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Advancing fish tissue reference material development for Ag nanoparticles through a perfusion approach

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There is a regulatory need for reliable methods to quantify nanoparticles in biological samples for the hazard assessment and bioaccumulation potential of engineered nanomaterials (ENMs). Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) is a promising technique, but its application is hindered by the lack of validated sample preparation procedures (e.g., solubilisation) and suitable biological reference materials. This study aimed to develop an in-house biological reference material for ENMs to support analytical method validation. Silver nanoparticles (Ag NPs) were introduced into rainbow trout via cardiac perfusion, with the gills and liver collected as target tissues. Tissues were homogenised using a blade grinder, followed by additional milling. Pre- and post-milling preparations were compared to evaluate the level of tissue processing required prior to SP-ICP-MS analysis. Tissue samples were solubilised using tetramethylammonium hydroxide before analysis. Background Ag NP concentrations in control tissues were low ($0.2\text{--}0.7 \times 10^9$ nanoparticles per g), consistent with previous studies. In contrast, fish exposed to Ag NPs showed a clear increase in particle concentration, reaching up to $33.8 \pm 4.4 \times 10^9$ nanoparticles per g, with median particle sizes of 74–81 nm. The additional milling significantly improved data quality. In the liver, nanoparticle concentrations increased from $10.7 \pm 4.4 \times 10^9$ nanoparticles per g before milling to $15.9 \pm 1.4 \times 10^9$ nanoparticles per g after milling. Precision also improved, with relative standard deviation (RSD%) reducing from 40.7% to 9.1%, and all nanoparticle metrics achieving RSD values below 15% after combined blade grinding and milling. These results demonstrate that perfusion, followed by blade grinding and milling, produces a homogenous in-house biological reference material containing ENMs. This approach is simple, cost effective, and ensures analytical robustness for SP-ICP-MS measurements. Future work should assess the material's shelf life and apply this approach in inter-laboratory testing to support broader method validation.

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Environmental significance

Reliable methods to quantify nanoparticles in biological tissues are essential for assessing the hazard and bioaccumulation potential of engineered nanomaterials (ENMs). Single particle ICP-MS is a powerful tool for this purpose, but its wider application is limited by the absence of validated preparation methods and suitable biological reference materials. This study demonstrates that cardiac perfusion of rainbow trout with silver nanoparticles, followed by homogenisation and milling, produces a reproducible in-house biological reference material. The approach is simple, cost-effective, and yields precise nanoparticle measurements. By improving analytical robustness, this work provides a practical foundation for regulatory testing and environmental monitoring of ENMs, with the potential to be extended to a wider range of nanomaterials.

Introduction

Assessing the hazards and bioaccumulation potential of engineered nanomaterials (ENMs) requires reliable methods to detect and quantify nanoparticles in biological tissues. Single particle inductively coupled plasma mass spectrometry (SP-ICP-MS) has gained traction as a powerful tool for the detection of metal-containing particles, especially for identifying and quantifying the presence of particles in tissues.^{1–4} The advantage of SP-ICP-MS over other ENM-

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characterisation tools is the mass-derived particle size, whereas other techniques measure the larger hydrodynamic diameter thus providing the hydrated particle radius.⁵ The core steps for a tissue involve sample collection/storage, solubilisation of the tissue and extraction of the ENMs as a suspension in a liquid phase, then dilution and analysis *via* SP-ICP-MS.⁴ While general guidelines exist for analysing ENM suspensions to determine the particle number concentration, mass concentration, size and size distribution,⁶ no standardised protocols are available for the critical upstream steps, especially tissue solubilisation/ENM extraction, when working with complex biological matrices.

From the perspective of total metal analysis using conventional ICP-MS, certified reference materials (CRMs) are available for validation of the analytical procedure. A CRM should be matrix matched to the test sample and have similar analyte properties (*i.e.* species) and concentration(s). For biological samples, a wide variety of CRMs are available for total metal content and elemental species but not for ENMs. For ENMs, CRM availability is limited to particle suspensions or powders, with only one of these (gold nanoparticles) characterised by SP-ICP-MS.^{7,8} Some progress has been made toward developing in-house biological reference materials (RMs), for example, homogenised chicken breast paste spiked with silver nanoparticles (Ag NPs).⁹ However, this approach prevents ENMs from being biologically incorporated into the cells/tissues and leads to a loss of the original tissue architecture, both of which are important for evaluating extraction efficiency and are therefore less well suited for toxicological studies.

As a result, researchers often rely on in-house spike-and-recovery experiments to validate the tissue preparation steps. This typically involves spiking the solubilisation fluid with ENMs and assessing particle recovery throughout the process.¹⁰ While this approach provides an estimate of method accuracy, it has significant limitations. Most importantly, it sidesteps the critical factor of how effectively the tissue matrix itself breaks down to release the ENMs, an issue that becomes especially relevant when working with (eco)toxicological tissue samples, where particle levels in the tissue are often a small fraction of the exposure dose, typically in the $\mu\text{g g}^{-1}$ range.¹¹ Furthermore, such spike recovery tests fail to capture the particle transformations that may occur inside the organism (*e.g.*, agglomeration in biological fluids, corona formation *in vivo*, *etc.*), which further undermines their validity. Regardless, many studies selectively report favourable outcomes without full disclosure of validation procedures. A recent review of studies on biological extraction and quantification of ENMs using SP-ICP-MS⁴ found that only 42% reported particle mass recovery, 18% reported particle number concentration, and just 11% reported both. Alarming, over half (51%) simply referred to previous methods without presenting validation data. This poor uptake of method validation is, in part, due to the lack of suitable particle and matrix-specific CRMs.

The aim of this study was to develop an in-house, or representative,¹² biological RM for ENMs, specifically Ag NPs, to support analytical method validation. Determining ENMs in fish is critical for both environmental regulation and food safety, yet no validated method currently exists to support mandatory bioaccumulation testing.¹³ Silver NPs were chosen for this study due to their partial solubility in environmental and biological systems,¹⁴ and because dissolved silver is among the most toxic metals to aquatic animals,¹⁵ making it essential to differentiate between ionic and particulate effects. To create the in-house biological RM, fish were perfused with Ag NPs and tissues taken for SP-ICP-MS analysis. A secondary objective was to evaluate the level of tissue processing required to achieve consistent and precise measurements through the use of a commercial blade grinder and an additional milling step afterwards.

Methods

Reagents, materials and nanomaterial characterisation

Reagents were analytical grade or higher (tetramethylammonium [TMAH, 25% in H_2O], CaCl_2 , and trace metal grade nitric and hydrochloric acids were obtained from Fisher Scientific, UK). High purity water (HPW) was used throughout ($18.2 \text{ M}\Omega \text{ cm}$; Elga Ltd, UK). Gold nanoparticle (60 nm) suspensions were obtained from BBI Solutions, UK, whilst Ag NPs, suspended in 5.5 mmol L^{-1} of sodium citrate and $25 \mu\text{mol L}^{-1}$ tannic acid, were obtained from Applied Nanoparticles (Barcelona, Spain). These Ag NPs have been extensively characterised in previous work.^{5,11,14,17} Briefly, the Ag NPs were supplied at a nominal size and concentration of 60 nm and 10.8 g L^{-1} , respectively (manufacturer's information). Measurements were made at the University of Plymouth to confirm the composition of the stock. The measured primary particle size (*via* transmission electron microscopy) and total Ag concentration (*via* ICP-MS) was $55 \pm 3 \text{ nm}$ and $9.9 \pm 0.3 \text{ g L}^{-1}$, respectively. The dissolution in saline is very low (<1%, ref. 14), ensuring a particle exposure was achieved. A commercially available dissolved Ag standard (QMX Laboratories Ltd, UK) at a concentration of $10\,000 \pm 5 \mu\text{g mL}^{-1}$ in 2% HNO_3 was used for routine instrument calibration after dilution to appropriate concentrations. All suspensions and solutions were prepared daily before use. All labware used was pre-cleaned by soaking in 10% HCl for 24 hours followed by rinsing in HPW.

Fish perfusion, tissue collection and sample processing

The use and care of animals in this study was in line with institutional husbandry guidelines and underwent internal ethical approval *via* a Local Ethics Committee prior to starting (reference number ETHICS-51-2022). Rainbow trout (*Oncorhynchus mykiss*) weighing $220 \pm 44 \text{ g}$ (mean \pm standard deviation, $n = 12$) were obtained from Exmoor Fisheries, UK. These fish were kept in a recirculating system containing dechlorinated Plymouth tap water until sampling and fed a commercially available diet (Aller Futura, Kaliningrad,



Russia). Subsequently, healthy fish were euthanised by induced concussion followed by pithing of the brain (schedule 1 method in accordance with ethical approvals, Home Office, UK, and in compliance with EU directive 2010/63/EU). A cut was then made horizontally in front of the dorsal fin to expose the kidney and the fish were then cut open ventrally and the vasculature by the gastrointestinal tract was cut to allow blood to drain from the animal during the perfusion. With the heart exposed, 40 ml of heparinised saline (0.9% NaCl, heparin Li salt at 1 mg ml⁻¹, unbuffered, pH 6.1 ref. 18) was injected through the base of the ventricles to distribute around the body. There were two treatments: an unexposed control (no added Ag NPs) or 200 mg L⁻¹ Ag NPs. The unexposed control allowed the background concentration of Ag NPs in the tissues to be quantified, and to confirm the presence of new material in the Ag NP exposed fish. The liver and gills were selected as target organs due to their established role in metal and ENM toxicity studies,¹⁶ as well as their contrasting fat content, which allowed assessment of method performance across different tissue types. Following the perfusion, the whole brachial basket (containing the gills) and the liver were removed from the fish. The gill filaments were removed from the brachial basket and, along with the liver tissues, stored at -20 °C until preparation for analysis.

The whole gill and liver tissues ($n = 6$ /treatment) were then freeze dried (Labryo freeze dryer) for 24 h and the dry mass recorded. To homogenise the samples, the individual gill or liver tissue samples from each treatment were pooled to create four bulk samples (one per treatment). Each bulk sample was then ground with a blade grinder to produce an in-house RM for that tissue and treatment. The fine powder was then sampled ($n = 3$ /treatment) and processed for SP-ICP-MS analysis and termed “pre-milling” samples. To further homogenise the sample, the remaining ground tissue was placed in a centrifuge tube that contained 2–3 small stainless-steel balls (cleaned in acetic acid, high purity water and then acetone to remove impurities) and milled (Retsch MM 400, Verder Scientific). The milling was conducted three times for 60 seconds at 30 Hz. The final milled product was sampled ($n = 3$ /treatment) and processed for SP-ICP-MS analysis. Each replicate was analysed three times, producing nine SP-ICP-MS data sets per treatment (total $n = 4$). This resulted in 36 samples each for “pre milling” and “post milling”, totalling 72 samples.

A previously validated and well documented extraction protocol to routinely breakdown tissues and liberate Ag NPs as a suspension from fish tissues was applied for the sample preparation here.^{5,17} Approximately 50 mg of the powdered tissue (chosen as this is the typical mass of whole tissue samples from smaller fish used in ecotoxicological studies conducted at Plymouth), was accurately weighed into a 15 mL centrifuge tube. Tissue solubilisation was achieved by the addition of 0.8 mL of 25 mmol L⁻¹ CaCl₂ in HPW, allowing the tissue to wet completely and then vortex mixing for 30–60 seconds, then a subsequent addition of 3.2 mL of 25% TMAH

and further vortex mixing for approximately 30 seconds. Procedural blanks ($n = 3$), *i.e.*, the extraction reagents alone added to a centrifuge tube, were also produced. Additionally, another set of procedural blanks spiked with dissolved silver (as Ag⁺, 100 μL of 100 μg L⁻¹ stock solution) were prepared to check the extraction procedure did not produce false-positives (see ref. 4 for details). The samples were left at room temperature in a dark, dry storage cupboard overnight and then diluted as appropriate, to ensure the final sample gave around 1500 particle events per time scan (approximately 50 ng L⁻¹ ref. 5), before being analysed by SP-ICP-MS.

Single particle ICP-MS analysis

For all sample analysis by SP-ICP-MS, an iCAP TQ ICP-MS instrument (Thermo Fisher), fitted with a Micromist nebuliser (400 μL min⁻¹ uptake rate, Glass Expansion, Australia) and a quartz cyclonic spray chamber cooled to 2 °C was operated in KED mode (with a helium gas flow rate of 5.1 mL min⁻¹). The plasma power was 1550 Watts, and the plasma, nebuliser and auxiliary gas flow rates were 14.0, 1.06 and 0.8 L min⁻¹, respectively. Nickel-plated sampler and high sensitivity insert skimmer cones were also used. Before each analytical session, the ICP-MS instrument was tuned, using a solution of 1 μg L⁻¹ Ba, Bi, Ce, Co, In, Li and U so that it performed according to the manufacturer's specifications. Sensitivity, stability, and oxide formation ratio (CeO/Ce), with the emphasis on the latter two, to give RSDs of <2% and an oxide ratio <0.6%, as an indication of the extent of polyatomic interference removal, were the key parameters for instrument tuning. A dwell time of 3 milliseconds was used throughout this work, with a total sampling time of 60 seconds using the ¹⁰⁷Ag *m/z* ratio. The sample wash out time was 60 seconds, using a solution of 4% HCl and 2% HNO₃, to ensure no carryover between samples. In addition, before the samples from each treatment were analysed solutions of 4% HCl and 2% HNO₃ and 0.1% TMAH were analysed to check instrumental cleanliness. The sample uptake rate was determined gravimetrically by difference daily by aspirating HPW over 5 min ($n = 5$) and was between 0.3 and 0.4 mL min⁻¹ for all analysis. The transport efficiency was also calculated daily using 60 nm Au NPs, in each sample matrix used ($n = 5$), and according to the particle mass method outlined by Pace *et al.*¹⁹ The instrument was calibrated using a series of dissolved Ag standards ($n = 5$) ranging from 0 to 4 μg L⁻¹. Quality control measures of procedural blanks ($n = 3$) and Ag⁺ spiked procedural blanks ($n = 3$), as well as check standards every 10–15 samples, were included. All solution/suspension preparation and ICP-MS analyses were undertaken in a laboratory under an ISO 9001 certified quality management system. The raw data for each sample was exported from the instrument (as counts per second, calculated from the acquisition in 3 millisecond dwell times) and entered into a bespoke Excel® spreadsheet which calculates the signal distribution, and from this the particle mass concentration, particle number concentration (as ×10⁹



particles) and median particle size, according to ref. 20 and assessed in ref. 5 (Fig. 1). Each time scan was visually inspected to determine the particle count threshold and ranged between 2000 and 10 000 counts per second (depending on daily sensitivity and the ionic Ag signal).

Total Ag content by ICP-MS

All fish tissues prepared post milling were analysed for their total Ag content. Each sample, along with DOLT-5 (Dogfish liver) CRM (NRCC, Canada) was subjected to a hot HNO₃ digestion in PTFE vessels. These digests were analysed using an iCAP TQ ICP-MS instrument (Thermo Fisher) which was tuned, checked for performance and operated as described previously, with the only difference being that a dwell time of 10 ms was used.

Statistical analysis

Statistical analyses were performed using SigmaPlot 14.5. Data were assessed for normality (Shapiro–Wilk test) and equal variance (Brown Forsythe) prior to the statistical tests. Statistical differences were assessed using a one-way ANOVA for analysis of effects between sample preparation within treatments, followed by a Holm–Sidak *post hoc* test. Where data were non-parametric, the Kruskal–Wallis test was used, followed by Tukey's *post hoc* test. The *P* values presented correspond with the appropriate *post hoc* test.

Results and discussion

Confirming the presence of Ag NPs in the perfused organs

The perfusion procedure post-blade grinding produced a combine set of four samples (total dry weight): control gill (1.42 g) and liver (2.54 g) and Ag NP loaded gill (1.59 g) and liver (2.59 g). For the Ag NP treatment, the measured particle mass concentrations were several hundred $\mu\text{g g}^{-1}$ (Table 1) and were similar for the both the gill and the liver. The gill Ag NP particle mass concentration was 226 ± 129 and $561 \pm$

$77 \mu\text{g g}^{-1}$ whilst for the liver the values obtained were 201 ± 41 and $632 \pm 89 \mu\text{g g}^{-1}$ for pre- and post-milling samples, respectively (Table 1). The presence of Ag NPs in the gill and liver confirmed the perfusion passed throughout the vasculature to reach these organs. Each of these tissue concentrations are perhaps towards the upper end of accumulated material for ecotoxicology studies. For example, a 4 week *in vivo* dietary study feeding fish 100 mg kg^{-1} of Ag NPs at a 2% body weight ratio resulted in the liver reaching a particle mass concentration of $\sim 268 \mu\text{g g}^{-1}$.¹⁷

Whilst the circulatory system can distribute ENMs from the blood into the tissues, for example, *via* the fenestrated endothelial cells in the liver,²⁸ it remains unlikely that the short perfusion had resulted in cellular uptake. Instead, the Ag NPs likely remained in the extracellular space which is a similar volume in trout gill and liver.²⁹ This likely is a cause for the similar particle mass concentrations in both the gills and the liver arising from the perfusion method (Table 1). This lack of uptake may be advantageous for developing in-house RMs. NPs are well known to undergo endocytosis into membrane-bound vesicles, where they may dissolve in acidic compartments such as lysosomes. This dissolution would alter the measurable NP concentration and compromise the limit of detection. Although these transformations depend on NP type, it is important for the perfusion technique that the NPs are embedded within a tissue matrix so that downstream sample handling (such as drying and homogenising) resembles that of genuine *in vivo* tissues.

In this respect, the retention of Ag NPs within the biological matrix (*i.e.*, inside the cardiovascular system of the tissue) provides meaningful in-house tool and represents a clear advancement over other approaches that simply spike NPs into the liquid arising from homogenising a piece of tissue.⁹ Importantly, in-house RMs produced using this approach should incorporate the relevant particle of interest in the (eco)toxicological study, as the primary goal is to support analytical validation rather than replicate all biological processes governing NP uptake.

Milling is an important step in sample preparation for determining particle metrics

Producing a homogenous powder is a critical step in the development of a biological RM. The blade grinding process serves two main purposes: first, to ensure sub-samples taken from the bulk tissue are representative, and second, to facilitate consistent tissue breakdown during any subsequent solubilisation or ENM extraction step. The former (sample homogeneity) can be evaluated through the RSD% values obtained for various measurements made on sub-samples. In this study, a two-step process was assessed, initially with a commercial blade grinder, followed by additional milling.

Although no obvious visual differences were observed between the solubilised pre- and post-milled samples, milling significantly altered both the measured Ag NP particle mass concentration and particle number concentration (Table 1) in



Fig. 1 Size distribution of Ag NPs in ultrapure water, corresponding to the suspension used during the tissue perfusion.



Table 1 Particle number concentration, mass concentration and size recovered from 50 mg of the Ag NP in-house RM

Sample preparation	Particle number concentration ($\times 10^9$ nanoparticles per g)	RSD (%)	Particle mass concentration ($\mu\text{g g}^{-1}$)	RSD (%)	Particle size (nm)	RSD (%)
Gill						
Pre-milling	12.9 ± 6.0^A	46.9	226 ± 129^A	57.1	79 ± 6.0^A	7.7
Post-milling	33.8 ± 4.4^B	13.0	561 ± 77^B	13.7	81 ± 3.3^A	4.1
Liver						
Pre-milling	10.7 ± 4.4^A	40.7	201 ± 41^A	20.6	74 ± 4.1^A	5.6
Post-milling	15.9 ± 1.4^B	9.1	632 ± 89^B	14.1	75 ± 2.1^A	2.8

Data are mean \pm standard deviation ($n = 3$ technical replicates that were measured 3 times). Different upper-case letters denote statistically significant difference between pre- and post-milling sample preparation within the same tissue (e.g., control gill *etc.*). The technical replicates are formed from the collection of three distinct samples from the exposed and homogenised tissues.

the tissues perfused with Ag NPs. For example, the Ag NP gill particle mass concentration doubled after milling, increasing from 226 to $561 \mu\text{g g}^{-1}$ (one-way ANOVA, $P < 0.001$). A similar trend was observed for the Ag NP gill particle number concentration, which rose more than 2.5-fold to $33.8 \pm 4.4 \times 10^9$ particles per g (Kruskal–Wallis, $P < 0.001$, Table 1). Similar results were observed for the liver whereby there was a significant increase in particle mass concentration (one-way ANOVA, $P < 0.001$) and particle number concentration (Kruskal–Wallis, $P = 0.003$) post-milling of the samples (Table 1). Crucially, the particle size (Table 1) and particle size distribution (Fig. 2) did not alter through milling in the Ag NP-perfused gills or liver, suggesting that the particle sizes measured before milling was accurate. Therefore, the increase in both particle number and mass concentrations after milling, in combination with no change in particle size, supports the idea that Ag NPs are bound to larger cellular aggregates/components not composed of Ag. Milling breaks these larger non-Ag components down, increasing homogeneity in the produced tissue sample without affecting Ag NP particle size.

Importantly, the sample milling step improved the RSD% of all particle metrics in the Ag NP treatment by up to 4.5-fold (Table 1). For example, the Ag NP exposed liver particle number concentration reduced from 40.7% RSD ($10.7 \pm 4.4 \times 10^9$ particles per g) to 9.7% RSD ($15.9 \pm 1.4 \times 10^9$ particle per g). Whilst there is still debate over an acceptable limit for RSD% value obtained for particle measurements in a sample, there have been suggestions of allowing up to 20%.³⁰ Whilst this is still above the typical uncertainties associated with CRMs, e.g. DORM-5, where the expanded uncertainty of the certified values for a suite of elements ranges between approximately 5–10%, quality control ranges can be expanded to $\pm 20\%$ for complex procedures.³¹ Importantly, the individually perfused fish were pooled together, and so the inter-fish variation in the final tissue concentrations is absent. However, the two-step sample process was able to homogenise any variability to produce the observed RSD% values. Therefore, the RSD% values obtained here are fit for purpose, and fall in the range of that reported in other studies.³²

Assessing the reliability of the Ag NP measurements

A mass balance approach was also undertaken to assess the reliability of the results presented in Table 1 and these data are presented in Table 2. Statistical assessment of the data was conducted according to the approach of Linsinger,³³ which compares the difference in two values (ΔM) with the expanded uncertainty of that difference ($U_{\Delta M}$). Here, ΔM represents the difference between the mean measured values and the certified values, while $U_{\Delta M}$ reflects the variation arising from independent measurements. For Ag NP data ΔM was the difference between the values obtained for the total Ag concentration from the acidic digestion and the particle mass concentration determined by SP-ICP-MS (Table 2). According to this approach, agreement between the two measured values is confirmed when $U_{\Delta M} \geq \Delta M$, indicating no statistically significant difference (Table 2). For the Ag NP-perfused gill and liver tissues, no significant difference was observed between the measured particle mass concentrations and the total Ag content (denoted as “yes” in Table 2). For the DOLT-5 CRM, used to validate the total acidic digestion procedure, the measured and certified values were consistent ($U_{\Delta M} > \Delta M$). These results provide further evidence that the in-house RM has been well characterised regarding the Ag NP metrics.

Trace amounts of nanosilver is present in the control tissues

Although no Ag NPs were intentionally introduced into the control fish (Table 3), nanosized Ag particles were still detected in their tissues. There are three possible sources for these nanoparticles. Firstly, contamination during sample solubilisation; however, this is unlikely as procedural blanks showed negligible Ag NP content (< 10 particle events per time scan) and the median sizes are around 50% of those found in the Ag NP treatment (Table 3). Secondly, the fish may have accumulated incidental Ag NPs from natural water (e.g., at the fish farm) prior to the study, though this is unlikely in rural locations with no point sources of ENMs but cannot be excluded because ENMs are not monitored in surface waters in the UK. Thirdly and most likely, the nanoparticles may have formed biogenically prior to the study. The biogenic formation of silver nanoparticles is





Fig. 2 Particle size distributions of control (A–D) and Ag NP (E–H) exposed gill and liver tissues pre-milling (left panels) and post-milling (right panels). Note, the effect of milling had negligible effect on the particle size distributions of the Ag NPs.



Table 2 The total Ag and particle mass concentrations ($\mu\text{g g}^{-1}$) in the Ag NP-perfused liver and gill samples, along with their associated standard uncertainty (u) and relative standard uncertainty values (RSu%)

	Ag ($\mu\text{g g}^{-1}$)	u ($\mu\text{g g}^{-1}$)	RSu%
Liver			
Ag NP acidic total digestion	483	9.8	2.0
Ag NP particle mass concentration	632	89	14
ΔM	149		
$U_{\Delta M}$	179		
Agreement	Yes		
Gill			
Ag NP acidic total digestion	674	80	12
Ag NP particle mass concentration	561	77	14
ΔM	113		
$U_{\Delta M}$	222		
Agreement	Yes		
DOLT-5			
Certified values	2.05	0.04	2.0
Measured values	1.97	0.09	4.4
ΔM	0.078		
$U_{\Delta M}$	0.192		
Agreement	Yes		

Statistical assessment of the data was according to the approach of Linsinger,³³ which compared the difference in two values, ΔM , with the expanded uncertainty of that difference, $U_{\Delta M}$; with agreement being shown by the latter being larger than the former, indicating no significant difference (*i.e.*, agreement, “yes”). The DOLT-5 CRM was used to verify the accuracy of total Ag concentration measurements, showing agreement between measured and certified values.

Table 3 Particle number concentration, mass concentration and size recovered from the control sample 50 mg of the in-house RM

Sample preparation	Particle number concentration ($\times 10^9$ nanoparticles per g)	RSD (%)	Particle mass concentration ($\mu\text{g g}^{-1}$)	RSD (%)	Particle size (nm)	RSD (%)
Control Gill						
Pre-milling	0.7 ± 0.5^A	79.3	0.1 ± 0.1^A	107.5	24 ± 0.8^A	3.5
Post-milling	0.9 ± 0.7^A	80.3	0.2 ± 0.2^B	81.1	19 ± 1.4^B	7.2
Liver						
Pre-milling	0.2 ± 0.1^A	53.8	0.4 ± 0.2^A	59.7	39 ± 6.1^A	15.5
Post-milling	0.4 ± 0.2^B	42.7	1.0 ± 1.3^B	139.8	39 ± 3.3^A	8.5

The technical replicates are formed from the collection of three distinct samples from the exposed and homogenised tissues.

plausible given that both invertebrates, fish and humans are known to convert dissolved silver into particulate silver,^{17,21,22} a process also observed with copper.²³ Physiological regions with high pH, such as those in the intestinal lumen of trout ($\text{pH} > 8$)^{24,25} can cause dissolved silver to precipitate as silver oxide (Ag_2O).^{5,26,27} In such cases, the nanoparticles are not engineered, but rather, are naturally formed nanosilver. Previous studies reported similar nanoparticles in control fish weighing ~ 18 – 32 g, with particle mass concentrations between 0.003 and $0.061 \mu\text{g g}^{-1}$.¹⁷ In the current study, fish were larger (~ 220 g), and background concentrations were higher ($\sim 0.4 \mu\text{g g}^{-1}$). This suggests that background nanosilver levels may increase (bioaccumulate) with fish size/age, either due to environmental exposure (from Ag NPs or naturally occurring nanosilver) or through biogenic formation. Regardless, the control tissues are still a necessity to inform on the background in the tissue and confirm the exposure of the Ag NP treatment.

In the control tissues, the overall effect of the milling process caused some modest changes in the RSD% of the

particle measurements, and in some cases, made the measurements more variable. For example, the control gill particle size RSD% increased from 3.5 to 7.2% post-milling (Table 3). The tendency for the larger RSD% values in milled control gill indicates the background nano silver may not be homogeneously distributed throughout the tissue (Table 1). It would be expected that milling may enhance the breakup of any agglomerates, and whether any Ag-containing crystals in any residual branchial cartilage, and that smaller particles would increase the number concentration and decrease the particle size, yet this was not observed. Instead, the post-milling control gill particle number concentration remained similar to pre-milling, despite an apparent unexplainable increase in the particle mass concentration and a concomitant (minor) reduction in particle size. It appears that milling to quantify background nano silver may not be necessary although a confirmatory technique to demonstrate the state of the particles in the tissues would be required to supplement this data.



Considerations for the perfusion technique when using nanomaterials

It is likely that some degree of NP agglomeration occurred during the perfusion, as the Ag NPs were suspended in 0.9% NaCl solution. Saline is known to promote agglomeration, with the hydrodynamic diameter of Ag NPs increasing from 66 nm in high purity water to approximately 115 nm in physiological gut saline (~1.5% saline solution).¹⁴ While SP-ICP-MS measurements of the pristine particles yield a size around 57 nm,⁵ the particle sizes measured in the perfused RM ranged from 74 to 81 nm (Table 1) and with particles in bin sizes exceeding this initial size (Fig. 1 and 2), suggesting some agglomeration occurred. This is further supported by the isoelectric point of Ag NPs, which is around pH 8.6.³⁴ Since the saline pH here was 6.1, it is below the isoelectric point which would have favoured particle aggregation. Regardless, the particle sizes were predominantly below 100 nm and would not have hindered the perfusion of the tissue or the penetration of the perfusate into the vasculature (*e.g.*, the typical diameter of venules in adult trout liver is ~60 μm).³⁵

The perfusion and solubilisation method, with appropriate milling, has been demonstrated for Ag NPs, but further validation for other nanomaterials is required. Additional steps, such as extended sonication (*e.g.*, ref. 36) of the solubilised material could also be explored to improve recovery or further reduce agglomeration. Importantly, this work has shown that an in-house biological RM for ENMs can be produced using the perfusion technique. This approach is simple and cost-effective, relying on readily available adult trout from fish farms to allow for in-house RM generation. Additional testing is required to verify the repeatability of the perfusion technique and to ensure that it reliably generates Ag NP concentrations like those found here in the in-house RM. Alternatively, larger fish species, such as Atlantic salmon, are also a commercially viable alternative to generate larger tissue masses in a single step. This approach would be more cost effective than the alternative of feeding fish a NP containing diet, which would have to be conducted under licence of the appropriate national authority, and could be assessed and adopted by organisations accredited to ISO 17025 and 17034 for the development and acquisition of a starting material, and processing thereof, for the production of NP containing fish tissue CRMs.

Conclusions

In conclusion, the perfusion technique successfully produced a biological in-house RM containing Ag NPs, with sufficient material for 20 to 40 replicates at a 50 mg sample size. Using a two-step tissue preparation process, measurement precision (RSD%) ranged from 2.8% to 14%, with particle size showing the highest consistency. It has been shown that sample homogenisation by milling is essential to produce representative 50 mg sub-samples from the original tissue. This has important implications for ecotoxicological studies,

where subsampling tissues (*e.g.*, splitting during dissection for multiple endpoints) should be avoided if accurate NP mass and number quantification is required. The perfusion approach to producing an in-house RM offers a fast, more appropriate alternative to traditional spike-and-recovery tests for solubilisation validation. The resulting in-house RM provides a valuable foundation for developing analytical methods to monitor particles (and potentially other contaminants) in environmental samples. The next step is to assess the stability and appropriate storage (shelf life) of the milled sample over time, to determine whether it must be prepared fresh for each analysis or if it can be stored and used in a similar way to a CRM. Additionally, interlaboratory testing will be important to evaluate differences in solubilisation, data handling and analysis. Future work should also explore adapting the perfused dose for materials with lower accumulation rates (*e.g.*, titanium dioxide) and testing more soluble particles (*e.g.*, zinc oxide) to assess the method's limits.

Conflicts of interest

The authors declare no conflicts of interest.

Data availability

Data can be made available on request.

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