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Expression of *merA*, *amoA* and *hao* in Continuously Cultured *Nitrosomonas europaea* Cells Exposed to Zinc Chloride Additions

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ABSTRACT: The effects of $ZnCl_2$ additions on a mercuric reductase, *merA*, ammonia monooxygenase, *amoA*, and hydroxylamine (NH_2OH) oxidoreductase, *hao*, gene expression were examined in continuously cultured *Nitrosomonas europaea* cells. The reactor was operated for 85 days with a 6.9 d hydraulic retention time and with four successive additions of $ZnCl_2$ achieving maximum concentrations from 3 to 90 μM Zn^{2+}. Continuously cultured *N. europaea* cells were more resistant to Zn^{2+} inhibition than previously examined batch cultured cells due to the presence of Mg^{2+} in the growth media, suggesting that Zn^{2+} enters the cell through Mg^{2+} import channels. The maximum *merA* up-regulation was 45-fold and expression increased with increases in Zn^{2+} concentration and decreased as Zn^{2+} concentrations decreased. Although Zn^{2+} irreversibly inactivated ammonia oxidation in *N. europaea*, the addition of either 600 μM $CuSO_4$ or 2250 μM $MgSO_4$ protected *N. europaea* from $ZnCl_2$ inhibition, indicating a competition between Zn^{2+} and Cu^{2+}/Mg^{2+} for uptake and/or AMO active sites. Since $ZnCl_2$ inhibition is irreversible and *amoA* was up-regulated at 30 and 90 μM additions, it is hypothesized that de novo synthesis of the AMO enzyme is needed to overcome inhibition. The up-regulation of *merA* during exposure to non-inhibitory Zn^{2+} levels indicates that *merA* is an excellent early warning signal for Zn^{2+} inhibition.

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KEYWORDS: *Nitrosomonas europaea*; chemostat; Zn^{2+} inhibition; *merA*; *amoA*; ammonia-oxidizing bacteria

Introduction

Heavy metals, such as Zn^{2+}, enter wastewater treatment plant (WWTP) influents primarily from industrial discharges and urban storm water runoff (Bitton, 1999; Davis et al., 2001; EPA, 2005) and are potent inhibitors of the nitrification process (EPA, 1993; Hu et al., 2003; Juliastuti et al., 2003). The oxidation of ammonia (NH_3) to nitrite (NO_2^-) by ammonia oxidizing bacteria (AOB) in WWTPs is often considered the most sensitive step in the nitrification process (EPA, 1993). Zn^{2+} has been shown to be highly inhibitory towards AOB, including *Nitrosomonas europaea* (Park and Ely, 2008; Radniecki and Ely, 2008).

N. europaea, an obligate chemolithoautotroph, derives all if its energy for growth solely from the oxidation of NH_3 to NO_2^- (Wood, 1986). This two-step process utilizes ammonia-monooxygenase (AMO) to oxidize NH_3 to hydroxylamine (NH_2OH) and hydroxylamine oxidoreductase (HAO) to oxidize NH_2OH to NO_2^-. The second oxidation releases four electrons, two of which are used to reduce AMO and two of which are shuttled further down the electron transport chain for cell growth and maintenance (Arp et al., 2002). The relatively low number of electrons generated from ammonia oxidations leads to a slow doubling time of 8–12 h even under ideal conditions (Watson et al., 1981). The sensitivity of *N. europaea* to metal toxicity combined with the slow growth rate can result in substantial down-time after a nitrification failure event. The early detection of Zn^{2+}-mediated nitrification inhibition in AOB may be useful in preventing nitrification failure at WWTPs.

This work examined the physiological and transcriptional responses of continuously cultured *N. europaea* cells upon long-term exposure to $ZnCl_2$. Several genes that may act as sentinel genes to indicate Zn^{2+} inhibition were chosen to be evaluated throughout the course of the experiment including *merA*, a mercury resistance protein found to be highly up-regulated in batch tests with *N. europaea* (Park and Ely, 2008). Because of the importance of AMO and HAO in *N. europaea*'s metabolism, *amoA* and *hao* gene expressions were also monitored throughout the experiment. Physiological responses such as growth and metabolic activity were examined throughout the experiment.

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There were several advantages of using continuously cultured *N. europaea* cells, as opposed to batch studies, such as the ability to examine the long-term physiological and transcriptional responses of *N. europaea* to Zn^{2+} inhibition. Through this long-term exposure study, we were able to evaluate the sensitivity of *merA*, *amoA* and *hao* to a variety of Zn^{2+} concentrations and determine how quickly these genes responded to Zn^{2+}, how long the response lasted as Zn^{2+} concentrations decreased and what were the upper limits of Zn^{2+} concentrations to which they responded. Additionally, the format of continuously cultured *N. europaea* cells allowed for the investigation of possible inhibition and recovery mechanisms exhibited in response to Zn^{2+}.

Pure cultures of *N. europaea* were continuously grown in a bioreactor under chemostat conditions and exposed to increasing concentrations of Zn^{2+} added as pulse inputs to mimic a pulse of Zn^{2+} that may come through WWTP. The effect of $ZnCl_2$ on nitrification activity, in particular to AMO and HAO activity, was monitored throughout the experiment. Because the mechanism of nitrification inhibition by Zn^{2+} remains unclear, experiments were conducted to help elucidate possible inhibition mechanisms.

AOB have been studied in continuous culture to identify the physiological responses to alterations in steady-state parameters including NH_3 (Tappe et al., 1999), NO_2, NO and O_2 levels (Sliekers et al., 2005; Zart and Bock, 1998), pH shifts (Groeneweg et al., 1994) and addition of organohydrazines (Logan and Hooper, 1995). Although a few studies have examined the gene expression of continuously cultured AOB in response to starvation conditions (Bollmann et al., 2005) and biofilm formation (Schmidt et al., 2004), to our knowledge, this research describes the first characterization of the dynamic gene expression by *N. europaea* under continuous culture conditions in response to long-term heavy metal exposure.

Methods

Organism and Cultivation

N. europaea (ATCC 19718) was cultivated in 3 L of minimal media in a 7 L water-jacketed Bioflo 110 bioreactor (New Brunswick Scientific, Edison, NJ) operated in batch mode. The bioreactor was operated in the dark at 30°C with agitation set to 500 rpm. Dissolved O_2 levels were kept at air saturation levels (8 mg/L) with the addition of filtered compressed air at 200 mL/min. The minimal medium contained the following: 12.5 mM $(NH_4)_2SO_4$, 25 mM NH_4OH, 10 μM KH_2PO_4, 730 μM $MgSO_4$, 200 μM $CaCl_2$, 9.9 μM $FeSO_4$, 0.65 μM $CuSO_4$ and 30 mM HEPES as a pH buffer. The pH of the minimal medium was maintained at 7.8 with the metered addition of 0.5 M Na_2CO_3 which also supplied *N. europaea* with an inorganic carbon source. Prior to inoculation, the bioreactor and minimal medium were autoclaved at 121°C for 40 min.

Experimental Design (Bioreactor)

The bioreactor was operated in batch mode until *N. europaea* cells lowered the NH_4^+ concentration below 5 mM. The bioreactor was then switched to a continuous culturing reactor. All conditions were kept as described above except the agitation was raised to 700 rpm and the dilution rate was set at 0.14 d^{-1} using an influent feed rate of 432 mL/day. The minimal medium feed was the same as that described above. Once steady-state was achieved within the bioreactor, as determined by OD_{600}, NO_2^- and NH_4^+ concentrations, $ZnCl_2$ was added as a spike addition and the OD_{600}, NO_2^-, NH_4^+, and Zn^{2+} concentrations along with ammonia-monooxygenase specific oxygen uptake rates (AMO-SOUR) and hydroxylamine oxidoreductase specific oxygen uptake rates (HAO-SOUR) were measured. Total RNA was extracted 1 h after the injection of the $ZnCl_2$, the same length of time that batch *N. europaea* cells were exposed to $ZnCl_2$ in previous experiments (Park and Ely, 2008), and every 7 days (1 hydraulic residence time) thereafter. This process was repeated with 3, 10, 30, and 90 μM $ZnCl_2$ spikes, over a period of operation from 35 to 85 days.

Analytical Procedures

Total NH_4^+ concentrations were measured from 3 mL bioreactor samples using a VWR SympHony Ammonia Concentration Electrode (VWR International Inc., West Chester, PA) per manufacturer's instructions. NO_2^- concentrations were determined by measuring the absorbance at 325 and 400 nm using a Beckman Coulter DU530 spectrophotometer (Fullerton, CA) and the following equation: NO_2^- (mM) $= (A_{352}–A_{400})/0.0225$. Nitrite concentrations for reversibility experiments were determined with a colorimetric assay. One milliliter cell suspensions were centrifuged at 16,000 rpm for 1 min. Triplicate 10 μL aliquots of supernatant were removed and placed in 890 μL of 1% w/v sulphanilamide in 1 M HCl and 100 μL of 0.2% w/v N(1-naphthyl) ethylenediamine dihydrochloride. The absorbance at 540 nm was measured spectrophotometrically (Hageman and Hucklesby, 1971). Zn^{2+} concentrations were measured at 213.9 nm using a Varian Liberty 150 ICP Emission Spectrometer (Varian, Inc., Palo Alto, CA) using standard procedures (APHA, 1995). A standard curve correlating the protein count of cells with OD_{600} was created using the biuret assay (Gornall et al., 1949).

AMO- and HAO-SOUR Measurements

AMO activity, AMO-SOUR, was measured as described by Ely et al. (1995) using a water-jacketed glass cell fitted with a Clark microelectrode (Yellow Springs Instrument Co, Model # 5331) attached to a YSI Model 5300 Biological Oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) and a flatbed strip chart recorder. To measure

HAO activity, HAO-SOUR, AMO activity was blocked with the addition of allyl thiourea (ATU; 100 μM) and hydrazine (750 μM) was added as an alternative substrate for HAO. The oxygen consumption rates were measured in the same manner as AMO-SOUR. Triplicate AMO- and HAO-SOUR measurements were made at each time point.

Reversibility Experiments

Five milliliters of *N. europaea* cells continuously cultured at a 0.14 d^{-1} dilution rate were withdrawn from the bioreactor, prior to the addition of $ZnCl_2$, and placed into a 25 mL vial and sealed with a Teflon coated cap. $(NH_4)_2SO_4$ was added to the bottles to a final concentration of 2.5 mM. $ZnCl_2$ was added to achieve a concentration of either 30 or 90 μM. Bottles without the addition of $ZnCl_2$ served as controls. The bottles were shaken at 250 rpm in the dark at 30°C for 15 min. After 15 min of exposure, the AMO-SOUR of the cells was measured, as described above. The cells were then centrifuged and washed five times in 30 mM HEPES buffer pH 7.8 to remove Zn^{2+}. The washed cells were placed into fresh medium containing 2.5 mM $(NH_4)_2SO_4$ and AMO-SOURs were measured after 15 min of incubation to determine whether the Zn^{2+} dependent inhibition of ammonia oxidation was reversible. Tests were performed in triplicate.

$CuSO_4$ and $ZnCl_2$ Competition Experiments

To determine if $CuSO_4$ would protect *N. europaea* from Zn^{2+} inhibition a series of AMO-SOUR experiments were also conducted with $CuSO_4$ present. Continuously cultured *N. europaea* cells were removed from the bioreactor at day 35 and placed into empty batch reactor bottles and had a cell concentration of 10 mg/L. $(NH_4)_2SO_4$ was added to the bottles to achieve a concentration of 2.5 mM. Various amounts of $CuSO_4$ were added to control bottles and various amounts of $CuSO_4$ and 120 μM $ZnCl_2$ were added to the treatment bottles. All bottles were incubated for 15 min prior to measuring the AMO-SOURs.

Reverse Transcriptase-Quantitative PCR (RT-qPCR) Analysis

Gene Runner v. 3.00 (Hastings Software, Inc., Hastings on Hudson, NY) was used to generate RT-qPCR primers from *16S*, *merA*, *amoA*, and *hao* DNA sequences. The RT-qPCR primers are as follows; 16S For: 5′-GGCTTCACACGTAATACAATGG-3′, 16S Rev: 5′-CCTCACCCCAGTCATGACC-3′, merA For: 5′-GCTTTATCAAGCTGGTCATC-3′, merA Rev: 5′-ACATCCTTGTTGAAGGTCTG-3′, amoA For: 5′-TGGCGACATACC-TGTCACAT-3′, amoA Rev: 5′-ACAATGCATCTTTGGCTTCC-3′, hao For: 5′-CAAACTTGCCGAAATGAACC-3′, and hao Rev: 5′-GCTGGTGATGTTCTCTGCAA-3′. The RT-qPCR primers were optimized for concentration and annealing temperature via PCR to achieve high efficiency with only one product detected. The optimal annealing temperature for each primer was 55°C. The optimal primer concentration, in each RT-qPCR reaction, was 125 nM for all primers used. Total RNA from cells, 200 mg wet-weight protein, was stabilized in Trizol (Invitrogen Co., Carlsbad, CA) and extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) per manufacturer's instructions. Total RNA concentrations and quality were determined spectrophotometrically using the Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) per manufacturer's instructions. cDNA was generated from 1 μg of total RNA using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA) per manufacturer's instruction and diluted 100-fold in TE buffer at pH 8. RT-qPCR was carried out in triplicate on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the iQ SYBR Green Supermix kit (Bio-Rad) as described previously (Park and Ely, 2008). The relative expression values for the reactions were determined using DART (Data Analysis for Real Time)-PCR analysis (Peirson et al., 2003) taking into account the efficiency of the reaction and normalizing the data to the amount of 16S RNA quantified in each reaction. For these experiments, the transcriptional responses of the bioreactor cells exposed to various Zn^{2+} concentrations were compared with the transcriptional responses of bioreactor cells that had achieved steady-state prior to Zn^{2+} addition.

Trace Metal Protection

To determine the protective properties of trace metal nutrients on *N. europaea* to Zn^{2+} inhibition, tests with batch grown cells were conducted. *N. europaea* cells were cultured as previously described (Park and Ely, 2008) and harvested at mid-exponential growth phase. Cells were washed with 30 mM HEPES buffer, pH 7.8 and suspended in 5 mL of media in a 25 mL vial. The medium consisted of 30 mM HEPES buffer, pH 7.8, 2.5 mM $(NH_4)_2SO_4$, 5 μM $ZnCl_2$ and a combination of the following growth medium salts; 10 μM KH_2PO_4, 270–2250 μM $MgSO_4$, 270–2250 μM $CaCl_2$, 18 μM $FeSO_4$, 1 μM $CuSO_4$, and 3.77 mM Na_2CO_3. Controls were run in the same medium without exposure to Zn^{2+}. Samples were shaken at 250 rpm at 30°C in the dark for 2 h and colorimetric NO_2^- measurements were taken every 0.5 h as described above.

Results

Continuously cultured *N. europaea* cells achieved steady state within a month. On day 37, $ZnCl_2$ was added to achieve a maximum concentration of 3 μM in the bioreactor (Fig. 1B). However, continuously cultured *N. europaea* cells did not appear to be affected by the addition of 3 μM $ZnCl_2$ nor to the addition of 10 μM $ZnCl_2$, based on the

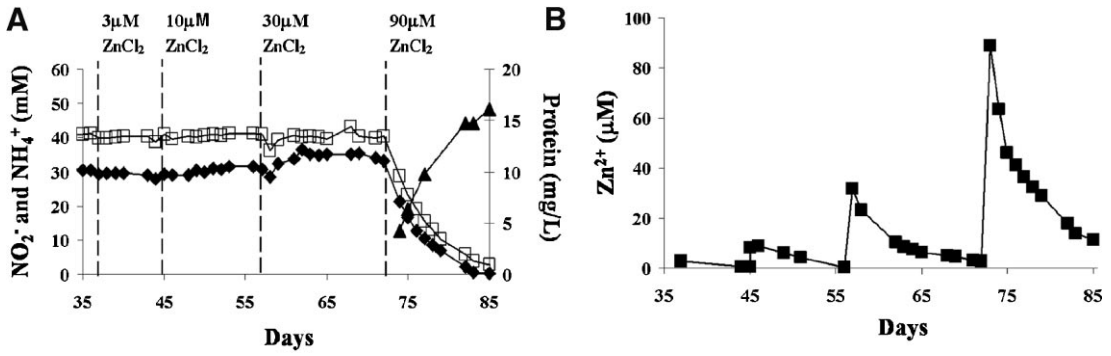

Figure 1. **A**: Physiological parameters of the chemostat monitored during the experiment. (◆) Protein, (□) NO_2^- and (▲) NH_4^+. Dashed lines represent when $ZnCl_2$ was spiked into the chemostat. **B**: The concentration of Zn^{2+} measured in the chemostat throughout the experiment.

measured NO_2^- and protein concentrations, 40 mM and 10 mg/L, respectively (Fig. 1A). After most of the Zn^{2+} had washed out of the reactor (Fig. 1B), a third addition of $ZnCl_2$ to a maximum concentration of 30 μM resulted in a 12% decrease in NO_2^- concentration and 9% decrease in protein concentration. Three days after the $ZnCl_2$ addition, however, the NO_2^- concentration returned to 100% of steady-state levels and the protein concentration increased to 114% of steady-state levels. Addition of $ZnCl_2$ to a concentration of 90 μM resulted in the complete washout of *N. europaea* from the bioreactor as indicated by the increase in NH_4^+ concentrations toward influent levels and decreases in protein and NO_2^- concentrations.

The expression of *merA* was monitored during the experiment (Fig. 2A). The expression of *merA* increased with each $ZnCl_2$ addition, peaking at 46-fold up-regulation after exposure to 30 μM $ZnCl_2$. As Zn^{2+} was washed out of the reactor after each addition, *merA* expression decreased toward background levels. The only exception was after spiking the bioreactor with 90 μM $ZnCl_2$. This lower expression of *merA* likely resulted from the lack of energy available in the *N. europaea* cells during this period due to the inhibition of AMO as shown below.

AMO and HAO activities were measured throughout the experiment using the SOUR method. The AMO- and HAO-SOUR did not significantly change upon exposure to 3 μM Zn^{2+} (Fig. 3A and B). However, unlike NO_2^- and protein concentrations, decreases in AMO (12%) and HAO (29%) activities were observed after the addition of 10 μM $ZnCl_2$. Background levels of HAO activity were regained 11 d after the $ZnCl_2$ addition. After the addition of 30 μM $ZnCl_2$, a 92% decrease in AMO activity and a 49% decrease in HAO activity were observed. Only small decreases in NO_2^- and protein concentrations were observed in the bioreactor (Fig. 1A). Once again, recovery of 81% of AMO activity and 100% of HAO activity were achieved 14 d after the $ZnCl_2$ addition. The addition of 90 μM $ZnCl_2$ created a 94% decrease in AMO activity with no recovery observed. This result was consistent with the increase in NH_4^+

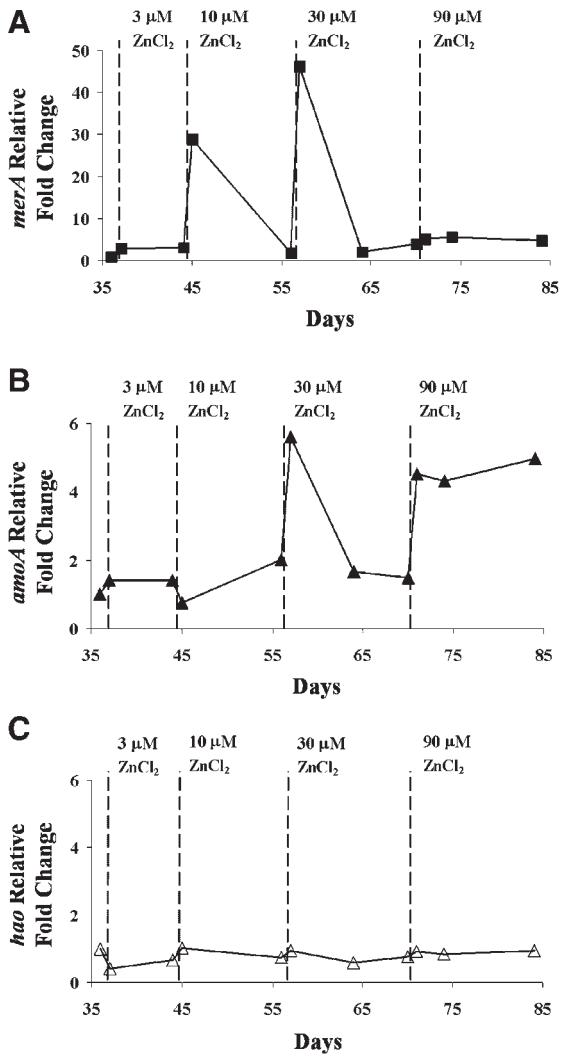

Figure 2. The relative fold change of *merA* (**A**); *amoA* (**B**); and *hao* (**C**) in continuously cultured *N. europaea* cells exposed to $ZnCl_2$ additions. Dashed lines indicate when $ZnCl_2$ was pulsed into the bioreactor.

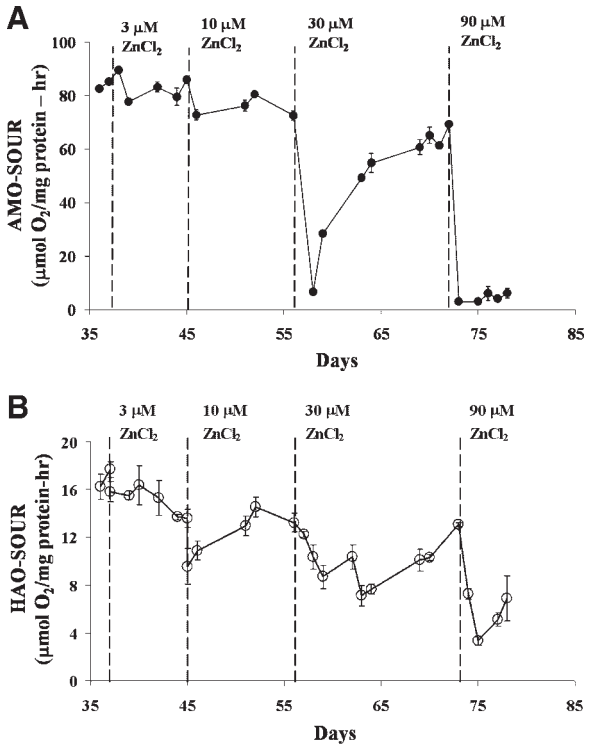

Figure 3. The (**A**) AMO-SOUR (●) and (**B**) HAO-SOUR (○) of continuously cultured cells exposed to $ZnCl_2$ additions. The dashed line indicates when $ZnCl_2$ was pulsed into the bioreactor. Error bars represent 95% confidence intervals.

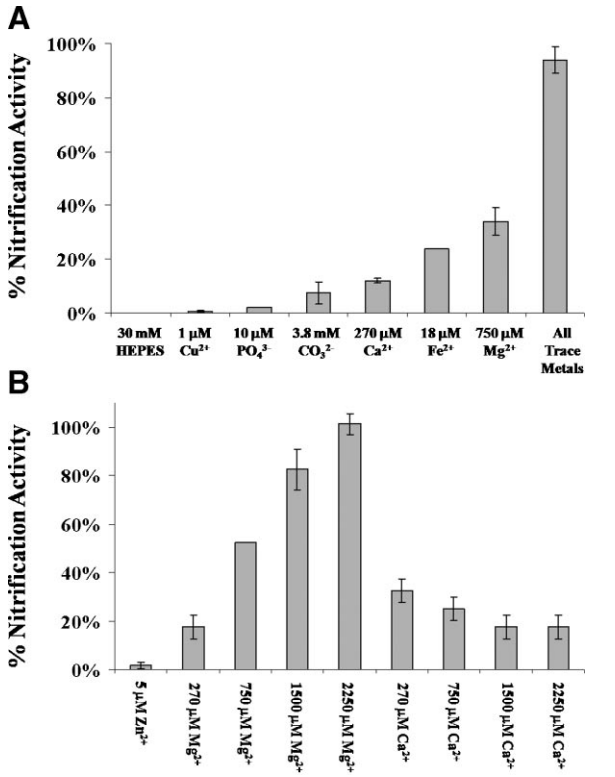

Figure 4. The effect of media composition (**A**) and specifically Mg^{2+} and Ca^{2+} concentrations (**B**) on the nitrite production inhibition of *N. europaea* caused by 5 μM $ZnCl_2$ was examined. *N. europaea* cells were placed in 30 mM HEPES, pH 7.8, 5 mM NH_4^+ and various trace elements as shown above and incubated at 30°C for 120 min with the nitrification activity measured and compared to the control every 30 min. Error bars represent 95% confidence intervals.

concentration in the bioreactor and the washout of NO_2^-. HAO activity decreased 75%, however, a slight recovery to 53% of steady-state activity was observed. The lack of recovery of AMO activity is assumed to be responsible for the washout of *N. europaea* cells observed after the 90 μM $ZnCl_2$ spike. AMO- and HAO-SOUR were measured for the full 85-day experiment. However, after day 78, cell concentrations within the chemostat dropped to such a degree that AMO- and HAO-SOUR could not be quantified.

The expression of *amoA* and *hao* were monitored throughout the experiment (Fig. 2B and C). Significant up-regulation of *amoA*, 5.6- and 4.5-fold, occurred after 30 and 90 μM spikes of $ZnCl_2$, respectively. The expression of *hao* was not significantly up-regulated throughout the experiment.

The role of the trace metals in protecting *N. europaea* from Zn^{2+} inhibition was studied in greater detail (Fig. 4A). Batch *N. europaea* cells were harvested in the mid-exponential growth phase, washed, and placed into fresh HEPES-buffered medium containing $(NH_4)_2SO_4$ and various trace metals at the concentrations found in the continuous culture media. The NO_2^- production rate was measured over a 2-h time period as described above. Each trace metal evaluated resulted in an increase in activity in the presence of 5 μM Zn^{2+} with the divalent cations, Ca^{2+}, Fe^{2+} and Mg^{2+}, providing the greatest percentage of inhibition protection at 14%, 24% and 34%, respectively. However, the protective properties of Fe^{2+} can be largely attributed to the EDTA added with the Fe^{2+} (data not shown). EDTA is necessary to bring Fe^{2+} into solution and is thought to be binding Zn^{2+} and reducing its bioavailability. The protection by each trace metal was cumulative with 100% activity being observed in *N. europaea* cells exposed to 5 μM $ZnCl_2$ with all trace metals and carbonate present.

To further investigate the protective properties of the divalent cations Mg^{2+} and Ca^{2+} to Zn^{2+} inhibition, addition batch tests were conducted as described above with medium containing 5 mM NH_4^+, 5 μM $ZnCl_2$ and 270–2,250 μM Ca^{2+} or Mg^{2+} (Fig. 4B). As Mg^{2+} concentrations increased from 270 to 2,250 μM, the nitrifying activity of *N. europaea* increased from 18% to 100%. However, as the Ca^{2+} concentrations increased from 270 to 2,250 μM, the nitrifying activity decreased slightly from 33% to 18%.

Batch tests were performed to evaluate the reversibility of Zn^{2+} inhibition. Continuously cultured *N. europaea* cells were harvested prior to $ZnCl_2$ additions and were spiked with 5 mM NH_4^+ and various concentrations of Zn^{2+}. The cells were incubated for 15 min and the AMO-SOURs were measured. The cells were then washed five times with HEPES

buffer and placed into fresh continuous culture medium. The cells were incubated for 15 additional min and the AMO-SOURs were measured. Exposure of continuously cultured *N. europaea* cells to 30 and 90 μM $ZnCl_2$ resulted in 23% and 45% inhibition, respectively (Fig. 5). After removing the Zn^{2+}, these cells showed 34% and 57% inhibition, respectively. These experiments demonstrated that Zn^{2+} inhibition is not reversible.

Whole-cell Cu^{2+}/Zn^{2+} competition experiments with continuously cultured cells were performed (Fig. 6). Due to the short time-span of each incubation and measurement, 15 min, along with the decreased sensitivity of continuously cultured cells to Zn^{2+}, a high Zn^{2+} concentration of 120 μM was necessary to cause 60% inhibition. Increases in Cu^{2+} concentration from 0.5 to 600 μM resulted in increased AMO enzyme activity confirming the protective property of Cu^{2+} against Zn^{2+} inhibition of the AMO enzyme.

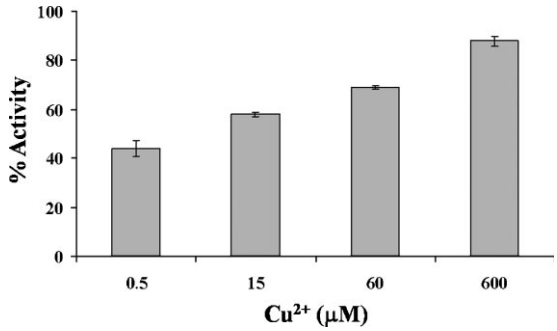

Figure 6. Cu^{2+}/Zn^{2+} competition experiments were conducted on continuously cultured *N. europaea* cells. Cells were incubated with 120 μM $ZnCl_2$ and various amounts of $CuSO_4$ for 15 min before the AMO-SOURs were measured. Error bars represent 95% confidence intervals.

Discussion

In previous batch studies with pure cultures of *N. europaea*, 3.4 μM Zn^{2+} was responsible for a 46-fold up-regulation of *merA*, a mercuric reductase in the mercury resistance pathway, and a 61% decrease in nitrite production rates (Park and Ely, 2008). Exposure of continuously cultured *N. europaea* cells to 3 and 10 μM Zn^{2+} resulted in the 3- and 29-fold up-regulation of *merA*, respectively. However, no significant physiological changes of protein content, ammonia and nitrite levels were observed and the AMO-SOURs remained essentially constant. This demonstrates the sensitivity of *merA* to the presence of Zn^{2+} and reveals that significant physiological changes in growth rate and activity do not have to occur for the up-regulation of *merA*. The up-regulation of *merA* to sub-inhibitory levels of Zn^{2+} makes it a useful indicator of nitrification inhibition from heavy metals such as Zn^{2+} and may be a valuable early warning signal to WWTP operators.

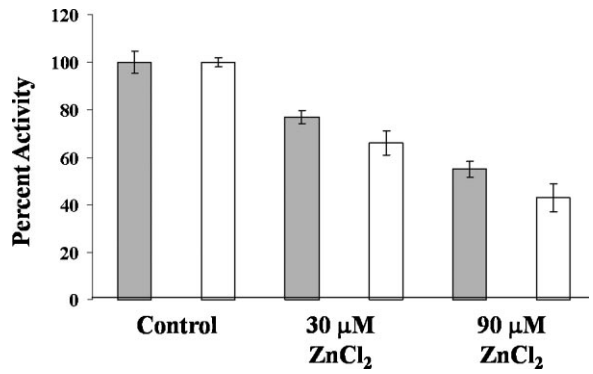

Figure 5. Reversibility of $ZnCl_2$ inhibition of nitrite production was examined with continuously grown *N. europaea* cells. Cells were exposed to 0, 30 or 90 μM $ZnCl_2$ for 15 min and AMO-SOURs were measured (▩). After 15 min, control and $ZnCl_2$ exposed cells were washed five times in 30 mM HEPES buffer, pH 7.8 and placed into fresh media. AMO-SOURs were measured after 15 min of exposure to fresh media (□). Error bars represent 95% confidence intervals.

Continuously cultured cells exposed to 30 μM Zn^{2+} experienced an immediate 89% decrease in nitrite production rates and up-regulated *merA* 46-fold. This demonstrates that significant Zn^{2+}-induced inhibition in both batch and continuously cultured *N. europaea* cells resulted in similar *merA* expression profiles. It is not yet known why *N. europaea* would up-regulate a mercury reductase in response to Zn^{2+}, a non-redox active divalent cation. It may be possible that *merA* is up-regulated in a general cascade response to heavy metals. More research is needed to clarify the role of *merA* in response to non-redox active heavy metals.

In the previous batch studies, exposure to 3.4 μM Zn^{2+} resulted in 67% inhibition of AMO-SOUR in *N. europaea* (Park and Ely, 2008). In our studies, 30 μM Zn^{2+} was required to cause significant inhibition of the AMO-SOUR. Medium composition was the major difference between the two studies. The batch tests of Park and Ely (2008) used a buffered HEPES system with $(NH_4)_2SO_4$ and contained neither trace metals nor carbonate. In the continuous cultured system both trace metals and carbonate were used in addition to HEPES and $(NH_4)_2SO_4$. Measured Zn^{2+} concentrations of filtered chemostat samples were the same as the unfiltered chemostat samples (data not shown) indicating that Zn^{2+} precipitation with OH^-, CO_3^{2-} and PO_4^{3-} was not occurring within the chemostat. Solubility calculations supported this observation that precipitation did not occur.

The protective properties of trace nutrients on Zn^{2+} inhibition were investigated further in batch experiments. These experiments revealed that Mg^{2+} and Ca^{2+} provided the most protection from Zn^{2+} inhibition (Fig. 4A) and increases in Mg^{2+} provided greater protection from Zn^{2+} inhibition, while increases in Ca^{2+} provided less protection from Zn^{2+} inhibition (Fig. 4B). This suggests that Zn^{2+} may

be competing with Mg^{2+} for entrance into the cell via *N. europaea*'s Mg^{2+} transport systems, such as *mgtE* (Chain et al., 2003), or more general divalent cation channels that Mg^{2+} uses but Ca^{2+} does not. Divalent heavy metals, such as Zn^{2+}, have been shown to enter cells via Mg^{2+} uptake systems in other gram-negative bacteria (Smith and Maguire, 1995; Tao et al., 1995). These results demonstrate the importance of media composition on the sensitivity of *N. europaea* to heavy metals such as Zn^{2+}.

Continuously cultured *N. europaea* cells demonstrated the ability to recover activity after inhibition from Zn^{2+} addition. The recovery of activity mirrored the washout of Zn^{2+} from the reactor (Figs. 1B and 3A and B) and is best illustrated by the response to the 30 μM $ZnCl_2$ addition. Additional tests were performed to determine the mode of recovery.

Zn^{2+} inhibition was found to be irreversible (Fig. 5). Therefore, moderate Zn^{2+} inhibition in the chemostat (30 μM $ZnCl_2$) was likely overcome by the generation of new cells and de novo synthesis of key enzymes, such as AMO. However, due to the irreversibility of $ZnCl_2$ inhibition, long-term exposure to severe $ZnCl_2$ inhibition results in the failure of the continuous culture. This was the case for *N. europaea* cells exposed to 90 μM $ZnCl_2$ which resulted in exposure to severe inhibitory levels of Zn^{2+} for 2 weeks.

To determine if de novo AMO synthesis was occurring in continuously cultured *N. europaea* cells exposed to $ZnCl_2$, the expression of *amoA* was monitored throughout the experiment. Batch $ZnCl_2$ inhibition studies with *N. mobilis* demonstrated an up-regulation of *amoA* in response to 10 μM Zn^{2+} (Radniecki and Ely, 2008). Similar results were observed with our continuously cultured *N. europaea* cells, but at a $ZnCl_2$ concentration of 30 μM. The expression of *amoA* increased significantly at 30 μM Zn^{2+} and decreased as the Zn^{2+} was washed out of the chemostat (Fig. 2A). This may indicate that Zn^{2+} concentrations determine the need for de novo AMO synthesis. Interestingly, the lower up-regulation of *amoA* after the addition of 90 μM $ZnCl_2$ likely resulted from the severe reduction in AMO activity. *N. europaea* cells likely did not have enough energy, due to the inhibition of AMO and the resulting lack of ammonia oxidation capability, to maintain as high a level of *amoA* expression as they did when exposed to 30 μM $ZnCl_2$. However, after the 90 μM $ZnCl_2$ addition, the level of *amoA* expression remained high compared to steady state levels, as the Zn^{2+} levels remained at highly inhibitory levels.

Based on the severe inhibition of AMO-SOURs (Fig. 3A) at doses of 30 and 90 μM Zn^{2+}, it is hypothesized that Zn^{2+} may have either competed with Cu^{2+} for uptake, and thus hinder synthesis of active AMO, or for the metal active site in AMO. Several heavy metals have been shown capable of displacing essential divalent cations in biologically important enzymes, rendering the enzyme nonfunctional (Chvapil, 1973; Nies, 1999). Zn^{2+} is unable to undergo redox reactions (Amor et al., 2001; Nies, 1999) and thus the replacement of Cu^{2+} by Zn^{2+} in the active site of AMO would make AMO unable to oxidize NH_3 to NH_2OH. Zn^{2+} was found to inhibit particulate methane monooxygenase (pMMO) enzymes, a monooxygenase with high homology to AMO, in methanotrophs by replacing Cu^{2+} in the pMMO active site (Cook and Shiemke, 1996). To investigate if Zn^{2+} was also replacing Cu^{2+} in AMO, whole-cell Cu^{2+}/Zn^{2+} competition experiments with continuously cultured cells were performed. An increased Cu^{2+} concentration protected *N. europaea's* AMO activity from $ZnCl_2$ inhibition (Fig. 6) and was consistent with the hypothesis that Zn^{2+} was replacing Cu^{2+} in the binding site of AMO. The de novo synthesis of AMO was likely insignificant due to the short time-scale of the experiment (15 min) and disfavors the notion of Zn^{2+} inhibition resulting from a shortage of intracellular Cu^{2+} for the synthesis of active AMO.

These competition results combined with the irreversibility of Zn^{2+} inhibition and the up-regulation of *amoA* support the hypothesis that Zn^{2+} is irreversibly replacing Cu^{2+} in the active site of AMO and that *N. europaea* is generating de novo AMO in response to the inhibition. The recovery is most evident in the results from the 30 μM addition of $ZnCl_2$ to the chemostat. Recovery occurs as the Zn^{2+} concentration decreases in the chemostat in response to washout. AMO-SOURs increase over a period of 15 days after the addition, which is long enough for de novo AMO generation and new cell growth.

Zn^{2+} is also known to be a potent inhibitor of the electron transport chain and is known to disrupt DNA repair mechanisms (Beard et al., 1995; Chvapil, 1973). Decreases in HAO activity after exposure to Zn^{2+} could have resulted from Zn^{2+} inhibition of the electron transport chain of *N. europaea*. AMO activity was more severely inhibited than HAO activity (Fig. 3A and B) indicating that Zn^{2+} imposed multiple inhibition mechanisms that included direct inhibition of the AMO enzyme and lesser inhibition of HAO activity.

This work is the first to examine the dynamic physiological and transcriptional responses of *N. europaea* exposed to heavy metal inhibition in a continuous culture and is among the first to examine gene expression in continuously cultured *N. europaea* cells. Previous transcriptional response experiments in continuously cultured *N. europaea* include changes in protein expression in biofilm formation (Schmidt et al., 2004) and changes in *amoA* expression during starvation conditions (Bollmann et al., 2005). To the best of the authors' knowledge, this is the first work with AOB that directly compares the physiological and transcriptional results of continuously cultured cells to batch cells and highlights the importance of such subtle factors as growth medium composition.

Future work with continuously cultured *N. europaea* cells and $ZnCl_2$ includes observing how excess Cu^{2+} and Mg^{2+} in the chemostat affects the sensitivity of the cells to Zn^{2+} inhibition and their corresponding gene expression. Also, further analysis of continuously cultured *N. europaea* cell exposed to Zn^{2+} concentrations between 30 and 90 μM would be of great interest to determine the threshold at

which cells can no longer be maintained in the chemostat. Another interesting question to investigate is whether the pulse addition of low concentrations Zn^{2+} in this study made the continuously cultured *N. europaea* cells more resistant to higher Zn^{2+} pulse additions later in the experiment. Other potential studies include examining the physiological and transcriptional responses of continuously cultured *N. europaea* cells to continuous additions of Zn^{2+}. Finally, it remains to be seen if all AOB exhibit the same sensitivity and gene expression profiles to Zn^{2+} inhibition as *N. europaea* has demonstrated in this study. Perhaps RT-qPCR primers can be developed for the detection of Zn^{2+}-induced nitrification inhibition that would be applicable to all AOB commonly found in WWTPs.

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