The strong biocidal effect of free nitrous acid on anaerobic sewer biofilms

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Inhibition
Nitrite
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Methane

Abstract

Several recent studies showed that nitrite dosage to wastewater results in long-lasting reduction of the sulfate-reducing and methanogenic activities of anaerobic sewer biofilms. In this study, we revealed that the quick reduction in these activities is due to the biocidal effect of free nitrous acid (FNA), the protonated form of nitrite, on biofilm microorganisms. The microbial viability was assessed after sewer biofilms being exposed to wastewater containing nitrite at concentrations of 0–120 mg-N/L under pH levels of 5–7 for 6–24 h. The viable fraction of microorganisms was found to decrease substantially from approximately 80% prior to the treatment to 15% after 6–24 h treatment at FNA levels above 0.2 mg-N/L. The level of the biocidal effect has a much stronger correlation with the FNA concentration, which is well described by an exponential function, than with the nitrite concentration or with the pH level, suggesting that FNA is the actual biocidal agent. An increase of the treatment from 6 to 12 and 24 h resulted in only slight decreases in microbial viability. Physical disrupted biofilm was more susceptible to FNA in comparison with intact biofilms, indicating that the biocidal effect of FNA on biofilms was somewhat reduced by mass transfer limitations. The inability to achieve 2-log killing even in the case of disrupted biofilms suggests that some microorganisms may be more resistant to FNA than others. The recovery of biofilm activities in anaerobic reactors after being exposed to FNA at 0.18 and 0.36 mg-N/L, respectively, resembled the regrowth of residual sulfate-reducing bacteria and methanogens, further confirming the biocidal effects of FNA on microorganisms in biofilms.

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1. Introduction

Hydrogen sulfide production and emission is a ubiquitous problem in sewer systems (Hvitved-Jacobsen, 2002; US EPA, 1974, 1991). Sulfate-reducing bacteria (SRB) proliferate in anaerobic sewers, reducing sulfate to sulfide. Sulfide is a major cause for sewer corrosion and odor, and is also a source of health hazard (Boon, 1995; Pomeroy, 1990; Thistlethwayte, 1972; US EPA, 1991; WERF, 2007). An increasing trend of sulfide production in sewers has been observed over the last 20 years (WERF, 2007).

Methane production in sewers has also been recognized recently. Field measurements conducted by Foley et al. (2009) and Guisasola et al. (2008, 2009) showed that a substantial amount of methane could be formed in pressure sewers. Given the low solubility of methane, it would be released at locations where a gas and liquid interface occurs including pumping stations, gravity sewers and also...
the inlet work of a wastewater treatment plant (GWRC, 2011). Methane is a potent greenhouse gas, whose global warming potential is about 21–23 times that of CO₂ (IPCC, 2006). Methane emission in sewers could thus contribute significantly to the greenhouse gas emissions from wastewater systems (Guisasola et al., 2009; GWRC, 2011). Besides, methane is also an explosive gas with a lower explosive limit of approximately 5%.

To control the production and release of hazardous sulfide in sewers, many chemical dosing strategies have been developed and used in practice (US EPA, 1992; Zhang et al., 2008). Commonly used chemicals include oxygen/air, nitrate, metal salts and alkali. These chemicals either remove dissolved hydrogen sulfide through oxidation (Bentzen et al., 1995; Gutierrez et al., 2008; Ochi et al., 1998), or precipitation reactions (Jameel, 1989; Padival et al., 1995), or reducing the transfer of molecular H₂S from wastewater to air through pH elevation (Gutierrez et al., 2009; Yongsiri et al., 2005). Some of these strategies have also been found to reduce methane formation in sewers (Gutierrez et al., 2009; Mohanakrishnan et al., 2009; Zhang et al., 2009a). These traditional dosing strategies involve constant addition of chemicals to remove sulfide already formed. The constant dosing requirement causes large chemical consumption and high operational costs.

It is favorable to reduce sulfide and methane production rather than to remove them after their production. To this end, more cost-effective strategies were under investigation using antimicrobial agents, including metabolic inhibitors and broad-spectrum biocides. Several biocidal agents have been reported to be effective in controlling sulfide and methane generation. Gardner and Stewart (2002) reported that a pulse dose of glutaraldehyde at 500 mg/L for 7 h completely stopped sulfide production by a mixed-culture biofilm. However, sulfide production resumed after 60 h Zhang et al. (2009b) found that sulfide generation in sewage was decreased by 90% by formaldehyde at 19 mg/L Greene et al. (2006) combined six broad-spectrum biocides (glutaraldehyde, bronopol, tetraphos hydroxymethyl phosphonium sulfate, benzalkonium chloride, cocodiamine, formaldehyde) with nitrite to inhibit sulfide production. Nitrite was found to be synergistic with biocides and the combination allows more efficient and cost-effective control of SRB. As a metabolic inhibitor for SRB, molybdate was employed to control H₂S production by a pure culture of SRB, in swine manure treatment, and in anaerobic digestors (Nemati et al., 2001; Predicala et al., 2008; Tanaka and Lee, 1997). Although many of the microbial biocides and inhibitors have been proven to be effective, they may impose adverse impacts on the environment due to their generic toxicity and low degradability. For the application in sewers, the residual biocides in sewage may have detrimental effects on the microbial processes in the downstream wastewater treatment plants.

Nitrite, known as a specific SRB inhibitor (Greene et al., 2003), has also been shown to be able to suppress both sulfide and methane production in lab-scale sewer reactors (Jiang et al., 2010; Mohanakrishnan et al., 2008). The continuous dosing of nitrite for four weeks was found to achieve lasting (1–2 months) reduction in sulfide and methane production in the sewer reactors. The lasting effectiveness was suggested to be due to the decreased or suppressed growth of sulfate-reducing and methanogenic populations in biofilms during the extended dosing period, and the recovery of these populations required 1–2 months.

However, in the field trial conducted by Jiang et al. (2010), a long-lasting reduction in sulfide and methane production (for weeks and months, respectively) was achieved by dosing nitrite at 100 mg-N/L for only 33 h. The recovery of sulfide and methane production resembled the regrowth of sulfate-reducing bacteria and methanogens. This could not be explained by the commonly observed inhibiting role of nitrite. Inhibitors like nitrite (sulfite analog) or molybdate (sulfate analog) interrupt the sulfate reduction pathway by blocking the dissimilatory sulfite or sulfate reductase (Greene et al., 2006, 2003). SRB may survive the inhibition if the exposure time is short, and resume their activity when the inhibitor is removed. Long-term application of an inhibitor is thus required in order to decrease the SRB population, through which to achieve long-lasting effectiveness.

We hypothesize that the lasting sulfide and methane control effects achieved by a short dosage of nitrite was due to a biocidal rather than an inhibitory effect. The aim of this study is to investigate this potential biocidal effect of nitrite and its derivative free nitrous acid (FNA, the protonated form of nitrite) on anaerobic wastewater biofilms. Laboratory-scale anaerobic reactors were used to grow sewer biofilms with real wastewater. Intact and disrupted biofilm samples taken from the reactors were incubated in wastewater containing nitrite at concentrations of 0, 30, 60, 90, and 120 mg-N/L, at pH levels of 5, 6, 6.5, and 7, and for 6, 12, and 24 h. These combinations of nitrite and pH levels give rise to FNA concentrations of 0–3 mg-N/L. The abundance of viable microorganisms in biofilms both prior to and after the treatment was measured using a LIVE/DEAD staining assay, which assesses microbial viability by verifying cell membrane integrity. Simultaneous dosing of nitrite and acid was also directly conducted with the sewer reactors, during which the suppression and recovery of sulfide and methane production activities of the reactors were measured.

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2. Material and methods

2.1 Anaerobic sewer biofilm reactors

Three lab-scale sewer reactors, namely R1, R2, and R3, were set up for growing biofilms with real wastewater under anaerobic conditions. These reactors were made of Perspex™. Each reactor had a volume of 0.75 L, with a diameter of 80 mm and a height of 149 mm (Fig. S1). Plastic carriers (Anox Kaldnes, Norway) of 1 cm diameter were clustered on four stainless-steel rods inside each reactor to provide additional surfaces for biofilm growth and to allow sampling of intact biofilms (see below). The total volume of the carriers used for each reactor was about 15 mL (2% of the reactor volume). The total biofilm area in each reactor, including both the reactor wall and carrier surfaces, was approximately 0.05 m². The area to volume ratio (A/V) was therefore 70.9 m²/m³.

Domestic wastewater, collected weekly from a wet well in Brisbane, Australia, was stored in a cold room at 4 °C. A water bath before each pumping event. The sewage typically contained sulfide at concentrations of <3 mg-S/L, sulfate at concentrations between 10 and 25 mg-S/L, and volatile fatty acid (VFA) at 50–120 mg-COD/L. Nitrite was below detection limits in the fresh sewage.

The reactors were fed with sewage through a peristaltic pump (Masterflex 7520-47) every 6 h. Every feed pumping event lasted for 2 min, delivering one reactor volume of sewage into each reactor. Mixing (200 rpm) was provided continuously with magnetic stirrers (Heidolph MR3000) to produce a moderate shear force, and also to avoid solids settling at the bottom.

Batch tests were carried out regularly (every 1–2 weeks) to measure the sulfide and methane production rates of each reactor. The tests were started by pumping fresh sewage into reactors for 6 min (three hydraulic retention times) to ensure a thorough replacement of liquid in reactors with fresh sewage. Wastewater samples were taken at 0, 30, 60, 90, and 120 min after pumping, for the analysis of dissolved inorganic sulfur (sulfide, sulfite, thiosulfate, and sulfate) and dissolved methane using methods to be further described below. The sulfide and methane production rates were calculated using linear regression of the sulfide and methane concentrations.

The reactors were operated for 9 months to reach stable performance (as indicated by the sulfide and methane production rates) before the FNA tests described below commenced. The three reactors displayed very similar sulfide and methane production rates.

2.2 Viability tests on the biocidal effect of FNA

2.2.1 FNA toxicity to intact biofilms

Twelve sets of tests were carried out under conditions summarized in Table 1. In each set, 1 L of wastewater taken from the cold room was heated up to 20 °C. The wastewater pH was then adjusted to the designated pH level (Table 1) with 1 M hydrochloric acid. The pH-adjusted wastewater was then used to fill up five 75 mL single-use sterile bottles (Borstedt, Australia). Pre-determined amounts of a sodium nitrite stock solution (12 g-N/L) were added to the five bottles to achieve the designated nitrite concentrations, i.e. 0, 30, 60, 90, and 120 mg-N/L (Table 1). For each set of tests, one more bottle was filled with fresh sewage (pH = 7.6) as the control. A plastic carrier with attached biofilm was transferred from the biofilm reactors into each bottle. The bottle was then capped and kept anaerobic by avoiding air bubbles. Gentle mixing was provided by an orbital shaker at 60 rpm. The duration of incubation used in each set of tests is as described in Table 1. The pH level and nitrite concentration in each bottle were measured at the end of each test. For the 6- and 12-h tests, the nitrite concentration decreased marginally (<5%), and pH increased only slightly (<0.2 unit). For the 24-h tests, the wastewater was replaced after 12 h to ensure that the designated pH and nitrite levels were maintained. At the end of each test, the biofilm on the carrier was sampled for LIVE/DEAD staining, with methods to be further described.

2.2.2 FNA toxicity to disrupted biofilms

In addition to the above tests with intact biofilms, one set of tests was carried out with disrupted biofilms. The biofilm carrier was transferred from a reactor to a 10 mL plastic tube

<table>
<thead>
<tr>
<th>Test NO.</th>
<th>Exposure time (hour)</th>
<th>pH</th>
<th>Nitrite (mg-N/L)</th>
<th>FNAα (mg-N/L)</th>
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<td>7</td>
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<td>0, 0.008, 0.015, 0.023, 0.031</td>
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<td>6</td>
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<td>0.077, 1.53, 2.3, 3.07</td>
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<td>5</td>
<td>0, 30, 60, 90, 120</td>
<td>0.77, 1.53, 2.3, 3.07</td>
</tr>
</tbody>
</table>

α FNA was not measured but calculated from the nitrite and pH levels applied/measured: FNA = NO₂⁻ / N_0 / (K_a × 10^{pH})

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filed with wastewater to avoid exposure to air. The biofilm was then detached from the plastic carrier with an ultrasonic bath (Soniclean 250 HT, 6 L, 120 W pulse swept power) at 45 kHz for 60 s, followed by 30 s of vortexing. The disrupted biofilm was exposed to pH = 6 and nitrite concentrations of 0, 30, 60, 90, and 120 mg-N/L, for 6 and 24 h. Microbial viability was analyzed with LIVE/DEAD staining prior to and after the exposure to nitrite. The particle size distribution of the disrupted biofilms was measured with Mastersizer 2000 (Malvern Instruments).

2.3. Direct FNA dosing to sewer biofilm reactors

Upon the completion of the above-described toxicity tests, Reactors R2 and R3 were dosed with nitrite and acid to reach FNA at 0.18 and 0.36 mg-N/L, respectively for 24 h (four pumping cycles). This was achieved by adding nitrite to these two reactors immediately after each pumping event to achieve nitrite concentrations of 70 and 140 mg-N/L, respectively. The pH in both dosed reactors was maintained at 6.0 ± 0.1 by adding 1 M hydrochloric acid. R1 was not dosed with nitrite or acid, and therefore served as a control reactor. This experiment aimed to verify the biocidal effect of FNA revealed in the viability tests through monitoring the loss of biofilm sulfide and methane producing activity after FNA dosage and its subsequent recovery. These dosing conditions (FNA levels and duration) were chosen based on the results of the viability tests.

Batch tests as described in a previous section were conducted immediately after the 24 h dosage and continued for 60 days with intervals of 2 days to two weeks, to monitor the sulfide and methane production rates of the three reactors. Biofilm carriers were taken from all reactors prior to and immediately after dosing events to determine the viability of microorganisms in biofilms.

2.4. LIVE/DEAD staining

The viability of microorganisms in biofilms was determined using the LIVE/DEAD® BacLight™ bacterial viability kits (Molecular Probes, L-7012). The viability kits utilize two nucleic acid stains, namely green-fluorescent SYTO-9 and red-fluorescent Propidium Iodide (PI) (Invitrogen Molecular Probes, 2004). The SYTO-9 stain generally labels all microorganisms in a population with intact or damaged membranes. In contrast, PI stain penetrates only those microorganisms with damaged membranes, causing a reduction in the SYTO-9 stain fluorescence when both dyes are present. Thus, microorganisms with intact cell membranes (viable cells) are stained green, whereas microorganisms with damaged membranes (dead cells) are stained red.

Biofilm on a plastic carrier was detached in filtered (0.22 μm) sewage with vigorous shaking and vortex mixing. Biofilm suspension (125 μL for each test) was transferred into 2-mL plastic centrifuge tubes with 50 μL of SYTO-9 and PI mixture solution. The tubes were incubated in a dark place for 15 min at the room temperature (20 °C), allowing the staining reactions to complete. Then, microscope slides with stained biofilm samples were photographed using a confocal laser scanning microscope (Zeiss LSM 510 META), equipped with a Krypton–Argon laser (488 nm) and two He–Ne lasers (543 and 633 nm).

Twenty images were taken for randomly chosen areas of each sample. Quantification of live and dead microorganisms was done by determining the relative abundance of green and red pixels. The pixel area counting was conducted with the ImageJ (National Institute of Health, USA). The percentage of green fluorescence to the total fluorescence (red + green fluorescence) was assumed to be equal to the percentage of viable cells to the total cells (viable + dead) in the biofilm.

2.5. Chemical analysis

For the analyses of dissolved inorganic sulfur species, 1.5 mL wastewater was filtered (0.22 μm membrane) into 0.5 mL preserving solution of sulfide anti-oxidant buffer (SAOB) (Keller-Lehmann et al., 2006). Samples were analyzed within 24 h on an ion chromatograph (IC) with a UV and conductivity detector (Dionex ICS-2000). For the analysis of nitrogen species (nitrite), 1 mL of wastewater was filtered and diluted 10 times. It was analyzed using a Lachat QuikChem 8000 (Milwaukee) flow-injection analyzer (FIA). VFA was measured by gas chromatography (PerkinElmer, Inc.). For the measurement of dissolved methane, 5 mL wastewater was filtered into vacuumed BD vacutainer tubes using a hypodermic needle attached to a plastic syringe. The tubes were allowed to reach gas–liquid equilibrium overnight. Methane in the gas phase was measured by gas chromatography (Shimadzu GC-9A) equipped with a flame ionization detector (FID). Concentrations of methane in wastewater were calculated using mass balance and Henry’s law (Guisasola et al., 2008).

3. Results

3.1. Toxicity of FNA to anaerobic sewer biofilm

Fig. 1A & B shows the percentage of viable microorganisms in biofilm plotted against the nitrite and pH levels, respectively, for the 12-h exposure tests (Tests 5–7). Similar results were obtained with the 6-h and 24-h tests (Figs. S2 and S3). The dependence of viable percentage on FNA concentration for the three exposure times, i.e. 6 (Tests 1–3), 12 (Tests 5–7), and 24 h (Tests 9–11), are shown in Fig. 1C–E respectively. The data obtained in Tests 4, 8 and 12 (i.e. Tests with pH = 5) are not included in these figures and will be presented separately.

There is a general negative impact of nitrite on the microbial viability. Higher nitrite concentration induced lower microbial viability. However, microbial viability varied in a wide range for each of the nitrite levels except for the case without nitrite addition, suggesting that nitrite is not the sole factor contributing to the loss of microbial viability and pH likely played a role in conjunction with nitrite. A similar observation can be made on the relationship between microbial viability and pH. Microbial viability was reduced to very different levels at the same pH but different nitrite levels. This suggests that pH within the range of 6.0–7.0 was not the main toxic factor either.

Fig. 1C–E shows that the level of microbial viability had a much stronger dependence upon the FNA concentration, indicating that FNA may directly cause the inactivation of
microorganisms in biofilms. Also shown in the figure is the fit between the experimental data and the predictions by an exponential model ($y = y_0 + ae^{-bx}$). It is seen that the toxicity of FNA to sewer biofilm could be well described by the exponential function.

Microbial viability decreased sharply with increased FNA concentration in the range of $0-0.1$ mg-N/L. Microbial viability decreased by 50% after being exposed to FNA at a concentration of $0.045$ mg-N/L (equivalent to a nitrite concentration of $18$ mg-N/L at pH 6) for all three exposure times, i.e. 6, 12 and 24 h. The decrease in microbial viability slowed down when the FNA concentration further increased.

The loss of microbial viability also depended on the duration of the FNA treatment. In the exponential model, parameters $b$ and $y_0$ indicate the overall decreasing rate of microbial viability and the residual percentage of viable microorganisms, respectively. When the exposure time increased from 6 to 12 and 24 h, $b$ increased from 17.1 to 19.7 and 22.5, respectively, while $y_0$ decreased from 13.6 to 10.2 and 7.6, respectively. Thus, longer exposure time could increase the microbial killing efficiency. Also, longer exposure resulted in lower residual microbial viability.

Fig. 2 shows that a pH level of 5 alone (in the absence of nitrite) can reduce the biofilm viability from 70 to 80% measured prior to the treatment to around 20% after 6–24 h exposure (Tests 4, 8, and 12). The presence of nitrite further enhanced the biocidal effect. It is worthwhile to note, however, that microbial viability was still 2–3% when the FNA concentration was greater than 3 mg-N/L (120 mg-N/L at pH 5). This indicates that a small portion of microorganisms is highly resistant to FNA.

### 3.2. Toxicity of FNA to disrupted biofilms

The physical disruption (ultra-sonication and vortex mixing) detached biofilms and broke them down to particles with a median size of $310 ± 5$ μm (See Fig. S4 for the particle size distribution). The microbial viability after sonication was at the same level of intact biofilms, i.e. approximately 70% (The leftmost data points in Fig. 3), implying that sonication did not cause significant microbial death by itself. It was found that disruption made the biofilm more susceptible to pH change. In the absence of nitrite, pH change from 7.6 to 6 decreased the viability by 25% in the disrupted biofilms. In comparison, this decrease was negligible for the intact biofilm samples (Fig. 3).

Fig. 3 also shows that disrupted biofilm was more sensitive to FNA toxicity. The viable microorganisms in disrupted
biofilm after exposure to FNA for 6 h were 10–20% lower than that in the intact biofilm. It is clear that the FNA toxicity to biofilms can be augmented by physical disruption of biofilms. For the 24-h exposure, the percentage of viable microorganisms (%) in intact and disrupted biofilms reached similar levels at high FNA concentrations. As shown in Fig. 3, about 4–5% of microorganisms survived the exposure to FNA at 0.31 mg-N/L for 24 h. This implies that some microorganisms are resistant to FNA no matter in intact or in disrupted biofilms.

### 3.3. Effect of pH on biofilm viability

Biofilms in the reactors were developed with real wastewater, which had a pH around 7.6. Fig. 4 shows the viability test results using intact biofilms at different pH in the absence of nitrite. The viable microorganisms in the anaerobic biofilms incubated with fresh wastewater after 6–24 h were about 70–80% in all tests. No discernable decrease of microbial viability was observed when pH was lowered to 7. For pH adjustments to 6.5 and 6, there was a slight decrease (4–9%) of microbial viability for all exposure times. However, pH level at 5 was found to be strongly biocidal. It reduced the biofilm viability to 20% after 6-h exposure, and even lower for longer exposure times.

![Fig. 4 – Effect of pH on microbial viability in biofilms after incubation at the specified pH for 6, 12 and 24 h.](image)

**Fig. 3**—The viable percentages (%) in intact (filled symbols) and disrupted (empty symbols) biofilms after being exposed to FNA at pH = 6 for 6 and 24 h. The leftmost data points are the viability for biofilm samples taken from the reactors directly or sonicated biofilms. Regression lines were obtained with exponential decay equation $y = y_0 + ae^{-bx}$. The parameters are summarized in Table S2.

**Fig. 4**—Effect of pH on microbial viability in biofilms after incubation at the specified pH for 6, 12 and 24 h.

### 3.4. Biofilm activity after FNA dosage to reactors

Fig. 5A shows the sulfide production rates in the FNA dosed reactors, i.e. R2 and R3, relative to that in the control reactor R1. The sulfide and methane production rates of R1 remained stable during the entire experimental period. Both R2 and R3 had sulfide and methane production rates similar to those of R1 before the commencement of FNA dosing on Day 0. Although R2 and R3 were dosed with different levels of FNA, i.e. 0.18 and 0.36 mg-N/L, respectively, sulfide and methane production were both completely suppressed at the end of the 24-h dosing. The viable microorganisms in the R2 and R3 biofilms decreased to 8.0 ± 1.3% and 5.6 ± 2.9%, respectively after FNA dosing, compared to 82.1 ± 2.3% in R1, consistent with the results of viability tests reported above.

After the FNA dosing, sulfide and methane production in R2 and R3 started to recover without an obvious lag phase. No prominent differences could be found between R2 and R3 in terms of the rates of recovery. The Gompertz growth equation was employed to fit the recovery phase (jiang et al., 2010). It is clear that the recovery processes resemble that of microbial regrowth. 50% recovery of the sulfide production rates took 9.2 and 7.7 days, respectively, for R2 and R3. In comparison, the methane production rates took 58.0 and 52.3 days for R2 and R3, respectively, to reach 50% recovery. The recovery of methane production was about 7 times slower compared to the recovery of sulfide production.

### 4. Discussion

#### 4.1. Biocidal effect of FNA

Previous work has demonstrated the inhibitory effects of FNA on a broad range of microbial metabolism. Zhou et al. (2008)

![Fig. 5 - Sulfide (A) and methane (B) production rates of R2 and R3, relative to the corresponding R1 rates, prior to and after being exposed to free nitrous acid at 0.18 or 0.36 mg-N/L, respectively, on Day 0 (vertical arrows). The solid and dashed lines are regressions with the Gompertz growth equation, see details in Supplementary information.](image)
reported that FNA at a concentration of 0.004 mg-N/L or above could completely inhibit the N₂O reduction activity of a denitrifying enhanced biological phosphorus removal sludge. Vadivelu et al. (2006a,b) revealed that the anabolic processes (growth) of an enriched Nitrobacter and an enriched Nitrosomonas culture were stopped by FNA at concentrations of 0.023 and 0.4 mg-N/L, respectively. Similarly, Pijuan et al. (2010) and Ye et al. (2010) showed that the growth of Candidatus Accumulibacter phosphatis (a known polyphosphate accumulating organism) and Candidatus Competibacter phosphatis (a known glycogen accumulating organism) was completely suppressed by FNA at 0.006 mg-N/L and 0.007 mg-N/L, respectively. The above reported FNA-caused inhibition on microbial metabolism was found to be reversible, with the microbial activities resuming immediately or within hours after the removal of the inhibitor (Pijuan et al., 2010; Vadivelu et al., 2006a; Ye et al., 2010). It is noteworthy that FNA applied in most of these studies (with the exception of the study on Nitrosomonas) were at parts per billion (ppb) levels.

In this study, we revealed for the first time the strong biocidal effect of FNA on microorganisms in anaerobic wastewater biofilms. This was demonstrated through measuring both membrane integrity of microorganisms and biological activities of biofilm reactors before and after FNA treatment. After being exposed to FNA for 6–24 h at concentrations of 0.1–0.3 mg-N/L, viable microorganisms in anaerobic biofilms reduced from approximately 80% to 5–15%. In fact, the biocidal effect initiated at FNA levels far lower than 0.1 mg-N/L, and increased sharply with FNA concentration in the range of 0–0.1 mg-N/L. The biocidal effect was found to be strongly dependent on the FNA concentration rather than the nitrite concentration or the pH level separately (for the pH range of 6.0–7.6). The biocidal effect revealed by microbial viability tests was strongly supported by the loss of biofilm activity after FNA treatment and its subsequent slow recovery following termination of FNA dosages. Mathematical modeling showed that the activity recovery was likely due to the regrowth of the residual viable microorganisms.

Our experimental results suggest that acidified conditions promote the formation of FNA, which kills microorganisms in the anaerobic biofilms. While the detailed mechanism is yet to be revealed in future studies, a number of potential contributing factors are discussed below.

Once FNA was formed from nitrite under acidic conditions, many reactive derivatives can be generated (Yoon et al., 2006):

\[
\text{HNO}_2 + \text{HNO}_2 \rightarrow \text{N}_2\text{O}_3 + \text{H}_2\text{O} \rightarrow \text{NO} + \text{NO}_2 + \text{H}_2\text{O}
\]

Small molecules such as dinitrogen trioxide (N₂O₃), nitrogen dioxide (NO₂) and nitric oxide (NO) can readily cross cell membranes where they can react with reduced thiols to form nitrosothiols, which are thought to be important in microbial killing (Heaselgrave et al., 2010; Phillips et al., 2004). NO is a well-known antimicrobial agent (Fang, 1997), and has also been demonstrated to be able to cause dispersal of Pseudomonas aeruginosa biofilms and multi-species biofilms from water distribution and treatment systems (Barraud et al., 2006, 2009). The strong nitrosating intermediate, N₂O₃, is capable of modifying the function of proteins (Yoon et al., 2006). Also, NO₂ can induce lipid peroxidation, resulting in cell membrane damage (Halliwell et al., 1992).

FNA can cause oxidative deamination of the NH₂ group of adenine or cytosine to an ether group (Klug et al., 2009; Malling, 2004). It converts adenine to hypoxanthine (which pairs with C), cytosine to uracil (which pairs with A) and guanine to xanthine (which still pairs with C). FNA alters a DNA base pair directly to a “micsoding” form and thus does not require subsequent DNA synthesis for its effect. The change of DNA base pairing (mutagenesis) is lethal to microorganisms.

A further possible cause of microbial death could be the metabolic inhibition and the adverse change of their living environment. Interrupted metabolisms combined with the acidic pH can be lethal to microorganisms, especially after being exposed for 6 h or longer. The exposure time (6, 12, and 24 h) used in this study is much longer than those (0.5–3 h) in the FNA inhibition studies previously conducted (Pijuan et al., 2010; Vadivelu et al., 2006b; Ye et al., 2010; Zhou et al., 2008).

In the pH 5 tests, 2–3% of microorganisms remained viable after being treated at FNA concentrations of up to 3 mg-N/L. It is possible that some microorganisms residing in deep layers could have been protected against the FNA toxicity to a certain degree due to mass transfer limitations. However, a similar level of residual viable microorganisms was also observed in the disrupted biofilm studies, suggesting that some microbial species may be more tolerant to FNA than others. Indeed, a nitritation reactor treating anaerobic digestion liquor, where ammonia oxidizing bacteria (AOB, e.g. Nitrosomonas) proliferate, nitrite is typically at 500–600 mg-N/L and pH easily reaches 6.0–6.5, giving rise to FNA in the range 0.4–1.5 mg-N/L (Vadivelu et al., 2006a). These results clearly indicate that some AOB can tolerate these levels of FNA. The potentially different abilities of different microbial species to tolerate FNA toxicity require further investigation. Similarly, it is also important to study if microorganisms are able to adapt to high FNA concentrations.

4.2. Potential applications of FNA toxicity

The biocidal effect of FNA on anaerobic sewer biofilms revealed in this study implies that FNA could be used as a biocidal agent to control the growth and activities of detrimental anaerobic biofilms. One potential application is the control of sulfide and methane formation and emission from sewers, to replace or supplement conventionally used chemicals such as oxygen, nitrate, ferric/ferrous salts and magnesium hydroxide. Based on the experimental results obtained in this study, we propose an FNA dosing strategy comprising a short period of FNA dosing and a longer period of recovery. This will substantially reduce the amount of chemicals required to achieve a desirable control of sulfide production and emission from sewers.

There are many other potential benefits associated with the proposed intermittent FNA dosing. For example, continuous oxygen or nitrate addition to sewers causes consumption of organic carbon in sewers thus reduces its availability for nutrient removal at the downstream wastewater treatment plant (Gutierrez et al., 2008; Mohanakrishnan et al., 2009). In contrast, with the intermittent FNA dosing strategy, only a small amount of electron acceptor (i.e. nitrite) is supplied, and consequently the amount of carbon to be oxidized due to nitrite reduction will be small. In addition, given the very slow recovery of methanogenic activities of sewer biofilms, intermittent dosages of FNA will likely permanently suppress...
methylene production, reducing greenhouse gas emissions from sewers on the one hand, and preserving carbon sources for nutrient removal on the other hand (methane is not available for denitrification or phosphorus removal).

Like other broad-spectrum biocides having multiple targets in terms of regulating gene expressions (Lee et al., 2010), FNA was demonstrated to be a biocide to different microorganisms. However, the adverse impacts of FNA to environment and the downstream wastewater treatment processes are expected to be negligible if added appropriately. It is not expected that FNA would be added to an entire sewer network at the same time. Instead, it should be added to different sections of a network at different time. At downstream sewer sections or at the treatment plant, nitrite will be diluted and pH neutralized, leading to non-toxic and non-inhibitory levels of FNA, which can be removed microbiologically. NOx (NO and NO2), form as FNA derivatives at acidic pH in the closed sewer pipes (rising main). Primarily, they can easily cross cell membranes and react with cell DNA, proteins and lipids. The residual NOx will be diluted in downstream pipes of selected dosing sites. Thus, NOx release from sewer system is not expected to be a significant problem. However, it is recognized that further study should be carried out to quantify the impacts of NOx release.

The intermittent FNA dosing strategy discussed above is likely applicable to many other anaerobic environments, e.g., for odor management of landfill and souring control of oil fields. The dosing frequency of an intermittent strategy will depend on the microbial growth rate. For faster growing microorganisms, a more frequent dosing of FNA will be required to keep the activity low at all times. Research is required for optimizing the dosing strategy under different environments.

5. Conclusions

The biocidal effect of free nitrous acid on anaerobic sewer biofilms was investigated through both viability tests and reactor studies. The key findings are:

- Free nitrous acid showed strong biocidal effect toward microorganisms residing in anaerobic sewer biofilms. The level of the biocidal effect has a much stronger correlation with the FNA concentration than with the nitrite concentration or with the pH level, suggesting that FNA is the actual biocidal agent.
- The biocidal effect of FNA is reduced by the limitation of mass transfer in the thick sewer biofilm. Some microorganisms in the biofilms might be resistant to FNA at concentrations up to 3 mg-N/L for exposure time up to 24 h.
- Based on the biocidal effects of FNA, intermittent dosing of nitrite with acid (to form FNA) is potentially a cost-effective strategy to control sulfide and methane production in sewers.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.watres.2011.04.026.

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