Nanoscale Chemical Imaging of Nanoparticles under Real-World Wastewater Treatment Conditions

Miguel A. Gomez-Gonzalez,* Mohamed A. Koronfel, Huw Pullin, Julia E. Parker, Paul D. Quinn, Maria D. Inverno, Thomas B. Scott, Fang Xie, Nikolaos Voulvoulis, Marian L. Yallop, Mary P. Ryan, and Alexandra E. Porter*

Understanding nanomaterial transformations within wastewater treatment plants is an important step to better predict their potential impact on the environment. Here, spatially resolved, in situ nano-X-ray fluorescence microscopy is applied to directly observe nanometer-scale dissolution, morphological, and chemical evolution of individual and aggregated ZnO nanorods in complex “real-world” conditions: influent water and primary sludge collected from a municipal wastewater system. A complete transformation of isolated ZnO nanorods into ZnS occurs after only 1 hour in influent water, but larger aggregates of the ZnO nanorods transform only partially, with small contributions of ZnS and Zn-phosphate (Zn$_3$(PO$_4$)$_2$) species, after 3 hours. Transformation of aggregates of the ZnO nanorods toward mixed ZnS, Zn adsorbed to Fe-oxy-hydroxides, and a large contribution of Zn$_3$(PO$_4$)$_2$ phases are observed during their incubation in primary sludge for 3 hours. Discrete, isolated ZnO regions are imaged with unprecedented spatial resolution, revealing their incipient transformation toward Zn$_3$(PO$_4$)$_2$. Passivation by transformation(s) into mixtures of less soluble phases may influence the subsequent bioreactivity of these nanomaterials. This work emphasizes the importance of imaging the nanoscale chemistry of mixtures of nanoparticles in highly complex, heterogeneous semi-solid matrices for improved prediction of their impacts on treatment processes, and potential environmental toxicity following release.

1. Introduction

Nanostructures of zinc oxide (ZnO) are at the forefront of application-driven nanotechnology because of their unique optical, piezoelectric, semiconducting, and antibacterial properties. However, their exceptional properties including high surface reactivity—engineered at the nanoscale to render their usage so advantageous for many applications—may also increase their environmental toxicity in the course of their synthesis, use, and disposal. Thus, there is a concomitant need to predict their fate and transformations within aquatic environmental media to inform management practices, including the safe-design of these materials. One of the main pathways for environmental exposure to ZnO engineered nanomaterials (ENMs) is via discharges from wastewater treatment plants (WWTPs) following their use and disposal to wastewaters. During wastewater treatment, ENMs have been shown to partition largely to sludge,[1,2] which is often recycled to soils as biosolids or fertilizers[3,4] with some ENMs reaching freshwaters through treated effluent discharges.[5]

The potential environmental toxicity of ENMs depends upon their physicochemical state—and specifically their chemical speciation—at the point of exposure (distinct from the native “as-processed” state), which is influenced by a range of environmental factors, such as temperature, pH, and interactions with other compounds and colloids during its life-cycle.[6,7] Hence, it is important to fully elucidate the extent to which ZnO ENMs transform, and to identify the intermediate species generated, as these...
intermediates may partition during wastewater treatment, over relevant (usually short) time and length-scales. This will allow us to both assess their impacts on treatment processes, as well as potential environmental toxicity following release. For example, zinc ions released after ENM dissolution can be highly toxic above a threshold dose, but other chemical processes such as sulfidation or complexation with organic carbon can diminish Zn$^{2+}$-related toxicity to microorganisms by “trapping” free Zn$^{2+}$ ions.

The fate of ZnO nanomaterials has mostly been studied in synthetic and simulated wastewater solutions, at a range of pH, ionic strengths, and organic contents. Bulk studies have focused on ZnO nanomaterial transformations within the anaerobic treatment of sewage sludge and their sub-products. Whilst the anaerobic digestion of sewage sludge is an important stage in the life cycle of ENMs, nanomaterial concentrations in undigested sludges are determined by the primary treatment of wastewater (generating primary sludges) and the biological treatment that follows (secondary sludges and activated sludge). During primary wastewater treatment only, ZnO ENMs undergo a range of possible chemical transformations. Musee et al. showed high Zn accumulation in primary sludges of a simulated WWTP bioreactor after only 6 h of hydraulic residence time, suggesting that biosorption and biosolid settling of these ENMs occurs during primary treatment. Brunetti et al. evaluated the transformation of ZnO nanoparticles within sewerage transfer networks using laboratory-scale systems fed with raw wastewater. These authors performed bulk X-ray absorption spectroscopy (XAS) analysis in selected samples from key sewer compartments and times (ranging from 4 h of reaction-time up to 3 days from spiking) and found that sulfidic forms of background Zn dominated most of the outlet sewers. Lombi et al. and Ma et al. also used bulk XAS analysis, to study transformations after the addition of ZnO nanomaterials to anaerobically digested sludges and influent wastewater of a pilot WWTP, respectively. These authors detected ZnS phases, as well as mixtures of Zn$_4$(PO$_4$)$_3$ and Zn-associated with Fe-(oxy)hydroxides in biosolids. However, with bulk XAS, only volume-averaged information is achieved and no spatially resolved information is provided regarding the extent of the ZnO ENMs’ transformation at the length scale of the particles. This is particularly relevant when using real (vs model or simulated) systems where the environment itself may be heterogeneous. Furthermore, the timescale over which different transformed Zn-phases evolve in each intermediate cleaning stage in a municipal WWTP has not been considered which will impact partitioning behavior. In this regard, Brunetti et al. showed that ZnO contributed to 20% of the total Zn species found in the inlet sewerage systems after 4 h. Therefore, there is a significant knowledge gap regarding how the other ~80% of Zn-phases transformed and over which timescales. These rapid incubation times in influent wastewaters (less than 4 h) may be representative of domestic and industrial sewage inlets being discharged close to municipal WWTPs. Because of the complexity of the environment, incubation in municipal sewage and primary sludge solutions would be expected to cause heterogeneous transformations over the ZnO nanorod surfaces, including preferential dissolution in some sterically available locations.

2. Results and Discussion

The wastewater influent and sewage sludge samples were analyzed using inductively coupled plasma-optical spectroscopy (ICP-OES) to obtain the elemental distribution. These results, together with other physicochemical parameters of both media, are listed in Table 1.

2.1. ZnO Nanorods Speciation after Incubation within Influent Water

Speciation maps (SM) were calculated by applying the 9-E methodology after short-term incubations in the collected wastewater media (Figure 1, middle). These maps provided a percentage for the reshaped array of spectra from each pixel of the aligned discrete X-ray absorption near edge structure (XANES) images. All pixel percentages within an individual image were averaged and subsequently plotted as a pie chart, showing the mean contribution of the four expected Zn-species (Figure 1, right). A grayscale fluorescence image at the Zn-peak maximum ($E_{\text{max}} = 9669$ eV, Figure 1, left) was also provided for visual comparison with the SM.

Some ZnO nanorods incubated in influent water showed an almost complete transformation to ZnS within only 1 h—these appear as discrete sites (as opposed to the heavily populated regions with flower-like arrays of ZnO nanorods in the "as-synthesized" silicon nitride window, Figure S1c, Supporting Information) surrounded by a homogeneous background signal (white color, SM in Figure 1a, indicating XANES peak intensities eight times lower than the background Zn signal, which were therefore treated as negligible for the SM calculations). When all the individual pixel percentages were averaged in this source with low emittance, providing superb energy resolution (~0.5 eV) and tunability, as well as a high penetration capacity into the analyte, making them ideal characterization techniques for in operando imaging at the (sub)micrometric scale. Micro-X-ray absorption and fluorescence spectromicroscopy have been applied in the hard X-ray range to quantify the Zn accumulation within biological and environmental samples, such as cells, plants, and soil sediments. There are only a few studies that have mapped the Zn distribution and/or concentration at the nanoscale. Here, for the first time, we use in situ nano-XFM to evaluate the dynamics of ZnO nanomaterial transformations within real-world samples of municipal wastewater media with sufficient temporal resolution to observe effects at short timescales (<4 h) representative of domestic and industrial sewage inlets being discharged close to municipal WWTPs. Because of the complexity of the environment, incubation in municipal sewage and primary sludge solutions would be expected to cause heterogeneous transformations over the ZnO nanorod surfaces, including preferential dissolution in some sterically available locations.
Table 1. Elemental composition (quantified by ICP-OES) and physicochemical parameters of the influent water and primary sludge WWTP media used for the ZnO nanomaterial incubations.

| WWTP media           | pH  | TOC\[^a\] [mg C L\(^{-1}\)] | Elemental quantification [mg L\(^{-1}\)]\[^b\] |
|----------------------|-----|-----------------------------|-----------------------------------------------|
|                      |     |                             | Zn    | P      | S\[^c\] | Fe      |
| Influent water       | 8.3 | 11.3                        | 0.23 ± 0.03 | 17.6 ± 0.1 | 64.9 ± 5.8 | 2.02 ± 0.05 |
| Primary sludge       | 5.2 | 336                         | 2.48 ± 0.08 | 497 ± 3   | 114 ± 8  | 298 ± 2  |

\[^a\]Total organic carbon (TOC, in milligrams of carbon per liter) was obtained by using a Dohrmann DC80 analyzer; \[^b\]All samples were digested by applying a two-step digestion process: i) 30% hydrogen peroxide (H\(_2\)O\(_2\)) to digest the organic fraction followed by, ii) 15.8 m nitric acid (HNO\(_3\)) addition (1:1 ratio v/v), as described by Fabricius et al.\[^36\] All digestions were performed in triplicate and the standard deviation of these quantifications is presented. Calibration standards and experimental blanks were run in matrix-matched solutions; \[^c\]The sulfide content of the influent water was screened weekly by Anglian Water Group Ltd. providing values lower than the detection limit (<0.03 mg L\(^{-1}\)), hence not being a critical parameter in comparison with the total sulfur content (≈65 mg L\(^{-1}\)); this value measured by Anglian Water is in agreement with the available literature, describing sulfide contents of ≈0.24 mg L\(^{-1}\) in some influent sewerage systems.\[^16\]

Figure 1. 9-Energy XFM of ZnO nanorods incubated in influent water after: a) 1 and b) 3 h, and in primary sludge after: c) 1 and d) 3 h. [Left] Greyscale fluorescence image acquired at the maximum of Zn K-edge (E\(_{\text{max}}\) = 9669 eV). [Middle] Speciation maps (SM) were calculated with the expected Zn-species: ZnO, ZnS, Zn\(_2\)(PO\(_4\))\(_2\), and Zn adsorbed to Fe-oxyhydroxides (Zn-Fe(ox)) (from left to right), where the red color equals a 100% compound contribution and the blue color corresponds to 0%, and the white pixels correspond to the pure background only (no-Zn detected). Similar speciation analysis confirmed the initial ZnO nature of the “as-synthesized” nanorods.\[^19\] [Right] Pie charts with the average of all individual (pixel-by-pixel) percentages were also generated,\[^37\] representing the main contribution of each Zn-species. The greyscale bars on the left map represent fluorescence intensity (arbitrary units) and the color bars at the right of the SM show the ratio of the different Zn species. All the scale bars (white and black) are equal to 1 μm.
region, a ≈93% contribution of ZnS was obtained (Figure 1a, pie chart, right). In contrast, other locations on the same window presented larger aggregates (not shown, pre-inspection of the samples only via optical microscope). A different silicon nitride window incubated within 3 h showed only relatively diffuse and overlapping ZnO nanorod structures remaining along the silicon nitride surface, whereas the more isolated small structures were no longer detected (Figure 1b). This large aggregated structure detected after 3-h incubation still conserved about ≈64% ZnO speciation, with increasing contributions of both ZnS (≈16%) and Zn-phosphate (≈13.5%) species in some sterically available locations (SM, Figure 1b, middle).

Our results indicate that isolated nanostructures of ZnS are observed at short incubation times (≈1 h), whereas only larger aggregated structures are detected at longer times (up to 3 h, Figure 1b), which exhibit more complex chemistry and “incomplete” transformation. Therefore, ZnO species that are protected inside larger structures may potentially reach the primary sludge tanks after short transportation times (less than 3 h) within drainage stream waters. This is in agreement with Brunetti et al., who reported around 20% contribution of initial ZnO speciation after addition to their lab-scale sewerage system with 4 h reaction time. This behavior may be enhanced by the ability of ZnO nanoparticles to form aggregates when they are incubated in wastewater, as described by Smeraldi et al.[11] The organic matter present in the influent water (total organic carbon (TOC), Table 1), including humic and fulvic acids, may also sorb to the surface of the nanomaterials altering their surface properties. This will influence their agglomeration which, in turn, will affect the nanomaterial settling, transport, physical-chemistry, and behavior within drainage wastewater.[38]

Sun et al.[39] suggested that the majority of ZnO ENMs that enter the environment via wastewater (and hence through WWTPs) come from their use in cosmetics. In line with this, ZnO rods with lengths ranging from 687 to 1512 nm are usually present in consumer products,[40] and hence these aggregated ZnO rods may potentially reach the primary sludge tanks of a municipal WWTP.

### 2.2. Transformation of ZnO Species in Primary Sludge Medium

Some ZnO nanorods exposed to sewage sludge maintained ZnO as the major species after 1 h incubation (≈55%, Figure 1c, right), while only about ≈8% of ZnS was detected (SM, Figure 1c, middle). Furthermore, Zn₃(PO₄)₂ had appeared as the main transformed phase in those areas depleted in ZnO (≈34%, Figure 1c, left). After 3 h of incubation in primary sludge medium, the percentage of the Zn-phosphate phase was as high as ≈63% in some sterically available locations, with complementary ZnS co-located (≈21%), whereas the initial ZnO almost fully transformed (≈6.5% of ZnO, Figure 1c).

To confirm the validity of these results, additional images along the Zn K-edge were acquired on these two samples, generating a more accurate XANES array with a similar spatial resolution (Figure 2; see also Figure S2, Supporting Information for further explanation). Analyses of the same spot after incubation in sewage sludge at 1 h revealed the dominant ZnO contribution in the larger aggregate at the top-left part of the map (spot number 1, Figure 2a, XANES spectrum at Figure 2b), similar to the Zn-speciation already described by 9-E speciation (Figure 1c). Furthermore, the left-side of the Zn-hotspot (spot number 2, Figure 2a) showed a comparable contribution of ZnO and ZnS (XANES spectrum 2, Figure 2b), which was in agreement with the ZnO/ZnS distribution calculated by the 9-E data in the same region (Figure 1c, SM). Bulk XANES analysis with a larger beam would not have been able to differentiate a ≈50% transformation of this single hotspot versus, for example, a ≈50% of the particles transformed as a consequence of the incubation.

The contribution of Zn-phosphate phases became significant when analyzing another location of the ZnO nanorods’ window incubated within primary sludge solution for 3 h (Figure 2b), different than the region analyzed by 9-E (Figure 1d). Even in this alternative location more sterically constrained (spot number 3, Figure 2a), the dissolving clouds arising from the Zn-hotspots presented increasing Zn₃(PO₄)₂ contributions, ranging from ≈8% in some structures (spot number 3, Figure 2b), to about ≈15.5% in other locations (spot number 4, Figure 2b). These results support the application of spatially resolved cutting-edge methodologies for analyzing incipient nanomaterial transformations.

During incubation of ZnO nanorods in primary sludge, the ZnO contribution rapidly decreased to ≈55% after 1 h in some Zn-hotspots, with a significant transformation toward Zn-phosphate (≈34%, Figure 1c). After 3-h incubation, the more sterically impeded locations still showed a major ZnO contribution of ≈88–92% (Figure 2b), although the Zn-phosphate arose as the main secondary phase. Nonetheless, some of the more available Zn-hotspots dramatically decreased their ZnO content to only ≈6.5% after 3 h (Figure 1d), while both the ZnS and the Zn₃(PO₄)₂ contributions increased to ≈21% and ≈63%, respectively. The greater increment in Zn-phosphate speciation compared to ZnS (Figure 1a) can be explained by two factors: i) the high phosphorus content of the sludge in comparison with the sulfur concentration (Table 1) and ii) the fact that transformation to Zn-phosphate phases are thermodynamically favored,[41,42] while the formation of ZnS is kinetically preferred.[43] Le Bars et al.[44] demonstrated that, although nano ZnS was readily available in raw organic wastes within a more processed compost, the majority of Zn was found as amorphous phosphates and adsorbed to ferrihydrite phases. Here, the transformations of the individual ZnO nanorods, observed by 9-E speciation after 1 h (Figure 1c), were confirmed in spatially resolved maps by increasing the number of images acquired as a function of the energy (135-E speciation methodology, Figure 2). Incubation of these nanorods in undigested primary sludge for longer times, ≈3 h, generated a mixture of Zn-species along with the whole template-growth sample, with Zn₃(PO₄)₂ as the predominant transformed phase—rather than the ZnS (Figures 1 and 2). Nonetheless, the partial transformation toward ZnS and/or Zn-phosphate species may not fully avoid further Zn dissolution and the generation of ionic Zn⁺ into the sewage treatment system. Future work should address whether the partially transformed structures could continue to transform and release Zn⁺ in effluent wastewater and sludge treatment processes and impact their performance.
2.3. Temporal Morphology Changes During In Situ ZnO Dissolution

In addition to the 9-E speciation analysis, XFM images (10 µm × 10 µm or 15 µm × 15 µm size, 100 ms acquisition time per pixel, 12 keV) were acquired consecutively during the 1 and 3 h in situ experiments (with a 5–10 min sleep mode in between two consecutive fluorescence maps to minimize any potential beam effect; Figure 3). All live images were integrated using Dawn and Matlab/Python software, calculating the average intensity of the Zn-Kα X-ray emission line, which was subsequently plotted as a function of time (Figure 3). After 1 h incubation in the influent water, a logarithmic decrease in the Zn intensity was observed when transitioning from the initial (left) to the final experimental time-point (right), of the acquired fluorescence images (Figure 3a). After 3 h, the extent of Zn dissolution tended to stabilize in wastewater influent with some recorded fluctuations at longer incubation times (Figure 3b).

Previous work demonstrated rapid dissolution of nano-ZnO under different conditions. Jiang et al.\(^\text{[45]}\) used anodic stripping voltammetry to monitor the dissolution kinetics of ZnO nanomaterials and found that dissolution of ZnO reached reaction equilibrium within half an hour regardless of the presence of the organic matter. In addition, Leung et al.\(^\text{[46]}\) showed that ZnO nanomaterials reach equilibrium within ≈2 h in aquatic media. We also found a logarithmic decrease in the Zn intensity during 1 h in situ incubation in influent water, indicative of an ongoing Zn dissolution from the ZnO nanorods, which tended to stabilize after 3 h (Figure 3).

This work shows that direct sulfiding is likely happening over short timescales within influent sewage waters, with some sterically available ZnO nanorods transforming to ZnS in less than an hour (Figure 1a). The final transformation, however, will be a competition between this direct sulfiding and the ongoing ZnO dissolution (releasing free Zn\(^2+\)), with the dominant species being a function of the local microenvironments generated. Metal sulfides are generally less soluble in water than their corresponding oxides or chlorides.\(^\text{[43,47]}\) The solubility product of zinc sulfide varies between 2.5 \times 10\(^{-22}\) and 1.6 \times 10\(^{-24}\), depending on whether the formed mineral phase is wurtzite or...
sphalerite.\[^{[48]}\] These relatively insoluble species may present negligible toxicological effects to microorganisms involved in the anaerobic digestion of sewage sludges that follow; alternatively transformation to zinc phosphate—as evidenced in the primary sludge incubation—may ensue. Nonetheless, the solubility of these transformed species will strongly depend on their crystallinity. Ma et al.\[^{[43]}\] showed that amorphous ZnS phases generated in aqueous sulfur-rich environments had a solubility of many orders of magnitude higher than that expected for bulk crystalline sphalerite. They hypothesized that amorphous ZnS nanoparticles were predominantly attached to the core of the remaining ZnO nanoparticles. Kim et al.\[^{[49]}\] reported that ZnS nanocrystals are formed in situ by the reaction of dissolved Zn species with reduced sulfur in the anaerobic process, which aggregate on the ZnO surface, and postulated that this may hinder further ZnO dissolution. It is also possible that indirect sulfiding can occur in the more sterically compromised areas where ZnO cores were shown to be surrounded by ZnS, according to the 2D SM calculated by nano-XFM (Figure 1b), which would support our hypothesis of the influence of the local sample-environments.

During the primary sludge incubation, there was either a significant increase in Zn\(_3\)(PO\(_4\))\(_2\) phases after only 1 h (Figure 1c) in some locations or slight increments in other sterically impeded regions after 3 h (Figure 2), with a concomitant decrease in ZnS. The proportion of Zn-phosphate dramatically increased after longer incubation in the more sterically available locations, where there is thought to be a greater ongoing Zn dissolution (Figure 1d). According to other studies, the higher organic matter content of primary sludge (Table 1) might also promote the rate of ZnO nanoparticle dissolution in a concentration-dependent fashion that is consistent with an increased thermodynamic driving force of dissolution, as also found by Jiang et al.\[^{[45]}\] These authors showed that the kinetics of ZnO dissolution and the dissolved zinc concentration at equilibrium depends not only on the concentration, but also on the type of organic matter available, although these effects are not straightforward and would depend on the mechanisms in which the organic matter molecules interact with particle surfaces. We have not attempted to amend the organic matter content nor its nature, therefore heterogenous mixtures of organic matter are expected in these real-world WWTP solutions. The released ionic Zn\(^{2+}\) then could complex with the predominant aqueous phosphates available (Table 1), generating the Zn\(_3\)(PO\(_4\))\(_2\) phases detected by nano-XFM after both 1 and 3 h incubation (Figures 1c,d, and 2b). With a much lower nominal solubility product of \(\approx 9.0 \times 10^{-33}\),\[^{[48]}\] the arising Zn-phosphates are even less likely than ZnS to dissolve further and would subsequently become incorporated into the solid-phase of the sludge. In the absence of phosphate, ZnO nanoparticles have been shown to dissolve rapidly within the initial 3 h, reaching equilibrium within 12 h.\[^{[42]}\] There, the authors revealed that, under favorable conditions, in terms of phosphate content, the proportion of zinc phosphate phases analyzed by XAS increased up to \(\approx 59%\) within 3 h of kinetic-reaction.

The subtle increase in the Zn intensity measured in situ during the primary sludge 1 h incubation (Figure 3) is likely due to Zn\(^{2+}\) already present in the sludge (\(\approx 2.5\) mg L\(^{-1}\), Table 1) potentially sorbing to the surface of the Zn nanorods or precipitating on to the ZnO surface due to local pH variations around the particles. This suggests that: 1) the nanorods are in reasonable equilibrium with the sludge from the outset (due to
the background Zn content, and also to the limited diffusion between the thin window and the sludge within the in situ cell), but 2) the aqueous Zn already present in the sludge would tend to form \( \text{Zn}_3(\text{PO}_4)_2 \) species on the surface of the ZnO nanorods during the incubation process.

2.4. Environmental Implications

The rapid generation of Zn-phosphate in primary sludge shown in this study may have an immediate impact on biota within the WWTP influent water and primary sludge environments. More likely, the exclusion of free Zn\(^{2+}\) ions from the sewerage waters will have greater environmental implications, due to the retention of the transformed Zn-phosphate phases into the anaerobic digester stage in a municipal WWTP. The following post-sewage treatment stages will ultimately dictate the environmental impact of the transformed Zn-species when they are deposited in soils through the recycling of biosolids\(^{[30]}\) or released into watercourses through a liquid effluent stream.\(^{[5]}\) In this study, we have identified four discrete nanosized phases co-existing within the wastewater media (ZnO, ZnS, \( \text{Zn}_3(\text{PO}_4)_2 \), and Zn adsorbed to Fe-oxyhydroxides), and have observed significant agglomeration and/or accumulation of the original ZnO nanorods during the initial stages of municipal wastewater environments (i.e., influent sewage water and primary sludge). Therefore, it is probable that there is a considerable range of sizes and heterogeneity of the individual Zn particulate phases present (also mixed withionic Zn) in any post-treatment WWTP discharge.

Available sources suggest that the production and use of ZnO nanoparticles are increasing, rising from \( \approx 550 \) 000 kg in 2012\(^{[53]}\) to \( \approx 1 \) 600 000 kg in 2014,\(^{[39]}\) with the estimated current global production in the range of 10 000 000 kg.\(^{[52]}\) Therefore, elevated concentrations of ZnO in wastewaters have been described as inevitable in the near future.\(^{[53]}\) Here, it was shown that a significant fraction of ZnO nanomaterials was rapidly sulfided during their incubation in influent water. Due to the lower solubility of ZnS compared with ZnO, this transformation will potentially restrict the amount of free Zn\(^{2+}\) in WWTP discharges in the event that no further transformations occur, hence limiting their toxicity for example to certain species of algae.\(^{[54]}\) The ZnS phases will present a higher residence time in the primary sludge tank before the anaerobic digestion step, and therefore, they may be more pernicious sources of ionic zinc when reaching the land via biosolid spreading, potentially reaching agricultural soils and entering the food chain.\(^{[57]}\)

Using nano-XFM we have shown that ZnO species could potentially reach the primary sludge tanks as larger aggregates if the ZnO nanomaterials are discharged into influent sewage water in the vicinity of municipal WWTPs. We note that both nanosized ZnO\(^{[55]}\) and \( \text{Zn}_3(\text{PO}_4)_2 \)\(^{[56]}\) have been shown to have antibacterial properties, which could be detrimental to the functioning of the wastewater treatment system, and alter the diversity of WWTP’s microbial communities and methanogenic activity. Although Fomina et al.\(^{[57]}\) observed that nanoscale Zn-phosphate exhibited lower toxicity to certain fungi than other metal phosphates, the presence of the phosphate ion has been associated with increased rates and amounts of Zn\(^{2+}\) release from ZnO nanoparticles.\(^{[58]}\) This increased dissolution, possibly via the partial dissociation of secondary \( \text{Zn}_3(\text{PO}_4)_2 \) precipitates or via other unspecified mechanisms, led to significantly elevated levels of NIH/3T3 fibroblast cell death at lower ZnO nanoparticle doses. In our study, a significant proportion of ZnO was transformed into Zn-phosphate phases in the primary sludge, which was attributed to its high phosphate concentration and the fact that \( \text{Zn}_3(\text{PO}_4)_2 \) is thermodynamically more stable. Mixtures of ZnS and \( \text{Zn}_3(\text{PO}_4)_2 \) species would reduce the input of ZnO nanomaterials into the anaerobic sludge digester, decreasing the bioreactivity of the ZnO nanorods and production of ionic Zn in the solid and effluent water leaving the WWTP. The mitigation of metal ion release (Zn\(^{2+}\) in this case) due to the described nanomaterials’ transformation is directly related to the WWTPs ability to provide clean water and sanitation, listed as one of the United Nation’s Sustainable Development Goals.

3. Conclusion

Through the application of synchrotron-based hard XFM, spatially resolved transformations on template-grown ZnO nanorods were shown during in situ incubation in real-world influent sewage water and primary sludge for 1 and 3 h. Isolated nanostructures of ZnS were observed after 1 h incubation in influent water, whereas only larger aggregated structures were detected at longer times (up to 3 h). The incubation of these nanorods in undigested primary sludge showed a varied response with a significant increase in \( \text{Zn}_3(\text{PO}_4)_2 \) phases after only 1 h in some locations and slight increments in other sterically impeded regions after 3 h, with a concomitant decrease in ZnS. The proportion of Zn-phosphate dramatically increased after longer incubation times in more sterically available locations. In addition, a rapid nano-spectroscopy methodology based on the acquisition of only nine discrete energy XANES images along the Zn K-edge was shown to be successful for the identification of four intermediate nanosized phases existing within the wastewater media: ZnO, ZnS, \( \text{Zn}_3(\text{PO}_4)_2 \), and Zn adsorbed to Fe-oxyhydroxides.

The evidence provided in this study contributes an important piece of the puzzle that will allow us to better understand, with a spatial resolution close to the individual particles, the physicochemical changes and the behavior of ZnO nanomaterials not only within wastewater systems but also helps to predict their fate and potential impact in other complex natural environments. For example, if, during their lifecycle, the nanomaterials become deposited in benthic regions, for example, riverine sediments or intertidal regions, they may be exposed to extreme gradients in oxygen and pH, which are likely to further influence transformations, the impact(s) of which is(are) yet to be recognized. This research showcases how relevant data can be extracted from complex, heterogeneous real-world media. The next stage would be to use in situ nano-XFM techniques, focusing on nanomaterials transformation post-release from WWTP treatment plants.
4. Experimental Section

ZnO Nanorods: Flower-like arrays of ZnO nanorods were synthesized on to silicon nitride windows (2 mm × 2 mm membrane, 5 mm × 5 mm frame; 1000 nm thickness; Norcada) following the procedure described by Xie et al.\cite{59} and Wang et al.\cite{60} Rods had a typical length of 724 ± 53 nm and diameter of 139 ± 23 nm (n = 102), as revealed by SEM (Figure S1, Supporting Information). These sizes were selected to ensure that the ZnO nanorods could be detected under hydrated conditions by nano-XFM, to align with the resolution of the most advanced X-ray nanoprobes (I14 beamline, Diamond Light Source, UK and 3-ID beamline, National Synchrotron Light Source II, USA). The ZnO nanorods were deposited onto silicon nitride windows to fix the position of the rods for real-time mapping, so that ZnO transformations in situ in influent water and primary sludge could be compared.

In Situ Cell: Two “in-house” designed and built liquid cells were utilized for the in situ synchrotron nano-XFM analysis, in accordance with the size and geometry requirements of each beamline (Figure S1, Supporting Information). The ZnO nanorods were independently incubated in: i) wastewater influents and ii) non-digested sewage sludges, collected in October 2016 from a typical municipal WWTP located in the South East of England (Anglian Water Group Ltd.). A similar setup was developed for the previous studies of incubation in model (artificial) aquatic solutions,\cite{19} where the power of the technique to follow nanoscale transformations was demonstrated, providing a base-line methodology such that more complex materials-environments could then be addressed. This was the first time that this methodology was applied to a wastewater/sludge environment sampled directly from the treatment plants. The geometry of the cells constrained the sewage medium volume to 200 mL, pipetted or pumped into the cell at the beginning of each in situ short-range incubation (either for 1 or 3 h). Besides, and to provide a baseline assessment of their composition, both raw media were digested and quantified by ICP-OES (Agilent 710).

Synchrotron X-ray Analysis: High-resolution nano-XFM images were acquired using the hard X-ray nanoprobes at two international synchrotron facilities: a) I14 beamline at Diamond Light Source (DLS), Didcot, UK and b) 3-ID beamline at the National Synchrotron Light Source II (NSLS-II) at Brookhaven National Laboratory, New York, USA. At the DLS, the focused X-ray beam size for this experiment was focused to ≈ 100 nm using pre-shaped KB mirrors, while at the NSLS-II, a coherently illuminated X-ray beam size of ≈ 50 nm was used to focus the monochromatic X-rays to a size of ~80 nm. At each facility, fluorescent X-rays were captured and processed using bespoke fluorescent detectors.\cite{61,62}

Stacks of XANES images were acquired along the Zn K-edge at different photon energies by applying a reduced nano-spectroscopic methodology after draining the in situ cells based on the acquisition of only nine energy points (referred to as 9-E), as defined elsewhere.\cite{19} The nine discrete XANES images were carefully aligned, and a thorough normalization relative to the background intensity of each image was applied through Matlab and Python-based methods\cite{37} in order to extract relevant pixel-by-pixel information. This process enabled the data to be reshaped into an array of spectra from each pixel of the aligned XANES images, and SM could be calculated by fitting the absorption data from each pixel to the linear combination of the standard spectra.

A complementary higher energy resolution image stack was collected for the samples after in situ incubation in primary sludge using 135-energies (135-E) along the Zn K-edge for further validation of the 9-E technique. The mean intensity of the 135-E image stack was represented as a function of energy by using Dawn software,\cite{63} generating a “traditional” XANES spectrum in selected regions of interest (Figure S2b, Supporting Information), which was subsequently analyzed by linear combination fitting (LCF) via Athena software.\cite{64} The four Zn-standards used for LCF (ZnO, ZnS, ZnS2PO4·H2O, and Zn adsorbed to Fe-oxyhydroxides) were selected as the most likely Zn-species present in wastewater media after ZnO nanoparticles addition, according to previous studies performing bulk XAS.\cite{17,18} The background was subtracted by applying a linear fit through the Zn pre-edge region and using the autobk routine in Athena for the spline fit through the XANES region. The quality of the LCF analysis was evaluated by using the R-factor calculated by Athena ([Σ (data – fit)2]/Σ (data)2), also known as the normalized sum of the squared residuals. The R-factor values were additionally calculated for all of the fitting absorption data from each pixel included in the SM in Figure 1. The histograms showing the frequency of the distribution of these values are presented in Figure S3, Supporting Information, providing an error estimation of the 9-E speciation fittings.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work and MGG contract were supported by the Natural Environment Research Council project: #NE/N006518/1 and #NE/ N006402/1. M.R. acknowledges support from the RAEng/Shell via a Research Chair in Interfacial Nanoscience. The authors acknowledge Diamond Light Source Ltd. (Didcot, Oxfordshire, UK) for providing beamtime of Hard X-ray Nanoprobe at I14 beamline (proposal: sp17784). This research used the Hard X-ray Nanoprobe (HXN) Beamline at 3-ID of the National Synchrotron Light Source II, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Brookhaven National Laboratory under contract No. DE-SC0012704. Authors appreciatively thank the allocated HXN beamtime (General User’s proposal: 309198) and Dr. Mingyuan Ge, Dr. Xiaojing Huang, Dr. Hanfei Yan, and Dr. Yong Chu for their assistance during the experimental beamtime. The authors acknowledge Anglian Water Group Ltd. for providing the influent water and the primary sludge samples, and Ms. Chetna Vaghela for liaising with the Anglian Water Group on Imperial College’s behalf. The authors thank the Thin Film Technology laboratory (TFT, Imperial College London) for the use of the RIE system and Mr. Xiangyu Xie (Materials Department) for use of the instrumentation used during the ZnO nanorods’ synthesis. The authors also gratefully thank Prof. Gregory Lowry (Carnegie Mellon University, PA, USA) for providing the Zn adsorbed onto Fe-oxyhydroxide XAS standard.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

in situ X-ray imaging, wastewater treatment plants, XANES, X-ray fluorescence microscopy, ZnO nanomaterials

Received: February 2, 2021
Revised: April 6, 2021
Published online: May 5, 2021

\[ R = \frac{\sum (data - fit)^2}{\sum (data)^2}, \]
