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Iron Sulfide Attenuates the Methanogenic Toxicity of Elemental Copper and Zinc Oxide Nanoparticles and their Soluble Metal Ion Analogs

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Abstract

Elemental copper (Cu^0) and zinc oxide (ZnO) nanoparticle (NP) toxicity to methanogens has been attributed to the release of soluble metal ions. Iron sulfide (FeS) partially controls the soluble concentration of heavy metals and their toxicity in aquatic environments. Heavy metals displace the Fe from FeS forming poorly soluble metal sulfides in the FeS matrix. Therefore, FeS may be expected to attenuate the NP toxicity. This work assessed FeS as an attenuator of the methanogenic toxicity of Cu^0 and ZnO NPs and their soluble salt analogs. The toxicity attenuation capacity of fine (25–75 μm) and coarse (500 to 1200 μm) preparations of FeS (FeS-f and FeS-c respectively) was tested in the presence of highly inhibitory concentrations of CuCl_2 , ZnCl_2 , Cu^0 and ZnO NPs. FeS-f attenuated methanogenic toxicity better than FeS-c . The results revealed that $2.5\times$ less FeS-f than FeS-c was required to recover the methanogenic activity to 50% (activity normalized to uninhibited controls). The results also indicated that a molar FeS-f/Cu^0 NP, FeS-f/ZnO NP, FeS-f/ZnCl_2 , and FeS-f/CuCl_2 ratio of 2.14, 2.14, 4.28, and 8.56 respectively, was necessary to recover the methanogenic activity to $>75\%$. Displacement experiments demonstrated that CuCl_2 and ZnCl_2 partially displaced Fe from FeS . As a whole, the results indicate that not all the sulfide in FeS was readily available to react with the soluble Cu and Zn ions which may explain the need for a large stoichiometric excesses of FeS to highly attenuate Cu and Zn toxicity. Overall, this study provides evidence that FeS attenuates the toxicity caused by Cu^0 and ZnO NPs and their soluble ion analogs to methanogens.

Keywords

Nanomaterials; Elemental copper; Zinc Oxide; Toxicity attenuation; Anaerobic digestion; Inhibition constants

1 Introduction

Engineered nanoparticles (NPs) are man-made materials with at least one dimension <100 nm. Copper-based (Cu-based) and zinc oxide (ZnO) NPs are applied in several industrial

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processes or commercial products. Cu-based NPs are used in products such as wood preservatives, catalysts, printable electronics, or antimicrobials (Wang et al., 2013); likewise, Cu-based NPs are the byproduct of the chemical and mechanical polishing in the semiconductor industry (Golden et al., 2000). ZnO NPs are also applied in industrial processes and extensively used in consumer products such as sunscreens, cosmetics, and bottle coatings due to their ultraviolet blocking properties and visible transparency (Klaine et al., 2008).

The majority of NPs applied in consumer products are likely to be disposed into the sewer (Kim, 2014). Therefore, NPs will end up in biological processes such as aerobic activated sludge of wastewater treatment plants. Studies investigating the fate of NPs in wastewater treatment have found accumulation of NPs in activated sludge solids (Westerhoff et al., 2013). The accumulation of NPs may have toxic effects not only to the activated sludge microorganisms, but also in the microbial cultures involved in the stabilization of the waste sludge by anaerobic digestion, such as methanogens.

Recent research has consistently shown that elemental copper (Cu^0) and ZnO NPs are toxic to methanogens (Gonzalez-Estrella et al., 2015; Gonzalez-Estrella et al., 2013; Mu et al., 2012; Otero-González et al., 2014a). Results have also indicated that the soluble ions released by NPs cause toxicity (Luna-delRisco et al., 2011; Mu et al., 2011). Recently it was shown that biogenic sulfide (S^{2-}) produced by sulfate reduction decreased the toxic effect of Cu^0 and ZnO NPs (Gonzalez-Estrella et al., 2015). Soluble ions released from these types of NPs are hypothesized to precipitate with the sulfide. These findings establish that biogenic S^{2-} from sulfate reduction reliably attenuate Cu^0 and ZnO NP toxicity to methanogens. Other potential sources of S^{2-} present in anaerobic environments may also play a similar attenuating role.

Iron sulfides are one of the most common forms of sulfide on the Earth crust (Muyzer & Stams, 2008). Iron sulfides, along with manganese sulfide, is one of the largest reservoirs of sulfides in aquatic sediments (Di Toro et al., 1992). The oxidization of organic matter in anaerobic environments provides the conditions for the bio-reduction of sulfate and Fe (III) yielding S^{2-} and Fe^{2+} (Morse et al., 1987), which react with each other to form iron sulfide (FeS) and pyrite (FeS_2) (Haaijer et al., 2012; Morse et al., 1987). FeS is a poorly soluble mineral ($\log K_{s0} = -16.84$ (Benjamin, 2002)) that commonly interacts with other heavy metals in aquatic sediments (Besser et al., 1996); and therefore, it regulates the concentration of heavy metals in these environments (Casas & Crecelius, 1994).

When divalent metals with more affinity for S^{2-} such as cadmium, copper, lead, mercury, or zinc are present in anaerobic sediments, the Fe^{2+} of FeS can be displaced by these divalent metals forming more stable metal sulfides and releasing Fe^{2+} cations to the aqueous phase (Peng et al., 2009). For instance, Cu^{2+} and Zn^{2+} have higher stability constants for sulfide (CuS ($\log K_{s0} = -35.96$) and ZnS ($\log K_{s0} = -21.97$) (Benjamin, 2002)) than FeS; thus, Cu^{2+} and Zn^{2+} will displace the Fe of FeS to form CuS and ZnS. This mechanism illustrated in Eq.1

where Me^{2+} are heavy metals such as Cu^{2+} or Zn^{2+} . Simpson et al. (2000) experimentally demonstrated the immobilization of Cd, Cu, and Zn by reaction with FeS.

Reactions of FeS with heavy metals have a key role controlling the toxicity in aquatic sediments (Allen et al., 1993; Casas & Crecelius, 1994; Di Toro et al., 1992; Di Toro et al., 1990). Consequently, a similar approach could be taken to attenuate the methanogenic toxicity of Cu^0 and ZnO NP and their soluble metal ion analogs by adding FeS to anaerobic reactors with presence of these inhibitors. If the toxic Cu^{2+} and Zn^{2+} ions released by Cu^0 and ZnO nanoparticles displace the Fe^{2+} in FeS to form stable metal sulfides, Cu^0 and ZnO toxicity can be expected to be attenuated by FeS. The purpose of this work was to evaluate the attenuation of Cu^0 and ZnO NP toxicity to methanogens by FeS.

2 Material and Methods

2.1 Chemicals

All NPs were acquired as powders. Cu^0 NPs (40–60 nm, 99%) were purchased from Sky-Spring Nanomaterials Inc. (Houston, TX), ZnO NPs (100 nm, 99%), $\text{CuCl}_2 \cdot \text{H}_2\text{O}$ (99%), ZnCl_2 (98%), sodium acetate (99.9%), and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (>98%) were acquired from Sigma Aldrich (St. Louis, MO, USA). $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ (>99%) was acquired from Fisher-Scientific. CH_4 standard gas (99%) was acquired from Air Liquid America (Plumstedsville, PA, USA).

2.2 Nanoparticle dispersions and metal solutions

ZnO and Cu^0 NP stock dispersions were sonicated (DEX® 130, 130 Watts, 20 kHz, Newtown, CT) at 70% amplitude for 5 min. No dispersant agent was supplied to the stock solutions. ZnO and Cu^0 NP stability in anaerobic media has been previously described (Gonzalez-Estrella et al., 2013; Otero-González et al., 2014a). The studies report a PSD of 685 ± 64 and 958 ± 34 for ZnO and Cu^0 NP, respectively; and a z-potential of -26.3 ± 0.8 and -14.9 ± 0.9 for ZnO and Cu^0 NP, respectively. Previous transmission electron microscopy (TEM) analysis reported an average particle size of 48.5 ± 27.8 nm for ZnO NPs (Otero-González et al., 2014a). TEM analysis applying the same methodology showed an average particle size of 50 ± 15.0 nm for Cu^0 NP (Figure S1, Supporting Information). Stock CuCl_2 and ZnCl_2 solutions were prepared by dissolving the salts in 0.01 M HCl.

2.3 FeS synthesis and characterization

FeS was synthesized by adapting a methodology previously used (Patterson et al., 1997). FeS was prepared by mixing an equimolar concentration of sodium sulfide and iron chloride (FeCl_2) for 10 min. Next, the suspension was centrifuged in 50 mL vials at 4000 rpm for 20 min. The supernatant was discarded and the pellet of FeS was resuspended with ~50 mL ethanol. Subsequently, the suspension was subjected twice to centrifuging (4000 rpm for 20 min) and rinsing procedure to eliminate the majority of the water. Next, the supernatant was discarded and the pellet was rapidly transferred to test tubes. The test tubes were sealed and flushed with N_2 gas until the pellet was completely dry. After drying the pellet, the product was composed of a coarse preparation of FeS particles (FeS-c). Half of the FeS-c was agitated in the test tube vigorously with a vortex mixer until the particle size was decreased to a fine preparation of FeS particles (FeS-f). The synthesized FeS preparations were kept in

sealed flask with N₂ atmosphere to prevent oxidation. The particle size of FeS-c and FeS-f particles was evaluated by SEM analyses. The images of the SEM analyses of FeS-c and FeS-f are shown in Figure 1. The analyses indicated that FeS-c had a particle size range of 500 to 1200 µm, whereas the range FeS-f particles were 25–75 µm. It should be noted that a few coarse pieces of FeS can be observed in FeS-f as shown in Figure 1B. Fe content of FeS was determined by measuring the soluble Fe after a microwave digestion assisted treatment. The samples revealed a content of 0.45 mg Fe mg solid⁻¹. Therefore, assuming that the concentration of Fe and S in the solid was stoichiometric, the purity of the synthesized FeS is 71.3%. A sample of each fraction was characterized by X-ray diffraction (XRD) analyses. XRD analysis revealed an amorphous composition by the lack of any clear X-ray diffraction signals in the samples (Figure S2). An additional energy-dispersive X-ray spectroscopy (EDS) analysis, revealed that the surface of the material contained 12.5 ± 2.07% (atomic weight) of S and 21.3 ± 7.96% of Fe. The mass ratio of Fe to S on the surface of 1.72 is consistent with the theoretically expected mass ratio of 1.74 for FeS. EDS analysis showed that the impurities of FeS were caused by oxygen in oxides formed during drying of sample and Na and Cl residues on the material (Table S1).

2.4 Anaerobic sludge

Anaerobic granular sludge was obtained from a full-scale upflow anaerobic sludge bed reactor treating brewery wastewater (Mahou, Guadalajara, Spain). The sludge was stored at 4 °C. Volatile suspended solids (VSS) were 7.0% of the wet weight. The maximum acetoclastic methanogenic activity determined was 280.5 ± 10.1 mg CH₄-COD g⁻¹ VSS d⁻¹ (where COD refers to chemical oxygen demand).

2.5 General bioassay description

Table 1 describes the specific sludge concentration, range of FeS concentrations used, and metal salt or NP applied as an inhibitor in each bioassay performed. All experiments were performed in 160 mL serum bottles with a work volume of 100 mL in a sulfate-free basal medium containing (mg L⁻¹): NH₄Cl (280), NaHCO₃ (3,000), K₂HPO₄ (250), CaCl₂•2H₂O (10), MgCl₂•6H₂O (183), and yeast extract (100) with 1 mL L⁻¹ of trace elements previously described in Gonzalez-Estrella et al. (2013). All bottles were flushed with N₂/CO₂ (80:20, v/v) and incubated overnight at 30 ± 2 °C in an orbital shaker at 115 rpm in 95 mL of basal medium. Next morning, all assays were supplied with 5 mL of NP stock dispersion or salt stock solution, flushed with the N₂/CO₂ mix, and incubated at the same conditions. The bioassays were provided with three feedings of sodium acetate (1 g chemical oxygen demand (COD) L⁻¹ each). The second and third acetate feedings were provided after the control reached the expected methane production (1.0 g CH₄-COD produced L⁻¹ liquid). Methane samples (100 µL) were taken from the headspace several times during the incubation. All experiments included a control free of toxic metals in order to have a reference of the control specific methanogenic activity. The treatments also included a control amended with only FeS to investigate the toxic effect of FeS on the methanogenic activity.

Specific methanogenic activities (SMA) were calculated as the maximum specific methanogenic rate using linear regression for each four or more consecutive points that

represented at least 50% of the substrate consumption. The normalized methanogenic activity (NMA) was calculated as follows:

$$NMA(\%) = \left(\frac{SA_t}{SA_c} \right) \cdot 100 \quad [2]$$

where, SA_t and SA_c are the specific activities of the treatment and control experiments, respectively.

The details and objectives of each bioassay performed are described below.

2.5.1 Attenuation of CuCl_2 and ZnCl_2 methanogenic toxicity by FeS—The experiments were performed with the objective of determining the attenuation of CuCl_2 and ZnCl_2 toxicity to methanogens by FeS. Our previous study indicated >90% loss of the methanogenic activity in a sulfate-free medium by 0.2 mM of CuCl_2 and ZnCl_2 (Gonzalez-Estrella et al., 2015). Therefore, a concentration 0.2 mM of both CuCl_2 and ZnCl_2 was selected for this experiment. The bioassays were pre-incubated overnight at the conditions described previously with a range of FeS concentrations (Table 1). After pre-incubation, 0.2 mM of CuCl_2 or ZnCl_2 were provided to the bottles, the bottles were reflushed with the N_2/CO_2 gas mix, and CH_4 production was monitored over the course of the experiment. Finally, with that information the concentration of FeS-c and FeS-f that recovers the NMA to 50% (R-NMA_{50}) was estimated.

2.5.2 Impact of FeS on the inhibition constants—A series of concentrations of CuCl_2 and ZnCl_2 were provided to quantify the inhibition effect on the NMA by the calculating inhibition constant (K_i). The K_i was calculated as follows:

$$NMA = NMA_{\max} \cdot \frac{1}{1 + \left(\frac{1}{K_i} \right)^n} \quad [3]$$

where $NMA_{\max}$ is the maximum NMA (%), I and K_i are the inhibitor (metal) concentration and inhibition constant, respectively (mM), and n is the inhibition order (dimensionless). The derivation of the equation and interpretation of each variable has been previously described in Gonzalez-Estrella et al. (2015).

2.5.3 Attenuation of NP toxicity—The experiments were performed with the objective of studying the effect of FeS-f on the attenuation of highly inhibitory concentrations of Cu^0 and ZnO NPs. Our previous study indicated >85% loss of the methanogenic activity in a sulfate-free medium by 0.24 and 0.18 mM of Cu^0 NPs and ZnO NPs, respectively (Gonzalez-Estrella et al., 2015). Thus, concentrations of 0.24 mM Cu^0 NPs and 0.18 mM of ZnO NP mM were selected for this experiment. The bioassays were pre-incubated overnight at the conditions with the FeS concentrations (described in Table 1). After pre-incubation, 0.24 and 0.18 mM of Cu^0 and ZnO NPs were provided to the bottles, respectively.

2.5.4 Effect of long-term FeS pre-exposure to NPs and salts before sludge addition—The experiments were carried out to determine if a long-term pre-exposure FeS-

f to highly inhibitory concentrations of Cu^0 NPs, ZnO NPs, CuCl_2 and ZnCl_2 would increase the toxicity attenuation effect of FeS. For that purpose, different concentrations (Table 1) of Cu and Zn (NPs and salts) were pre-exposed to a concentration of FeS which corresponded to an stoichiometric ratio of $\text{FeS}/\text{Me}=2.14$ for five days in the absence of sludge. After five days of exposure of the metals to FeS, the sludge and electron donor were added to initiate the assay.

2.5.5 Fe displacement mechanism—An experiment was performed to demonstrate that soluble Cu^{2+} and Zn^{2+} cations can release Fe^{2+} cations from FeS by a displacement mechanism. The assays were performed in deionized water (pH 6 adjusted with HCl) with 0.71 mmol $\text{FeS-f L}^{-1}_{\text{liq}}$, and CuCl_2 , and ZnCl_2 additions ranging from 0.25 to 2 mmol $\text{L}^{-1}_{\text{liq}}$. Liquid samples (1.4 mL) were taken after 2, 24, and 120 h. The samples were filtered through a 0.4 μm filter and immediately acidified with one 10 μL of concentrated HNO_3 (>90%). The samples were later analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES) to determine the content of soluble Fe, Cu, and Zn. The soluble concentration of sulfide was also measured.

2.6 Analytical methods

Methane was quantified by gas chromatography with flame ionization detection (Hewlett Packard 5890 Series II). The GC was fitted with a Nukol fused silica capillary column (30 m length, 0.53 mm ID, Supelco, St. Louis, MO). Details of the measurement parameters are described in Karri et al. (2006). Analysis of soluble metals by inductively coupled plasma-optical emission spectroscopy (ICP-OES Optima 2100 DV, Perkin-Elmer TM, Shelton, CT). Fe concentration in FeS was quantified by firstly adding 45 mg of solid to 10 mL of a digestion solution containing 6.66 mL of HNO_3 (70%) and 3.3 mL of HCl (37%). Secondly, the samples were subjected to microwave-assisted acid digestion (MSD2100, CEM Corp. Matthews, NC). The digestion was performed by applying a ramp for 30 min until 150 $^{\circ}\text{C}$ were reached, then the temperature was held for 30 min. Finally, the digested solution was analyzed by ICP-OES to measure the soluble Fe. The wavelengths used for ICP-OES analysis were 324.754, 259.940, and 206.200 for Cu, Fe and Zn, respectively. All samples were centrifuged at 13,000 rpm and filtered (0.4 μm VSWP, Millipore, Billerica, MA, USA), acidified with concentrated HNO_3 and frozen prior ICP analysis. Sulfur and Iron content in FeS was measured by Energy-dispersive X-ray spectroscopy (EDS). Sulfide was analyzed by spectrophotometry using the methylene blue method (Truper & Schlegel, 1964).

3 Results

3.1 FeS attenuation of methanogenic inhibition caused by CuCl_2 and ZnCl_2 salts

A range of FeS-c and FeS-f concentrations were supplied to attenuate the methanogenic toxicity over three feedings of acetate. Figures 2A and 2C show the NMA response as a function of increasing concentrations of FeS-c and FeS-f; respectively, in the presence of 0.2 mM of CuCl_2 . CuCl_2 decreased the NMA to <10% in the absence of FeS. However, the NMA activity was recovered by FeS-c and FeS-f over three feedings of acetate. FeS-c attenuated CuCl_2 toxicity by restoring the NMA to a range of 20–100% corresponding to a range of 0.64 to 5.13 mmol $\text{FeS L}^{-1}_{\text{liq}}$ (Figure 2A) supplied. Similarly, FeS-f attenuated

CuCl₂ toxicity by restoring the NMA to a range of 25 to 75% corresponding to a range of 0.14 to 1.71 mmol FeS L⁻¹_{liq} added. CuCl₂ toxicity was fully manifested in the assay lacking FeS after the 2nd feeding; therefore, the attenuation effect of FeS can also be better appreciated in the 2nd and 3rd feeding. The lowest FeS concentrations tested improved the NMA in the latter two feedings compared to the CuCl₂-only treatments. The attenuation effect increased in the second and third feedings of acetate when 1.28 mmol FeS L⁻¹_{liq} as FeS-c was added; whereas the attenuation was more immediate with FeS-f. The R-NMA₅₀ were estimated from interpolation of the response curves in the 3rd feeding in Figures 2A and 2C. The R-NMA₅₀ estimated values were 1.07 and 0.43 mmol FeS L⁻¹_{liq} for the experiments amended with FeS-c and FeS-f, respectively. These results indicated that a 2.5 lower concentration of FeS-f was required compared to FeS-c to attenuate CuCl₂ toxicity to methanogens to the same extent. Finally, even though a minor decrease on the methanogenic rate was observed in the assays supplied with only FeS-c or FeS-f, no substantial toxicity to methanogens was observed over the three feedings of acetate (Figure S3). Overall, the results revealed that FeS attenuated CuCl₂ toxicity. An analogous experiment was carried out by supplying ZnCl₂ as the inhibiting metal.

Figure 2B and 2D shows the NMA as a function of increasing concentrations of FeS-c and FeS-f; respectively, in presence of 0.2 mM of ZnCl₂. The NMA decreased to <15% when ZnCl₂ was added in the absence of FeS. FeS successfully decreased the toxicity of ZnCl₂ to methanogens over three feedings of acetate. FeS-c increased the NMA in the range of 25 to 100% corresponding to a range of 0.64 to 5.13 mmol FeS L⁻¹_{liq} added (Figure 2B). Likewise, Figure 2D shows that FeS-f attenuated ZnCl₂ toxicity by increasing the NMA in the range of 23 to 98% corresponding to a range of 0.14 to 1.71 mmol FeS L⁻¹_{liq} supplied. ZnCl₂ gradually and remarkably increased its toxicity in absence of FeS after three feedings of acetate; therefore, a full attenuation effect of FeS on ZnCl₂ toxicity is better appreciated after the 3rd feeding of acetate. Thus the most prudent comparisons of the FeS attenuation effect of ZnCl₂ toxicity should be based on those of the 3rd feeding. The lowest FeS-f concentration tested (0.14 mmol FeS L⁻¹_{liq}) significantly improved the NMA in the 2nd and 3rd feedings compared to the ZnCl₂ only treatments. A similar significant effect required a much higher concentration of FeS-c (1.43 mmol FeS L⁻¹_{liq}). The estimated R-NMA₅₀ for attenuating the ZnCl₂ methanogenic inhibition was observed to be 1.43 and 0.57 mmol FeS L⁻¹_{liq} for the assays supplied with FeS-c and FeS-f, respectively, which also indicated that a 2.5 lower FeS-f concentration was needed to attenuate ZnCl₂ toxicity to the same extent compared to FeS-c (Figure 2B and 2D). Thus, these results confirmed that FeS effectively attenuates ZnCl₂ toxicity. Finally, after determining the R-NMA₅₀ for CuCl₂ and ZnCl₂, a corresponding concentration of 1.28 and 0.43 mM of FeS-c and FeS-f was selected for further attenuation experiments since these concentrations corresponded approximately to the R-NMA₅₀ values for CuCl₂ and ZnCl₂.

3.2 Impact of FeS on the K_i

A range of increasing concentration of CuCl₂ and ZnCl₂ was supplied to evaluate the effect FeS on the K_i estimated from the third acetate feeding. Figure 3 shows the calculated K_i of CuCl₂ and ZnCl₂ in presence and absence of FeS of after three feedings of acetate. Addition of 1.28 mmol FeS L⁻¹_{liq} as FeS-c increased the K_i values (thus decreased the inhibition) of

both CuCl_2 and ZnCl_2 by approximately 2-fold (Figure 3A). Addition of $0.43 \text{ mmol FeS L}^{-1}_{\text{liq}}$ as FeS-f increased the K_i values of CuCl_2 and ZnCl_2 by 1.3 and 2.2-fold respectively (Figure 3B). Both observations demonstrated that the estimated R-NMA_{50} values used in these experiments have a beneficial effect by decreasing the toxicity by approximately 2-fold of both metal salts in almost all of the assays.

The inhibition can be interpreted by the n value, which indicates the inhibition order. The value of n increases with the steepness of the inhibition response to the inhibitor concentration. An n value equal to 1 would indicate that the inhibition changes proportionally to the inhibitor concentration. If $n \gg 1$, this indicates the microbial activity abruptly decreases in response to small increase in the inhibitor concentration (Gonzalez-Estrella et al., 2015). The n value of the assays amended with FeS regardless of its size was larger than the assays lacking FeS (Table S2). This indicates that the assays amended with FeS provided a toxicity buffer; however, once the concentration of metal overcame the toxicity attenuation capacity of FeS, the toxicity increased abruptly accounting for n values $\gg 1$.

3.3 Attenuation of NP toxicity

The effect of FeS-f on the attenuation of Cu^0 and ZnO NP toxicity over three feedings of acetate was also investigated. Concentrations of 0.24 mM of Cu^0 NPs and 0.18 mM ZnO NPs in absence of FeS-f decreased the NMA after three feedings of acetate to only 27 and 10%, respectively. FeS-f effectively decreased the toxic effect of Cu^0 NPs with increasing concentrations of FeS-f (Figure 4A). FeS-f prevented large losses in activity after three feedings of acetate, the NMA was decreased only to 63 and 80% when 0.17 to $0.51 \text{ mmol FeS L}^{-1}_{\text{liq}}$ were respectively provided. Thus, the estimated R-NMA_{50} value for Cu^0 NPs was $0.11 \text{ mmol FeS L}^{-1}_{\text{liq}}$. Likewise, Figure 4B shows that FeS-f attenuated ZnO NPs methanogenic toxicity by increasing the NMA to 25 and 90% NMA with the addition of 0.13 and $0.39 \text{ mmol FeS L}^{-1}_{\text{liq}}$, respectively. In this case, the effect was less evident when the lowest concentration of FeS-f was supplied ($0.13 \text{ mmol FeS L}^{-1}_{\text{liq}}$). However, an almost full recovery was observed in the assay amended with the highest concentration of FeS-f ($0.39 \text{ mmol FeS L}^{-1}_{\text{liq}}$). The progressive inhibitory impact of ZnO NPs was clearly observed in the assay lacking FeS as evidenced by a NMA which decreased from approximately 50 to 10 % in the 1st to the 3rd feedings of acetate. Also at the highest FeS-f concentration, the attenuation effect of FeS towards the added ZnO NP progressively increased over the course of the three feedings. The estimated R-NMA_{50} value was $0.23 \text{ mmol FeS L}^{-1}_{\text{liq}}$ as FeS-f for the attenuation ZnO NPs. Therefore, these experimental results demonstrate that the toxic effect of Cu^0 NPs and ZnO NPs could be successfully attenuated by providing excess concentrations of FeS.

3.4 Effect of long-term FeS pre-exposure to NPs and salts before sludge addition

An experiment was performed to evaluate whether a pre-exposure of FeS-f for five days to highly inhibitory concentrations of metal salts and NPs before the incubation with methanogens could increase the attenuation effect of FeS-f. Figure 5 shows the NMA as a function of the long-term pre-exposure and regular incubation treatment (FeS added simultaneously with methanogens) with either no FeS-f or an exposure to FeS-f provided at

a molar ratio of FeS/metal = 2.14. The pre-exposure of FeS to NPs or salts can only be performed without a pre-incubation of sludge to substrate, contrary to the procedure carried out in all the other assays of the present study in which the sludge was activated prior contact with the toxic metals. The results show that the absence of pre-incubating sludge with substrate and FeS overnight resulted in a more severe toxic effect of the metals. Nonetheless, the pre-exposure to FeS-f and the metals increased the NMA to 65 and 63% in the assays amended with CuCl₂ and ZnCl₂, respectively; compared to a recovery of the NMA to 50 and 40% shown in a regular incubation in which CuCl₂ and ZnCl₂ were introduced after the sludge was incubated overnight (Figure 5A and 5C), respectively. Even though the pre-exposure of FeS-f to Cu⁰ NPs and ZnO NPs recovered the NMA to 90 and 80% (Figure 5B and 5D), the improvement in recovery compared to the regular incubation was not as obvious as in the assays supplied with the metal salts. There was a small improvement in the NMA in the case of Cu⁰ NP due to pre-exposure; however, the longer exposure of Cu⁰ NPs alone to the methanogens (in absence of FeS) also increased the NMA as well.

3.5 FeS displacement mechanism

An experiment was carried out to investigate if Fe can be displaced from FeS to different extents by supplying a range of CuCl₂ and ZnCl₂ concentrations. Figure 6 shows the variation of the soluble concentration of Fe at different incubation times and the final concentration of soluble S²⁻ as a function of increasing concentrations of added salts in presence of 0.71 mmol FeS L⁻¹_{liq}. The concentration of soluble Fe²⁺ increased as a function of CuCl₂ concentration. The most evident increase was observed after 24 h of incubation (Figure 6A). The measured soluble Fe after 24 h indicated that the fraction of Fe displaced was only 28.5, 32.1, and 15.1% of the maximum Fe that could have been theoretically displaced when concentrations of 0.25, 0.5, and 2.0 mM of CuCl₂ were supplied. The experiments supplied with ZnCl₂ showed a similar pattern of increasing concentration of Fe²⁺ as a function of the added ZnCl₂ concentration. Likewise, the most obvious increase of Fe²⁺ was observed after 24 h of incubation (Figure 6B). In this case, the measured soluble Fe indicated that the fraction of Fe displaced was only 32.4, 24.7, and 5.6% of the maximum Fe that could have been theoretically displaced when concentrations of 0.25, 0.5, and 2.0 mM of ZnCl₂ were supplied.

Additionally, sulfide was measured to corroborate that the S⁻² of FeS was not in solution after 120 h of incubation when Cu and Zn have fully reacted in the displacement reaction with FeS. The measurements confirmed little correlation of increasing CuCl₂ concentrations with S²⁻ (Figure S4). The assays amended with ZnCl₂ showed a very small increase of the concentration of S²⁻ as a function of the added ZnCl₂. Nevertheless, the concentration of S²⁻ found was very low (<3.5 µM) (Figure S4). Overall, these findings confirm that Fe is displaced from FeS in presence of Cu²⁺ and Zn²⁺ cations and that for the most part S²⁻ remains in solid form.

4 Discussion

4.1 Main findings

FeS is an effective attenuator of methanogenic toxicity caused by Cu and Zn. Attenuation occurred regardless of whether the metals were added as chloride salts or as NPs. The particle size of FeS influenced the attenuation effect since 2.5-fold less FeS-f than FeS-c was needed for the restoration of the NMA to 50% when assays were exposed to 0.2 mM of CuCl₂ or ZnCl₂. Additionally, methanogenic toxicity caused by Cu⁰ NPs and ZnO NPs was also decreased by FeS-f. Results also indicated that molar ratios of FeS-f/Cu⁰, FeS-f/ZnO, FeS-f/ZnO, FeS-f/ZnCl₂ and FeS-f/CuCl₂ of 2.14, 2.14, 4.28, and 8.56 respectively, was necessary to show a high recovery of the methanogenic activity (>75%). Finally, a displacement mechanism was demonstrated by measuring progressively greater releases of Fe²⁺ in response to increasing added concentrations of CuCl₂ or ZnCl₂. This demonstrated that toxic divalent metals with a greater affinity for S²⁻ could be removed from the solution by exchanging with Fe in the amorphous FeS.

4.2 Cu and Zn toxicity to methanogenesis and attenuation approaches

Cu and Zn are well-known inhibitors of methanogenesis (Chen et al., 2014). The toxicity of these heavy metals has been attributed to their binding to protein structures which results in the disruption of essential enzymes of these organisms (Chen et al., 2008). In fact, Cu is used as a biocide due to its lethal effect of different microorganisms (Karlsson et al., 2015). The toxic effect of Cu on wastewater microorganisms usually is observed in the order of hours (Ochoa-Herrera et al., 2011). Zn also is toxic to methanogens and other microorganisms involved in the anaerobic digestion process (Chen et al., 2014). Interestingly, Zn toxicity has generally been considered as less lethal than Cu toxicity (Chen et al., 2008). However, Zn toxicity has a tendency of increasing overtime which could lead to a reactor collapse in the long-term (Gonzalez-Estrella et al., 2015; Otero-González et al., 2014a). The present research reaffirmed that ZnCl₂ toxicity gradually increases over time.

Recent studies found that Cu-based NPs (of Cu⁰ and CuO) and ZnO NPs are highly toxic to acetoclastic methanogens (Gonzalez-Estrella et al., 2015; Gonzalez-Estrella et al., 2013; Otero-González et al., 2014a; Otero-González et al., 2014b). In these studies, acetoclastic methanogenic activity was inhibited by 50% in absence of a sulfide source with concentrations ranging from 0.17 to 0.30 mM for CuO NPs, 0.11 mM for Cu⁰ NPs, and from 0.041 to 0.19 mM for ZnO NPs. There is an overall consensus that the methanogenic toxicity caused by Cu⁰ NPs and ZnO NPs is due to the release of soluble ions during dissolution and corrosion of the NPs (Gonzalez-Estrella et al., 2013; Luna-delRisco et al., 2011; Mu et al., 2011). Given Cu and Zn salts have been shown to be toxic to methanogens, the ions released by their NP analogs should also be expected to be toxic as well. Thus, the attenuation of the toxicity may be likely reached by controlling the soluble concentration Cu and Zn ions.

The methanogenic toxicity of soluble Cu and Zn salts has been successfully attenuated by precipitating these metals as CuS or ZnS with biogenic sulfides via sulfate reduction (Lawrence & McCarty, 1965) or by directly supplying sulfide as a salt (e.g. Na₂S) (Jin et al.,

1998; Zayed & Winter, 2000). By that same principle, biogenic sulfide effectively decreased the methanogenic toxicity caused by Cu^0 and ZnO NPs due to precipitation of metal ions released from the NPs forming metal sulfides (Gonzalez-Estrella et al., 2015). These findings indicated a substantial decrease in toxicity as evidenced by the 7 and 14-fold increase of the K_i for Cu^0 and ZnO NPs, respectively, in assays where biogenic sulfide was formed compared to the K_i of assays lacking biogenic sulfide. The present study agreed with the previous findings. The methanogenic toxicity of Cu^0 and ZnO NPs and their corresponding salts could be significantly attenuated by another important source of S^{2-} , namely FeS, commonly present in anaerobic digesters and anaerobic sediments (Gerardi, 2003; Morse et al., 1987).

4.3 The mechanism of toxicity attenuation by FeS

FeS is commonly formed by the reaction of S^{2-} and Fe^{2+} in anaerobic environments where the oxidation of organic matter is linked to SO_4^{2-} and Fe^{3+} reduction generating S^{2-} and Fe^{2+} which precipitate with each other (Allen et al., 1993; Morse et al., 1987). This phenomena takes place mostly in anaerobic marine sediments where there is a high content of sulfate (Morse et al., 1987). Even though FeS is a poorly soluble mineral, the amorphous monosulfide (FeS) is considered one of the most reactive phases of sulfide in the sediments (Di Toro et al., 1992). The reactive S^{2-} fraction of FeS, also known as acid-volatile sulfide (AVS), is a key phase that controls the soluble concentration of metal cations (Ankley et al., 1994) and thus, has important implications regarding heavy metal toxicity in aquatic environments (Casas & Crecelius, 1994).

Heavy metals such as Cu or Zn, with more affinity to sulfide than Fe are expected to react with sulfide and become incorporated into insoluble sulfide minerals by displacing Fe^{2+} (Simpson et al., 2000). The presence of soluble heavy metal ions should displace the Fe from FeS to form CuS and ZnS according to the solubility constants of FeS, CuS, and ZnS previously mentioned in the introduction of this document. If true, the displacement of Fe from FeS by either Cu^{2+} or Zn^{2+} should result in increasing concentrations of soluble Fe as the concentration of added Cu^{2+} and Zn^{2+} increases. The present study showed that the concentration of soluble Fe increased as a function of added Cu or Zn concentrations; however, in neither case was 120 h enough for the full displacement of Fe from 0.71 mmol FeS L^{-1}_{liq} initially added even with excess of Cu and Zn. This experiment showed that even when an excess of metal salt was provided (2 mM of CuCl_2 or ZnCl_2 per 0.71 mmol FeS L^{-1}_{liq}) the fraction of actual Fe displaced was only 15.1 and 5.6% of the maximum that could have been theoretically displaced. Our findings are in agreement with Simpson et al. (2000) which also found increasing soluble Fe concentrations as a function of Zn and Cd added to a sulfidic estuarine sediments. FeS was used as well to immobilize Hg^{2+} (Han et al., 2014). The study observed 100% of Hg removal at concentrations <0.5 mM of Hg in 10 min; however, concentrations >1 mM of Hg required up to 800 minutes to achieve the same results. Such results may be explained partially by a low availability of the sulfide for the total metal added in the first minutes of the experiment

The toxicity of Cd, Cu, and Ni to aquatic microorganisms has been successfully attenuated when a known reactive fraction of FeS has been observed to be present in a molar ratio

greater than 1 (Di Toro et al., 1992; Di Toro et al., 1990). If a ratio of FeS/Me is applied to analyze our experiments, assuming that the total FeS added is reactive, the ratio FeS/Me of 1 fails to predict the total attenuation of heavy metal toxicity to methanogens. Figure 7 shows the NMA response as of function of the ratio of FeS-f/Me. In the assays amended with Cu, a molar ratio of FeS/Cu⁰ NPs and FeS/CuCl₂ of 2.14 and 8.56 respectively was needed to obtain a high recovery (>75%) of the methanogenic activity (Figure 7). Likewise, a molar ratio of FeS/ZnO NPs and FeS/ZnCl₂ of 2.14 and 4.28 respectively was needed to observe a high level of recovery response (Figure 7). According to the prediction, all the points displayed in the provided chart inside the grid with grey diagonal lines should have shown a NMA around 100%. Therefore, in our experiments it can be assumed that not all of the total FeS added was readily available for reaction with added heavy metals (and corresponding NPs). This behavior is distinct from biogenic sulfide that we have observed in our previous study to immobilize Zn²⁺ and Cu²⁺ (Gonzalez-Estrella et al., 2015). Such immobilization depended on the toxic effect of heavy metals and NPs on sulfate reducers. Stoichiometric immobilization was only observed in assays supplied with ZnCl₂ and ZnO NPs. Conversely, Cu was toxic for sulfate reducers as well; thus, the attenuation by biogenic sulfide was not stoichiometric for assays supplied with CuCl₂ and Cu⁰ NPs.

Our findings demonstrated that FeS-f improved the metal toxicity attenuation effect compared to FeS-c, most likely by increasing the availability of sulfide due to the increase the total surface area. The total surface area increase from FeS-c to FeS-f was approximately 17-fold (assuming average spherical particle sizes for FeS-f and FeS-c of 50 and 850 μm , respectively and a particle density of 4.84 g cm⁻³ (Dana & Hurlbut, 1959). However, the increase of attenuation of FeS-f due to its particle size was only 2.5-fold. This discrepancy maybe caused by the microporosity of FeS-c which may increase the sulfide availability and occurrence of coarse particles within the fine fraction.

5 Conclusions

FeS was found to be effective in attenuating the methanogenic toxicity caused by Cu⁰ NPs and ZnO NPs and their soluble chloride salt analogs. The attenuation effect of FeS on Cu and Zn toxicity was increased by decreasing the particle size. At least 2.5 times less FeS-f was needed to observe the same attenuation effect of FeS-c. The R-NMA₅₀ values for the attenuation of metal salt toxicity were 0.43 and 0.57 mmol FeS L⁻¹_{liq} for CuCl₂ and ZnCl₂, respectively; when FeS-f was used. Whereas 1.07 and 1.43 mmol FeS L⁻¹_{liq} were the R-NMA₅₀ values for CuCl₂ and ZnCl₂ respectively, when FeS-c was supplied. Likewise, the R-NMA₅₀ values were 0.09 and 0.23 mmol FeS-f L⁻¹_{liq} for the attenuation of Cu⁰ NPs and ZnO NPs toxicity, correspondingly. The results also demonstrate that the Fe from FeS is displaced in the presence of soluble Cu²⁺ and Zn²⁺ cations. In order to achieve nearly complete attenuation of Cu and Zn toxicity, FeS had to be added in stoichiometric excess, indicating that not all the sulfide was readily available for sequestering the metals. The results taken as a whole indicate that the toxicity caused by the release of Cu-based and ZnO NPs and their soluble metal analogs could be effectively attenuated by FeS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
SEM analysis of FeS-c (A) and FeS-f (B) The whole scale bar represent an scale of 1.0 and 0.5 mm in panel A and B, respectively.

Figure 2.

CuCl₂ (A and C) and ZnCl₂ (B and D) toxicity attenuation by coarse FeS (top panels) and fine FeS (bottom panels) in the 1st Feeding (□), 2nd feeding (○), and 3rd feeding (▲) of acetate. The solid trend line represents the response of the NMA of the third feeding, the intersection of the dotted lines represent the concentration of FeS that recovers 50% of the methanogenic activity (R-NMA₅₀), and the dashed-dotted line represents the NMA activity of the control without addition of metals or FeS.

Figure 3. Inhibition constant values in the assays supplied with no-FeS (empty bars) and FeS (filled) after three feedings of acetate. Assays were supplied with $1.28 \text{ mmol FeS L}^{-1}_{\text{liq}}$ as FeS-c (A) and $0.43 \text{ FeS L}^{-1}_{\text{liq}}$ as FeS-f (B)

Figure 4.

Toxicity attenuation of a high inhibitory concentration of Cu⁰ NPs (A) and ZnO NPs (B) to methanogenesis over the 1st Feeding (□), 2nd feeding (○), and 3rd feeding (▲) of acetate by concentrations ranging from 0.71 to 0.51 mmol FeS L⁻¹_{liq}. The solid trend line represents the response of the NMA of the third feeding, the dotted line represents the 50% toxicity attenuation concentration of FeS, and the dashed line represents the NMA activity of the control without metals or FeS. Assays were amended with 0.24 mM and 0.18 mM of Cu⁰ and ZnO NPs, respectively.

Figure 5.

Effect of pre-exposure of FeS-f to a high inhibitory concentration of CuCl₂ (A), Cu⁰ NPs (B), ZnCl₂ (C), and ZnO (D) of on the methanogenic activity after three feedings of acetate. Empty bars represent the NMA from the assays pre-exposed to FeS for five days prior to addition of sludge and filled bars represent the NMA from assays where FeS and the sludge were added simultaneously before the addition of toxic metals. The NMA represents the activity after three feedings of acetate. Assays were amended with 0.2, 0.24, 0.2, and 0.18 mM of, CuCl₂ (A), Cu⁰ (B), ZnCl₂ (C), and ZnO NPs (D), respectively

Figure 6.

Fe release as function of added CuCl_2 (A) and ZnCl_2 (B) after 2 (□), 24 (○), and 120 (△) h of incubation. Assays were supplied with $0.71 \text{ mmol FeS L}^{-1}_{\text{liq}}$ and performed in acidic deionized water (pH 6) with a N_2 headspace.

Figure 7. NMA following three feedings of acetate as a function of increasing molar ratios of FeS-f/Me. The symbols represent Cu ($\square$) and Zn ($\circ$) either as chloride salts (A) or NPs (B). The diagonal lines show the theoretical area where the toxicity attenuation should approach 100%. This analysis was made with the data of the assays amended with 0.2, 0.2, 0.24 and 0.18 mM of CuCl_2 , ZnCl_2 , Cu^0 , and ZnO NPs. Me (Metal): CuCl_2 , ZnCl_2 , Cu^0 , and ZnO NPs

Table 1

Experimental design of salt and NP toxicity attenuation and displacement assays.

Experiments	FeS (mmol L ⁻¹ _{liq})	Inhibitor (mM)
Effective 50% attenuation concentration by FeS-c*	0.43–2.57	CuCl ₂ and ZnCl ₂ (0.05–0.6)
Effective 50% attenuation concentration by FeS-f*	0.14–1.71	CuCl ₂ and ZnCl ₂ (0.05–0.6)
NP toxicity attenuation by FeS-f*	Cu ⁰ assays (0.17–0.51) ZnO assays (0.13 and 0.39)	Cu ⁰ NP (0.24) ZnO NP (0.18)
Effect of long-term FeS-f pre-exposure to NPs and salts before sludge addition*	CuCl ₂ and ZnCl ₂ assays (0.43) Cu ⁰ assays (0.51) ZnO assays (0.39)	CuCl ₂ and ZnCl ₂ (0.2) Cu ⁰ NP (0.24) ZnO NP (0.18)
FeS-f displacement	0.71	CuCl ₂ and ZnCl ₂ (0.25–2.0)

* Experiments were inoculated with 1.5 g VSS L⁻¹.

FeS-c: FeS coarse fraction; FeS-f: FeS fine fraction