Effects of the chemical characteristics and concentration of inorganic suspended solids on nitrification in freshwater
Quynh Nga Le, Chihiro Yoshimura and Manabu Fujii

ABSTRACT
The effect of inorganic suspended solids (ISS) on nitrification in freshwater samples has been described inconsistently and remains unclear. This study therefore investigated the effects of the chemical characteristics and concentration of ISS on the nitrification rate by focusing on Nitrosomonas europaea and Nitrobacter winogradskyi as the two most dominant nitrification species in freshwater. Batch-wise experiments were conducted using three chemically well-characterized ISS (i.e. the clay minerals montmorillonite, sericite, and kaolinite in the concentration range 0–1,000 mg L$^{-1}$). The results show that the ammonium oxidation rate constant ($k_{NH_4}$) was significantly affected by the ISS type, whereas changes in the ISS concentration had an insignificant effect on $k_{NH_4}$, except for kaolinite. The highest $k_{NH_4}$ was observed in samples containing sericite ($k_{NH_4}$, 0.067 L mg$^{-1}$ day$^{-1}$), followed by samples containing montmorillonite ($k_{NH_4}$, 0.044 L mg$^{-1}$ day$^{-1}$). The ammonium oxidation rate was low in the control and kaolinite samples. Nitrite oxidation was enhanced in the presence of all types of ISS. The rate constants of ISS-mediated nitrite oxidation ($k_{NO_2}$, 0.13–0.21 L mg$^{-1}$ day$^{-1}$) were not significantly different among the three types of ISS, but $k_{NO_2}$ was significantly affected by ISS concentration. Overall, our study indicated various effects of the ISS type and concentration on nitrification and, in particular, a notable positive effect of sericite.

Key words | inorganic suspended solids, nitrification, Nitrobacter winogradskyi, Nitrosomonas europaea, sericite

INTRODUCTION
Nitrogen (N) is a major nutrient for primary production in freshwater ecosystems (Elser et al. 2007), so understanding the factors that affect N transformations in freshwater columns is necessary to effectively manage and conserve water environments. Nitrification is an important biologically mediated process as it is the fundamental mechanism for removing N from natural and engineered aqueous systems (Kim et al. 2013), thereby helping prevent water quality degradation (Piehler et al. 2004). Nitrification can be divided into two processes: ammonium (NH$_4^+$) oxidation mediated by ammonia oxidizing bacteria (AOB), and nitrite (NO$_2^-$) oxidation driven by nitrite oxidizing bacteria (NOB). AOB and NOB are often restricted to a few genera, often referred to as Nitrosomonas and Nitrobacter, respectively (Purkhold et al. 2000; Grunditz & Dalhammar 2001; Cébron & Garnier 2005). The nitrification rate is affected by several factors, including temperature, pH (Strauss & Lamberti 2000), and the concentrations of dissolved oxygen (DO) (Triska et al. 1990), inhibitors (e.g. nitrous acid (Anthonisen et al. 1976)) and suspended solids (SS) (Campos et al. 2002; Xia et al. 2009; Le et al. 2015a, 2015b).

SS are considered important components of water bodies and affect physical, chemical, and biological processes. High inorganic SS (ISS) concentrations (up to 29 g L$^{-1}$) are found naturally in many rivers worldwide, and the dominant size fractions are silt and clay (Meybeck et al. 2003). The major components of ISS minerals are illite, smectite, chlorite, and kaolinite (Georg 1991). These minerals are primarily composed of crystalline hydrous aluminum silicates that are classified as type 2:1 or 1:1, according to the arrangement of the tetrahedral sheet of silicate (Si) and the octahedral sheet of aluminium (Al).
hydroxide (OH) (Carroll 1959). These aluminosilicate minerals exhibit high cation exchange capacity (CEC), which relates to their ability to provide macro- and micro-nutrients for microorganisms and pH buffering capacity to the aqueous environment. Hence, we anticipate direct and indirect interactions between nitrification and ISS.

Several previous studies have reported the effects of clay mineral particles on the metabolic and enzymatic activities of microorganisms, and on biochemical transformations. For example, earlier studies indicated that the presence of kaolinite in an aquatic environment very negatively affects bacterial colonization, probably because the surface characteristics of kaolinite are unfavourable for bacterial attachment (e.g. nanometer-sized pores (Deflaun & Mayer 1985; Yamamoto & Lopez 1985)). In contrast, other studies suggested positive effects of montmorillonite and kaolinite on bacterial respiration, both in aqueous (Stotzky & Rem 1966) and soil environments (Macura & Stotzky 1980). More recently, it was reported that the addition of kaolinite enhances both ammonium (NH₄⁺) and nitrite (NO₂⁻) oxidation in activated sludge (Campos et al. 2002) and air-lift reactors (Vieira et al. 2001). These authors argued that this positive effect is associated with the incorporation of kaolinite particles into biofilms, resulting in an increased pH buffering capacity of the biofilms. In contrast, the effect of N species adsorbing onto kaolinite particles on biofilm development may be negligible (Vieira et al. 2001; Campos et al. 2002). The conclusion from these previous studies is that montmorillonite enhanced the respiration of nitrifiers, whereas such effects are not consistently observed with kaolinite.

In short, there are some discrepancies between reports regarding the effect of ISS on the growth of microorganisms and on nitrification, and the exact roles of ISS remain unclear. Given that the two sub-processes of nitrification are mediated by different bacterial species, the presence of these two bacterial species in the same reactor, an approach typically employed in previous studies, could have resulted in ambiguous interpretations of the interactions between SS, nitrifying bacteria, and nitrification. Moreover, the use of natural river samples for these experiments may also contribute to these discrepancies because river water quality widely varies and samples often contain unknown organic substances that could affect nitrification transformation. Thus, it could be advantageous to conduct experiments investigating individual nitrification processes under defined conditions to enhance our understanding of the interactions between nitrification and ISS.

The aim of this study was to investigate the effects of the chemical characteristics and concentration of ISS on each of the two sub-processes of nitrification. Batch-wise laboratory experiments were conducted to investigate the nitrification rate in the presence of chemically well-characterized standard clay minerals (i.e. montmorillonite, sericite, and kaolinite). We tested two hypotheses: (i) nitrification in freshwater samples is affected by the chemical characteristics and concentration of ISS, with the magnitude of the effect depending on the sub-process; and (ii) the nitrification rate partly depends on the interaction of nitrifying bacteria and nutrients with the surface of the ISS.

**MATERIALS AND METHODS**

**Cultivation of nitrifiers**

We used two bacterial species, *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (hereinafter referred to as *N. europaea* and *N. winogradskyi*), which are, respectively, AOB and NOB widely dominant in the environment (Purkhold et al. 2000; Cébron & Garnier 2005). These strains were purchased from National Institute of Technology and Evaluation Biological Resource Center (NBRC), Japan, and were initially supplied as L-dried cells (i.e. in a vacuum-sealed glass container). Each strain was transferred to culture medium containing a mixture of inorganic salts specified as NBRC medium 829 and medium 239 for *N. europaea* and *N. winogradskyi*, respectively. Medium 829 contains 2.5 g L⁻¹ (NH₄)₂SO₄, 0.50 g L⁻¹ KH₂PO₄, 11.9 g L⁻¹ HEPES, 0.50 g L⁻¹ NaHCO₃, 100 mg L⁻¹ MgSO₄.7H₂O, 5.0 mg L⁻¹ CaCl₂.2H₂O, and 75 mg L⁻¹ Fe-EDTA (i.e. 217 μM Fe). Medium 239 contains 1.0 g L⁻¹ NaNO₂, 0.50 g L⁻¹ NaCl, 0.50 g L⁻¹ K₂HPO₄, 0.50 g L⁻¹ MgSO₄.7H₂O, 2.0 mg L⁻¹ MnSO₄.H₂O, and 5.0 mg L⁻¹ Fe₂(SO₄)₃. The cultures were incubated aerobically in the dark at 28 °C for over 2 weeks before being used in experiments.

**Characteristics of ISS**

Three types of clay minerals commonly found in rivers globally were purchased from the Clay Science Society of Japan (CSSJ): montmorillonite (smectite group), sericite (illite group), and kaolinite (Table 1). Montmorillonite and sericite are representative of the 2:1 clay type whereas kaolinite belongs to the 1:1 clay group. The specific weights of the three clay minerals were estimated by measuring the volume of a specified amount of clay mineral in a 100 mL graduate
cylinder (SANSYO Co., Ltd). Total alkalinity was measured by titration (APHA-AWWA-WEF 2005). Other characteristics of the three clay minerals (e.g. particle size distribution, specific surface area, chemical composition, zeta potential) were provided by CSSJ (Miyawaki et al. 2010). Sericite exhibited the lowest specific surface area (10.21 m² g⁻¹) and the highest total alkalinity (2.96 g CaCO₃ g⁻¹). Kaolinite was the least active mineral, with a low CEC, large average particle size, and low specific surface area and total alkalinity.

**Experimental design**

**Ammonium oxidation (Series-1)**

Sample solutions were generated by diluting medium 829 to an approximate NH₄⁺ concentration of 12 mg-N L⁻¹ (dilution factor: 45). A culture of *N. europaea* was centrifuged at 1,500 × g for 20 min, the supernatant was removed, and the bacteria were used to inoculate the sample solution. The activity of AOB is strongly affected by pH (Jiménez et al. 2011), and the three types of ISS used in our study have different total alkalinity values (Table 1); therefore, the experiment was designed to maintain the pH at 7.5, which is within the optimal pH range for *N. europaea* (Antoniou et al. 1990). HEPES (10 g L⁻¹) was used as the pH buffer and the pH was adjusted with sodium hydroxide and hydrochloric acid. The inoculated sample (100 mL) was transferred to polypropylene tubes and the three types of ISS were separately added to each tube to final ISS concentrations of 200, 500, 800, and 1,000 mg L⁻¹. Tubes without ISS were prepared as controls. The experiments were run in duplicate on compact shakers (ROCKERS CR95, FINEPCR, South Korea) to mix and aerate the samples. The DO concentration was determined to be nearly saturated (7–8 mg L⁻¹). Experiments were conducted in a temperature-controlled room at 23 ± 2 °C. All glassware was sterilized by autoclaving at 121 °C for 30 min and plastic vessels were soaked in 10% hydrochloric acid for at least 1 day before use.

NO₂⁻ and NO₃⁻ concentrations were determined by removing an aliquot of sample at 4, 7, 9, 13, 14, 15, and 19 d post-inoculation, followed by filtration through a 0.22 μm membrane filter. The density of *N. europaea* cells in suspension was measured at day 15. Sampling was performed on a biological clean bench to avoid sample contamination. All samples were kept in a refrigerator at 4 °C and NO₂⁻ and NO₃⁻ concentrations were measured within 3 days of sampling. N concentrations were analyzed by a colorimetric method using a Bran + Luebbe Traacs 2000 Autoanalyzer (SEAL Analytical, Ltd, UK). All samples were measured in duplicate. The relative errors of measured N concentration were less than 5%.

**Nitrite oxidation (Series-2)**

Nitrite oxidation experiments were conducted using a method identical to that used for the NH₄⁺ oxidation.

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**Table 1 | Characteristics of ISS used in this study**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Montmorillonite</th>
<th>Sericite</th>
<th>Kaolinite</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical composition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SiO₂</td>
<td>66.4</td>
<td>SiO₂</td>
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<td>SiO₂</td>
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<tr>
<td>Al₂O₃</td>
<td>11.9</td>
<td>Al₂O₃</td>
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<tr>
<td>MgO</td>
<td>2.6</td>
<td>K₂O</td>
<td>9.2</td>
<td>K₂O</td>
</tr>
<tr>
<td>Na₂O</td>
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<td>Fe₂O₃</td>
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<tr>
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<td>CaO</td>
<td>2.0</td>
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</tr>
<tr>
<td>K₂O</td>
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<td>MgO</td>
<td>1.0</td>
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</tr>
<tr>
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<td>9.7</td>
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</tr>
<tr>
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<td>CaO</td>
</tr>
<tr>
<td>CO₂</td>
<td>0.19</td>
<td>CO₂</td>
<td>1.53</td>
<td>CO₂</td>
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<tr>
<td>Clay type</td>
<td>2:1</td>
<td>2:1</td>
<td>1:1</td>
<td>Miyawaki et al. (2010)</td>
</tr>
<tr>
<td>Zeta potential (mV) at pH = 6–8</td>
<td>−40</td>
<td>−24 to −28</td>
<td>−40 to −40</td>
<td>Miyawaki et al. (2010)</td>
</tr>
<tr>
<td>CEC (me g⁻¹)</td>
<td>70–100</td>
<td>10–40</td>
<td>3–15</td>
<td>Carroll (1959)</td>
</tr>
<tr>
<td>Average particle diameter (μm)</td>
<td>0.1–30</td>
<td>0.5–30</td>
<td>0.5–50</td>
<td>Miyawaki et al. (2010)</td>
</tr>
<tr>
<td>Specific weight (g mL⁻¹)</td>
<td>2.78</td>
<td>2.00</td>
<td>2.50</td>
<td>Present study</td>
</tr>
<tr>
<td>Specific surface area (m² g⁻¹)</td>
<td>27.35</td>
<td>10.21</td>
<td>14.82</td>
<td>Miyawaki et al. (2010)</td>
</tr>
<tr>
<td>Total alkalinity (mg CaCO₃ g clay⁻¹)</td>
<td>937.5</td>
<td>2,962.5</td>
<td>12.5</td>
<td>Present study</td>
</tr>
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</table>
experiment, except that medium 239 and *N. winogradskyi* were used, and there were some minor procedural modifications, as noted below. Briefly, medium 239 was diluted to provide a NO$_2^-$ concentration of 10 mg-N L$^{-1}$. The pH of the medium was adjusted to 8.2, which is within the optimal pH range for *N. winogradskyi* growth (Park et al. 2007). Although NO$_2^-$ oxidation does not change the pH of the environment, a pH buffer (i.e. HEPES) was nonetheless used to keep the pH constant. A culture of *N. winogradskyi* was centrifuged, and the cells were inoculated into the above medium to provide the sample. The inoculated sample (100 mL) was transferred to polypropylene tubes and the three types of ISS were separately added to each tube to final ISS concentrations of 100, 500, and 800 mg L$^{-1}$. Aliquots were removed at 3, 3.5, 4, 4.5, 5, 6, and 7 days post-inoculation for the determination of NO$_2^-$ and NO$_3^-$ concentrations, and additional samples were taken at day 6 to measure the density of the *N. winogradskyi* cells. The temperature and DO concentration were maintained at approximately 23 ± 2 °C and 7–8 mg L$^{-1}$, respectively. In both Series-1 and -2, experiments conducted using samples containing no ISS, or containing montmorillonite, sericite, or kaolinite, are referred to as Control-ex, Mont-ex, Seri-ex, and Kao-ex, respectively. Furthermore, in the two series, blank samples (two replicates) without ISS and bacteria were also prepared as negative controls for the targeted nitrogen transformation determinations.

**Determination of bacterial cell density**

Bacterial samples were preserved with sterile 10% glutaraldehyde (GTA) in phosphate-buffered saline (PBS) at a final GTA concentration of 1% (Kepner & Pratt 1994) in a refrigerator in the dark at 4 °C. The densities of *N. europaea* and *N. winogradskyi* were estimated by direct enumeration using epifluorescence microscopy (ECLIPSE 80i, Nikon, Japan) using the 4',6-diamidino-2-phenylindole (DAPI) staining method. Both total cell density (i.e. including both free and attached bacteria) and free cell density were estimated to obtain the ratios of free and attached cells to the total cell density. Samples were diluted 5-, 7-, or 14-fold with ultrapure water to determine the best dilution according to the bacterial cell density of each sample. Total bacterial cell density was estimated by physicochemically detaching and dispersing attached cells from the particles by adding methanol 99.8% (final methanol concentration: 10%) and sonicating for 15 min at 35 °C (Lunau et al. 2005). The ISS particles in the total and free cell density samples were removed by centrifuging at 40 × g for 1 min. Control samples containing no ISS were also centrifuged to obtain a correction factor (1.15) for centrifugation. It should be noted that this centrifugation speed and time was determined by trial-and-error to remove ISS as much as possible while keeping bacteria in the supernatant. Due to the similar size of some ISS particles and bacterial cells, centrifugation cannot completely remove all ISS particles from bacterial suspensions.

 Supernatants from 5 mL samples were stained with DAPI solution (0.5 μg mL$^{-1}$) for 30 min in the dark at 28 °C. The stained sample solutions were mixed well using a vortex mixer and then gently poured into a filter tower that contained black polycarbonate membrane filters (25 mm diameter, 0.2 μm pore size, Advantec Nissei Kaisha, Ltd, Japan). The unincorporated stain was removed by vacuum filtration, followed by rinsing with 5 mL of PBS. The filter membranes were removed from the filter tower and placed on sterile microscope slides, and then covered with non-fluorescent immersion oil and a glass cover slip. The prepared slides were kept at 4 °C and the stained bacterial cells were counted using an epifluorescence microscope (light filter: 340–580 nm for excitation; 435–480 nm for emission). All these procedures were conducted in the dark. Final bacterial densities were calculated using the following equation (Wetzel & Likens 1991):

$$\text{Number of cells (mL}^{-1}) = \text{Membrane conversion factor} \times N \times D,$$

where the membrane conversion factor is the ratio of the filtration area and the area of the micrometer field. $N$ is the ratio of the total number of bacteria counted and the number of micrometer fields counted. $D$ is the dilution factor calculated as the ratio of the volume of sample stained and the total volume of the sample. Both *N. europaea* and *N. winogradskyi* in samples containing sericite and kaolinite were enumerated using this DAPI staining method and epifluorescence microscopy. However, bacterial counting was not performed on samples containing montmorillonite because residual montmorillonite particles (remaining in the stained sample after centrifugation) emit fluorescent light at wavelengths similar to that emitted by the stained cells, resulting in significant noise in the microscopic images.

**Adsorption experiment**

NH$_4^+$ has high affinity with some types of ISS particles, so the abiotic adsorption of NH$_4^+$ onto ISS particles was
determined at various NH$_4^+$ and ISS concentrations. In this experiment, sample solutions containing different initial NH$_4^+$ concentrations were generated by diluting with medium 829 at pH 6.5. Next, 100 mL of each prepared solution was transferred to polypropylene tubes and a specific amount of ISS was added to each tube to achieve final ISS concentrations of 0, 200, 500, 600, 800, 1,000, and 1,500 mg L$^{-1}$. After shaking for 60 min (Xia et al. 2004) using a compact shaker (ROCKERS CR95, FINEPCR) at room temperature (25 ± 2°C), the samples were filtered through 0.22 μm membrane filters, followed by measurement of the NH$_4^+$ concentration using an auto-analyzer.

The amount of NH$_4^+$ adsorbed was calculated by subtracting the NH$_4^+$ concentration in the filtrate after the adsorption experiment from the initial NH$_4^+$ concentration. This value was then divided by the ISS concentration to determine the amount of NH$_4^+$ adsorbed per unit mass of ISS (Q, mg-N mg-ISS$^{-1}$). Adsorption parameters were estimated by fitting the Langmuir isotherm equation (Equation (2)), which is commonly used in this process (Buragohain et al. 2013), to the experimental data (i.e. Q and equilibrated concentration of NH$_4^+$ [$C_d$, mg-N L$^{-1}$]):

$$Q = \frac{Q_mKC_d}{1 + KC_d},$$

where $Q_m$ is the maximum adsorption capacity (mg-N mg-SS$^{-1}$) and $K$ is the equilibrium constant for NH$_4^+$ adsorption (L mg$^{-1}$).

**Model application and statistical analysis**

A logistic model was applied to investigate nitrification kinetics (Simkins & Alexander 1984). The model is described by two differential equations, as follows:

$$\frac{dC_{NH4}}{dt} = k_{NH4}C_{NH4}(C_{NH40} + X_0 - C_{NH4}),$$

$$\frac{dC_{NO2}}{dt} = k_{NO2}C_{NO2}(C_{NO20} + X_0 - C_{NO2}),$$

where $k_{NH4}$ and $k_{NO2}$ are the oxidation rate constants for NH$_4^+$ and NO$_2$ (L mg$^{-1}$ day$^{-1}$), respectively; $C_{NH4}$ and $C_{NO2}$ are the concentrations of NH$_4^+$ and NO$_2$, respectively, during the experiment (mg-N L$^{-1}$); $C_{NH40}$, $C_{NO20}$ are the initial concentrations of NH$_4^+$ and NO$_2$; and $X_0$ is the bacterial population at time zero (μg L$^{-1}$). The integrated forms of Equations (3) and (4) are

$$C_{NH4} = \frac{C_{NH40} + X_0}{1 + (X_0/C_{NH40})e^{k_{NH4}(C_{NH40} + X_0)/T}},$$

$$C_{NO2} = \frac{C_{NO20} + X_0}{1 + (X_0/C_{NO20})e^{k_{NO2}(C_{NO20} + X_0)/T}},$$

The kinetic parameters $k_{NH4}$ and $k_{NO2}$ were estimated by fitting Equations (5) and (6) to the observed concentrations of NH$_4^+$ and NO$_2$ during the experiments, respectively, using the nonlinear least squares method. The values of $X_0$ corresponding to the highest correlation coefficient between the observed and modeled $C_{NH4}$ were used and varied in the range 0.5 to 50 μg L$^{-1}$. The nonlinear least squares method was used to estimate the adsorption parameters. Correlation analysis was applied to examine the effect of nitrifier cell density on the oxidation rate constants (i.e. $k_{NH4}$ and $k_{NO2}$). Two-way analysis of variance (ANOVA) followed by Tukey’s test was applied to test the effects of ISS concentration and type on bacterial cell density and $k_{NO2}$. Statistical analyses and model fitting were conducted using R software (version 3.2.1, R Foundation for Statistical Computing, Austria).

**RESULTS**

Ammonium oxidation

In Series-1, an increase in NO$_2^-$ concentration was observed in the Seri-ex samples after 7 days’ incubation, whereas longer periods (e.g. 9–13 days) were required to observe discernible increases in NO$_2^-$ in the Mont-ex and Kao-ex samples (Figure 1). In the Seri-ex samples, all NH$_4^+$ introduced into the system (i.e. 12 mg-N L$^{-1}$) was oxidized to NO$_2^-$ by day 19 (i.e. 11 days after the lag time), whereas in the Mont-ex and Kao-ex samples, the NO$_2^-$ concentrations at day 19 were 4.9–7.2 mg-N L$^{-1}$ and 1.0–6.5 mg-N L$^{-1}$, respectively. There was no clear trend in the effect of ISS concentration on the NO$_2^-$ concentration in the Mont-ex and Seri-ex samples over the duration of the experiment. In contrast, the Kao-ex samples generated the highest concentration of NO$_2$ at the lowest ISS concentration (i.e. 200 mg L$^{-1}$), and the degree of NO$_2^-$ formation decreased as the ISS concentration increased. NO$_2^-$ formation was not observed during the experimental period. Since NO$_2^-$

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was not detected in the blank samples, we could confirm that \( \text{NH}_4^+ \) oxidation cannot occur in the absence of bacteria. The average ammonium oxidation rates \( k_{\text{NH4}} \) were determined to be 0.037, 0.035–0.044, 0.064–0.072, and 0.01–0.06 L mg\(^{-1}\) day\(^{-1}\) for the Control-ex, Mont-ex, Seri-ex, and Kao-ex samples, respectively (Figure 2). The regression coefficients for the observed \( \text{NH}_4^+ \) concentration and predicted \( \text{NH}_4^+ \) concentration (using \( k_{\text{NH4}} \) from the model fitting) over time were all larger than 0.96 (\( p < 0.001 \)). Two-way ANOVA indicated a significant effect of ISS type but an insignificant effect of ISS concentration on \( k_{\text{NH4}} \) (\( F_{24,2} = 244.4 \) [\( p < 0.01 \]) for ISS type, \( F_{24,3} = 1.7 \) [\( p = 0.2 \]) for ISS concentration, and \( F_{24,6} = 3.8 \) [\( p = 0.02 \]) for the interaction of ISS type \( \times \) concentration). According to the subsequent Tukey’s test, \( k_{\text{NH4}} \) in Seri-ex samples was significantly higher than that observed in the other ISS systems. In

Figure 1 | \( \text{NO}_2^- \) concentrations in the \( \text{NH}_4^+ \) oxidation experiment in the presence of (a) montmorillonite, (b) sericite, and (c) kaolinite. Error bars indicate standard deviation from duplicate experiments.

Figure 2 | The ammonium oxidation rate (\( k_{\text{NH4}} \), L mg\(^{-1}\) day\(^{-1}\)) averaged over the experimental period for different clay types and concentrations. Lower-case letters indicate a significant difference in \( k_{\text{NH4}} \) at all conditions examined (Tukey’s test). Error bars indicate standard deviation from duplicate experiments.
addition, $k_{\text{NH}_4}$ values for the Mont-ex and Seri-ex samples were higher than that for the Control-ex sample and were not affected by ISS concentration. Kao-ex samples showed a very low $k_{\text{NH}_4}$ (about 0.03 L mg$^{-1}$ day$^{-1}$) for all ISS concentrations (i.e. 200–1,000 mg L$^{-1}$) (Figure 2).

The total cell densities of *N. europaea* at day 15 were determined to be $6.1\times10^8$, $5.9\times10^8$, and $1.8\times10^8$ (mL$^{-1}$) for the Seri-ex, Control-ex, and Kao-ex samples, respectively (Figure 3). A negative correlation between *N. europaea* cell density and ISS concentration was observed for kaolinite ($R = -0.86$ [$p = 0.007$]), whereas an insignificant correlation was found in the case of sericite ($R = 0.1$ [$p = 0.8$]). In the Seri-ex samples, almost all nitrifier cells were found in the free form rather than attached to sericite particles. In the Kao-ex samples, the *N. europaea* cell density was very low (lower than in the Control-ex samples) at all ISS concentrations (i.e., 200–1,000 mg L$^{-1}$). Subsequently, Tukey’s test indicated significantly higher densities at concentrations of $\geq 500$ mg L$^{-1}$ of sericite (Seri-ex) compared with other cases. Positive correlation was found between nitrifier cell density and $k_{\text{NH}_4}$ ($R = 0.9$ [$p < 0.001$]) for all experimental data available in the absence and presence of ISS (Figure 4).

The adsorption experiment showed that among the three ISS types tested, only montmorillonite particles clearly adsorb NH$_4^+$ (Figure 5(a)–5(c)), and the NH$_4^+$ equilibrated concentration in solution decreased as the montmorillonite concentration increased. In contrast, the free NH$_4^+$ concentration was independent of the sericite and kaolinite concentrations. The observed amount of NH$_4^+$ adsorbed onto montmorillonite particles was fitted well by the Langmuir adsorption isotherm, yielding parameters of $Q_m = 0.0049$ (mg mg$^{-1}$ SS$^{-1}$) ($p < 0.001$) and $K = 0.54$ ($p > 0.05$) (Figure 5(d)).

**Nitrite oxidation**

In Series-2, the NO$_2^-$ concentration decreased with time irrespective of the ISS added (Figure 6). NO$_2^-$ oxidation rate was relatively slow in the Control-ex samples; the total amount of NO$_2^-$ oxidized to NO$_3^-$ was only 3.2 mg-N L$^{-1}$ after 1 week. In contrast, the NO$_2^-$ concentration decreased faster in the presence of ISS. In addition, faster NO$_2^-$ oxidation was observed as the concentration of ISS increased (Figure 6), although there was little difference in the rate of NO$_2^-$ oxidation at 100 and 500 mg L$^{-1}$ ISS. After 1 week, all NO$_2^-$ introduced was oxidized to NO$_3^-$ in samples containing 500–800 mg L$^{-1}$ sericite or 800 mg L$^{-1}$ montmorillonite or kaolinite. In blank samples, NO$_2^-$ remained constant and was similar to the initial concentration introduced, which implies that NO$_2^-$ oxidation cannot occur in the absence of bacteria.

The values for $k_{\text{NO}_2}$ were determined to be 0.13, 0.14–0.19, 0.14–0.21, and 0.14–0.18 L mg$^{-1}$ day$^{-1}$ for the Control-ex, Mont-ex, Seri-ex, and Kao-ex samples, respectively (Figure 7). The $k_{\text{NO}_2}$ value was higher for samples containing ISS compared to the control medium, with the exception of some lower ISS concentrations (e.g. 100 mg L$^{-1}$). The correlation coefficients for NO$_2^-$ concentrations observed and predicted (using $k_{\text{NO}_2}$ from the model fit) in all systems were higher than 0.95 ($p < 0.001$). Two-way
ANOVA indicated a significant effect of ISS concentration, while the effect of ISS type on $k_{NO2}$ was statistically insignificant ($F_{18,2} = 4.07 \ [p = 0.06]$ for ISS mineral type, $F_{18,2} = 48.86 \ [p < 0.001]$ for ISS concentration, and $F_{18,4} = 1.50 \ [p = 0.28]$ for the interaction). Subsequent Tukey’s tests showed that the highest $k_{NO2}$ values were obtained for systems containing 800 mg L$^{-1}$ ISS for all three ISS types. While there was no significant increase in NO$_2^-$ oxidation rate (relative to Control-ex) at ISS concentrations of 100 and 500 mg L$^{-1}$, simple correlation analysis indicated positive and significant correlation between $k_{NO2}$ and ISS concentration for montmorillonite ($R = 0.88 \ [p = 0.02]$), sericite ($R = 0.99 \ [p < 0.001]$) and kaolinite ($R = 0.89 \ [p = 0.02]$).

The total cell densities of N. winogradskyi at day 6 in the Seri-ex and Kao-ex samples were up to 6.6 times higher than those in the Control-ex samples. The highest cell density was obtained with 800 mg L$^{-1}$ of sericite (Figure 8). The percentage of N. winogradskyi cells in free form ranged between 53–85% for Seri-ex and 100% for Kao-ex samples. Two-way ANOVA indicated a significant effect of ISS type and concentration on N. winogradskyi cell density: $F_{12, 1} = 22.92 \ [p = 0.03]$ for ISS type, $F_{12, 2} = 23.64 \ [p = 0.001]$ for ISS concentration, and $F_{18, 4} = 1.02 \ [p = 0.415]$ for the interaction (ISS type × ISS concentration). The results of subsequent Tukey’s tests showed that the cell densities observed in samples containing 800 mg L$^{-1}$ sericite or kaolinite were significantly higher than that in samples containing 100 mg L$^{-1}$ ISS (Figure 8). Furthermore, positive correlation was observed between the total cell density and $k_{NO2}$ (Figure 9).
DISCUSSION

Effect of ISS on ammonium oxidation

In general, the retention of cations, such as the adsorption of NH$_4^+$ onto ISS, is associated with the concentration of exchangeable cations on the ISS (Carroll 1959; Ranjbar & Jalali 2013). Therefore, the high CEC of montmorillonite and the low CEC of sericite and kaolinite (Table 1) may explain the adsorption results obtained in this study (i.e. substantial adsorption of NH$_4^+$ to montmorillonite and little adsorption to the other two ISS types). Previous studies reported that adsorbed NH$_4^+$ was oxidized preferentially compared with free NH$_4^+$ in soil (Lees & Quastel 1946) and in aqueous conditions (Xia et al. 2009). Our study, however, indicated that the oxidation rates in the Seri-ex (free NH$_4^+$-dominated system) samples are faster than those for the Mont-ex samples, which contain a mixture of free and adsorbed NH$_4^+$. Furthermore, the predominance of _N. europaea_ in the free form in Seri-ex samples suggests that the surface of sericite particles is not suitable for colonization by _N. europaea_. Thus, it is most likely that the NH$_4^+$ oxidation process observed in our study mainly occurred in the bulk solution rather than on the surface of the ISS particles, and the adsorption of NH$_4^+$ does not necessarily accelerate the NH$_4^+$ oxidation rate, at least under the conditions examined here. One plausible reason for the discrepancy between this and previous studies may be differences in ISS quality, which affects the adsorption of bacteria and substrates (Khan et al. 2015). Lees & Quastel (1946) argued that nitrification on soil particle surfaces increases when both the bacteria and substrate attach onto solid.

Figure 6 | NO$_2^-$ concentrations in the NO$_2^-$ oxidation experiment in the presence of (a) montmorillonite, (b) sericite, and (c) kaolinite. Error bars indicate standard deviation from duplicate experiments.
sites near each other. Lees & Quastel (1946) and Xia et al. (2009) used natural soil and river SS containing various types of organic and inorganic particles that likely created optimal conditions for bacteria and nitrification on solid surfaces. In contrast, in this study we used a single type of clay mineral in each treatment and, thus, the substrate and the bacteria might not adsorb simultaneously. The results of our study suggest that a high specific SS surface area does not always result in a high nitrification rate, especially in the case of ISS, as was previously pointed out by Xia et al. (2009).

The initial lag phase for NH$_4^+$ oxidation is likely the period during which the oxidation rate is negligible because the cell density is low and/or the nitrifiers begin to adapt to a new environment in which the cells lack some biochemical substrates required for NH$_4^+$ oxidation (Hooper 1969). Our results indicate that the adaptation time (i.e. the time until N. europaea starts to catalyze NH$_4^+$ oxidation at a significant level) in the presence of sericite is shorter than those for the other cases (i.e. Control-ex, Mont-ex, and Kao-ex). Furthermore, the nitrifier cell densities in the Seri-ex samples were higher than in the Control-ex and Kao-ex samples. These results consistently indicated that sericite enhances the metabolic and enzymatic activities of N. europaea, resulting in the acceleration of the NH$_4^+$ oxidation process.

One possible reason for the stimulation of NH$_4^+$ oxidation by sericite is that sericite particles are an important iron source for enzymatic NH$_4^+$ oxidation by N. europaea. Among the three ISS types tested in the present study, sericite has the highest iron content (i.e. Fe$_2$O$_3$ accounted for 1.6, 4.0, and 0.0% of montmorillonite, sericite, and kaolinite, respectively, Table 1). Previous studies have shown that N. europaea has a high demand for iron because of its large number of cytochromes and its octaheme-containing hydroxylamine oxidoreductase (Vajrala et al. 2003; Upadhyay et al. 2003; Wei et al. 2006). Furthermore, it has been shown that N. europaea can efficiently acquire not only Fe$^{2+}$ but also free Fe$^{3+}$ (Wei et al. 2006; Pérez et al. 2015). The initial iron concentration in the medium used in the present experiment was about 4.8 $\mu$M Fe (the initial solution of 217 $\mu$M Fe-EDTA diluted by a factor 45), which is lower than the optimal concentration of Fe for N. europaea’s growth (i.e. 10–250 $\mu$M Fe (Wei et al. 2006)). Below this optimal concentration, the growth of N. europaea is positively
correlated with the iron concentration (Wei et al. 2006), and therefore it is likely that iron supplied from sericite particles stimulated *N. europaea* growth and thus NH$_4^+$ oxidation.

**Effect of ISS on nitrite oxidation**

It was previously reported that the rate of NO$_2^-$ oxidation catalyzed by *N. winogradskyi* is closely related to bacterial density (Knowles et al. 1965; Ossenbruggen et al. 1996; Xia et al. 2009). The present study also found a significant correlation between cell density and $k_{NO2}$. In our experiment, the cell density of *N. winogradskyi* was determined to be higher in systems containing ISS compared with Control-ex samples, and statistically, the total cell density was not significantly different between the sericite and kaolinite treatments. In addition, the cell density increased as the ISS concentration increased. These results consistently indicated that the presence of ISS stimulates the NO$_2^-$ oxidation rate due to the enhancement of NOB growth. Although *N. winogradskyi* also has a high demand for iron, this bacterial species is less sensitive to iron concentration than *N. winogradskyi* (Meiklejohn 1955), thereby resulting in the observed insignificant difference between cell number and $k_{NO2}$ at the same concentration of the different ISS types.

The attachment of nitrifying bacteria onto solid surfaces is believed to enhance their growth (Underhill & Prosser 1987; Diab & Shilo 1988; Xia et al. 2009). The effect of the solid–water interface on microbial growth may be explained by indirect mechanisms in which the surrounding environment of the cell is modified in the presence of solid surfaces (Vanloosdrecht et al. 1990). One reported mechanism is that the solid surface possibly provides physical support for the stable growth of nitrifying bacteria (Kholdebarin & Oertli 1977). Another possible mechanism is the formation of a stable extracellular layer on the solid surface, facilitating the growth of bacteria (Keen & Prosser 1987). In our study, the attachment of *N. winogradskyi* onto sericite (but not onto kaolinite) resulted in higher bacterial cell densities in Seri-ex samples compared with Control-ex and Kao-ex samples, suggesting that the physical support of sericite particles has an effect on *N. winogradskyi* growth. Furthermore, given that NO$_2^-$ and NO$_3^-$ ions are not adsorbed onto the sericite particle surface, *N. winogradskyi* likely uses the sericite surface simply for attachment. Our results also suggest that the surface of kaolinite particles is unsuitable for colonization by *N. winogradskyi*, consistent with previous reports by Deflaun & Mayer (1985) and Yamamoto & Lopez (1985).

**Potential mechanisms for the enhanced nitrification rate in the presence of ISS**

Several previous studies indicated that the ability of ISS to maintain an optimal aqueous pH and provide essential nutrients for the growth of bacteria are the primary mechanism underlying the stimulatory effect of ISS on bacterial interactions (Stotzky & Rem 1966; Macura & Stotzky 1980). In the present study, we maintained the pH of the medium at an optimal level throughout the experiment. Thus, the various oxidation rate constants ($k_{NH4}$ and $k_{NO2}$) determined using different ISS types and concentrations suggest that the observed effects of ISS on bacterial cell density and oxidation rate ($k_{NH4}$ and $k_{NO2}$) are due to mechanisms unrelated to the pH buffer capacity of ISS. Possible mechanisms may include the role of ISS as an iron source for *N. europaea* and *N. winogradskyi* and as a physical support for NO$_2^-$ oxidation by *N. winogradskyi*. Moreover, numerous spectroscopic studies have shown that clay particles adsorb water molecules, especially in the presence of exchangeable cations, resulting in changes in the properties of water molecules near the particle surface (Russell & Farmer 1964; Johnston et al. 1992). Accordingly, water molecules coordinated to exchangeable cations are more strongly polarized than bulk water, with the H–O–H bending mode of adsorbed water being up to three times greater than that of bulk water. Thus, it is also likely that these adsorbed water molecules are more chemically active than molecules in bulk water. According to reactions (2) and (3), substrate must react with H$_2$O molecules to complete nitrification (Abeliovich 2006). Thus, nitrification may proceed at a faster rate in the presence of ISS.

**SUMMARY**

This study investigated the interaction between ISS and nitrification in freshwater samples; based on the experimental results, we suggested potential mechanisms involved in this interaction. Our results pointed out that the ISS type and concentration exert important effects on the nitrification processes. Interestingly, two sub-processes of nitrification proceeded in a different manner, depending on the ISS type and concentration. The type of ISS was found to be more important than ISS concentration for NH$_4^+$ oxidation, whereas ISS concentration was the more important factor for NO$_2^-$ oxidation. Sericite was found to enhance two sequential nitrification processes at all concentrations studied (i.e. 100–1,000 mg L$^{-1}$). Although the
sericite surface did not support the growth of and \( \text{NH}_4^+ \) oxidation by AOB, sericite may nonetheless provide a potential iron source for the growth of AOB. However, \( \text{NO}_2^- \) oxidation by NOB is enhanced, likely via physical adsorption of NOB onto sericite particles. Therefore, this study suggests a potential application of sericite as an additional material to stimulate nitrification in water treatment processes. Thus, it will be valuable to investigate the nitrification process in further detail in the presence of sericite.

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