

Isotopic Tracing of Engineered Nanomaterials in Environmental Samples

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Declaration of Originality

I declare that the content of this thesis is my own work unless otherwise stated, and that contributions to this thesis from others are fully and appropriately acknowledged and referenced.

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Abstract

The use of engineered nanomaterials (ENMs) in consumer products is becoming ever more prevalent due to their unique properties. As a consequence of their increasing use, the discharge of nanoparticles (NPs) to the environment is expected to rise rapidly in the following decades. Due to their novel material properties, the behaviour and fate of ENMs in natural compartments is unclear and there is insufficient knowledge regarding potential hazards which warrant further study. However, the study of ENM ecotoxicity is hampered by analytical challenges in resolving ENMs at environmentally relevant levels in complex natural samples.

Over the past decade, the method of stable isotope tracing has been established as a useful and efficient tool for the detection and quantification of ENMs at environmentally realistic exposure concentrations. The basis of the technique is the application of NPs, which are prepared from and hence labelled with an enriched stable isotope of a constituent element. Isotopic measurements can then be applied for sensitive and selective detection and quantification of the labelled material.

The work presented in this thesis covers different aspects of the stable isotope tracing methodology, as applied to Ag, ZnS and TiO₂ (nano-) materials. In detail, the thesis presents (i) a new analytical technique, which applies precise isotopic analyses by multiple collector inductively-coupled plasma mass spectrometry, for the detection of isotopically labelled Ag NPs in diverse environmental samples at high yields and with low associated Ag blanks; (ii) results of a multigenerational exposure study that shows the uptake of Ag NPs in gonad and liver tissue of zebrafish and the transgenerational maternal transfer of Ag NPs; (iii) a scalable synthesis of sub-micron sized ZnS particles at yields of 10-40%; and (iv) an in-depth evaluation of the stable isotope tracing technique, in support of future application to the detection of TiO₂ NPs.

Table of contents

	<i>Page</i>
List of figures.....	11
List of tables.....	18
Chapter 1 - Introduction.....	19
1.1. Background.....	20
1.2. Nanoecotoxicology – current knowledge.....	22
1.3. Stable isotope tracing.....	24
1.4. Thesis overview.....	28
Chapter 2 - Synthesis and characterization of sub-micron isotopically labelled zinc sulphide particles for tracing studies.....	30
Abstract.....	31
2.1. Introduction.....	32
2.2. Experimental.....	38
2.2.1. Chemicals and reagents.....	38
2.2.2. Synthesis.....	39
2.2.3. Characterization of particle size and crystal structure.....	41
2.2.3.1. Dynamic light scattering.....	41
2.2.3.2. Transmission electron microscopy.....	42
2.2.3.3. X-ray diffraction.....	42
2.3. Results and discussion.....	43
2.3.1. Size characterization of particles.....	43
2.3.1.1. DLS analysis.....	43
2.3.1.2. TEM analysis.....	45
2.3.2. Energy dispersive X-ray spectrometry.....	47

2.3.3. Crystallographic analysis.....	48
2.3.4. Synthesis yields.....	50
2.3.5. Use of ^{68}ZnS as stable isotope tracer.....	51
2.4. Conclusions.....	52
2.5. Acknowledgements and declaration.....	53
Chapter 3 - Robust and efficient analytical methods for stable isotope tracing of silver nanoparticles in complex environmental samples.....	54
Abstract.....	55
3.1. Introduction.....	56
3.2. Experimental.....	60
3.2.1 Samples.....	60
3.2.2. Materials and reagents.....	60
3.2.3. Synthesis and characterization of isotopically enriched Ag species.....	61
3.2.4. Sample preparation.....	62
3.2.4.1. Sample digestion.....	62
3.2.4.2. Ion exchange separation of Ag.....	63
3.2.5. Mass spectrometry.....	64
3.2.5.1. Instrumentation and protocols.....	64
3.2.5.2. Initial sample assessment.....	67
3.2.5.3. Preparation of sample solutions.....	68
3.2.5.4. Preparation of Ag standard solutions.....	68
3.2.5.5. Ag isotope measurements.....	69
3.2.6. Data reduction.....	71
3.3. Results and discussion.....	75
3.3.1. Analytical scenarios.....	75

3.3.2. Evaluation of analytical procedures.....	77
3.3.2.1. Sample preparation and separation procedure.....	77
3.3.2.2. Correction of instrumental memory.....	80
3.3.3. Results for standards solutions.....	84
3.3.3.1. Standard solutions with natural Ag isotope compositions.....	84
3.3.3.2. Standard solutions containing Ag-En.....	85
3.3.4. Results for samples.....	87
3.3.4.1. Samples with natural Ag isotope compositions.....	87
3.3.4.2. Analyses of natural samples that contain Ag-En.....	91
3.3.5. Application of the methods to samples from exposures.....	95
3.4. Conclusions.....	97
3.5. Acknowledgments and declaration.....	99
Chapter 4 - Multigenerational study of isotopically labelled Ag nanoparticles in Danio rerio – transgenerational effects of maternal exposure.....	100
Abstract.....	101
4.1. Introduction.....	102
4.2. Experimental.....	104
4.2.1. Synthesis and characterization of isotopically enriched Ag species.....	104
4.2.2. Multigenerational exposure of ¹⁰⁹ Ag nanoparticles to zebrafish.....	105
4.2.2.1. Flake preparation and dosing.....	105
4.2.2.2. Fish source, culture and husbandry.....	105
4.2.2.3. Sexing, weighing and measuring the zebrafish.....	106
4.2.2.4. Experimental set-up.....	107
4.2.2.5. Measuring egg output and fertilisation.....	108
4.2.2.6. Biological sampling.....	109

4.2.3. Isotopic analysis of samples.....	110
4.2.3.1. Materials and reagents.....	110
4.2.3.2. Sample digestion.....	111
4.2.3.3. Ion exchange separation of Ag from environmental matrix...	112
4.2.3.4. Mass spectrometry.....	112
4.2.3.4.1. Preparation of sample and standard solutions.....	112
4.2.3.4.2. Measurement instrumentation and protocols.....	113
4.2.3.4.3. Sample measurements and data reduction.....	114
4.2.3.5. Isotopic mass balance calculations.....	115
4.2.3.6. Statistical analysis.....	117
4.3. Results and discussion.....	118
4.3.1. Nanoparticle characterisation.....	118
4.3.2. Ag nanoparticle dissolution.....	118
4.3.3. Multigenerational exposure of enriched Ag nanoparticles to adult zebrafish.....	121
4.3.3.1. Mass difference of tissue samples.....	121
4.3.3.2. Zebrafish embryo samples (F1).....	122
4.3.3.3. Zebrafish tissue samples (F0)	123
4.3.3.4. Intergenerational trends (F0 & F1).....	126
4.3.3.5. RNA-sequencing (F1)	131
4.4. Conclusions.....	132
4.5. Acknowledgments and declaration.....	133
Chapter 5 - Development and evaluation of stable isotope tracing of TiO₂ nanoparticles.....	135
Abstract.....	136

5.1. Introduction.....	137
5.2. Tracing of TiO ₂ nanoparticles in environmental samples.....	140
5.2.1. Sample preparation.....	140
5.2.2. TiO ₂ nanoparticle tracing by Ti concentration measurements.....	143
5.2.3. TiO ₂ nanoparticle tracing by SP-ICP-MS.....	144
5.2.4. Stable isotope tracing of TiO ₂ nanoparticles – status quo.....	146
5.3. Experimental.....	147
5.3.1. Synthesis of TiO ₂ nanoparticles.....	147
5.3.2. Characterisation of TiO ₂ nanoparticles.....	148
5.3.3. Isotopic tracing of TiO ₂ NPs - modelling study.....	148
5.4. Results and discussion.....	151
5.4.1. Preliminary results for TiO ₂ synthesis route	151
5.4.2. Stable isotope tracing of TiO ₂ nanoparticles.....	152
5.4.2.1. Sample preparation and isobaric interferences.....	152
5.4.2.2. Stable isotope tracing of TiO ₂ nanoparticles based on intrinsic differences in Ti stable isotope compositions.....	154
5.4.3. Stable isotope tracing using isotopically labelled nanoparticles.....	156
5.4.3.1. Key considerations and issues.....	156
5.4.3.2. Instrumental scenarios.....	158
5.4.3.3. Choice of considered tracer isotopes and diagnostic isotope ratios.....	160
5.4.3.4 Modelling results.....	162
5.4.3.4.1. Detection sensitivity.....	162
5.4.3.4.2 Performance for determining proportion of spike present.....	166

5.4.4. Final evaluation.....	167
5.5. Conclusions.....	169
5.6 Acknowledgements and declaration.....	170
Chapter 6 - Summary and outlook.....	171
Bibliography.....	176
Appendices.....	200
Appendix 1: Additional information relating to synthesis of ZnS particles.....	200
Appendix 2: Isotopic and Ag mass fraction data obtained for samples in the study for Chapter 4.....	204

List of figures

	Page
1.1. Simplified schematic depicting the potential release pathway of ENMs during their lifetime. At almost any point during production, transportation, consumption and disposal, ENMs can enter the different compartments of the natural environment by way of point sources (landfills, WWTP) or non-point sources (wear and tear).	22
1.2. Isotopic composition of natural Ti, Zn and Ag as well as the isotope spikes ^{46}Ti , ^{68}Zn and ^{109}Ag .	25
1.3. Accumulation of an isotopic tracer in an organism (in this case ^{109}Ag) with the corresponding change in the diagnostic isotope ratio ($^{109}\text{Ag}/^{107}\text{Ag}$).	27
2.1. Schematic showing the process of ZnO NP release from consumer products into the environment and their subsequent transformation to ZnS during anaerobic digestion processes in wastewater treatment plants.	33
2.2. Examples of typical exposure concentrations reported for ZnO NPs in the literature (right) compared to natural Zn backgrounds (left) and predicted environmental concentrations for anthropogenic ZnO NPs released into soils (middle).	34
2.3. Isotopic compositions of natural Zn (red) and commercially available Zn enriched in the ^{68}Zn isotope (blue).	37
2.4. Evolution of DLS particle diameter for experiment Nat-2 (80°C reflux temperature) as a function of nucleation time. Panel A (left) shows the signal intensity for individual DLS measurements conducted at different times. Panel B (right) summarises the average particle diameters (red) and PDIs (black) vs. time. Both particle size and PDI continuously increase up to a nucleation time of 30 minutes. At 40 minutes the particles reach a size of 944 nm with a PDI of 0.1.	43
2.5. Three batches of ZnS particles (one containing natural Zn, two with enriched ^{68}Zn) were synthesised at 70°C temperature with a nucleation time of 10 minutes (Label-1, red) and 15 minutes (Nat-1 in black and Label-2 in blue). Shorter nucleation times produce smaller NPs of ~105 nm size whereas a longer nucleation time yields NPs of 147-167 nm size.	45
2.6. Measured particle size distributions (top) and representative TEM images (bottom) where (A) are natural ZnS NPs (Nat-1) and (B) and (C) are images of	46

isotopically enriched ^{68}ZnS (Label-1 and Label-2 respectively). Scale bars of TEM images are 0.5 μm . (A) Average particle size = 188.7 ± 7.7 nm (1 SD), $n = 189$; (B) average particle size = 162.0 ± 5.9 nm (1 SD), $n = 96$; (C) average particle size = 145.0 ± 4.8 nm (1 SD), $n = 369$.

2.7. EDS spectra of experiments Nat-A (black), Label-1 (red) and Label-2 (blue). 48
EDS analysis confirms the formation of ZnS and the absence of impurities in the sample solution. Peaks indicating the presence of carbon and copper are due to the use of a carbon coated Cu grid for sample mounting.

2.8. XRD patterns of natural ZnS NPs (black) and ZnS NPs enriched in ^{68}Zn (blue). 49
The peak patterns confirm formation of cubic ZnS (zinc blende) with no differences between ZnS and ^{68}ZnS .

3.1. Comparison of natural Ag levels (left of each panel), predicted environmental concentrations (PECs) from engineered Ag NPs (middle of each panel) and examples of Ag NP concentrations used in ecotoxicity studies (right of each panel) for (A) soils, 57
(B) waters and (C) biological organisms. For biological organisms, no PEC values are available in the literature and exposure concentrations are not applicable as they are typically applied through indirect exposure (e.g. dermally or orally). Typical exposure concentrations are 3 to 4 times higher than PECs in pristine waters and soils and 1 to 2 orders of magnitude higher than PECs in sludge-treated soils and waste water treatment plant effluents, which carry a higher burden of anthropogenic nano-waste.

3.2. Illustration of a regular analytical sequence with corresponding correction 70
procedures. Standard and sample analyses are preceded by a memory measurement to correct for memory effects. Two sample analyses are bracketed by the analysis of a standard solution, which is used to determine the corresponding standard correction (details in text).

3.3. Calibration line method that was employed to determine molar concentrations of 74
Ag present in the MC-ICP-MS sample solutions. The $^{109}\text{Ag}/^{105}\text{Pd}$ ratio of a mixed Ag-Pd standard solution is measured and together with the origin, this defines the slope of a correlation line in a plot of ^{109}Ag concentration versus $^{109}\text{Ag}/^{105}\text{Pd}$ ratio. The $^{109}\text{Ag}/^{105}\text{Pd}$ ratio determined for a sample that has the same Pd concentration as a sample can then be employed to determine the sample ^{109}Ag concentration.

- 3.4.** Change of the $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratio with the relative proportion of Ag-En present, as defined by the Ag-En/Ag-Tot ratio. Also shown is the uncertainty that arises for a calculated Ag-En/Ag-Tot ratio when the measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratio is uncertain to 0.2% (purple curve), 2% (green curve) and 20% (red curve). 76
- 3.5.** Results acquired in two subsequent measurement sessions. (A) Isotopic composition of the instrumental memory effect measured on wash solutions that were run in between sample analyses. (B) The ^{107}Ag and ^{109}Ag ion beam intensities of the environmental samples that were analysed in the measurement sessions. (C) Approximate relative contribution (in %) of the instrumental memory effect to the ^{107}Ag and ^{109}Ag ion beam signals measured for the samples. 81
- 3.6.** Relative deviation (in %) of measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratios from the reference value obtained in analyses of Ag standard solutions with a non-natural isotope composition and Ag concentrations of about 0.02 to 2 ng ml⁻¹. The plot compares the isotopic results that were obtained without use of a memory correction during data reduction (open symbols) and with addition of a memory correction (filled symbols). 83
- 3.7.** Relative deviation (in %) of measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratios for standard solutions of natural Ag from the mean result obtained for bracketing standard solutions with Ag concentrations from the reference value obtained in analyses of Ag standard solutions. The red symbols denote the results of individual analyses, which show no trend with changing Ag solution concentrations. The blue symbols denote the mean results obtained for multiple analyses of 5 ng ml⁻¹ and 30 ng ml⁻¹ Ag standard solutions, which served as the bracketing standard solutions for the other analyses. These mean values have a deviation that is identical to 0%, within error (see text for details). Error bars (often smaller than the symbol size) denote the 1 SE within-run error of the measurements. 85
- 3.8.** (A) Relative deviation and (B) absolute number of the relative deviation (both in %) of $^{109}\text{Ag}/^{107}\text{Ag}$ data obtained in individual analyses of Ag-En + Ag-Nat solutions at various concentrations from the $^{109}\text{Ag}/^{107}\text{Ag}$ reference value that was determined by runs at high Ag concentrations. Error bars (often smaller than the symbol size) denote the 1 SE within-run error of the measurements. 87
- 3.9.** (A) Relative deviation and (B) absolute number of the relative deviation (both in %) of $^{109}\text{Ag}/^{107}\text{Ag}$ data obtained in analyses of environmental samples with only Ag-Nat from the reference value of the natural $^{109}\text{Ag}/^{107}\text{Ag}$ ratio. Red symbols denote 89

results obtained for individual sample solutions (see Table 3.4). The blue symbol is the mean value obtained for analyses of several aliquots of a single Lufa 2.2 Soil digest (Soil Digest 1; Table 3.4). The black symbol is the mean value for multiple analyses of a 5 ng ml⁻¹ natural Ag standard solution. Error bars (often smaller than the symbol size) denote the 1 SD calculated from the results of multiple analyses (see Table 3.4).

3.10. Detection sensitivity for the detection of Ag-En for samples that have low (red line), medium (blue line) and high (green line) background contents of Ag-Nat, where the measurement errors are about 7%, 1% and 0.1%, respectively (see text for details). The results demonstrate that 1 pg of Ag-En can be detected in 10 pg, 100 pg and 1 ng of natural Ag. 91

3.11. Data obtained from the analyses of environmental samples that contain variable quantities of Ag-En to produce variable isotope compositions (Table 3.5). Panel (A) shows the ¹⁰⁹Ag/¹⁰⁷Ag data, (B) the Ag-En/Ag-Tot ratios and (C) the Ag-En mass fractions. 92

3.12. Data obtained from exposure samples that acted as control lines. Their natural isotope composition was confirmed to within an error of ±1% for samples containing ≥20 pg Ag. For Ag contents lower than 10 pg, ¹⁰⁹Ag/¹⁰⁷Ag ratios can be recovered to within ±5%. 96

3.13. Data from biological tissues sampled and analysed from a ¹⁰⁹Ag NPs exposure to zebrafish. Each data point represents a set of samples collected from separate zebrafish that were reared under identical conditions and each set of samples represents a different parameter (e.g. male/female, type of tissue). 97

4.1. Experimental set up of the multigenerational diet-borne exposure of zebrafish to casein-coated and citrated-coated ¹⁰⁹Ag NPs. Each treatment line and a control line were set up with six replicates in generation F0. Some of the embryos (F1) were reared to adulthood and used in a further exposure generation under the same conditions as in F0. Additionally, in F1 a further 3x4 aquariums were set up that contained offspring of fish that had been exposed to ¹⁰⁹Ag NPs in F0 but were not exposed in F1 (F1 Recovery generation, in red). 108

4.2. TEM micrographs of isotopically labelled (A) casein-coated ¹⁰⁹Ag NPs (2-4 nm) and (B) citrate-coated ¹⁰⁹Ag NPs (8-10 nm). 118

4.3. Molecular structure of citric acid (left) and α-casein (right). 120

- 4.4.** Mass of analysed liver and gonad samples grouped by sex and exposure generation. Error bars (not always visible) denote 1 SD. 121
- 4.5.** (A) Mass fraction of Ag-En in embryo samples collected in Weeks 1, 2 and 3 of the F1 generation exposure. Plotted are the mean values $\pm$ 1 SD for 6 samples, except Week 2 of cas-¹⁰⁹AgNP treatment (n = 4) and Weeks 2 and 3 for the cit-¹⁰⁹AgNP treatment (n = 5). There are no observable differences between embryos from the cas-¹⁰⁹AgNP treatment (green triangles) and the cit-¹⁰⁹AgNP treatment (black squares). 123
- 4.6.** Mass fraction of enriched Ag present in (A) liver and (B) gonad tissue of male (circles) and female (triangles) zebrafish from exposure generation F0 for treatments with casein-coated Ag NP (red) and citrate-coated Ag NP (white). Plotted are the mean values $\pm$ 1SD for livers and gonad samples of five female and six male zebrafish. (Note the different scale of the y-axes.) 124
- 4.7.** Absolute deviation of measured ¹⁰⁹Ag/¹⁰⁷Ag ratios from the natural reference value of 0.929 for (A) livers and (B) gonads of unexposed F1 generation zebrafish. Samples are from zebrafish that are descendants of fish that were either pre-exposed to cas-¹⁰⁹AgNPs (F1 Recovery Cas-¹⁰⁹AgNPs) or cit-¹⁰⁹AgNPs (F1 Recovery Cit-¹⁰⁹AgNPs) in F0 or acted as control group in F0 (clean media). Plotted are mean values $\pm$ 1 SD for two to four individual samples. Liver and gonad tissues that were collected from either treatment group and the control line in F1 show no significant difference in isotopic signature. (Note the different scale of the y-axes.) 126
- 4.8.** Mass fraction of Ag-En in embryo samples of the F1 (black and green) and F2 (yellow and purple) generation. Plotted are mean values $\pm$ 1 SD for 4–6 individual samples of each treatment group and week, except for cit-¹⁰⁹AgNP in F1 Week 2 with n = 3. Whilst Ag-En was detected in all F1 samples, Ag-En was not detected in embryos from Week 1 of the F2 exposure (left). The overall uptake trends are similar in Weeks 2 and 3 for either exposure generation (middle and right). 128
- 4.9.** Comparison of the Ag-En contents of livers for male (circles) and female (triangles) zebrafish after exposure to cit-¹⁰⁹AgNPs (right) and cas-¹⁰⁹AgNPs (left) in F0 (red and white) and F1 (black and blue). Plotted are the mean values $\pm$ 1SD for 4 to 6 individuals of each group. 129
- 4.10.** Comparison of the Ag-En contents of gonads for male (circles) and female (triangles) zebrafish after exposure to cit-¹⁰⁹AgNPs (right) and cas-¹⁰⁹AgNPs (left) in 130

F0 (red and white) and F1 (black and blue). Plotted are the mean values ± 1 SD for 5–6 individuals per group for F0 and 5–10 individuals per group for F1.

4.11. Cumulative viable egg output for exposure generation F0 (A) and F1 (B). The decrease in egg output observed in F1 is statistically significant within the 95% confidence interval for cis-¹⁰⁹AgNP and cas-¹⁰⁹AgNP treatment group. (Note different scale of the y-axes in the panels.)

5.1. Comparison of natural Ti levels (left of each panel), predicted environmental concentrations (PECs) from engineered TiO₂ NPs (middle of each panel) and examples of TiO₂ NP concentrations used in ecotoxicity studies (right of each panel) for (A) soils, (B) waters and (C) biological organisms. For biological organisms, PEC values are currently not available in the literature and exposure concentrations are not applicable as they are typically applied through indirect exposure.

5.2. The five stable isotopes of Ti and their natural abundance (black). Also shown are the isotopic abundances of different enriched Ti (Ti-En) isotopes that were considered in this study - ⁴⁶Ti-En in red, ⁴⁷Ti-En in green, ⁴⁸Ti-En in blue and ⁵⁰Ti-En in yellow.

5.3. TEM images of the synthesised TiO₂ NPs at different magnifications that show dense clusters of spherical particles.

5.4. Natural stable isotope variations for Ti in terrestrial geological materials, reported as $\delta^{49/47}\text{Ti}$ (black) and $\delta^{49/46}\text{Ti}$ (red). The samples show an overall isotopic variability of nearly 2‰.

5.5. Calculated detection sensitivities, which are defined as the smallest amount of engineered Ti that can be detected against the natural Ti background. The calculations assume that (i) engineered TiO₂ NPs have an intrinsic isotopic signature that differs from natural Ti by 2‰ (black line) and 10‰ (red line) and (ii) an analytical uncertainty of $\pm 0.05\%$ for the MC-ICP-MS measurements.

5.6. Detection sensitivities offered by different enriched Ti isotope labels for the analytical scenarios of Case 1 to 5 (Table 5.3). The detection sensitivity is defined as the minimum amount of enriched Ti that can be resolved against a given natural Ti background. The black (top), red (middle) and green (bottom) lines represent the use of isotopically enriched ⁴⁸Ti, ⁴⁷Ti and ⁵⁰Ti, respectively. No data are shown for ⁴⁷Ti/⁴⁹Ti as the results are essential identical to those obtained for ⁴⁷Ti/⁴⁸Ti.

5.7. Error propagation in the quantification of the (Ti-En/Ti-Total) ratio when 168
variable levels of enriched Ti are present. The black (top), red (middle) and green
(bottom) lines represent the use of isotopically enriched ^{48}Ti , ^{47}Ti and ^{50}Ti ,
respectively, for the five analytical scenarios (Cases 1 to 5) and corresponding
analytical uncertainties of 0.01%, 0.1%, 1%, 5% and 25%. No data are shown for
 $^{47}\text{Ti}/^{49}\text{Ti}$, as the results are essentially identical to those obtained for $^{47}\text{Ti}/^{48}\text{Ti}$.

List of tables

	Page
1.1: Estimated worldwide production quantities for six ENMs based on a company survey published by Piccinno et al.	21
2.1: Summary of the synthesised ZnS and ⁶⁸ ZnS batched together with associated yield and size characterization.	40
2.2: ZnS reference values and measured Bragg's angles for natural ZnS and ⁶⁸ ZnS with corresponding normalised spectral intensities and crystal planes.	50
3.1: Anion exchange chromatography for the separation of Ag from environmental samples.	64
3.2: Operating conditions of the Nu Plasma MC-ICP-MS and CETAC Aridus II desolvating nebulizer system.	65
3.3: Faraday cup arrangement for Ag isotope measurements with Nu Plasma MC-ICP-MS including all monitored elements and potential spectral interferences.	66
3.4: Ag isotope data obtained for environmental samples that contain only natural Ag and a pure standard solution.	90
3.5: Ag isotope and concentration data for environmental samples spiked with isotopically enriched ¹⁰⁹ Ag at varying levels and their influence on measurement trueness and precision.	93
4.1: Summary of main exposure and sampling parameters of the exposure.	110
4.2: Results of analysed water sample collected at the end of week 3 of the exposure.	119
5.1: Information on natural abundances, enrichment factors and cost of Ti isotopes.	139
5.2: Isobaric and spectral interferences for the five stable isotopes of titanium.	142
5.3: Overview of different analytical scenarios considered for modelling the stable isotope tracing of TiO ₂ .	159
5.4: Results of modelling study.	163

Chapter 1

Introduction

1.1. Background

In 2011, the European Commission defined a nanomaterial (NM) as a material that is comprised of at least 50% particles with at least one external dimension in the size range of 1-100 nm, whereby the particles may be in an unbound state or exist as aggregates or agglomerates [1]. This definition encompasses (i) materials that are produced naturally, such as primary particles in atmospheric aerosols; (ii) materials from anthropogenic sources, for example incidental emissions due to human activity, such as release of nanoparticulate carbon in soot from the burning of fossil fuels; and (iii) engineered NMs (ENMs) that are intentionally manufactured for a certain purpose.

Due to their small size, nanoparticles (NPs) have higher surface to volume ratios than their bulk counterparts, which results in unique physico-chemical properties. As such, the European Commission recommends that a material may be categorised as a NM if its specific surface to volume ratio exceeds $60 \text{ m}^2 \text{ cm}^{-3}$ even if the above stated criteria are otherwise not fulfilled [1]. As experimental and production capabilities have improved over the last decades, so has the ability to synthesise and mass-produce NMs to precise specifications in order to meet specialist applications. Increasingly, the use of ENMs is shifting from niche purposes, such as scientific laboratory equipment, towards a broad market for consumer products in the areas of personal hygiene, fitness related products, cosmetics or within home appliances.

The Nanodatabase (<http://nanodb.dk/>), a database developed and established by the Technical University of Denmark, the Danish Ecological Council and the Danish Consumer Council, attempts to build and maintain an online inventory of consumer products containing nanoparticles available on the European market. There are currently (January 2018) over 3030 products listed in the Nanodatabase, up from 2200 in 2015 [2]. Companies do not have to disclose if their products contain ENMs, which implies that any database that relies on publicly

available information is likely to vastly underestimate the actual number of ENMs circulating on the market. Estimates assume stark increases in the output of ENMs with projected rises from 2000 tons produced in 2004 to 58,000 tons between 2011-2020 [3].

Although products that contain ENMs often do not list very much detail regarding their nano-components, an estimate based on a company survey by Piccinno et al. [4] lists SiO₂, TiO₂, and ZnO as the three ENMs with the highest median worldwide production outputs of 5500, 3000 and 550 tons per year, respectively. In contrast, Ag NPs are produced at much lower capacities of 55 tons year⁻¹ (Table 1.1) [4].

Table 1.1: Estimated worldwide production quantities for six ENMs based on a company survey published by Piccinno et al. [4].

ENM	25 th /50 th percentile [t/year]	Median [t/year]
SiO ₂	55-55,000	5,500
TiO ₂	500-5,500	3,000
ZnO	55-550	550
Carbon nanotubes	55-550	300
Ag	5.5-550	55
CeO _x	5.5-550	55
All values are reproduced from a study published by Piccinno et al. [4].		

Various studies that investigate the use of ENMs in consumer products agree that one of the main application fields of nano-TiO₂, nano-ZnO and nano-Ag are cosmetics, including sunscreens and deodorants, as well as cleaning and medical purposes for the latter due the bactericidal properties of Ag NPs and paints for nano-TiO₂ [2, 4, 5]. The waste streams

associated with these types of products do not currently allow for the separate removal or recycling of ENMs, thus enabling their transport into the natural environment by way of wastewater treatment plants (WWTPs) or landfills (point sources) (Fig. 1.1). Additionally, it is likely that there will be accidental release of ENMs during production and transport as well as ENM release from (unintentional) non-point sources, due to normal use of the products (e.g. release into natural waters from sunscreens) (Fig. 1.1) [6]. Once they are in the environment, NPs will aggregate, dissolve and/or undergo transformation reactions. However, as they behave differently than bulk materials, it is necessary to investigate the specific behaviour, fate and transport mechanisms of ENMs once they are released into the environment in order to be able to rationally evaluate potential risks associated with these materials.

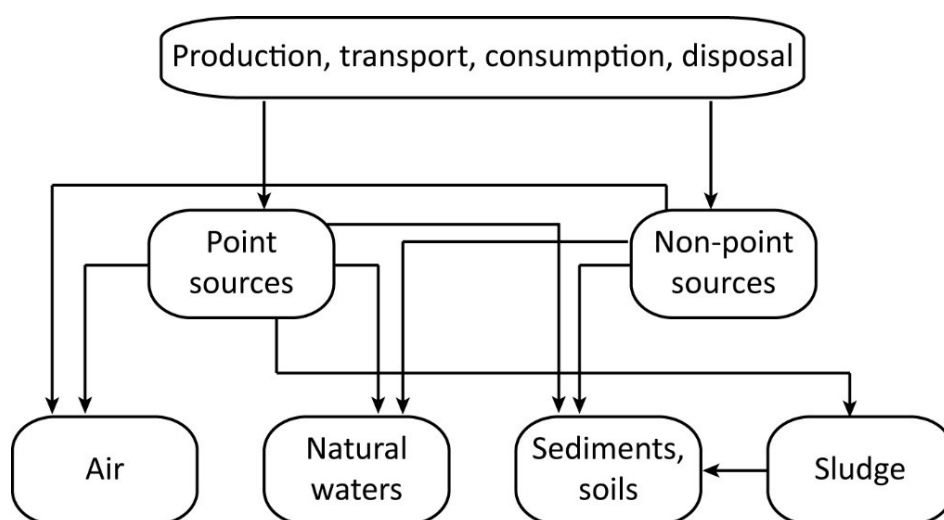


Fig. 1.1. Simplified schematic depicting the potential release pathway of ENMs during their lifetime. At almost any point during production, transportation, consumption and disposal, ENMs can enter the different compartments of the natural environment by way of point sources (landfills, WWTP) or non-point sources (wear and tear).

1.2. Nanoecotoxicology – current knowledge

Life-cycle assessments (LCAs) of products containing ENMs have concluded that these materials enter the environment at varying endpoints [7]. Over the past decade, the rising

interest in the fate, behaviour and transport mechanisms of ENMs has resulted in an increase in the number of published studies that investigate various types of NPs (e.g., Ag, ZnO, TiO₂, CeO₂, Fe_xO_x, carbon nanotubes). Such work has demonstrated that ENMs are taken up by a wide variety of cell types in human and animal tissues and that they have the ability to cross cell membranes, although this behaviour is dependent on material properties such as particle size. It has furthermore shown that NPs often facilitate the production of reactive oxygen species with resulting negative effects for the organisms [6]. However, observations often focus on toxicity endpoints and exposure, effect and/or susceptibility biomarkers (BMs). In detail, exposure BMs relate the exposure of a specific substance to its level of exposure, effect BMs monitor early health effects, whilst disease and susceptibility BMs inform on the ability of an organism to react or respond to a specific substance [8]. There is, however, a lack of comprehensive and detailed analyses of NP fate, behaviour and transport of ENMs within organisms [6].

Furthermore, studies tend to underreport NP properties and there continues to be a lack of information regarding how ENMs are actually used in consumer products on the market [2, 9]. In addition, almost all exposure studies focus on ENM concentrations that vastly exceed the predicted environmental concentrations (PECs) due to difficulties in distinguishing ENMs from natural elemental background levels and to induce biological reactions which allow, for example, the determination of toxicity endpoints [10]. In detail, such investigations typically apply the NPs at concentrations that exceed the natural background values by two to three orders of magnitude so that the ENM fraction can be reliably resolved from its natural elemental counterpart and to induce clearly measurable biological effects. For example, Zn levels in natural and urban soils typically range from 10 - 500 mg kg⁻¹ [11-13], whereas the burden from anthropogenic ZnO NPs in pristine soils is predicted to be at levels of about 3 – 30 mg kg⁻¹ [14]. In contrast, ecotoxicological studies often require the introduction of

100 - 1000 mg kg⁻¹ of nano-ZnO [15-17]. This results in questions of knowledge transferability as it is uncertain if the information on nano-specific toxicity and behaviour gained from these studies can be transferred to realistic systems where ENMs are present in much lower quantities [18].

In order to fully assess the possible impacts of ENMs on human and environmental health and safety, it is therefore necessary to study the behaviour and effects of ENMs at low and realistic concentrations in natural systems. To overcome the analytical challenges posed by distinguishing between ENMs and natural elemental backgrounds within such realistic exposure systems, Gulson and Wong proposed applying the method of stable isotope tracing to the detection and analysis of ENMs [19, 20].

1.3. Stable isotope tracing

Originally proposed in 2006 by Gulson and Wong [20], the efficacy and reliability of stable isotope tracing using enriched isotopic materials has since been shown in a variety of pioneering studies by a handful of research groups, which worked with ⁶⁸ZnO, ⁶⁷ZnO, ¹⁰⁷Ag, ¹⁰⁹Ag, ⁶⁵Cu and ⁴⁷TiO₂ NPs [21-28]. The work conducted in this area so far has shown that stable isotope tracing, particularly when coupled with multiple collector ICP-MS (MC-ICP-MS), is an extremely sensitive detection method that allows the detection of ENMs even at parts per trillion concentrations, and which produces results characterised by excellent accuracy and precision [22, 24, 29-31].

The stable isotope tracing procedure for ENMs encompasses four distinct steps:

- 1) Isotopically labelled NPs are synthesised from precursor material that is artificially enriched in a single isotope of the target element. This material is often referred to as an isotopic tracer or spike. Whereas NP synthesis procedures usually focus on optimising properties such

as size and shape, for the production of enriched NPs special attention has to be paid to the synthesis yield. Due to the high cost of enriched materials (e.g., ~US-\$2 per mg for ^{68}Zn enriched to >99%) reliably high yields or the ability to recycle the reaction products for further synthesis are an imperative condition. In addition, it is desirable that the synthesis can be scaled to produce different amounts of NM to specifically meet the demands of environmental studies. As yields are almost always unreported in the literature, it is unfortunately routinely necessary to not just conduct an extensive literature review but to also test and adapt different published procedures to identify, confirm and optimise a suitable NP synthesis protocol. Prior to use, the NPs are characterised and compared to their counterparts synthesised from natural material to show that their behaviour and properties are indistinguishable from each other. When NPs are prepared from an isotopically enriched precursor material, they intrinsically feature a distinct, non-natural isotope signature, which can then be traced with high sensitivity and selectivity when introduced to a natural system (Fig. 1.2)

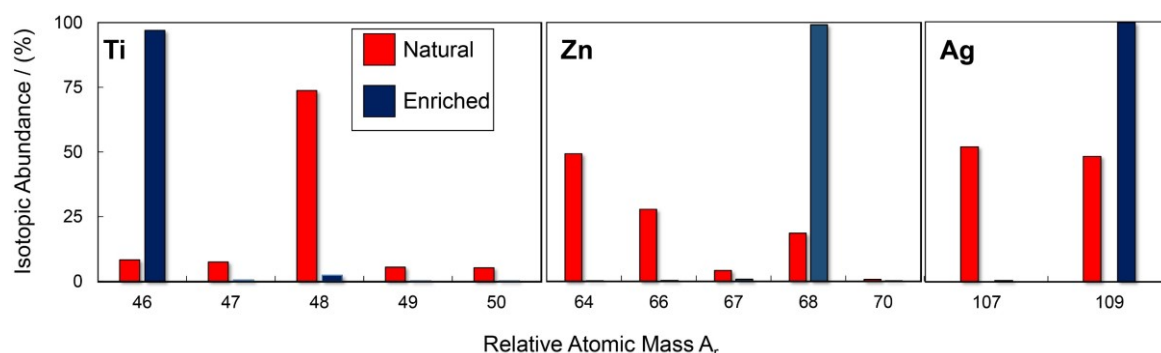


Fig. 1.2. Isotopic composition of natural Ti, Zn and Ag as well as the isotope spikes ^{46}Ti , ^{68}Zn and ^{109}Ag .

2) The stable isotope tracer is employed in an exposure study where it is introduced into an experimental system, which typically aims to reproduce key features of natural systems. Such studies can employ various natural compartments (e.g., waters, soils, sewage sludge, urine, etc.) and the artificially enriched NM can be introduced at various points (e.g., food, spiked into sludge, endermic), allowing for versatile experimental set-ups. Most importantly,

the ENMs can be introduced to the system at low and realistic concentrations that match the known PECs, and this renders the results of such investigations environmentally relevant. Both exposed and control samples can be analysed in the same manner by measuring suitable diagnostic isotopic ratios instead of the elemental concentrations.

3) The most sensitive detection of the non-natural isotope composition and the most precise quantification of the amount of labelled material present is afforded if the isotopic analyses of the samples are carried out at high precision. Such high precision isotopic analyses are most readily carried out by MC-ICP-MS. However, in order to utilise the superior precision and accuracy that is provided by this technique, the element of interest must be separated from the sample matrix. For many elements, this is most conveniently achieved by ion exchange chromatography, as a broad range of literature on the separation of numerous elements from complex natural matrices is available. As environmental exposures routinely produce large sample numbers (>100) it can be useful to invest time into the development of specifically targeted ion exchange procedures, which optimise the overall sample throughput and streamline the time-intensive laboratory work. Although such fundamental method development work requires a significant initial investment of time, this is likely to pay off later with the time that is saved during the processing of the many samples that are produced in environmental exposure studies.

4) Finally, the purified elemental solutions obtained from the ion exchange separation are analysed by MC-ICP-MS for the precise determination of the diagnostic isotope ratios. The amount of isotopic label present in a sample (mass and/or moles) can then be calculated based on the observed diagnostic isotopic ratio. For example, if Ag NPs, prepared from and hence labelled with isotopically enriched ^{109}Ag , are present in a sample, this would generate a non-natural $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratio. In detail, the deviation of the isotope ratio from the natural reference value is proportional to the amount of isotopic spike present in the sample.

The principle of how the diagnostic isotope ratio changes with an increasing proportion of isotopic tracer present is illustrated for the example of mixing between natural Ag and a ^{109}Ag spike in Fig. 1.3. Natural Ag (~48.2% ^{109}Ag abundance) and a pure ^{109}Ag spike (99.7% ^{109}Ag abundance) have associated $^{109}\text{Ag}/^{107}\text{Ag}$ ratios of 0.929 and 323.33, respectively (Fig. 1.3). The introduction of an enriched Ag tracer thereby both increases the overall Ag content of a sample and changes the isotope composition of the total Ag present, which can be quantified through measurement of the diagnostic $^{109}\text{Ag}/^{107}\text{Ag}$ ratio. For example, an increase of the total Ag concentration [Ag] in a sample by 10%, from the addition of the ^{109}Ag spike, results in a $^{109}\text{Ag}/^{107}\text{Ag}$ ratio of 1.121 compared to the initial, natural reference value of 0.929. This is a direct effect of the corresponding increase in ^{109}Ag present in the sample relative to ^{107}Ag , which results from the mixing of the highly enriched ^{109}Ag spike with natural Ag. Importantly, these principles also allow the precise quantification of the absolute and relative amounts of isotopic spike present in a sample, using ‘isotope dilution’ mass balance equations.

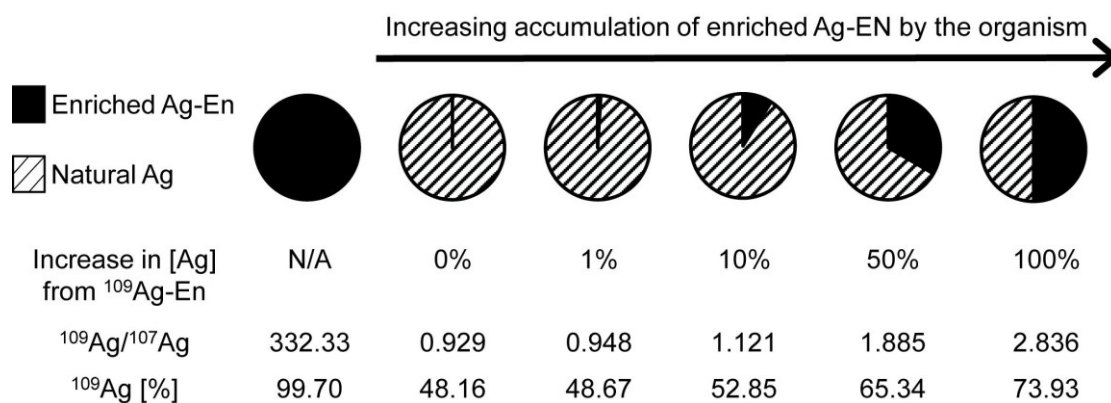


Fig. 1.3. Accumulation principle of an isotopic tracer in an organism (in this case ^{109}Ag) with the corresponding change in the diagnostic isotope ratio ($^{109}\text{Ag}/^{107}\text{Ag}$).

Isotope fractionation is a common process in nature and results in biological samples often displaying isotope ratios that are not identical to laboratory reference standards. Such fractionations, however, are typically very small for elements other than H, C, O, N and S. Considering the elements that are of interest for stable isotope tracing of ENMs, including Si, Ti, Fe, Cu, Zn, Se, Cd and Ce, natural variations in isotope ratios generally do not exceed $\pm 2\text{‰}$

[32-34]. The introduction of even small amounts of isotopic spike, on the other hand, produces large isotopic variability that often exceeds $\pm 100\%$ and hence renders natural isotope fractionations essentially negligible.

1.4. Thesis overview

This thesis covers all aspects of the application of the stable isotope tracing technique in conjunction with detection by MC-ICP-MS. A particular focus is thereby the tracing and application of Ag NPs and the preparation of isotopically labelled ZnS and TiO₂ NPs. The objective of this work is thereby to further develop the method of stable isotope tracing for the study of ENMs by expanding its applicability to further types of ENMs (Ag, TiO₂ and ZnS) and to show its specific advantages for the study of large, multi-generational exposure systems.

Chapter 2 presents a protocol for the synthesis of sub-micron ZnS particles, which has been adapted and optimized for the preparation of isotopically labelled material. Such particles are of interest to trace the fate of ZnS phases, which are formed in WWTPs from effluents that carry Zn and ZnO NPs. Particle size, shape and crystallographic structure of the ZnS particles were characterised both for labelled and unlabelled material. In addition, the synthesis yields were evaluated and are reported in terms of cost to produce specific amounts of labelled ZnS. The new protocol allows reliable, small scale production of labelled ZnS at yields that afford cost-effective synthesis of the material.

A novel analytical method that enables measurement of $^{109}\text{Ag}/^{107}\text{Ag}$ by MC-ICP-MS after Ag separation using a simple one-step ion exchange chromatography protocol is presented in Chapter 3. The new separation procedure allows for vastly improved sample throughput and was tested using a variety of natural materials, including soils and biological tissues that had both natural and non-natural Ag isotope composition. The validation measurements show that

the technique allows reliable $^{109}\text{Ag}/^{107}\text{Ag}$ and Ag concentration measurements of samples even for Ag contents of less than 50 pg. As such, the new methodology now allows the technique of stable isotope tracing combined with MC-ICP-MS to be applied for the detection of isotopically labelled Ag NPs. This is significant because the application of engineered Ag NPs in consumer products has risen considerably in recent years [4].

Chapter 4 present the results of collaborative research with the Universities of Exeter and Birmingham. In this study, zebrafish were exposed to two types of ^{109}Ag NPs through their diet and different types of tissue (livers, gonads), embryos and water samples were analysed. Environmentally relevant low dosing concentrations were employed and the exposures were conducted over three generations. Importantly, this is the first time that adult zebrafish were exposed to Ag NPs in a diet-borne, multigenerational exposure.

Chapter 5 present preliminary work on the development of a synthesis protocol for TiO_2 NP that is tailored to the production of isotopically labelled material. Importantly, the work identified a new small-scale, high yield synthesis method for the production of TiO_2 NPs. In addition, the chapter provides a detailed evaluation of the potential of stable isotope tracing for the analysis of TiO_2 NPs in the environment. A model was developed that allows calculation of detection sensitivities for a variety of analytical and sampling scenarios and the analytical possibilities of stable isotope tracing are presented for different enriched Ti isotopes.

Chapter 2

Synthesis and characterization of sub-micron isotopically labelled zinc sulphide particles for tracing studies

Abstract

Zinc sulphide (ZnS) is a product of transformation reactions of anthropogenically released ZnO NPs and is formed when these particles undergo anaerobic digestion in wastewater treatment plants (WWTPs). Their behaviour in the environment is currently not well understood and novel tracing methods are required in order to study their environmental fate at realistically low concentrations. Stable isotope tracing is well suited for this purpose as it allows tracing materials in complex natural samples from exposures at high sensitivity and accuracy.

In this work, a method for the synthesis of sub-micron ZnS particles of sizes between 100 and 200 nm diameter, dependent upon nucleation time and temperature, is presented. Particles were produced both using an isotopically natural Zn source as well as Zn enriched in the ^{68}Zn isotope (>99%) in order to demonstrate that isotopically modified materials exhibit the same basic physico-chemical properties and synthesis behaviour as their counterparts of natural isotopic composition. The particles were characterised using DLS and TEM imaging as well as XRD and EDS to obtain crystallographic and compositional information which confirmed the formation of cubic ZnS (sphalerite, zinc blende). The synthesis yield was 9-20% for smaller NPs (<150 nm) and up to 40% for larger NPs (~190 nm) that were produced via long nucleation at room temperature. The presented synthesis allows stable isotope tracing to be used to analysis the behaviour, fate and transport mechanisms of ZnS particles upon their release into the natural environment.

2.1. Introduction

The field of nanotechnology has undergone rapid growth in recent decades and as a result the production and use of engineered nanomaterials (ENMs) in consumer products has seen stark increases globally. The rise of ENM proliferation is associated with the spread of ENMs in the natural environment where their behaviour and accumulation in organisms is not yet well understood. Environmental health and safety research is increasingly focused on gaining an understanding of the most important sources of anthropogenic release of ENMs, their release pathways into the biosphere as well as their behaviour, fate and transport mechanisms once they have reached environmental compartments such as natural waters and agricultural soils.

One of the compounds of interest is zinc sulphide (ZnS), which finds use as a photocatalyst and, more importantly, has been shown to be a transformation product of zinc oxide nanoparticles (NPs) that are released into sewage sludge through waste water treatment plants (WWTPs) (Fig. 2.1) [35-38]. Zinc oxide NPs (ZnO NPs) are widely used in products such as cosmetics or sunscreen due to their optical properties and are one of the top five most widely manufactured ENMs [4, 39]. Due to the type of products that ZnO NPs are used for, the most important release pathway into the environment is through the sewage system into WWTPs where they may accumulate in sewage sludge (Fig. 2.1). The chemical environment in WWTP sewage sludge is complex with manifolds of organic and inorganic compounds as well as bacteria present. Chemical transformations are further stimulated by elevated temperatures and an aqueous environment, which facilitate material exchange within the sludge, whereby various parameters, such as time, temperature, redox potential and pH can impact the nature and kinetics of the chemical process. Whilst it is therefore difficult to predict the behaviour of NPs within WWTPs, a number of studies, particularly for ZnO, Ag and

CeO₂ NPs, have obtained systematic results that enable useful predictions for studies of the environmental fate and impact of ENMs [36-38, 40-43].

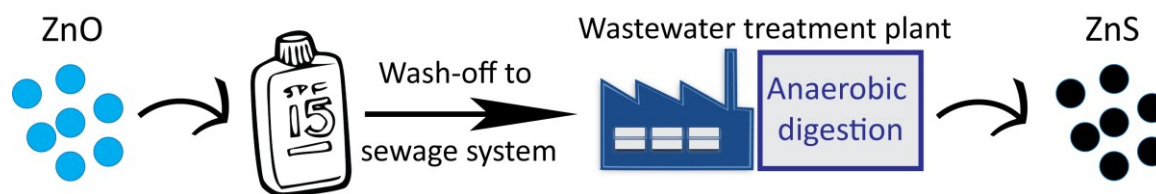


Fig. 2.1. Schematic showing the process of ZnO NP release from consumer products into the environment and their subsequent transformation to ZnS during anaerobic digestion processes in wastewater treatment plants.

Two recent investigations are particularly relevant for ZnO NPs. Lombi et al. [38] added ZnO NPs of 30-40 nm size to a mixture which mimicked the composition of sludge commonly used to feed anaerobic digesters in WWTP. The mixture was left to age for 30 days in a bioreactor at anaerobic conditions and then analysed using X-ray absorption near edge structure (XANES) measurements. These analyses revealed that 90% of the initial ZnO NPs had undergone transformation to ZnS particles [38]. A 2014 study by Ma et al. [37] used XANES to determine the fate of ZnO NPs (30 ± 10 nm) in a pilot WWTP, which simulated the actual release pathway of the NPs through the sewage system. After 15 days of anaerobic digestion they determined that the admixed ZnO NPs had undergone complete transformation to $\text{Zn}_3(\text{PO}_4)_2$, Zn-Fe oxide and hydroxide phases, and ZnS (23-38%) [37]. While neither of these studies investigated the produced species further, another study found that Zn speciation in sewage sludge results in ZnS nanocrystals of 3-8 nm size that form aggregates of up to 200 nm size [44].

The dried biosolids, which are produced from sewage sludge, are commonly used as fertilisers on farmland. Given that ZnS of sub-micron size is the main product formed in WWTPs from released anthropogenic ZnO NPs, it is important to study the behaviour and fate of such ZnS particles in natural systems. In a recent study, Boxall et al. [14] determined the

mean predicted environmental concentrations (PECs) of various ENMs for three scenarios covering 10%, 50% and 100% market penetration of consumer products with ENMs. For these three scenarios, they predict levels of ZnO NPs in the UK of 3-32 mg kg⁻¹ for soils, 2-22 mg kg⁻¹ for sludge and 76-760 µg l⁻¹ for natural waters [14]. Assuming that, as found by Ma et al. [37], about a quarter of ZnO NPs from these sources undergo transformation to ZnS, this would result in PECs of 0.6-5.4 mg kg⁻¹ for ZnS in sludge-treated soils.

Zn is an abundant and ubiquitous element in the environment, with natural concentrations in unpolluted soils and sediments commonly ranging from a few mg kg⁻¹ to a few hundred mg kg⁻¹ (Fig. 2.2) [11]. Anthropogenic emissions affect the presence of Zn in the environment, especially in urban areas where significant amounts of Zn are released by industrial activities. Luo et al. [12] summarised the findings of various studies that investigated

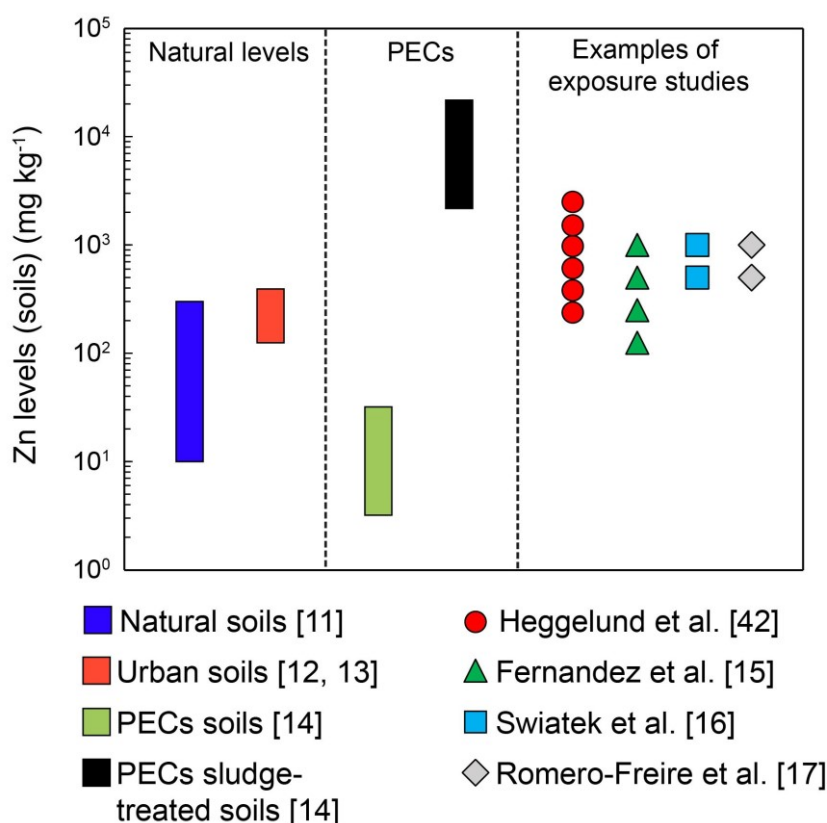


Fig. 2.2. Examples of typical exposure concentrations reported for ZnO NPs in the literature (right) compared to natural Zn backgrounds (left) and predicted environmental concentrations for anthropogenic ZnO NPs released into soils (middle).

Zn pollution and reported an average Zn concentration of 130 mg kg^{-1} for the soils of 34 European cities, while Cannon and Horton reported a mean Zn concentration of 397 mg kg^{-1} for urban soils from Chicago, Illinois (USA) [12, 13]. These and other studies highlight that the Zn background found in various environmental compartments is up to two to three orders of magnitude higher than the PECs of ZnS released from anthropogenic ZnO NP (Fig. 2.2). This large concentration contrast and the highly variable Zn background complicate the study of ZnO and ZnS NP fate upon release into the natural environment.

Most investigations of ZnO NPs and ZnS ecotoxicology employ Zn concentration measurements to quantify the presence of the NPs in samples. Such analyses, however, require the addition of ENMs at levels far exceeding the PECs to reliably resolve the presence of the engineered NPs from the corresponding natural elemental background (Fig. 2.2) [15-17, 45]. Ecotoxicity studies carried out at such high and unrealistic NP concentrations may potentially induce effects that would not be observable at lower and environmentally relevant exposure concentrations. It is therefore necessary to overcome the limitations of conventional concentration measurements to allow the study of ENMs at or close to the PECs.

The most common methods that have been employed for the study of ENMs at low concentrations against high natural backgrounds are fluorescent labelling [46-48], labelling with a radioactive isotope [49, 50] and stable isotope labelling. The first technique involves coating an ENM with a fluorescent dye prior to an exposure and analysing the samples using fluorescence microscopy or spectroscopy [46-48]. However, the application of a fluorescent coating can influence the behaviour of ENMs by affecting their surface chemistry, which could have unintended consequences for the ecosystem that the particles are introduced to. Additionally, it cannot easily be monitored if the fluorescent coating sticks to the NPs throughout the exposure in complex environmental systems where numerous chemical,

biological and physical processes are affecting the NPs. Furthermore, such a coating does not allow the tracing of the particles (or their products) once they undergo dissolution [51].

Radioactive tracers overcome the problems associated with fluorescent coatings, as they introduce an intrinsic rather than extrinsic property to the produced ENM – radioactivity. Radiolabelled NPs can be produced through activation in a nuclear reactor or by direct introduction of a radionuclide through in-diffusion at elevated temperatures [49, 50]. However, radioactive tracers can only be used in combination with expensive specialist equipment and within specific health and safety frameworks that require extensive training of personnel involved in the experiment. They also require a radioactive isotope of suitable half-life for the element of interest and put specific time constraints on the exposure experiment. This is due to the fact that the NPs have to be used in an exposure and the resulting samples have to be analysed before radioactive decay has reduced the amount of radiolabel present to below the detection limit.

A different labelling technique, first proposed by Gulson and Wang, is stable isotope tracing which allows overcoming the limitations of other tracing methods and has successfully been applied to a range of ENMs in pioneering exposure studies [20-24, 26, 52]. Considering all elements with at least one stable isotope, approximately 80% consist of two or more isotopes and the isotopic compositions of nearly all of these elements remain fixed within a fairly narrow window (<10‰) in the natural environment [30]. A material with an artificially altered isotopic composition can therefore be used to introduce disturbances to the isotopic signature of a system. Enriched stable isotopes are commercially available in bulk form for almost all elements as well as some of the most common composite ENMs such as ZnO. They can be purchased and used to synthesise NPs with a distinct isotopic composition that can be introduced into an exposure system where the isotopically labelled NPs behave identical to the NPs with natural isotopic composition. The diagnostic isotope ratio of samples resulting from

the exposure can then be measured to trace the movement of the NPs through the system and therefore allow conclusions about pathways and behaviours of a material. Such isotopic analyses provide a detection sensitivity and selectivity for the produced isotopically labelled species that is superior to that achievable with conventional concentration measurements [53].

In the case of Zn, any of the five naturally occurring stable Zn isotopes is commercially available in its enriched form. For more abundant isotopes such as ^{68}Zn essentially isotopically pure material (> 99%) can be purchased for use as a precursor to synthesise ZnO or ZnS NPs (Fig. 2.3). Larner et al. [30] conducted a detailed study on the use of Zn as a stable isotope tracer where they evaluated the choice of isotope label through modelling of an array of diagnostic isotope ratios and tracer isotopes. They show that using the enriched forms of the naturally least abundant isotopes (^{67}Zn or ^{70}Zn) coupled with analysis via multiple-collector ICP-MS (MC-ICP-MS) results in the highest detection sensitivity but is also associated with the highest cost of purchasing the enriched material. Larner et al. [30] further show that for typical applications the use of enriched ^{68}Zn and monitoring of the $^{68}\text{Zn}/^{66}\text{Zn}$ ratio provides sufficient detection sensitivity and allows utilisation of the superior accuracy and precision of MC-ICP-MS [30].

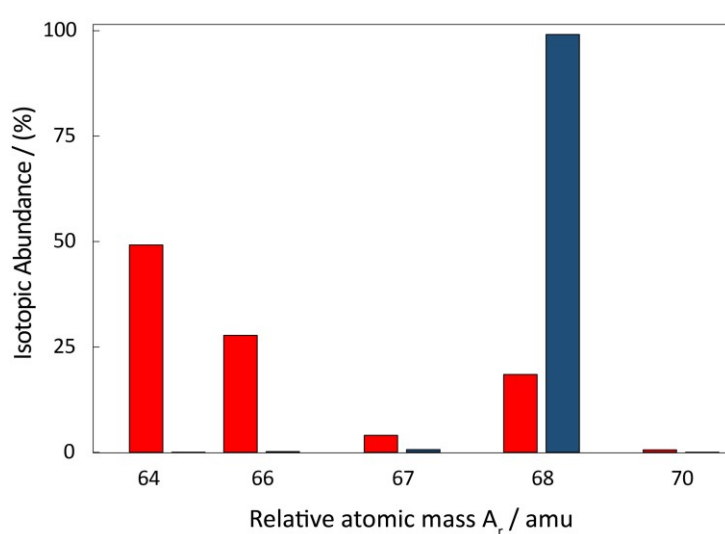


Fig. 2.3. Isotopic compositions of natural Zn (red) and commercially available Zn enriched in the ^{68}Zn isotope (blue).

However, existing NP synthesis protocols often lack the detail and documentation required for their application to synthesise stable isotope tracers. Due to the relatively high price of commercially available enriched stable isotopes (~US-\$4 per mg for ^{68}Zn compared to ~US-\$0.02 per mg for natural Zn) the methods need to focus on reliability, suitability for small-scale synthesis and high yields, whereby the latter are usually not reported in the literature where focus is put on properties such as shape and size [24].

In this study, the literature was carefully reviewed and procedures for the synthesis of sub-micron sized ZnS were tested, adapted and evaluated for their suitability according to the aims outlined above with the objective of developing a reliable small-scale synthesis of ZnS particles for use in isotopic labelling studies. Both natural ZnS and labelled ^{68}ZnS were synthesised to establish that the introduction of a stable isotope label does not produce particles that differ in their physico-chemical properties from their counterparts with natural isotope composition, which is essential to validate findings from ENM exposure studies using isotopically modified NPs.

2.2. Experimental

2.2.1. Chemicals and reagents

Powdered zinc metal with natural isotopic composition was purchased from Aldrich. Zinc metal enriched to 99.09% in ^{68}Zn was obtained from Isoflex, USA. Thioacetamide (TAA) (reagent grade) was purchased from Sigma-Aldrich. Water of >18.2 M Ω cm quality (Millipore, UK) was used throughout for experiments. AnalaR grade 15.3 mol l $^{-1}$ nitric acid purified by sub-boiling distillation in a quartz still was used to prepare all required dilutions of HNO $_3$. Reagent grade acetone and ethanol were purchased from VWR Chemicals.

2.2.2. Synthesis

All experimental work was performed in the MAGIC Laboratories at Imperial College London, UK. The synthesis protocol was developed from a procedure originally published by Celikkaya et al. [54] and is based on the precipitation of ZnS from $\text{Zn}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$ through addition of a sulphur source. Modifications and adjustments to the protocol were introduced to allow for the small desired amounts to be synthesised. The synthesis was repeated several times to produce both unlabelled ZnS with a natural Zn isotope composition and labelled ^{68}ZnS , which will be denoted as Nat-1, Nat-2, etc. and Label-1, Label-2, etc. All experiments, the associated synthesis conditions and characterisation results are summarised in Table 2.1.

Zinc nitrate was prepared by dissolution of 82 mg metallic Zn powder (1.25 mmol) in 8 ml 4 mol l⁻¹ HNO₃ in a Savillex® Teflon beaker and subsequent evaporation of unreacted nitric acid to dryness. MQ water (10 ml) was added to the beaker and the suspension placed in an ultrasonic bath for 20 seconds to disperse the $\text{Zn}(\text{NO}_3)_2$ flakes. The zinc nitrate suspension was transferred to a round-bottom flask and a further 10 ml of MQ water and 2.5 ml of 0.1 mol l⁻¹ HNO₃ were added under stirring. Nitric acid was added to adjust the final solution pH to 2, which leads to the complete dissolution of $\text{Zn}(\text{NO}_3)_2$ and the formation of a transparent solution. Solid TAA was added (0.75 g, 0.01 mol) to the solution and the total volume adjusted to 25 ml with MQ water. The beaker was then submerged in a pre-heated silicone oil bath and the reaction solution was refluxed at 70°C or 80°C depending on the target particle size. The reaction was stopped at the onset of particle nucleation, which is indicated by a white-blueish tint forming and quickly deepening in the previously transparent solution. Onset of nucleation occurred within 30-40 minutes after the round-bottom flask was transferred to the silicone oil bath. Upon removal of the flask from the heat source, the solution was quenched in an ice bath.

Table 2.1: Summary of the synthesised ZnS and ⁶⁸ZnS batched together with associated yield and size characterization.

	Synthesis			DLS		TEM			Yield
	Nucleation time	Reflux temperature [°C]	Synthesis date	Z-size average ± 1 SD [nm]	PDI ^b ± 1 SD	Diameter ± 1 SD [nm]	CV ^a	Particle number n ^{\$}	Yield [%]
Nat-1	15 min	70	2017-02-20	146.9 ± 1.6	0.16 ± 0.02	188.7 ± 7.7	0.04	189	15
Nat-2	40 min	80	2017-02-16	944 ± 36	0.06 ± 0.09	N/A [*]	N/A [*]	N/A [*]	N/A [*]
Nat-3	15 min	70	2017-06-28	N/A [*]	N/A [*]	N/A [*]	N/A [*]	N/A [*]	20
Label-1	10 min	70	2017-02-22	104.7 ± 0.3	0.09 ± 0.02	162.0 ± 5.9	0.04	96	8
Label-2	15 min	70	2017-02-27	167.3 ± 0.3	0.14 ± 0.04	145.0 ± 4.8	0.03	369	9
Label-3	72 h [#]	70	2017-02-27	189.7 ± 0.3	0.18 ± 0.02	N/A [*]	N/A [*]	N/A [*]	40

^bPolydispersity index. ^aCoefficient of variance = Standard deviation/TEM diameter. [#]Nucleated for 72 hours at room temperature whereas all other experiments were nucleated at the reflux temperature given in this table. ^{\$}For each experiment, several images were recorded during one TEM session. This number reflects the amount of distinct particles that were imaged, counted and added to a histogram analysis that was used to determine the average TEM size of an experiment. *: Data or information not available.

Apart from experiment Label-3, all suspensions were centrifuged and washed on the same day (Table 2.1). In contrast, the Label-3 suspension was kept at room temperature for 72 hours and further nucleation of particles was observed. In order to study nucleation and determine the optimal endpoint for the reaction, aliquots were taken at different time points throughout the synthesis by pipetting 1 ml fractions from the round-bottom flask, transferring them to Eppendorf centrifuge tubes and quenching in an ice bath.

The final suspensions were centrifuged for 45 minutes at 4000 RPM in a Thermo Scientific Heraeus Megafuge and the aqueous fractions containing excess TAA were pipetted off. The produced ZnS was washed once with MQ and twice with acetone before being placed in a ventilated oven to dry for >12 hours at 40°C to yield a white powder. Synthesis yields were assessed by weighing the final dried products using a microbalance (Mettler Toledo AX205).

2.2.3. Characterization of particle size and crystal structure

2.2.3.1. Dynamic light scattering

Dynamic light scattering (DLS) was performed at the Department of Bioengineering at Imperial College London. The Z-size average (hydrodynamic diameter) and polydispersity index (PDI) of the ZnS particles were determined at 25°C by DLS using a Malvern Zetasizer Nano range instrument (Malvern Instruments Ltd. Malvern, UK). For this, 1 ml aliquots of the samples were sonicated for 1-2 minutes prior to analysis and analysed in three cycles, each with 15 individual measurements to account for sedimentation. All reported hydrodynamic diameters and PDI values represent the average of the three separate measurement cycles with their associated errors reported as one standard deviation (± 1 SD). The Z-averages were calculated using the instrument software.

2.2.3.2. Transmission electron microscopy

For transmission electron microscopy (TEM) analysis, a few drops of sample suspension prior to drying were diluted with ethanol and a drop of this suspension was deposited onto a Formvar/carbon coated Cu-grid and left to dry for ten minutes. TEM images were recorded at the Department of Materials at Imperial College London. TEM micrographs were obtained from at least three areas on each grid at varying magnifications using a JEOL STEM/TEM 2100Plus (Jeol Ltd.) equipped with an Oxford Instruments ultra-thin window energy dispersive X-ray spectrometer (EDS) to allow for elemental analysis as well as microscopic capabilities. The particle diameters were calculated from particle areas determined from the micrographs using ImageJ software.

2.2.3.3. X-ray diffraction

The X-ray diffraction (XRD) analyses of all samples were conducted at the Natural History Museum in London, UK. For analysis, at least 10 mg of dry powder were mixed with acetone. A drop of acetone-sample suspension was placed onto a flat sapphire substrate (zero background holder) and following drying the sample was analysed using an Enraf-Nonius diffractometer (PDS120). Measurements were conducted in reflection mode with a fixed tube-sample-detector geometry at an angle of 6.1° between incident X-ray beam and sample. The Enraf-Nonius diffractometer has a copper source, a germanium 111 primary monochromator and an INEL 120° curved position sensitive detector with the tube operating at 40 kV/35 mA. The slits after the monochromator were set to 0.24×5 mm to confine the X-ray beam to pure copper K α_1 radiation. The sample was rotated during the measurements to increase the number of crystallites in the X-ray beam and Y_2O_3 was used as external standard

to calibrate the angular linearity of the detector. Sample data was fitted to reference data obtained from the PDF-2 database from the International Centre for Diffraction Data (ICDD).

2.3. Results and discussion

2.3.1. Size characterization of particles

2.3.1.1. DLS analysis

In order to synthesise ZnS particles of a desired size, the effect of nucleation time on particle size was investigated using DLS analyses. In experiment Nat-2 (Table 2.1) particles were synthesised at 80°C, nucleated for 40 minutes and suspension aliquots were collected in ten minute intervals during nucleation. Right at the nucleation point, the particles were smallest with an average hydrodynamic diameter of 213 ± 5 nm ($t = 0$ min, Fig. 2.4).

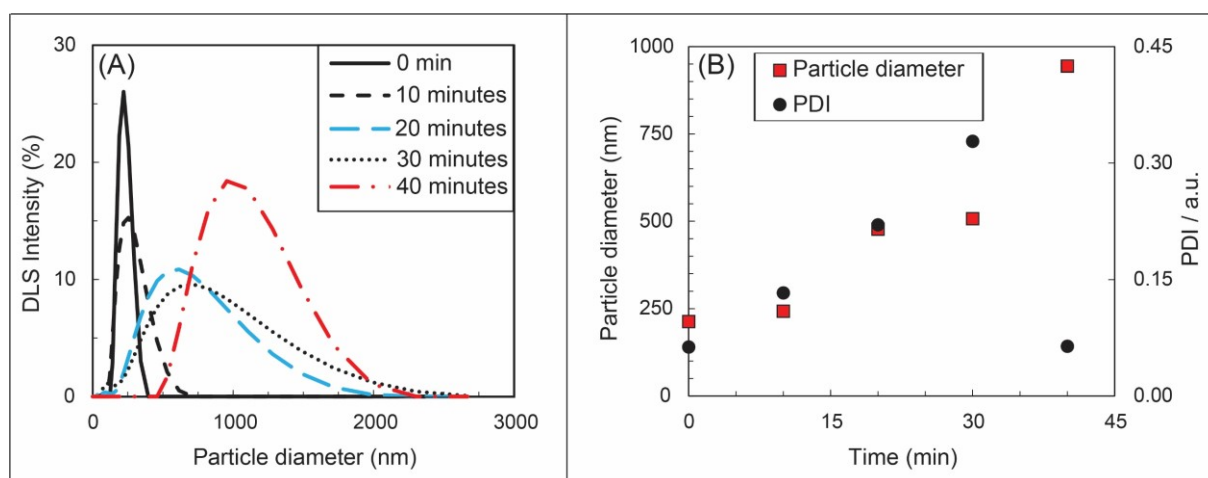


Fig. 2.4. Evolution of DLS particle diameter for experiment Nat-2 (80°C reflux temperature) as a function of nucleation time. Panel A (left) shows the signal intensity for individual DLS measurements conducted at different times. Panel B (right) summarises the average particle diameters (red) and PDIs (black) vs. time. Both particle size and PDI continuously increase up to a nucleation time of 30 minutes. At 40 minutes the particles reach a size of 944 nm with a PDI of 0.1.

As shown in Fig. 2.4, the particle size then continuously increases with nucleation time until the particles reach a size of 944 ± 36 nm after 40 minutes. In Fig. 2.4A the complete

intensity weighed size distributions of the individual aliquots are shown, whereas Fig. 2.4B plots the average particle diameter (weighted as Z-size average) and PDI. At 0 minutes the particles have a narrow size distribution and low associated PDI of 0.06 ± 0.02 . At 80°C, an increased nucleation time increases the polydispersity of the particles (Fig. 2.4A) and the PDI reaches a maximum of 0.33 ± 0.01 after 30 minutes. After 40 minutes nucleation time, the PDI drops back to 0.06 ± 0.09 indicating a highly monodisperse suspension with particles at an average size of 944 nm.

In order to reduce overall particle size, the synthesis temperature was lowered by 10°C to 70°C in the remaining experiments, which produced smaller particles (Table 2.1). DLS analysis showed the lowest overall average diameter of 105 ± 1 nm coupled with high monodispersity ($\text{PDI} = 0.09 \pm 0.02$) for particle batch Label-1. Larger and slightly more polydisperse particles of 147 ± 2 nm ($\text{PDI} = 0.16 \pm 0.02$) and 167 ± 1 nm ($\text{PDI} = 0.14 \pm 0.04$) were found for experiments Nat-1 and Label-2 (Fig. 2.5). These two particle suspensions were synthesised with nucleation times of 15 minutes whereas the NPs of batch Label-1 were nucleated for only 10 minutes. This indicates that the nucleation time vs. particle size relationship described might also be valid for reactions at lower temperature, although this would need to be confirmed by further analyses. Enriched ^{68}ZnS particles (Label-2, 167 ± 1 nm) deviate in their size by 14% from natural ZnS NPs (Nat-1, 147 ± 2 nm) synthesised at the same conditions. This minor difference is probably due to accumulated minor variations in experimental conditions such as temperature, heating gradient and the time of sampling. Finally, a particle batch was allowed to react at room temperature for a further 72 hours after nucleating at 70°C for 15 minutes, which yielded particles of 190 ± 1 nm size (Label-3) and a PDI of 0.18 ± 0.02 .

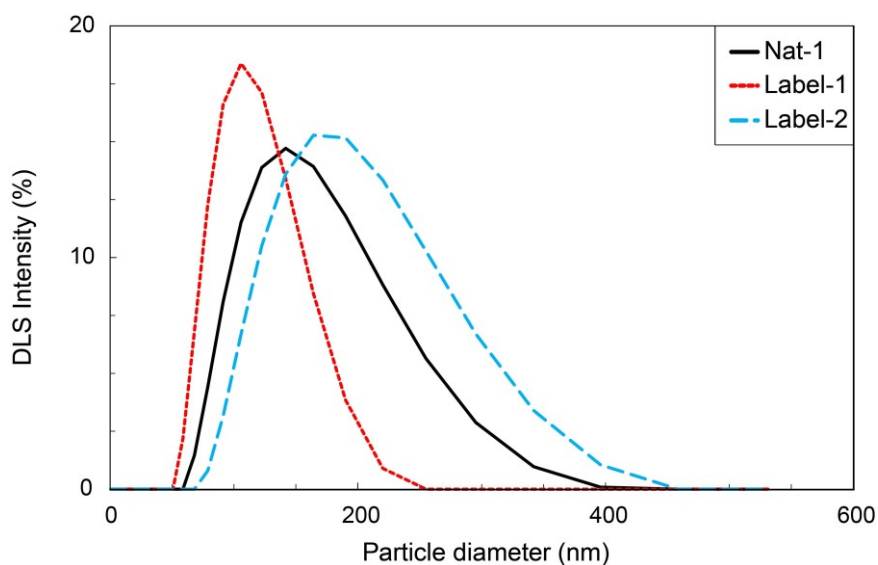


Fig. 2.5. Three batches of ZnS particles (one containing natural Zn, two with enriched ^{68}Zn) were synthesised at 70°C temperature with a nucleation time of 10 minutes (Label-1, red) and 15 minutes (Nat-1 in black and Label-2 in blue). Shorter nucleation times produce smaller NPs of ~ 105 nm size whereas a longer nucleation time yields NPs of 147-167 nm size.

In accord with the law of Arrhenius the nucleation process is slowed down considerably by decreasing the reaction temperature. Allowing particles to nucleate at room rather than at elevated temperature might therefore offer the option of producing NPs of a more targeted size. Whilst NP batches that were produced using longer nucleation periods at higher temperatures (70 - 80°C) were characterised by relatively high polydispersity, nucleation at room temperature appears to result in more monodisperse suspensions.

2.3.1.2. TEM analysis

Particles from three experiments were imaged by TEM and analysed with EDS (Nat-1, Label-1 and Label-2). Representative TEM micrographs are shown in Fig. 2.6 alongside histograms compiled by evaluating particles from all images collected for each experiment. The synthesised ZnS NPs are spherical in shape and are solely found as agglomerates rather than as discrete particles [55]. It is possible that the particles aggregated during the drying

process on the TEM grid, however, this would likely leave some discrete particles on the grid, but none were found in this study. It is therefore more probable that the particles imaged by TEM are representative of their form in suspension, such that they already aggregate during the nucleation or the work-up process.

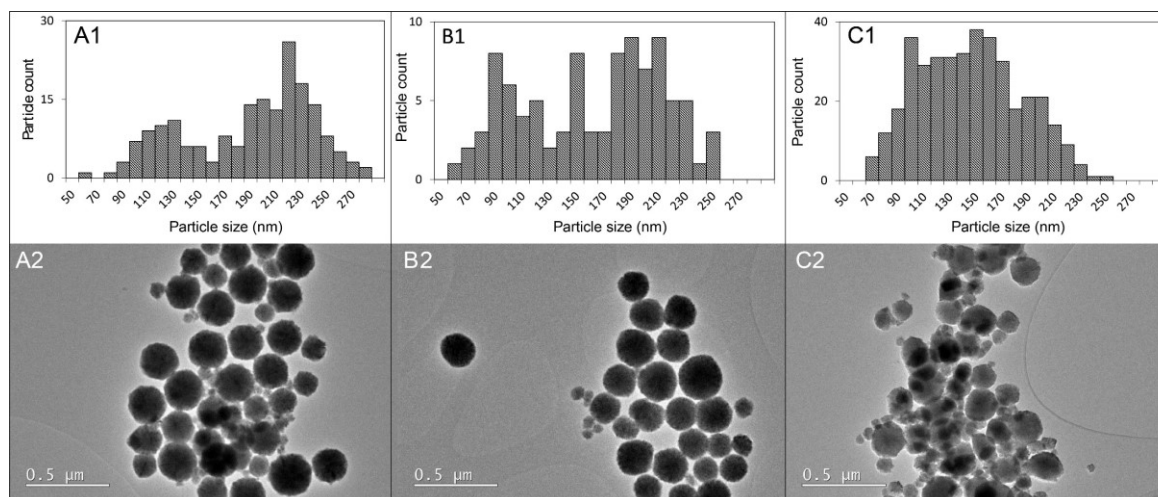


Fig. 2.6. Measured particle size distributions (top) and representative TEM images (bottom) where (A) are natural ZnS NPs (Nat-1) and (B) and (C) are images of isotopically enriched ^{68}ZnS (Label-1 and Label-2 respectively). Scale bars of TEM images are $0.5\ \mu\text{m}$. (A) Average particle size = $188.7 \pm 7.7\ \text{nm}$ (1 SD), $n = 189$; (B) average particle size = $162.0 \pm 5.9\ \text{nm}$ (1 SD), $n = 96$; (C) average particle size = $145.0 \pm 4.8\ \text{nm}$ (1 SD), $n = 369$.

TEM data for experiments Label-1, Label-2 and Nat-1 show particles with sizes of $162 \pm 6\ \text{nm}$, $145 \pm 5\ \text{nm}$ and $189 \pm 8\ \text{nm}$ respectively. The observed variability in size (1 SD) corresponds to a relative standard deviation of between 3 and 4% for all experiments, so that the particles can be classified as nearly monodisperse, which is in good agreement with the DLS results (Table 2.1) [56].

It is noticeable that the particles of experiments Nat-1 and Label-1 do not follow the generally observed trend, whereby the hydrodynamic diameters determined by DLS are larger than the particle sizes determined by TEM [57]. At particle sizes of $189 \pm 8\ \text{nm}$ (Nat-1) and $145 \pm 5\ \text{nm}$ (Label-2), the TEM sizes are 22% and 35% larger, respectively, than the DLS

measurements of the same samples (Table 2.1). The DLS measurement protocol might be responsible for this discrepancy. The DLS analyses involve a 90 second equilibration period and during this time large particles could settle at the bottom of the DLS cuvette, thereby biasing the results towards smaller particles. Alternatively, larger particles are easier to distinguish in TEM image analyses and might cover smaller particles in dense suspensions, and this can distort the TEM results slightly towards larger particles. It is also possible that an insufficient number of particles were imaged and included in the TEM size analysis for experiments Nat-1 and Label-1, such that only the TEM data for experiment Label-2, which follows the expected trend, includes a relevant amount of particles ($n = 369$).

Compared to the original protocol by Celikayya and Akinc, the particles produced in this study are smaller in diameter. Celikayya and Akinc used scanning electron microscopy (SEM) for their particle analysis and using the same experimental conditions (70°C reflux temperature, 10-15 minutes nucleation time) produced spherical particles of 200-250 nm size [54]. The observed differences might be caused by the difference in reaction volume employed here. Although the reagent concentrations are identical, this work decreased all reagent volumes by a factor of ten, so that the amount of Zn metal required and the quantity of ZnS produced are better matched to the requirements of environmental exposure studies, that aim to employ realistic ZnS concentrations.

2.3.2. Energy dispersive X-ray spectrometry

All particle batches that were imaged in TEM, namely Nat-1, Label-1 and Label-2, were also analysed using EDS to verify the composition of the produced material. All spectra confirm the prominent presence of Zn and S through the detection of the signature $L\alpha$ and $K\alpha$ Zn peaks at ~ 1.01 keV and ~ 8.63 keV and the $K\alpha$ peak of S at ~ 2.31 keV (Fig. 2.7). Additional

peaks in the EDS analysis from the presence of Cu and C are due to the use of a carbon coated Cu-grid to mount the samples in the microscope. The excellent agreement between the spectra corroborates that there are no differences in particle composition induced by using enriched ^{68}Zn compared to natural Zn. The EDS results furthermore confirm that the experiments produced pure ZnS particles with no significant by-products.

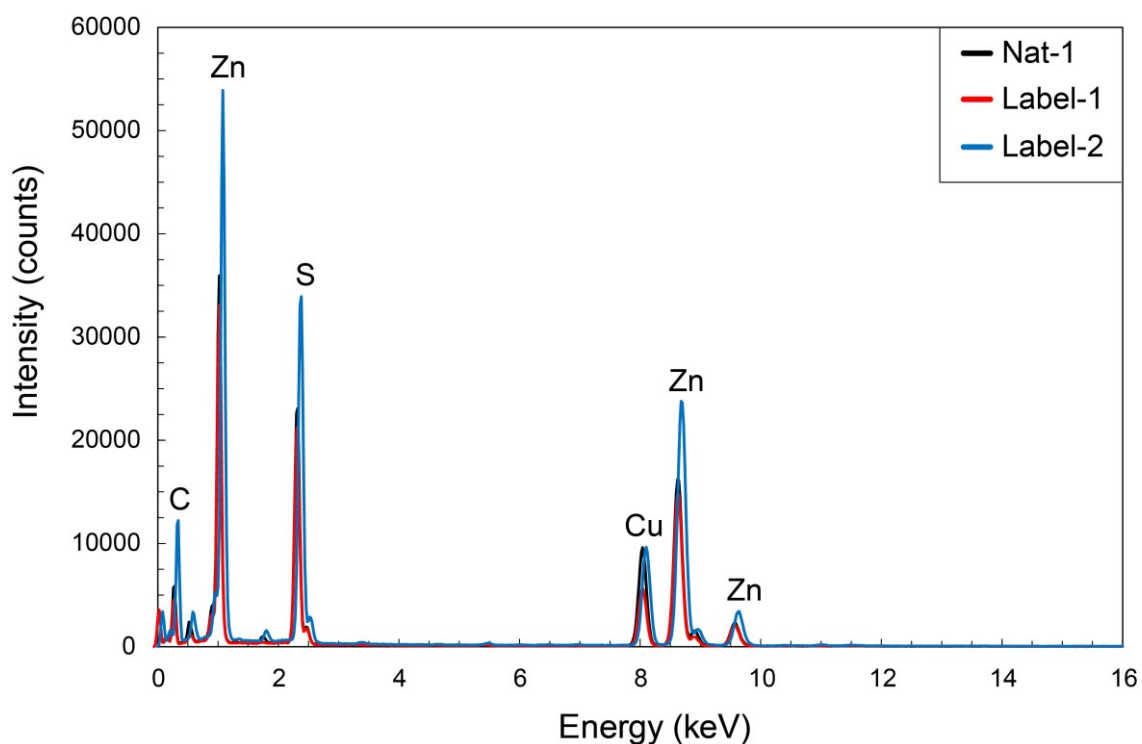


Fig. 2.7. EDS spectra of experiments Nat-A (black), Label-1 (red) and Label-2 (blue). EDS analysis confirms the formation of ZnS and the absence of impurities in the sample solution. Peaks indicating the presence of carbon and copper are due to the use of a carbon coated Cu grid for sample mounting.

2.3.3. Crystallographic analysis

Zinc sulphide exists naturally in two crystal structures, sphalerite (also known as zincblende) and wurtzite. Sphalerite is the more abundant, low temperature phase and represents the most important Zn ore, which is found and exploited in numerous locations worldwide. It is often denoted as $\alpha\text{-ZnS}$ and has the space group F-43m and space group

number 216. Wurtzite, or β -ZnS, is the hexagonal variant that forms at high temperatures and has the space group $P6_3mc$.

To confirm the crystallisation structure and ensure that the use of enriched ^{68}Zn did not alter the crystallisation behaviour, one sample of natural ZnS (Nat-2) and one sample of enriched ^{68}ZnS (Label-3) were analysed using XRD (Fig. 2.8).

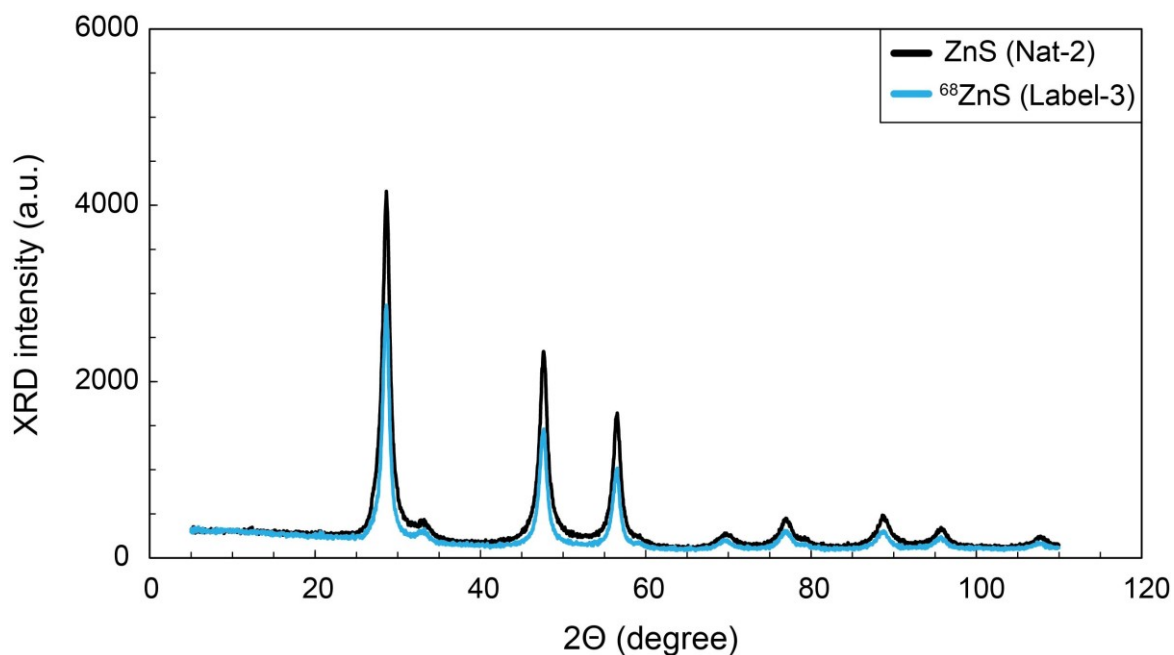


Fig. 2.8. XRD patterns of natural ZnS NPs (black) and ZnS NPs enriched in ^{68}Zn (blue). The peak patterns confirm formation of cubic ZnS (zincblende) with no differences between ZnS and ^{68}ZnS .

Peak intensities for the ^{68}ZnS sample are considerably lower than for natural ZnS, which reflects the difference in sample amount available for the XRD analyses. Both samples show precise alignment with the cubic ZnS (sphalerite) reference structure (ICDD 03-065-0309) and all reference peaks above 2% intensity (relative to (111) plane at 100%) between a Bragg's angle of 5° and 110° were confirmed (Table 2.2), with reflections of the (111), (220) and (311) planes featuring most prominently. The peaks show the widening that is typical of NPs in XRD patterns, although to a lesser degree than reported previously for NPs

of smaller size [58, 59]. The average size of the crystallites present in a sample can be calculated using the Scherrer formula:

$$D = k \lambda \beta^{-1} (\cos \theta)^{-1} \quad (\text{Equation 2.1})$$

where D is the crystallite size, k the geometric factor (close to unity, here 0.9), λ is the X-ray wavelength (1.54 nm), β is the full width at half maxima (FWHM) of the diffraction peak (in radian), and θ is the diffraction angle (in radian). Using the (111) plane, calculations yielded crystallite sizes of 7 nm for ZnS and of 7.8 nm for ^{68}ZnS (Table 2.2).

Table 2.2: ZnS reference values and measured Bragg's angles for natural ZnS and ^{68}ZnS with corresponding normalised spectral intensities and crystal planes.

Reference value [degree]	Bragg's angle ZnS (Nat-2) [degree]	Bragg's angle ^{68}ZnS (Label-3) [degree]	Plane (hkl)	Intensity [%]
28.61	28.63	28.63	(111)	100
33.15	33.18	33.22	(200)	9
47.59	47.74	47.74	(220)	50
56.58	56.55	56.58	(311)	29
69.58	70.31	70.05	(400)	6
76.89	76.84	76.84	(331)	8
88.67	88.79	88.79	(422)	9
95.67	95.72	95.72	(511)	5
107.60	107.76	107.83	(440)	3

	FWHM [rad]	Average crystallite size [nm]
ZnS (Nat-3)	0.020	7.02
^{68}ZnS (Label-3)	0.018	7.83

2.3.4. Synthesis yields

Reliable and suitably high synthesis yields are a requirement for the production of particles containing isotopically labelled Zn due to the cost of highly enriched stable isotopes. The total particle yield, as measured by the dry powder weight of the reaction products, was

assessed for several of the synthesis experiments. The experiments to synthesize natural ZnS at 70° which were nucleated for 15 minutes gave yields of between 15 and 20% (Nat-1 and Nat-3; Table 2.1). In contrast, the experiments to produce isotopically labelled ^{68}ZnS particles at the same temperature with nucleation time of 10 and 15 minutes achieved yields between 8 and 9% (Label-1 and Label-2; Table 2.1). The synthesis of Label-3 which utilized a nucleation time of 72 hours resulted in a significantly higher yield of 40% (Label-3, Table 2.1).

2.3.5. Use of ^{68}ZnS as stable isotope tracer

As outlined in the Introduction, the studies of Lombi et al. [38] and Ma et al. [37], among others, showed that ZnS is the main transformation product of anthropogenic ZnO NPs in WWTPs [60, 61]. At this point, only one study was found which characterised the physico-chemical properties of the ZnS particles present in sewage sludge from a WWTP. Kim et al. [44] identified ZnS nanocrystals of 3-8 nm size that formed aggregates of up to a few hundred nanometres. As such, the sub-micron sized ZnS particles synthesised in the present study are likely to be a realistic experimental proxy for the ZnS particles that are produced from ZnO NPs during treatment in WWTPs [62].

Assuming a realistic cost of US-\$1.5 per mg of highly enriched ^{68}Zn (March 2012, Isoflex, 1000 mg purchased at ~US-\$1400) and an average synthesis yield of 13%, ZnS particles with a diameter of 100-150 nm can be produced for a price of about US-\$10 per mg of labelled ^{68}ZnS . If the application allows for the use of larger particles with diameters of about 200 nm, the yield can be increased significantly by allowing the synthesis suspensions to nucleate for a prolonged period of time at room temperature (Table 2.1). The production cost for 1 mg of ^{68}ZnS can then be reduced to only about US-\$4.

2.4. Conclusions

In this study, a published method for the synthesis of sub-micron sized ZnS particles was altered and then assessed for its suitability to produce small quantities of isotopically labelled ZnS particles that can be applied to stable isotope tracing. To this end, the synthesis was scaled down in reagent volumes by about a factor of 10 and the physico-chemical properties of the produced particles were thoroughly characterised. In particular, particles produced with natural Zn were compared to material synthesised from artificially enriched ^{68}Zn to confirm the absence of differences between the produced particles. Particle sizes of between 14 ± 2 (DLS) – 189 ± 8 (TEM) for ZnS and 145 ± 5 (TEM) and 167 ± 1 nm (DLS) for ^{68}ZnS were found. The produced particles were monodisperse with a relative standard deviation in the particle size of less than 5% and an average PDI value of 0.13 ± 0.04 (1 SD). The results furthermore indicate no systematic difference in size and shape between particles produced from natural Zn and enriched ^{68}Zn . The syntheses produced cubic ZnS (zincblende/sphalerite) with a composition and structure, as confirmed by XRD and EDS analyses, which is identical for labelled and unlabelled particles.

With an average yield of 13%, the synthesis enables the production of ^{68}ZnS at a cost of about US-\$10 per mg, with the possibility to reduce the cost to about US-\$4 per mg of ^{68}ZnS if slightly larger particles are acceptable. Our synthesis protocol thus extends the routine application of the stable isotope tracing methodology to ZnS particles. Such applications are of particular interest because ZnS particles are the main transformation product of ZnO NPs in WWTPs. Hence, there is significant interest in investigations that study the behaviour, fate and transport properties of ZnS at environmentally relevant exposure concentrations and such studies will be facilitated through stable isotope labelling of ZnS.

2.5. Acknowledgements and declaration

Andrew Tye of the British Geological Survey and Claus Svendsen of the Centre for Ecology and Hydrology suggested the study. Tabea Junk (TJ) carried out the literature review and all laboratory work related to the development of the synthesis. Jens Najorka carried out XRD analysis at the Natural History Museum, London and Mahmoud Ardakani recorded TEM micrographs at the Department of Materials at Imperial College London. Mark Rehkämper (MR) and Adam Laycock provided feedback during the synthesis development. TJ wrote the chapter with input from MR.

Chapter 3

Robust and efficient analytical methods for stable isotope tracing of silver nanoparticles in complex environmental samples

Abstract

Silver nanoparticles (Ag NPs) are among the most commercialised engineered nanomaterials due to their antimicrobial properties. However, there is much uncertainty concerning their behaviour when they enter natural systems, which necessitates investigations into the behaviour of Ag NPs in the environment.

This chapter presents a novel, simplified method for the separation of Ag from diverse natural samples prior to isotopic analysis. Also presented are optimized techniques for high precision $^{109}\text{Ag}/^{107}\text{Ag}$ analyses of samples that contain variable quantities of labelled ^{109}Ag NP by multiple-collector ICP-MS. Average column yields were $104 \pm 13\%$ (1 SD) and blanks were typically at a level of 7 pg Ag or lower, thus enabling analysis of samples even with extremely low Ag contents. The method was validated using Ag standard solutions with natural and non-natural $^{109}\text{Ag}/^{107}\text{Ag}$ isotope composition as well as realistic environmental samples in the form of soils and biological tissues. Careful measurement of and correction for the instrumental memory was found to be an important component of overall data quality.

Natural Ag standard solutions were measured with an average offset and precision of 0.001 ± 0.001 for samples with Ag concentrations of $\leq 5 \text{ ng ml}^{-1}$ and of 0.0002 ± 0.0003 for Ag concentrations of 30 ng ml^{-1} . Non-natural Ag standard solutions were measured to within $\pm 5\%$ error for solutions containing less than 50 pg total Ag. Several analytical scenarios were investigated through the analysis of natural samples, encompassing those that contain (i) only natural Ag, (ii) a mix of natural and enriched Ag, and (iii) almost exclusively enriched ^{109}Ag . For all three scenarios, the isotopically labelled Ag was detected and distinguished from the natural background with sufficient accuracy and precision to facilitate use of stable isotope tracing for investigating the behaviour, fate and transport mechanisms of isotopically labelled Ag NPs in complex environmental systems at low dosing concentrations.

3.1. Introduction

Engineered silver nanoparticles (Ag NPs) are one of the most widely manufactured engineered nanomaterials (ENMs). They are applied for diverse purposes due to their unique antimicrobial properties [5], including consumer products such as textiles, food packaging and health supplements, and for medical applications [63-66]. As a consequence, Ag NPs are continuously released into the natural environment, especially into aquatic systems, where they can propagate into soils and accumulate in the food chain. Here, their antimicrobial properties may have adverse effects on natural systems and their dissolution releases Ag^+ ions which are toxic to most aquatic organisms [5].

At present, there is only scant information on the fate, behaviour and transport mechanisms of Ag NPs at environmentally relevant concentrations. At least in part, this reflects analytical difficulties that are related to the limitations of conventional measurement techniques. In particular, high concentrations of Ag NPs are often used in exposures so that (i) Ag concentration measurements are not carried out at or close to the detection and quantification limits of the instrumental technique and (ii) concentration effects from the presence of Ag NPs can be readily resolved from natural variations in the background concentration. Most published exposure studies that investigate the impact of Ag NPs upon release hence employ Ag concentrations of $\mu\text{g l}^{-1}$ to mg l^{-1} in waters and of mg kg^{-1} in soils [67-74]. In contrast, the predicted environmental concentrations (PECs) of Ag NPs are much lower, with values in the range of ng l^{-1} to $\mu\text{g l}^{-1}$ for natural waters and ng kg^{-1} to $\mu\text{g kg}^{-1}$ for soils, whereby the latter are similar to the natural Ag background concentrations (Fig. 3.1) [5, 39, 75, 76].

Previous work has shown that stable isotope tracing is a powerful technique for the sensitive and selective detection of metal-based ENMs in complex exposure systems. The methodology involves preparation of the NPs from an isotopically enriched form of the

element. For example, isotopically labelled ^{68}ZnO NPs can be prepared from metallic Zn that consists to >99% of ^{68}Zn , compared to a natural abundance of ~25% [30]. This distinct non-natural isotopic signature can then be readily traced even against high natural background concentrations by mass spectrometric measurements of a diagnostic isotope ratio. The technique of stable isotope tracing has successfully been developed for ZnO, Cu, CeO_2 and TiO_2 NPs [20, 26, 27, 30, 52] and its efficiency has been shown in a number of recent studies [21, 22, 53].

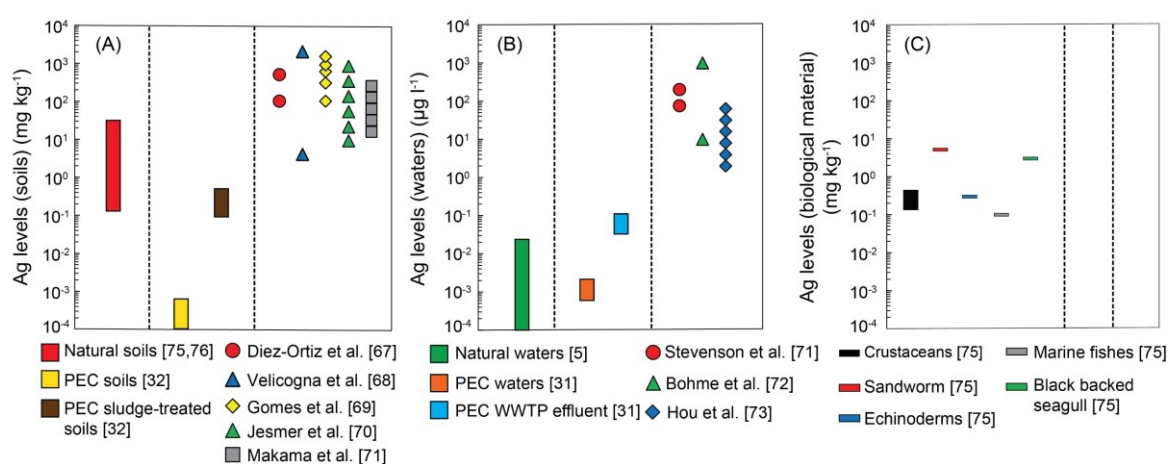


Fig. 3.1. Comparison of natural Ag levels (left of each panel), predicted environmental concentrations (PECs) from engineered Ag NPs (middle of each panel) and examples of Ag NP concentrations used in ecotoxicity studies (right of each panel) for (A) soils, (B) waters and (C) biological organisms. For biological organisms, no PEC values are available in the literature and exposure concentrations are not applicable as they are typically applied through indirect exposure (e.g. dermally or orally). Typical exposure concentrations are 3 to 4 times higher than PECs in pristine waters and soils and 1 to 2 orders of magnitude higher than PECs in sludge-treated soils and waste water treatment plant effluents, which carry a higher burden of anthropogenic nano-waste.

In a pilot investigation, Croteau et al. [25] were the first to employ stable isotope tracing to Ag NPs, thereby exploiting that natural Ag is comprised of the two stable isotopes ^{107}Ag and ^{109}Ag , with relative abundances of 51.8% and 48.2% respectively. In their study, Croteau et al. [25] investigated the uptake of soluble $^{109}\text{AgNO}_3$ and ^{109}Ag NPs by the freshwater snail

Lymnaea stagnalis. Using quadrupole ICP-MS for detection, they found that the stable isotope labelling improved the sensitivity of detection (compared to exposures using natural Ag) by a factor of 2 for waterborne exposures and by a factor of 40 for diet-borne exposures.

The current study also investigates the application of stable isotope tracing to enhance detection of Ag NPs in complex environmental samples, such as soils and biological tissue. In contrast to Croteau et al. [25], the new methodology employs multiple collector ICP-MS (MC-ICP-MS) for the isotopic measurements. Given that ecotoxicological studies routinely involve large (>100) sample numbers, a particular advantage of quadrupole ICP-MS is that the analyses are typically conducted without any prior elemental pre-concentration. In contrast, chemical separation of the target element is a pre-requisite for analysis by MC-ICP-MS. The latter technique, however, can provide unparalleled precision for the determination of diagnostic isotope ratios. For the determination of the $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratio, quadrupole ICP-MS typically achieves a precision of $\pm 5\%$ (2 SD), whilst MC-ICP-MS can provide such data with a precision of up to $\pm 0.05\%$ (2 SD), a vast improvement which might be critical for some types of studies [24]. The excellent performance of MC-ICP-MS thereby reflects the unique instrumentation and the absence of matrix in the final sample solutions. Together, this enables highly sensitive and precise analyses that are not compromised by spectral interferences and signal suppression, which can severely impact analytical accuracy, particularly if target element concentrations are low.

High precision MC-ICP-MS analyses of Ag isotope compositions are most commonly carried out in cosmochemistry, to search for isotope variation from the decay of now extinct ^{107}Pd to ^{107}Ag ($t_{1/2} \approx 7$ Ma) as well as in geochemistry and archaeology to resolve $^{109}\text{Ag}/^{107}\text{Ag}$ variations from stable isotope fractionation [77-83]. The largest isotopic variability was thereby identified in meteorites, which show radiogenic $^{107}\text{Ag}/^{109}\text{Ag}$ ratios of up to 10. In contrast, Ag stable isotope fractionation is more limited. [80, 84] Analyses of native Ag metal from coins

revealed $^{109}\text{Ag}/^{107}\text{Ag}$ ratios that vary by up to 0.6‰. A study of environmental samples such as domestic and industrial sludge found $^{109}\text{Ag}/^{107}\text{Ag}$ variations of about 0.3‰ while a recent investigation of naturally formed Ag NPs identified enrichments in ^{109}Ag of up to about 1‰ [85, 86].

The application of MC-ICP-MS necessitates the complete digestion and full recovery of the Ag present in a natural sample as well as the separation of Ag from the sample matrix. This in turn requires balancing optimal data quality and the envisioned sample throughput. The pre-concentration of Ag from geological or biological samples is usually achieved using ion exchange chromatography and numerous methods have been published [77, 80, 81]. Given that investigations of natural Ag isotope fractionation require the resolution of very small (<0.1‰) variations in $^{109}\text{Ag}/^{107}\text{Ag}$, multiple ion exchange chromatography steps are typically used to obtain an extremely pure Ag fractions. A simpler, less laborious procedure is desirable for the current study, as stable isotope tracing of Ag NPs generally involves samples that exhibit much larger differences in $^{109}\text{Ag}/^{107}\text{Ag}$ ratios that are easier to resolve. For the same reason, dedicated mass spectrometric procedures that apply faster measurement protocols are desirable, but this will need to be balanced with compromises in data quality.

Given these circumstances, the aim of this work is to develop an ion exchange procedure that allows optimised sample throughput and separation of Ag from other matrix elements, to enable sample analysis via MC-ICP-MS with sufficient precision and accuracy. In detail, this chapter presents optimized Ag separation and measurement techniques that enable highly sensitive detection of stable isotope labelled Ag at low concentrations. The technique utilises the superior precisions of MC-ICP-MS while allowing for the high sample throughput required by environmental exposure studies. The application of the methods for key analytical scenarios is carefully tested and demonstrated. This includes analyses of Ag samples of

variable size and Ag content, encompassing both samples that have natural Ag isotope compositions and those that contain intermediate to high level of enriched ^{109}Ag (Ag-En).

3.2. Experimental

3.2.1 Samples

Two natural reference materials were repeatedly analysed during this study to assess the performance of the methods –LUFA Speyer 2.2 soil and biological sample ERM-BB422®. The standard test soil LUFA-Speyer 2.2 (Sp 2121, Germany, 2009) has a $\text{pH}_{\text{CaCl}_2}$ of 5.5, a total organic carbon content of 2.09%, a cation exchange capacity of $10.0 \text{ meq } 100^{-1} \text{ g}^{-1}$ and a water holding capacity (WHC) of 46.5%. The ERM-BB422® material is a lyophilised, powdered fish muscle prepared from the species *Pollachius virens* (Saithe). These two samples were processed and analysed repeatedly, both as received and following addition of variable quantities of Ag-En, to assess the performance of the methods for unspiked and spiked samples. In addition, as part of the study presented in Chapter 4 of this work, zebrafish (*Danio rerio*) were exposed to Ag NPs enriched in the ^{109}Ag isotope through their diet. From such fish, liver and gonad tissue as well as embryos were sampled and analysed. Data in this Chapter that is marked as originating from zebrafish gonad, liver or embryonic tissue of fish was collected as part of the work presented in Chapter 4. A detailed description of the corresponding exposure and sampling process can be found in the experimental section of Chapter 4.

3.2.2. Materials and reagents

The analytical work was conducted in the MAGIC Laboratories at the Department of Earth Science and Engineering of Imperial College London. All work aside from the preparation of labelled NPs was thereby carried out in an ISO 6 clean room equipped with ISO 4 laminar flow hoods to prevent contamination. The concentrated and dilute mineral acids

used for sample preparation and analysis were prepared from AnalaR grade 15.3 mol l⁻¹ HNO₃ and 6 mol l⁻¹ HCl by sub-boiling distillation in quartz stills. Milli-Q water of >18 MΩ cm quality (Millipore, UK) and 30% Suprapur H₂O₂ (VWR UK) were used throughout. All lab-ware was carefully cleaned prior to every use in one or several steps with mineral acids (HNO₃ and/or HCl) of different concentration and purity (reagent grade or distilled), as appropriate, with intermittent and final rinses using Milli-Q H₂O.

Solutions with natural Ag were prepared by dilution from a 1000 µg ml⁻¹ Ag solution obtained from Alfa Aesar. Isotopically enriched ¹⁰⁹Ag (99.7 ± 0.1%) was purchased from Isoflex USA (CA, USA) as a metallic powder and used for the preparation of isotopically labelled ¹⁰⁹Ag NPs and ¹⁰⁹AgNO₃. To simplify preparation, reference samples that contain non-natural Ag were prepared by addition of ¹⁰⁹AgNO₃ to the sample material prior to dissolution. The doping of sample and standard solutions with Pd employed a 100 ng ml⁻¹ Pd solution in 0.1 mol l⁻¹ HNO₃, which was prepared freshly every four to six weeks in two stages of dilution from a 20 µg ml⁻¹ Pd solution using 0.1 mol l⁻¹ HNO₃.

3.2.3. Synthesis and characterization of isotopically enriched Ag species

Isotopically enriched ¹⁰⁹Ag NPs were synthesised following Laycock et al. [24] In short, Ag metal was oxidised with 15.3 mol l⁻¹ HNO₃ to yield AgNO₃. An aqueous solution of AgNO₃ and sodium citrate (C₃H₄OH(COOH)₂COONa, Sigma-Aldrich) was then reduced at elevated temperatures with sodium borohydride (NaBH₄, Sigma-Aldrich) to produce mono-dispersed citrate-stabilised Ag NPs. Particle size, shape and size distribution were imaged at the Department of Materials at Imperial College London using a transmission electron microscope (JEOL STEM/TEM 2100Plus with EDS capabilities) and Gatan Microscopy Suite DigitalMicrograph software (Version 2.32.888.0). Histogram data of TEM images were collected and analysed using ImageJ. Dynamic light scattering (Malvern Zetasizer

Nano ZS with Zetasizer Software Version 7.11) measurements were conducted to analyse primary particle size and polydispersity of the stock Ag NP solution. Synthesis yield was assessed by MC-ICP-MS, whereby instrumental details are given below.

3.2.4. Sample preparation

3.2.4.1. Sample digestion

Up to 500 mg of sample were weighed (Mettler Toledo AX205) in TFM microwave digestion vessels, whereby a procedural blank was processed with every sample batch of six to nine samples to monitor contamination. A mixture of 3 ml H_2O_2 and 7 ml distilled 15.3 mol l^{-1} HNO_3 was added to each vessel and samples were then pre-digested for 14-20 hours at room temperature. This was followed by microwave digestion in an Ethos EZ microwave system (Milestone Inc., 1200 W) using a step-wise heating protocol. This ramped the temperature to 200°C within 45 minutes and remained at 200°C for a further 30 minutes. Samples were then transferred to Savillex® Teflon beakers and evaporated to dryness. Samples were then dissolved repeatedly in approximately 0.5 – 1 ml 6 mol l^{-1} HCl and evaporated to achieve complete conversion to the chloride form. Finally, each sample was taken up in about 0.5 to 2 ml of 0.5 mol l^{-1} HCl and refluxed for >12 hours. Stock solutions of reference materials dissolved in 0.5 mol l^{-1} HCl were kept for later use in pre-cleaned HDPE containers. Samples of the LUFA-Speyer 2.2 soil were centrifuged at this stage for 10 minutes at 4000 RPM (Thermo Scientific Heraeus Megafuge) and any undigested silicate material was discarded. Samples of biological tissue were fully digested during the microwave digestion procedure and did not require further centrifugation.

3.2.4.2. Ion exchange separation of Ag

Silver was separated from the sample matrix by anion exchange chromatography. The separation procedure was modified from the methods of Woodland et al. [80], primarily to improve sample throughput. In detail, this was achieved by adopting a one-step rather than the original three-step procedure for Ag isolation and purification (Table 3.1).

The procedure of Woodland et al. [80] was designed to yield Ag sample fractions of high purity to enable isotopic analyses by MC-ICP-MS that are able to resolve natural variations in Ag isotope compositions at the 1‰ level or less. This in turn requires the use of multiple ion exchange separation steps for the isolation and purification of Ag. The application of MC-ICP-MS to the measurement of larger isotopic deviations, as found in tracing studies with enriched Ag, does not require such a lengthy separation protocol and a one-step ion exchange technique was found to be sufficient and hence preferable. The high Zn/Ag ratios of environmental samples are problematic, however, as spectral interferences from Zn-argides (especially $[^{67}\text{Zn}^{40}\text{Ar}]^+$) can bias the Ag isotope measurements. The method was therefore further modified by addition of a Zn elution step with $0.005 \text{ mol l}^{-1} \text{ HCl}$, which successfully removes Zn from the resin prior to Ag elution. Further changes were made to the acid volumes that were used during the elution procedure. Of particular importance is the amount of $0.5 \text{ mol l}^{-1} \text{ HNO}_3$ that is employed prior to and for Ag recovery, as this acid elutes both Cd and Ag, whereby biological and geological samples have widely different Cd/Ag ratios.

The sample solutions in $0.5 \text{ mol l}^{-1} \text{ HCl}$ were loaded onto Bio-Rad *Poly-Prep*® chromatography columns containing 1 ml AG1-X8 (100-200 μm mesh) resin that had been cleaned and pre-conditioned with $9 \text{ mol l}^{-1} \text{ HCl}$ and $0.5 \text{ mol l}^{-1} \text{ HCl}$, respectively (Table 3.1). Following sample loading, the columns were washed sequentially with HCl of decreasing concentration (0.1 mol l^{-1} , 0.005 mol l^{-1} and 0.001 mol l^{-1}) and with $0.5 \text{ mol l}^{-1} \text{ HNO}_3$ to

remove matrix elements. Silver was then collected by elution with 12 ml 0.5 mol l⁻¹ HNO₃ and, after letting the resin rest for at least an hour, a further 5 ml of 0.5 mol l⁻¹ HNO₃.

Table 3.1: Anion exchange chromatography for the separation of Ag from environmental samples.

Process	Medium	Volume / ml
Resin loading	AG1-X8, 100-200 mesh	1
Cleaning	9 mol l ⁻¹ HCl	16
Conditioning	0.5 mol l ⁻¹ HCl	24
Sample loading	0.5 mol l ⁻¹ HCl	0.5-2
Matrix elution	0.1 mol l ⁻¹ HCl	27
	0.005 mol l ⁻¹ HCl	28
	0.001 mol l ⁻¹ HCl	16
	0.5 mol l ⁻¹ HNO ₃	3.5
Ag elution	0.5 mol l ⁻¹ HNO ₃	12
		≥1 hour break
		5

3.2.5. Mass spectrometry

3.2.5.1. Instrumentation and protocols

All isotopic measurements were performed in the MAGIC Laboratories at Imperial College London with a Nu Plasma HR MC-ICP-MS. Sample introduction utilised a CETAC Aridus II desolvation system equipped with glass MicroMist nebulizers. Details of the operating parameters and typical measurement conditions are summarised in Table 3.2. The Ag isotope analyses were carried out by static multiple collection with Faraday cups fitted with 10¹¹ Ω resistors to monitor masses 107 and 109 for Ag, as well as 104 and 105 for Pd (Table 3.3). As Ag has only two naturally occurring stable isotopes, internal normalisation cannot be applied for the correction of the instrumental mass bias that is typically observed in

Table 3.2: Operating conditions of the Nu Plasma MC-ICP-MS and CETAC Aridus II desolvating nebulizer system.

Nu plasma MC-ICP-MS	
RF Power [W]	1300
Acceleration potential [V]	~6000
Ar coolant gas flow [l min ⁻¹]	13
Ar auxiliary gas flow [l min ⁻¹]	1
Faraday cup resistors [Ω]	10 ¹¹
Expansion chamber vacuum [mbar]	~0.75
Analyser vacuum [mbar]	~2 x 10 ⁻⁹
Sensitivity for Ag [V ($\mu\text{g ml}^{-1}$) ⁻¹]	250 – 350
Sensitivity for Pd [V ($\mu\text{g ml}^{-1}$) ⁻¹]	180 – 220
CETAC Aridus II desolvating nebulizer system	
Argon sweep gas [l min ⁻¹]	~3
Nebulizer pressure [psi]	~60
Sample uptake rate [$\mu\text{l min}^{-1}$]	~110

plasma source mass spectrometry [87]. The simultaneous measurement of ¹⁰⁵Pd and ¹⁰⁴Pd enables the ‘raw’ measured ¹⁰⁹Ag/¹⁰⁷Ag ratios of samples and standards to be corrected for the time-dependent instrumental mass bias of about 2‰ per amu mass difference by external normalisation relative to ¹⁰⁵Pd/¹⁰⁴Pd using the exponential law [87]. Similar approaches of mass bias correction were previously applied in high precision measurements of small natural Ag isotope fractionation [24, 78, 80, 81, 85, 88, 89], and this demonstrates that the technique is suitable for the less stringent analytical requirements of the current methodology. In detail, this approach assumes that Ag and Pd experience (nearly) identical mass bias during mass spectrometry due to their overlapping mass ranges so that their mass fractionation coefficients β are essentially in accord with $\beta_{\text{Ag}} = \beta_{\text{Pd}}$ [80, 87]. As it was found that the quality of the Ag

Table 3.3: Faraday cup arrangement for Ag isotope measurements with Nu Plasma MC-ICP-MS including all monitored elements and potential spectral interferences.

Faraday cup	H5	H4	H2	Ax	L2	L3
Atomic mass	109	(108)	107	(106)	105	104
Elements & isobaric interferences (abundance)	Ag (48.6%)	Pd (26.7%) Cd (0.9%)	Ag (51.4%)	Pd (27.1%) Cd (1.2%)	Pd (22.6%)	Pd (9.3%) Ru (18.3%)
Molecular interferences	$[^{93}\text{Nb}^{16}\text{O}]^+$		$[^{91}\text{Zr}^{16}\text{O}]^+$		$[^{89}\text{Y}^{16}\text{O}]^+$	$[^{88}\text{Sr}^{10}\text{O}]^+$
	$[^{95}\text{Mo}^{14}\text{N}]^+$		$[^{93}\text{Nb}^{14}\text{N}]^+$		$[^{91}\text{Zr}^{14}\text{N}]^+$	$[^{90}\text{Zr}^{14}\text{N}]^+$
	$[^{69}\text{Ga}^{40}\text{Ar}]^+$		$[^{67}\text{Zn}^{40}\text{Ar}]^+$		$[^{65}\text{Cu}^{40}\text{Ar}]^+$	$[^{64}\text{Zn}^{40}\text{Ar}]^+$

isotope measurements is mainly influenced by memory effects and therefore the signal intensity during sample analysis, Pd was added at a constant concentration of 10 ng ml^{-1} to produce a sufficiently high signal for the precise quantification of the $^{105}\text{Pd}/^{104}\text{Pd}$ ratio. During the course of the study, sensitivities of $250 \text{ to } 350 \text{ V } (\mu\text{g ml})^{-1}$ and $180 \text{ to } 220 \text{ V } (\mu\text{g ml})^{-1}$ were routinely achieved for Ag and Pd, respectively (Table 3.2). Whilst different analytical protocols were used for the initial assessment of the Ag concentrations and the subsequent Ag isotope measurements, automatic peak centring routines were not employed in any of the analyses to ensure optimised use of sample solutions with only little Ag. Instead, the peak centre position was routinely checked and adjusted manually based on manual mass scans that were carried out with Ag standard solutions.

3.2.5.2. Initial sample assessment

Following evaporation of the Ag fractions from the column chemistry to dryness, the residues were dissolved in 1 ml of $0.1 \text{ mol l}^{-1} \text{ HNO}_3$ for mass spectrometric analysis. A small 50 to 100 μl aliquot of the solutions was used for an initial assessment of the samples solutions. To this end, the aliquot was diluted to a volume of 500-1000 μl with $0.1 \text{ mol l}^{-1} \text{ HNO}_3$ and Pd was added to a concentration of typically 10 ng ml^{-1} . Samples were then analysed using a ~2 min measurement protocol comprised of a single block with an initial 15 s determination of the electronic baselines whilst the ion beams were deflected in the electrostatic analyser and 10 data acquisition cycles of 5 s each.

The Ag ion beam intensities that were obtained in these analyses served three purposes. (i) They enabled a first ‘rough’ determination of the Ag contents available in the remaining sample solutions, so that they could be appropriately diluted for the isotopic analyses. (ii) Based on the estimated Ag content and preferred dilution, the sample solutions were ‘ranked’ either in order of increasing total Ag contents (for samples with natural Ag isotope compositions) or

increasing ^{107}Ag content (for samples containing Ag-En) so that the subsequent isotopic analyses are carried out in the most efficient fashion. (iii) The results also advised on the preparation of ‘matching’ Ag standard solutions that suitably match to the Ag concentrations of the samples, which are bracketed by the standard runs

3.2.5.3. Preparation of sample solutions

Based on the results of the initial sample assessment, the remaining 900 to 950 μl of the purified Ag sample solutions from the column chemistry were diluted to an appropriate volume with $0.1 \text{ mol l}^{-1} \text{ HNO}_3$ to prepare the final sample solution for the determination of the Ag isotope composition. In practise, most ‘real’ samples were diluted to the minimum volume of 0.9 to 1.0 ml due to the limited amounts of Ag available for analysis. As part of the dilution, the solutions were doped with Pd to 10 ng ml^{-1} . All final run solutions of samples (and Ag standards; see below) with Ag concentrations of less than $1 \text{ } \mu\text{g ml}^{-1}$ were thereby prepared fresh on the day of use, to ensure that results are not significantly biased by minor changes in Ag content from adsorption onto container walls or blank addition.

3.2.5.4. Preparation of Ag standard solutions

To monitor instrument performance and for standardisation, all sample analyses were bracketed with regular measurements of dilute Ag standard solutions. For samples with Ag of natural or near-natural isotope composition, an appropriate standard solution with natural Ag (Ag-Nat) was prepared by dilution of the concentrated $1000 \text{ } \mu\text{g ml}^{-1}$ Ag stock solution with $0.1 \text{ mol l}^{-1} \text{ HNO}_3$. This method of standardisation is not useful for measurement sessions that involve analyses of samples that contain larger contents of Ag-En. Instead, such analyses applied a standard solution that was prepared as a mixture of Ag-Nat and Ag-En with $^{109}\text{Ag}/^{107}\text{Ag} \approx 18$. This was prepared as a mixture of $\sim 130 \text{ ng Ag-Nat}$, from a 1000 ng ml^{-1}

Ag-Nat solution in $0.1 \text{ mol l}^{-1} \text{ HNO}_3$, and $\sim 1100 \text{ ng Ag-En}$ from a $\sim 68 \text{ ng ml}^{-1}$ solution of Ag-En, also in $0.1 \text{ mol l}^{-1} \text{ HNO}_3$. This mixture was diluted to $\sim 100 \text{ ml}$ volume with $0.1 \text{ mol l}^{-1} \text{ HNO}_3$ to obtain a final total Ag-Tot concentration of $\sim 12.5 \text{ ng ml}^{-1}$. A precise reference value for the $^{109}\text{Ag}/^{107}\text{Ag}$ ratio of the mixed Ag-En + Ag-Nat solution was subsequently established by repeated analyses of the essentially undiluted solution. Similarly, updated internal reference values for the isotope composition of natural Ag were established regularly during the study based on repeated analyses of $10 \mu\text{g ml}^{-1}$ Ag-Nat standard solutions.

Like the final sample solutions, all Ag standard solutions for isotopic analysis were doped to 10 ng ml^{-1} Pd using a 100 ng ml^{-1} Pd solution in $0.1 \text{ mol l}^{-1} \text{ HNO}_3$.

3.2.5.5. Ag isotope measurements

The Ag isotope compositions were determined using a $\sim 6 \text{ min}$ long measurement protocol that encompasses 3 blocks of 20 integration cycles of 5 s each, whereby each block is preceded by an electronic baseline measurement of 15 s. Following tuning of the instrument, several runs of appropriate Ag standard solutions were carried out at the beginning of each measurement session to ensure and document that the instrument provided the expected performance. Following this, sample analyses were commenced. To ensure that results were not compromised by large memory effects, blank samples were analysed first. Following this, sample solutions were analysed in order of increasing total Ag concentration for samples with only natural Ag, whilst samples containing Ag-En were analysed in order of increasing concentration for the non-spike ^{107}Ag isotope. Furthermore, samples with natural and non-natural Ag isotope compositions were preferably analysed on separate measurement sessions or such analyses were separated by a long ($>1 \text{ h}$) washout period. Usually, the sample introduction system of the instrument was washed with $0.1 \text{ mol l}^{-1} \text{ HNO}_3$ for at least 2 to 3 minutes to reduce all signal intensities to below 5 mV between runs of samples and standard

solutions (Fig. 3.2). The machine memory was carefully monitored during measurement sessions to ensure that it was appropriately low (<5 mV) and with the expected isotope composition. To this end, on-peak measurements of the instrumental memory were conducted using a designated $0.1 \text{ mol l}^{-1} \text{ HNO}_3$ solution after completion of the normal ‘wash’ procedure and prior to each sample or standard run (Fig. 3.2). The machine memory was determined using a measurement protocol of a single block with 30 integration cycles of 5 s each that was preceded by an electronic baseline measurement of 15 s.

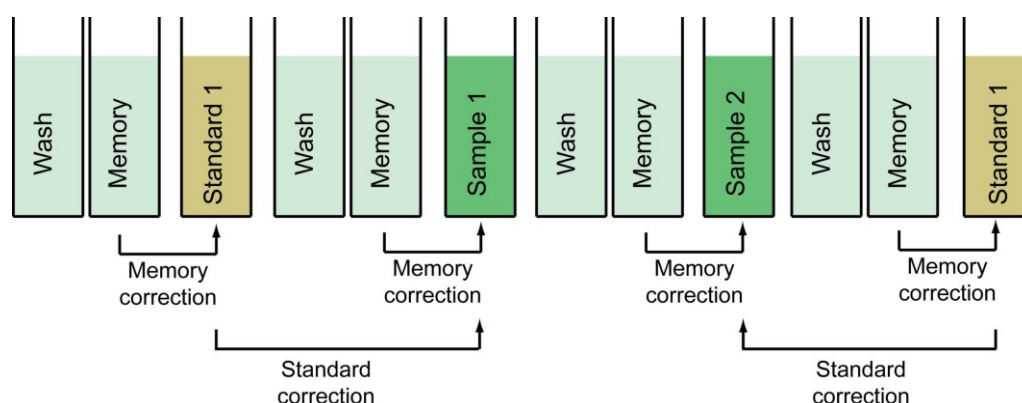


Fig. 3.2. Illustration of a regular analytical sequence with corresponding correction procedures. Standard and sample analyses are preceded by a memory measurement to correct for memory effects. Two sample analyses are bracketed by the analysis of a standard solution, which is used to determine the corresponding standard correction (details in text).

Each block of two subsequent sample runs was bracketed by analyses of a ‘matching’ Ag standard solution (Fig. 3.2). The $^{109}\text{Ag}/^{107}\text{Ag}$ ratios of samples with natural (or near-natural) Ag isotope compositions were measured relative to dilute solutions of natural Ag that were prepared to match sample concentrations to within $\pm 25\%$. In contrast, samples that feature a significant contribution of Ag-En were bracketed by runs of the mixed Ag-En + Ag-Nat solution with $^{109}\text{Ag}/^{107}\text{Ag} \approx 18$, whereby the ^{107}Ag ion beam intensities of the sample and standard solutions were matched to within $\pm 25\%$ or better. The procedure of matching the Ag concentrations of sample and standard solutions helps to minimise memory effects in the

sample introduction system and ensures that the standard results are applicable to monitor and characterise the quality of data acquisition for samples.

With this, the normal analytical sequence during a measurement session was wash - memory run - standard run – wash – memory run – sample run – wash – memory run – sample run - wash – memory run – standard run, etc. which is shown in Fig. 3.2.

3.2.6. Data reduction

The data recorded by the mass spectrometer during the memory, sample and standard runs were fed into a stepwise data reduction process, which was carried out offline after the completion of a measurement. The ‘Results’ files, which were downloaded from the mass spectrometer, provide various parameters of which the ‘raw’ ^{107}Ag , ^{109}Ag and ^{105}Pd signal intensities and the $^{105}\text{Pd}/^{104}\text{Pd}$ ratio are important for further processing. Importantly, all four values are already corrected for the offset of the electronic baseline values of the individual Faraday cups by the ‘online’ data processing that is carried out during the runs.

(1) In a first step, the raw ^{107}Ag and ^{109}Ag signal intensities were corrected for residual instrumental memory by subtracting the ^{107}Ag and ^{109}Ag signal intensities that were recorded during the preceding on-peak memory measurement (Fig. 3.2). This procedure serves to determine the memory corrected ^{107}Ag and ^{109}Ag ion beam intensities and from this, the memory corrected $(^{109}\text{Ag}/^{107}\text{Ag})_{mc}$ and $(^{109}\text{Ag}/^{105}\text{Pd})_{mc}$ ratios of samples and standard solutions. No background correction was carried out for the raw ^{105}Pd signal, as Pd was present at a relatively high concentration of 10 ng ml^{-1} in all sample and standard solutions. In this case, the memory corrections are essentially inconsequential and were thus omitted for simplicity.

(2) Following this, the $(^{109}\text{Ag}/^{107}\text{Ag})_{mc}$ and $(^{109}\text{Ag}/^{105}\text{Pd})_{mc}$ ratios were corrected for instrumental mass bias by external normalisation with the admixed Pd, using the ‘raw’ $^{105}\text{Pd}/^{104}\text{Pd}$ ratios that were determined during each individual sample or standard run. A

reference ratio of $^{105}\text{Pd}/^{104}\text{Pd} = 2.00377$ [90] was thereby employed for the correction of the $^{109}\text{Ag}/^{107}\text{Ag}$ ratio with the exponential law [85]. This produces mass bias corrected $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ ratios.

(3) The $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ ratios of the matching standards, that were measured interspersed between samples, were continuously monitored during each analytical session. Normally, the $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ ratios obtained in the standard runs should deviate from the internal $^{109}\text{Ag}/^{107}\text{Ag}$ reference values, which were determined by repeated analyses at high Ag concentrations, by less than 1% for standard solutions with Ag concentrations $>0.5 \text{ ng ml}^{-1}$ and by less than about 5% for solutions with less than 0.05 ng ml^{-1} Ag (see below). These deviations are primarily a consequence of measurement (im-) precision and are therefore concentration-dependent. However, such deviations can also be produced by spectral or non-spectral interferences that are generated by residual matrix material from samples in the sample introduction system. In particular, the $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ data for the preceding and bracketed sample runs are also likely to be affected by the same analytical artefacts and are thus best disregarded. In practise, large deviations for the $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ data of bracketing standard solution runs were only observed very rarely, affecting less than 2% of the sample analyses that were conducted during the course of about 2 years.

Given that even small, normal deviations of $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ ratios for standards from the internal reference values are likely to be caused, at least in part, by residual sample matrix in the MC-ICP-MS introduction system, a simple procedure was adopted for the correction of $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ ratios that were obtained for samples. To this end, the mean relative offset that was recorded for the two runs of matching Ag standard solutions that bracketed two subsequent sample runs was determined. This relative offset factor was subsequently applied to correct the $(^{109}\text{Ag}/^{107}\text{Ag})_{mbc}$ result determined for the bracketed sample, to obtain the final ‘standard offset corrected’ $(^{109}\text{Ag}/^{107}\text{Ag})_{soc}$ ratio (Fig 3.2).

(4) Using the data reduction methods summarized by Laycock et al. [24], the final $(^{109}\text{Ag}/^{107}\text{Ag})_{\text{soc}}$ ratio determined for a sample was used to calculate the mass and molar ratio of enriched Ag-En in a sample relative to the total amount of Ag that is present ($\text{Ag-En}/\text{Ag-Tot}$). The only additional information required for this is the $^{109}\text{Ag}/^{107}\text{Ag}$ ratio of the enriched Ag that is present in the sample and of the natural background, which can be gathered from the isotopic analyses of the Ag-En tracer material and the natural Ag reference standard solution at high concentration. As mass dependent fractionation of $^{109}\text{Ag}/^{107}\text{Ag}$ in natural materials is known to be small ($<0.1\%$), these calculations can readily apply the Ag isotope composition determined for the Ag standard solution to characterise the $^{109}\text{Ag}/^{107}\text{Ag}$ ratio of the natural sample background.

(5) Finally, the total molar or mass-based Ag content of a sample was determined from the $(^{109}\text{Ag}/^{105}\text{Pd})_{\text{mbc}}$ ratios that were calculated for each sample and standard solution in Step (2). To this end, a two-point calibration line was established for each individual sample, based on the $(^{109}\text{Ag}/^{105}\text{Pd})_{\text{mbc}}$ data that were obtained for a bracketing standard that best matches the Ag concentration of the sample of interest and which was analysed in close temporal proximity (Fig. 3.2 and 3.3). With this calibration, the $(^{109}\text{Ag}/^{105}\text{Pd})_{\text{mbc}}$ ratio determined for a sample can be applied to determine the ^{109}Ag concentration of the sample solution following Equation 3.1, as both sample and standard solutions feature the same concentration of Pd and hence ^{105}Pd .

$$c_n(^{109}\text{Ag in sample}) = \frac{c_n(^{109}\text{Ag in standard})}{\left(\frac{^{109}\text{Ag}}{^{105}\text{Pd}}\right)_{\text{mbc,std}}} \times \left(\frac{^{109}\text{Ag}}{^{105}\text{Pd}}\right)_{\text{mbc,sam}} \quad (\text{Equation 3.1})$$

The ^{109}Ag concentration of the sample solution can be readily used to calculate the amount of substance $n(^{109}\text{Ag})$ present in the sample by considering the solution volume V and dilution factor DF (Equation 3.2)

$$n(^{109}\text{Ag}) = \frac{c_n(^{109}\text{Ag in sample}) \times V_{\text{sam}}}{DF} \quad (\text{Equation 3.2})$$

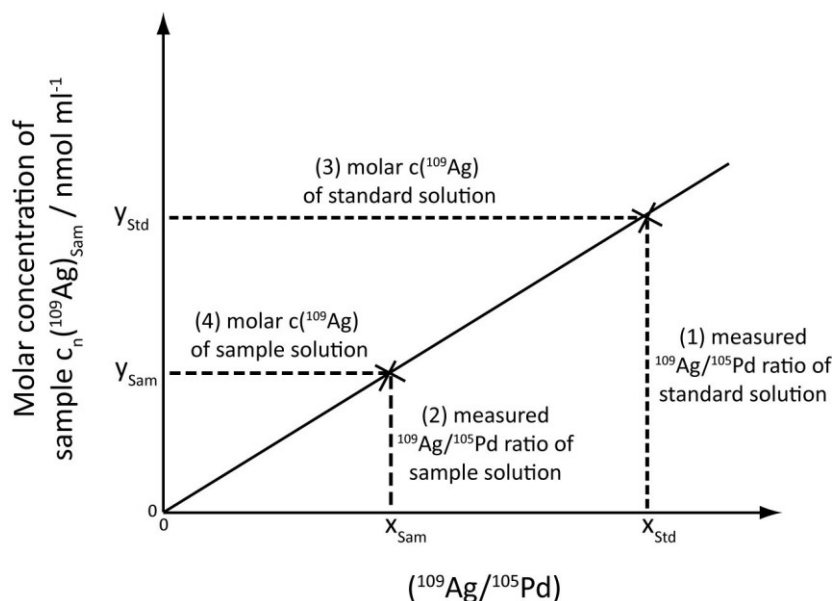


Fig. 3.3. Calibration line method that was employed to determine molar concentrations of Ag present in the MC-ICP-MS sample solutions. The $^{109}\text{Ag}/^{105}\text{Pd}$ ratio of a mixed Ag-Pd standard solution is measured and together with the origin, this defines the slope of a correlation line in a plot of ^{109}Ag concentration versus $^{109}\text{Ag}/^{105}\text{Pd}$ ratio. The $^{109}\text{Ag}/^{105}\text{Pd}$ ratio determined for a standard that has the same Pd concentration as a sample can then be employed to determine the sample ^{109}Ag concentration.

This, combined with the known isotope compositions of Ag-En and Ag-Nat (see above) as well as the $(^{109}\text{Ag}/^{107}\text{Ag})_{\text{soc}}$ ratio of a sample then yields the total Ag content of a sample. Together with the sample weight and the $(\text{Ag-En}/\text{Ag-Tot})$ ratio calculated in Step (4), this can be applied to determine both the total Ag mass fraction of the sample (in units of mol g^{-1} or ng g^{-1}) as well as the Ag-En sample mass fraction, again either in units of mol g^{-1} or ng g^{-1} . All equations necessary for these calculations are provided in Section 4.2.3.5.) of this thesis.

(6) In a last step, the $(^{109}\text{Ag}/^{107}\text{Ag})_{\text{soc}}$ and $(\text{Ag-En}/\text{Ag-Tot})$ ratios as well as the total Ag and Ag-En contents can be corrected for the procedural blank contribution. To this end, the data recorded for the procedural blank was treated identical to any other sample measurement, so that the ^{107}Ag and ^{109}Ag content of the blank as well as its isotopic composition is determined. The total amount of substance $n(\text{Ag})$ of the procedural blank was subtracted from

the amount of substance $n(\text{Ag})$ of the corresponding sample measurement. The final, blank corrected Ag content of the samples can then be calculated following Step (5).

3.3. Results and discussion

3.3.1. Analytical scenarios

The application of stable isotope tracing for the detection and quantification of Ag NPs encompasses variable analytical conditions with two distinct endmember scenarios. On the one hand, there are samples with little or no labelled material that have natural or near-natural isotope compositions. On the other hand, there are samples with variable but generally low natural Ag contents that contain variable but possibly quite high proportions of added isotopically enriched Ag. The consequences of these scenarios are illustrated in Fig. 3.4, which shows how $^{109}\text{Ag}/^{107}\text{Ag}$ changes as a function of the mass fraction of isotopically enriched material present relative to the total amount of Ag in a sample or system ($\text{Ag-En}/\text{Ag-Tot}$). The calculations employ ^{109}Ag as the enriched spike material, with a composition similar to the actual Ag-En used in this study. The ($\text{Ag-En}/\text{Ag-Tot}$) parameter is a key result of the tracing analyses for samples that is derived from the measurement of $^{109}\text{Ag}/^{107}\text{Ag}$. Also shown in the Fig. 3.4 is the error that results for ($\text{Ag-En}/\text{Ag-Tot}$) if the measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratio is offset from the true value by an analytical error of 0.2%, 2% and 15%.

At low values of ($\text{Ag-En}/\text{Ag-Tot}$), which denote natural or near-natural Ag isotope ratios, the curves reveal that high precision measurements are necessary to resolve whether a sample contains a small amount of the isotopic spike or not. The precision of such analyses thereby determines the smallest amount of isotopic tracer that can be resolved from the natural background with confidence, termed ‘detection sensitivity’ in the following. To a first order, this is primarily a function of the amount of Ag available for measurement.

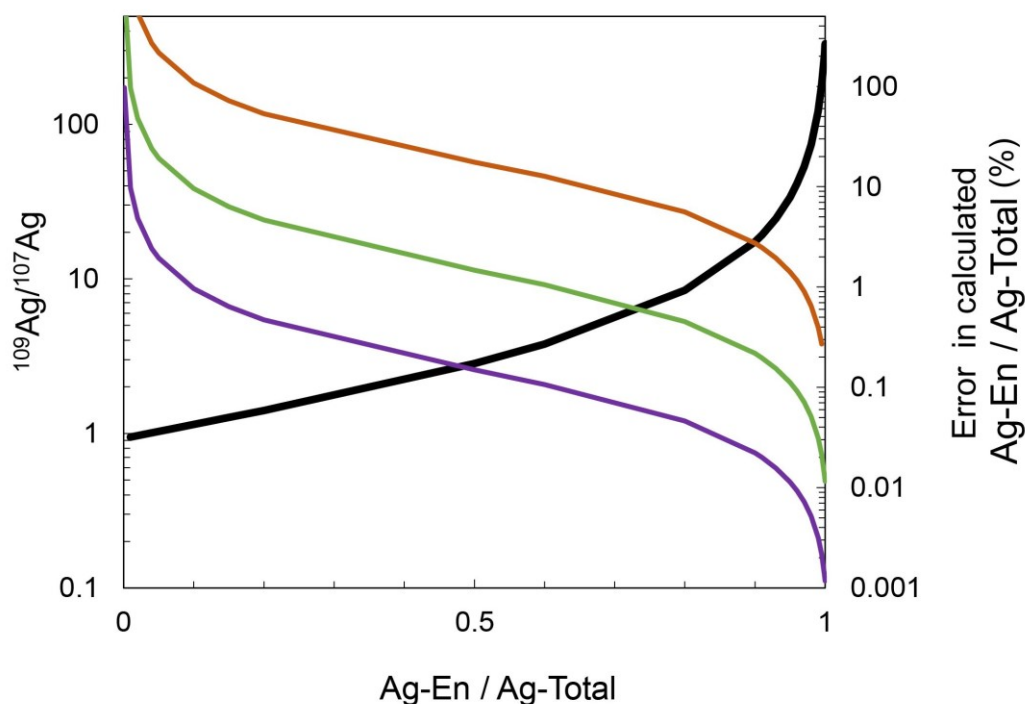


Fig. 3.4. Change of the $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratio with the relative proportion of Ag-En present, as defined by the Ag-En/Ag-Tot ratio. Also shown is the uncertainty that arises for a calculated Ag-En/Ag-Tot ratio when the measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratio is uncertain to 0.2% (purple curve), 2% (green curve) and 20% (red curve).

When higher proportions of isotopically enriched Ag are present, uncertainties in the measurement of $^{109}\text{Ag}/^{107}\text{Ag}$ ratios for samples have a reduced impact on the determination of (Ag-En/Ag-Tot). Specifically, for samples that contain primarily enriched Ag at (Ag-En/Ag-Tot) $\geq 90\%$, even errors of 20% or more in the determination of $^{109}\text{Ag}/^{107}\text{Ag}$ have little impact on (Ag-En/Ag-Tot) and result in errors of less than 5%. Even though such analyses do not require highly precise $^{109}\text{Ag}/^{107}\text{Ag}$ measurements, they are difficult for other reasons. In particular, they may involve measurements of very small ion beam intensities for the unspiked ^{107}Ag isotope. Highly variable ^{109}Ag ion beam intensities from variably spiked samples furthermore require careful control of memory effects in the instrument. In addition, a key result of such analyses is the determination of the mass fraction of enriched ^{109}Ag present in a sample which are subject to additional errors from incomplete Ag yields during sample preparation.

Less problematic in contrast are analyses of samples that feature intermediate Ag isotope compositions and hence intermediate levels of mixing between Ag-Nat and Ag-En. Such conditions, particularly at $(Ag-En/Ag-Tot) \approx 0.8$ or less (equivalent to $^{109}Ag/^{107}Ag \approx 10$ or less) are, in principle, conducive for precise $^{109}Ag/^{107}Ag$ measurements (Fig. 3.4). In particular, problems with the instrumental memory are de-magnified when variations in the $^{109}Ag/^{107}Ag$ ratio are less extreme. Statistical principles thereby indicate that such measurements should achieve better reproducibility than analyses of natural Ag, for a given ion beam intensity of ^{107}Ag . The impact of any error in the isotopic measurement on the determination of $(Ag-En/Ag-Tot)$ thereby decreases steadily with increasing $^{109}Ag/^{107}Ag$ (Fig. 3.4).

3.3.2. Evaluation of analytical procedures

In the following the role of key analytical procedures on the performance of the methods is assessed.

3.3.2.1. Sample preparation and separation procedure

The advantage of the presented anion exchange chromatography protocol (Table 3.1) compared to previously published Ag separation methods by ion exchange chromatography [77, 80, 81] is that it greatly reduces sample processing time. Whereas a three-stage ion exchange chromatography requires at least 2-3 days of experimental work, the developed method enables the processing of up to 30 samples through anion exchange chromatography in six hours. Its disadvantage is that the purity of the Ag fraction is lower than in previously developed multi-stage procedures [77, 80, 81]. However, the present work shows that sample solutions are sufficiently pure so that the isotope compositions of samples with both natural and spiked Ag isotope signatures can be analysed with appropriate accuracy. In particular, this

requires that spectral interferences are not present in the final sample solutions for the isotope ratios of interest, which are $^{109}\text{Ag}/^{107}\text{Ag}$ and $^{105}\text{Pd}/^{104}\text{Pd}$, whereby the latter is used for mass bias correction.

There are no isobaric interferences for either isotope of Ag whilst ^{104}Pd overlaps with ^{104}Ru (Table 3.3). It was reported previously that Ru is not fully separated from the sample during the anion exchange chromatography [80]. However, the interfering Ru isotope ^{104}Ru has a natural abundance of only 0.19% and the 10 ng ml^{-1} Pd concentration that was employed in this study ensured that any trace amounts of natural Ru in the final sample solutions had no impact on the accuracy of the measured $^{105}\text{Pd}/^{104}\text{Pd}$ ratios and subsequently, after mass bias correction, on $^{109}\text{Ag}/^{107}\text{Ag}$ data.

Obtaining a complete separation of Ag from Cd is difficult, as the latter is efficiently eluted from the anion exchange resin prior to Ag with the same $0.5\text{ mol l}^{-1}\text{ HNO}_3$ eluent. Cadmium has the minor isotopes ^{106}Cd and ^{108}Cd (with abundances of roughly 1%) which are isobaric to ^{106}Pd and ^{108}Pd (Table 3.3). To ensure high Ag yields and accurate mass bias correction, ^{106}Pd and ^{108}Pd were hence not employed for the external normalization of the Ag isotope data.

Isotope ratio measurements can furthermore be biased by polyatomic spectral interferences. Considering the potential spectral interferences (Table 3.3) $^{68}\text{ZnAr}^+$, $^{66}\text{ZnAr}^+$ and $^{64}\text{ZnAr}^+$ are probably the most problematic, and can cause interferences on $^{108}\text{Pd}^+$, $^{106}\text{Pd}^+$ and $^{104}\text{Pd}^+$, which are used for external normalisation. The anion exchange chromatography was modified to include a $0.005\text{ mol l}^{-1}\text{ HCl}$ elution step that efficiently decreased the overall Zn content of the samples [91]. Spectral interferences were thereby reduced and mass scans revealed signal intensities of $<50\text{ mV}$ at mass number 68, which was deemed tolerable considering the application of the method for stable isotope tracing of labelled ENMs.

Over the course of the study, procedural blanks were carefully monitored. These analyses document a blank that varied between 1 pg and 26 pg with a mean value of 7 ± 7 pg (1 SD, $n = 32$). Careful control of the procedural blank is important as it limits the accuracy to which small Ag samples can be analysed with confidence to resolve isotopic differences. Careful acid cleaning of labware, particularly if this comes into contact with processed Ag, is important to prevent a gradual build-up of blank. An even more important issue arises from contamination of the samples by the natural Ag from jewellery worn in the laboratory. The highest blanks that were measured here arose when a laboratory user, who did not handle any of the samples, inadvertently wore Ag jewellery in the same laboratory. The high blank levels of >20 pg dropped immediately once Ag jewellery was rigorously excluded from the laboratory and all vessels had been carefully acid cleaned. Excluding these anomalously high blanks, the average procedural blank was 5 ± 4 pg (1 SD, $n = 28$). As such, the method is suitable for robust analyses of samples even with Ag contents of 20 pg or less.

The overall Ag yields of the procedure are important as they have an impact on the accuracy to which the Ag-En and total Ag mass fractions of samples can be determined. Most concerning are Ag losses during sample dissolution and, in particular, the column chemistry for Ag separation. To assess the procedural Ag yields, known quantities of Ag-En of between 70 pg and 6 ng, were added to 7 soil samples and the amount of recovered Ag-En was determined from measurements of the $^{109}\text{Ag}/^{105}\text{Pd}$ elemental ratio and $^{109}\text{Ag}/^{107}\text{Ag}$. The determined Ag yields varied between 77% and 118%, whereby results above 100% most likely reflect uncertainties in the calibration that is applied to determine the ^{109}Ag concentrations of the analysed sample solution. The assessed sample yields give a mean result of 104 ± 13 % (1 SD), which compares favourably to yields of 75 to 100% and 60% to 105% reported by Woodland et al. [80] and Theis et al. [88], respectively, and is in line with yields of ~99% achieved after careful calibration by Schönbächler and co-workers [81].

3.3.2.2. *Correction of instrumental memory*

Memory effects are always present in ICP-MS. They typically stem primarily from the sample introduction system and are known to be particularly severe for the high sensitivity desolvation sample introduction system that was employed here, due to the large surface area that comes into contact with samples. In general terms, such memory effects are of little consequence if (i) the isotopic signature of the memory is identical or very similar to the isotopic composition of the sample and (ii) if the memory effect comprises only a small fraction of the overall recorded sample signal. As such, in most cases memory effects are not problematic for isotopic analyses that focus on resolving small natural variations in isotope compositions for various elements using relatively large samples (generally >10 ng) to obtain high precision data. In such cases, simple washing protocols, generally involving the use of dilute HNO₃, are employed between sample measurements to sufficient effect. Some studies, particularly of Pb isotope compositions, which show relatively large natural isotope variations, additionally employ short on-peak measurements of the instrumental memory to improve data accuracy [92].

The conditions encountered in the current work differed from this in two key ways. First, it involves analyses of samples that have extremely variable Ag isotope compositions that can vary from natural (~1) or near-natural (~2-3) to highly non-natural ¹⁰⁹Ag/¹⁰⁷Ag (>10). Furthermore, the Ag-Nat and Ag-En content of the samples can be highly variable depending on sample size as well as the amount of Ag-En present in a sample relative to Ag-Nat. As a consequence, measurements revealed that the memory effects, which are encountered in analytical sessions that involve analyses of sample solutions with variable Ag concentrations and variable extents of Ag-En present, have a key impact on the quality of the results.

This is illustrated by data that were collected in two consecutive analytical sessions (Fig. 3.5). These featured analyses of samples that had ¹⁰⁹Ag/¹⁰⁷Ag ratios between the natural

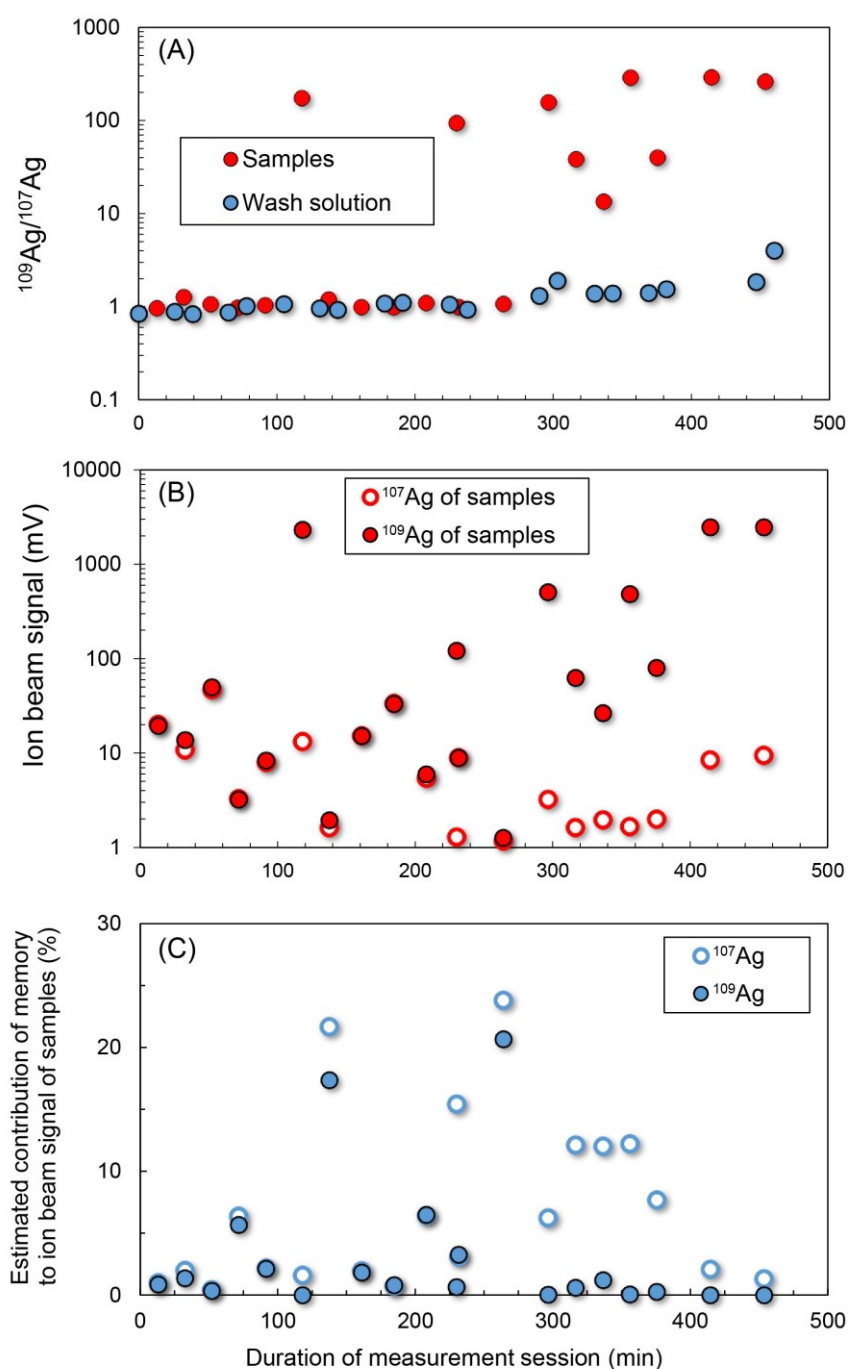


Fig. 3.5. Results acquired in two subsequent measurement sessions. (A) Isotopic composition of the instrumental memory effect measured on wash solutions that were run in between sample analyses. (B) The ^{107}Ag and ^{109}Ag ion beam intensities of the environmental samples that were analysed in the measurement sessions. (C) Approximate relative contribution (in %) of the instrumental memory effect to the ^{107}Ag and ^{109}Ag ion beam signals measured for the samples.

value of about 0.95 and nearly 300, whilst the ^{107}Ag and ^{109}Ag ion beam intensities varied between 1 to 50 mV and 3 to 2500 mV respectively, which is equivalent to ^{107}Ag and ^{109}Ag concentrations of about 4 to 200 pg ml^{-1} and 10 pg ml^{-1} to 10 ng ml^{-1} (Fig. 3.5). During the course of the sessions, the $^{109}\text{Ag}/^{107}\text{Ag}$ isotopic signature of the background shifted from ~ 0.95 , which mirrors the natural isotopic composition of Ag, to values of up to 4-5 (Fig. 3.5A).

While the overall signal intensity of the background is typically only equivalent to $<5 \text{ pg ml}^{-1}$ it contributes disproportionately to the non-enriched isotope with especially prominent consequences for samples with low Ag content. The amount of ^{109}Ag that is introduced to the system is higher than for ^{107}Ag and the background signal retains more memory of ^{109}Ag ions compared to ^{107}Ag , thus showing an increasingly non-natural $^{109}\text{Ag}/^{107}\text{Ag}$ ratio (Fig. 3.5). However, samples that contain mainly enriched ^{109}Ag will have a small ^{107}Ag fraction which might only be equivalent to 1 pg of ^{107}Ag present in a sample containing 1 ng total Ag. Even small disturbances introduced by the background signal are therefore able to double or triple the total ^{107}Ag signal and thereby skew the calculated isotopic ratio enormously. For samples with enriched ^{109}Ag , the background signal contributed up to 20% of the overall non-enriched ^{107}Ag isotopic signal whereas it was the cause for less than 1% of the enriched ^{109}Ag signal (Fig. 3.5C).

As such, an accurate procedure for monitoring and correcting memory effects, as adopted here, is instrumental for ensuring data quality. The successful approach of the presented protocol for the correction of analytical artefacts that stem from memory effects is highlighted in Fig. 3.6. Here, the mixed Ag-En + Ag-Nat standard solution was analysed at varying solution concentrations of $\sim 0.02 \text{ ng ml}^{-1}$ to $\sim 2 \text{ ng ml}^{-1}$ and the recovered $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratios with (black circles) and without (white circles) the memory correction are shown.

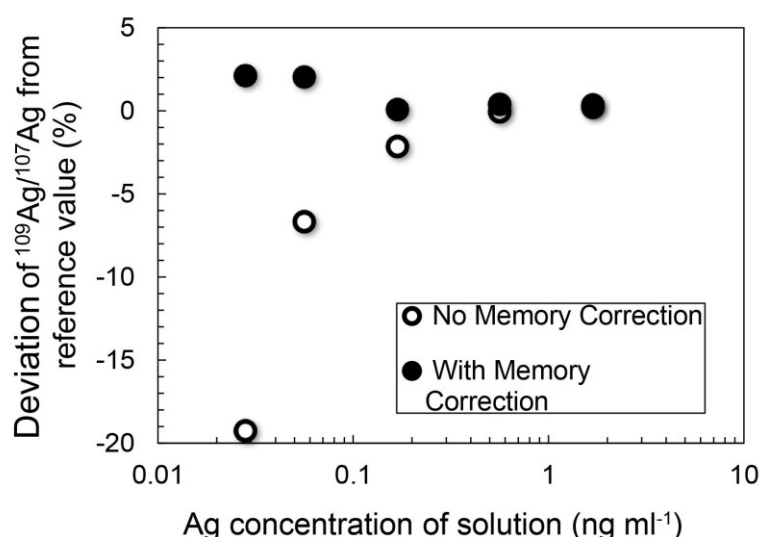


Fig. 3.6. Relative deviation (in %) of measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratios from the reference value obtained in analyses of Ag standard solutions with a non-natural isotope composition and Ag concentrations of about 0.02 to 2 ng ml⁻¹. The plot compares the isotopic results that were obtained without use of a memory correction during data reduction (open symbols) and with addition of a memory correction (filled symbols).

In this, the separately measured isotope ratio of the undiluted, high concentration Ag-En + Ag-Nat standard is assumed to be unaffected by memory effects and thus used as a reference value. As Fig. 3.6 shows, correcting for memory effects does not influence the recovery of the $^{109}\text{Ag}/^{107}\text{Ag}$ ratio for concentrations over >1 ng ml⁻¹, but the improvements in data quality become very prominent as the concentration of Ag in the standard solution decreases. For the lowest analysed concentration of 0.02 ng ml⁻¹ a simple correction for memory effects improves the ability to recover the diagnostic $^{109}\text{Ag}/^{107}\text{Ag}$ ratio by approximately 20%.

Furthermore, it is helpful if analyses of samples with natural Ag isotope compositions and very high $^{109}\text{Ag}/^{107}\text{Ag}$ ratios are conducted in different measurement sessions and in order of increasing concentration of Ag or natural ^{107}Ag , for samples with predominantly Ag-Nat and Ag-En, respectively.

3.3.3. Results for standards solutions

3.3.3.1. Standard solutions with natural Ag isotope compositions

Standard solutions with natural Ag isotope compositions and variable concentrations were analysed to characterise the performance of the mass spectrometric data acquisition protocols. In detail, 5 $\mu\text{g ml}^{-1}$ and 30 $\mu\text{g ml}^{-1}$ Alfa Aesar Ag solutions were analysed repeatedly and were further used to bracket single runs with Ag concentrations of 0.1 to 2 ng ml^{-1} and 5 to 15 ng ml^{-1} , respectively.

Fig. 3.7 reports the relative deviation of the $^{109}\text{Ag}/^{107}\text{Ag}$ data for each sample from the mean value measured for the bracketing standards. This ‘standard-sample bracketing’ approach, also commonly used for Ag stable isotope analyses, produces data of excellent accuracy. A key observation is that the isotopic deviations are larger for solutions with lower Ag contents. In detail, the observed deviations are ubiquitously smaller than 0.05% for solutions with Ag concentrations of 1 $\mu\text{g ml}^{-1}$ or more but somewhat worse (at 0.03 to 0.18%) for more dilute solutions with 0.01 to 2 ng ml^{-1} Ag. This result is most likely a consequence of poorer counting statistics and a larger impact of detector noise at low ion beam intensities. Aside from this, the data for the most dilute Ag solutions (with 0.01 to 2 ng ml^{-1}) reveal no systematic trend of isotopic deviations with Ag content (Fig. 3.7). Whilst unsurprising, this result is important, as it demonstrates that accurate results can be obtained for $^{109}\text{Ag}/^{107}\text{Ag}$ analyses of samples even if the Ag concentrations of the bracketing standards are not well matched to the concentration of a sample.

The repeated analyses of solutions with Ag concentration of 5 and 30 ng ml^{-1} yielded mean offsets and precisions (quoted here and below as 1 SD) of 0.0010 ± 0.0011 ($n = 21$) and 0.0002 ± 0.0003 ($n = 4$), respectively (Fig. 3.7). The new methods can therefore provide Ag isotope data which feature a trueness and precision that is similar to that reported for Ag

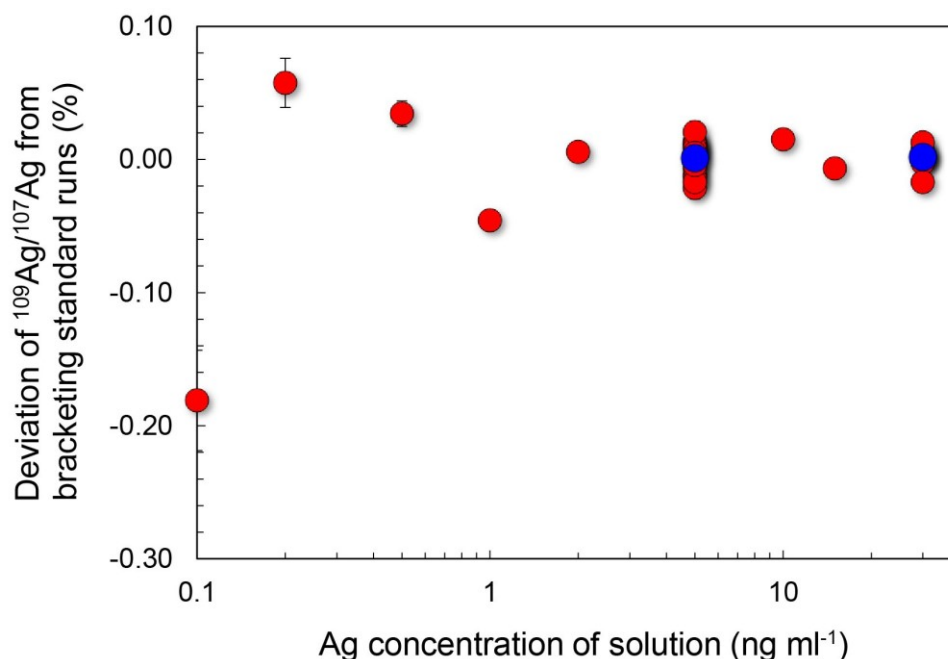


Fig. 3.7. Relative deviation (in %) of measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratios for standard solutions of natural Ag from the mean result obtained for bracketing standard solutions with Ag concentrations from the reference value obtained in analyses of Ag standard solutions. The red symbols denote the results of individual analyses, which show no trend with changing Ag solution concentrations. The blue symbols denote the mean results obtained for multiple analyses of 5 ng ml^{-1} and 30 ng ml^{-1} Ag standard solutions, which served as the bracketing standard solutions for the other analyses. These mean values have a deviation that is identical to 0%, within error (see text for details). Error bars (often smaller than the symbol size) denote the 1 SE within-run error of the measurements.

standard solution runs in the previous Ag isotope studies of Schönbächler et al. [78, 81] and Woodland et al. [80] As such, the methods are suitable for the less stringent analytical requirements posed by the stable isotope tracing technique.

3.3.3.2. Standard solutions containing Ag-En

An evaluation of data collected for Ag standard solution runs that were conducted interspersed between sample analyses on a number of different measurement sessions informs on the trueness and precision of the methods, with results also relevant for samples. This

follows from the observation that bracketing standard runs are, albeit to a lesser extent, also subject to the spectral and non-spectral interferences, which can affect samples in the presence of matrix [87]. However, in contrast to samples, standard solutions can be analysed multiple times and at variable dilution and a precise reference value for $^{109}\text{Ag}/^{107}\text{Ag}$ can be obtained even for non-natural isotope compositions from replicate analyses of solutions with high Ag concentrations.

The result of such analyses, obtained from runs of an Ag-En + Ag-Nat solution with $^{109}\text{Ag}/^{107}\text{Ag} \approx 18$ that bracketed analyses of real natural samples, with variable Ag-Tot and Ag-En present, are summarized in Fig. 3.8. Notably, the data were acquired in about 15 separate measurement sessions over a period of six months. They reveal a precision and trueness of better than about 0.5 to 1% for run solutions with Ag-Tot concentrations of $\geq 500 \text{ pg ml}^{-1}$, approximately equivalent to a consumption of $>500 \text{ pg Ag}$ for a single measurement. The accuracy of the data is poorer at lower concentrations, as a consequence of counting statistics, but even Ag-En + Ag-Nat solutions with Ag-Tot concentrations of about 30 to 50 pg ml^{-1} are sufficient to enable $^{109}\text{Ag}/^{107}\text{Ag}$ ratios to be determined with a precision and accuracy of about 5% (Fig. 3.8).

These results demonstrate the optimal analytical performance that can be achieved for samples with non-natural Ag isotope compositions in measurement sessions with normal run conditions. Data from actual samples, however, are expected to return results of somewhat lower accuracy and/or precision for a given Ag concentration, as spectral and non-spectral interferences are likely to be worse and blank problems might occur for very small samples. Furthermore, it is important to check the quality of the standard solution data obtained in the analyses bracketing samples, as they may indicate instrument- and sample-related analytical problems that can impact accuracy. As such, the bracketing standard runs constitute an important quality control mechanism during data collection.

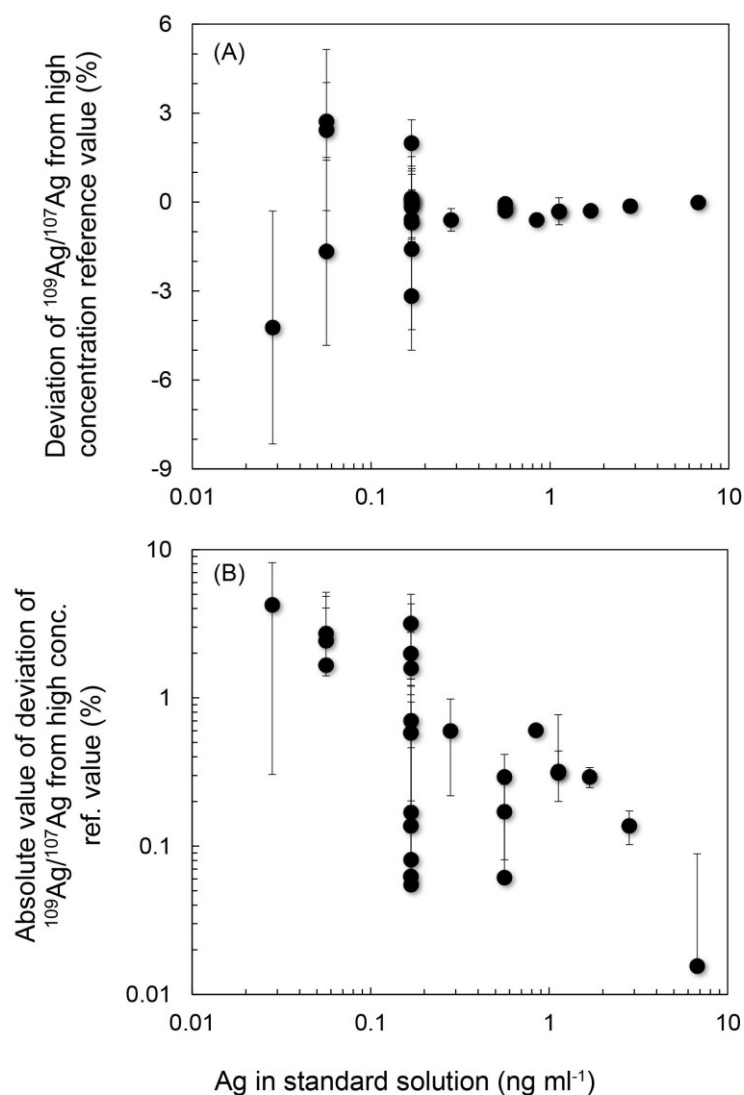


Fig. 3.8. (A) Relative deviation and (B) absolute value of the relative deviation (both in %) of $^{109}\text{Ag}/^{107}\text{Ag}$ data obtained in individual analyses of Ag-En + Ag-Nat solutions at various concentrations from the $^{109}\text{Ag}/^{107}\text{Ag}$ reference value that was determined by runs at high Ag concentrations. Error bars (often smaller than the symbol size) denote the 1 SE within-run error of the measurements.

3.3.4 Results for samples

3.3.4.1. Samples with natural Ag isotope compositions

Although the current method was developed primarily for analyses of samples that contain variable quantities of Ag-En, it is also important that high quality data can be obtained for samples with natural and near-natural Ag isotope compositions. In particular, the accuracy

of such measurements is important as it determines the minimum amount of added enriched Ag which can be detected in a sample against the natural Ag background. Furthermore, environmental studies routinely feature one or several control lines alongside the actual exposures to confirm that the exposure system is not compromised by any cross-contamination between the different lines and/or to establish the elemental background concentration and its natural variability.

Both soil and biological samples with different Ag contents were processed through the sample preparation procedure and subsequently analysed for their Ag isotope composition relative to an unprocessed Ag standard solution (Table 3.4, Fig. 3.9). The data reveal that offsets from the natural isotope composition are increasingly smaller as more Ag is available for isotopic analysis. In detail, the offsets from the natural reference value are less than $\pm 1\%$ for samples that contain more than 20 pg Ag while they increase to 5% and even up to 15% for samples with less than 20 pg Ag content. This reflects both the poorer counting statistics of analyses for small samples and larger systematic errors in the measurements, e.g., from spectral and non-spectral interferences or memory effects, which are more problematic at low ion beam intensities.

Given the results presented in Fig. 3.9, the smallest isotopic offsets in the $^{109}\text{Ag}/^{107}\text{Ag}$ ratio that can be robustly ascribed to the presence of Ag-En in a sample (as opposed to the inaccuracy of a measurement) is about 15% for samples with ≤ 20 pg Ag, about 2% for samples with about 20 to 200 pg Ag and about 0.2% for samples with ≥ 1 ng Ag. The observed uncertainties in the measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratio of samples can be used to constrain the detection sensitivity of the method, as the smallest addition of Ag-En that can be reliably resolved against a given quantity of natural background Ag (Fig. 3.10). The detection sensitivity is about 7% for samples with ≤ 20 pg Ag, about 1% for samples with 20 to 200 pg Ag and about 0.1% for samples with ≥ 1 ng Ag, which is equivalent to the addition of 1 pg

Ag-En to roughly 10 pg, 100 pg and 1 ng of natural Ag, respectively (Fig. 3.10). This implies that the detection sensitivity is primarily governed by the accuracy of Ag isotope measurements, which in turn is mainly dependent on the amount of Ag available for measurement.

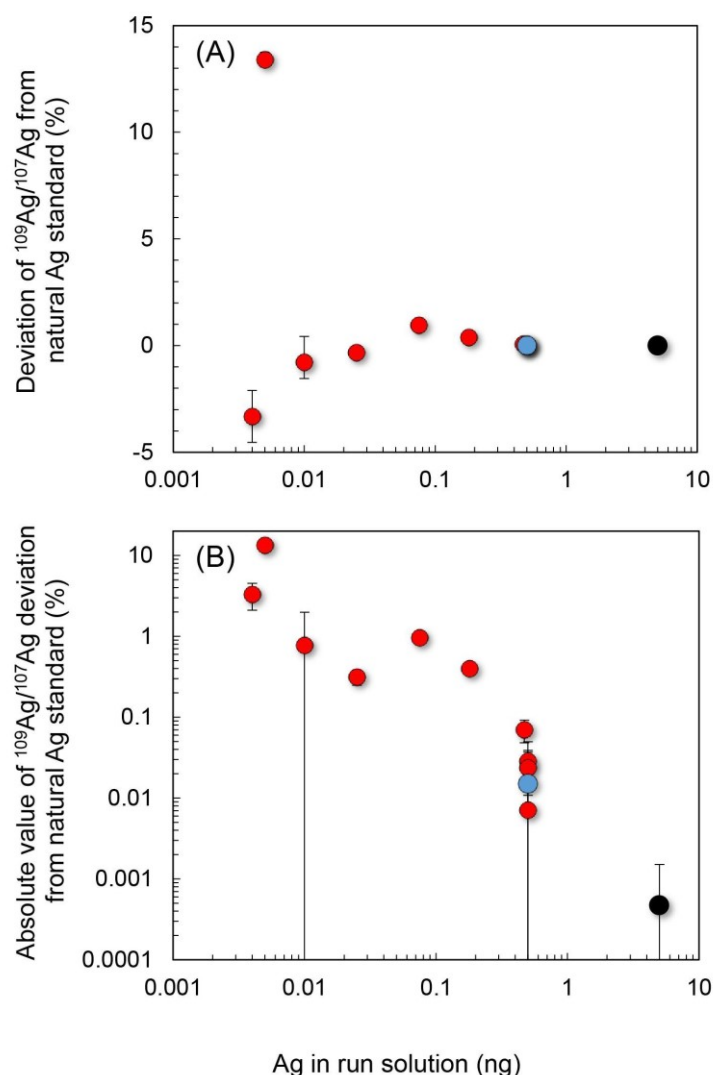


Fig. 3.9. (A) Relative deviation and (B) absolute number of the relative deviation (both in %) of $^{109}\text{Ag}/^{107}\text{Ag}$ data obtained in analyses of environmental samples with only Ag-Nat from the reference value of the natural $^{109}\text{Ag}/^{107}\text{Ag}$ ratio. Red symbols denote results obtained for individual sample solutions (see Table 3.4). The blue symbol is the mean value obtained for analyses of several aliquots of a single Lufa 2.2 Soil digest (Soil Digest 1; Table 3.4). The black symbol is the mean value for multiple analyses of a 5 ng ml⁻¹ natural Ag standard solution. Error bars (often smaller than the symbol size) denote the 1 SD calculated from the results of multiple analyses (see Table 3.4).

Table 3.4: Ag isotope data obtained for environmental samples that contain only natural Ag and a pure standard solution.

Sample	n	Deviation of $^{109}\text{Ag}/^{107}\text{Ag}$ from nat. Ag standard [%]	Relative standard deviation (RSD) [%]	Ag in sample aliquot [pg]	Ag consumed in single run [pg]	^{107}Ag ion beam [mV]
5 ng ml ⁻¹ Ag Standard	19	0.000	0.001		5000	~1250
Lufa Soil Digest 1 Aliquot 1	2	0.029	0.021	26,000	500	~60
Lufa Soil Digest 1 Aliquot 2	2	-0.007	0.019	26,000	500	~60
Lufa Soil Digest 1 Aliquot 3	2	0.024	0.013	26,000	500	~60
<i>Lufa Soil Digest 1 Aliquots 1-3</i>	<i>6</i>	<i>0.015</i>	<i>0.024</i>	<i>26,000</i>	<i>500</i>	<i>~60</i>
Lufa Soil Digest 2 Aliquot 1	4	0.070	0.022	1630	470	~80
Lufa Soil Digest 2 Aliquot 2	3	0.40	0.03	640	180	~30
Lufa Soil Digest 2 Aliquot 3	3	13	0.34	17	5	~1
Lufa Soil Digest 2 Aliquot 4	4	-3.3	1.2	15	4	~1
ERM BB422 fish muscle Digest 1	3	0.96	0.10	270	75	~10
ERM BB422 fish muscle Digest 2	4	-0.77	1.2	35	10	~1.5
Zebrafish Liver	3	-0.31	0.07	75	25	~5

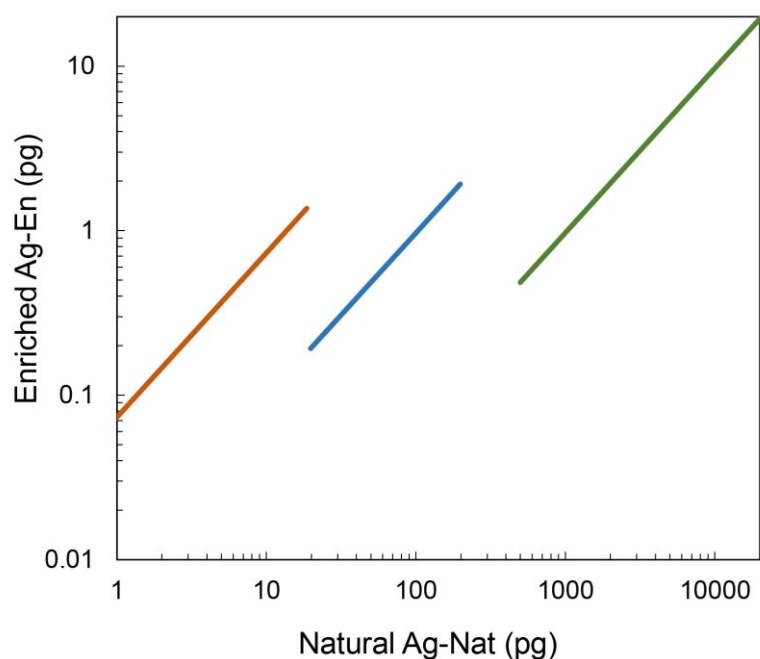


Fig. 3.10. Detection sensitivity for the detection of Ag-En for samples that have low (red line), medium (blue line) and high (green line) background contents of Ag-Nat, where the measurement errors are about 7%, 1% and 0.1%, respectively (see text for details). The results demonstrate that 1 pg of Ag-En can be detected in 10 pg, 100 pg and 1 ng of natural Ag.

3.3.4.2. Analyses of natural samples that contain Ag-En

The results of analyses of environmental samples that contained variable quantities of Ag-En and were processed through the sample preparation procedure are summarised in Fig. 3.11 and Table 3.5. Three types of spiked samples were analysed, Lufa 2.2 soil (Soil 1-3), ERM-BB422 (Fish muscle) and Zebrafish Liver, whereby the amount of Ag consumed in a single analysis varied between about 2 pg and 1.1 ng. The samples had $^{109}\text{Ag}/^{107}\text{Ag}$ ratios that varied from about 3 to 70 compared to a natural Ag ratio of ~ 0.9 . As such, the data shown here encompass two of the analytical scenarios discussed earlier in Section 3.1., with analyses of samples with a relatively low $^{109}\text{Ag}/^{107}\text{Ag}$ ratio of ~ 3 as well as samples that feature $^{109}\text{Ag}/^{107}\text{Ag} > 40$. As discussed in Section 3.1., samples with very high $^{109}\text{Ag}/^{107}\text{Ag}$ consist primarily of Ag-En and hence have $\text{Ag-En}/\text{Ag-Tot}$ ratios of > 0.9 (Fig. 3.11B, blue and black

circles). This stands in contrast to samples with $^{109}\text{Ag}/^{107}\text{Ag} \approx 3$, which contain only about 55% Ag-En (Fig. 3.11B, red circles).

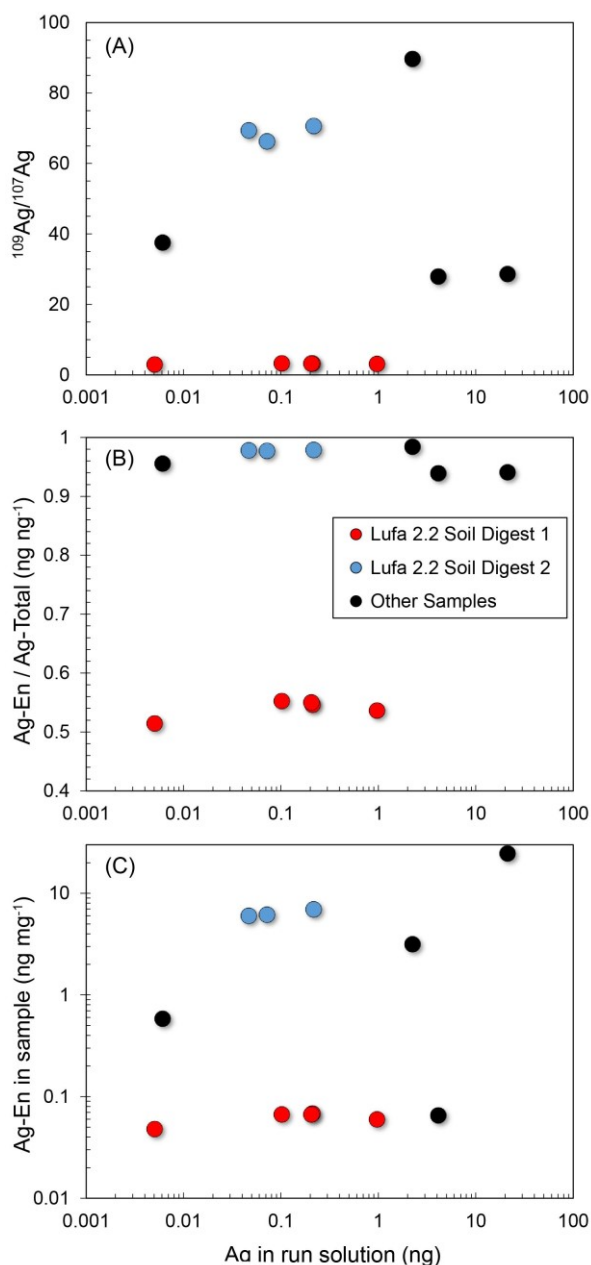


Fig. 3.11. Data obtained from the analyses of environmental samples that contain variable quantities of Ag-En to produce variable isotope compositions (Table 3.5). Panel (A) shows the $^{109}\text{Ag}/^{107}\text{Ag}$ data, (B) the Ag-En/Ag-Tot ratios and (C) the Ag-En mass fractions.

Table 3.5: Ag isotope and concentration data for environmental samples spiked with isotopically enriched ^{109}Ag at varying levels and their influence on measurement trueness and precision.

Sample	n	$^{109}\text{A}/^{107}\text{Ag}$	1 SD	1 RSD	Ag-En / Ag-Total	1 SD	1 RSD	Ag-En level	1 SD	1 RSD	Ag in aliquot	Ag consumed	^{107}Ag ion beam
				[%]	[ng ng ⁻¹]		[%]	[ng mg ⁻¹]		[%]	[ng]	[ng]	[mV]
Soil 1 A1	2	3.10	0.00	0.04	0.536	0.0001	0.02	0.060	0.0003	0.43	4.2	1.0	100
			1										
Soil 1A2	3	2.92	0.02	0.6	0.514	0.002	0.4	0.048	0.001	1.1	0.7	0.005	0.8
Soil 1A3	1	3.25			0.552			0.067			0.9	0.1	11
Soil 1A4	1	3.19			0.546			0.068			1.9	0.21	21
Soil 1A5	1	3.23			0.550			0.067			1.9	0.21	22
<i>Soil 1 mean</i>	<i>5</i>	<i>3.14</i>	<i>0.11</i>	<i>3.5</i>	<i>0.54</i>	<i>0.013</i>	<i>2.3</i>	<i>0.062</i>	<i>0.007</i>	<i>11.2</i>			
Soil 2 A1	1	69.4			0.978			6.00			0.05	0.04	4
Soil 2 A2	1	70.6			0.979			6.95			0.22	0.19	1.5
Soil 2 A3	3	66.3	1.6	2.5	0.977	0.001	0.07	6.15	0.02	0.3	0.07	0.03	0.2
Soil 3	3	37.5	0.6	1.7	0.9557	0.0008	0.1	0.584	0.001	0.2	0.006	0.002	0.1
Fish muscle	3	27.9	0.04	0.1	0.9391	0.0001	0.01	0.065	0.001	1.5	4.1	1.10	10
Zebrafish liver 1	4	28.6	0.1	0.4	0.9407	0.0002	0.03	24.61	0.06	0.2	21	0.30	4
Zebrafish liver 2	3	89.7	0.3	0.4	0.9843	0.0001	0.01	3.16	0.03	0.8	2.2	0.78	9

Regardless of the relative Ag-En content, it is important to assess the quality of the analytical data based on (i) repeated runs of the same sample Ag solution that was prepared for mass spectrometry and (ii) repeated sample analyses, whereby several aliquots of a single sample digest were processed and analysed separately. The data presented in Fig. 3.11 and Table 3.5 enable such an evaluation of data quality and are hence discussed in the following.

(i) Multiple mass spectrometric runs of the same sample Ag solution give results that display excellent precision (Table 3.5). For the $^{109}\text{Ag}/^{107}\text{Ag}$ and $\text{Ag-En}/\text{Ag-Tot}$ ratios, the observed precision roughly scales with the amount of Ag consumed in a single analysis. In detail, the analyses of Soil 1 (Soil 1 A2), ERM-BB422 (Fish muscle) and Zebrafish Liver 2, which consumed about 0.8 to 1.1 ng Ag per run have a reproducibility of better than 0.4% for $^{109}\text{Ag}/^{107}\text{Ag}$ and better than 0.01% for $\text{Ag-En}/\text{Ag-Tot}$. In contrast, the data for Soil 2 (Soil 2 A3) and Soil 3, where each run consumed less than about 30 pg Ag, have a precision of about 2% for $^{109}\text{Ag}/^{107}\text{Ag}$ and about 0.1% for $\text{Ag-En}/\text{Ag-Tot}$. This rough correlation between data quality and Ag consumption per run is not, however, apparent for the Ag-En mass fraction results. Presumably this reflects that the precision of the Ag-En mass fraction data is more readily affected by the determination of the ^{109}Ag solution concentrations. In detail, replicate sample solution runs provide Ag-En mass fraction data with a reproducibility of about 0.2 to 1.5%, regardless of how much Ag is used in a single measurement (Table 3.5).

(ii) For samples that were split into separate aliquots that were processed and then analysed individually, the precision of the data is clearly somewhat worse than the results that were obtained in repeat measurements of the same sample solution (Fig. 3.11, Table 3.5). In detail, results are available for multiple aliquots of two Lufa 2.2 soil samples, Soil 1 and Soil 2. These two samples have isotope ratios of ~ 3 and ~ 70 and were split into 5 and 3 separate aliquots, respectively, that were subsequently processed and analysed separately. For both samples, the $^{109}\text{Ag}/^{107}\text{Ag}$ isotope data have a comparable precision of roughly 3%. Due to the

different $^{109}\text{Ag}/^{107}\text{Ag}$ ratios of the samples and the resulting error propagation, the uncertainties of the *Ag-En/Ag-Tot* results, however, are very different at 2.3% and 0.07% (Fig. 3.11, Table 3.5). Relatively large errors of about 11% and 6% were obtained for the Ag-En mass fraction data of these samples. This result is not surprising, however, given that the variable Ag yields of the column chemistry also have a direct impact on the precision of the measured Ag-En mass fractions.

A further important observation follows from a comparison of the individual aliquot results that were obtained for the Soil 1 and Soil 2 samples. For the former, the Ag mass per aliquot and individual run varied from 0.7 to 4.2 ng and 5 pg to 1 ng, respectively. For Soil 2, this variability was 50 to 220 pg and 30 to 90 pg. Importantly, for both samples, the results obtained for $^{109}\text{Ag}/^{107}\text{Ag}$, *Ag-En/Ag-Tot* and the Ag-En mass fractions do not vary systematically with the Ag content of the aliquots or the amount of Ag consumed per run. This demonstrates that the new analytical techniques are able to deliver accurate analytical data in analyses of samples that contain variable quantities of Ag-En, even if the samples contain only 50 pg Ag and/or if less than 10 pg Ag are consumed in a single mass spectrometric run.

3.3.5. Application of the methods to samples from exposures

The new methods that are presented here were used to process and analyse samples that were obtained from an aquarium exposure of zebrafish to isotopically labelled ^{109}Ag NPs. As part of this work, various types of biological tissue from both unexposed control and exposed zebrafish lines were analysed and selected results are presented in Fig. 3.12 and Fig. 3.13.

For the control samples, it is apparent that deviations from the expected natural Ag isotope ratio are smaller when more Ag is available for analysis. In detail, the deviation from the natural Ag isotope composition are $\leq 1\%$ for runs with ≥ 20 pg Ag and still better than 5% even for runs that consume only 0.2 to 10 pg Ag. Notably, for the smallest samples that contain

less than 10 pg Ag, the average deviation from the natural Ag reference standard is $-1.2 \pm 2.1\%$ (1 SD), which is equivalent to a measured $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratio of 0.941 ± 0.019 , compared to the NIST reference value of 0.929 (Fig. 3.12) [90].

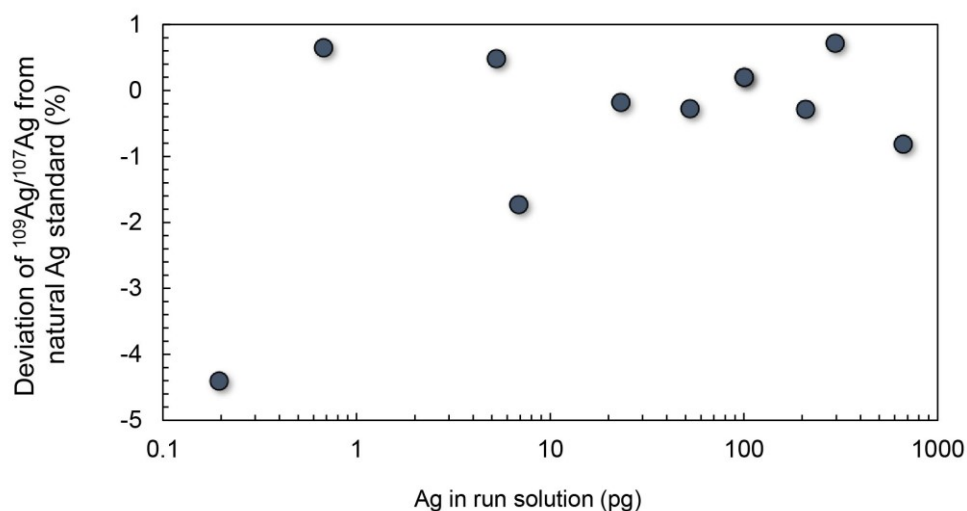


Fig. 3.12. Data obtained from exposure samples that acted as control lines. Their natural isotope composition was confirmed to within an error of $\pm 1\%$ for samples containing ≥ 20 pg Ag. For Ag contents lower than 10 pg, $^{109}\text{Ag}/^{107}\text{Ag}$ ratios can be recovered to within $\pm 5\%$.

In addition to control samples, two types of biological tissues from male and female zebrafish exposed to ^{109}Ag NPs were analysed (Fig. 3.13). Each data point represents a set of samples comprised of tissue samples collected from 4-6 individual zebrafish. While the details of the exposure are negligible at this point, suffice it to say that each sample set differs from the others in at least one specific parameter (e.g. only contains results obtained from male fish). The amount of Ag that was available for analysis of individual samples varied widely from approximately 10 pg to >20 ng and measured $^{109}\text{Ag}/^{107}\text{Ag}$ ratios ranged from 10 to ~300.

The data shown in Fig. 3.13 demonstrate that the here presented methodology is able to successfully analyse samples that result from the use of a ^{109}Ag tracer in an environmental exposure where low dosing concentrations were applied. Exposed and control lines can readily be distinguished (Fig. 3.12 and 3.13). Differences between exposure groups are resolvable by

the $^{109}\text{Ag}/^{107}\text{Ag}$ isotope measurements (Fig. 3.13A) and can be used to calculate the Ag levels present in samples (Fig. 3.13B).

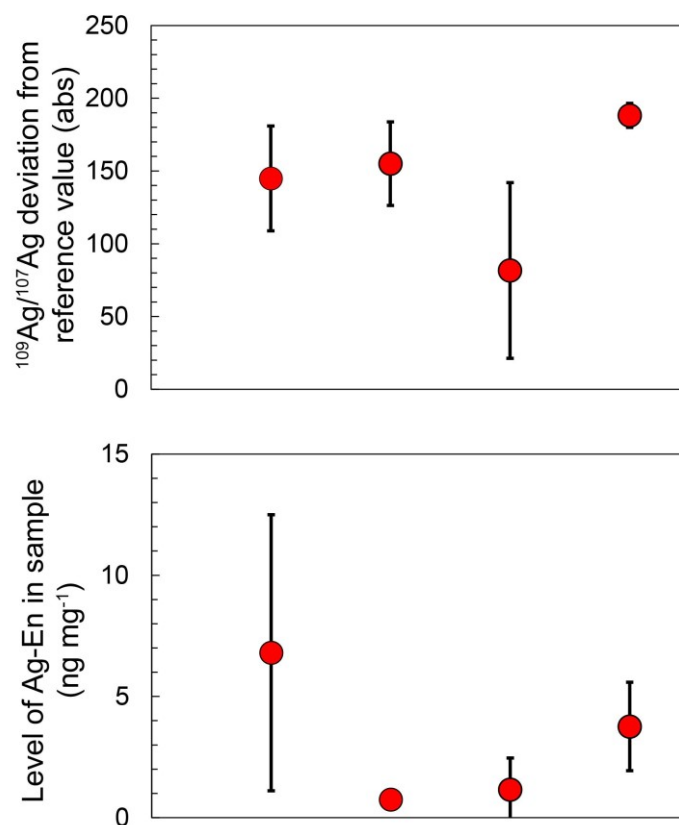


Fig. 3.13. Data from biological tissues sampled and analysed from a ^{109}Ag NPs exposure to zebrafish. Each data point represents a set of samples collected from separate zebrafish that were reared under identical conditions and each set of samples represents a different parameter (e.g. male/female, type of tissue).

3.4. Conclusions

The new ion exchange procedure that was developed for the separation of Ag from environmental samples facilitates the high sample throughput that is required to apply the stable isotope tracing technique to Ag NPs, which constitute one of the most industrially relevant ENMs. The one-step separation procedure efficiently separates Ag from the environmental

matrix at high yield ($104 \pm 13\%$, 1 SD) and produces a solution of sufficient purity to enable accurate Ag isotope measurements by MC-ICP-MS.

The ability to carefully correct for instrumental memory effects was found to be a key factor for improving data quality, as the combination of low Ag concentrations in many environmental samples and the introduction of Ag with widely varying $^{109}\text{Ag}/^{107}\text{Ag}$ ratios can otherwise readily generate erroneous results.

Standard solutions as well as natural soil and tissue samples with either natural Ag or Ag with a non-natural isotope composition were analysed to establish and document the analytical performance of the methodology. These analyses define a detection sensitivity, as the smallest addition of Ag-En that can be resolved against a given natural background, of about 7% for samples with ≤ 20 pg Ag, about 1% for samples with 20 to 200 pg Ag and about 0.1% for samples with ≥ 1 ng Ag. This is approximately equivalent to the addition of 1 pg Ag-En to 10 pg, 100 pg and 1 ng of natural Ag. This implies that the detection sensitivity is primarily governed by the accuracy of the Ag isotope measurements, which in turn is mainly dependent on the amount of Ag available for analysis.

Analyses of replicate aliquots of natural samples that contain variable quantities of Ag-En reveal that the $^{109}\text{Ag}/^{107}\text{Ag}$ ratios of such materials can be determined with a precision of better than 5%, even if only 30 to 50 pg of Ag are available for analysis. The precision of the Ag-En mass fraction data is worse at about 10%, which primarily reflects the variable yields of the sample preparation chemistry.

In summary, the comprehensive results that were obtained in this study show that the new analytical methods enable sensitive and accurate detection and quantification of a stable Ag isotope label in complex environmental samples. Furthermore, the methods also enable relatively rapid processing of samples, such that they are suitable for processing the large sample numbers that are often generated in environmental exposure studies involving Ag NPs.

3.5. Acknowledgments and declaration

Mark Rehkämper (MR) and Adam Laycock (AL) provided guidance during the development of the ion exchange chemistry and mass spectrometric methods. Tabea Junk (TJ) carried out all analytical work and prepared the text, with guidance from MR for the latter.

Chapter 4

Multigenerational study of isotopically labelled Ag nanoparticles in *Danio rerio* – transgenerational effects of maternal exposure

Abstract

The antimicrobial properties of silver nanoparticles (Ag NPs) have led to a wide spectrum of applications, of which many are related to medical and personal hygiene products. Their increasing use results in their widespread and often uncontrolled release into the natural environment, where their behaviour is not yet fully elucidated. Specifically, the long-term effect and potential bioaccumulation of silver nanoparticles (Ag NPs) are not well studied or understood, especially at low and environmentally relevant dosing concentrations.

In this work, the results of a diet-borne exposure of the zebrafish *Danio rerio* to isotopically labelled ^{109}Ag NPs are presented. The exposure was conducted over consecutive generations and Ag NPs were added to the feed of *D. rerio* at concentrations that match their expected release levels. Ag NPs were successfully detected in the livers and gonads of male and female zebrafish after diet-borne exposure over three weeks. The results indicate that at these low exposure levels, Ag NPs do not accumulate in the liver and gonad tissue of *D. rerio* over consecutive generations of exposure. It was further shown that within one generation of non-exposure, Ag NP levels in the zebrafish tissues are sufficiently low that they can no longer be detected by means of isotopic tracing. In addition, for the first time, this work conclusively shows that Ag NPs can be transferred through maternal exposure, such that they can be detected in zebrafish embryos that did not experience any direct contact with Ag NPs. This suggests that zebrafish embryos are exposed to Ag NPs during the earliest and most vulnerable stages of their development.

4.1. Introduction

Recent developments in the ability to design and manufacture nanoparticles (NPs) have led to a stark global increase in industrial output of engineered nanomaterials (ENMs) [4]. Compared to their bulk counterparts, NPs show unique properties due to their small size and comparatively high resulting surface area. In addition, the use of ENMs allows well-known properties of a material to be exploited in targeted applications at lower concentrations, thereby reducing the associated costs. This is especially true for Ag, which, due to its antimicrobial properties, has long been used for medical applications. More recently Ag NPs have found use in a variety of commercial applications such as water filters, cosmetics, deodorants, textiles, wound dressings and food packaging [93-101].

As the market for applications in consumer products grows, it is expected that the annual global production and consumption of Ag NPs will increase, with output estimates ranging from 55 ton year⁻¹ to 450 ton year⁻¹ [4, 102]. This expected rise in Ag NP production will be accompanied by an upsurge in the discharge of the material to the environment, often in an uncontrolled manner. Due to the typical uses of Ag NPs, such as in cosmetics and deodorants, a main release pathway into the environment is the direct discharge into freshwaters as well as indirect release to water bodies, e.g. through washing of textiles.

Research into the environmental fate and implications of Ag NPs has intensified in recent years and numerous studies have been published that investigate the behaviour of Ag NPs in geological compartments (e.g., soils, natural waters) and biological tissues (e.g., freshwater animals, humans) [103]. There is a wide range of literature that covers the effects of Ag NPs on crustaceans such as *Daphnia magna* in acute, chronic and multigenerational exposure studies, which involve different types of Ag NPs and a variety of exposure conditions, such as the influence of high and low food rations [72, 74, 104-107]. However, there is less information available regarding the effects of Ag NPs on freshwater fish, including for

experimental models such as *Danio rerio* (zebrafish), which has been adopted widely in ENM ecotoxicity study [108]. Most research focuses on the potential of acute toxicity of Ag NPs on the development of zebrafish embryos [73, 109-111], with few published studies investigating adult zebrafish [112-116].

As it has been shown that zebrafish embryos are more sensitive to the effects of Ag NPs and Ag⁺ than adult fish, it is reasonable to focus research efforts on early developmental stages [117]. However, McGillicuddy et al. [103] recently published a review paper on the toxicity of Ag NPs to freshwater biota, where they conclude that acute toxicity in natural systems is not to be expected due to the relatively low predicted environmental concentrations (PECs) for Ag NPs. It is widely recognised that chronic exposure studies and exposures conducted over multiple generations can result in different outcomes compared with acute exposure studies, especially for toxicants that have the potential to bioaccumulate. In a 2013 paper, Völker et al. [107] showed the importance of conducting multigenerational exposures for understanding ENM effects. These workers treated three species of daphnids with polyvinylpyrrolidone (PVP) coated Ag NPs as well as AgNO₃ over five generations and compared the results to acute (48 h) and chronic (21 d) exposures. While AgNO₃ was the most toxic material to all *Daphnia* species in acute exposures, Ag NPs were shown to have the same level of toxicity as AgNO₃ to *Daphnia magna* in chronic exposures. In addition, they found that effects on reproduction did not necessarily appear in the first generation and were not linear over subsequent generations. This exemplifies the importance of studying the effect of ENMs on the natural environment over several generations, in exposures that more realistically mirror the release patterns of biota to anthropogenic nanomaterials.

The current study provides results obtained in a multigenerational diet-borne exposure of the zebrafish *Danio rerio* to isotopically labelled citrate-coated ¹⁰⁹Ag NPs and casein-coated ¹⁰⁹Ag NPs. The study encompasses zebrafish that were exposed to Ag NPs for one and for two

generations and the samples cover tissue material (gonads and livers) as well as embryos to assess transgenerational effects of Ag NP exposure. To the best of knowledge of the author, no studies that include multigenerational exposures of zebrafish to Ag NPs have been published to date. The present study therefore aims to (i) investigate the application of isotopically labelled tracer materials in multigenerational exposure studies and (ii) assess the intergenerational transfer of Ag NPs in aquatic systems.

The use of isotopically labelled ^{109}Ag NPs in this study is accompanied by isotopic analyses of the samples using multiple-collector ICP-MS (MC-ICP-MS). The techniques help to overcome the problems that are associated with distinguishing ENMs from the natural elemental background even at low and realistic exposure concentrations. In addition, the analyses by MC-ICP-MS following separation of Ag from the sample matrix enables an interference free detection and quantification of isotopically labelled Ag NPs even at very low total Ag contents, thereby allowing analyses of small samples such as zebrafish embryos.

4.2. Experimental

4.2.1. Synthesis and characterization of isotopically enriched Ag species

Nanoparticle synthesis and characterisation was conducted at the Facility for Environmental Nanoscience Analysis and Characterisation (FENAC) at the University of Birmingham. Citrate-coated ^{109}Ag NPs were synthesised following an appropriately modified standard reduction method [118, 119] specifically detailed in Laycock et al. [24]. In short, ^{109}Ag was oxidised with $15.3 \text{ mol l}^{-1} \text{ HNO}_3$ to yield $^{109}\text{AgNO}_3$. An aqueous solution of $^{109}\text{AgNO}_3$ and sodium citrate ($\text{C}_3\text{H}_4\text{OH}(\text{COOH})_2\text{COONa}$) was then reduced at elevated temperatures with sodium borohydride (NaBH_4) to yield mono-dispersed citrate-stabilised ^{109}Ag NPs.

For the synthesis of casein stabilised Ag NPs, a stock solution of casein (0.6% wt V⁻¹) was prepared by dissolution in warm water and 3 ml of this solution were then mixed with 0.1 ml sodium hydroxide NaOH ($c = 1 \text{ mol l}^{-1}$). The total volume of the reaction solution was adjusted to 18 ml through addition of deionised water and the mixture was stirred at 60°C for five minutes. At this point, 2 ml of a ¹⁰⁹AgNO₃ solution ($c = 20 \text{ mmol l}^{-1}$) were added and the reaction was maintained at 60°C for one hour. The suspension was then allowed to cool to room temperature and the produced casein-coated ¹⁰⁹Ag NPs were characterised for size and shape.

Both ¹⁰⁹Ag NP particle suspensions were characterised at FENAC by dynamic light scattering (DLS) using a Malvern Instruments ZetaSizer HPPS and by transmission electron microscope (TEM) with a Jeol-2100 LaB6. For storage, the suspensions were kept at -4°C in the dark.

4.2.2. Multigenerational exposure of ¹⁰⁹Ag nanoparticles to zebrafish

4.2.2.1. Flake preparation and dosing

Zebrafish of the species *Danio rerio* were exposed to ¹⁰⁹Ag NPs through their diet. To this end, TetraMin Tropical Flakes were ground to a fine powder and mixed with flour and a ¹⁰⁹Ag NPs suspension. The exposure concentration was 100 µg_{Ag} g_{food}⁻¹ at 4% body weight for both casein-coated and citrate-coated ¹⁰⁹Ag NPs. The food mixture was spread evenly onto baking paper and dried in an oven at 50°C. Once dried, flakes were crumbled and stored in a Falcon tube at -4°C.

4.2.2.2. Fish source, culture and husbandry

Wild type zebrafish embryos (WIK strain) were obtained from the Max Planck Institute of Tübingen (Germany) and maintained at the University of Exeter. From approximately two days post fertilisation (dpf) fry were fed on a microencapsulated diet (ZM advanced fry feed; ZM Ltd. Hampshire, UK) which was supplemented from approximately seven dpf with freshly hatched *Artemia nauplii*. From 21 dpf fish were fed twice daily to satiation with freshly hatched *A. nauplii* and then throughout to adulthood with freshly hatched *A. nauplii* in the morning and with TetraMin Tropical Flake Food in the afternoon. Once the ^{109}Ag NP exposure had been initiated the breeding fish were fed TetraMin Tropical Flake with added ^{109}Ag NPs in the morning and afternoon.

Fish were maintained in reconstituted water. Mains tap water was filtered by reverse osmosis (Environmental Water Systems (UK) Ltd) and reconstituted with AnalaR grade mineral salts to standardised synthetic freshwater (final concentrations to give a conductivity of 300 mS: 58 mg l⁻¹ CaCl₂ · 2 H₂O, 24.65 mg l⁻¹ MgSO₄ · 7 H₂O, 12.95 mg l⁻¹ NaHCO₃, 1.15 mg l⁻¹ KCl, 12.5 mg l⁻¹; Tropic Marin Sea Salt). This water was aerated and heated to 28°C in a reservoir before it was supplied to each aquarium using a flow-through system. Aquarium water was routinely monitored for pH and conductivity as well as ammonia, nitrate and nitrite levels, all of which were well within acceptable limits of U.S. EPA guidelines. The photoperiod was set to 13:11 h light:dark, with an artificial dawn to dusk transition of 30 min. Prior to the experiment, fish of mixed sex were maintained in large holding aquaria (900x500x300 mm in dimension) with a working volume of 112 l (approximately 100 fish per aquarium).

4.2.2.3. Sexing, weighing and measuring the zebrafish

Fish were sexed using differences in their colouration and differences in the behaviours that exist between males and females. Individual sexually mature fish were placed in a beaker of water to obtain an accurate body weight measurement. They were then placed on laminated graph paper to measure fork length.

4.2.2.4. Experimental set-up

A pre-exposure week was used to study breeding compatibility and conduct a reproductive assessment to ensure that all zebrafish pairs were matched effectively, i.e. that fish were spawning and behaving normally. In both exposure generations, F0 and F1, adult zebrafish were used that had reached sexual maturity (>3 months old).

In generation F0, six tanks (30 cm x 30 cm) were set up per treatment (casein-coated ^{109}Ag NPs, citrate-coated ^{109}Ag NPs and control line) each containing a total of eight fish (four males and four females) (Fig. 4.1). Each tank held a volume of 12 l and the system was set up for five full water replacements every 24 h. The diet-borne exposure of zebrafish to Ag NP lasted for 21 days, during which embryos were collected every seven days. Liver and gonad tissue from adult zebrafish was sampled at the end of the exposure after 21 days.

The exposure was set up identically in generation F1 with six separate fish tanks per treatment (Fig 4.1, F1 black treatments). In each tank, fish reared to adulthood (3 months) from the offspring of the previous generation were placed and exposed to the same conditions as their F0 predecessors after seven days equilibration time. As in F0, embryos were collected at the end of each exposure week and liver and gonad tissue was sampled at the end of the exposure after 21 days.

In F1, 12 additional tanks were set up in three lines of four replicates. Offspring of fish that had been exposed to either casein-coated or citrate-coated ^{109}Ag NPs or acted as control treatment in F0 were placed in these tanks and are subsequently labelled as “F1 Recovery” generation (Fig. 4.1, F1 red treatments). In F1, none of these fish were exposed to ^{109}Ag NPs (Fig. 4.1) and no embryos were collected from these fish. At the end of the experiment (after 21 days), liver and gonad samples were collected from these fish.

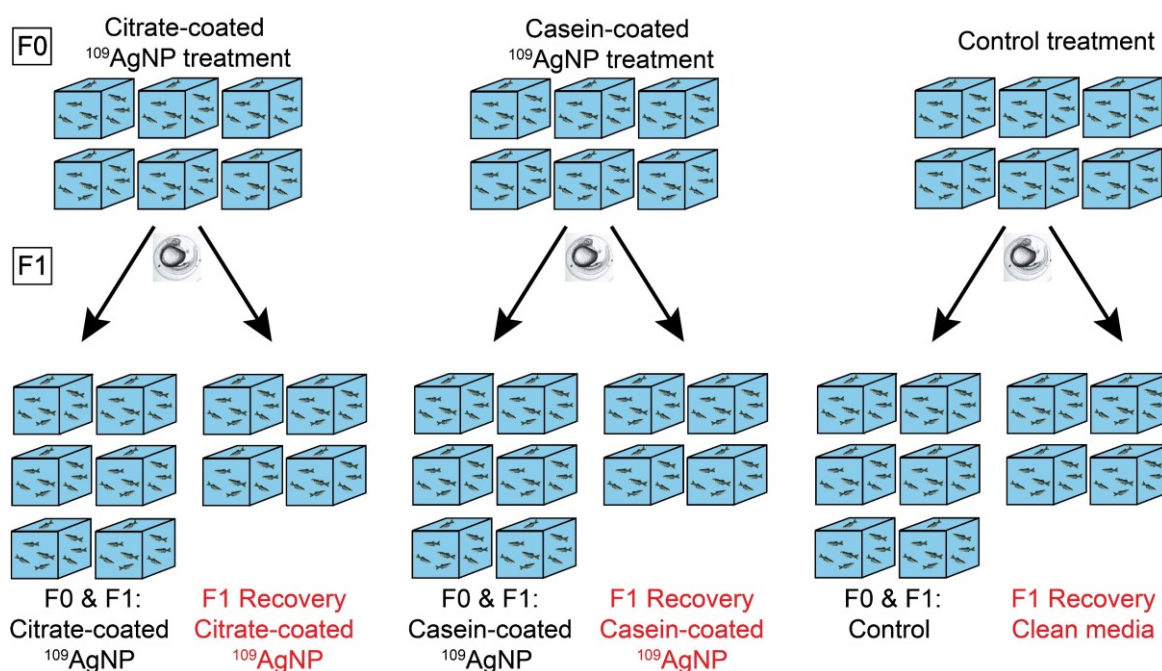


Fig. 4.1. Experimental set up of the multigenerational diet-borne exposure of zebrafish to casein-coated and citrated-coated ^{109}Ag NPs. Each treatment line and a control line were set up with six replicates in generation F0. Some of the embryos (F1) were reared to adulthood and used in a further exposure generation under the same conditions as in F0. Additionally, in F1 a further 3x4 aquariums were set up that contained offspring of fish that had been exposed to ^{109}Ag NPs in F0 but were not exposed in F1 (F1 Recovery generation, in red).

4.2.2.5. Measuring egg output and fertilisation

Throughout the exposure, fish were allowed to breed naturally and eggs were collected via collection chambers roughly one hour post fertilisation (hpf). To this end, the glass aquaria

were designed with sloping sides to form a ‘funnel’ that channels any spawned eggs into an egg-collecting chamber. This facilitated daily embryo collection whilst minimising disturbance to the fish.

Glass marbles of 10 mm diameter were placed in the ‘funnel’ section at the base of the aquaria at a depth of 3-4 marbles to act as a spawning substrate and to minimize oophagy (egg eating). An artificial weed was placed in the tanks rooted in the marbles to act as a refuge and spawning stimulus. Each day, eggs were collected 1 h after dawn by opening a valve at the base of the egg collecting chamber, which is fitted with a fine mesh sieve, drawing water through the marbles where the eggs were trapped. The eggs were captured on the fine mesh sieve and washed to remove any food waste and/or faeces before being transferred into a Petri dish with embryo culture water.

Fertilisation success was calculated using all embryos collected from each tank. Infertile eggs and dead embryos were separated from the live embryos at approximately two hpf. The numbers of live and dead eggs were counted to give a record of total egg output and overall egg viability for each test tank and for each pair of fish.

4.2.2.6. Biological sampling

After 21 days of exposure fish from all treatments were terminated by anaesthesia (Benzocaine, Sigma Poole, UK) and measurements of fork length and weight were recorded in both F0 and F1 (Table 4.1). Fish were dissected and gonads and livers were weighed and stored in Eppendorf tubes at -80°C . Embryos of the F1 and F2 generation were collected at the end of each exposure week (7 days, 14 days and 21 days) (Table 4.1).

For each tank, pools of 10 embryos were weighed collectively and frozen in Eppendorf tubes at -80°C until further use. In addition, water samples were collected from each tank at the end of each exposure week in F0 and F1 (Table 4.1). To this end, ~ 50 ml of water was collected in a Falcon tube which was wrapped in Parafilm and stored at -4°C until further use.

Prior to shipment from the University of Exeter to Imperial College London, all tissue and embryo samples designated for mass spectrometric analysis were freeze dried. Each Eppendorf tube was wrapped in Parafilm to minimise potential contamination.

Table 4.1: Summary of main exposure and sampling parameters of the exposure.

F0	
Exposure time	21 days
Collected samples	Livers (day 21)
	Gonads (day 21)
	Water (day 21)
F1	
Exposure time	21 days
Collected samples	Livers (day 21)
	Gonads (day 21)
	Embryos (day 7, day 14, day 21)
	Water (day 21)
F2	
	Embryos (day 7, day 14, day 21)

4.2.3. Isotopic analysis of samples

4.2.3.1. Materials and reagents

All samples were processed and analysed at the MAGIC Laboratories at Imperial College London in an ISO 6 clean room equipped with ISO 4 laminar flow hoods to prevent contamination.

Prior to use, Savillex® sample beakers underwent a strict cleaning procedure to prevent Ag contamination and to minimise the procedural blank. To this end, all sample beakers were soaked in soapy water for 24 hours at room temperature followed by 48 hours cleaning in AnalaR grade HNO₃ and 48 hours in AnalaR grade HCl at sub-boiling temperatures. Beakers were then submerged in a mixture of MQ water and distilled HCl and HNO₃ for an additional 48 hours at elevated temperatures. Vials for mass spectrometric analysis were cleaned for >12 h in 1 mol l⁻¹ distilled HNO₃ and samples were not kept in these vials for longer than six hours.

AnalaR grade 15.3 mol l⁻¹ HNO₃ and 6 mol l⁻¹ HCl were purified by sub-boiling distillation in a quartz still prior to use or further dilution. Milli-Q water of >18 MΩ cm quality (Millipore, UK) and 30% Suprapur H₂O₂ (VWR UK) were used throughout.

4.2.3.2. Sample digestion

Water samples (40-50 ml) were dried on a hotplate in open Savillex® containers and hotplate digested at 140°C for 120 hours using 5 ml of 6 mol l⁻¹ distilled HCl. This was followed by dilution with MQ water to give a concentration of 0.5 mol l⁻¹ HCl for column loading.

The freeze-dried weights of all tissue samples (livers, gonads and embryos) were recorded individually by differential weighing (Mettler Toledo AX205, max 81 g/220 g, d = 0.01 mg/0.1 mg) before and after transfer from the Eppendorf tube. They were then transferred to a Savillex® container. If tissue remnants were sticking to the Eppendorf tube, vials were flushed with 4 mol l⁻¹ HNO₃ which was subsequently evaporated at 160°C. A mixture of 0.15 ml H₂O₂ and 0.35 ml distilled 15.3 mol l⁻¹ HNO₃ was added to each Savillex® beaker which was then placed in a modified PTFE (TFM) microwave digestion vessel. A procedural blank was processed with every sample batch of nine samples to monitor contamination.

All tissue samples were pre-digested for 14-20 hours at room temperature followed by microwave digestion in an Ethos EZ microwave system (Milestone Inc., 1200 W). A step-wise heating protocol was applied that ramped the temperature to 200°C within 45 minutes and held at this temperature for a further 30 minutes. Savillex® Teflon beakers were then removed from the TFM microwave digestion vessels and placed on hotplates where solutions were evaporated to dryness at 120-160°C. Dried samples were subsequently dissolved drop wise and evaporated repeatedly in approximately 0.2-0.5 ml 6 mol l⁻¹ HCl to achieve complete conversion to a chloride matrix. Finally, each sample was taken up in 0.5 mol l⁻¹ HCl and refluxed for >12 h.

4.2.3.3. Ion exchange separation of Ag from environmental matrix

Silver was separated from other matrix elements by performing an anion exchange column chemistry procedure modified from Woodland et al. [80] to allow for the high throughput necessary for the analysis of multiple samples from environmental exposures. The one-step separation is presented in detail in Chapter 3.2.4.2. The column chemistry was carried out in batches of 20-30 samples at a time. Columns were cleaned for at least 48 hours in 2 mol l⁻¹ distilled HNO₃ between use and the cleaning acid was exchanged after ten uses.

4.2.3.4. Mass spectrometry

4.2.3.4.1 Preparation of sample and standard solutions

The purified Ag fractions obtained from the column chemistry were evaporated to dryness and dissolved in 1 ml of 0.1 mol l⁻¹ HNO₃ for mass spectrometric analysis. Of this solution, 5% were used to prepare a 0.5 ml dilution with 0.1 mol l⁻¹ HNO₃ that was used for an initial assessment of the Ag concentration and isotopic composition of a sample. Subsequently, 80-90% of the remaining sample solution were used to prepare a 0.9 ml dilution that contained

10 ng ml⁻¹ Pd standard in 0.1 mol l⁻¹ HNO₃ for precise measurements of both the diagnostic ¹⁰⁹Ag/¹⁰⁷Ag ratio and the ¹⁰⁹Ag concentration.

A new 1000 µg ml⁻¹ Alfa Aesar Ag standard solution was purchased prior to analysis of the samples in this study. It was used to prepare a 10 µg ml⁻¹ Ag standard from which a 1 µg ml⁻¹ Ag working stock solution was made up by further dilution. The latter solution was used to prepare any specific Ag standard solution for mass spectrometry on the measurement day. A 100 µg ml⁻¹ in-house Pd standard was used to prepare 1 µg ml⁻¹ and 100 ng ml⁻¹ working standards from which Pd was added to all sample and Ag standard solutions that were used for MC-ICP-MS measurements.

An in-house Ag standard solution with non-natural isotopic signature was prepared by mixing a natural Ag standard solution and a ¹⁰⁹AgNO₃ solution gravimetrically. This solution was isotopically characterised by MC-ICP-MS analyses and used as a reference standard to mimic the isotopic signature of samples containing isotopically enriched ¹⁰⁹Ag as outlined in Chapter 3.2.5.4. For this study, two of these standards were prepared and used to analyse all samples from the first (F0) and second (F1) generation of the exposure.

4.2.3.4.2. Measurement instrumentation and protocols

All isotopic measurements were performed in the MAGIC Laboratories at Imperial College London with a Nu Plasma HR MC-ICP-MS. Sample introduction utilised a CETAC Aridus II desolvation system equipped with glass MicroMist nebulizers. Detailed operating parameters and the measurement set up are summarised in Table 3.2 in Chapter 3 of this work. Silver isotope analyses were carried out by static multiple collection with four Faraday cups fitted with 10¹¹ Ω resistors to monitor masses 104 and 105 for Pd as well as 107 and 109 (for Ag). Sensitivities of 250 to 350 V (µg ml)⁻¹ for Ag and 180 to 220 V (µg ml)⁻¹ for Pd were

routinely achieved. In between measurements the sample introduction system was washed with 0.1 mol l⁻¹ HNO₃ for 2-3 minutes to reduce all signal intensities to below 5 mV intensity.

4.2.3.4.3. Sample measurements and data reduction

All samples were analysed following the methods laid out in detail in Chapter 3.2.5. In summary, samples first underwent a short analysis that was used to obtain a first indication of the Ag concentrations and isotope compositions of the Ag fraction solutions of the samples. This information was subsequently used to appropriately set up the final, longer measurements of the samples along with appropriate bracketing standard solutions. To this end, the initial measurement protocol recorded one block with 10 integration cycles of 5 s each, prior to which the electronic baseline was determined for 15 s. The longer measurement protocol for precise isotopic analysis recorded a total of 60 measurement cycles in three blocks of 20 integration cycles. In this, the electronic baselines were recorded for 15 s prior to each block.

The instrumental memory was monitored throughout each measuring session using a separate 0.1 mol l⁻¹ HNO₃ solution. This solution was analysed in a single block measurement protocol with 30 integration cycles of 5 s each prior to which the electronic baseline was determined for 15 s. The solution to measure the memory effect was freshly prepared for each measurement session and replaced if any contamination appeared. The instrumental memory was recorded right before each sample and standard measurement with the following measurement sequence (Fig. 3.2):

Wash - memory run - standard run – wash – memory run – sample run – wash – memory run – sample run - wash – memory run – - standard run

An external mass bias correction was employed by monitoring the ¹⁰⁵Pd/¹⁰⁴Pd ratio and applying the exponential mass fractionation law [80, 87]. Due to the small size and mass of the samples collected in this study and their low overall Ag content, the impact of the instrumental

memory had to be considered for each sample measurement during the data reduction procedure. To this end, all sample analyses underwent several offline correction steps. The multi-step data reduction process is presented and discussed in detail in Chapter 3.2.6. and it was used to treat all data recorded during the course of this study.

To calculate the amount of dissolved Ag present in the water tanks, the amount of Ag measured in 50 ml water aliquots was extrapolated to the amount of enriched Ag (Ag-En) present in a 12 l aquarium. The dissolution rate was then calculated as the fraction of Ag-En found in the tank compared to the total Ag-En added through food in a 24 hour period.

4.2.3.5. Isotopic mass balance calculations

Measuring the diagnostic $^{109}\text{Ag}/^{107}\text{Ag}$ isotope ratio of a sample with unknown composition allows the calculation of the overall Ag content as well as the determination of the amount of enriched tracer present in the sample. The diagnostic $^{109}\text{Ag}/^{107}\text{Ag}$ ratio (R_{meas}) is comprised of the natural and the enriched Ag fraction which can both be expressed through their fractional molar abundances:

$$\begin{aligned} \frac{^{109}\text{Ag}}{^{107}\text{Ag}} &= R_{meas} \\ &= \frac{fr^{109}\text{Ag}_{nat} \times \text{Ag}_{nat} + fr^{109}\text{Ag}_{en} \times \text{Ag}_{en}}{fr^{107}\text{Ag}_{nat} \times \text{Ag}_{nat} + fr^{107}\text{Ag}_{en} \times \text{Ag}_{en}} \end{aligned} \quad (\text{Equation 4.1})$$

Where $fr^{xxx}\text{Ag}_{nat}$ and $fr^{xxx}\text{Ag}_{en}$ denote the fractional molar abundances of the ^{xxx}Ag isotope in natural Ag (Ag_{nat}) and enriched Ag (Ag_{en}) respectively. The fractional molar abundances of natural Ag were determined through analyses of the Alfa Aesar Ag standard solution that bracketed the sample analyses. For enriched Ag, they were determined by repeated ($n>5$) analyses of a pure solution of the enriched isotopic material (0.99714 for $^{109}\text{Ag}_{en}$ and 0.00286

for $^{107}\text{Ag}_{\text{en}}$). Equation 4.1 can be recast to give the molar ratio of enriched Ag to natural Ag in the sample:

$$\frac{Ag_{\text{en}}}{Ag_{\text{nat}}} = frAg_{\text{en},\text{sam}} = \frac{R_{\text{meas}} \times fr^{107}Ag_{\text{nat}} - fr^{109}Ag_{\text{nat}}}{fr^{109}Ag_{\text{en}} - R_{\text{meas}} \times fr^{107}Ag_{\text{en}}} \quad (\text{Equation 4.2})$$

The molar ratio of enriched to natural Ag can be used to determine the fractional molar abundance of each Ag isotope ($fr^{109}Ag_{\text{sam}}$ and $fr^{107}Ag_{\text{sam}}$) in the sample, e.g. for ^{109}Ag this is:

$$fr^{109}Ag_{\text{sam}} = (fr^{109}Ag_{\text{en}} \times {}^{109}Ab_{\text{en}}) + ((1 - fr^{109}Ag_{\text{en}}) \times {}^{109}Ab_{\text{nat}}) \quad (\text{Equation 4.3})$$

The total amount of substance $n(\text{Ag})$ present in the sample can then be calculated by dividing the amount of substance $n(^{109}\text{Ag})$ by the fractional molar abundance of ^{109}Ag calculated using Equation 4.3.

$$n(Ag_{\text{sam}}) = \frac{n(^{109}Ag_{\text{sam}})}{fr^{109}Ag_{\text{sam}}} \quad (\text{Equation 4.4})$$

The amount of substance $n(^{109}\text{Ag})$ in the measured sample was determined by external calibration using the analyses of the Ag standard solutions that bracketed all sample measurements as described in Chapter 3.2.5.5.

The amount of enriched isotopic tracer present in the sample in mols can then be calculated by multiplying the molar ratio of enriched to natural Ag in the sample (Equation 4.2) and the amount of Ag in the sample as calculated in Equation 4.4.

$$n(Ag_{\text{en},\text{sam}}) = frAg_{\text{en},\text{sam}} \times n(Ag_{\text{sam}}) \quad (\text{Equation 4.5})$$

Similarly, for the amount of natural Ag present in the sample $n(Ag_{\text{nat},\text{sam}})$ (in mol):

$$n(Ag_{\text{nat},\text{sam}}) = n(Ag_{\text{sam}}) - n(Ag_{\text{en},\text{sam}}) =$$

$$= n(Ag_{sam}) - [frAg_{en,sam} \times n(Ag_{sam})] \quad (\text{Equation 4.6})$$

The mass m of natural and enriched Ag present in the sample can be determined using the relationship:

$$n = \frac{m}{M} \quad (\text{Equation 4.7})$$

The molar mass M of the sample can be calculated from the sum of the stable Ag isotope abundances multiplied by their atomic mass m_A .

$$M_{sam} = \sum fr^{109}Ag_{sam} \times m_A \quad (\text{Equation 4.8})$$

4.2.3.6. Statistical analysis

Unless otherwise stated all data are presented as mathematical mean $\pm$ 1 SD. All statistical analyses were performed in Microsoft Excel and within each treatment group, data points outside of the inner fence boundaries were treated as outliers (11% and 8% in F0 and F1 respectively). Differences between treatment groups were analysed by two-sample t-tests using all data points apart from outliers. Two-sample t-tests were calculated for populations (i) male vs female fish, (ii) casein-coated ^{109}Ag NPs vs. citrate-coated ^{109}Ag NPs, (iii) generation F0 vs. generation F1 and (iv) gonad tissue samples vs. liver tissue samples. For embryo samples, two-sample t-tests were performed to compare the following populations: (i) samples from week 1 vs. samples from week 2, (ii) samples from week 2 vs. samples from week 3, (iii) casein-coated ^{109}Ag NPs vs. citrate-coated ^{109}Ag NPs and (iv) samples from generation F0 vs. generation F1. All measurement results acquired in this study as well as t-test analyses can be found in the Appendix of this work.

4.3. Results and discussion

4.3.1. Nanoparticle characterisation

Analyses by TEM confirm the presence of spherical Ag NPs for both synthesised particle suspensions (Fig. 4.2) with particle diameters between 2 and 4 nm for casein-coated ^{109}Ag NPs (cas- ^{109}Ag NPs; Fig. 4.2A) and between 8 and 10 nm for citrate-coated ^{109}Ag NPs (cit- ^{109}Ag NPs; Fig. 4.2B). The hydrodynamic diameter as measured by DLS is identical within uncertainty for both particle types, with results of 21 ± 1 nm for cas- ^{109}Ag NPs and 18 ± 2 nm for cit- ^{109}Ag NPs.

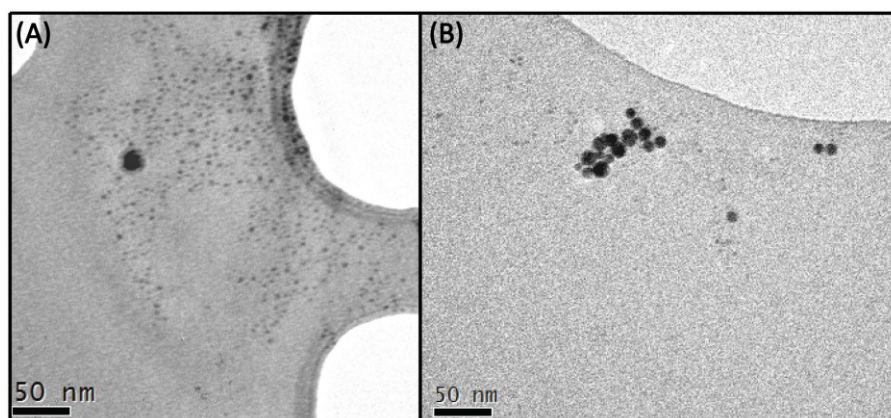


Fig. 4.2. TEM micrographs of isotopically labelled (A) casein-coated ^{109}Ag NPs (2-4 nm) and (B) citrate-coated ^{109}Ag NPs (8-10 nm).

4.3.2. Ag nanoparticle dissolution

Fish were exposed to isotopically enriched Ag NPs (Ag-En) through their diet at a low and environmentally relevant Ag concentration in food of $100 \mu\text{g g}^{-1}$, which was provided at 4% body weight per day, equivalent to about $0.02 \mu\text{g Ag}$ per fish per day. However, Ag from the Ag NPs in the feed can dissolve at any point after addition to the water and before it is consumed by the fish, thereby opening up the possibility of exposure routes that are not diet-borne. In order to minimise dissolution effects, the water in all aquariums was replaced five times every 24 hours, resulting in a complete water replacement on average every

4.2 hours. To assess Ag dissolution, water from each tank was sampled at the end of week 3 to determine its Ag content and isotope composition.

The analysed water from tanks of the control lines did not contain any enriched material, indicating no cross-contamination between the treatment lines at this point. However, the isotopic signature of water collected from aquaria with the cit-¹⁰⁹AgNP and the cas-¹⁰⁹AgNP treatments show the presence of Ag-En in F0 and F1 (Table 4.2). The average ¹⁰⁹Ag/¹⁰⁷Ag ratio over both generations for cit-¹⁰⁹AgNP and cas-¹⁰⁹AgNP water samples was 17.9 ± 15.2 (1 SD) and 1.4 ± 1.1 (1 SD), respectively, with average Ag-En concentrations over both generations of 34.0 ± 19.9 pg ml⁻¹ (1 SD) and 7.0 ± 1.5 pg ml⁻¹ (1 SD). These results indicate higher dissolution rates for citrate-coated Ag NPs than for casein-coated Ag NPs. The molecular structures of citric acid and α -casein are shown in Fig. 4.3. Whereas citric acid is a compact molecule with only polar functional groups attached to each atom of the carbon-chain, casein is a large organic molecule that contains both polar and unpolar structural components and it has a lower overall polarity than citric acid. Casein-stabilised Ag NPs have been reported to be highly stable in aqueous media, which is likely due to the moderate polarity of water which does not facilitate the dissolution of casein. In addition, casein has been reported to form micelles that further stabilise the cas-¹⁰⁹AgNPs [120]. In contrast, citric acid readily dissolves

Table 4.2: Results of analysed water sample collected at the end of week 3 of the exposure.

Generation	Treatment	¹⁰⁹ Ag/ ¹⁰⁷ Ag (relative to reference value) [abs]	Ag-En per ml water [pg ml ⁻¹]
F0	Casein	4.9	5.8
F0	Casein	8.3	9.3
F0	Citrate	25.2	26.2
F0	Citrate	15.3	16.3
F1	Casein	7.8	8.7
F1	Citrate	61.6	62.5



α -Casein

The highest measured Ag concentration for the water of 39.4 pg ml⁻¹ for cit-¹⁰⁹AgNPs (Table 4.2) indicates that 2.9% of the added Ag NPs dissolved in water, when the volume of the aquariums and the total daily amount of added Ag-En are taken into consideration. This is in line with the results of dissolution experiments undertaken by Laycock and co-workers, who reported that between 2 and 5% of citrate-coated Ag NPs dissolved within the first eight hours following addition to water [24].

In conclusion, although Ag-En was detected in the water of the exposure system, the average detected amount is equivalent to a dissolution of $\sim 1\%$ for cit- $^{109}\text{AgNP}$ and $\sim 0.1\%$ for cas- $^{109}\text{AgNP}$ treatments (Table 4.2). The regular exchange of the water in the aquariums therefore successfully ensured that the exposure of zebrafish happened orally through their diet rather than, for example, dermal absorption caused by the presence of high Ag-En concentrations in the water.

4.3.3. Multigenerational exposure of enriched Ag nanoparticles to adult zebrafish

4.3.3.1. Mass difference of tissue samples

The masses of the analysed liver and gonad tissue samples are summarised in Fig. 4.4. Female tissue samples (Fig. 4.4B and Fig. 4.4D) were generally larger and heavier, with the ovaries of female zebrafish (Fig. 4.4D) weighing almost 10 times as much as the male (testis) counterparts (Fig. 4.4C) and livers from female zebrafish weighing approximately twice as much as livers of male zebrafish (Fig. 4.4A and Fig. 4.4B).

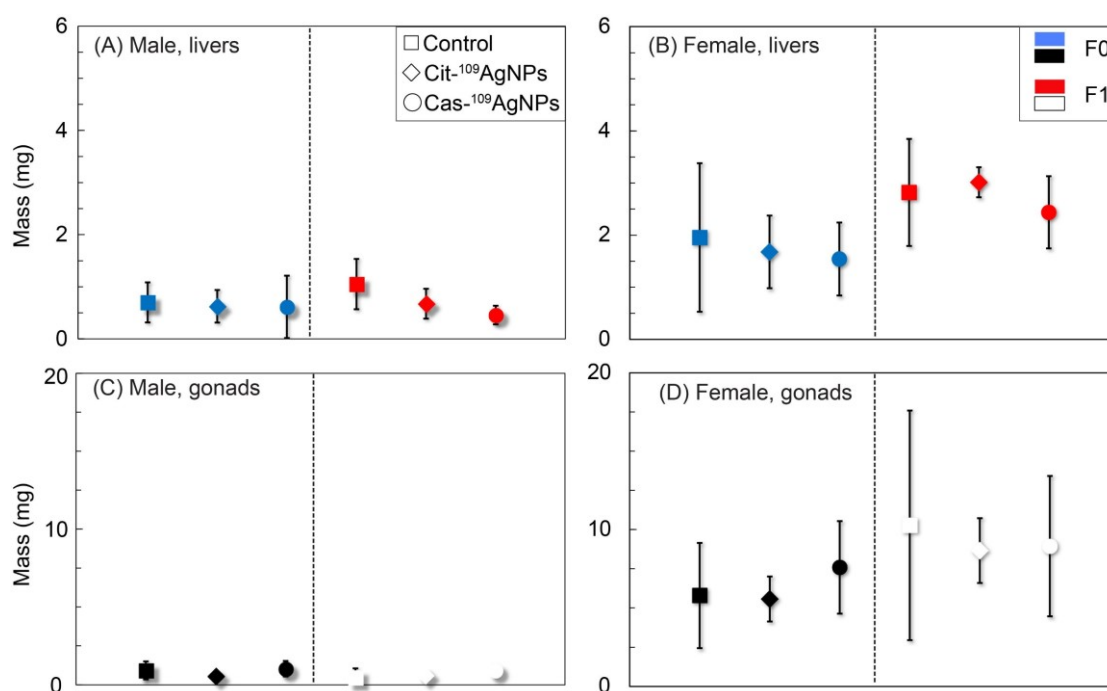


Fig. 4.4. Mass of analysed liver and gonad samples grouped by sex and exposure generation. Error bars (not always visible) denote 1 SD.

The tissue mass of gonads and livers is essentially unaffected by exposure to Ag-En and the sample weights for all treatment groups, regardless of sex or exposure generation, are in good agreement with the corresponding control group. The only trend that can be inferred from this data is possibly a tendency towards slightly higher total tissue mass for the livers of female zebrafish in exposure generation F1 compared to F0 (Fig. 4.4D). Whereas the results of the t-tests indicate no statistically relevant differences between F0 and F1 in the control group samples ($p = 0.30$), p values for $\text{cas-}^{109}\text{AgNP}$ ($p = 0.09$) and in particular $\text{cit-}^{109}\text{AgNP}$ ($p = 0.02$) appear to suggest a trend towards heavier livers in F1. This was cross-examined through a t-test comparison of the F1 control and F1 $\text{cit-}^{109}\text{AgNP}$ populations, where results indicate no statistically relevant difference ($p = 0.72$).

4.3.3.2. Zebrafish embryo samples (F1)

Embryos that were produced by adult zebrafish, which had been exposed to Ag NPs through their diet, were collected at the end of each exposure week (W1, W2 and W3, Table 4.1) and analysed for their Ag-En content (Fig. 4.5). While the presence of Ag-En was confirmed for each analysed sample, there are no discernible differences between the two treatment types or the time of embryo collection. This conclusion is corroborated by t-test results, which revealed no significant difference between the datasets at the 95% confidence level ($p = 0.05$), whereby all tests except one (with $p = 0.08$) recorded p values of between 0.29 and 1.00 (A2.5).

In this context, it is noteworthy that the embryos were not directly exposed to Ag NPs. Rather, the Ag-En was fed to adult zebrafish and passed on to the embryos through maternal transfer. These results therefore strongly suggest that exposure of adult zebrafish to Ag NPs suffices for the offspring to be affected by potentially adverse effects from the exposure. Previous work by Lee and co-workers found that zebrafish embryos are most susceptible to

damage induced by Ag NPs during the pre-hatching period of embryonic development [117]. Consequently, maternal transfer of Ag to embryos during the earliest developmental stages will likely affect the organism more than external exposure to Ag NPs during the larval stage.

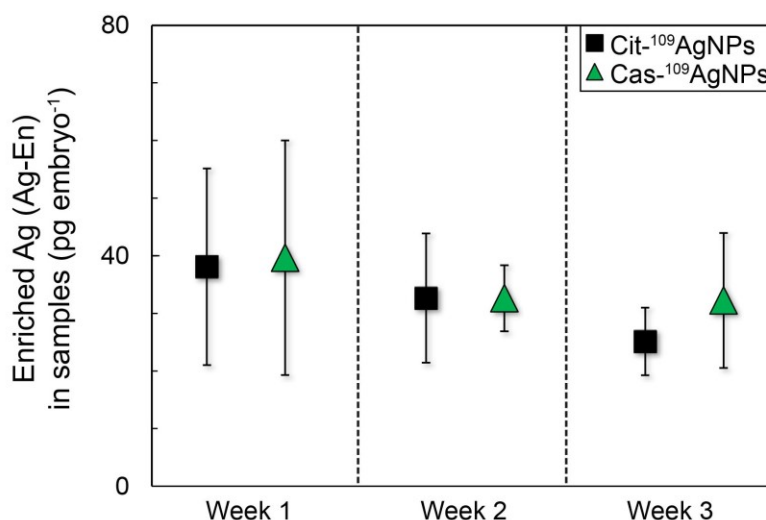


Fig. 4.5. (A) Mass fraction of Ag-En in embryo samples collected in Weeks 1, 2 and 3 of the F1 generation exposure. Plotted are the mean values ± 1 SD for 6 samples, except Week 2 of cas-¹⁰⁹AgNP treatment ($n = 4$) and Weeks 2 and 3 for the cit-¹⁰⁹AgNP treatment ($n = 5$). There are no observable differences between embryos from the cas-¹⁰⁹AgNP treatment (green triangles) and the cit-¹⁰⁹AgNP treatment (black squares).

4.3.3.3. Zebrafish tissue samples (F0)

The mass fraction of total Ag-En present in livers of the F0 zebrafish generation are presented in Fig. 4.6A and key observations are discussed in the following. For liver samples from the cit-¹⁰⁹AgNPs treatment, smaller variations within each population can be observed for both male and female fish in comparison to samples from the cas-¹⁰⁹AgNPs treatment. Interestingly, at 35 ± 24 ng mg⁻¹ livers from male fish exposed to cas-¹⁰⁹AgNPs contain, on average, almost six times as much enriched Ag compared to zebrafish exposed to cit-¹⁰⁹AgNPs (6 ± 5 ng mg⁻¹). The livers of females generally have lower Ag-En mass fractions than male livers, regardless of the coating material (Fig. 4.6A). However, as for male fish, the average Ag-En content for the livers of females exposed to cit-¹⁰⁹AgNPs (0.7 ± 0.3 ng mg⁻¹) is lower

compared to the cas-¹⁰⁹AgNPs ($1.0 \pm 0.9 \text{ ng mg}^{-1}$) treatment but the difference is much less striking. Statistical analysis using two-sample t-tests for the male vs. female populations returned p values of $p = 0.13$ (cit-¹⁰⁹AgNPs) and $p = 0.02$ (cas-¹⁰⁹AgNPs) which indicates statistically significant differences in the Ag-En uptake between males and females for cas-¹⁰⁹AgNPs.

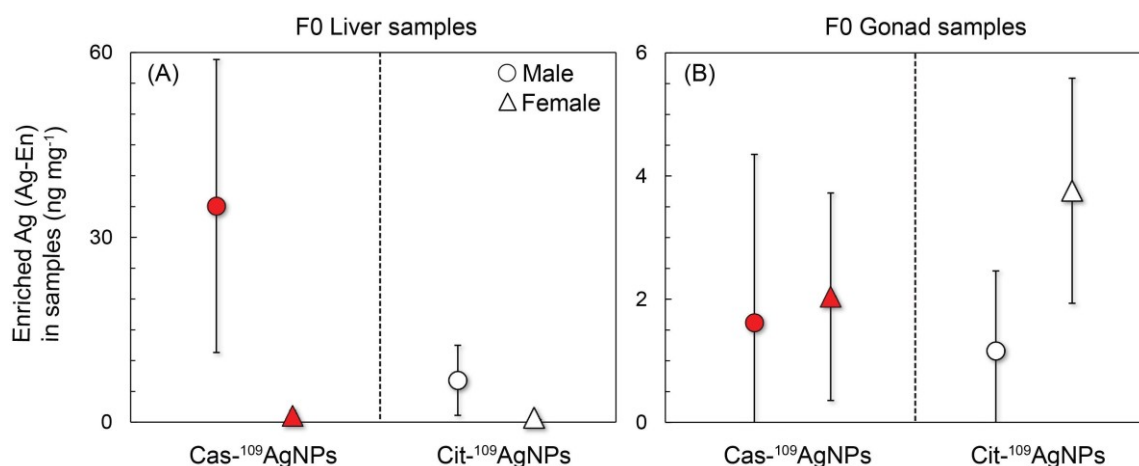


Fig. 4.6. Mass fraction of enriched Ag present in (A) liver and (B) gonad tissue of male (circles) and female (triangles) zebrafish from exposure generation F0 for treatments with casein-coated Ag NP (red) and citrate-coated Ag NP (white). Plotted are the mean values $\pm 1SD$ for livers and gonad samples of five female and six male zebrafish. (Note the different scale of the y-axes.)

Choi et al. [123] previously reported the presence of agglomerated Ag NPs in *Danio rerio* liver tissue at a level of $0.29 \text{ ng}_{\text{Ag}} \text{ mg}^{-1}_{\text{liver}}$ following a water-borne exposure to 5-20 nm sized Ag NPs at a concentration of $30 \text{ mg}_{\text{Ag}} \text{ l}^{-1}$. Even though Choi and co-workers employed exposure concentrations that exceeded the level of Ag NPs available to individual fish in this study by a factor of about 10^3 , they found lower overall levels of Ag NPs in the fish livers. This shows that even for Ag NPs of small size, which will facilitate dermal absorption, water-borne absorption appears to be a less relevant exposure route compared to dietary uptake of Ag NPs.

The data obtained for the gonads of male and female fish, as summarized in Fig. 4.6B and discussed below, differed from the results collected for liver tissue (Fig. 4.6A). Overall, the largest levels of Ag-En was detected in the reproductive system of female fish exposed to

cit-¹⁰⁹AgNPs ($4 \pm 2 \text{ ng mg}^{-1}$) with similar but slightly lower mean Ag-En contents for all other populations (Fig. 4.6A, A2.4). The level of Ag-En found in the livers of male zebrafish was approximately 8 to 10 times higher than in male gonad tissue for both Ag NP treatment groups. In contrast, slightly higher Ag-En levels were found in the gonad tissue of female zebrafish than in the livers. This may suggest that a diversion of Ag NPs to the liver, which acts as a detoxification system, is more effective in male zebrafish compared to females. This observation possibly reflects that female zebrafish have to provide nutrients including trace metals for the embryonic development of their offspring, and this might entail the enhanced transport of Ag NPs from the digestive to the reproductive system [124].

Overall, enriched Ag from ¹⁰⁹Ag NPs was found to be present in all analysed tissue samples, regardless of NP type, tissue material or zebrafish sex (Fig. 4.). Hence, the results demonstrate that Ag NPs are absorbed by zebrafish through the gut to subsequently enter organs (i.e. the liver) and the gonads even at the low and environmentally relevant dosing concentrations that were employed in this study. As the zebrafish were not exposed to Ag NPs continuously (e.g., by addition of Ag NPs to the aquarium water) but only ingested Ag NPs twice a day with their feed, the data further show that the fish were unable to eliminate Ag NPs from their body at a fast enough rate to counteract the repeated diet-borne exposure. Bioaccumulation of Ag NPs was previously reported for various species and was also observed by Pecoraro and co-workers for tissue samples of adult zebrafish after an exposure period of 30 days [116, 125]. However, while Pecoraro et al. [116] applied water-borne Ag NP exposure concentrations of 8 to 70 $\mu\text{g l}^{-1}$, the present study confirmed Ag NP uptake by adult zebrafish after 21 day exposure to only 2 $\mu\text{g Ag}$ per fish per day.

With an average result of $13 \pm 20 \text{ ng}_{\text{Ag}} \text{ mg}^{-1}_{\text{liver}}$, the level of Ag present in the livers of adult zebrafish at the end of the exposure was significantly higher than was previously reported for the water-borne Ag NP exposure of Choi et al. [123], who found only $0.29 \text{ ng}_{\text{Ag}} \text{ mg}^{-1}_{\text{liver}}$.

Whilst it is unclear if Ag NPs induce hepatotoxicity, such toxicity was previously detected for both TiO₂ and ZnO NPs. In detail, Xiong et al. [126] found that a 96 h exposure of zebrafish to 50 mg l⁻¹ TiO₂ NPs and 5 mg l⁻¹ ZnO NPs was associated with increased oxidative stress and tissue damage to the fish livers. However, an exposure study with 1.5 mg l⁻¹ Cu NPs reported adverse effects primarily for the gills rather than the liver tissue of the exposed zebrafish [127]. Further studies, such as histological examinations, will be required to assess liver health in mature zebrafish after exposure to Ag NP at low dosing concentrations.

4.3.3.4. Intergenerational trends (F0 & F1)

As it was shown that Ag from diet-borne Ag NPs can be detected in the offspring, it was studied if the Ag was still detectable in the fish once they had reached maturity. The ¹⁰⁹Ag/¹⁰⁷Ag isotope ratios determined for liver and gonad tissue of F1 generation zebrafish that were not exposed to ¹⁰⁹Ag NPs, but which were reared from the offspring of F0 zebrafish exposed to enriched ¹⁰⁹Ag NPs are shown in Fig. 4.7. There are no significant differences

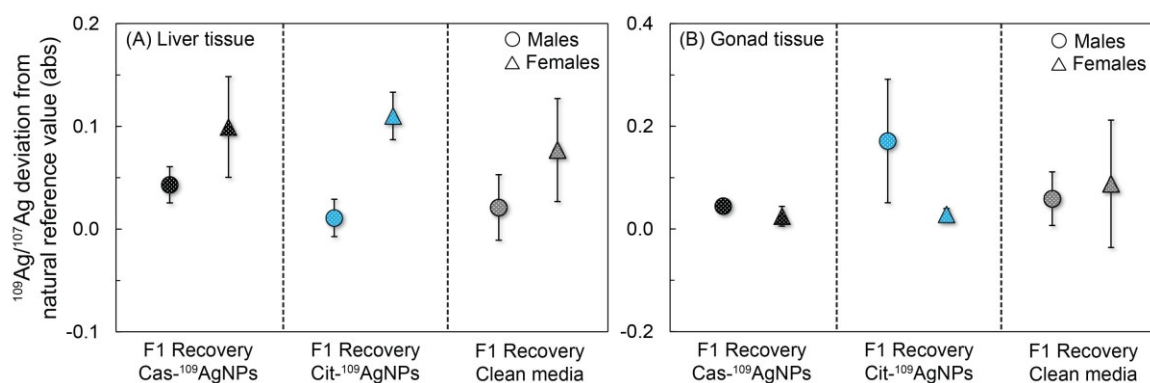


Fig. 4.7. Absolute deviation of measured ¹⁰⁹Ag/¹⁰⁷Ag ratios from the natural reference value of 0.929 for (A) livers and (B) gonads of unexposed F1 generation zebrafish. Samples are from zebrafish that are descendants of fish that were either pre-exposed to cas-¹⁰⁹AgNPs (F1 Recovery Cas-¹⁰⁹AgNPs) or cit-¹⁰⁹AgNPs (F1 Recovery Cit-¹⁰⁹AgNPs) in F0 or acted as control group in F0 (clean media). Plotted are mean values ± 1 SD for two to four individual samples. Liver and gonad tissues that were collected from either treatment group and the control line in F1 show no significant difference in isotopic signature. (Note the different scale of the y-axes.)

between the two treatments and the corresponding control group. This suggests (i) that Ag NPs were eliminated from the bodies of the zebrafish during the evolution from larval to adult stage, once the Ag NP exposure source was removed and/or (ii) that any Ag-En still present was no longer detectable, as it is diluted by the greater mass of the adult zebrafish. In addition, genetic stress markers were analysed for the adult zebrafish that were raised from the embryos of organisms exposed to ^{109}Ag NPs. An initial assessment of this data indicates that gene expression was not altered in the F1 generation zebrafish, which suggests that the Ag NP exposure had little or no impact on the genome of the embryos.

During the F1 generation exposure, embryos were collected on a weekly basis for all treatment groups to study potential similarities or differences in Ag-En uptake to embryos produced during exposure generation F0. Interestingly, none of the F1 embryos collected after week 1 of the exposure were found to contain Ag-En (Fig. 4.8, yellow and purple symbols). For exposure weeks 2 and 3, similar Ag-En levels of about 1 to 50 pg per embryo were found in F0 and F1 embryos (Fig. 4.8). With t-tests returning p values of between 0.18 and 0.83, the minor variations in the level of Ag-En present in F0 and F1 embryos are not statistically significant (A2.5). While the data show few if any differences between the F0 and F1 embryos in regard to Ag content, there is some indication that Ag-En uptake in the analysed tissue samples of adult zebrafish might differ between the two generations (Fig. 4.9). For livers of female fish exposed to either cit- ^{109}Ag NPs or cas- ^{109}Ag NP as well as male fish from the cit- ^{109}Ag NPs treatment, higher levels of Ag-En were found for the F1 generation than in F0 samples (Fig. 4.9). In female fish from the cas- ^{109}Ag NP treatment, the overall increase of Ag-En present in tissue was fairly low, from $1.0 \pm 0.9 \text{ ng mg}^{-1}$ in F0 to $1.6 \pm 1.1 \text{ ng mg}^{-1}$ in F1 (A2.4), and the corresponding t-test p value of 0.54 indicates that the difference is not statistically significant (A2.5). In the cit- ^{109}Ag NP treatment, however, the average Ag-En

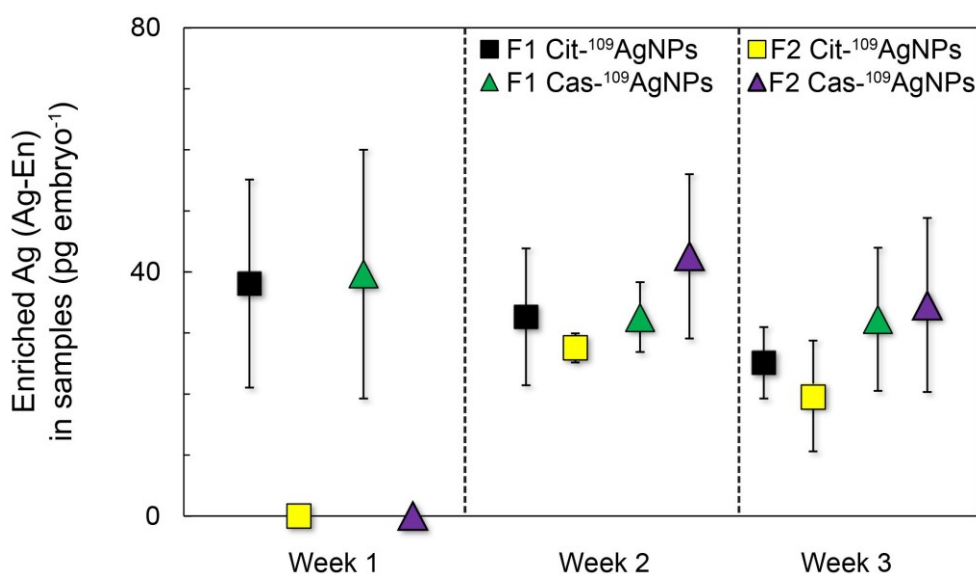


Fig. 4.8. Mass fraction of Ag-En in embryo samples of the F1 (black and green) and F2 (yellow and purple) generation. Plotted are mean values ± 1 SD for 4–6 individual samples of each treatment group and week, except for cit-¹⁰⁹AgNP in F1 Week 2 with $n = 3$. Whilst Ag-En was detected in all F1 samples, Ag-En was not detected in embryos from Week 1 of the F2 exposure (left). The overall uptake trends are similar in Weeks 2 and 3 for either exposure generation (middle and right).

content of liver tissue increased by a factor of three to four from 0.7 ± 0.3 ng mg⁻¹ (F0) to 3.1 ± 3.1 ng mg⁻¹ (F1) in female fish and from 6.2 ± 4.9 ng mg⁻¹ (F0) to 20.4 ± 13.5 ng mg⁻¹ (F1) in male fish (A2.4). For these cases, t-tests returned p values of 0.15 (for F0 versus F1 male zebrafish livers) and 0.16 (for F0 versus F1 female zebrafish livers), which may be indicative of minor differences in Ag distribution between the generations that could be investigated in further, specifically targeted, studies (A2.5).

In accord with the possible increase of Ag-En in liver tissue, most gonad samples show lower Ag-En levels in F1 compared to F0 (Fig. 4.10). In detail, the mean Ag-En content of gonads for all the analysed groups (female/male, cas-¹⁰⁹AgNPs/cit-¹⁰⁹AgNPs) decreases from the F0 to the F1 generation. The intergenerational differences are thereby larger for the gonads of female zebrafish ($p = 0.18$ for cas-¹⁰⁹AgNPs and $p = 0.04$ for cit-¹⁰⁹AgNPs respectively) than for the male counterparts ($p = 0.90$ for cas-¹⁰⁹AgNPs and $p = 0.23$ for cit-¹⁰⁹AgNPs

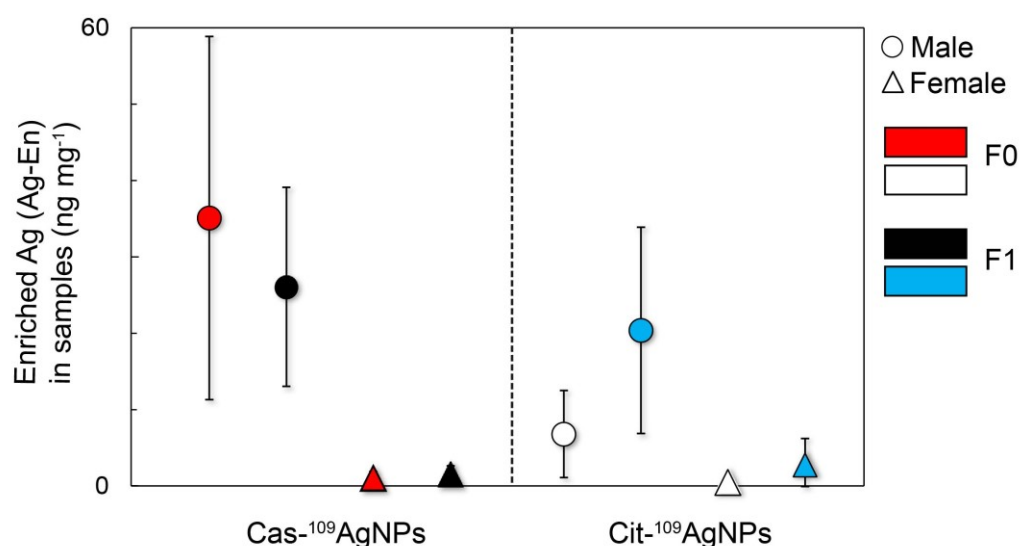


Fig. 4.9. Comparison of the Ag-En contents of livers for male (circles) and female (triangles) zebrafish after exposure to cit-¹⁰⁹AgNPs (right) and cas-¹⁰⁹AgNPs (left) in F0 (red and white) and F1 (black and blue). Plotted are the mean values $\pm$ 1SD for 4 to 6 individuals of each group.

respectively) (A2.5). Also of interest is the observation that the within-group variability of the data decreases considerably from F0 to F1, whereby all populations show very uniform Ag-En levels in the gonads of F1 organisms. In addition, the variability between groups is also lower for the gonads of F1 fish. This is demonstrated by the observation that the average Ag-En content for all F1 gonad groups of 0.5 ± 0.4 ng mg⁻¹ has a much smaller standard deviation than the corresponding F0 mean (2.10 ± 2.15 ng mg⁻¹; Fig. 4.10).

In summary, for both liver and gonad tissue the largest difference in Ag-En content between the F0 and F1 generations was observed for the cit-¹⁰⁹AgNP treatment groups. The general increase for the Ag-En content of liver tissue was thereby accompanied by a decrease of Ag-En in the gonad tissue of both male and female zebrafish. This trend appears to be more pronounced for female than for male fish. There are only a few published studies which investigate uptake into, and effect within, gonads of zebrafish after exposure to ENMs and none of these cover transgenerational effects [128-130]. Data obtained in a chronic water-borne exposure (28 d) of zebrafish to Ag NPs at a concentration of $8 \mu\text{l}_{\text{Ag}} \text{l}^{-1}$ thereby reports follicular

damage and atresia in ovarian tissue [128]. Furthermore, a general decrease in reproduction rates following exposure of mature zebrafish to Ag NPs was reported in multiple studies [114, 115]. For example, Orbea et al. [115] investigated the effect of water-borne Ag NPs at concentrations of 0.1 and 10 $\mu\text{g l}^{-1}$ on both *D. rerio* embryos and the reproduction of adult fish. These workers found a decrease in cumulative viable egg output of 58% and 68% for the low and high dose treatment groups, respectively. In the present study, significant differences in the total number of embryos and the overall cumulative viable egg output between the Ag NP treatment and control groups were observed in the F1 but not the F0 generation (Fig. 4.11). In F1, the decrease in cumulative viable egg output was 17% and 25% for the cas-¹⁰⁹AgNP and cit-¹⁰⁹AgNP treatment groups, respectively (Fig. 4.11).

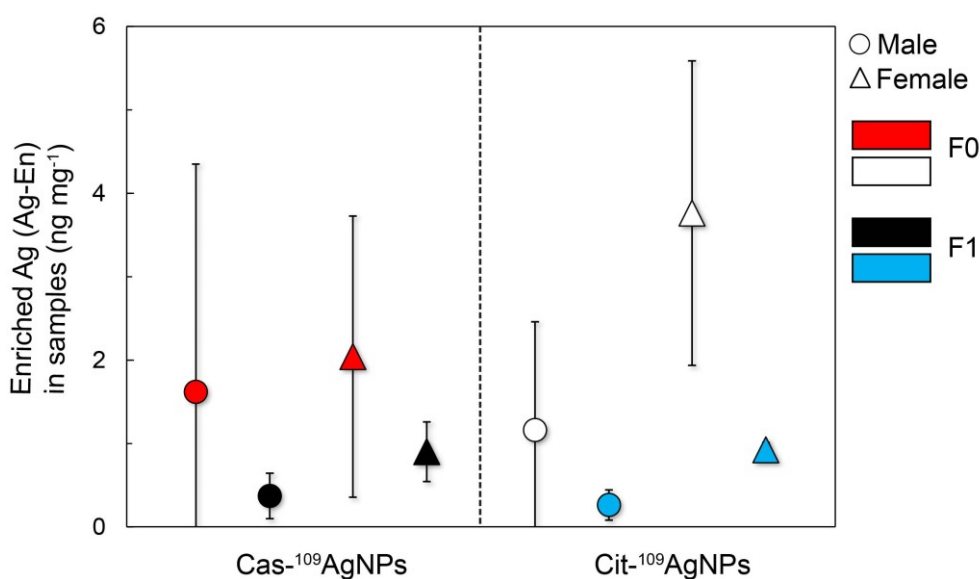


Fig. 4.10. Comparison of the Ag-En contents of gonads for male (circles) and female (triangles) zebrafish after exposure to cit-¹⁰⁹AgNPs (right) and cas-¹⁰⁹AgNPs (left) in F0 (red and white) and F1 (black and blue). Plotted are the mean values ± 1 SD for 5–6 individuals per group for F0 and 5–10 individuals per group for F1.

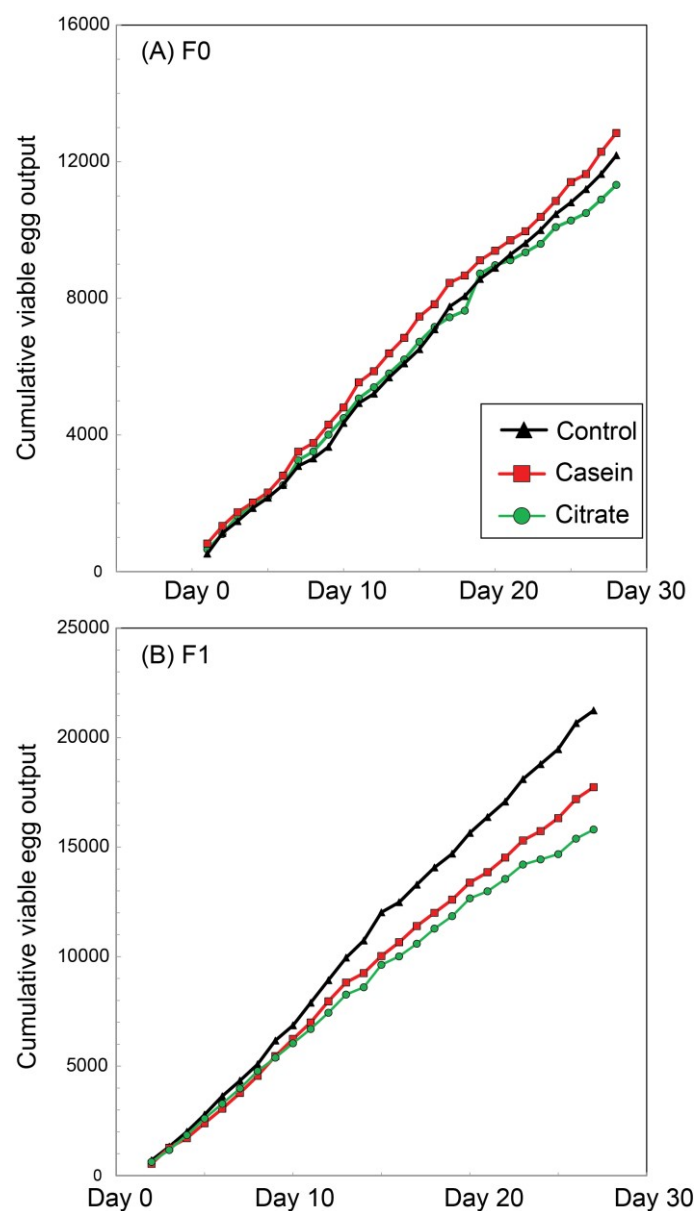


Fig. 4.11. Cumulative viable egg output for exposure generation F0 (A) and F1 (B). The decrease in egg output observed in F1 is statistically significant within the 95% confidence interval for *cis*-¹⁰⁹AgNP and *cas*-¹⁰⁹AgNP treatment group. (Note different scale of the y-axes in the panels.)

4.3.3.5. RNA-sequencing (F1)

In addition to the stable isotope tracing analyses that were performed to quantify the presence of Ag NPs in the exposed zebrafish, measurements of differential gene expression were carried out at the University of Exeter via RNA sequencing. An initial assessment of the

results reveals differential expression for only a small number of samples, with differences that are not significant compared to the ‘normal’ biological variability that was detected for individuals within each treatment line, including the control groups. In particular, the data provide no indication that gene expression was altered in the F1 offspring of zebrafish that were exposed to Ag NPs in the F0 generation, which suggests that the Ag NP exposure had little or no impact on the genome of the embryos. As such, the results of the RNA sequencing thus far provide no conclusive evidence that the exposure to Ag NPs had any effects on the gene expression of the zebrafish.

In conclusion, the data of this study does not show accumulation of Ag-En in the liver or gonad tissue of zebrafish that were exposed to cit-¹⁰⁹AgNPs or cas-¹⁰⁹AgNPs at PECs through their diet. In addition, gene expression in the zebrafish was not found to be altered by the exposure to Ag NPs over two consecutive generations. However, a decline in the spawning of healthy offspring for both treatments in generation F1 indicates that chronic exposure to Ag NPs at PECs may induce transgenerational effects on reproduction that warrant further study.

4.4. Conclusions

In the present exposure, zebrafish *D. rerio* were exposed to citrate-coated and casein-coated Ag NPs through their diet over two generations. Silver derived from the Ag NPs was successfully detected in the liver and gonad tissue of exposed fish and traced in embryos which themselves had not been affected by primary exposure to Ag NPs. The highly sensitive tracing analyses were enabled through the use of Ag NPs labelled with enriched ¹⁰⁹Ag and subsequent high precision Ag isotope analyses of samples by MC-ICP-MS.

The results of the analyses show, for the first time, that NP-derived Ag is detectable in the offspring of adult zebrafish that were exposed to Ag NPs through their diet at a relevant

environmental concentration. Given the previously reported high sensitivity of zebrafish embryos to the presence of Ag NPs, it is hence possible that the observed maternal transfer may have an impact on the fitness of the offspring [103, 117], specifically after multi-generational exposure. The analyses also revealed that Ag-En was not detectable in zebrafish livers and gonads within one generation of non-exposure and that the Ag levels of the organs do not increase over two generations of exposure. However, whilst reproduction appeared unaffected after one generation of exposure to Ag NPs, the cumulative viable egg output dropped significantly in the second exposure generation.

The presented data demonstrate that observations and conclusions drawn from exposures performed with Ag NPs at high dosing concentrations cannot be directly extrapolated to low exposure levels. In addition, the effects that Ag NPs can have on ecosystems may be different in successive exposure generations. To fully assess the potential environmental impact of ENMs, it is therefore necessary to conduct multigenerational exposures, to explore the accumulation and transfer of NPs in the food chain, and to evaluate the effect of simultaneous introduction of multiple contaminants [113, 131, 132].

4.5. Acknowledgments and declaration

The project was devised and planned by Mark Rehkämper (MR), Adam Laycock (AL), Charles Tyler (CT) (University of Exeter), Rhys Goodhead (RG) (University of Exeter), Inder Kaur (IK) (University of Nottingham) and Eva Valsami-Jones (EVJ) (University of Birmingham). AL, RG and IK performed all experimental work related to a pilot project that informed the set up and details of the exposure discussed in this work. Any experimental information related to the synthesis and characterisation of Ag NPs in this work was provided by IK. RG, with guidance from CT, planned, set up and performed the exposures and conducted work related to the zebrafish husbandry. RG also collected all samples analysed in this work

and analysed the data related to the zebrafish reproductive assay. RNA sequencing was performed by Bas Verbruggen (University of Exeter) who also provided an initial assessment of the data. Tabea Junk (TJ) digested, processed and analysed all samples for the isotopic and sample mass analysis provided in this chapter. TJ also wrote the text and produced the figures with guidance by MR and helpful comments by CT.

Chapter 5

Development and evaluation of stable isotope tracing of TiO₂ nanoparticles

Abstract

Engineered TiO₂ nanoparticles (NPs) are one of the most ubiquitous engineered nanomaterial (ENM) in the world. Their use in consumer products is becoming ever more widespread, which has raised concerns about the unclear transport mechanisms and fate of these materials in the natural environment. Detection of ENMs at environmentally relevant levels against a high and varying natural background is hindered by analytical limitations and has required the development of novel analytical methods. One such method is stable isotope tracing, which has already been successfully applied to the study of ZnO, Ag and CeO₂ NPs.

The work presented here assesses if stable isotope tracing is a suitable method for the study of engineered TiO₂ NPs in natural environments at predicted environmental concentrations. To this end, the current approaches towards studying TiO₂ NPs in the environment are reviewed and the method of stable isotope tracing is fully evaluated. Commonly encountered issues with concentration and isotopic measurements of Ti are discussed and previously established analytical uncertainties were used to constrain the detection sensitivities that can be achieved for the determination of labelled TiO₂ NPs by quadrupole ICP-MS, sector-field ICP-MS and multiple collector ICP-MS. The best measurement accuracy is offered by multiple collector ICP-MS analyses, which allow the addition of isotopically labelled TiO₂ NPs to be detected even at contributions of only 0.03% to the overall Ti budget of a sample.

5.1. Introduction

Titanium dioxide nanoparticles (TiO₂ NPs) are the second-most produced engineered nanomaterial worldwide according to a recent survey by Piccinno et al. [4]. They have long been used as semi-conductors, disinfectants and drinking water treatment agents, for surface coatings and in cosmetics such as sunscreens, partly due to their photocatalytic properties. Specifically, due to their widespread use in sunscreens, waste streams associated with TiO₂ NPs cannot easily be controlled and the increase in their use in consumer products is likely to be accompanied by a corresponding rise of their release into natural environments.

Titanium is an abundant metal that is ubiquitous in nature and has been classified as non-toxic in its elemental and in its most commonly found oxide form [133]. Regarding TiO₂ NPs, there are concerns about nano-specific effects, which may be caused by the small size and resulting high surface to volume ratio of the material as well as their ability to cross biological barriers such as cell membranes [134, 135]. As summarised in a recent review by Tan et al. [136], the information available on nano-TiO₂ and its interaction with the biosphere is often contradictory and further hindered by a lack of data on the actual release concentrations of TiO₂ NPs to the environment.

Analysing the findings of seven different published reports, Gottschalk and co-workers predicted concentrations of 0.01 – 10 µg l⁻¹ for TiO₂ NPs in natural surface waters in industrialised countries [10]. For soils, the same report found an even wider range of predicted environmental concentrations (PECs) from 10⁻⁵ µg kg⁻¹ for a local release scenario in Johannesburg (South Africa) to 10³ µg kg⁻¹ for soils treated with biosolids (Fig. 5.1) [10]. This shows that the actual environmental concentrations of TiO₂ NPs are likely to be highly variable, depending on the time and place of sampling as well as the type of environmental sample that is analysed. In addition, natural Ti background concentrations are of similar variability

although they are typically much higher than PECs and concentrations of 0.1 to $1200 \mu\text{g l}^{-1}$ for surface waters and up to 3500 mg kg^{-1} for soils have been reported (Fig. 5.1) [137-139].

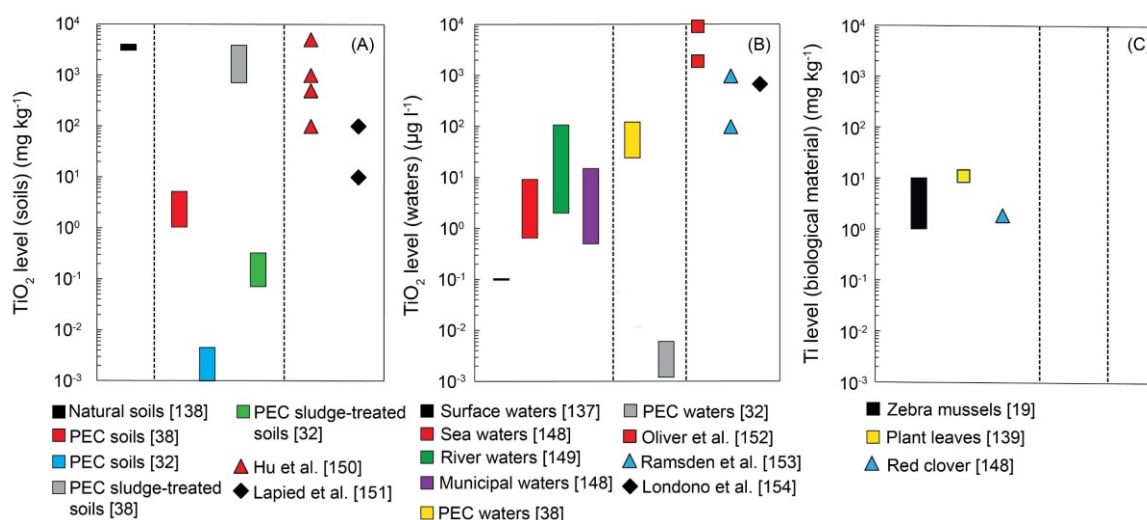


Fig. 5.1. Comparison of natural Ti levels (left of each panel), predicted environmental concentrations (PECs) from engineered TiO₂ NPs (middle of each panel) and examples of TiO₂ NP concentrations used in ecotoxicity studies (right of each panel) for (A) soils, (B) waters and (C) biological organisms. For biological organisms, PEC values are currently not available in the literature and exposure concentrations are not applicable as they are typically applied through indirect exposure.

Resolving small additions of engineered nano-TiO₂ from a high and variable natural background presents unique analytical challenges for conventional concentration measurements. However, in recent years stable isotope labelling and tracing techniques have proven their ability to successfully detect engineered nanomaterials (ENMs) at environmentally relevant concentrations in a variety of studies that employed ¹⁰⁹Ag, ⁶⁷ZnO, ⁶⁸ZnO and ⁶⁵CuO NPs [21, 22, 25, 31, 52, 140]. The method has recently been extended to TiO₂ NPs by Bourgeault et al. [26] using isotopically labelled ⁴⁷TiO₂ NPs for the investigation of ecotoxicological effects at PECs. The synthesis procedure they employed to produce nano-⁴⁷TiO₂ from bulk-⁴⁷TiO₂ utilised hotplate HCl digestion followed by reaction with NH₅F₂ in a microwave oven to generate a suitable Ti-precursor for alkaline precipitation of titanium hydroxide Ti(OH)₄ [26]. This method had a final synthesis yield of only 14%, which can render

labelled NPs extremely expensive, given that the enriched minor Ti isotopes ^{46}Ti , ^{47}Ti , ^{49}Ti and ^{50}Ti have a cost of about US-\$10 to US-\$15 per mg (Table 5.1). This implies that the synthesis of 100 mg of TiO_2 NPs from these isotopes costs about US-\$10,000-15,000, which is prohibitively expensive for many research budgets and may impede the wider use of Ti isotope tracing.

Table 5.1: Information on natural abundances, enrichment factors and cost of Ti isotopes.

Isotope	^{46}Ti	^{47}Ti	^{48}Ti	$^{49}\text{Ti}^\#$	^{50}Ti
Natural abundance [%]	8.25	7.44	73.72	5.41	5.18
Isotopic enrichment [%]	97	95.7	96.2	N/A	50.4
Price of tracer [US-\$ mg⁻¹]	15.9	15.8	1.1	N/A	13.5
Cost to produce 1 mg of enriched TiO_2 NPs at...					
14% yield [US-\$]	114	113	8	N/A	96
50% yield [US-\$]	32	32	2	N/A	27
90% yield [US-\$]	18	18	1	N/A	15

[#]: Although ^{49}Ti is commercially available, Isoflex was unable to provide details on its enrichment levels or cost at the time this study was prepared.

It is therefore desirable to explore alternative synthesis routes, to improve the overall yield and to identify a scalable preparation protocol that allows the production of specific enriched TiO_2 quantities, as required for a particular exposure experiment. In addition, a detailed evaluation of the different Ti isotopes, analytical requirements and considerations in light of cost-effectiveness has not been carried out to date.

The stable isotopes of Ti and their natural abundances, available enrichment levels and associated cost of the isotopically enriched isotopes are shown in Fig. 5.2 and Table 5.1. Titanium has five stable isotopes with abundances of between ~5% and ~73% and isotope

tracers are typically available at enrichment levels of up to >95% (Table 5.1, Fig. 5.2). As the isotopes of Ti differ so strongly in their natural abundance, the choice of isotope label greatly influences the detection sensitivity, but also the cost of the TiO₂ NP synthesis. It is therefore worthwhile to investigate the capabilities that are offered by the use of different Ti isotopes in order to be able to choose the most suitable isotopic tracer for a specific application. The present study aims to provide this information and to offer a detailed evaluation of the detection abilities of isotopically labelled Ti when used in combination with different types of analytical instrumentation and for a range of analytical scenarios. In addition, preliminary data for the development of a high yield, small-scale wet TiO₂ NP synthesis suitable for the preparation of isotopically labelled tracer material is presented.

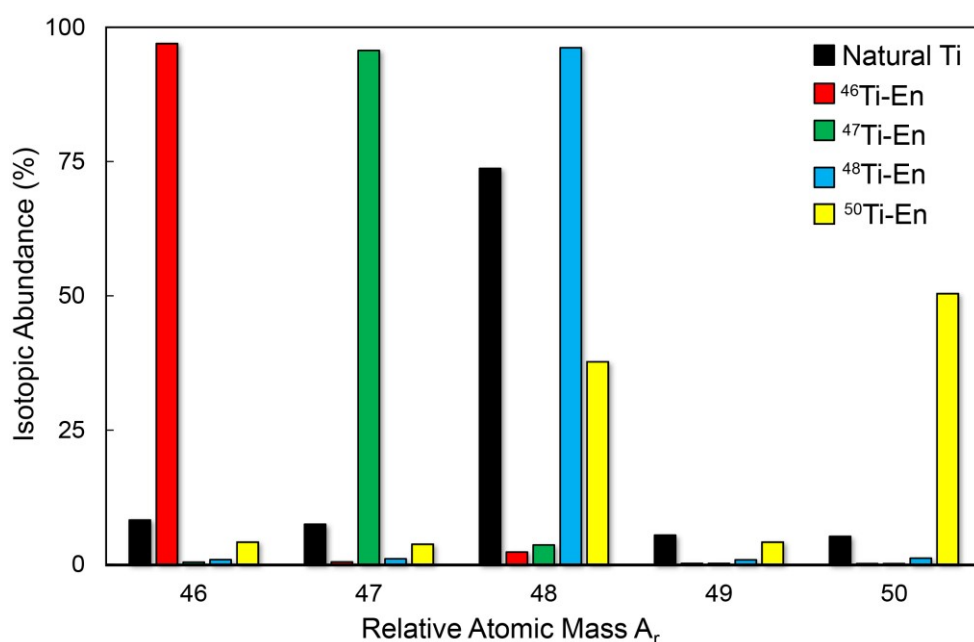


Fig. 5.2. The five stable isotopes of Ti and their natural abundance (black). Also shown are the isotopic abundances of different enriched Ti (Ti-En) isotopes that were considered in this study - ⁴⁶Ti-En in red, ⁴⁷Ti-En in green, ⁴⁸Ti-En in blue and ⁵⁰Ti-En in yellow.

5.2. Tracing of TiO₂ nanoparticles in environmental samples

5.2.1. Sample preparation

As isotopically enriched Ti is mainly available as TiO₂, a synthesis to produce labelled TiO₂ NPs needs to first dissolve adequate amounts of bulk TiO₂, which is hindered by the refractory nature of the oxide. Most commonly, TiO₂ is dissolved via (i) fusion methods, (ii) using hydrofluoric acid or (iii) with sulphuric acid.

Fusion procedures can effectively digest TiO₂, but at the cost of introducing large quantities of salts. If high dissolved solid concentrations are present in the final solutions for ICP-MS analysis without prior pre-concentration of Ti, this can hinder efficient sample nebulisation and cause blockages of sampler and/or skimmer cones. In addition, the fusion reagents may raise blank levels considerably [141]. To circumvent the former issues, significant sample dilution is generally employed, but this affects sensitivity as it decreases the Ti concentration in solution. In addition, fusion procedures introduce elements to the solution that produce interferences during ICP-MS analysis. This includes ³¹P, which can interfere with measurements of any Ti isotope through formation of [³¹P¹⁵N]⁺, [³¹P¹⁶O]⁺, [³¹P¹⁷O¹H]⁺, [³¹P¹⁸O]⁺ and [³¹P¹⁹O¹H]⁺ (Table 5.2) [26]. Such effects are also relevant for digestion methods that employ sulphuric acid or fusion digestion with sulphate species, as S isotopes in combination with isotopes of C, N, and O can impede accurate measurements of all Ti isotopes (Table 5.2). The use of such reagents is thus best avoided or suitable measures must be taken to ensure that analyses, which may suffer from such molecular interferences, are not significantly affected.

Conventional acid digestion methods with concentrated HCl or HNO₃ are unable to fully dissolve TiO₂, even at elevated temperatures during hotplate or microwave digestion [26, 141]. Whilst this is unproblematic and even desirable for the detection of NPs using single particle ICP-MS (SP-ICP-MS), it is a matter of concern for analyses that aim to quantify the presence of TiO₂ NPs in bulk samples following sample digestion, based on Ti concentration measurements or stable isotope tracing.

Table 5.2: Isobaric and spectral interferences for the five stable isotopes of titanium.

Isotope	⁴⁶ Ti	⁴⁷ Ti	⁴⁸ Ti	⁴⁹ Ti [#]	⁵⁰ Ti
Isobaric interferences (Abundance)	⁴⁶ Ca (0.004%)		⁴⁸ Ca (0.2%)		⁵⁰ Cr (4.6%) ⁵⁰ V (0.3%)
Molecular interferences[26]	[¹⁴ N ³² S] ⁺	[¹⁶ O ³¹ P] ⁺	[³⁶ Ar ¹² C] ⁺	[⁴⁰ Ar ⁹ Be] ⁺	[³⁶ Ar ¹⁴ N] ⁺
	[⁴⁰ Ar ⁶ Li] ⁺	[⁴⁰ Ar ⁷ Li] ⁺	[¹⁶ O ³² S] ⁺	[¹⁴ N ³⁵ Cl] ⁺	[⁴⁰ Ar ¹⁰ B] ⁺
	[¹² C ³⁴ S] ⁺	[¹² C ³⁵ Cl] ⁺	[¹ H ⁴⁷ Ti] ⁺	[¹ H ⁴⁸ Ti] ⁺	[¹ H ⁹ Ti] ⁺
	[¹⁶ O ³⁰ Si] ⁺	[¹ H ⁴⁶ Ti] ⁺	[¹⁴ N ³⁴ S] ⁺	[¹² C ³⁷ Cl] ⁺	[¹⁶ O ³⁴ S] ⁺
	[¹⁵ N ³¹ P] ⁺	[¹⁴ N ³³ S] ⁺	[¹³ C ³⁵ Cl] ⁺	[¹⁶ O ³³ S] ⁺	[¹⁵ N ³⁵ Cl] ⁺
	[¹⁸ O ²⁸ Si] ⁼⁺	[¹⁵ N ³² S] ⁺	[¹² C ³⁶ S] ⁺	[¹⁸ O ³¹ P] ⁺	[¹³ C ³⁷ Cl] ⁺
	[¹⁹ O ¹ H ²⁷ Al] ⁺	[³⁶ Ar ¹¹ B] ⁺	[¹⁷ O ¹ H ³¹ P] ⁺	[¹ H ⁴⁸ Ca] ⁺	[¹⁸ O ³² S] ⁺
	[¹⁷ O ¹ H ²⁹ Si] ⁺	[¹⁹ O ¹ H ²⁸ Si] ⁺		[¹⁷ O ¹ H ³² S] ⁺	[¹⁴ N ³⁶ S] ⁺
		[¹⁷ O ¹ H ³⁹ Si] ⁺			[¹⁷ O ¹ H ³³ S] ⁺
					[¹⁹ O ¹ H ³¹ P] ⁺

While HF allows quantitative hotplate digestion of bulk TiO₂, the resulting solution contains anionic fluorotitanate [TiF₆]²⁻ complexes, which would release gaseous HF during TiO₂ NP synthesis. This digestion method is therefore associated with significant safety concerns and is further hampered by a lack of published methods for the synthesis of TiO₂ NPs from fluorotitanate species.

Dissolution of TiO₂ in H₂SO₄ results in the formation of titanyl sulphate TiOSO₄, which has previously been used in aqueous solution to synthesise different types of TiO₂ NPs [142-146]. During the course of this study, it was found that the dissolution of TiO₂ in H₂SO₄ is only successful if concentrated H₂SO₄ is used in excess quantities and at high temperature. At room temperature and hotplate digestion temperatures of up to 120°C, even TiO₂ concentrations as

low as 0.02 mol l^{-1} (1.8 g l^{-1}) were not readily dissolved in H_2SO_4 . In contrast, 0.63 mol l^{-1} (50 g l^{-1}) TiO_2 were successfully and quantitatively dissolved when refluxed in H_2SO_4 in a silicone bath at 130°C (hotplate temperature of 300°C). This produces a highly acidic ($\text{pH} < 1$) stock solution of dissolved TiOSO_4 that can be used for further synthesis.

5.2.2. TiO_2 nanoparticle tracing by Ti concentration measurements

Studies of ENMs often employ concentration measurements whereby NPs are added to an exposure system and detection relies on tracing of concentration changes. This approach is particularly useful for rare elements such as gold, where natural background levels are very low and only small amounts of Au NPs are required for successful detection [147]. Concentration tracing, however, is not well suited to the study of materials such as TiO_2 where detection at environmentally relevant exposure levels is hindered by comparatively high natural backgrounds. For example, the average natural background level for Ti in soils has been reported as 3500 mg kg^{-1} , with large regional differences in this value [138].

Several studies have modelled the fluxes of ENMs to the environment and published PECs for TiO_2 NPs [14, 39]. Depending on the modelling parameters and the studied region, PECs ranging from 10^{-5} mg kg to 10^3 mg kg have been reported for soils (Fig. 5.1). Notably, these PECs are within the range of or orders of magnitude lower than the environmental background levels of Ti (Fig. 5.1). Although Ti concentrations are lower in aquatic systems, the same relationship between natural background levels and TiO_2 NP PECs exists in surface waters and sea-water (Fig. 5.1). While Ti concentrations between $10^{-2} \text{ } \mu\text{g l}^{-1}$ to $10^2 \text{ } \mu\text{g l}^{-1}$ have been reported for different water bodies, the relevant PECs are in the range of $10^{-2} \text{ } \mu\text{g l}^{-1}$ or $10\text{--}100 \text{ } \mu\text{g l}^{-1}$ (Fig. 5.1) [14, 39, 137, 148, 149].

To study the environmental behaviour and effects of TiO₂ NPs, Ti concentration measurements require the addition of large amounts of Ti in order to successfully resolve the added nano-Ti fraction against a high natural Ti background. As a consequence, exposure studies that employ Ti concentration measurements for detection typically use TiO₂ NP quantities that exceed the PECs by several orders of magnitude (Fig. 5.1) [150-154]. While such investigations may reveal the behaviour of TiO₂ NPs in ecosystems, the results may not reliably inform on the consequences that TiO₂ exposure will have on organisms or the rate at which TiO₂ NPs accumulate in the food-chain. It is therefore desirable to develop instrumental methods that allow the determination of TiO₂ NPs at realistically low and environmentally relevant exposure concentrations.

5.2.3. TiO₂ nanoparticle tracing by SP-ICP-MS

The application of SP-ICP-MS for the characterisation of NPs was first established in a series of papers by Degueldre and co-workers, who applied the technique to the study of Al₂O₃, ZrO₂ and TiO₂ NPs [155-158]. While ICP-MS methods generally rely on the measurement of a steady-state signal for a solution containing dissolved species, SP-ICP-MS detects the ion cloud that is produced by the introduction of a particle into the plasma. Such analyses are difficult because detection of the complete ion cloud produced by a single particle in an individual integration cycle requires careful calibration of the timing parameters. During measurement, both the number and intensity of the ionic pulses are recorded and used to infer the NP concentration of the solution and the size and/or size distribution of the NPs. The dissolved material is monitored simultaneously as a steady-state background signal that usually has a low intensity and defines the detection sensitivity of the measurement. As such, the analyses allow determination of the concentration of the dissolved fraction at the same time as

resolving the pulses produced by the NPs against the background signal. The SC-ICP-MS technique requires that samples are introduced at low analyte concentrations, which renders it attractive for the study of ENMs at environmentally relevant concentrations.

In recent years, the analytical advantages of SP-ICP-MS have led to increased use for the study of ENMs in general and TiO₂ in particular. As such, SP-ICP-MS has been used to characterise TiO₂ NPs in wastewater effluent [159], surface water samples [160, 161], drinking water [162] and in sunscreens [163]. To a lesser extent, the technique was also employed to study samples from TiO₂ NP exposure experiments in aquatic environments. In one such study, TiO₂ NPs were added to river water at a concentration of 700 µg l⁻¹ and were found to have no statistically significant effect on the microbial community [154].

A drawback of TiO₂ NPs analyses by SP-ICP-MS is the isobaric overlap of ⁴⁸Ti with ⁴⁸Ca, which is problematic because Ca is an abundant element in most environmental compartments. Published methods therefore usually rely on the measurement of ⁴⁷Ti, which has a much lower natural abundance at 7.3% than ⁴⁸Ti at 73.7% (Table 5.1). This increases the particle size limit of detection (LOD) for Ti in environmental samples compared to, for example, laboratory standard solutions. In detail, reported LOD values for environmental samples are 55-65 nm for ⁴⁷Ti, 37 nm for ⁴⁸Ti and 100 nm for ⁴⁹Ti [164]. As such, these size LODs severely limit SP-ICP-MS studies of TiO₂ NPs in the environment, especially as many TiO₂ NP applications rely primarily on small particles [165]. Another drawback of the technique is that it does not differentiate between natural and engineered TiO₂ NPs. It therefore requires careful monitoring of the natural NP background, which is not possible in most dynamic environmental systems.

5.2.4. Stable isotope tracing of TiO₂ nanoparticles – status quo

The use of an isotopically enriched tracer for the study of TiO₂ NPs was pioneered by Bourgeault et al. [26]. These workers used ⁴⁷Ti enriched to >95% to prepare ⁴⁷TiO₂ NPs that were employed in an exposure of zebra mussels at environmentally relevant dosing levels [26]. As they analysed whole sample solutions without pre-concentration of Ti by quadrupole ICP-MS (Q-ICP-MS), their choice of isotope label was strongly dictated by the presence of matrix elements from the sample and sample preparation.

In order to minimise contaminant levels, a sample digestion procedure that employed H₂O₂/HCl/HNO₃ was tested but found to recover only about 70% of Ti. Fusion procedures with either ammonium persulfate or di-hydrogen phosphate/di-sodium hydrogen phosphate, provided an almost quantitative digestion yield of >95% [26]. However, both the persulfate and phosphate fusion methods introduced large quantities of salts into the sample solutions, which can produce problematic spectral interferences. Bourgeault and co-workers found that phosphate fusions produced sample solutions that featured particularly problematic interferences from [³¹P¹⁶O]⁺, which rendered accurate measurements of ⁴⁷Ti at low Ti concentrations impossible (Table 5.2) [26].

Although the persulfate fusions introduce S into sample solutions, S-based polyatomic ions (Table 5.2) were found to be less problematic and resulted in better performance during Q-ICP-MS analyses. For samples with high Ti concentrations, sample dilution by up to a factor of 200 decreased the salt contents in the solutions to tolerable levels. However, samples with low Ti contents could not be diluted to this extent and significant spectral interferences were thus observed for all relevant isotopes (Table 5.2). To assess the impact of the interferences, Bourgeault et al. [26] analysed a series of solutions with variable Ti and S concentrations, whereby the diagnostic ⁴⁷Ti/⁴⁶Ti and ⁴⁷Ti/⁴⁹Ti ratios were monitored. These measurements

showed that the Ti isotope analyses were not biased by S-based spectral interferences if the solutions had S/Ti ratios of less than 100,000. The $^{47}\text{Ti}/^{46}\text{Ti}$ and $^{47}\text{Ti}/^{49}\text{Ti}$ measurements were furthermore reported to have similar 1 RSD errors of roughly $\pm 1\%$ and $\pm 15\%$ for solutions with Ti concentrations of about 45 and $1\text{ }\mu\text{g l}^{-1}$, respectively.

The study of Bourgeault and co-workers did not discuss the advantages and disadvantages of using different Ti isotopes for tracing purposes. However, as Ti features five stable isotopes of variable natural abundance, a range of tracing approaches with different diagnostic isotope ratios are possible. Furthermore, the cost of the different enriched isotopes ranges from US-\$1 to more than US-\$10 per milligram and may influence decisions regarding the choice of isotope label, especially given that protocols for the synthesis of TiO_2 NPs may not provide perfect yields.

5.3. Experimental

5.3.1. Synthesis of TiO_2 nanoparticles

Bulk TiO_2 was purchased in powder form from Sigma Aldrich and dissolved in a round-bottom flask at elevated temperatures ($>130^\circ\text{C}$) in AnalaR grade concentrated H_2SO_4 to form the synthesis precursor titanyl sulphate TiOSO_4 . Typically, 300 mg TiO_2 (3.8 mmol) were dissolved in 6 ml H_2SO_4 to prepare a stock solution of TiOSO_4 ($c = 0.62\text{ mol l}^{-1}$). Of this solution, 2 ml were transferred to a reaction beaker and diluted with Milli-Q (MQ) water (Millipore, UK) to give a TiOSO_4 concentration of 0.25 mol l^{-1} . The synthesis then followed a procedure published by Sakthivel and co-workers [146]. In short, TiO_2 NPs were precipitated from TiOSO_4 by dropwise addition of NaOH under vigorous stirring in a round bottom flask at room temperature. The final pH of the solution was adjusted to ~ 5 . The suspension was then aged under stirring at room temperature for 36 hours. The suspension was centrifuged for

60 minutes at 4000 RPM in a Thermo Scientific Heraeus Megafuge and the precipitate washed twice with MQ water to remove excess sulphate ions. Finally, the precipitate was dried in an oven at 100°C and the solid product was weighed to determine the synthesis yield (Mettler Toledo AX205).

5.3.2. Characterisation of TiO_2 nanoparticles

For transmission electron microscopy (TEM), 1 ml of sample suspension was removed before the solution was washed and dried. A drop of this suspension was deposited undiluted onto a Formvar/carbon coated Cu-grid and dried in a desiccator overnight. TEM micrographs were obtained at varying magnifications using a JEOL STEM/TEM 2100Plus (Jeol Ltd.) equipped with an Oxford Instruments ultra-thin window energy dispersive X-ray spectrometer (EDS) to allow for elemental analysis as well as microscopic capabilities.

5.3.3. Isotopic tracing of TiO_2 NPs - modelling study

Titanium has five natural stable isotopes with abundances between 5.2% and 73.7% (Fig. 5.2, Table 5.1), and the multiple isotopes enable stable isotope tracing of Ti, and by inference, TiO_2 NPs. In principle, there are two pathways available for stable isotope tracing of engineered TiO_2 NPs – this can be based on an intrinsic difference in stable isotope composition or make use of enriched stable isotopes. Stable isotope studies of Ti quote differences in Ti isotope compositions using $\delta^{X/Y}Ti$ values, which are based on the difference in the diagnostic $^{X}Ti/^{Y}Ti$ isotope ratio of a sample compared to a standard reference material and are reported as a per mil deviation:

$$\delta^{X}Ti(\text{‰}) = \left[\frac{(^{X}Ti/^{Y}Ti)_{\text{Sample}}}{(^{X}Ti/^{Y}Ti)_{\text{Standard}}} - 1 \right] \times 1000 \quad (\text{Equation 5.1})$$

If an enriched stable isotope is used to trace TiO₂ NPs, the nanomaterial would be manufactured from a single enriched isotope of Ti. The tracing of this material is then based on measurements of a diagnostic isotope ratio, which is changed by the variable presence or absence of the labelled material. As such, the diagnostic ratio most conveniently features the labelled isotope in the numerator and a suitable other, non-labelled stable reference isotope of the element in the denominator.

For example, if ⁴⁹Ti is used as the enriched label and ⁴⁷Ti as the non-labelled reference isotope, the addition of labelled material to a natural sample will produce a mixture with a novel, non-natural ⁴⁹Ti/⁴⁷Ti isotope ratio. This ratio (R_{Sam}) can be predicted based on mass bias calculations as outlined in detail for Ag in Chapter 4.2.3.5. In the case of ⁴⁹Ti/⁴⁷Ti, the isotope ratio of the sample is given by

$$\frac{{}^{49}\text{Ti}}{{}^{47}\text{Ti}} = R_{Sam} = \frac{fr^{49}\text{Ti}_{Nat} \times \text{Ti}_{Nat} + fr^{49}\text{Ti}_{En} \times \text{Ti}_{En}}{fr^{47}\text{Ti}_{Nat} \times \text{Ti}_{Nat} + fr^{47}\text{Ti}_{En} \times \text{Ti}_{En}} \quad (\text{Equation 5.2})$$

where $fr^{XX}\text{Ti}_{XXX}$ are the fractional molar abundances of the ^{XX}Ti isotope in natural Ti (Ti_{Nat}) and enriched Ti (Ti_{En}) respectively.

The fraction of enriched Ti to natural Ti ($\text{Ti}_{En}/\text{Ti}_{Nat}$) in a sample is thus given by:

$$\frac{\text{Ti}_{En}}{\text{Ti}_{Nat}} = \frac{R_{Sam} \times fr^{47}\text{Ti}_{Nat} - fr^{49}\text{Ti}_{Nat}}{fr^{49}\text{Ti}_{En} - R_{Sam} \times fr^{47}\text{Ti}_{En}} \quad (\text{Equation 5.3})$$

The mass and molar amounts of natural titanium present in a sample ($m_{Ti,Sam}$ and $n_{Ti,Sam}$, respectively) can be determined through the sample mass (m_{Sam}), the natural background levels of Ti (Ti_{BG}) and the atomic mass of Ti, $M(\text{Ti}_{Nat})$.

$$m_{Ti,Sam} = m_{Sam} \times \text{Ti}_{BG} \quad (\text{Equation 5.4})$$

$$n_{Ti,Sam} = \frac{m_{Ti,Sam}}{M(\text{Ti}_{Nat})} \quad (\text{Equation 5.5})$$

The molar ratio of enriched to natural Ti in combination with the amount of natural Ti present in the sample can be used to quantify the increase in Ti level (Δn_{Ti}) that is required to resolved enriched Ti from the Ti background, for a given uncertainty in the measurement of R_{Sam} .

$$\Delta n_{Ti} = \frac{Ti_{En}}{Ti_{Nat}} \times n(Ti_{Nat}) \quad (\text{Equation 5.6})$$

Based on the result of Equation 5.6, the increase in Ti in a sample required for detection can now be calculated as a mass increase (Δm_{Ti}), a percentage or per mil increase ($\Delta\%_{Ti}$) or an increase in the mass fraction of Ti relative to the total Ti in the sample ($\Delta\omega_{Ti}$).

$$\Delta m_{Ti} = \Delta n_{Ti} \times M(Ti_{En}) \quad (\text{Equation 5.7})$$

$$\Delta\%_{Ti} = \frac{\Delta n_{Ti}}{n(Ti_{Nat})} \times 1000 \quad (\text{Equation 5.8})$$

$$\Delta\omega_{Ti} = \frac{\Delta m_{Ti}}{m_{Sam}} \quad (\text{Equation 5.9})$$

The NIST database was used as a source for the relative atomic weights and isotopic abundances of natural Ti [90]. Information about available enriched Ti isotopes, particularly purity and their associated cost, were obtained from Isoflex, U.S.A for ^{46}Ti , ^{47}Ti , ^{48}Ti and ^{50}Ti . Analytical uncertainties for different measurement techniques and natural Ti background concentrations for soils and biological tissues were gathered from relevant published literature. Altogether, these data were used to evaluate isotopic tracing considering sensitivity of detection as well as cost of production of labelled TiO_2 NPs for a variety of analytical scenarios.

5.4. Results and discussion

5.4.1. Preliminary results for TiO_2 synthesis route

As part of this work, studies of TiO_2 NP precipitation from an acidic solution containing dissolved TiOSO_4 were carried out. A detailed evaluation of TiO_2 NP precipitation under varying conditions by Sakthivel and co-workers was used as a template and adapted for the fact that TiOSO_4 was not present in aqueous solution but rather in a highly acidic H_2SO_4 solution [146]. The use of NaOH as hydrolysing agent at $\text{pH} \approx 5$ and ageing of particles at room temperature for 36 hours produced a solution of TiO_2 particles that were characterised by TEM (Fig. 5.3).

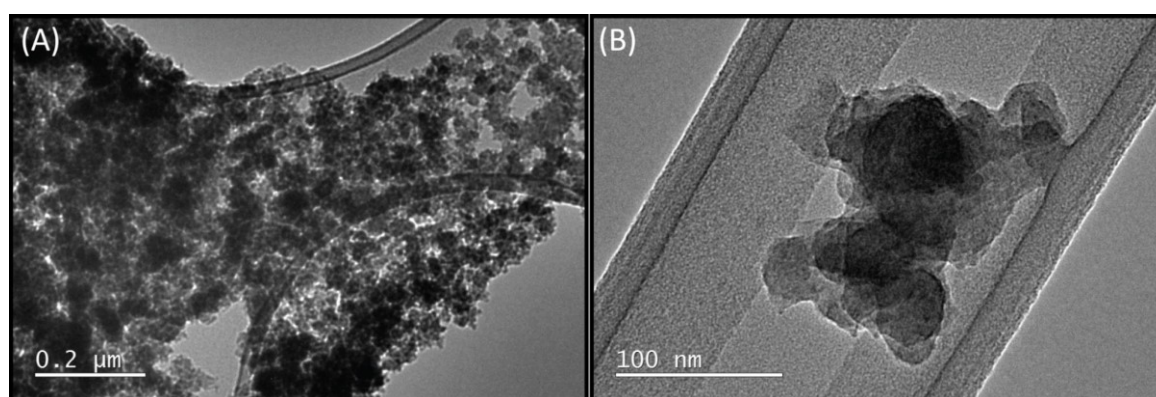


Fig. 5.3. TEM images of the synthesised TiO_2 NPs at different magnifications that show dense clusters of spherical particles.

The TEM suspension was densely populated (Fig. 5.3A) by spherical particles of <100 nm diameter (Fig. 5.3B). Precise analysis of particle size was not possible, however, due to the high concentration of particles in the suspension, which resulted in more than one particle layer being imaged during transmission mode. Nonetheless, the images show that nano-sized particles were produced in large quantities during synthesis, which is a promising starting point for further method development. At this point, the final product is likely to be hydrolysed TiO_2 in the form of $\text{Ti}(\text{OH})_4$ [26] or $\text{TiO}(\text{OH})_2$. For these two cases, the synthesis product of 132 mg is equivalent to a yield 91% for $\text{Ti}(\text{OH})_4$ or 108% for $\text{TiO}(\text{OH})_2$. To produce the final TiO_2 NPs

of ~13 nm size, the intermediate products are normally heated at 500°C for four hours to ensure full removal of water [146].

5.4.2. Stable isotope tracing of TiO₂ nanoparticles

5.4.2.1. Sample preparation and isobaric interferences

Stable isotope tracing involves the measurement of a diagnostic isotope ratio and as such requires a precise and unbiased measurement of the relative abundance of two Ti isotopes. As, in principle, any of the Ti isotopes can be used for tracing, it is useful to consider possible spectral interferences, which may render such analyses particularly difficult for individual Ti isotopes. Such considerations are obviously most important for isotopic analyses that are conducted without pre-concentration of the target element. However, spectral interferences are also a potential issue even after chemical separation. This reflects that no chemical separation is perfect and that procedures that generate the purest solutions are often compromised in their yield and tend to require time-intensive, multi-stage processing. For environmental studies that require the processing of many samples (>100), it is hence important that simple separation techniques are applied, which in turn are commonly compromised in the elemental purity that is achieved. Considering Ti, isobaric interferences from ⁴⁶Ca, ⁴⁸Ca, ⁵⁰Cr and ⁵⁰V are possibly problematic for ⁴⁶Ti, ⁴⁸Ti and ⁵⁰Ti, especially for sample solutions without any Ti pre-concentration (Table 5.1). In addition, polyatomic interferences from argides such as [¹²C³⁶Ar]⁺ or [¹⁴N³⁶Ar]⁺ (Table 5.1), should be considered as argon is the ICP-MS plasma gas and therefore present in abundance.

Whilst isotopic measurements by Q-ICP-MS or single collector sector field ICP-MS (SF-ICP-MS) are typically conducted without any chemical separation of the target element, analyses by multiple collector ICP-MS (MC-ICP-MS) are usually performed on pure elemental

solutions. Such a chemical separation is an absolute pre-requisite if a high-sensitivity desolvating sample introduction system (such as the CETC Aridus II or Nu Instruments DSN-100) is used for sample introduction. Chemical separation of the target element has the added advantage that this improves the overall sensitivity of the isotopic measurements, as relatively concentrated pure elemental solutions can be analysed. As such, the detection limit and analytical precision that can be achieved following chemical separation are far superior to those attainable without elemental pre-concentration. The most precise isotopic measurements are thereby achieved on pure elemental solutions using SF-ICP-MS and, in particular, MC-ICP-MS.

A number of protocols have been published for the separation of Ti from geological samples for high precision measurements of natural variations in stable Ti isotope compositions [166-170]. These procedures typically employ multiple (2-3) stages of ion exchange chromatography to generate a very pure Ti solution. In conjunction with MC-ICP-MS, these methods have been used to determine variations in $^{49}\text{Ti}/^{47}\text{Ti}$ and $^{49}\text{Ti}/^{46}\text{Ti}$ ratios (reported as $\delta^{49}\text{Ti}$) with an external precision of $\pm 0.02\text{‰}$ (2 SD) [171, 172]. The separation procedures of such stable isotope studies are, in principle, directly applicable to the pre-concentration of Ti from soil, water or biological samples for isotopic tracing of TiO_2 NPs. However, whilst the use of several column separation steps produces very pure Ti solutions it also constitutes a very laborious process. Given that isotopic tracing of NPs labelled with enriched stable isotopes generally does not require analyses at the same level of precision as natural stable isotope investigations, it will be useful to modify and simplify such procedures for tracing studies. If done with care, such modifications will allow significantly better sample throughput with only a limited sacrifice in sample purity and data quality, as demonstrated by the Ag separation method presented in Chapter 3.

5.4.2.2. Stable isotope tracing of TiO₂ nanoparticles based on intrinsic differences in Ti stable isotope compositions

Stable isotope tracing of TiO₂ NPs based on the intrinsic Ti stable isotope composition is possible if there is a sufficient isotopic difference from the natural background. Previous work has thereby established precise techniques to resolve even small natural variation in Ti stable isotope compositions. Based on this, a number of studies have revealed a natural variability of up to about 2‰ in $\delta^{49/47}\text{Ti}$ and $\delta^{49/46}\text{Ti}$ for terrestrial rocks, whilst investigations of biological and anthropogenic samples are non-existent (Fig. 5.4) [171-174]. However, given that Ti is bio-inert as well as highly refractory it is unlikely that biological processes and industrial processing will generate Ti isotope variations that significantly exceed the geological variability.

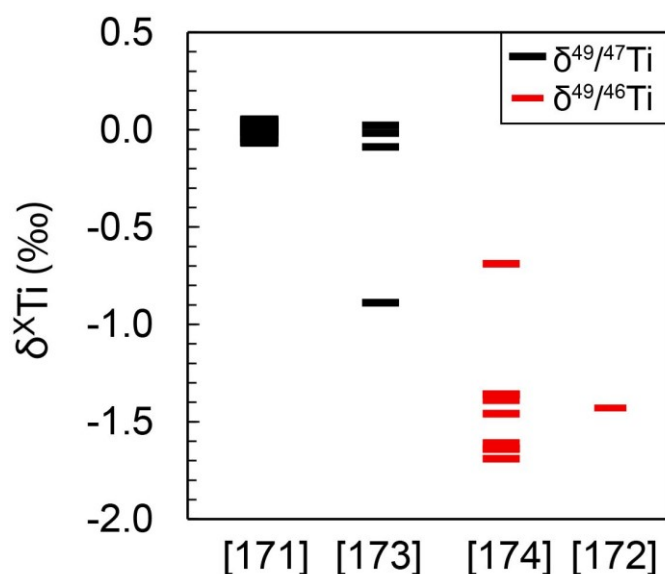


Fig. 5.4. Natural stable isotope variations for Ti in terrestrial geological materials, reported as $\delta^{49/47}\text{Ti}$ (black) and $\delta^{49/46}\text{Ti}$ (red). The samples show an overall isotopic variability of nearly 2‰.

In the following, mass balance modelling is applied to assess the potential use of stable isotope tracing of TiO₂ NPs based on an intrinsic isotope difference. To this end, the modelling

assumes that the engineered TiO₂ NPs have an isotopic signature in $\delta^{49/47}\text{Ti}$, which differs from the natural background by either +2‰ or +10‰. Such a difference is too small to be resolved reliably using Q-ICP-MS or SF-ICP-MS but can be detected using MC-ICP-MS techniques, which are assumed to have a precision of $\pm 0.05\text{‰}$. These parameters can be used to calculate the associated detection sensitivities, hereby defined as the smallest addition of enriched Ti (Ti-En) from engineered TiO₂ NPs that can be reliably resolved in the presence of a given background level of natural TiO₂ (Fig. 5.5). The results of the calculations (Fig. 5.5) demonstrate that an isotopic shift from the introduction of isotopically heavy TiO₂ NPs, characterized by $\delta^{49/47}\text{Ti}$ of +2‰ and 10‰, can be detected if the increase in total Ti content is equivalent to 10‰ and 2‰, respectively.

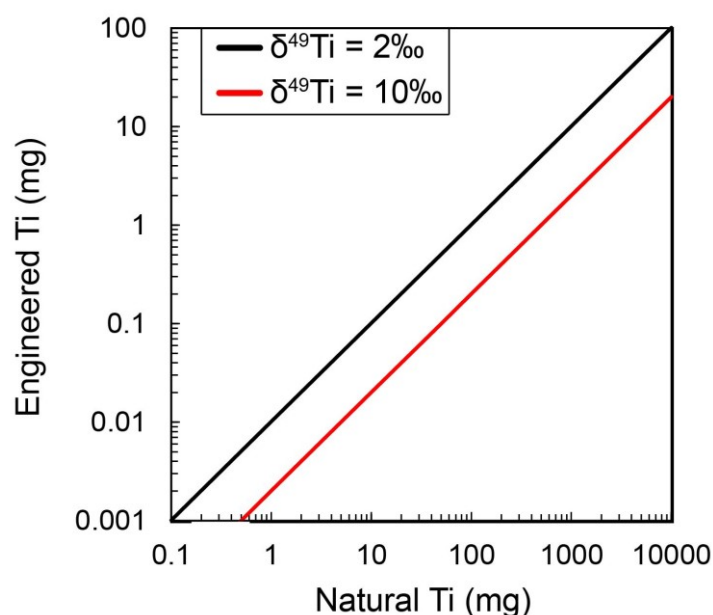


Fig. 5.5. Calculated detection sensitivities, which are defined as the smallest amount of engineered Ti that can be detected against the natural Ti background. The calculations assume that (i) engineered TiO₂ NPs have an intrinsic isotopic signature that differs from natural Ti by 2‰ (black line) and 10‰ (red line) and (ii) an analytical uncertainty of $\pm 0.05\text{‰}$ for the MC-ICP-MS measurements.

While no data have yet been published for engineered TiO₂ NPs, isotope fractionation in commercially available ENMs was previously investigated for ZnO, Ag and CeO₂ NPs [30,

86, 175]. These studies found that various analysed ENMs featured less isotopic variability than the natural background, as characterised by data for rock, soil, water and biological samples. Intrinsic stable isotope variability was therefore deemed to be unsuitable for tracing of ZnO, CeO₂ and Ag NPs. Given the documented natural variability in Ti stable isotope compositions of possibly up to 2‰ in terrestrial samples (Fig. 5.4), it seems unlikely that TiO₂ NPs have sufficiently fractionated isotope compositions to enable intrinsic stable Ti isotope tracing.

5.4.3. Stable isotope tracing using isotopically labelled nanoparticles

Given the previous discussion, stable isotope tracing of TiO₂ NPs is only viable if the NPs are prepared from and hence labelled with a highly enriched single Ti isotope ‘spike’. The application of this technique, in particular the optimal choice of the isotopic label considering the sensitivity and robustness of the tracing as well as the cost of preparing isotopically labelled TiO₂ NPs is considered in the following.

5.4.3.1. Key considerations and issues

Two key considerations in the choice of the isotope label are the detection sensitivity that is achieved with a particular isotope and the cost of the material. In general, the best detection sensitivities are achieved through the use of a highly enriched isotope that has a low natural abundance (e.g., ⁴⁹Ti or ⁵⁰Ti, Table 5.1, Fig. 5.2). For such isotopes, even minor additions of enriched material induce changes in the isotopic composition of the system that can be resolved analytically. In such a scenario, it is more likely that changes in the diagnostic isotope ratio caused by the introduction of labelled NPs can be traced even when the overall amount of spike present is low compared to the background content of the system. This can be

important for larger experimental systems, such as mesocosms or field studies, particularly for elements with high natural background concentrations such as Ti. However, isotopes with low natural abundances are usually the most expensive to purchase in enriched form, thereby increasing the cost associated with the synthesis of the isotopically labelled NPs.

Aside from this, there are a few additional factors that should be considered in the choice of the isotope label as well as the reference isotope, which together make up the diagnostic isotope ratio, which is measured by mass spectrometry to detect and quantify the presence of isotopically enriched material. These factors follow from the consideration that precise and unbiased measurements of the diagnostic ratio improve the performance of the tracing approach:

(i) Both isotopes should, as far as possible, be free from possibly problematic spectral interferences, which could bias the results.

(ii) Non-spectral interferences from matrix effects can generate erratic changes in the instrumental mass bias that can alter results. Such effects are mass dependent and are hence minimised if the diagnostic isotope ratio features isotopes that have only a small difference in atomic mass number, ideally 1.

(iii) Precise measurements of the diagnostic isotope ratio for small samples that contain primarily the spike isotope are rendered easier if the natural reference isotope has a relatively high natural abundance. In contrast, monitoring a highly abundant natural isotope for large samples that contain only little isotopic label can be unfavourable, as the signal of the reference isotope may exceed the dynamic range of the detection system.

5.4.3.2. Instrumental scenarios

Given the above considerations, it is apparent that different isotope labels and diagnostic isotope ratios produce highly variable results. As such, these parameters are best chosen based on an application-specific evaluation of the analytical requirements. In detail, the best choice depends on both the instrumental analytical approach and the nature of the samples that will be analysed for tracing purposes. The instrumental approach thereby has a key impact on the analytical precision that can be achieved for the determination of the diagnostic isotope ratio for a given sample. Due to the highly variable Ti background concentrations of environmental samples like soils, waters and biological materials, it is important to consider the sample type prior to analysis (Fig. 5.1). Furthermore, exposure studies may involve analyses of very small biological samples or much larger soil samples, which may feature highly variable ratios of $\text{TiO}_2\text{-En}$ to natural Ti.

In the following, a quantitative evaluation of these factors and their impact on the tracing sensitivity for labelled TiO_2 NPs is presented. To produce an evaluation of tracing methods that encompasses this diverse set of conditions, the modelling considers a wide range of analytical uncertainties, ranging from a maximum of 25% to an optimum of 0.01% (Table 5.3). Notably, these conditions cover a realistic range of uncertainties that can be achieved with different instrumental techniques and approaches. Analyses with ‘conventional’ Q-ICP-MS instrumentation typically use whole sample solutions without any chemical separation. As a consequence, the corresponding analytical uncertainties are comparatively high. For the purpose of this study, an analytical uncertainty of 5% was chosen for the optimum measurement conditions of Q-ICP-MS. This is based on the results reported by Bourgeault et al. [26], for their analyses of environmental samples to trace labelled $^{47}\text{TiO}_2$ NPs at realistic environmental concentrations. For a worst-case scenario, whereby analyses are

complicated by small sample size (<1 ng Ti content), an analytical uncertainty of 25% was considered for Q-ICP-MS (Table 5.3).

Table 5.3: Overview of different analytical scenarios considered for modelling the stable isotope tracing of TiO₂.

Analytical uncertainty	Type of isotopic analysis
25%	Q-ICP-MS analysis of small samples without prior chemical separation
5%	Q-ICP-MS analysis of samples with higher Ti contents, no chemical separation
1%	(i) SF-ICP-MS analysis without prior chemical separation (ii) MC-ICP-MS analysis of very small samples with chemical separation
0.1%	(i) SF-ICP-MS at optimum performance including chemical separation (ii) MC-ICP-MS analysis at routine conditions for the study of samples from environmental exposures
0.01%	MC-ICP-MS at optimum performance

For SF-ICP-MS, analytical uncertainties of between 0.1%, denoting optimum performance for analyses of sample solutions with relatively high concentrations of purified Ti, and 1% for analyses without prior chemical separation of Ti are considered [176]. An analytical uncertainty of about 1% is also applicable for analysis of very small samples with <<1 ng Ti by MC-ICP-MS, even if Ti is chemically isolated from the sample matrix prior to the measurement. A measurement error of about 0.1% is reasonable for routine MC-ICP-MS analyses of environmental samples with a Ti isotope tracer, assuming that matrix elements are largely removed from sample solutions prior to analysis. At optimum measurement conditions,

an improved uncertainty of 0.01% is further considered for MC-ICP-MS. Notably, this uncertainty is still worse than the best MC-ICP-MS data quality of about $\pm 0.02\text{‰}$ (2 SD), which was reported for analyses to resolve small natural Ti isotope fractionations [171, 172].

5.4.3.3. Choice of considered tracer isotopes and diagnostic isotope ratios

The isotope tracers and diagnostic isotope ratios that will be evaluated by detailed modelling are discussed and justified first, based on a consideration of possible spectral interferences as well as the isotope abundance of natural and enriched Ti (Table 5.2).

The ^{46}Ti isotope suffers from potentially problematic interferences from ^{46}Ca and $^{30}\text{Si}^{16}\text{O}^+$, whereby Ca and Si are both major constituents of sediments and soils. In addition, the major S isotope ^{32}S , with an abundance of $\sim 95\%$, can produce interfering polyatomic $^{32}\text{S}^{14}\text{N}^+$ (Table 5.1). Another challenge encountered in the measurement of Ti isotopes is the use of Ar gas in plasma mass spectrometry, which results in a flooding of the mass signal associated with the ^{40}Ar ion beam and baseline instabilities for nearby masses. As ^{46}Ti is the closest Ti isotope in mass to ^{40}Ar , it suffers most from this scattering.

Isobaric interferences are also caused by ^{50}Cr and ^{50}V on ^{50}Ti (Table 5.2). The former elements are, however, typically only present in the environment at trace levels and therefore less likely to affect isotope measurements. In addition, the presence of Cr and V in sample solutions can be monitored during analysis by measuring the isotopes ^{52}Cr (83.8%) and ^{51}V (99.7%) and applying appropriate corrections if necessary. The ^{50}Ti isotope further suffers from a range of polyatomic interferences (Table 5.2), of which $^{34}\text{S}^{16}\text{O}^+$ is likely to be the most problematic due to the high S levels in many environmental compartments.

Accurate measurements of ^{48}Ti can be hindered by isobaric ^{48}Ca (0.2%) and monitoring as well as correcting for the presence of Ca in samples is recommended, even after chemical

separation [171]. The presence of $[^{32}\text{S}^{16}\text{O}]^+$ is also possibly problematic for ^{48}Ti measurements, particularly as ^{32}S is the main S isotope of sulphur (Table 5.2). Whilst there are no isobars to ^{47}Ti and ^{49}Ti , there are a number of potential molecular interferences. Of these, $[^{31}\text{P}^{16}\text{O}]^+$ and $[^{33}\text{S}^{16}\text{O}]^+$ are likely the most problematic for ^{47}Ti and for ^{49}Ti , respectively, whereby the latter is mitigated by the low natural abundance of ^{33}S (Table 5.2).

Given these constraints and considering the natural Ti isotope abundances as well as the composition and cost of enriched Ti isotopes the model calculations will evaluate the following isotopes and diagnostic isotope ratios:

(i) ^{47}Ti because it is a minor natural isotope (7%) available at high enrichment (96%). It will be considered in the diagnostic ratios $^{47}\text{Ti}/^{48}\text{Ti}$ and $^{47}\text{Ti}/^{49}\text{Ti}$, as analyses that apply a minor and the major natural Ti nuclide as reference isotope are favourable in different conditions and both ratios can be measured simultaneously by MC-ICP-MS. Essentially identical modelling results are obtained if highly enriched ^{49}Ti is used instead of ^{47}Ti to form the diagnostic ratios $^{49}\text{Ti}/^{48}\text{Ti}$ and $^{49}\text{Ti}/^{47}\text{Ti}$. This reflects that the natural abundance of ^{49}Ti (~5%) is similar to that of ^{47}Ti and no separate model calculations are hence carried out in the following.

(ii) ^{48}Ti is the major natural Ti isotope (74%) and also the least expensive enriched nuclide, with a cost about an order of magnitude lower compared to other enriched Ti isotopes (Table 5.1). The $^{48}\text{Ti}/^{49}\text{Ti}$ isotope ratio is considered in the following but essentially identical results are obtained for $^{48}\text{Ti}/^{47}\text{Ti}$, due to the similar natural abundances of ^{47}Ti and ^{49}Ti (Table 5.1).

(iii) ^{50}Ti is considered primarily to investigate the effect of using a minor natural isotope that is available at a lower enrichment of only 50%. It was included in this study to explore if the approach of employing an isotope that is only moderately enriched offers a more favourable

balance between detection sensitivity and tracer cost. Whilst the use of an isotope tracer enriched to >90% is usually associated with abundances of <1% for all non-enriched isotopes, ^{49}Ti is still present at 4.1% in the moderately enriched ^{50}Ti material (Fig. 5.2). This will produce slightly higher signal intensities for the ^{49}Ti isotope during measurements, which may be beneficial, particularly for analyses of samples with low natural Ti contents. Therefore, the diagnostic ratio $^{50}\text{Ti}/^{49}\text{Ti}$ is investigated in the following.

Although various other measurement protocols are conceivable, results are not provided for additional models, as they yield detection sensitivities that are either essentially identical (e.g., $^{48}\text{Ti}/^{47}\text{Ti}$ for $^{48}\text{Ti}/^{49}\text{Ti}$) or less favourable (e.g., $^{48}\text{Ti}/^{46}\text{Ti}$ and $^{48}\text{Ti}/^{49}\text{Ti}$) compared to those considered here.

5.4.3.4 Modelling results

5.4.3.4.1. Detection sensitivity

The modelling study considers (i) how the addition of isotopically labelled TiO_2 NPs changes the Ti isotope composition of a sample and (ii) that this change can be resolved and quantified if it exceeds the uncertainty of the measurement. The results of the modelling are presented in Table 5.4 and Fig. 5.6, whereby three different isotope tracers and four diagnostic isotope ratios are evaluated. The model calculations determine the detection sensitivities for the five analytical scenarios (Cases 1-5, Table 5.3), whereby two distinct sample scenarios are evaluated, a soil and a biological tissue (zebra mussels) with contrasting Ti background levels of 3500 mg kg^{-1} and 8 mg kg^{-1} , respectively.

Cases 1 and 2 encompass the detection sensitivities for sample analysis by Q-ICP-MS, for a worst-case and best-case analytical scenario. For samples with high Ti contents (Case 2), relative detection sensitivities of 4‰ or 44‰ are achieved with a ^{47}Ti and ^{48}Ti tracer,

Table 5.4: Results of modelling study.

Enriched / Non-enriched	$^{47}\text{Ti}/^{48}\text{Ti}$	$^{47}\text{Ti}/^{49}\text{Ti}$	$^{48}\text{Ti}/^{49}\text{Ti}$	$^{50}\text{Ti}/^{49}\text{Ti}$
Case 1: Analytical uncertainty = 25%				
Detectable increase in Ti level				
Relative [‰]	20	20	224	29
In soils [#] [mg kg ⁻¹]	67	67	784	102
In biological tissue ^{\$} [µg kg ⁻¹]	153	153	1792	233
Case 2: Analytical uncertainty = 5%				
Detectable increase in Ti level				
Relative [‰]	3.9	3.9	44	5.6
In soils [#] [mg kg ⁻¹]	13	13	153	20
In biological tissue ^{\$} [µg kg ⁻¹]	31	31	349	46
Case 3: Analytical uncertainty = 1%				
Detectable increase in Ti level				
Relative [‰]	0.78	0.78	8.7	1.1
In soils [#] [mg kg ⁻¹]	2.7	2.7	30	4.0
In biological tissue ^{\$} [µg kg ⁻¹]	6.1	6.1	70	9.1
Case 4: Analytical uncertainty = 0.1%				
Detectable increase in Ti level				
Relative [‰]	0.078	0.078	0.87	0.11
In soils [#] [mg kg ⁻¹]	0.27	0.27	3.0	0.40
In biological tissue ^{\$} [µg kg ⁻¹]	0.61	0.61	6.9	0.91
Case 5: Analytical uncertainty = 0.01%				
Detectable increase in Ti level				
Relative [‰]	0.008	0.008	0.087	0.011
In soils [#] [mg kg ⁻¹]	0.027	0.027	0.30	0.040
In biological tissue ^{\$} [µg kg ⁻¹]	0.061	0.061	0.69	0.091

[#]: Assumes a natural background level of titanium of 3500 mg kg⁻¹ in soils [138].
^{\$}: Natural background level of 10 µg g⁻¹ Ti in zebra mussels [26].

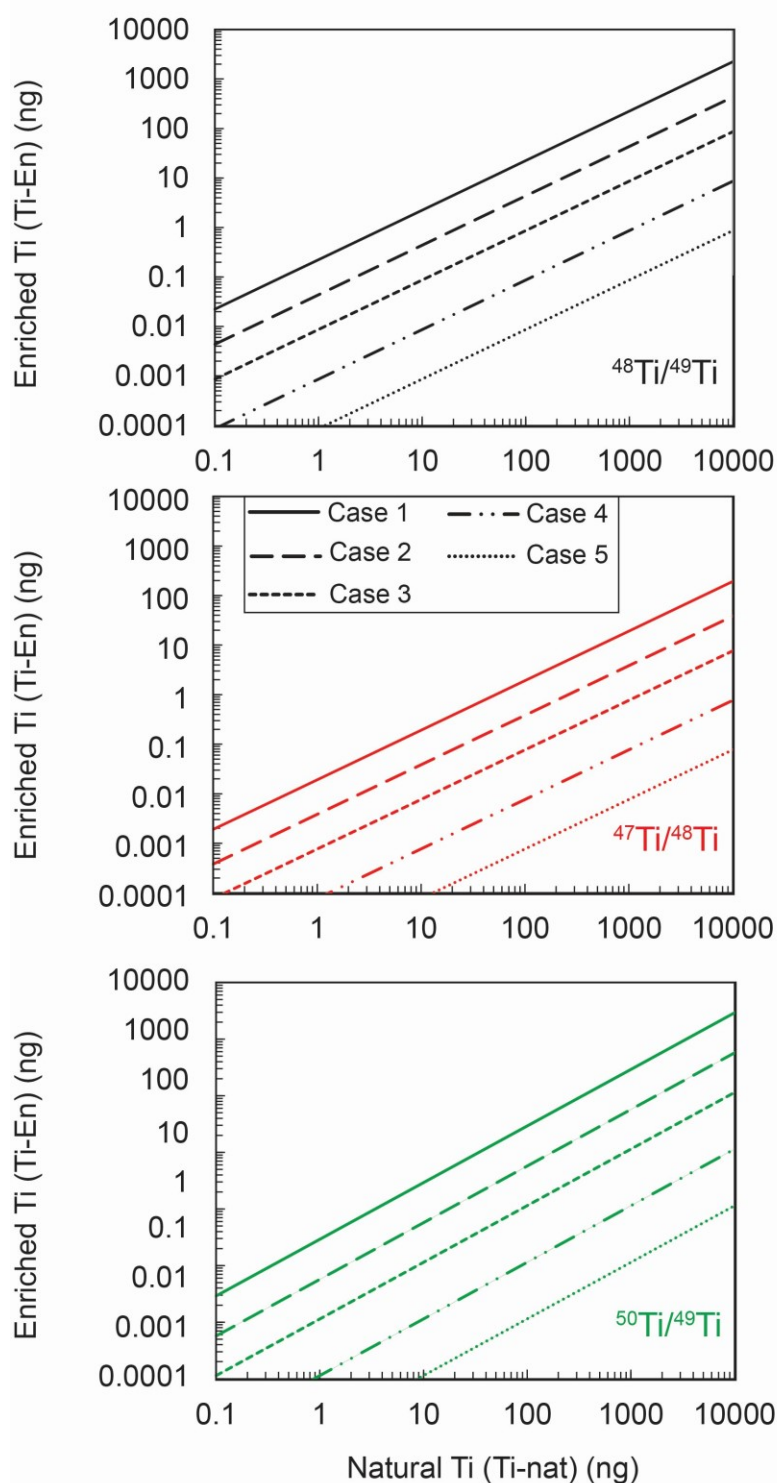


Fig. 5.6. Detection sensitivities offered by different enriched Ti isotope labels for the analytical scenarios of Case 1 to 5 (Table 5.3). The detection sensitivity is defined as the minimum amount of enriched Ti that can be resolved against a given natural Ti background. The black (top), red (middle) and green (bottom) lines represent the use of isotopically enriched ^{48}Ti , ^{47}Ti and ^{50}Ti , respectively. No data are shown for $^{47}\text{Ti}/^{49}\text{Ti}$ as the results are essential identical to those obtained for $^{47}\text{Ti}/^{48}\text{Ti}$.

respectively. For the soils, this implies that at least 13 and 153 mg kg⁻¹ enriched Ti needs to be added to the samples to enable detection (Table 5.4). In comparison, most studies predict an annual engineered TiO₂ NPs flux of less than 10 mg kg⁻¹ into soils (Fig. 5.1), and this implies that Q-ICP-MS is not precise enough for robust analyses of soil samples from exposures performed at environmentally realistic concentrations.

It is possible to resolve increases in Ti levels of <10 mg kg⁻¹ against a natural Ti background of 3500 mg kg⁻¹ by analysis with SF-ICP-MS (Table 5.4, Case 3). As shown by the modelling, use of ⁴⁷Ti or ⁵⁰Ti yields fairly similar results, whereby increasing Ti levels from tracer addition can be detected if these exceed 0.8‰ and 1.1‰, respectively. This is equivalent to the addition of Ti-En at 2.7 mg kg⁻¹ and 4.0 mg kg⁻¹ for the soil scenario. In contrast, the use of ⁴⁸Ti, which has the lowest associated cost of the Ti-En isotopes (Table 5.1), enables a detection sensitivity of 9‰ for an analytical uncertainty of 1% (Table 5.4, Case 3). This is equivalent to increases in Ti levels of 30 mg kg⁻¹ and 70 µg kg⁻¹ for the soil and the biological tissue, respectively, and these detection sensitivities likely exceed the quantities of TiO₂ NPs that are released into the environment (Fig. 5.1).

With MC-ICP-MS, Ti isotope ratios can be measured with a precision that is 10 to 100 times better compared to SF-ICP-MS (Table 5.4), and this provides detection sensitivities that are improved to a similar extent (Table 5.4, Case 4). If the measurements have uncertainties of 0.1%, additions of Ti-En are detectable if they increase total Ti contents by 0.08‰ (⁴⁷Ti-En), 0.9‰ (⁴⁸Ti-En) and 0.11‰ (⁵⁰Ti-En). Notably, analytical uncertainties of ≤0.1% render Ti-En additions to soils detectable at ≤3 mg kg⁻¹, even if inexpensive ⁴⁸Ti is employed as tracer. As such, the application of MC-ICP-MS enables cost-effective tracing of TiO₂ NPs using ⁴⁸Ti, with detection sensitivities that allow realistic exposure levels similar to published PECs, even for soils.

At optimum conditions, Ti isotope ratios can be determined with an uncertainty approaching 0.01% by MC-ICP-MS (Table 5.4, Case 5). Such analyses can provide detection sensitivities as low as 0.008‰ using enriched ^{47}Ti and this is equivalent to a Ti addition from TiO_2 NPs of only 0.03 mg kg^{-1} for the soil scenario. If less expensive ^{48}Ti is used, Ti-En additions can be resolved if they exceed 0.9‰ of the total Ti budget, equivalent to increases in Ti contents of 0.3 mg kg^{-1} and $0.7 \mu\text{g kg}^{-1}$ for the soil and biological tissue, respectively. Notably, both results are well within the PECs, which indicates that tracing is possible at environmentally relevant exposure levels.

In conclusion, the analytical and sample scenarios discussed above showcase the versatility of the stable isotope tracing approach, whereby different enriched isotopes and analytical techniques can be used to match the demands of a specific tracing application.

5.4.3.4.2. Performance for determining proportion of spike present

The addition of a Ti-En tracer changes the Ti isotope composition of a sample in proportion to the amount of spike present. Based on the analytical uncertainty of an instrumental method, this isotopic change can be measured and the amount of enriched Ti in a sample can be quantified, for example by calculating the ratio of enriched Ti to total Ti (Ti-En/Ti-Tot) present. The error associated with this quantification is governed by the analytical uncertainty of the measurement, which can be propagated through the calculations.

Notably, the final uncertainty for (Ti-En/Ti-Tot) does not scale linearly with the analytical error but is a function of the isotopic enrichment of the spike and the proportion of enriched to natural Ti present. In detail, the (Ti-En/Ti-Tot) ratio can be determined with an improved uncertainty at a given measurement error for the diagnostic isotope ratio, when the proportion of enriched material present is large (Fig. 5.7). For example, assuming an analytical

error of 0.1% for the determination of $^{48}\text{Ti}/^{49}\text{Ti}$, the associated uncertainty for $(\text{Ti-En}/\text{Ti-Tot})$ is 0.11% and 0.03% at $(\text{Ti-En}/\text{Ti-Tot})$ ratios of 0.5 and 0.9, respectively. The data of Fig. 5.7 furthermore show a distinctly less favourable error propagation for the enriched ^{50}Ti at high $(\text{Ti-En}/\text{Ti-Tot})$ ratios, which reflects that this spike has only a moderate enrichment of about 50% rather than >95%.

5.4.4. Final evaluation

Assuming a synthesis yield of 90%, 1 mg of $^{47}\text{TiO}_2$ NPs can be produced at a tracer cost of US-\$18 (Table 5.1). Notably, introduction of 1 mg TiO_2 NPs to 1 kg of soil represents a dosing concentration that falls within the PECs for TiO_2 NPs in soils (Fig. 5.1). However, detection of $^{47}\text{TiO}_2$ at a concentration of 1 mg kg^{-1} in soils is only possible if the isotopic analyses have uncertainties of $\ll 1\%$ and this requires that the measurements are conducted by MC-ICP-MS (Table 5.4, Case 4 and Case 5).

The cost of 96% enriched ^{47}Ti is only US-\$2 higher compared to ^{50}Ti enriched to 50% (Table 5.1), such that the potential savings associated with the latter are only noticeable if large amounts of tracer material are required. The model calculations show that ^{50}Ti enriched at ~50% affords detection sensitivities that are similar to those achievable with highly enriched material, such as 96% enriched ^{47}Ti (Table 5.4). However, the use of moderately enriched ^{50}Ti is associated with a less favourable error propagation in the calculation of $(\text{Ti-En}/\text{Ti-Tot})$, when large proportions of spike are present (Fig. 5.7) As such, a different spike isotope will be preferable for exposures that generate samples that have high $(\text{Ti-En}/\text{Ti-Tot})$ ratios, for example biological tissues with low Ti background levels.

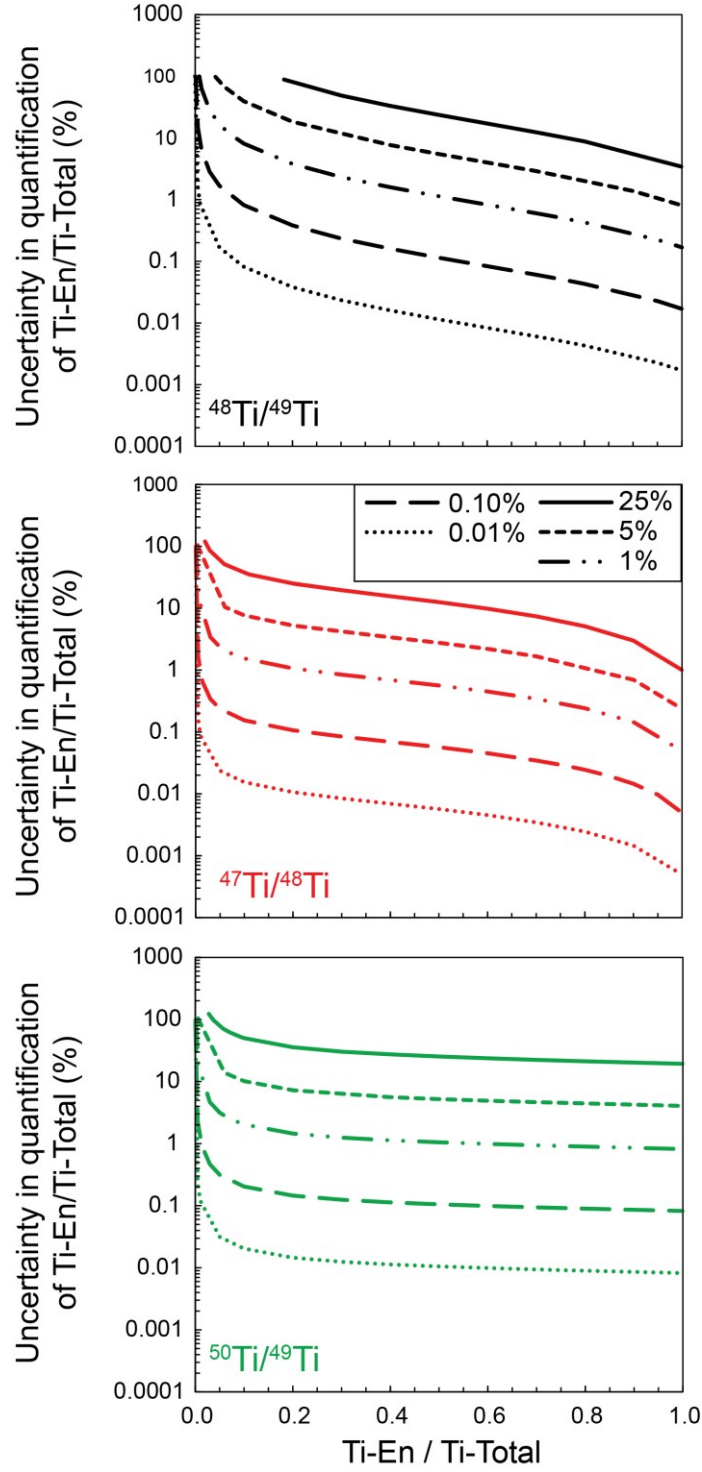


Fig. 5.7. Error propagation in the quantification of the (Ti-En/Ti-Total) ratio when variable levels of enriched Ti are present. The black (top), red (middle) and green (bottom) lines represent the use of isotopically enriched ^{48}Ti , ^{47}Ti and ^{50}Ti , respectively, for the five analytical scenarios (Cases 1 to 5) and corresponding analytical uncertainties of 0.01%, 0.1%, 1%, 5% and 25%. No data are shown for $^{47}\text{Ti}/^{49}\text{Ti}$, as the results are essentially identical to those obtained for $^{47}\text{Ti}/^{48}\text{Ti}$.

The lowest costs are associated with the use of ^{48}Ti as tracer material, which can be purchased enriched to 96% at a cost of US-\$1.10 per mg. Even at low synthesis yields of <20%, 1 mg of $^{48}\text{TiO}_2$ NPs can hence be produced for less than US-\$10 (Table 5.1). For exposure systems where Ti background levels are low, such as many biological organisms and aquatic systems (Fig. 5.1), the detection of the labelled material is comparatively straightforward. In such cases, the application of a ^{48}Ti tracer is a suitable and cost-effective option, especially if MC-ICP-MS is employed for sample analysis (Table 5.4).

5.5. Conclusions

The modelling presented and discussed in this chapter provides a detailed evaluation of the stable isotope tracing technique for the detection and quantification of isotopically labelled TiO_2 NPs in the environment. The results show that application of MC-ICP-MS provides detection sensitivities that can resolve the addition of TiO_2 NPs to natural systems at realistically low exposure concentrations for all enriched isotopes considered in this study. In comparison to MC-ICP-MS, the detection sensitivities of SF-ICP-MS and Q-ICP-MS are about one and two orders of magnitude lower, respectively. However, if the natural Ti background levels are appropriately low, the addition of TiO_2 NPs can also be resolved even by SF-ICP-MS and Q-ICP-MS, if a highly sensitive tracer isotope such as ^{47}Ti is used.

Stable isotope tracing can be applied to the study of ENMs in a wide range of exposure systems that feature different conditions and requirements, such as amount of spike needed, Ti background levels, the required size and number of samples, as well as the budget available for the experiments. A careful consideration of these issues will be particularly important for longer and more complex experiments, including multigenerational exposures or exposures

that examine multiple parameters at the same time, and such investigations may become more routine as the study of ENM ecotoxicity evolves and progresses [103, 131].

5.6. Acknowledgements and declaration

All laboratory work and the modelling study presented in this chapter were performed by Tabea Junk (TJ). TEM images were recorded with support provided by Miguel Gomez Gonzalez at the Department of Materials at Imperial College London. The manuscript was written by TJ with suggestions and support from Mark Rehkämper.

Chapter 6

Summary and outlook

6. Summary and outlook

The aim of this research project was to further expand the method of stable isotope tracing to include more types of engineered nanomaterials (ENMs), whereby work was carried out that covers different aspects of the methodology.

Zinc sulphide particles are the most likely transformation product of ZnO NPs in waste water treatment plants. As such, the environmental behaviour and fate of ZnS has recently attracted significant attention. Chapter 2 presents a modified protocol for the synthesis of isotopically labelled ZnS particles, which allows scalable production of sub-micron sized ZnS at the moderate yields which are required to ensure the economic feasibility of stable isotope tracing. The isotopically labelled particles are shown to be indistinguishable from their counterparts with natural isotope composition, whilst the synthesis is demonstrated to be straightforward and reliable.

Furthermore, the research successfully builds on a previously established synthesis procedure for labelled Ag NPs to extend the method of stable isotope tracing to one of the most commonly used NPs in consumer products. In detail, Chapter 3 presents an efficient and robust analytical procedure that was developed for the separation of Ag from matrix elements and subsequent measurements of $^{109}\text{Ag}/^{107}\text{Ag}$ ratios by multiple collector ICP-MS (MC-ICP-MS). The technique was extensively tested using a variety of relevant environmental samples with natural and spiked isotopic signatures to demonstrate and document its performance. Notably, the simplified separation chemistry facilitates the high sample throughput that is required to analyse the many samples that are commonly produced in environmental exposures whilst providing a Ag separate of sufficient purity to allow unbiased and precise Ag isotope measurements by MC-ICP-MS.

Collaborative work from a multigenerational exposure of zebrafish to two different types of isotopically labelled Ag NPs is presented in Chapter 4. Even at dosing levels that are within the predicted environmental concentrations, Ag NPs were readily detected in the livers and tissues of all exposed fish. It is further shown that Ag NPs can cross barriers in the gonads of female fish, such that they can be passed on transgenerationally and are detectable in their offspring. However, the analysed data also demonstrate that at environmentally realistic dosing concentrations, zebrafish can eliminate Ag originating from Ag NPs within one generation of non-exposure. Our results therefore provide no evidence for bioaccumulation of Ag NPs over several generations.

Finally, Chapter 5 provides a preliminary assessment of procedures for the synthesis and application of isotopically enriched TiO₂ NPs. In particular, it encompasses a modelling study that evaluates the performance that can be obtained for stable isotope tracing of TiO₂ NPs, using different tracer isotopes and instrumental analytical techniques, including quadrupole ICP-MS and MC-ICP-MS. The modelling demonstrates that TiO₂ NPs can be detected if they contribute as little as 0.01‰ to the total Ti content of a sample. As TiO₂ is widely used in pigments, paints and cosmetic products, it is among the three most produced ENMs [4]. Studies of the environmental behaviour and fate of TiO₂ NP are, furthermore, of particular difficulty due to high and highly variable background concentrations of natural, bulk and nano-TiO₂.

The use of isotopic tracers for the study of ENMs offers unique advantages. The technique has been established in the last decade as one of various methods that have helped to improve the understanding of the behaviour and potential health and safety risks of ENMs. A particular advantage of the use of isotopic tracers is their ability to track ENMs at environmentally realistic dosing concentrations and their use thereby offers distinct possibilities in furthering the understanding of the behaviour of ENMs in the environment. As

such, stable isotope tracing can be used to test the findings of studies that were performed at high dosing concentrations to investigate if differences in behaviour and/or ENM fate occur for low exposure concentrations.

As natural environments will increasingly accumulate various ENMs over a prolonged timeframe, isotopic tracers could also be used as a key tool to investigate the fate of different ENMs within the same system and at realistically low exposure concentrations. For example, the same ecosystem can be treated with ^{109}Ag , ^{68}ZnO and $^{48}\text{TiO}_2$ NPs whereby stable isotope tracing allows the investigation of the effects of simultaneous exposure to several ENMs. This type of contamination is more closely matched to real life scenarios, where natural environments are expected to be exposed to several ENMs simultaneously.

This work, among others, has shown that multigenerational studies are of particular importance in order to fully understand ENM behaviour in the environment. As a consequence, nano-ecotoxicity studies should attempt to investigate subsequent exposure generations where possible. Similarly, accumulation and/or transfer of ENMs in the food chain is not yet understood due to the difficulty in studying transfer mechanisms across species through concentration measurements. Stable isotope tracing is uniquely suited to this pursuit, as it is an endeavour similar to the transgenerational transfer that was shown to occur in zebrafish in this work. The isotopic label on an ENM is an intrinsic property of the particle and is therefore not lost during transfer, either between species or as part of the movement of material within an organism. Isotopic labelling could therefore be applied to the study of bioaccumulation of ENMs in the food-chain by exposure of microorganisms to the isotope tracer. Through the design of an exposure system that mimics the organisms and compartments in a naturally occurring food-chain, the movement of the tracer could be studied by analysis of each component of the food-chain, which would then inform on the potential accumulation of

ENMs. The technique of stable isotope tracing therefore has more to offer still and the potential to further contribute to the understanding of ENM ecotoxicity.

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Appendices

Appendix 1: Additional information relating to syntheses of ZnS

A1.1 Synthesis following Vacassy et al.

Following Vacassy et al. [177], 200 mg of a zinc acetate powder were dissolved in 18.2 ml of Milli-Q water ($c = 0.05 \text{ mol l}^{-1}$). Thioacetamide (TAA) was added at a ratio of 1/8 Zn^{2+} /TAA from a 0.4 mol l^{-1} stock solution. Whereas Vacassy et al. [177] filtered both the zinc acetate and TAA stock solutions prior to their use, this step was not performed due to a potential loss of Zn during filtration. The aqueous Zn acetate and TAA solutions were heated separately under stirring to 80°C and combined once they had reached the target temperature for further heating under reflux at 80°C for 30 minutes. The original synthesis was performed in a batch reactor without stirring rather than using a reflux system. Subsequently, the solution was quenched in an ice bath and centrifuged for 30 minutes at 4000 RPM in a Thermo Scientific Heraeus Megafuge. The suspension was washed twice with water and three times with isopropylalcohol and finally dried overnight in an oven at 50°C to evaporate excess isopropylalcohol. Prior to drying, a 1 ml aliquot was removed for analysis by dynamic light scattering (DLS).

DLS analysis was performed at the Department of Bioengineering at Imperial College London. Analyses were carried out in three cycles, each with 15 individual measurements to account for sedimentation. The results showed the formation of monodisperse particles within the micrometer size range and a Z-size average diameter of 3142 nm.

A1.2 Synthesis following Panda et al.

According to a method published by Panda et al. [178], aqueous zinc nitrate $\text{Zn}(\text{NO}_3)_2 \cdot 6 \text{H}_2\text{O}$ was added at a concentration of 2 mmol l^{-1} to an aqueous solution of 2.9 g of poly(N-vinyl-2-pyrrolidone) (PV) in 60 ml of Milli-Q water. To produce a Zn nitrate stock solution, 400 mg of Zn acetate powder were dissolved in 10 ml concentrated HNO_3 at 80°C

for 24 hours. While the original method by Panda et al. [178] employed PVP at a molecular weight of 40,000, PVP at a molecular weight of 58,000 was used in this synthesis due to its availability in the laboratory and as such the mass of PVP used was changed to be equimolar to Panda et al. The suspension of PVP and Zn nitrate was stirred at room temperature for three hours followed by the addition of 0.159 g (2 mmol l⁻¹) and further stirring under reflux for two hours at 80°C. The suspension was cooled to room temperature and centrifuged at 4000 RPM for 30 minutes in a Thermo Scientific Heraeus Megafuge. The precipitate was washed once with water and once with propanol followed by filtration through a 100 nm filter to remove larger particles. Large amounts of a white substance were removed during the filtration and the remaining suspension was almost transparent. A 1 ml aliquot for analysis by dynamic light scattering (DLS) was removed and the remaining suspension was dried at 50°C in an oven.

DLS analysis was performed at the Department of Bioengineering at Imperial College London. Analyses were carried out in three cycles, each with 15 individual measurements to account for sedimentation. The DLS results (Fig. A1.2) show two separate peaks, both of which broaden over the course of the analysis (Run 1 in red, Run 3 in black). This is indicative of large sedimenting particles skewing the analysis and inhibiting the precise measurement of the

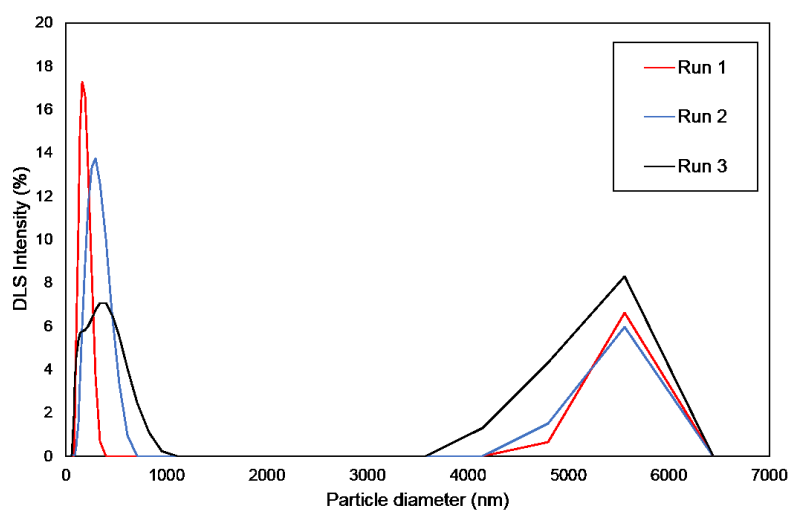


Fig.A1.2. DLS analysis of ZnS particles.

hydrodynamic particle diameter. Although the suspension was filtrated prior to analysis, these results suggest that the synthesis suspension is highly monodisperse and therefore unsuitable for the production of sub-micron or nanoparticles of ZnS. The reason for this difference to the original procedure, which produced particles of 20-50 nm size [178] may be the use of PVP at a higher molecular weight and therefore a larger size which may prevent the formation of smaller, monodisperse particles.

A1.3 Synthesis following Wang et al.

Following a solid-state synthesis published by Wang et al. [179], equimolar amounts of zinc acetate and TAA powders were milled separately for approximately 20 minutes, mixed in a mortar and milled for a further 30 minutes. The powder mixture was then placed in an oven at 100° for four hours to induce a solid-state reaction before analysis by transmission electron microscope (TEM).

TEM images were recorded at the Department of Materials at Imperial College London. TEM analysis (Fig. A1.3) showed that the synthesis did not result in the formation of distinct nanoparticles or aggregates of nanoparticles.

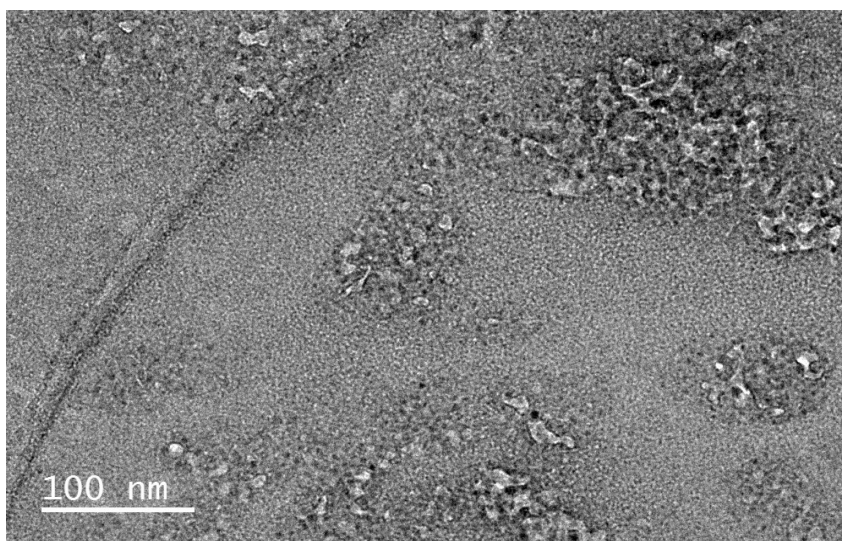


Fig. A1.3. TEM image of the product of solid-state ZnS synthesis.

**Appendix 2: Isotopic and Ag mass fraction data obtained
for samples in the study for Chapter 4**

A2.1. Complete Ag isotope data and Ag mass data collected from embryos exposed to Ag NPs in generation F0 and F1 (excluding outliers).

Sample	Generation	Collection	Treatment	Total Ag mass	Mass of Ag-En	Mass of Ag-En	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En
				in sample [ng]	in sample [ng]	per embryo [pg/em]		rel to Total-Ag [ng/ng]
1.1	F1	Week 1	Cit- ^{109}Ag NPs	0.216	0.212	21.2	88.49	0.98
1.2	F1	Week 1	Cit- ^{109}Ag NPs	0.158	0.155	15.5	73.71	0.98
1.3	F1	Week 1	Cit- ^{109}Ag NPs	0.562	0.555	55.5	112.45	0.99
1.4	F1	Week 1	Cit- ^{109}Ag NPs	0.291	0.288	28.8	122.08	0.99
1.5	F1	Week 1	Cit- ^{109}Ag NPs	0.603	0.598	59.8	137.44	0.99
1.6	F1	Week 1	Cit- ^{109}Ag NPs	0.479	0.476	47.6	159.51	0.99
2.1	F1	Week 2	Cit- ^{109}Ag NPs	0.462	0.455	45.5	89.33	0.98
2.2	F1	Week 2	Cit- ^{109}Ag NPs	0.119	0.117	11.7	111.71	0.99
2.3	F1	Week 2	Cit- ^{109}Ag NPs	0.374	0.371	37.1	144.79	0.99
2.4	F1	Week 2	Cit- ^{109}Ag NPs	0.345	0.343	34.3	146.62	0.99
2.5	F1	Week 2	Cit- ^{109}Ag NPs	0.349	0.347	34.7	156.99	0.99
3.1	F1	Week 3	Cit- ^{109}Ag NPs	0.347	0.339	33.9	63.66	0.98
3.2	F1	Week 3	Cit- ^{109}Ag NPs	0.288	0.286	28.6	159.57	0.99
3.3	F1	Week 3	Cit- ^{109}Ag NPs	0.167	0.167	16.7	176.84	0.99
3.4	F1	Week 3	Cit- ^{109}Ag NPs	0.247	0.246	24.6	181.99	0.99
3.5	F1	Week 3	Cit- ^{109}Ag NPs	0.219	0.218	21.8	251.23	1.00
1.7	F1	Week 1	Cas- ^{109}Ag NPs	0.017	0.016	1.6	67.33	0.98
1.8	F1	Week 1	Cas- ^{109}Ag NPs	0.445	0.436	43.6	76.40	0.98

A2.1. (continued)

Sample	Generation	Collection	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per embryo [pg/em]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
1.9	F1	Week 1	Cas- ^{109}Ag NPs	0.270	0.267	26.7	102.05	0.99
1.10	F1	Week 1	Cas- ^{109}Ag NPs	0.473	0.468	46.8	116.13	0.99
1.11	F1	Week 1	Cas- ^{109}Ag NPs	0.573	0.570	57.0	161.73	0.99
1.12	F1	Week 1	Cas- ^{109}Ag NPs	0.626	0.622	62.2	166.57	0.99
2.6	F1	Week 2	Cas- ^{109}Ag NPs	0.255	0.254	25.4	186.72	1.00
2.7	F1	Week 2	Cas- ^{109}Ag NPs	0.294	0.293	29.3	189.66	1.00
2.8	F1	Week 2	Cas- ^{109}Ag NPs	0.356	0.355	35.5	202.99	1.00
2.9	F1	Week 2	Cas- ^{109}Ag NPs	0.404	0.403	40.3	221.35	1.00
3.6	F1	Week 2	Cas- ^{109}Ag NPs	0.518	0.516	51.6	201.59	1.00
3.7	F1	Week 3	Cas- ^{109}Ag NPs	0.266	0.266	26.6	241.94	1.00
3.8	F1	Week 3	Cas- ^{109}Ag NPs	0.303	0.302	30.2	229.31	1.00
3.9	F1	Week 3	Cas- ^{109}Ag NPs	0.395	0.394	39.4	273.99	1.00
3.10	F1	Week 3	Cas- ^{109}Ag NPs	0.325	0.325	32.5	348.58	1.00
3.11	F1	Week 3	Cas- ^{109}Ag NPs	0.132	0.132	13.2	367.62	1.00
1.1	F2	Week 1	Cit- ^{109}Ag NPs		Below detection limit		0.95	N/A
1.2	F2	Week 1	Cit- ^{109}Ag NPs		Below detection limit		0.96	N/A
1.3	F2	Week 1	Cit- ^{109}Ag NPs		Below detection limit		0.97	N/A
1.4	F2	Week 1	Cit- ^{109}Ag NPs		Below detection limit		0.99	N/A

A2.1. (continued)

Sample	Generation	Collection	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per embryo [pg/em]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
1.5	F2	Week 1	Cit- $^{109}\text{AgNPs}$		Below detection limit		1.30	N/A
2.1	F2	Week 2	Cit- $^{109}\text{AgNPs}$	0.308	0.301	30.1	71.91	0.98
2.2	F2	Week 2	Cit- $^{109}\text{AgNPs}$	0.249	0.244	24.4	73.97	0.98
2.3	F2	Week 2	Cit- $^{109}\text{AgNPs}$		Below detection limit		129.69	N/A
2.4	F2	Week 2	Cit- $^{109}\text{AgNPs}$	0.284	0.282	28.2	138.73	0.99
3.1	F2	Week 3	Cit- $^{109}\text{AgNPs}$	0.246	0.240	24.0	62.18	0.98
3.2	F2	Week 3	Cit- $^{109}\text{AgNPs}$	0.057	0.056	5.6	72.64	0.98
3.3	F2	Week 3	Cit- $^{109}\text{AgNPs}$	0.192	0.190	19.0	106.21	0.99
3.4	F2	Week 3	Cit- $^{109}\text{AgNPs}$	0.305	0.302	30.2	130.01	0.99
1.6	F2	Week 1	Cas- $^{109}\text{AgNPs}$		Below detection limit		0.93	N/A
1.7	F2	Week 1	Cas- $^{109}\text{AgNPs}$		Below detection limit		0.95	N/A
1.8	F2	Week 1	Cas- $^{109}\text{AgNPs}$		Below detection limit		0.95	N/A
1.9	F2	Week 1	Cas- $^{109}\text{AgNPs}$		Below detection limit		1.00	N/A
1.10	F2	Week 1	Cas- $^{109}\text{AgNPs}$		Below detection limit		0.99	N/A
2.5	F2	Week 2	Cas- $^{109}\text{AgNPs}$	0.197	0.196	19.6	146.58	0.99
2.6	F2	Week 2	Cas- $^{109}\text{AgNPs}$	0.361	0.359	35.9	153.05	0.99
2.7	F2	Week 2	Cas- $^{109}\text{AgNPs}$	0.359	0.357	35.7	166.77	0.99
2.8	F2	Week 2	Cas- $^{109}\text{AgNPs}$	0.565	0.563	56.3	208.24	1.00

A2.1. (continued)

Sample	Generation	Collection	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per embryo [pg/em]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
2.9	F2	Week 2	Cas- ^{109}Ag NPs	0.570	0.568	56.8	240.18	1.00
2.10	F2	Week 2	Cas- ^{109}Ag NPs	0.512	0.511	51.1	243.01	1.00
3.5	F2	Week 3	Cas- ^{109}Ag NPs	0.472	0.439	43.9	24.27	0.93
3.6	F2	Week 3	Cas- ^{109}Ag NPs	0.128	0.124	12.4	45.40	0.96
3.7	F2	Week 3	Cas- ^{109}Ag NPs	0.509	0.499	49.9	71.14	0.98
3.8	F2	Week 3	Cas- ^{109}Ag NPs	0.444	0.435	43.5	75.00	0.98
3.9	F2	Week 3	Cas- ^{109}Ag NPs	0.236	0.234	23.4	124.25	0.99

A2.2. Complete Ag isotope data and Ag mass data collected from tissue samples exposed to Ag NPs in generation F0 and F1 (excluding outliers).

Sample	Generation	Type/Sex	Treatment	Total Ag mass	Mass of Ag-En	Mass of Ag-En	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En
				in sample [ng]	in sample [ng]	per mg tissue [ng/mg]		rel to Total-Ag [ng/ng]
1	F0	Gonad/Male	Cit- ^{109}Ag NPs	0.114	0.097	0.21	11.55	0.85
2	F0	Gonad/Male	Cit- ^{109}Ag NPs	0.056	0.054	0.11	46.12	0.97
3	F0	Gonad/Male	Cit- ^{109}Ag NPs	0.069	0.068	0.08	57.73	0.97
4	F0	Gonad/Male	Cit- ^{109}Ag NPs	0.553	0.539	2.99	58.80	0.97
5	F0	Gonad/Male	Cit- ^{109}Ag NPs	0.377	0.373	0.64	127.70	0.99
6	F0	Gonad/Male	Cit- ^{109}Ag NPs	2.437	2.426	2.96	193.45	1.00
7	F0	Liver/Male	Cit- ^{109}Ag NPs	0.097	0.095	0.53	71.30	0.98
8	F0	Liver/Male	Cit- ^{109}Ag NPs	0.568	0.564	0.58	152.70	0.99
9	F0	Liver/Male	Cit- ^{109}Ag NPs	10.553	10.485	9.71	159.91	0.99
10	F0	Liver/Male	Cit- ^{109}Ag NPs	1.396	1.388	3.23	169.27	0.99
11	F0	Liver/Male	Cit- ^{109}Ag NPs	7.912	7.867	15.43	170.16	0.99
12	F0	Gonad/Female	Cit- ^{109}Ag NPs	16.349	16.264	4.91	178.94	1.00
13	F0	Gonad/Female	Cit- ^{109}Ag NPs	11.044	10.987	1.45	180.44	1.00
14	F0	Gonad/Female	Cit- ^{109}Ag NPs	29.458	29.330	6.04	193.84	1.00
15	F0	Gonad/Female	Cit- ^{109}Ag NPs	29.948	29.815	4.66	191.89	1.00
16	F0	Gonad/Female	Cit- ^{109}Ag NPs	10.040	10.000	1.75	200.76	1.00
17	F0	Liver/Female	Cit- ^{109}Ag NPs	0.580	0.573	0.62	101.80	0.99
18	F0	Liver/Female	Cit- ^{109}Ag NPs	0.466	0.463	0.28	153.49	0.99

A2.2. (continued)

Sample	Generation	Type/Sex	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per mg tissue [ng/mg]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
19	F0	Liver/Female	Cit- $^{109}\text{AgNPs}$	3.215	3.195	1.21	166.83	0.99
20	F0	Liver/Female	Cit- $^{109}\text{AgNPs}$	0.818	0.813	0.87	175.59	0.99
21	F0	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.042	0.040	0.09	34.77	0.95
22	F0	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.125	0.123	0.14	83.02	0.98
23	F0	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.306	0.304	0.33	166.80	0.99
24	F0	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.430	0.744	0.46	220.92	1.73
25	F0	Gonad/Male	Cas- $^{109}\text{AgNPs}$	4.039	4.032	7.07	263.23	1.00
26	F0	Liver/Male	Cas- $^{109}\text{AgNPs}$	0.276	0.267	1.40	45.62	0.96
27	F0	Liver/Male	Cas- $^{109}\text{AgNPs}$	2.355	2.342	39.03	175.91	1.00
28	F0	Liver/Male	Cas- $^{109}\text{AgNPs}$	5.737	5.722	47.69	239.00	1.00
29	F0	Liver/Male	Cas- $^{109}\text{AgNPs}$	72.606	72.422	41.15	238.14	1.00
30	F0	Liver/Male	Cas- $^{109}\text{AgNPs}$	8.590	8.568	9.11	238.79	1.00
31	F0	Liver/Male	Cas- $^{109}\text{AgNPs}$	45.505	45.433	72.12	270.65	1.00
32	F0	Gonad/Female	Cas- $^{109}\text{AgNPs}$	39.932	39.896	5.01	298.59	1.00
33	F0	Gonad/Female	Cas- $^{109}\text{AgNPs}$	3.187	3.183	0.50	295.16	1.00
34	F0	Gonad/Female	Cas- $^{109}\text{AgNPs}$	8.225	8.219	0.99	310.77	0.999
35	F0	Gonad/Female	Cas- $^{109}\text{AgNPs}$	5.911	5.906	0.83	307.93	0.999
36	F0	Gonad/Female	Cas- $^{109}\text{AgNPs}$	15.964	15.953	1.24	308.88	0.999

A2.2. (continued)

Sample	Generation	Type/Sex	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per mg tissue [ng/mg]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
37	F0	Liver/Female	Cas- $^{109}\text{AgNPs}$	1.225	1.201	0.83	75.32	0.98
38	F0	Liver/Female	Cas- $^{109}\text{AgNPs}$	0.189	0.186	0.21	85.43	0.98
39	F0	Liver/Female	Cas- $^{109}\text{AgNPs}$	0.483	0.479	0.68	136.76	0.99
40	F0	Liver/Female	Cas- $^{109}\text{AgNPs}$	6.738	6.716	2.47	218.79	1.00
41	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.130	0.085	0.14	4.44	0.65
42	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.091	0.87	0.58	40.59	0.96
43	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.129	0.126	0.382	63.00	0.98
44	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.153	0.151	0.15	80.83	0.98
45	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.494	0.488	0.60	106.20	0.99
46	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.201	0.199	0.25	106.48	0.99
47	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.103	0.101	0.12	69.53	1.00
48	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.088	0.085	0.17	61.72	0.98
49	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.049	0.047	0.10	42.36	0.91
50	F1	Gonad/Male	Cit- $^{109}\text{AgNPs}$	0.101	0.092	0.14	19.95	1.00
51	F1	Liver/Male	Cit- $^{109}\text{AgNPs}$	19.616	19.296	21.21	86.99	0.98
52	F1	Liver/Male	Cit- $^{109}\text{AgNPs}$	8.757	8.632	18.37	96.14	0.99
53	F1	Liver/Male	Cit- $^{109}\text{AgNPs}$	27.127	26.778	39.97	103.55	0.99
54	F1	Liver/Male	Cit- $^{109}\text{AgNPs}$	2.051	2.038	1.92	163.86	0.99

A2.2. (continued)

Sample	Generation	Type/Sex	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per mg tissue [ng/mg]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
55	F1	Gonad/Female	Cit- $^{109}\text{AgNPs}$	9.137	9.082	0.82	165.84	0.99
56	F1	Gonad/Female	Cit- $^{109}\text{AgNPs}$	8.059	8.013	1.03	171.09	0.99
57	F1	Gonad/Female	Cit- $^{109}\text{AgNPs}$	4.586	4.563	0.90	183.83	1.00
58	F1	Gonad/Female	Cit- $^{109}\text{AgNPs}$	8.286	8.248	0.84	188.69	1.00
59	F1	Gonad/Female	Cit- $^{109}\text{AgNPs}$	9.868	9.828	1.02	201.53	1.00
60	F1	Liver/Female	Cit- $^{109}\text{AgNPs}$	3.238	3.173	1.13	74.39	0.98
61	F1	Liver/Female	Cit- $^{109}\text{AgNPs}$	3.737	3.674	1.32	84.91	0.98
62	F1	Liver/Female	Cit- $^{109}\text{AgNPs}$	2.611	2.581	0.82	112.96	0.99
63	F1	Liver/Female	Cit- $^{109}\text{AgNPs}$	3.053	3.030	1.10	147.59	0.99
64	F1	Liver/Female	Cit- $^{109}\text{AgNPs}$	13.935	13.832	4.63	148.19	0.99
65	F1	Liver/Female	Cit- $^{109}\text{AgNPs}$	34.071	33.867	9.46	166.24	0.99
66	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.083	0.073	0.18	14.80	0.88
67	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.301	0.277	0.38	21.50	0.92
68	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.301	0.289	0.17	42.18	0.96
69	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.234	0.225	0.22	42.64	0.96
70	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.626	0.621	1.00	155.20	0.99
71	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.010	0.010	0.02	25.62	0.99
72	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.450	0.445	0.42	107.67	0.92

A2.2. (continued)

Sample	Generation	Type/Sex	Treatment	Total Ag mass in sample [ng]	Mass of Ag-En in sample [ng]	Mass of Ag-En per mg tissue [ng/mg]	$^{109}\text{Ag}/^{107}\text{Ag}$	Mass fraction Ag-En rel to Total-Ag [ng/ng]
73	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.660	0.656	0.57	154.39	0.99
74	F1	Gonad/Male	Cas- $^{109}\text{AgNPs}$	0.436	0.427	0.38	77.96	0.99
75	F1	Liver/Male	Cas- $^{109}\text{AgNPs}$	6.066	5.972	8.53	90.15	0.98
76	F1	Liver/Male	Cas- $^{109}\text{AgNPs}$	3.290	3.247	19.10	103.66	0.99
77	F1	Liver/Male	Cas- $^{109}\text{AgNPs}$	5.727	5.671	13.19	123.36	0.99
78	F1	Liver/Male	Cas- $^{109}\text{AgNPs}$	12.385	12.298	39.67	152.04	0.99
79	F1	Liver/Male	Cas- $^{109}\text{AgNPs}$	22.775	22.646	41.18	171.38	0.99
80	F1	Liver/Male	Cas- $^{109}\text{AgNPs}$	20.694	20.614	34.94	205.02	1.00
81	F1	Gonad/Female	Cas- $^{109}\text{AgNPs}$	10.292	10.272	1.06	255.30	1.00
82	F1	Gonad/Female	Cas- $^{109}\text{AgNPs}$	10.093	10.082	1.35	290.90	1.00
83	F1	Gonad/Female	Cas- $^{109}\text{AgNPs}$	1.873	1.872	0.29	321.17	1.00
84	F1	Gonad/Female	Cas- $^{109}\text{AgNPs}$	18.934	18.927	1.03	326.74	1.00
85	F1	Livers/Female	Cas- $^{109}\text{AgNPs}$	1.095	1.069	0.76	63.52	0.98
86	F1	Livers/Female	Cas- $^{109}\text{AgNPs}$	2.090	2.046	0.77	70.82	0.98
87	F1	Livers/Female	Cas- $^{109}\text{AgNPs}$	1.291	1.275	0.49	106.75	0.99
88	F1	Livers/Female	Cas- $^{109}\text{AgNPs}$	5.367	5.340	2.61	180.56	0.99
89	F1	Livers/Female	Cas- $^{109}\text{AgNPs}$	10.982	10.926	3.13	180.68	0.99

A2.3. Complete Ag isotope data and Ag mass data collected from samples exposed to Ag NPs in the parental generation F0. Samples were collected in generation F1.

Sample	Type/Sex	F0 Parental	Mass of Ag per	
		Treatment	mg tissue [ng mg ⁻¹]	¹⁰⁹ Ag/ ¹⁰⁷ Ag
90	Gonad/Male	Cit- ¹⁰⁹ AgNPs	0.02	0.93
91	Gonad/Male	Cit- ¹⁰⁹ AgNPs	0.13	1.15
92	Gonad/Male	Cit- ¹⁰⁹ AgNPs	0.01	1.22
93	Liver/Male	Cit- ¹⁰⁹ AgNPs	0.29	0.91
94	Liver/Male	Cit- ¹⁰⁹ AgNPs	0.42	0.95
95	Liver/Male	Cit- ¹⁰⁹ AgNPs	0.14	0.96
96	Gonad/Female	Cit- ¹⁰⁹ AgNPs	0.01	0.95
97	Gonad/Female	Cit- ¹⁰⁹ AgNPs	0.01	0.95
98	Gonad/Female	Cit- ¹⁰⁹ AgNPs	0.01	0.98
99	Livers/Female	Cit- ¹⁰⁹ AgNPs	0.02	1.05
100	Livers/Female	Cit- ¹⁰⁹ AgNPs	0.06	1.01
101	Livers/Female	Cit- ¹⁰⁹ AgNPs	0.02	1.06
102	Gonad/Male	Cas- ¹⁰⁹ AgNPs	0.11	0.96
103	Gonad/Male	Cas- ¹⁰⁹ AgNPs	0.01	0.96
104	Gonad/Male	Cas- ¹⁰⁹ AgNPs	0.01	0.98
105	Gonad/Male	Cas- ¹⁰⁹ AgNPs	0.04	0.99
106	Liver/Male	Cas- ¹⁰⁹ AgNPs	0.33	0.99
107	Liver/Male	Cas- ¹⁰⁹ AgNPs	1.54	0.95
108	Gonad/Female	Cas- ¹⁰⁹ AgNPs	0.01	0.93
109	Gonad/Female	Cas- ¹⁰⁹ AgNPs	0.01	0.97
110	Livers/Female	Cas- ¹⁰⁹ AgNPs	0.02	1.00
111	Livers/Female	Cas- ¹⁰⁹ AgNPs	0.02	1.00
112	Livers/Female	Cas- ¹⁰⁹ AgNPs	0.02	1.11
113	Liver/Male	Control	0.12	0.93
114	Liver/Male	Control	0.46	0.93
115	Liver/Male	Control	1.68	0.94
116	Liver/Male	Control	0.14	1.01
117	Gonad/Male	Control	0.01	0.95
118	Gonad/Male	Control	0.03	0.96
119	Gonad/Male	Control	0.00	0.97

A2.3. (continued)

Sample	Type/Sex	F0 Parental Treatment	Mass of Ag per	
			mg tissue [ng mg ⁻¹]	¹⁰⁹ Ag/ ¹⁰⁷ Ag
			Below quantification	
120	Gonad/Male	Control	limit	1.08
121	Gonad/Female	Control	0.01	0.92
122	Gonad/Female	Control	0.01	0.96
123	Gonad/Female	Control	0.01	0.96
124	Gonad/Female	Control	0.01	1.23
125	Livers/Female	Control	0.15	0.92
126	Livers/Female	Control	0.07	1.02
127	Livers/Female	Control	0.13	1.03
128	Livers/Female	Control	0.01	1.05

A2.4. Average levels of enriched Ag and number of analysed samples for each treatment group.

Sample type	Treatment group	Generation	Average enriched Ag [pg embryo ⁻¹]	1 SD [pg embryo ⁻¹]	Number of analysed samples
Embryo	Casein, Week 1	F1	39.6	20.4	6
	Casein, Week 2		32.6	5.7	4
	Casein, Week 3		32.2	11.7	6
	Citrate, Week 1		38.1	17.1	6
	Citrate, Week 2		32.6	11.2	6
	Citrate, Week 3		25.1	5.9	6
	Casein, Week 1	F2	N/A	N/A	5
	Casein, Week 2		42.6	13.5	6
	Casein, Week 3		34.6	14.3	5
	Citrate, Week 1		N/A	N/A	5
	Citrate, Week 2		27.6	2.4	3
	Citrate, Week 3		19.7	9.1	4
Sample type	Treatment group	Generation	Average m(Ag-En) [ng mg ⁻¹]	1 SD [pg mg ⁻¹]	Number of analysed samples
Livers Male	Casein	F0	35.08	23.77	6
	Citrate		6.80	5.69	6
	Casein	F1	26.07	13.04	6
	Citrate		20.37	13.50	4
Livers Female	Casein	F0	1.05	0.85	4
	Citrate		0.75	0.34	4
	Casein	F1	1.55	1.09	5
	Citrate		3.07	3.14	6
Gonads Male	Casein	F0	1.62	2.73	5
	Citrate		1.16	1.30	6
	Casein	F1	0.37	0.27	9
	Citrate		0.26	0.18	10
Gonads Female	Casein	F0	2.04	1.69	6
	Citrate		3.76	1.83	5
	Casein	F1	0.90	0.36	5
	Citrate		0.92	0.09	5

A2.5. Results of the student t-test analysis as p-values calculated.

Compared population	Parameters	p-value: Ag content	p-value: Ag ratio
F0 Male vs Female	Cit- ¹⁰⁹ AgNPs, livers	0.13	0.47
	Cit- ¹⁰⁹ AgNPs, gonads	0.05	0.01
	Cas- ¹⁰⁹ AgNPs, livers	0.02	0.37
	Cas- ¹⁰⁹ AgNPs, gonads	0.78	0.02
F0 Citrate vs casein	Male, livers	0.04	0.14
	Male, gonads	0.78	0.10
	Female, livers	0.61	0.98
	Female, gonads	0.18	0.00
F1 Male vs Female	Cit- ¹⁰⁹ AgNPs, livers	0.11	0.76
	Cit- ¹⁰⁹ AgNPs, gonads	0.00	0.00
	Cas- ¹⁰⁹ AgNPs, livers	0.01	0.53
	Cas- ¹⁰⁹ AgNPs, gonads	0.04	0.00
F1 Citrate vs casein	Male, livers	0.64	0.30
	Male, gonads	0.36	0.28
	Female, livers	0.35	0.95
	Female, gonads	0.91	0.00
F1 Embryos			
Cit- ¹⁰⁹ AgNPs vs Cas- ¹⁰⁹ AgNPs	Week 1	0.90	0.01
	Week 2	1.00	0.00
	Week 3	0.08	0.06
Week 1 vs Week 2	Cit- ¹⁰⁹ AgNPs	0.59	0.41
Week 2 vs Week 3	Cit- ¹⁰⁹ AgNPs	0.29	0.14
Week 1 vs Week 2	Cas- ¹⁰⁹ AgNPs	0.50	0.00
Week 2 vs Week 3	Cas- ¹⁰⁹ AgNPs	0.67	0.03
F2 Embryos			
Cit- ¹⁰⁹ AgNPs vs Cas- ¹⁰⁹ AgNPs	Week 1	N/A	N/A
	Week 2	0.04	0.00
	Week 3	0.14	0.21

A2.5. (continued)

Compared population	Parameters	p-value: Ag content	p-value: Ag ratio
F2 Embryos			
Week 1 vs Week 2	Cit- ¹⁰⁹ AgNPs	N/A	N/A
Week 2 vs Week 3	Cit- ¹⁰⁹ AgNPs	0.75	0.41
Week 1 vs Week 2	Cas- ¹⁰⁹ AgNPs	N/A	N/A
Week 2 vs Week 3	Cas- ¹⁰⁹ AgNPs	0.41	0.00
	Cit- ¹⁰⁹ AgNPs, female, livers	0.15	0.37
	Cit- ¹⁰⁹ AgNPs, female, gonads	0.02	0.47
	Cas- ¹⁰⁹ AgNPs, male, livers	0.69	0.45
	Cas- ¹⁰⁹ AgNPs, male, gonads	0.31	0.67
	Cas- ¹⁰⁹ AgNPs, female, livers	0.12	0.60
	Cas- ¹⁰⁹ AgNPs, female, gonads	0.32	0.45
F1 vs F2 Males			
	Cit- ¹⁰⁹ AgNPs, livers	0.15	0.28
	Cit- ¹⁰⁹ AgNPs, gonads	0.23	0.23
	Cas- ¹⁰⁹ AgNPs, livers	0.84	0.57
	Cas- ¹⁰⁹ AgNPs, gonads	0.90	0.43
F1 vs F2 Females			
	Cit- ¹⁰⁹ AgNPs, livers	0.16	0.14
	Cit- ¹⁰⁹ AgNPs, gonads	0.04	0.39
	Cas- ¹⁰⁹ AgNPs, livers	0.54	0.46
	Cas- ¹⁰⁹ AgNPs, gonads	0.18	0.99