Spatially Resolved Dissolution and Speciation Changes of ZnO Nanorods during Short-Term *in Situ* Incubation in a Simulated Wastewater Environment

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ABSTRACT: Zinc oxide engineered nanomaterials (ZnO ENMs) are used in a variety of applications worldwide due to their optoelectronic and antibacterial properties with potential contaminant risk to the environment following their disposal. One of the main potential pathways for ZnO nanomaterials to reach the environment is via urban wastewater treatment plants. So far there is no technique that can provide spatiotemporal nanoscale information about the rates and mechanisms by which the individual nanoparticles transform. Fundamental knowledge of how the surface chemistry of individual particles change, and the heterogeneity of transformations within the system, will reveal the critical physicochemical properties determining environmental damage and deactivation. We applied a methodology based on spatially resolved *in situ* X-ray fluorescence microscopy (XFM), allowing observation of real-time dissolution and morphological and chemical evolution of synthetic template-grown ZnO nanorods (~725 nm length, ~140 nm diameter). Core-shell ZnO-ZnS nanostructures were formed rapidly within 1 h, and significant amounts of ZnS species were generated, with a corresponding depletion of ZnO after 3 h. Diffuse nanoparticles of ZnS, Zn₃(PO₄)₂, and Zn adsorbed to Fe-oxyhydroxides were also imaged in some nonsterically impeded regions after 3 h. The formation of diffuse nanoparticles was affected by ongoing ZnO dissolution (quantified by inductively coupled plasma mass spectrometry) and the humic acid content in the simulated sludge. Complementary *ex situ* X-ray absorption spectroscopy and scanning electron microscopy confirmed a significant decrease in the ZnO contribution over time. Application of time-resolved XFM enables predictions about the rates at which ZnO nanomaterials transform during their first stages of the wastewater treatment process.

KEYWORDS: ZnO nanomaterials, X-ray fluorescence microscopy, scanning electron microscopy, *in situ* ZnO dissolution, spatially resolved ZnO transformations

Zinc oxide engineered nanomaterials (ZnO ENMs) are incorporated into a wide range of consumer products, such as sunscreens, personal care products, and antibacterial agents, raising concerns about their potential impact as a pollutant following their inevitable release into the environment. The annual production of ZnO ENMs was estimated to be 1600 tons in EU countries, a marked increase on previous estimates.[\(1\)](#) One of the primary pathways for environmental exposure to ZnO ENMs is *via* their passage through municipal sewage systems.[\(2,3\)](#) Current concentrations of ZnO ENMs in municipal waters are predicted to be of the order of mg L^{-1} .[\(4\)](#) Variations in the physicochemical properties (such as size, surface chemistry, and aggregation state) of ZnO ENMs in aqueous environmental media will generate a highly complex mixture of transformed ENMs with very varied properties and behavior.[\(5\)](#) Transformation of ZnO ENMs may inhibit bacterial processes within urban wastewater treatment plants (WWTPs) and may adversely affect the health of organisms in soils and waters once treated material is released back into the natural environment,[\(6,7\)](#) though some research indicates minimal impacts.[\(8\)](#) More detailed knowledge and understanding of the nature of these transformations and the time-scales over which transformations can occur is essential for predicting likely outcomes of the interactions between individual ZnO ENMs and biota and the overall implications for ecosystem health. The toxicity of ZnO ENMs to biota is expected to be strongly influenced by their surface chemistry, speciation, and transformation. These thermodynamically driven processes are influenced by a range of environmental factors, including temperature, pH, organic matter content, and the presence of other compounds and colloids.[\(9,10\)](#) Above a threshold concentration, zinc ions released from ENMs dissolution can be highly toxic,[\(11,12\)](#) but chemical reactions, such as sulfidation or complexation with organic carbon within environmental systems, can reduce their toxicity by effectively trapping free Zn^{2+} ions.[\(13,14\)](#) The speciation and transformation of ZnO ENMs within a pilot WWTP have been studied previously using bulk analytical techniques.[\(2,15\)](#) Applying X-ray absorption near edge spectroscopy (XANES), Ma *et al.*[\(2\)](#) found that ZnO particles transform into a complex mixture of ZnS, $\text{Zn}_3(\text{PO}_4)_2$, and Zn adsorbed to Fe oxy/hydroxides within the biosolids generated at the WWTPs. However, bulk analyses were conducted, with no spatially resolved information about the particle distribution, morphology, and size, or the local degree of ZnO particle transformation. One report showed a conversion of almost 100% of ZnO to ZnS in an aquatic medium with a high enough sulfide concentration and incubation time (5 days).[\(16\)](#) These transformations will all be critical determinants of the ZnO ENMs' end-point toxicity. Natural organic matter (NOM) is present in all urban WWTPs and therefore must be taken into account in the kinetics of ZnO ENM transformations and their behavior during wastewater treatment processes.[\(17\)](#) Organic matter, including humic and fulvic acids, may sorb to the surface of the ENMs, altering their surface properties. This will influence factors such as agglomeration of the ENMs and their dissolution, which, in turn, will affect the ENM's settling, transport, physico-chemistry, and behavior within sludges.[\(18,19\)](#) These organic molecules usually form ligands on the surface of the ENMs, however, their influence on subsequent chemical transformations of the ENMs is less clear. Bian *et al.*[\(20\)](#) reported that ZnO ENMs became more stable in the presence of humic acid ($10\text{--}100 \text{ mg L}^{-1}$). However, a neutral pH was

used for their analysis, which is in contrast to the acidic pH found in a primary sludge within a WWTP (pH ~ 4.5).

The dissolution rate for ZnO ENMs is not the sole factor that will influence their physico-chemistry (including morphology, size, and surface chemistry). Alterations to the bulk and surface chemistry of the nanoparticles and their transformation into so-called “daughter particles” will affect where they accumulate and their ultimate bioreactivity. These processes will occur dynamically within, for example, the aqueous sludges of WWTPs, and assessment of ZnO ENMs transformations requires the development of spatially resolved *in situ* methods to track any physicochemical changes in real-time. Few techniques are capable of visualizing dissolution kinetics and chemical transformations of the individual engineered nanomaterials or of measuring these properties over relatively short-range time scales (~hours). Chemical analysis of nanomaterials has mostly been limited to static techniques, such as nanoanalytical electron energy loss spectroscopy (EELS); these methods are limited by the fact that the samples must be imaged under high-vacuum conditions.[\(21\)](#) Dynamic information can now be generated using *in situ* electron microscopy holders, however, the samples will undergo electron beam damage induced by water hydrolysis that will cause artifacts.[\(22,23\)](#) Hard X-ray fluorescence microscopy (XFM),[\(24,25\)](#) has a spatial resolution ranging from around 25 to 100 nm, provided by the Fresnel X-ray zone plates or KB mirrors, and has higher energy resolution than nanoanalytical spectroscopy in electron microscopes.[\(26–28\)](#) This technique offers the capability to monitor dynamic changes to the morphology and surface chemistry of the ENMs, *in situ*, within relevant hydrated environments,[\(29,30\)](#) with an outstanding combination of energy and spatial resolution. In recent years, XFM has emerged as a promising technique for imaging environmental samples due to its high spatial resolution and sensitivity to a range of elements (depending on their absorption edges). This technique has been applied to study Zn accumulation and quantification within relevant biological[\(31–33\)](#) and environmental samples,[\(34,35\)](#) with only a few *in situ* micro-X-ray analyses previously reported.[\(36\)](#) Here, we report on ZnO speciation and morphological changes studied by *in situ* XFM within relevant simulated sludges. A relevant aqueous component of wastewater sludge, humic acid, was used to demonstrate that the XFM technique can be utilized to characterize chemical and morphological transformations of individual nanomaterials in aqueous environments *in situ*. It is only by applying this simplified system, instead of a more complex real sludge, that we can generate accurate proof-of-concept of the feasibility of the technique. An X-ray absorption spectrum was generated by acquiring multi-energy XFM images along the Zn K-edge, after plotting the mean Zn intensity of the image *versus* the energy. Here, the linear combination fitting of this spectrum against the Zn-standard spectra[\(37\)](#) was used to confirm the nature of the Zn species after the *in situ* incubation by XFM.

Previously published data showed that ZnO rods with lengths ranging from 687 to 1512 nm are present in consumer products, together with even bigger aggregates.[\(38\)](#) Therefore, we selected large ZnO nanorods deposited over silicon nitride windows to compare ZnO transformations *in situ* before and after short-time incubations in relevant simulated sludge to fix the position of the rods for real-time mapping. The aims were to acquire: (i) spatially resolved maps showing the transformations of ZnO nanorods in a simulated WWTP medium, (ii) identify the time scales over which the Zn species changed, and (iii) elucidate the temporal evolution of the ZnO surfaces, particle morphology, and size and formation of daughter

particles. Reacting nanoparticles were imaged in hydrated environments to generate mechanistic insights into the underlying pathways both for environmental damage and potential (de)activation of ZnO ENMs used in consumer products over short-time scales, as they transport through the urban WWTPs.

Results

ZnO Nanorod Characterization

Scanning electron microscopy (SEM) analysis of the initial nanorod material revealed star-like arrays of ZnO rods on the silicon nitride windows. Rods exhibited a typical length of 724 ± 53 nm and diameter of 139 ± 23 nm ($n = 102$) ([Figure 1a](#)). These diameters([39,40](#)) were selected to ensure that the ZnO nanorods could be detected under hydrated conditions by XFM, meeting the resolution of the hard X-ray nanoprobe capability on the I14 beamline (Diamond Light Source). Energy-dispersive X-ray spectroscopy (EDX) was performed on both the rods and silicon nitride window surface, to confirm their chemical composition ([Figure 1b](#)). Complementary X-ray photoelectron spectroscopy (XPS) analyses determined the surface chemistry of the ZnO nanorods, confirming that sulfates/sulfides and phosphates were absent in the “as-synthesized” structures ([Figure 1c](#)). Further details of the ZnO synthesis and their diameter distribution are presented in the Supporting Information ([Figure S1](#)).

Figure 1

Figure 1. Characterization of the synthesized ZnO nanorods. (a) SEM image of the “as-synthesized” ZnO nanorods grown in the silicon nitride membranes. The boxed region shows the higher magnification area selected for EDX analysis. (b) EDX spectra taken from the nanorods (circle) and the silicon nitride (open triangle, [Figure 1a](#)). (c) XPS spectrum acquired from the ZnO nanorods, indicating the presence of zinc and oxygen. (d) A stack of nine X-ray fluorescence microscopy images of the “as-synthesized” ZnO nanorods acquired at 9648.5, 9653, 9658, 9669, 9670.5, 9674, 9675, 9682, and 9702 eV (see further details in [Experimental Methods](#) section). (e) XANES spectrum calculated using the mean intensity of 135 energy images acquired along the Zn K-edge for the “as-synthesized” ZnO nanorods (as detailed in the [Experimental Methods](#) section).

Spatially Resolved Mapping of ZnO Dissolution and Evolution of Morphology As a Function of Time

During incubation of the ZnO nanorods in a simulated primary sludge environment, consecutive spatially resolved XFM maps were acquired to investigate the morphological evolution of the nanorods over 3 h. Primary sludge is one of the first physio-chemical barriers that influent or sewage waters encounter when entering the cleaning cycle of a regular WWTP. [\(2,41\)](#) This

sludge is primarily formed by organic matter and humic substances, and hence we selected humic acid as a relevant aqueous component of wastewater sludge. A total of four fluorescence maps were acquired during a 1 h incubation period (humic acid 10 mg L⁻¹ concentration, ~10 min/map, 5 min dead-time between measurements), and 18 maps were collected over the 3 h *in situ* incubation period (~7 min/map, 3 min dead-time). The consecutive maps were integrated into a single two-dimensional (2D) matrix, where the average Zn intensity was plotted as a function of time ([Figure 2](#)). A slight decrease in the Zn intensity was observed during the first hour of incubation in the simulated sludge ([Figure 2a](#)), corresponding to a minor decrease in intensity between the first (left) and last (right) fluorescence maps acquired. Over 3 h *in situ*, the Zn “hotspot” being monitored decreased gradually in size, until it is no longer detectable after ~150 min incubation time (as shown in the first (left) and last (right) XFM maps in [Figure 2b](#)). An irregular fluctuation of the Zn intensity was recorded during the 3 h *in situ* experiment ([Figure 2b](#)). This can be explained by flux within the medium having an influence on the acquired Zn mean intensity between consecutive images. The expected ZnO nanorod dissolution favors the release and transport of Zn²⁺ ions within the sludge solution, generating the diffusion clouds observed during the *in situ* imaging ([Video S1](#), Supporting Information).

Figure 2

Figure 2. ZnO dissolution as a function of time during the *in situ* experiments, showing a decrease of Zn intensity by X-ray fluorescence microscopy. Graphical representation of the Zn normalized intensity for the acquired XFM maps as a function of the incubation times: (a) 1 h and (b) 3 h. XFM maps corresponding to the first (left) and the last time map (right, dotted square) are also represented for each condition. Scale bars equal to 750 nm in all cases. *The variable intensities shown are a consequence of the fluctuating Zn signal arising from the flux in the internal solution, as presented in the [Video S1](#).

After *in situ* XFM analyses, the composition of the remaining simulated sludge solutions was quantified by inductively coupled plasma mass spectrometry (ICP-MS), in order to evaluate changes in zinc concentration between 1 and 3 h ([Table 1](#)). Iron and phosphorus were also

quantified since $\text{Zn}_3(\text{PO}_4)_2$ and Zn adsorbed to Fe oxy/hydroxides (Zn-Fe(ox)) are reported to be predominant Zn species in biosolids analyzed after WWTP cleaning processes.⁽²⁾ The sulfur content could not be quantified due to the limited volume of the sample and its known spectrometric interference with the protons and oxyanions from aquatic media in the ICP-MS.⁽⁴²⁾

Table 1. ICP-MS Quantification Performed on the Simulated Sludge Solutions Collected after *in Situ* XFM Analyses^a

		ICP-MS quantification ($\mu\text{g L}^{-1}$)		
<i>in situ</i> solutions		Zn	Fe	P
	blank^b	9.2 ± 0.1	102 ± 1	93 ± 10
simulated sludge	1 h	12391 ± 106	798 ± 9	264 ± 20
	3 h	27533 ± 216	772 ± 12	254 ± 33

^a

Presented as mean ± standard error.

^b

The simulated sludge solution (humic acid 10 mg L⁻¹ concentration) used for the *in situ* experiments was additionally quantified by ICP-MS in order to elucidate the background elemental composition.

The analyses performed by ICP-MS indicate that the ZnO nanorods begin dissolving within the first hour of exposure to the simulated sludge, releasing Zn²⁺ ions into the solution ([Table1](#)). A significant amount of Zn (~27.5 mg L⁻¹) was released from the nanorods after 3 h incubation in the simulated sludge solution, which is in qualitative agreement with the decrease in Zn intensity measured during the *in situ* XFM experiment ([Figure 2a,b](#)). Phosphorus and iron concentrations were slightly lower after 3 h compared with the 1 h incubation in the simulated sludge, possibly indicating that secondary P- and Fe-rich phases may have precipitated, though the measured variation was not statistically significant ([Table1](#)).

Speciation Analyses by Synchrotron-Based Techniques: Zn-Transformations Evaluated by Nine-Energy Scans

Speciation maps were calculated after acquiring nine-energy (9-E) imaging scans, generating a pixel-by-pixel contribution for each Zn species between 0 (dark blue) and 1 (red) ([Figure 3](#), detailed in the [Experimental Methods](#) section). The spectra taken using the 9-E maps for the “as-synthesized” ZnO showed a major ZnO contribution, as expected for both the speciation maps and the quantification maps (SM and QM, [Figure 3a](#)). QM were calculated by normalizing the SM to the isosbestic point (E_9 in [Figure S4](#)), producing a visual estimation of the real extent of the Zn species generation. A fluorescence map was also acquired at the maximum of the Zn K-edge XANES spectrum (E_{max} = 9669 eV, [left], [Figure 3](#)) for total Zn intensity contribution.

Figure 3

Figure 3. Nine-Energy X-ray fluorescence microscopy of: (a) “as-synthesized” ZnO, (b) sample after the 1 h incubation in a simulated sludge (humic acid 10 mg L⁻¹), and (c,d) two different regions analyzed in the sample after the 3 h incubation. (Left) Fluorescence map acquired at the maximum of Zn K-edge XANES spectrum ($E_{\text{max}} = 9669$ eV). (Right) SM of the expected Zn species: ZnO, ZnS, Zn₃(PO₄)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% (generated by [eq 1](#)). QM for the same species (generated by [eq 3](#)) were calculated after normalizing the SM with the isosbestic point (E_9 in [Figure S4](#)). The numbering on the scale bars on the QM provides an estimation of the Zn species contribution. Scale bars equal to 750 nm in all cases.

After 1 h incubation of the ZnO nanorods in the simulated sludge ([Figure 3b](#)), ZnS became the predominant species on the surface of the Zn hotspot according to the SM. The calculated QM confirmed the prevalence of ZnS at the top-left location of the Zn hotspot, although this species does not cover the whole high-intensity region. ZnO remained as a major phase in this case, both in the top-right location of the spot and in the background of the image. Minor contributions of both Zn₃(PO₄)₂ and Zn-Fe(ox) in relation to ZnS were found after 1 h incubation

in the simulated sludge solution.

After the 3 h incubation, different behavior of the ZnO nanorods was found depending on the region analyzed. In the first region ([Figure 3c](#)), in the SM, most of the Zn hotspots remained as ZnO. The quantification maps indicated a significant amount of ZnS species generated in areas depleted in ZnO. The second region analyzed after 3 h incubation ([Figure 3d](#)) showed a more disperse variation in the Zn species in the SM, with ZnS as the dominant species. Although ZnO was still a major component of the larger hotspots in the QM, there was a significant contribution of ZnS within the smaller hotspots. Trace amounts of diffuse $\text{Zn}_3(\text{PO}_4)_2$ and Zn-Fe(ox) were also mapped after the 3 h incubation, with different trends in their spatial variations. Compared to the sample incubated in a simulated sludge for 1 h ([Figure 3b](#)), after 3 h, in the first region analyzed, both the $\text{Zn}_3(\text{PO}_4)_2$ and Zn-Fe(ox) contributions decreased in the QM ([Figure 3c](#)). Conversely, in the second area analyzed after 3 h incubation ([Figure 3d](#)), the concentration of both species *increased* in the QM compared to the 1 h time point, and their distribution became highly diffuse. In region 2, both $\text{Zn}_3(\text{PO}_4)_2$ and Zn-Fe(ox), as well as the ZnS, were more prevalent within larger Zn hotspots (refer to the fluorescence map acquired at E_{max} , [Figure 3d](#), left).

Zn Transformations Evaluated by 135-Energy Scans

To confirm the validity of the spectra extracted from 9-E speciation, a higher energy resolution image stack was acquired using 135-energies (135-E) along the Zn K-edge (see energy-range description in [Section S4b](#), Supporting Information). The mean intensity of the 135-E image stack was measured and represented as a function of energy, generating a “traditional” X-ray absorption near edge spectroscopy (XANES) spectrum for each sample ([Figure 1e](#)). These XANES spectra were compared with the Zn-standards in both the k -space and the Fourier transform representations ([Figure S6](#)). A region of interest, selected from the k -space representation, was subsequently analyzed by linear combination fitting (LCF) ([Figure 4b](#)).

Figure 4

Figure 4. X-ray absorption near edge structure spectra comparison of both samples and standards; and LCF analyses. (a) Focused k -space (3–5 $\text{\AA}^{-1}$) of the k^3 -weighted XANES spectra plotted after 135-E acquisition for the Zn-standards, the “as-synthesized” nanorods, and the samples after *in situ* incubation in the simulated sludge (humic acid 10 mg L^{-1}) solution: 1 h and 3 h (second-region, see [Figure 3d](#)). (b) LCF of the XANES spectra for the indicated samples, showing the best three fits calculated by Athena software. The LCF values for fits number 1 (red dots), 2 (blue dots), and 3 (green dots) of each sample are presented in [Table2](#).

Comparison of the narrowed k -space region (3–5 $\text{\AA}^{-1}$) from the samples with the standards showed a transition from the ZnO features (two peaks, red spectrum) to other Zn species, which presented only a single peak approximately in the middle of the k region ([Figure 4a](#)), when going from the “as-synthesized” sample to the two simulated sludge incubations. An estimation of the sample composition was performed by applying LCF over the k -space region ([Figure 4b](#) and [Table2](#)). The LCF values of the “as-synthesized” ZnO showed a major ZnO contribution in all fittings (ranging 78–86%) with minor contributions of ZnS, $\text{Zn}_3(\text{PO}_4)_2$, and Zn-Fe(ox) ([Figure 4b](#), [Table2](#)). LCF data indicated a decrease in the ZnO contribution after only 1 h incubation in the simulated sludge (to 27–41%) ([Table2](#)). In this case, the best fitting statistical parameters (R-factor and red χ^2) were found for the $\text{Zn}_3(\text{PO}_4)_2$ as major species ([Table2](#)), although the graphical representation of the LCF indicated that both ZnS and Zn-Fe(ox) could also explain the sample features ([Figure 4b](#)). The accuracy of LCF results is generally limited by the accuracy of the standards in representing the chemical species present in the sample and the uniqueness of XANES spectral features between standards.⁽⁴³⁾ In any case, the LCF results

shown here can be used to complement the data obtained after the 9-E speciation study.

Table 2. Linear Combination Fit Results for Zn K-Edge XANES Spectra Acquired on Samples before and after *in Situ* Incubation in the Simulated Sludge Solution

fit number <a>	ZnO (%) b	ZnS (%) b	Zn ₃ (PO ₄) ₂ (%) b	Zn-Fe(ox) (%) b	R factor c	red χ^2 d
“As-Synthesized” ZnO						
1	78.0			22.0	0.3022	4.728
2	79.6		20.4		0.3027	4.737
3	86.6	13.4			0.3188	4.989
After 1 h <i>in Situ</i> Incubation in the Simulated Sludge						
1	29.7		70.3		0.1413	0.9937
2	26.8			73.2	0.1959	1.378
3	41.5	58.5			0.2201	1.548
After 3 h <i>in Situ</i> Incubation in the Simulated Sludge						
1	12.0		81.4	6.6	0.0856	0.5601
2	12.5	27.3		60.3	0.1177	0.7705
3	17.3	47.6		35.0	0.1553	1.017

Linear combination fit was applied over the k -range: 3–5 Å⁻¹ on the Zn K-edge XANES spectra. The graphical representation of fitting numbers 1 (red dots), 2 (blue dots), and 3 (green dots) is presented in [Figure 4](#).

The contribution of each Zn species is presented as a percentage. Fittings were constrained to fit 100%.

Normalized sum of the squared residuals of the fit [$R = \sum(\text{data} - \text{fit})^2 / \sum \text{data}^2$].

Goodness-of-fit between different fittings was assessed by the χ^2 parameter $[= N_{\text{idp}}/N_{\text{pts}} \sum ((\text{data} - \text{fit})/\epsilon)^2]$, where N_{idp} is the number of independent points in the model fit, N_{pts} is the number of data points, and ϵ is the measurement uncertainty.

Finally, a three-standard linear combination fitting was used in the sample incubation for 3 h in the simulated sludge, indicating a greater mixture of Zn species. The graphical representation of the LCF does not fit perfectly with the XANES spectrum ([Figure 4b](#)), possibly due to the generation of additional species different from the Zn standards used for the analysis. However, the ZnO contribution decreased significantly in all fittings when compared with the 1 h *in situ* incubation, to a 12–17% of the total Zn content ([Table 2](#)).

Additional Analyses on Residual solids: SEM and XFM

SEM images were acquired on the samples after *in situ* incubation in the simulated sludge solution ([Figure 5](#), after draining excess liquid). After 1 h incubation, most of the ZnO nanorods showed evidence of partial dissolution, exhibiting thinner rod structures of 164 ± 21 nm length and 18 ± 4 nm width ($n = 30$, [Figure 5a](#)). The rods had a hexagonal morphology after 3 h incubation, with a range of diameters between 200 and 390 nm (300 ± 48 nm average) and 439 ± 50 nm length ($n = 30$, [Figure 5b](#)). In both incubation times (1–3 h), residual ZnO nanorods with their original sizes and morphologies were found (positions 1 and 5 [Figure 5a](#); positions 6 and 7, [Figure 5b](#)).

Figure 5

Figure 5. Morphology changes and chemical phases of transformed ZnO nanorods after *in situ* incubation in a simulated sludge (10 mg L^{-1} humic acid). SEM images acquired after the *in situ*: (a) 1 h and (b) 3 h incubations. Numbers 1–9 represent the locations analyzed by energy-dispersive X-ray spectroscopy (raw EDX spectra and atomic percentages are shown in [Figure S2](#) and [Table S1](#)).

EDX spectra were also acquired from some of the imaged regions ([Figure 5a,b](#)), and the atomic percentages are presented in [Figure S2](#) and [Table S1](#). The thinner rods obtained after 1 h incubation (position 2, [Figure 5a](#)) had a comparable Zn/O ratio to the unreacted original ZnO

rods (position 1, [Figure 5a](#)), although these values were considerably larger than the “as-synthesized” ZnO nanorods (Zn/O ratio = 0.36, [Table S1](#), [Figure 1b](#)). However, the Zn/C ratio decreased significantly from the “unreacted” rods to the thinner rods generated during 1 h incubation in the simulated sludge solution ([Table S1](#)). This tendency changed as a function of time since the hexagon-shaped structures formed after 3 h incubation (position 9, [Figure 5b](#)) presented a much higher Zn/C ratio than the unreacted ZnO rods (positions 6 and 7, [Figure 5b](#)). Negligible concentrations of both sulfur and phosphorus were detected by EDX after 1 h incubation in all locations, according to the calculated standard deviations (RSD, [Table S1](#)). Nonetheless, minor sulfur contributions were obtained after the 3 h incubation in the simulated sludge, both for the unreacted ZnO rods (position 7, [Figure 5b](#)) and the nano hexagon-shaped structures (position 8, [Figure 5b](#)). Even though EDX is not sufficiently sensitive to detect minor contributions from these elements,⁽⁴⁴⁾ SEM-EDX analyses ([Figure S2](#), ESI) indicated ZnO speciation changes toward ZnS species after 3 h incubation in a simplified primary sludge medium.

Finally, XFM maps were acquired over larger Zn hotspot areas ([Figure 6a](#)), already analyzed by the 9-E speciation studies. [Figure 6b](#) shows a decrease in the mean Zn signal intensity from the recovered sample after the 1 h *in situ* incubation in a simulated sludge compared to the dry (“as-synthesized”) sample. This decrease was coupled with a slight increase in both S and P intensities. Note that it was not possible to perform the XFM analyses on the same drained region as the original Zn hotspot as the ZnO dissolved during acquisition ([Video S1](#)).

Figure 6

Figure 6. ZnO nanorod transformations measured by X-ray fluorescence microscopy. (a) XFM images acquired from ZnO nanorods on dry and drained conditions, before and after the 1 h *in situ* incubation in the simulated sludge (10 mg L⁻¹ humic acid). The numbering on the scale bars represents the maximum intensity measured by XFM. Scale bars equal to 750 nm in both cases.

(b) The calculated mean intensity of zinc (black), sulfur (red), and phosphorus (blue) of the previous XFM images.

Discussion

Using complementary techniques, we have studied the evolution of ZnO nanorod surfaces, morphology, and chemical transformations during short-term *in situ* incubations in a simplified simulated wastewater environment. We collected spatially resolved XFM maps in real time to monitor the chemical transformations of the ZnO nanorods in a simulated primary sludge, composed of humic acid 10 mg L⁻¹ solution at pH 4.5. It was observed that ZnS species were the predominantly forming compounds within the studied incubation times (1–3 h, [Figure 3](#)), which was confirmed by the incremental increase recorded in sulfur intensity measured by *ex situ* XFM analysis ([Figure 6b](#)) after an only 1 h incubation. Ma *et al.* [\(16\)](#) studied nano-ZnO transformations in a sulfur-rich environment; in this work, the authors found sphalerite (ZnS) nanoshell formation on the surface of ZnO nanomaterials. This ZnS shell did not completely prevent further ZnO dissolution to Zn²⁺, and a dissolution and reprecipitation process was suggested to explain the sulfidation of ZnO nanoparticles. We propose that a superficial ZnS nanoshell may be generated in our experiment within 1 h of incubation in a simulated wastewater environment, as shown by the ZnS diffuse cloud arising from the ZnO hotspot in [Figure 3b](#). The potential ZnS shell formation on the ZnO core has been reported to dramatically *decrease* subsequent Zn²⁺ release from ZnO nanomaterials, which will be more effective if higher degrees of sulfidation are achieved.

Nonetheless, a full transformation of ZnO was not observed here over the short-time scales studied (1–3 h), evidenced by the decreased, but nonzero, ZnO contribution as a function of time calculated by LCF of the XANES spectra generated after the 135-E speciation analyses ([Figure 4](#), [Table 2](#)). Compared to the precursor ZnO nanorods, the partial formation of less soluble (and potentially less bioreactive) ZnS phases in wastewater environments may have both direct and indirect impacts to microorganisms. Using a metagenomic approach to characterize the bacterial microbiome in activated sludge, Meli *et al.* [\(6\)](#) recorded a concentration-dependent reduction in the number of operational taxonomic units (OTU's) following the application of ZnO nanoparticles (NPs). Though speciation was not measured in that work it is likely that transformations would have occurred. Such negative impacts on microbial populations could seriously impact their vital role as bioremediators, reducing the nutrient concentrations of effluents. Furthermore, primary sludge, containing variously transformed Zn species may subsequently be applied to land as fertilizers, through biosolids generation during the anaerobic digestion. [\(15\)](#) Durenkamp *et al.* [\(8\)](#) found minimal impacts to the composition and functioning of soil bacteria growing in soil amended with WWTP sludge containing silver and zinc oxide nanoparticles compared with metals salt equivalents, though a reduction in fungi was recorded following metal exposure. However, they did not measure if transformations occurred in Zn forms once the sewage sludge was added to the soil. Short-term negative impacts to biota following the application of pristine ENMs to soils have not been taken into account, leading to erroneous assessments of the toxicity of ENMs. [\(8\)](#) Here we demonstrate that particle modification can occur over relatively short time scales, hence

impacts measured in the very short-term may not necessarily be realized in the long term, illustrating the highly dynamic nature of ENMs modification. The tendency for aggregation is increased in ZnS particles,[\(16\)](#) which may promote sedimentation not only in WWTPs but also in the natural environment. Once deposited in benthic regions, for example, river beds, ENMs may be exposed to extreme gradients in pH, H₂S, and redox conditions, which are likely to further influence transformation, affecting their toxicity.[\(6\)](#) With modifications, the proposed XFM detection and speciation methodology can be further utilized as a test bed to analyze ENMs impacts in aquatic media, over a suite of environmental regimes.

Our results also suggest the presence of daughter particles of both Zn₃(PO₄)₂ and Zn adsorbed to Fe-oxyhydroxides after the short-time *in situ* incubations in a simulated primary sludge, by traditional linear combination fitting of the Zn-standards spectra ([Figure 4b](#), [Table 2](#)) and X-ray fluorescence microscopy ([Figure 6b](#)). The formation of a ZnO-Zn₃(PO₄)₂ core-shell structure has been reported in phosphate-rich environments,[\(45\)](#) where Zn²⁺ can be complexed with aqueous PO₄³⁻, resulting in the formation of an amorphous zinc phosphate hydrate precipitate.[\(46\)](#) Although Zn₃(PO₄)₂ is the thermodynamically favored species, the full transformation to these species is kinetically limited to longer reaction times (15 days),[\(45,46\)](#) which were not studied here. The higher solubility reported by Ma *et al.*[\(16\)](#) for the ZnS-shell/ZnO nanomaterials than that expected for bulk ZnS ($K_{sp} = 2 \times 10^{-25}$ yields a solubility of 3.7×10^{-10} mM) would kinetically favor the transformation to ZnS phase rather than to Zn₃(PO₄)₂. This fact was confirmed by *ex situ* SEM-EDX analyses after 3 h incubation (positions 6 and 7, [Figure 5b](#), [Table S1](#)), highlighting the importance of the Zn-sulfide over the Zn-phosphate phases. Complementary XAS analyses were conducted on commercial smaller ZnO nanoparticles (10–30 nm size) incubated for 3 h in the simulated sludge solution ([Figure S5](#)). These results showed a transition on the maximum of peak for the three samples incubated in humic acid toward lower energy-values from the ZnO (red) to the ZnS (green) and Zn₃(PO₄)₂ species (blue) ([Figure S5a](#), yellow arrow), confirming that ZnO nanoparticles in solution will transform into the same phases as the fixed ZnO nanorods, which validates the use of the methodology presented in this work.

However, stability diagrams for the Zn-S-P system indicate that Zn₃(PO₄)₂ should be a stable product under oxidizing conditions.[\(16\)](#) Le Bars *et al.*[\(47\)](#) showed that nano-ZnS is a major Zn species in raw liquid organic wastes, but only a minor species in solid and more processed organic-rich wastes. In this case, most of the Zn phases transformed to either amorphous Zn-phosphate or Zn adsorbed to ferrihydrite in composts. A similar tendency has been reported in the biosolids formation at a pilot WWTP,[\(15\)](#) where the ZnS transformed mostly to Zn₃(PO₄)₂ after the anaerobic digestion process. The formation of Zn₃(PO₄)₂ species after secondary and tertiary wastewater treatment process would have potential environmental implications since their eco-toxicity to algae/bacteria would inherently change.

The presence of humic acid, used as simplified primary sludge during the *in situ* incubation, also has an influence on the ZnO nanorod behavior, by affecting the mechanisms in which the organic molecules interact with particle surfaces.[\(48\)](#) Natural organic compounds (*i.e.*, humic/fulvic acids) in aqueous environments may be adsorbed onto nanomaterial surfaces,[\(49\)](#) generating bridges between the nanomaterials and the organic molecules *via* multiple functional groups. Suwannee River humic and fulvic acids have been reported to promote ZnO nanoparticle dissolution, which increases linearly with their concentration (from 0 to 40 mg C L⁻¹),[\(50\)](#) although inhibition of ZnO dissolution could result

from steric protection following humic acid adsorption.⁽⁵¹⁾ Furthermore, the number of ZnO nanorods per unit area on the initial template and their geometry are thought to play a role in subsequent ZnO morphology and speciation changes. This would explain why ZnO transformation to $\text{Zn}_3(\text{PO}_4)_2$ species was sterically limited in the first region analyzed after 3 h incubation (SM, [Figure 3c](#)) in comparison with the more accessible second region (SM, [Figure 3d](#)).

An ongoing ZnO dissolution was monitored by XFM during the *in situ* experiments, which was also revealed by the increasing Zn^{2+} concentration in the incubation solutions analyzed by ICP-MS ([Table 1](#)). This process was initially slow but then speeded up rapidly after 130–140 min, in some regions, leading to the complete disappearance of the Zn hotspots ([Video S1](#)). This fact added more heterogeneity to the ZnO transformation, since the more “exposed” nanorods may be dissolved after the 3 h incubation. This heterogeneity was also observed on the high-resolution SEM images acquired ([Figure 5](#)), where ZnO nanorods underwent different compositional and structural changes according to the steric impedance. Our results suggest that at longer time scales (24–48 h), we might expect to see a complete dissolution of all the ZnO nanorods, with some Zn being released into solution as Zn^{2+} and some precipitating/transforming as ZnS particles or core–shell structures.

The acidic pH conditions tested here (4.5) are expected to promote ZnO dissolution, by generating soluble ions including Zn^{2+} and $\text{Zn}(\text{OH})^+$.⁽⁵²⁾ Fast dissolution rates have been reported in different environments such as soils (within only 1 h)⁽⁵³⁾ and artificial lysosomal fluid at a similar pH⁽⁵⁴⁾ (within 24 h). As a consequence, thinner ZnO nanorods were observed after 1 h incubation ([Figure 5a](#)), while larger hexagon-shaped ZnO assemblies, with faceting that resembles the Wurtzite crystalline structure, were generated after 3 h ([Figure 5b](#)). Similar hexagon-shaped ZnO structures have been reported after electrodeposition of ZnO layers by Illy *et al.*,⁽⁵⁵⁾ who also indicated that acidic pH (below 4.1) can dramatically change the film thickness and the crystallographic orientation of the ZnO.

The methodology described in this work allowed us to recreate “as-synthesized” ZnO nanorod transformations *in situ*, obtaining significant information within their early stages (1–3 h) of exposure in an environmentally relevant aquatic medium. Differences in the chemistry of real sludges, such as varied pH and increased phosphate ion concentrations, will alter the composition and morphology of the transformed ZnO nanomaterials, for example, we would expect to detect more zinc phosphate daughter particles in a real sludge. Here, we show marked changes to the structure and chemistry of these nanomaterials incubated in humic acid solution, in an experimental cell. These results can be extrapolated to wastewater systems containing humic acid as organic matter content, providing insights about the fate and partitioning of Zn species in two-stage conventional wastewater treatment plants. With modifications, this test-bed can now be utilized to measure impacts of ZnO ENMs in a wide range of environmental scenarios, including real wastewater sludges.

Conclusions

Here, spatially resolved maps of ZnO nanomaterial transformation within a simulated environmental medium have been acquired dynamically. Our findings demonstrated: (i) ZnO nanorods partially transformed to ZnS species predominantly within *in situ* short-time incubations (1–3 h) in a simulated primary sludge medium; (ii) minor $\text{Zn}_3(\text{PO}_4)_2$ and Zn adsorbed

to Fe-oxyhydroxides were also measured in some nonsterically impeded regions; and (iii) the ongoing ZnO dissolution and the humic acid presence have an influence in the preferential transformation of the remaining ZnO nanorods.

Nanomaterials transformations take place rapidly, as highlighted here by the generation of ZnS species after only 1 h incubation, and these transformations depend on several factors (*i.e.*, pH, elemental composition, temperature, redox conditions, and organic matter content).

Therefore, developing cutting-edge spatially resolved speciation analyses in real time, such as the described 9-E methodology, will illuminate the extent of nanomaterial transformations within relevant aquatic environments at relevant length scales, from the individual particles to micrometric aggregates. This could provide crucial information about the underlying pathways both for environmental damage and (de)activation of engineered nanomaterials used in consumer products and the implications for their passage through natural environments. More generally, this *in situ* methodology is potentially a widely applicable and powerful means to characterize transformations of nanomaterials within a range of complex hydrated systems, such as natural waters or biological environments (*e.g.*, bacteria, algae, macroinvertebrates, *etc.*). The XFM could also be utilized to study the kinetics of nanomaterials transformations *in situ* in a range of related fields, such as nanomedicine and biomineralization. As nanomaterials formulations become more complex, the XFM technique would offer the possibility to characterize chemical transformations of individual nanoparticles within these mixtures and coupled behavior between the particles. When studying the structural and functional impacts of transformed ENMs on organisms, this information could, in the future, be used to generate mechanistically underpinned insight into predict ENM behavior, to refine guidelines for safe use, and to inform remediation strategies of ENMs.

Experimental Methods

Synthesis and Characterization of ZnO Nanorods

Reproducible ZnO nanorod-like arrays were synthesized on silicon nitride windows (2 × 2 mm membrane, 5 × 5 mm frame; 1000 nm thickness; Norcada) following the procedure described by Xie *et al.* [\(39\)](#) and Wang *et al.* [\(40\)](#) The experimental steps are described in [Figure S1](#) and summarized as follows: (1) a non-close-packed polystyrene monolayer (~400 nm diameter) was formed using a liquid interface-mediated method; (2) a seed layer deposition of ZnO was applied on the polystyrene monolayer; and (3) a hydrothermal growth of ZnO nanorod-like arrays was performed onto the seed layer. All chemicals were purchased from Sigma-Aldrich, as reagent grade quality or higher. Ultrapure water (18 MΩ cm⁻¹; Millipore Corp.) was used for all solutions and dilutions.

SEM images of the ZnO nanorod-like arrays were collected using a LEO Gemini 1525 field emission gun scanning electron microscope (FEG-SEM), equipped with a Genesis 4000 EDAX for the collection of EDX spectra of the samples. Samples were sputter coated with a ~5 nm carbon layer before the SEM-EDX analyses to increase the conductivity of the samples without interfering with the compositional determinations performed by EDX.

The surface chemistry of the ZnO nanorods was characterized by XPS. The spectra were recorded on a Thermo Scientific K-Alpha⁺ X-ray photoelectron spectrometer (XPS) system (base

pressure 2×10^{-9} mbar), incorporating a monochromated, microfocused Al K α X-ray source ($h\nu = 1486.6$ eV) and a 180° double focusing hemispherical analyzer with a 2D detector. The X-ray source was operated at 6 mA emission current and 12 kV anode bias. Data were collected using a $400 \times 400 \mu\text{m}^2$ X-ray spot and a pass energy of 200 eV for the survey and a 20 eV pass energy for core level and valence band spectra, respectively. A flood gun was used to minimize sample charging, and spectra were aligned assuming the C 1s core line to be at a binding energy of 285.0 eV. All data were analyzed using the Advantage software package (Thermo Scientific).

Synchrotron X-ray Analyses

XFM analyses were carried out on the Hard X-ray Nanoprobe at the I14 beamline (Diamond Light Source Ltd., Didcot, UK). X-rays from an undulator source are preshaped using a pair of horizontal mirrors onto a secondary source, with a Si(111) horizontally deflecting double-crystal monochromator providing monochromatic X-rays, selectable over the energy range 5–23 keV. The beam propagates to an external building (source–sample distance 186 m) where it is focused using preshaped KB mirrors. The focused X-ray beam size for this experiment was 150 nm. The sample is raster scanned (continuous scanning) through the X-ray focus, and the fluorescent X-rays collected by a four-element silicon drift detector (Rayspec) with a solid collection angle of 0.8 sr.

In Situ Incubation Experiments: X-ray Fluorescence Microscopy and ICP-MS

An “in-house” liquid cell was designed for the *in situ* synchrotron XFM analyses, with specifications designed to fit the size and geometry requirements of the I14 beamline at the Diamond Light Source synchrotron facility. This polyhedral aluminum cell ($15 \times 10 \times 6$ mm, $h \times w \times d$), with an open space of $4 \text{ mm} \times 4 \text{ mm}$ in the beam trajectory ([Figure S3a](#)), was mounted in the beamline stage and fixed with a magnetic bottom located at the end of the cylindrical base ([Figure S3b](#)). There, the silicon nitride window, with the ZnO nanorods attached, was placed in the middle of the experimental window, aligned within the path of the X-ray beam, and secured with two Kapton polyimide foils ($\sim 80 \mu\text{m}$ width). According to the geometry of the cell and in order not to overfill it, a volume of 200 μL of synthetic simulated medium was pipetted in for every *in situ* experiment performed ([Figure S3c](#)).

As a simplified simulated primary sludge, a humic acid solution of 10 mg L^{-1} was selected, presenting a reasonable organic matter concentration for the experimentation, as previously studied by Bian *et al.* ([20](#)) This humic acid stock solution was prepared by dissolving solid humic acid (Sigma-Aldrich) in water for 12 h under stirring and then filtering the solution through a $0.45 \mu\text{m}$ filter cartridge. ([49](#)) Subsequent acidification to pH 4.5 with acetic acid 0.1 M was carried out to simulate the pH conditions of the real primary sludge. The total organic carbon of the solution was quantified before performing the *in situ* experiments, accounting for 8.65 mg C L^{-1} . This parameter was used as an indicator of the organic matter present in the solution regardless of the extent of humic acid solubility, which might be limited. ([20](#))

After the addition of 200 μL of the simulated sludge solution in the *in situ* cell, XFM maps ($10 \times 10 \mu\text{m}$ or $15 \times 15 \mu\text{m}$ size, 100 ms acquisition time per pixel, 75 nm pixel-size) were acquired consecutively at 12 keV during the course of the 1 h and 3 h *in situ* experiments (with a 2 or 5 min dead-time between consecutive measurements, respectively). The acquired XFM maps

were postprocessed, in order to measure the intensity signal of: P K α , S K α , Cl K α , Ca K α , Ti K α , Mn K α , Fe K α , Cu K α , Zn K α , and Ag L α . All live maps were processed using Dawn software,⁽⁵⁶⁾ generating an individual image for all acquired conditions and reprocessed elements ($\times 10$). Each individual map was subsequently integrated using Matlab software, obtaining the average intensity of every element in the XFM map.

These two short-time scales (1 h and 3 h) were selected to evaluate the ZnO nanorod morphology and speciation within the initial stages of their release to aquatic media containing organic matter. To calculate the extent of the ZnO nanorod dissolution in the simulated sludge, the residual solution was quantified for aqueous Zn concentration by ICP-MS (PerkinElmer NexION 350D). Upon completion of the *in situ* experiments, the liquid phase was collected with precleaned syringes, diluted to 2 mL with ultrapure water acidified to 1% HNO₃ (trace-metal grade), and stored at 4 °C before analyses.

Speciation Analyses Evaluated by Multi-Energy Scans: Methodology

To obtain chemical and speciation information along the Zn K-edge, XFM image stacks were collected using different photon energies.⁽⁵⁷⁾ These images were acquired on the incident photon flux I_0 to compute reference corrected absorbance images, $-\ln(I/I_0)$. Subsequently, XANES spectra were analyzed pixel-by-pixel and compared to the relevant standard X-ray absorption edges, providing spatially resolved chemical information. Speciation maps (SM) were then calculated through fitting the absorption data from each pixel to the linear combination of the standard (LCS) spectra, as represented by the following (eq 1):

$LCS = w_1ZnO + w_2ZnS + w_3Zn_3(PO_4)_2 + w_4Zn - Fe(ox)$ (1) where the least-squares fit weights (w_i) were calculated per pixel (where $\sum w_i = 1$), thus a 2D plot of w_i represents the spatial distribution maps of Zn species across the particle, defined here as SM.

Therefore, a SM of the sample, representing the ratio between Zn species could be formed by plotting the different w_i maps (as seen in Figure 3). Furthermore, the percentage of each species can be quantified by first considering the absorbance intensity of the sample at an isosbestic energy point. The isosbestic point is the incident X-ray energy where the absorption of all Zn species is equal (E_9 in Figure S4). Hence, absorbance intensity variations across the imaged sample are proportional to the total amount of Zn present irrespective of speciation in accordance with the Beer–Lambert law: $absorbance = a(\lambda) \times t \times c$ (2) where $a(\lambda)$ is a wavelength-dependent absorptivity coefficient, t is the X-ray path length, and c is the component concentration.

In addition, quantification maps (QM) can be calculated, including the overall amount of each

species, in the image through the following calculation (eq 3):
$$QM\% = \frac{\sum_n (P_n \times I_n)}{\sum_n (I)} \times 100$$
 (3) where “ P ” is the proportion of Zn species between 0 and 1 in an individual pixel (n), and “ I ” its absorbance intensity at the isosbestic point).

By applying the same equation to all the relevant Zn species included in the calculation, the amount of Zn in an individual pixel was obtained. The percentage calculated with this equation is subsequently (pixel-by-pixel) normalized by the intensity value obtained in the isosbestic point (ninth energy, Figure S4), where the absorbance is related to the total amount of Zn present irrespective of speciation.⁽⁵⁷⁾ Hence, the numbering on the scale bars on the QM provides an estimation of the Zn species contribution.

XANES Spectra Acquisition and Data Treatment

Nine different images were collected along the Zn K-edge at nine-energies (9-E) as follows: (i) for background removal, three energies (9648.5, 9653, and 9658 eV) at Zn pre-edge were acquired; then (ii) five postedge energies (9669, 9670.5, 9674, 9675, and 9682 eV) were recorded to map different Zn phases; and (iii) a spectrum image was acquired at (9702 eV), defined as the isosbestic point, at which all data were normalized (E_0 in [Figure S4](#)). An example of absorption data obtained from individual pixels across the indicated stack of 9-E is shown in [Figure 1d](#), while all the 9-E image stacks obtained for the samples are included in the [Figure S7](#). The use of only nine absorption energies is a trade-off between accurate chemical analysis *versus* temporal resolution and X-ray dose (explained in [Section S5a](#), Supporting Information). In order to validate these results, complementary XANES maps were also performed by imaging at 135 different energies along the Zn K-edge ([Figure 1e](#)). There, the mean intensity of each individual image was plotted as a function of the energy, generating an individual XANES spectrum which could be analyzed by linear combination fitting using Zn-standards and according to the Zn species previously described in WWTP systems.[\(2,58\)](#) Therefore, bulk ZnO, nano ZnO (80–200 nm, US nano), ZnS, $\text{Zn}_3(\text{PO}_4)_2$, and Zn adsorbed to Fe oxy-hydroxides were used as standards for data analysis ([Figure S4](#)). The k -space and the Fourier-transform representations of the XANES spectra of both standards and the samples analyzed in the study are presented in [Figure S5](#) ([Section S5](#), Supporting Information). Linear combination fitting (LCF) of the XANES k -region: 3–5 Å⁻¹ was performed by Athena from Demeter software,[\(37\)](#) in order to focus in the distinctive features on this region. The background was subtracted by using a linear fit through the Zn pre-edge region and the autobk routine in Athena for the spline fit through the XANES region. The LCF of the Zn spectra was carried out starting from the best fit with one component, and the number of components n was increased as long as the normalized sum of the squared residuals ($\text{NSSR} = \sum(\text{data}_i - \text{fit}_i)^2 / \sum(\text{data}_i)^2$) of best $n + 1$ – component fit was at least 10% lower than the NSSR of the best n – component fit and if no component accounted for <5% of total Zn. More details about the methodology followed in this study are shown in the Supporting Information ([Section S5](#)), including an evaluation of the accuracy of the 9-E speciation analyses ([Section S5c](#)). Complementary XAS analyses were conducted on commercial smaller ZnO nanoparticles (10–30 nm size, US nano, 1000 mg L⁻¹ concentration) incubated for 3 h in simulated sludge solution (10 mg L⁻¹ humic acid adjusted to pH 4.5). These results are presented in [Figure S5](#) ([Section S4](#), Supporting Information).

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