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EFFECT OF DIFFERENT OSMOLYTES ON BACTERIA AND ARCHAEA AMMONIA OXIDISERS

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Abstract

This study is aimed to investigate response of ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB) to different concentrations of NaCl or sorbitol using culture media. It was hypothesized that an increase in concentrations will reduce nitrification activity of AOA and AOB, irrespective of NaCl or sorbitol. Two AOA (Candidatus *(Ca.) Nitrosotalea sinensis,* Candidatus *Nitrososcosmicus franklandus*) and AOB (*Nitrosomonas europaea* (ATCC 19718) and *Nitrosospira multiformis* (ATCC 25196)) were grown aerobically without shaking in a freshwater medium (FWM) adjusted to seven different concentrations of either NaCl or sorbitol. AOB were incubated at 25°C while AOA were incubated at 35°C both in the dark. Aliquots of 100 µl were taken from each sample at 24 hours intervals for 250 hours and analysed for nitrite concentration. Both AOA and AOB responded to changes in NaCl or sorbitol concentrations in the culture media as nitrification activity decreased with an increase in NaCl or sorbitol concentrations in both cultures. AOA were more sensitive to the increase in NaCl or sorbitol concentrations than AOB. *Ca. Nitrosotalea sinensis* was the most sensitive while *N. europaea* is the least sensitive ammonia oxidisers to changes in increase in NaCl or sorbitol concentrations. This study confirmed that there were differences in the osmolytes tolerance between AOA and AOB upon exposure to similar concentration of NaCl or sorbitol.

Keywords: Archaea, bacteria, NaCl, sorbitol, nitrite, concentration, ammonia oxidation.

Introduction

Archaea and bacteria, both respond to changes in the concentrations of chemical substances in their environment by producing and acquiring compatible solutes referred to as osmolytes or osmo-protectant (RoeBler and Muller, 2001). When bacteria are exposed to a higher osmotic stress, they absorb high concentration of potassium ion (K^+) into the cell from the environment through turgor-responsive transport system. Within a minute of the exposure to hyperosmotic condition, glutamic acid (which provides counter ions for the high uptake of K^+) production in the cell is increased. This process helps to maintain a steady state of K^+ concentration within the cell (McLaggan *et al.*, 1994; Yan *et al.*, 1996). This mechanism is slightly adjusted in non-halophilic bacteria by *de novo* synthesis and/or accumulation of compatible solutes which permit the cell to release K^+ through specific and non-specific efflux systems back into the environment (Csonka and Epstein, 1996; Stumpe *et al.*, 1996).

The process of accumulating compatible solutes occurs in Gram positive bacteria at all environmental conditions while Gram negative bacteria do not absorb osmolytes from the environment unless they are subjected to the harsh environmental conditions such as hypertonic solutions (RoeBler and Muller, 2001). The possible reason for the differences in the turgor pressure between Gram positive and Gram negative bacteria (Peddie *et al.*, 1998). In general, Gram-negative bacteria are more

susceptible to dehydration than the Gram-positive bacteria. The reason for the higher resistance of Gram-positive bacteria could be attributed to their smoother surfaces i.e.; the thicker peptidoglycan layer and the lack of lipopolysaccharides (Peddie *et al.*, 1998; Miyamoto-Shinohara *et al.*, 2008). However, in most archaea K^+ is maintained at high intracellular concentrations under non-salt stress condition. The exposure of archaea to a high osmotic stress causes the accumulation of more K^+ and synthesis of glutamate (which provides the counterion) (RoeBler and Muller, 2001). Archaea proteins are rich in acidic amino acids (i.e., glutamate and aspartate) which help to neutralize excess K^+ in the cell. This mechanism is slightly altered at higher salt concentration (>1M NaCl) by replacement of glutamate with the synthesis of N-acetyl-β-lysine (Martin *et al.*, 2001; Zhao *et al.*, 2015).

Environmental microorganisms are also likely to experience hypotonic conditions (osmotic downshock) which are caused by rainfall and washout into a fresh water body. Microbial cell possesses an osmo-protectant efflux system which helps to pump out selective osmo-protectant from the cell within minutes of the hypotonic shock. This process reduces the intracellular pressure (turgor pressure), prevents cell lysis and maintains a homeostatic system in the cell (Ruffert *et al.*, 1997). There is a clear difference in the structure and types of osmolytes found in both archaea and bacteria. For example, polyol, phosphodiesters and glucosyl glycerateare uniquely found in archaea while glycerol, glucosyl-glycerol, proline and ectoine are found solely in bacteria (Bhaganna *et al.*, 2016; Tao *et al.*, 2016).

Several researches have been conducted to determine the quality and quantity of osmolytes that are either utilized or synthesized by microorganisms in response to different salt concentrations. A higher number of research on bacteria and archaea response to salt stress have been conducted using different strains of microorganisms such as *Salmonella* sp., *E. coli*, *Bacillus subtilis*, *Listeria monocytogenes, Pseudomonas* sp. and Methanotrophic archaea (Metris *et al.*, 2014; Zhao *et al.*, 2015; Bhaganna*et al.*, 2016). Besides, RoeBler *et al.* (2001) demonstrated that the growth of methanogen *Methanosarcina mazei* Gö1 decreased in response to an increase in NaCl concentration. However, Metris *et al.* (2014) observed a decrease in specific growth rate of *E. coli* with increasing NaCl concentration. Several studies were also conducted to observe the expression, upregulation or downregulation of gene(s) of interest in bacteria or archaea. Despite the differences in the compatible solutes found in bacteria and archaea in response to osmotic stress, there is no known literature yet that compared the response of both organisms (archaea and bacteria) to the same osmolytes nor compare the effect of different osmolytes (electrolyte and non-electrolyte osmolyte let alone the ammonia oxidising bacteria (AOB) and archaea (AOA). Thus, the exploration of other organism responses to environmental (osmotic) stress is equally important. This study is aimed at investigating the effect of different osmolytes on AOB and AOA, with a hypothesis that an increase in the concentration of NaCl or sorbitol will reduce the nitrification activity of both AOA and AOB in culture.

Materials and Methods

AOA and AOB Strains and Culture Conditions

All ammonia oxidisers (AOA and AOB) used for this experiment were obtained from continued sub-culturing of soil isolates. The ammonia oxidising archaea *ca. Nitrosotalea sinensis* and *ca. Nitrosocosmicus franklandus* and the ammonia oxidising bacteria *Nitrosomonas europaea* (ATCC 19718) and *Nitrosospira multiformis* (ATCC 25196) were grown aerobically without shaking in a freshwater medium (FWM). *ca.* N. sinensis (Nd2) and *ca. N. franklandus* were grown in FWM

according to Lehtovirta-Morley *et al.* (2011, 2016). The media for AOA contained ammonium chloride at concentrations of 1 mM for *ca. N. franklandus* and 0.5 mM for *ca. N. sinensis*. *N. europaea* and *N. multiformis* were cultured in 50 ppm Skinner and Walker medium (Skinner and Walker, 1961) adjusted to ~ pH 8.0 with Na_2CO_3. The culture media were inoculated with 2 % (v v^{-1}) of exponential growth phase cultures of AOA and AOB. AOB were incubated at 25°C while AOA were incubated at 35°C. AOA and AOB cultures were incubated in the dark and sampled (100 µl) at intervals of 24 hours for 250 hours. The purity of all ammonia oxidisers cultures used in this study was confirmed on 5% nutrient agar medium, incubated under the same condition as the liquid culture.

Experimental Design and Sample Analysis

The response of ammonia oxidising archaea and bacteria to different osmolyte was investigated using 50 ml of FWM (in a sterile 100 ml Duran bottles) adjusted with NaCl according to Metris *et al.,* (2014) and sorbitol separately. AOA and AOB culture media were adjusted to 0, 0.05, 0.1, 0.2, 0.25, 0.3 and 0.4 M of NaCl or sorbitol. Sorbitol (a non-electrolyte osmolyte) was used to distinguish the effects of different osmolyte and NaCl, for Na^+ was shown to have cytotoxic effect on microbial cells (Lanyi, 1979). The growth media with 0 molar NaCl concentrations was used as the control.

Determination of Nitrite concentration and maximum specific growth

Nitrite (NO_2^-) concentration was measured colourimetrically using a 96-well plate. The nitrite production was measured by diazotising and coupling with Griess reagent (Shinn, 1941). Duplicate standard ranged between 0 and 100 µM of $NaNO_2$was used. Absorbance was recorded at 540 nm using an Infinite F50_ microplate reader (Labtech, Uckfield, UK). The ammonia oxidising activity was determined by measuring increases in nitrite (NO_2^-) concentration over time for each culture with different NaCl treatment. The increase in the nitrite production by ammonia oxidisers correlates with increasing cell densities. This parameter has been routinely used to approximate the specific growth of ammonia-oxidisers (Powell and Prosser, 1992; Konneke *et al.*, 2005; Lehtovirta-Morley *et al.*, 2011: 2016). The maximum specific growth rate was estimated by calculating the slope during the exponential growth phase of semi-logarithmic plots of nitrite concentration over time, as previously described by Powell and Prosser (1992), Konneke *et al.* (2005) and Lehtovirta-Morley *et al.* (2011).

Statistical analysis

The results obtained were subsequently used to calculate the percentage growth rate of AOA and AOB with an increase in NaCl and sorbitol concentration. The nitrite produced by different organisms at different NaCl and sorbitol concentration were log transformed and the statistical analyses were done using the program R 3.2.2 (Sarkar *et al.*, 2015). The differences in growth rate were analyzed by One-way ANOVA. While Tukey HSD multiple comparisons of means were used to show the differences among the means at 95% family-wise confidence level.

Results

Growth of Ammonia Oxidisers in Response to Different Concentrations of NaCl and Sorbitol

All AOA and AOB strains used in this experiment grew exponentially during incubation in the dark. The initial increase in nitrite concentration obtained for *N. europaea*, *N. multiformis* and *ca. N. franklandus* were due to carryover of nitrite with inocula. The effect of different concentrations of NaCl or sorbitol on the four cultivated AOA and AOB strains was investigated by assessment of their (NaCl or sorbitol) influence on maximum specific growth rate of the tested AOA and AOB during exponential growth in liquid batch culture. Maximum specific growth rate

was estimated as the specific rate of increase in nitrite concentration and data were presented as absolute values (Fig. 1 - 4). The maximum specific growth rate of *N. europaea*, *N. multiformis*, *Ca. N. franklandus* and *Ca. N. sinensis* are 0.028, 0.024, 0.015 and 0.025 h^{-1} respectively in the control treatment (Fig. 1 - 4). Maximum specific growth rate of both AOA and AOB decreased with increasing NaCl concentration and AOA were more sensitive than AOB (Fig. 1 - 4). Within AOB, *N. multiformis* was more sensitive to NaCl than *N. europaea*, and both were almost completely inhibited by 0.4 M NaCl. Within AOA, *Ca. N. sinensis* was less sensitive than *Ca. N. franklandus* which produced no detectable nitrite in medium 0.1 M NaCl.

The growth of *N. europaea* was inhibited by sorbitol but was significantly less than that of NaCl ($p < 0.001$). Sorbitol did not reduce the growth of *N. multiformis* over the range tested. Both AOA were inhibited by sorbitol and both were significantly more sensitive than AOB ($p < 0.001$). *Ca. N. sinensis* was unable to grow at 0.05 M sorbitol, the lowest concentration investigated, and sensitivity was therefore greater than that of NaCl. Contrastingly, *Ca. N. franklandus* was less sensitive to inhibition by sorbitol than NaCl and relatively small increases in inhibition at higher sorbitol concentrations (Fig. 4). AOA were therefore more sensitive than AOB to the effects of different concentrations of both NaCl and sorbitol.

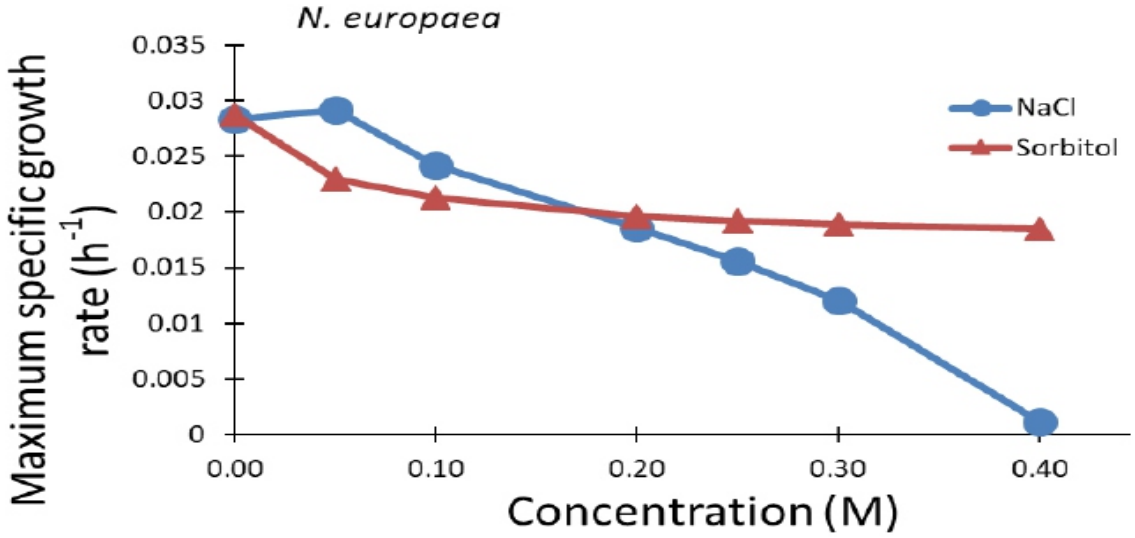

Fig. 1. The maximum specific growth rate (h^{-1}) of ammonia oxidising bacteria (*N. europaea*) during batch growth in liquid medium containing different concentrations of NaCl (blue colour) or sorbitol (red colour). Data are plotted as the mean and standard error of values calculated from triplicate cultures.

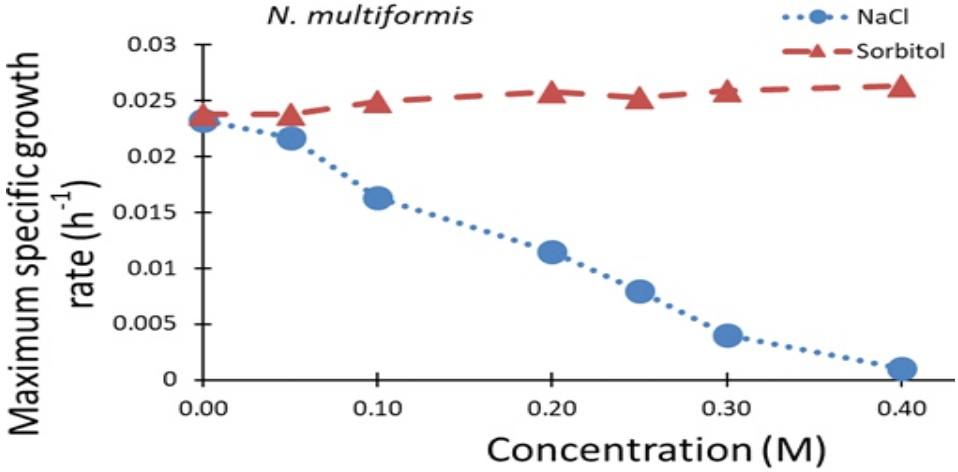

Fig. 2. The maximum specific growth rate (h^{-1}) of ammonia oxidising bacteria (*N. multiformis*) during batch growth in liquid medium containing different concentrations of NaCl (blue colour) or sorbitol (red colour). Data are plotted as the mean and standard error of values calculated from triplicate cultures.

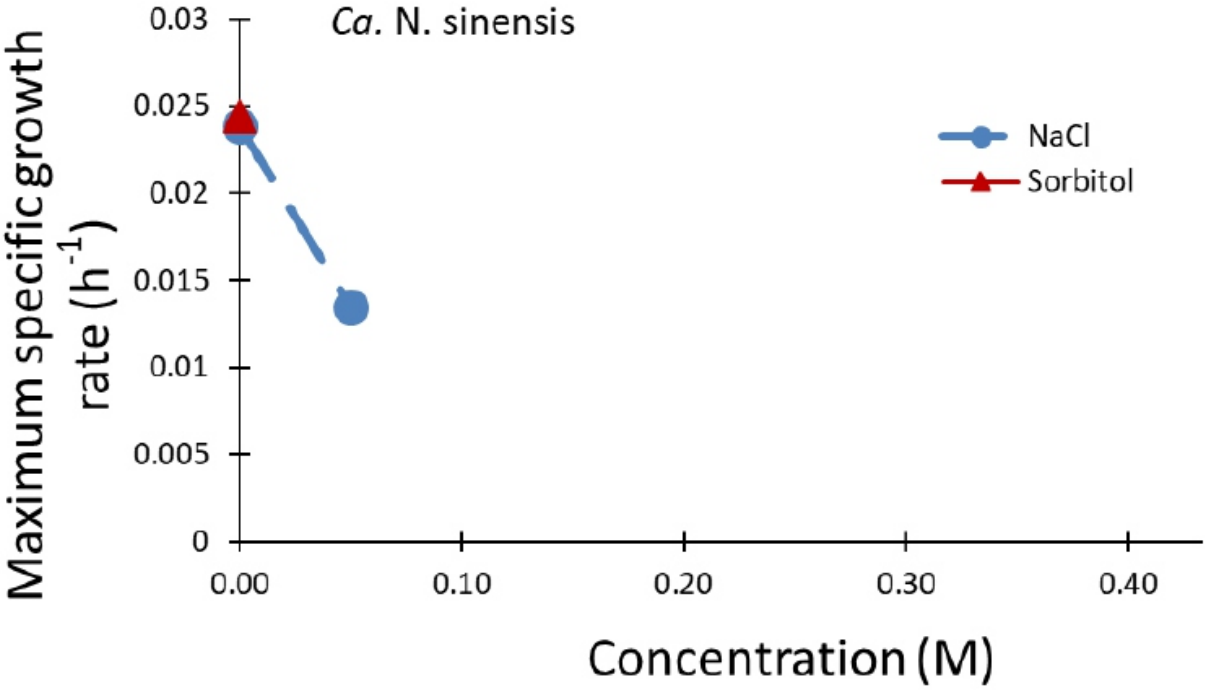

Fig. 3. The maximum specific growth rate (h^{-1}) of ammonia oxidising archaea (*N. sinensis*) during batch growth in liquid medium containing different concentrations of NaCl (blue colour) or sorbitol (red colour). Data are plotted as the mean and standard error of values calculated from triplicate cultures.

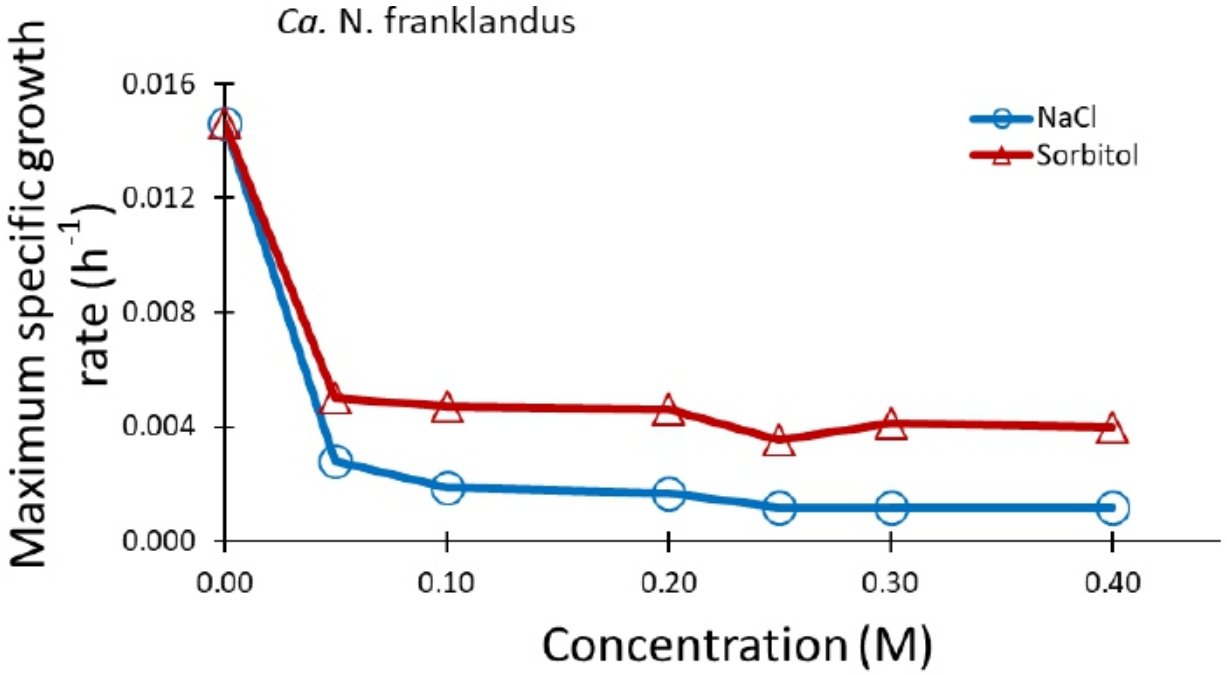

Fig. 4. The maximum specific growth rate (h^{-1}) of ammonia oxidising archaea (Ca. *N. franklandus*) during batch growth in liquid medium containing different concentrations of NaCl (blue colour) or sorbitol (red colour). Data are plotted as the mean and standard error of values calculated from triplicate cultures.

Discussion and Conclusion

The results obtained in this study showed that both AOA and AOB respond swiftly to changes in NaCl or sorbitol concentration in the culture media. This resulted to the decrease in both the nitrification activity and growth rate with an increase in NaCl or sorbitol concentrations. These results uphold our hypothesis that increase in NaCl or sorbitol concentrations will reduce the nitrification activity of ammonia oxidising archaea and bacteria.

The specific growth rate obtained for the *N. europaea* is lower than 0.125 h^{-1} obtained

from *N. europaea* in the culture medium by Sato *et al.* (1985). Contrastingly, the maximum specific growth rate obtained from *N. europaea* and *N. multiformis* are within the maximum specific growth rate of AOB that are isolated from soil 0.005–0.044 h^{-1} (Belser,1980; Jiang and Bakken, 1999; Prosser and Nicol, 2012). However, the low maximum specific growth rate obtained for both AOB could be attributed to the low incubating temperature of 25°C as previous studies have shown that the optimum growth temperature for AOB in culture is better still better still 28°C (Jones and Moritar, 1985; Koops and Harms 1985).

The maximum specific growth obtained for AOA (*Ca. N. sinensis* and *Ca. N. franklandus*) in the control samples are 0.025 and 0.015 h^{-1}. This result corroborates Lehtovirta-Morley *et al.* (2014; 2016). The sensitivity of ammonia oxidisers was determined using the maximum specific growth rate shows that AOA are more sensitive to increase in the concentrations of osmolytes (NaCl and sorbitol) than AOB. Based on the results obtained from this experiment, *Ca. N. sinensis* is the most sensitive while N. europaea is the least sensitive ammonia oxidisers to changes in the concentration of osmolytes. Using culture dependent technique, we have successfully shown that the growth and nitrification activity of ammonia oxidisers decreased with an increase in the concentration of osmolytes.

Conclusively, AOA were more sensitive to increase in the concentration of osmolytes (both electrolyte and non-electrolyte) than AOB, thus confirming the differential response of AOA and AOB to changes in the concentrations of osmolytes (which affects the availability of water for microbial metabolic processes) as a niche differentiating factor between AOA and AOB.

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