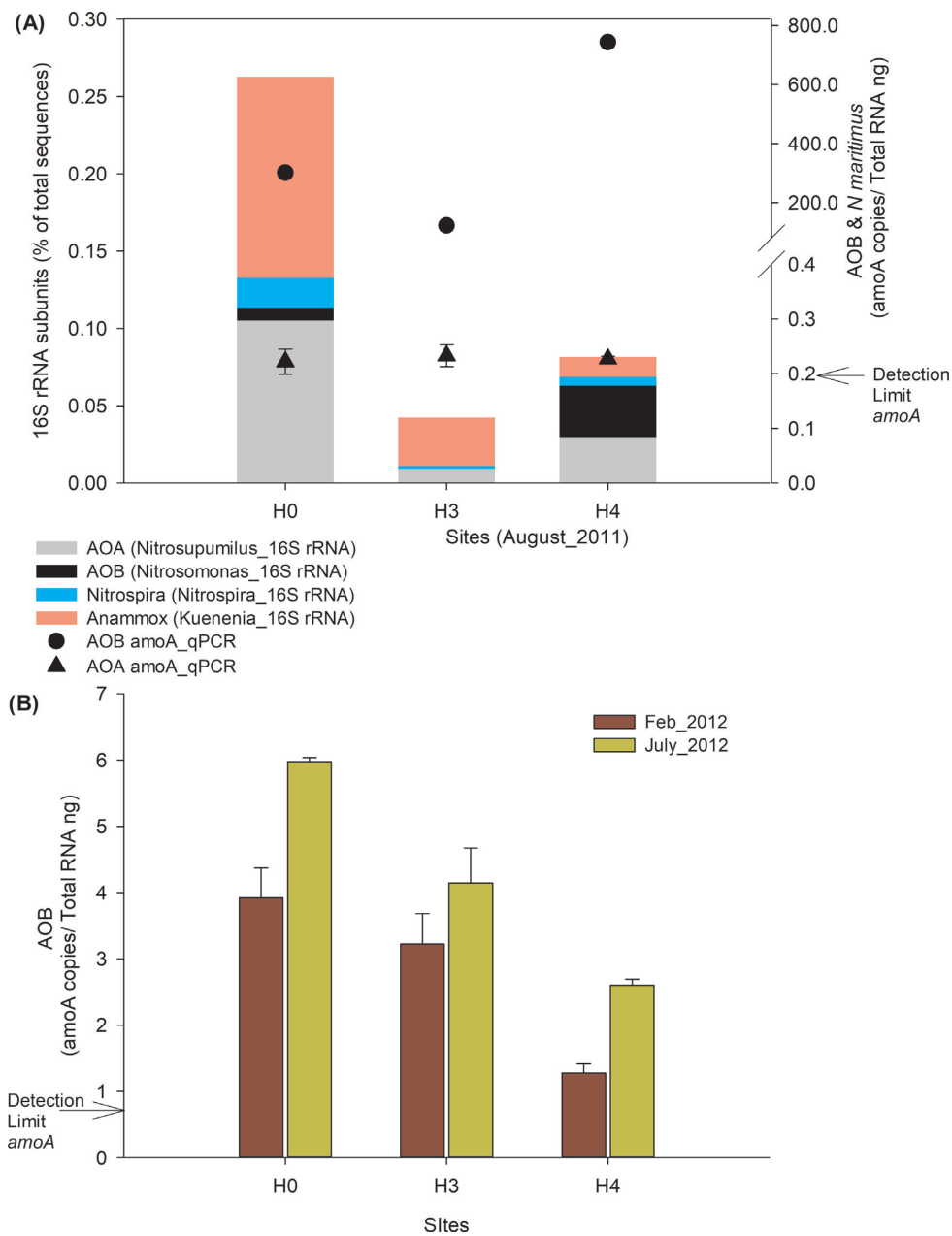


Figure 4. (A) Nitrifying microorganisms contribution (percentages) based on 16S rRNA subunit sequencing identification including anammox and AOB and *N. maritimus* amoA-qPCR counts in situ at the different sites during August 2011. (B) AOB contribution at the different sites determined during February and July 2012.

Nitrospira sequences were detected in all the sites and represented <0.09% of pyrolibraries. These sequences were associated to lineage 2, where comammox *Nitrospira* were affiliated, together with groundwater and other high altitude ecosystems sequences, such as the Tibetan plateau (Yuan et al. 2014) (Fig. S4, Supporting Information). Archaeal nitrifying groups represented between 0.95% and 100% of the total archaeal sequences and between 0.1% and 1% of total bacterial plus archaeal sequences (Table S2, Supporting Information; Fig. 4). The AOA sequences detected demonstrated a high similarity with *Nitrosopumilus* (94–96%) and only between 78.5% and 78.8% with other AOA isolates *Nitrosocaldus yellowstonii*, *Nitrososphaera gargensis* and *N. viennensis*. These *Nitrosopumilus*-like sequences grouped together in a separate subcluster affiliated with uncultured clones; for example, sequences derived from a drinking

water reservoir (Lympelopoulou et al. 2012), fresh water and sediments from aquatic ecosystems such as rivers and lakes (GenBank; e.g. Galand et al. 2008; Hugoni et al. 2013). These sequences were also found in a clone library from Salar de Huasco in a previous study (Dorador et al. 2010) (Fig. S5, Supporting Information). Anaerobic ammonia-oxidizing (anammox) bacteria, affiliated to *Kuenenia stuttgartiensis*, were also detected in situ at all sites and during our incubations, representing between 0.01% and 0.4% of total bacterial sequences (Table S2, Supporting Information; Fig. 4).

In general, if the nitrifying assemblages from the different treatments were pooled they represented a higher contribution to the total rRNA at freshwater and subsaline sites (spring and pond) compared with the saltier lagoon (Fig. 4). Similar trends among nitrifying groups were in general comparable with the

bacteria distribution at the different aquatic sites (Fig. 5A). In general, most of the changes within microbial communities were associated to the study site and not with the incubation and amendments (Fig. 5B; Table S3, Supporting Information). For example, some phyla were predominant at freshwater spring (e.g. Euryarchaeota and Cyanobacteria), whereas other abundant phyla were common to all aquatic sites and treatments (e.g. Verrucomicrobia, Proteobacteria and Bacteroidetes). However, within these phyla some classes, such as Betaproteobacteria (phylogenetic group where *Nitrospira* and *Nitrosomonas* are affiliated) represented a higher contribution to total Proteobacteria at the subsaline pond, followed by the spring and decreased at the lagoon during 2011. A similar trend was observed for other classes within Bacteroidetes (e.g. Cytophagia, Sphingobacteria, Fig. 5A). In general, higher similarities were observed between the freshwater spring and the subsaline pond during 2011, supported by clustering analyses at OTU levels with significant bootstrap values (Fig. 5B).

In terms of the specific nitrifying group contribution, bacterial *amoA*-qPCR counts peaked at the subsaline pond during 2011 coinciding with *Nitrosomonas* sequences contribution (%) to pyrolibraries (Fig. 4A and Table 1). During 2012, bacterial *amoA*-qPCR counts were lower than the transcripts detected during 2011 (Fig. 4B). Bacterial *amoA*-qPCR counts increased at sites with low salinity and higher ammonium concentrations (Fig. 3B; Fig. S2, Supporting Information), the later showing a significant correlation during 2012 ($r = 0.94$, $P = 0.004$). On the other hand, archaeal *amoA* genes were at the limit of detection during 2011. Archaeal *amoA* were undetected during 2012 using various of the PCR approaches selected based in 16S rRNA pyrolibraries from 2011 (*Nitrosopumilus*) and for general AOA, ecotypes A and B (data not shown).

Bacterial and archaeal *amoA*-qPCR counts measured in ^{15}N tracer endpoint experiments during 2011 showed a reduction of 6% and 31% in the NH_4^+ ATU treatment compared with NH_4^+ only at the pond and lagoon, respectively (Table S1, Supporting Information), showing a high similarity (>80%, data not shown). During 2012, bacterial *amoA*-qPCR counts were highly similar between treatments except for the lagoon during July 2012 (>60%, data not shown). The bacterial *amoA*-qPCR counts were reduced in the NH_4^+ ATU treatment compared to NH_4^+ only at the spring during February 2012, whereas the opposite was found during February and July 2012 experiments at the pond and lagoon (Table S1, Supporting Information). Flow cytometry data for picoplankton abundance were variable among sites and within the different treatments of the nitrification experiments (Table S1, Supporting Information). The spring site was characterized by low picoplanktonic abundance ($<134 \times 10^3$ cells mL^{-1}) compared with the saltier pond and lagoon ($446\text{--}21,953 \times 10^3$ cells mL^{-1}). During the incubations, changes in abundance were detected, but without a clear effect of the specific amendment.

DISCUSSION

Nitrification and nitrogen recycling at the high altitude plateau ecosystem

An intense nitrogen recycling, including nitrification was detected under the extreme conditions associated with the high-altitude plateau wetland showing a great variability potentially influenced by salinity and ammonium concentration at different aquatic sites (springs, ponds and main lagoon); well-known factors associated with the ecophysiology of nitrifying microorganisms (e.g. Oren 2001; Horz et al. 2004; Erguder et al.

2009; Hatzepichler 2012). During our study, salinity, was variable between the different aquatic sites and sampling periods supporting previous observations of a seasonal and interannual variability in high-altitude plateau aquatic ecosystems (Dorador, Pardo and Vila 2003). Conductivity changes were correlated with phosphate, nutrient that reached high levels at ponds and the salty lagoon during our sampling. This association was reported before for the same area of Salar de Huasco and other high-altitude wetlands and lakes considered as eutrophic conditions for this ecosystems linked with high biological activity including large birds colonies (Marquez-Garcia et al. ; Dorador et al. 2010) and possibly as a result of intense microbial activity (Hernández et al. 2016).

In addition, an increment in water ammonium concentration up to eight fold but not correlated with conductivity was observed between 2011 and 2012 samplings (Table 1). In fact, during 2012, higher DIN up to 21.1 and phosphate up to 220 μM environmental conditions were determined for this ecosystem, supporting the predominance of eutrophic conditions compared with high-altitude freshwater lakes characterized by oligotrophic conditions (DIN <0.78 and phosphate $<0.12 \mu\text{M}$, Hayden and Beman 2014). In accordance with the higher *in situ* ammonium concentration, a high ammonium consumption and potential turnover was notorious in the study area (Fig. 3), including high ammonium uptake at saltier sites and potentially a greater contribution of nitrification at low-salinity aquatic sites of the wetland.

Nitrification rates from high elevation lakes are scarce; however, the ammonia and nitrite oxidation rates determined here using the ^{15}N tracer approach ($12\text{--}85 \text{ nM d}^{-1}$) were much higher than prior reports ($\sim 0.015 \text{ nM d}^{-1}$) at other elevated ecosystems (Hayden and Beman 2014). In fact, using the ^{15}N tracer approach, our rates were in the range of rates measured from surface waters of saline environments such as Mono Lake ($<60 \text{ nM d}^{-1}$, Carini and Joye 2008) and marine ecosystems using similar methodology (e.g. $20\text{--}100 \text{ nM d}^{-1}$, Ward 2005) and were reliable considering the limit of detection obtain here (Fig. 2) compared with other studies in marine ecosystems (Bristow et al 2016). However, even greater nitrification potential was expected using DIN rates of change and inhibitors (up to 6000 nM d^{-1} , Fig. 3). The ammonia oxidation rates obtained here based in both approaches (^{15}N tracer and DIN rates of change) peaked at aquatic sites characterized by low salinity when higher ammonium was available (Fig. S2, Supporting Information). For example, ammonia oxidation rates determined using tracer approach were detected only at the pond site during 2011, a site characterized by subsaline conditions ($<3050 \mu\text{S cm}^{-1}$) and low ammonium concentration $\sim 0.1 \mu\text{M}$. Whereas during July 2012 ammonia and nitrite oxidation rates were detected at all sampling sites (Fig. 2), but reached higher magnitudes at freshwater and hyposaline sites also characterized by greater *in situ* ammonium concentration $0.24\text{--}0.83 \mu\text{M}$ compared to 2011 sampling. In agreement with tracer ammonia oxidation, a greater potential ammonium oxidation was observed at low salinity sites but were persistent at saltier pond and lagoons when ammonium was available during 2012 (Fig. 3). In fact, ammonia oxidation at Salar de Huasco estimated from DIN rates of change and bacterial ammonia oxidation rates from ($^{15}\text{NH}_4^+$ GC7 during July 2012) were positively and significantly correlated with *in situ* ammonium concentration. A negative correlation was observed with conductivity but was not significant.

Our results were supported by previous reports with variable salinity such as estuaries showing an increase of nitrification (planktonic and benthic) rates at low salinity, but in contrast to

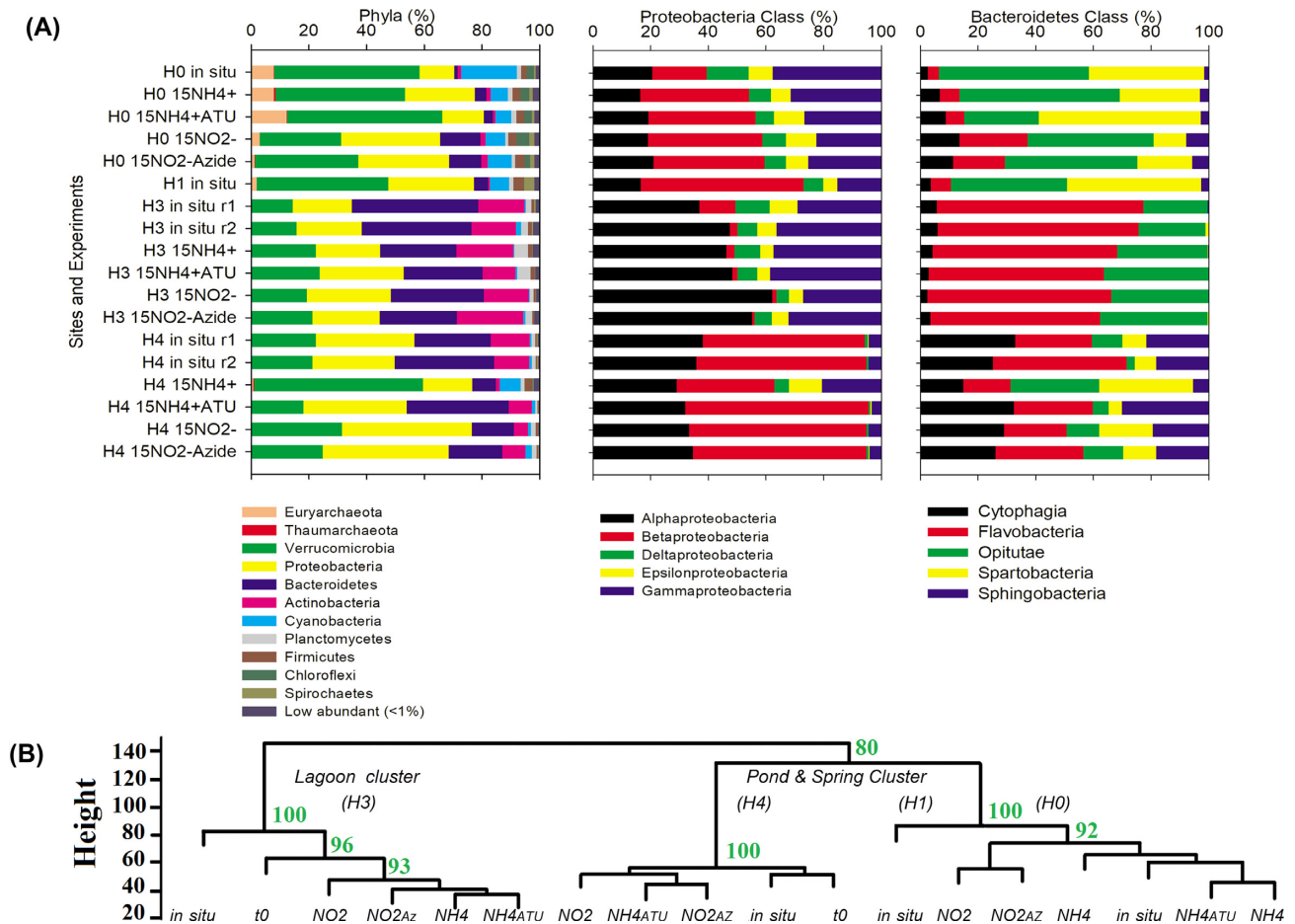


Figure 5. (A) Changes in abundant phyla, Proteobacteria and Bacteroidetes class within the different incubation bottles and treatments. (B) Clustering analyses based on OTUs.

our study in response to very high ammonium conditions, when oxygen is available; for example, from seawater (>35 PSU and $0.5 \mu\text{M}$ ammonium) towards the proximal portion of the river Rhone plume (5 PSU to $10 \mu\text{M}$ ammonium) or at contrasting benthic areas of Elkhorn Slough, characterized by 98–1135 porewater ammonium μM and variable salinity 0 to 60 PSU in sediment overlying water (Bianchi, Feliatra and Lefevre 1999; Caffrey et al. 2003).

Despite similar distribution or tendency of both approaches (^{15}N tracer versus DIN rates of change) to estimate ammonia oxidation, significant differences in terms of magnitude were determined (Figs 2 and 3). In general, rates derived from the use of inhibitors and DIN concentration change could be in the upper limit or higher than the ones detected by other approaches in marine ecosystems (Molina and Farias 2009). On the other hand, in the study area $^{15}\text{NH}_4^+$ and $^{15}\text{NO}_2^-$ tracer experiments could represent a lower limit of nitrification rates (gross nitrification), if we assume that rates derived from DIN concentration changes could give an expected (net nitrification) based also on nitrate accumulation rates (Fig. 3). The later includes nitrate originated from nitrification coupled to ammonification or to other sources of ammonia such as urea (Xiang et al. 2017) during the experiment, process not consider in our tracer ammonia and nitrite oxidation determinations. In fact, ammonification was found to be significant since

high magnitude of ammonium net consumption (negative rates) were determined during our study mainly at saltier ponds and lagoon (Fig. 3). Moreover, high ammonium uptake was determined during August 2011 and February 2012, which also peaked at the lagoon and increased between periods, coinciding with an increase in AO potential and *in situ* ammonium availability (Table 1). The use of multiple approaches (^{15}N tracer and DIN concentration changes) helped to determine ammonia and nitrite oxidation lower and upper limits, and in combination with ammonium uptake rates to identify the intense ammonium recycling in this ecosystem. However, future studies in this ecosystem must consider the use of other techniques such as dilution method ($^{15}\text{NO}_3^-$ incubations) to determine nitrate originated from ammonification during the incubation (Norton and Stark 2011).

Active nitrifying microorganisms at aquatic sites with variable conductivity and ammonium and their interaction with the microbial community

The 16S rRNA subunit survey carried out during August 2011 indicated that nitrifying assemblages were diverse and characterized by aerobic nitrifying bacteria (*Nitrosospira*, *Nitrosomonas* and *Nitrosospira* from lineage 2) and archaea (mainly *Nitrosopumilus maritimus*). Our study supports previous reports based on specific clone library surveys targeting archaea and enrichment studies focused on ammonia oxidizing bacteria (Dorador et al 2008;

2010). However, our 16S rRNA subunit survey indicated certain nitrifying groups had a low activity compared with the total active microbial community in terms their contribution (%) to pyrolibraries (Fig. 4). This result coincides with a recent 16S rDNA and rRNA result (March 2015) using MiSeq sequencing at H0 spring site, indicating the contribution of *Nitrosomonas* (0.02 vs 0%), *Nitrospira* (0.03 vs 0.4%), *Nitrosopumilus* (0.03 vs 0%) in 16S rDNA and rRNA, respectively. Sequences available from a recent published report also indicates that *Nitrosomonas* was rare (0.09%) at a pond site in H3 area based also in 16S rRNA gene study using pyrosequencing (Aguilar et al. 2016). Moreover, PCR independent methods such as cell counting (immunofluorescence and fluorescence in situ hybridization) also indicates that bacteria represent a low percentage (0.1%–0.8%) of the microbial assemblages in aquatic ecosystems (Bothe et al. 2000), in contrast to archaea that could account to a significant fraction (>30%) of total microbial community in marine ecosystems (Karner, DeLong and Karl 2001).

The detection of *Nitrospira* from lineage 2 in a cluster closely associated with *Nitrospira inopinata* (Daims et al. 2015), could potentially indicate the presence of comammox (complete ammonia oxidizing bacteria) in Salar de Huasco. However, 16S rRNA is not a specific gene marker for this group (Daims et al. 2015; van Kessel et al. 2015) and additional studies, for example, identifying functional genes related to comammox through metagenomics might unravel the presence of this group, as in other environments (Pinto et al. 2016). Also, the anammox bacterium *Kuenenia stuttgartiensis* was detected in the study area, suggesting that anaerobic ammonium oxidation (anammox) might occur in particularly at the spring site. It is worthwhile noting here that as part of the anammox metabolism nitrite acts as an electron donor in the carbon fixation step; hence, growth is associated with the production of nitrate anaerobically (Kartal et al. 2013).

The contribution of bacteria and archaea ammonia oxidizing groups

Pyrosequencing data suggest that both archaea and bacteria were present at Salar de Huasco during 2011. The contribution of archaea and bacteria to ammonia oxidation was determined within ^{15}N tracer experiments using the archaeal inhibitor (GC7) during 2012. The difference of ammonia oxidation between the control and GC7 indicates that archaeal activity represented between 49% and 67% of total ammonia oxidation at subsaline and hyposaline sites (spring and pond), whereas bacterial activity (NH_4^+ GC7) represented 71% of ammonia oxidation at the large mesosaline lagoon. The relevance of bacterial nitrification at the lagoon could be significant since this site represents one of the largest water-covered areas of the wetland, c.a. 175–597 ha, based on satellite images (Landsat, data not shown). Moreover, the lagoon was also characterized by significant benthic primary production, potentially able to sustain local fauna, including the conspicuous flamingo population (de la Fuente 2014).

The *amoA*-qPCR results suggest a higher transcription of bacteria compared with *N. maritimus* in situ and within the experiments, since this later group using qPCR were at the limit of detection (2011) or undetectable (2012). In total, these results support previous observations of higher abundance of AOB compared to AOA in saline estuarine waters e.g. (Mosier and Francis 2008; Santoro et al. 2008). Moreover, AOB *amoA*-qPCR counts during our study and ammonia oxidation associated with AOB

(NH_4^+ GC7) were correlated with *in situ* ammonium concentrations, suggesting a higher substrate dependency of AOB, a result supported by the ammonia oxidation kinetics of bacteria versus archaea (Martens-Habbenha et al. 2009). On the other hand, even when archaeal *amoA* sequences were undetectable (i.e. July 2012), ^{15}N tracer experiments conducted with inhibitors indicated that there was the potential for archaeal ammonia oxidation. This apparent contradiction might stem from a failure to amplify *amoA* genes from the active community during 2012. This could potentially be linked to a community shift between 2011 and 2012 to an AOA unknown group, which is possible considering the high archaeal diversity and novelty found in the wetland in a previous study (Dorador et al. 2010).

In summary, our results show that nitrification was an active process at Salar de Huasco, mediated by a diverse community of bacterial and archaeal microorganisms. Ammonia and nitrite oxidation rates were primarily dependent on ammonium availability and the rates were persistent at salty evaporitic areas suggesting shifts in the active nitrifying community can support nitrogen recycling in these high altitude plateau wetlands.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](https://www.femsec.org) online.

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Conflicts of interest. None declared.

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