Effect of salt stress on aerobic methane oxidation and associated methanotrophs; a microcosm study of a natural community from a non-saline environment.

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Abstract

We investigated the response of aerobic methane oxidation and the associated methanotrophs to salt-stress in a NaCl gradient ranging from 0 M (un-amended reference) to 0.6 M NaCl (seawater salinity) using a rice paddy soil as a model system. Salt-stress significantly inhibited methanotrophic activity at > 0.3 M NaCl; at 0.6 M NaCl amendment, methanotrophic activity fully ceased. MiSeq sequencing of the *pmoA* gene and group-specific qPCR analyses revealed that type Ia methanotroph (*Methylobacter*) appeared to be favored under salinity up to 0.3 M NaCl, increasing in numerical abundance, while the type Ib was adversely affected. This suggests niche differentiation within members of the gammaproteobacterial methanotrophs. Overall, rice paddy soil methanotrophs showed remarkable resistance to salt-stress.

Main text

Irrigated lowland rice cultivation is the most common rice production system worldwide (Maclean et al., 2002). Lowland rice is typically cultivated in coastal areas, particularly in the Asia-Pacific region, and hence, is threatened by seawater intrusion (salinization) with the projected rise in seawater level (Rahmstorf, 2007). Salinization affects belowground microbially-mediated biogeochemical processes, including methane-cycling in rice paddies. Rice paddies are a source of methane, a potent greenhouse gas, where methane emission is regulated by methane production and oxidation rates. Previous work showed the adverse (in)direct effects of salinization on methanogenesis and methane emission (Baldwin et al., 2006; van Dijk et al., 2015; Peng et al., 2017). However, less is known on the response of methane oxidation and the methanotrophs to salt-stress in wetland rice paddies.

Halophilic and halotolerant methanotrophs have been documented in widespread saline environments (e.g. soda lakes, mangrove, alkaline lake, estuary; de Angelis & Scranton, 1993; Antony et al., 2010; Sherry et al., 2016; Deng et al., 2017; Osundar et al., 2017; Shiau et al., 2018), where community members may possess specialized mechanisms to overcome salt-stress (Khmelenina et al., 1999). These methanotrophs are predominantly associated with type Ia subgroup belonging to the gammaproteobacteria, albeit an alphaproteoacterial methanotroph (*Methylocystis*) has recently been shown to cope with moderate salt-stress (<1% NaCl; Han et al., 2017). While methanotrophs indigenous to saline environments and their response to salt-stress at the cellular level have been the focus of previous studies (Khmelenina et al., 1999; Han et al., 2017), the response of methanotrophs from non-saline

environments to increasing salinity, and the resistance and threshold for methanotrophic activity have received little attention.

Considering that different methanotrophs may show different degrees of resistance to salt-stress (Osundar et al., 2017), we hypothesize that persistent salinity will favor the proliferation of salt-resistant methanotrophs, which will dominate the community over time. We addressed our hypothesis using a well-characterized paddy soil (see Krüger et al., 2001; Ho et al., 2013) collected during fallow from the CRA Agricultural Research Council, Rice research Unit, Vercelli, Italy (coordinates, 45° 20´N, 8° 25´W) as a model non-saline ecosystem. The paddy soil is of clay texture with a mean pH of 5.4, and contained a total carbon of 13.9 µg C mg g dw^{-1} and a total nitrogen of 1.3 µg N mg g dw^{-1} (Ho et al., 2015). Selected nutrients detected in the soil include NO_x (total of NO_2^- and NO_3^-; 34.4 µg g dw $soil^{-1}$), SO_4^{2-} (96 µg g dw $soil^{-1}$), and PO_4^{3-} (µg g dw $soil^{-1}$) (Ho et al., 2015); other nutrients (e.g., Fe_2^+, Fe_3^+, Cu_2^+, and multi-carbon compounds) are given elsewhere (Klüber and Conrad, 1998; Ho et al., 2013). We determined the response of the methane uptake rate and the methanotrophs to a salinity gradient ranging from 0 M (as reference) to 0.6 M NaCl (seawater concentration). Instead of artificial seawater, we used NaCl solution to determine the direct effect of salinity, and to eliminate potential confounding effects arising from the other components which may have been introduced along with the artificial seawater (e.g., sulphate, a component of the artificial seawater, may exert an effect on the methanotrophs; Krause et al, 2010). Additionally, bacterial respiration (CO_2 production) was shown to be most responsive to NaCl addition when compared to other salts (KCl, K_2SO_4, and Na_2SO_4; Rath et al., 2016). Na^+ and Cl^- ions were predominant in saline soils, correlating well to the conductivity measurement (EC) (Egamberdieva et al., 2010; Bissett et al., 2012). The methanotroph abundance and community composition were

monitored by targeting the *pmoA* gene (encoding for the particulate form of the methane monooxygenase enzyme) using group-specific qPCR assays and MiSeq sequencing, respectively. We focused on the *pmoA* gene because it is present in virtually all methanotrophs, with the exception of *Methylocella* and *Methyloferula* which harbor only the *mmoX* gene (gene encoding for the soluble form of the methane monooxygenase enzyme). However, *Methylocella* and *Methyloferula* are sensitive to salt-stress (Dedysh et al., 2011). Moreover, the transcription of the *mmoX* gene was not detected in the same soil when incubated under methane (Reim et al., 2013). The qPCR assays were performed as described in Kolb et al. (2003), with modifications (Ho et al., 2016a) to target aerobic gammaproteobacterial (subgroup type Ia and Ib) and alphaproteobacterial (subgroup type II) methanotrophs. Sequencing was performed after amplification of the *pmoA* gene using the A189f/A682r primer pair as described before (Reumer et al., 2018). Detailed methodology for the qPCR and MiSeq sequencing analyses are provided in the Supplementary Materials.

Response of the methanotrophic activity to salt-stress.

The effect of salt-stress on methane uptake was dose-dependent, with no discernable effect at 0.005 M NaCl and a complete inhibition at seawater concentration (0.6 M NaCl or 3.5%; Figure 1). Although methane uptake was immediately inhibited in the 0.3 M NaCl-amended incubation, activity partly recovered after three days (Figure 1). The NaCl threshold for methanotrophic activity appears to be approximately 0.3 M (~1.75% salinity) in the paddy soil, which is higher than the threshold exhibited by freshwater planktonic methanotrophs (0.1-0.5% salinity; Osundar et al., 2017), but lower than methanotrophs inhabiting an estuary (>3.5% salinity; Sherry et al., 2015). The salinities tested here are appreciably lower than salinities in hypersaline environments. Previously, aerobic methanotrophs were not detected

in a hypersaline microbial mat (> 8% salinity) despite of available methane and oxygen (Conrad et al., 1995). However, *Methylohalobius crimeensis* (type I) has since been isolated from a hypersaline lake, and shown to grow optimally at 87 g L^{-1} NaCl (8.7% salinity; Heyer et al., 2005). High salinity increases osmotic stress, decreasing water availability which dehydrates the cell, and limits methane oxidation (Khmelenina et al., 1999; Dalal et al., 2008; Rath & Rousk, 2015). Moreover, dissolved Na^+ and Cl^- ions can be toxic to bacterial cells (e.g. interaction with binding sites of enzyme; Serrano, 1996). Nevertheless, our results show that the methanotrophic activity in the Vercelli paddy soil is resistant to moderate salinity (<0.3 M NaCl).

The gradient in NaCl concentrations (0.005 M – 0.6 M) was relatively stable (Figure S1) and pH remained within a narrow range (6.3 - 6.5) during incubation. Hence, results showed a direct effect of salinity that was not confounded by a pH shift. However, increasing NaCl concentrations up to 0.3 M NaCl concurrently increases soluble ammonium concentration, likely caused by the displacement of NH_4^+ from binding sites (e.g., on clay minerals) by Na^+ (Table 1). Ammonium amendment selectively stimulated specific gammaproteobacterial methanotrophs (*Methylomicrobium* and *Methylocaldum*) in the same paddy soil (Noll et al., 2008). Conversely, elevated ammonium concentration may competitively inhibit methane oxidation under low methane availability (<100 ppm; King & Schnell, 1994). Therefore, salinization may have an indirect effect on the methanotrophic activity and community composition *via* increased ammonium availability.

Response of the methanotrophic abundance and community composition to salt-stress.

Methanotrophs respond to salt-stress not only at the cellular level (e.g. accumulation of compatible solutes; Khmelenina et al., 1999, Han et al., 2017; specific ion toxicities; Rath & Rousk, 2015; Rath et al., 2016) but also, at the community level over prolonged periods. Although salt-stress may immediately inhibit methanotrophic activity, a salt-tolerant community may emerge and thrive from the existing pool of methanotrophs or from the seedbank reservoir over time (Ho et al., 2016b), gradually replacing less salt-resistant members. The qPCR was performed to enumerate the *pmoA* gene copy numbers during incubation, to be used as a proxy to assess growth. Generally, we detected a decrease in all methanotroph populations (type Ia, Ib, and II subgroups) in both the NaCl- and un-amended incubations over time (Figure 2). The continuously high substrate supply (headspace methane at 0.5 – 5 $\%_{v/v}$) throughout the (pre-)incubation may have depleted the availability of other nutrients, as a result of a rapid population growth. Hence, nutrient limitation other than methane may have constrained the methanotroph population, as well as the methanotrophic activity during incubation (Figures 1 & 2). Focusing on the response of the individual methanotroph subgroups, the qPCR analyses revealed significant increase in type Ia *pmoA* gene copies with increasing salinity up to 0.3 M NaCl (Figure 2), indicating a shift in the methanotrophic community towards a predominance of type Ia methanotrophs. The increased dominance of type Ia methanotrophs in the 0.3 M NaCl-amended incubation corroborated with the recovery in methanotrophic activity and decrease in ammonium concentration (> 3 days) after pre-incubation (Figures 1 & S2). The apparent stimulatory effect on type Ia methanotrophs may be attributable to increased ammonium bioavailability, an indirect effect of NaCl amendment (Table 1), as has been shown before in this paddy soil (Noll et al., 2008). In contrast, the numerical abundance of type Ib methanotrophs was adversely affected with increasing salinity, while the type II methanotrophs were apparently unaffected

by the salt-stress up to 0.1 M NaCl amendment (Figure 2). Thereafter, values decreased and remained unchanged during incubation. Consistent with a recent study (Han et al., 2017), the type II methanotrophs were resistant to relatively low salt-stress (<1% NaCl). Hence, salt-stress differentially affected the methanotrophic population, favoring type Ia methanotrophs at salinities < 0.3 M NaCl.

The response of the methanotrophic community composition was further resolved by MiSeq sequencing of the *pmoA* gene, and visualized as a principal component analysis (PCA; Figures 3 & S3). Amplification of the *pmoA* gene using the A189f/A682r primer pair also captured sequences with ambiguous affiliation, falling between the methane- and ammonium-oxidizers (Figure 3A). Excluding these sequences from the ordination revealed a divergent community in the 0.3 M NaCl-amended soil after incubation, which could be separated from the community in the un-amended soil along PC axis 2 (Figure 3B). Consistent with the qPCR analysis, a type Ia methanotroph (*Methylobacter*) become more dominant and was indicative of the community in the 0.3 M NaCl-amended soil (Figure 3B & Table 2). *Methylobacter*, along with other members of the type Ia subgroup (*Methylosarcina*-, *Methylomonas*-, and *Methylomicrobium*-like methanotrophs) formed the predominantly metabolically active methanotrophs in saline environments (e.g., e.g. mangrove, alkaline lake, estuary; Antony et al., 2010; Deng et al., 2017; Osundar et al., 2017; Shiau et al., 2018). Hence, similar methanotroph subgroups/genera from both saline and non-saline environments showed resistance to salt-stress. Although the physiological response of type Ia methanotrophs to salt-stress may differ at the cellular level, future studies determining whether salt resistance is a universal trait among this subgroup/genus warrants attention.

Although we did not anticipate the methanotrophic community composition to remain unchanged during the incubation, the community in the soil amended with 0.6 M NaCl did not show a clear trend after incubation (Figure 3). In this incubation, the decreased *pmoA* gene copies along with non-detectable methane uptake strongly suggest that the methanotrophs were adversely affected by the salt-stress, and may not have survived the amendment (Figures 1 & 2). This was further supported by the steady increase in soluble ammonium concentration from ~3 (day 0) to ~10 mmoles g dw $soil^{-1}$ (day 7), which could have been caused by mineralization (Figure S2). Taken together, the methanotrophic community composition, determined at the DNA-level, may have been obscured by disproportionally persistent relic DNA in this incubation when compared to the other amendments, partly explaining the rather dispersed community in the PCA. The exact reason for this observation remains to be elucidated, and may be aided by transcript-based analyses, or viability PCR differentiating intact cells from extracellular DNA (Ho et al., 2013; Carini et al., 2016) for future work.

Overall, we showed that methanotrophs indigenous to a non-saline environment were remarkably resistant to salt stress. Supporting our hypothesis, a subgroup of methanotrophs (type Ia; *Methylobacter*) were favored and proliferated at increasing salinity up to 0.3 M NaCl, while others (type Ib) were adversely affected by the salt-stress, suggesting niche differentiation among gammaproteobacterial community members. Our results thus imply that the type Ia methanotrophs are relevant under moderate salt-stress, enabling methane oxidation under the incubation conditions at < 0.3 M NaCl; the loss of this subgroup may result in diminished resistance of the methanotrophic activity to salinization in paddy soils. Besides methanotrophs, the accompanying microorganisms may also be relevant members of the community (Ho et al., 2016c; Veraart et al., 2018), attenuating salt-stress.

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Tables

Table 1: Proportional increase in NaCl and soluble ammonium concentrations. Changes in soluble ammonium concentration over time is given in Figure S2.

Amendment (M NaCl)	Soluble NH_4^+ (mmoles g dw $soil^{-1}$)*
0 (reference)	1.00±0.10
0.005	1.43±0.42
0.5	1.54±0.12
0.1	1.94±0.05
0.3	3.48±0.13
0.6	2.95±0.12

*Soluble ammonium was determined in triplicate (mean ± s.d.) by a colorimetric method in autoclaved deionized water (1:5) after filtration (0.2 µm) as described before (Palmer and Horn, 2012). Total ammonium (soluble and adsorbed), determined in 2 M KCl (1:5), was approximately 3.6 mmoles g dry weight $soil^{-1}$. Total ammonium was solubilised after amendment with 0.3 M NaCl.

Table 2: Relative abundance (%) of the *pmoA* gene diversity. Only *pmoA* sequences with known affiliations are shown, corresponding to Figure 3B.

Amendment[§]	Type I (Unc.)	*Methylobacter* (type Ia)	*Methylomonas* (type Ia)	*Methylosarcina* (type Ia)	Type Ia (Unc.)	OSC-related (type Ib-related)	RPC (type I-related)	Type Ib (Unc.)	*Methylocystis* (type II)	Type IIa (Unc.)
0 M NaCl										
0 day	6.2±5.8	11.0±12.8	n.d.	2.4±4.1	n.d.	78.0±20.1	1.3±2.2	1.3±2.2	n.d.	n.d
3 day	14.3±19.9	1.3±2.3	n.d.	12.2±21.1	0.6±1.1	51.9±6.7	2.5±2.2	2.5±2.2	14.6±22.1	n.d.
7 day	11.5±4.7	n.d.	n.d.	3.3±5.8	n.d.	76.8±6.7	2.6±4.4	n.d.	5.9±5.2	n.d.
0.005 M NaCl										
3 day	n.d.	7.1±12.3	2.0±3.5	n.d.	n.d.	57.6±4.7	12.0±7.1	7.7±10.8	12.1±21.0	1.5±2.6
7 day	9.4±8.1	n.d.	n.d.	1.2±2.0	n.d.	68.8±22.6	14.3±24.7	n.d.	4.7±5.4	1.8±3.0
0.05 M NaCl										
3 day	3.7±6.4	n.d.	n.d.	3.7±6.4	n.d.	85.2±25.7	7.4±12.8	n.d.	n.d.	n.d
7 day	16.1±5.3	n.d.	n.d.	n.d.	n.d.	77.3±6.4	3.3±5.8	3.3±5.8	n.d.	n.d
0.1 M NaCl										
3 day	5.3±6.5	2.5±4.3	n.d.	n.d.	1.7±2.9	81.2±6.8	1.9±2.7	3.3±5.8	4.2±7.2	n.d
7 day	2.8±4.8	4.2±7.2	n.d.	n.d.	n.d.	83.3±14.4	7.0±6.4	2.8±4.8	n.d.	n.d
0.3 M NaCl										
3 day	6.1±5.4	10.5±13.0	n.d.	n.d.	n.d.	76.8±23.3	3.3±2.8	3.3±5.8	n.d	n.d
7 day	1.2±2.1	60.5±43.0	n.d.	n.d.	n.d	37.0±39.0	n.d	1.2±2.1	n.d	n.d
0.6 M NaCl										
3 day	0.8±1.3	n.d.	n.d.	n.d.	n.d.	67.7±18.3	21.7±13.6	9.8±13.3	n.d.	n.d
7 day	3.7±6.4	18.5±32.1	n.d.	n.d.	n.d.	44.4±51.0	n.d.	n.d.	n.d.	n.d

Abbreviations: n.d., not detected; Unc., Unclassified; OSC, organic soil clones originated from Finland; RPC, rice paddy cluster.

[§]Values are given in % derived from the mean ± s.d. of triplicate incubations.

Figure Legend

Figure 1: Methane depletion over time (a), and methane uptake rate (b) as determined from (a) by linear regression (mean ± s.d., *n* = 3). The incubation, performed in a 120 ml bottle, comprised of 5 g air-dried soil and 5 ml autoclaved deionized water, and the initial headspace methane was adjusted to 5 $\%_{v/v}$. Incubation was performed in the dark on a shaker (140 rpm) at 25^{O}C. After 5 days (pre-incubation), 5 ml NaCl solution was added to the soil slurry to achieve a final concentration of 0.005 M, 0.05 M, 0.1 M, 0.3 M, and 0.6 M, corresponding to a mean EC value of 0.6, 4.8, 9.4, 28.0, and 55.1 4 dS m^{-1}, respectively. In the reference incubation, 5 ml autoclaved deionized water was added instead of NaCl solution (mean EC, 0.2 dS m^{-1}). Generally, a soil is considered saline when EC > 4 dS m^{-1}. Incubation resumed for 7 days, during which headspace gas was replenished and methane adjusted to 5 $\%_{v/v}$ after 3 days. Headspace methane was measured using gas chromatography (GC) coupled to a thermal conductivity and pulsed discharge helium ionization detector (7890B, Agilent Technologies, JAS GC systems, Moers, Germany). In (a), arrows indicate methane replenishment. In (b), letters indicate the level of significance (ANOVA; $p<0.01$) between treatments per time.

Figure 2: qPCR analysis of MBAC (A), MCOC (B), TYPEII (C), and EUBAC (D) assays, targeting the type Ia, type Ib, and type II methanotrophs, and the total bacteria, respectively. The qPCR assays were applied to the samples after pre-incubation (designated as 0 d), and 3 and 7 days after pre-incubation. The letters indicate the level of significance (ANOVA; $p<0.01$) between treatments (un-amended, and NaCl-amended incubations) per time.

Figure 3: Principal component analysis showing the response of the total *pmoA* gene sequences, including sequences with ambiguous affiliation (A) and *pmoA* sequences of known affiliation (B) to NaCl amendments. The methanotrophic community composition was derived from Illumina MiSeq sequencing of the *pmoA* gene amplicon (A189f/A682r primer pair). Sequencing was performed for each DNA extract (*n*=3) per time and treatment. The vectors indicate predominant methanotrophic affiliations/genera. The distribution and affiliation of all methanotrophs are given in the Supplementary Material (Figure S3). Unclassified environmental sequences with ambiguous identity/affiliation (in between ammonium- and methane-oxidizers; e.g., 'unclassified methanotroph-like *pmoA*' and gp23), as well as ammonium-oxidizers in (A) were omitted for the analysis in (B). Abbreviation: RPC, rice paddy clusters (type I-related); OSC, organic soil clones originated from Finland (type Ib-related). The *pmoA* gene sequences were deposited at the EMBL European Nucleotide Archive (ENA) under the project accession number PRJEB25534.