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Uptake, distribution and elimination of palladium-doped polystyrene nanoplastics in rainbow trout (*Oncorhynchus mykiss*) following dietary exposure

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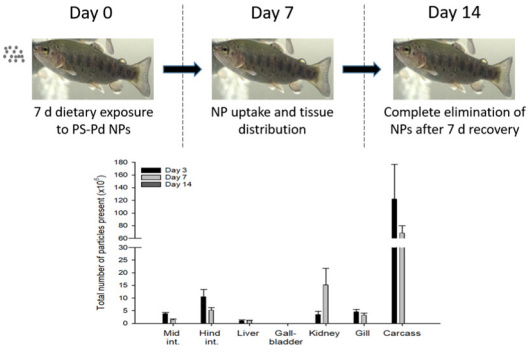
HIGHLIGHTS

- Quantitative data on the uptake and depuration of nanoplastics in fish is lacking.
- Palladium labelling of nanoplastics allows for assessments of biological fate.
- Feeding fish nanoplastics result in particle transfer to internal organs.
- After 7 days of exposure, most nanoplastics were in the carcass.
- Following a depuration period, nanoplastics were removed from the tissues.

GRAPHICAL ABSTRACT



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ABSTRACT

The ingestion of nanoplastics (NPs) by fish has led to concerns regarding fish health and food chain transfer, but analytical constraints have hindered quantitative data collection on their uptake and depuration. We used palladium-doped polystyrene nanoplastics (PS-Pd NPs, ~200 nm) to track particle fate in rainbow trout (*Oncorhynchus mykiss*) during a week-long dietary exposure and subsequent 7-day depuration period on a control diet (no added PS-Pd NPs). At Day 3 and 7 of the exposure, and after depuration, the mid intestine, hind intestine, liver, gallbladder, kidney, gill and carcass were sampled. All organs and the carcass were analysed for total Pd content by inductively couple plasma mass spectrometry. After 3 days of exposure, the mid (32.5 ± 8.3 ng g^{-1}) and hind (42.3 ± 8.2 ng g^{-1}) intestine had significantly higher total Pd concentrations compared to the liver and carcass (1.3 ± 0.4 and 3.4 ± 1.1 ng g^{-1}, respectively). At Day 7, there was no time-related difference in any organ (or the carcass) total Pd concentrations compared to Day 3. When the total Pd content was expressed as a body distribution based on mass of tissue, the carcass contained the highest fraction with 72.5 ± 5.2 % at Day 7, which could raise concerns over transfer to higher trophic levels. The total number of particles that entered the fish over the 7 days was $94.5 \pm 13.5 \times 10^6$ particles, representing 0.07 ± 0.01 % of the Pd the fish had been fed. Following depuration, there was no detectable Pd in any organ or the carcass, indicating clearance from the fish. These data indicate that these NPs are taken into the internal organs and carcass of fish, yet removal of the exposure results in substantial excretion to below the limit of detection.

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1. Introduction

Plastic pollution represents a global environmental crisis. The recently signed UN Environment Assembly (UNEA-5) Global Agreement to End Plastic Pollution, a forerunner to a legally binding international agreement by 2024, shows the grave level of concern among stakeholders (UNEP, 2022). The breakdown of larger plastic items and the direct release of smaller pieces termed microplastics (MPs, <5 mm) and nanoplastics (NPs, defined as <1 μm by ISO; Allan et al., 2021) is viewed as the most problematic end of the plastic size spectrum at least from the viewpoint of biological interactions. Smaller particles that can be consumed provide an entry point for plastics and their associated chemicals into organisms and food webs that can end with human consumption. MPs have been found within the gastrointestinal tracts (GIT) of numerous fish worldwide including many commercial species (Azevedo-Santos et al., 2019), but their translocation across the intestinal wall and into the internal tissues of the liver and carcass appears to remains low (Jovanović et al., 2018). The presence of NPs in the environment is beginning to be detected (Davranche et al., 2020; Materić et al., 2022a; Materić et al., 2022b; Ter Halle et al., 2017), with the mean concentrations in lakes and streams reaching around 563 μg L^{-1} (Materić et al., 2022b). However, the concentrations of nanoplastics in many environmental compartments remain elusive owing to constraints of detection, especially those relating to the gut contents of fish. There is increasing concern that NPs are potentially present in large concentrations in the environment and furthermore may have greater hazardous potential compared to their micro-sized counterparts because of their smaller size (Mitrano et al., 2021). Toxicologically, NPs present a different proposition to MPs (Gigault et al., 2021) in that they may be internalised by endocytosis mechanisms which are active in the nano-size range. This opens the possibility for NPs to translocate into parts of the fish that are consumed (e.g., the carcass).

NPs have been compared to MPs and engineered nanomaterials (ENMs; Abdolahpur Monikh et al., 2022), but it is becoming increasingly clear this may not be appropriate (Gigault et al., 2021). For instance, at such fine scales, NP behaviour is dominated by Brownian motion (Sun et al., 2021), rather than the sedimentation or buoyancy that determines MP fate (Horton and Dixon, 2018). In addition, NPs have a high proportion of its surface molecules exposed to the environment, which is also intrinsically linked to the speed at which plastic additives can leach from the NP (Gigault et al., 2021), leading to different biological effects compared to MPs (Yin et al., 2021). Similarly, NPs are distinguished from another well studied particle contaminant group, namely ENMs, that are produced to have certain properties that are not found in the same material but at larger (bulk materials) or smaller sizes (ions). However, only a fraction of NPs are intentionally engineered, with the greatest production through incidental breakdown from larger particles (i.e., MPs), ensuring they are more heterogeneous than ENMs (Gigault et al., 2021). Indeed, the processes that cause this degradation (e.g., UV; Song et al., 2020) can alter the physical and chemical properties of the final environmental NPs, such as crystallinity, polarity and eco-corona formation, which have inherent effects on reactivity and potentially biological uptake, which is absent from ENMs (Gigault et al., 2021).

As opposed to research with ENMs where the inorganic element could be used to trace the environmental bioavailability, internalisation and tissue or intracellular distribution of the particles (Luoma et al., 2014), investigations with NPs are hampered by analytical challenges of detecting carbon-based polymers against a high carbon background within biological samples. Fluorescent labels have been employed to trace NPs, but this necessitates the use of fluorophores which may separate from the particle during exposure or *in vivo*, or tissues themselves which may emit autofluorescence, rendering results inconclusive (Catarino et al., 2019). Using conservative tracers within the chemical backbone, or in the core during the synthesis process, has become a recognised approach to quantitatively assess the uptake and fate of NPs (Al-Sid-Cheikh et al., 2020; Mitrano et al., 2019). Nanoplastic particles synthesized with chemically entrapped palladium (Pd; Mitrano et al., 2019) allows for the detection and determination of particle uptake via analysis of the tissue Pd burdens. Such particles have been employed in various exposure scenarios demonstrating accumulation in terrestrial and aquatic compartments. For instance, earthworms have been shown to cause the movement of nanoplastics into the lower layers of the soil through bioturbation (Heinze et al., 2021), as well as accumulating into the earthworms (Lahive et al., 2021). Contaminated soils also result in nanoplastics entering the epidermis of plant roots (Del Real et al., 2022). Additionally, spiked aquatic sediments transferred nanoplastics into *Gammarus pulex* (Redondo-Hasselerharm et al., 2021).

In a previous study, we used palladium-doped nanopolystyrene particles (PS-Pd NPs) in combination with an *ex vivo* gut sac technique to demonstrate the that the intestinal regions of freshwater-adapted salmon were able to translocate up to 700,000 NPs across the gastrointestinal tract in as little as 4 h (Clark et al., 2022). This represented 0.6 % of the amount of PS-Pd NPs accumulated into the gut sac, with the main site of uptake being the anterior intestine. This study was first to employ the gut sac approach with NPs and both quantify uptake and localise uptake to a gut region. The gut sac technique is a well-established method to determine the translocation of contaminants from the gut lumen into the tissue (see Clark et al., 2022 and references therein), and can also represent an initial step in ethically determining if further work with vertebrates is required for chemical safety. However, the final fate of the internalised PS-Pd NPs was not addressed in our previous work. The present study aims to fill this knowledge gap by testing the hypothesis that nanoplastics can achieve dietary uptake, systemic distribution and retention *in vivo*, data which is currently lacking in salmonid fish. By exposing rainbow trout (*Oncorhynchus mykiss*) to PS-Pd NPs via their diet we aimed to quantify the uptake and excretion of nanoplastics within selected organs and on a whole-body basis (e.g., the carcass). By sampling the mid intestine, hind intestine, liver, gallbladder, kidney, gill and carcass, a body distribution of total Pd and the total number of particles present could be calculated. Secondly, if the PS-Pd NPs were bioavailable and entered the internal organs and/or the carcass, the subsequent aim was to understand whether these particles could be mobilised and excreted.

2. Materials and methods

2.1. Nanoplastics and diet formation

Palladium-doped polystyrene nanoplastics (PS-Pd NPs) were synthesized according to Mitrano et al. (2019) and the same batch used in this study was the same previously utilised and characterised within Clark et al. (2022). Briefly, a Pd concentration of 255.8 ± 3.9 mg Pd L^{-1} (mean ± SD, $n = 3$) was measured in the NPs by inductively coupled plasma mass spectrometry (iCAP RQ ICP-MS, Thermo Fisher) following acid digestion (3:1 HNO_3:HCl) of the stock suspension. This concentration was used for subsequent dosing of the feed pellets. To confirm the particle size between experiments, the PS-Pd NPs were checked a few months since the previous report, confirming a hydrodynamic diameter (Nanoparticle Tracking Analysis, LM10, Malvern, UK) of 205 ± 15 nm in high purity water (Fig. 1), similar to the previously reported 202 ± 7 nm (Clark et al., 2022). Full methods and results of PS-Pd NP characterisation are presented in Clark et al. (2022).

2.2. Diet preparation

PS-Pd NPs were incorporated into a commercially available diet (Aller Futura, EX, Kaliningrad, Russia) with a pellet size of 1–2 mm of the same composition as previously used (Clark et al., 2019). The target feed concentration was nominally 10 mg Pd as PS-Pd NPs per kg^{-1} fed. To achieve this food pellets were supplemented with either ultrapure water (control) or an equal volume suspension of PS-Pd NPs. The suspension (or ultrapure water) was added to 270 g of the diet and thoroughly mixed with a commercial food mixer (Kenwood KM810/KM816, 2004). A solution of 3 g of porcine gelatine (>98 %, Sigma-Aldrich) in 30 mL of high-purity water was prepared by heating to 40 °C, and then poured over the diet and mixed

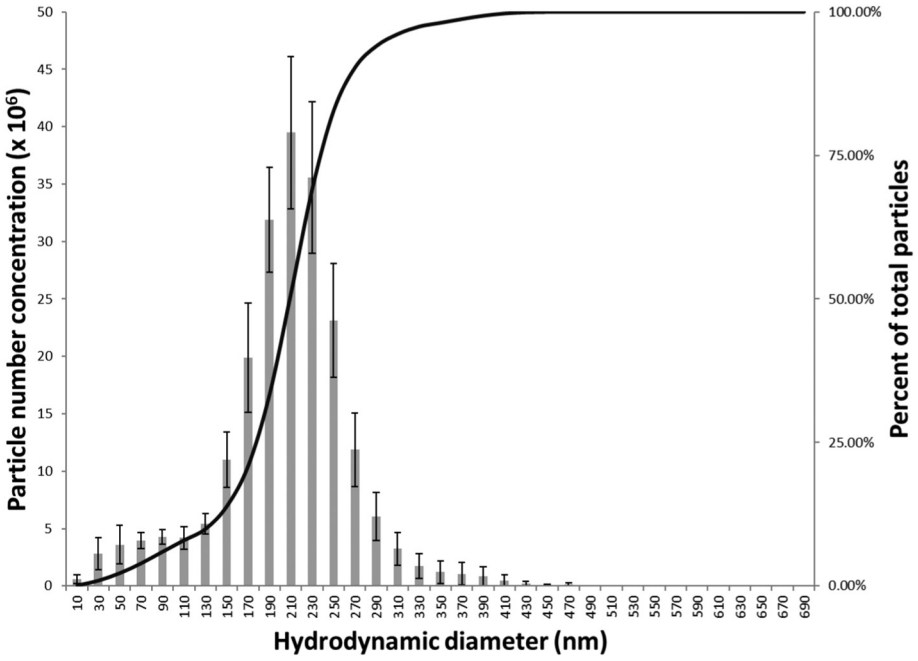

Fig. 1. The hydrodynamic diameter of PS-Pd NPs in high purity water was added to the fish diet. Data are mean ± S.D., $n = 3$. The percent of total particles is calculated from the particle number concentration.

(as above) for 5 min. The diets were then placed in an incubator at 45 °C and left to dry overnight.

2.3. Fish husbandry

Juvenile rainbow trout (weight approximately 5–10 g body mass) were obtained from Exmoor fisheries and housed within the aquatic facility at the University of Plymouth. Fish were initially held for 10 days in a quarantine tank prior to the experiment, with a 12:12 h photoperiod at 15 °C, fed a 1 % body ration. Fish ($n = 132$) were randomly assigned to 6 replicate 70 L flow through glass tanks ($n = 22$ per tank). The water flow rate remained at a constant 0.5 L min^{-1} throughout (Clark et al., 2019) and did not differ between treatments (one-way ANOVA, $P = 0.872$). The fish were acclimated for 2 days prior to the start of the experiment to allow social hierarchies to form. The entire experiment was conducted with ethical approval from the UK Home Office via a Project Licence held at the University of Plymouth (by N. Clark), under the Animals (Scientific Procedures) Act (1986), and its amendments, in compliance with Directive 2010/63/EU. Fish health checks were conducted three times per day against an in-house body conditioning score sheet that assessed the animal's general appearance, movement and swimming, the body, scale and fin, and any bone formation.

2.4. Dietary exposures to PS-Pd NPs

The 6 tanks were randomly assigned between the two treatment groups: an unexposed control (no PS-Pd NPs added to the diet), or nominally 10 mg Pd as PS-Pd NPs kg^{-1}. This exposure concentration was chosen based on a seven-day pilot study to determine the lowest exposure concentration required to ensure the gut epithelium integrity was not compromised to allow for active uptake, and not passive uptake across a damaged gut. The fish were fed these experimental diets for a total of one week at a ration of 2 % body weight day^{-1}, spread over three feeding times (9 am, 1 pm and 5 pm). The food was slowly added to the tanks to ensure it was all eaten, with no pellets left at the bottom of the tank. The photoperiod was 12 h light:12 h dark. The tanks were cleaned prior to each feed to remove any faecal material to ensure high water quality. Fish ($n = 3$) were sampled from each tank on days 3 and 7 of the exposure period. Following the exposure period, the remaining fish were depurated for a further 7 days to determine the retention of Pd. During this time both treatments were fed the control diet (i.e., no added Pd).

Throughout both the exposure and depuration period, water samples were taken daily for temperature, dissolved oxygen, pH and total ammonia. Additional samples were taken on alternative days and acidified for total Pd measurements.

2.5. Tissue collection

Dissection and tissue collection of the internal organs and carcass was conducted according to Clark et al. (2019). At each time point (days 3 and 7 of exposure, and after a 7 day depuration period), three fish were taken from each tank, giving a total of $n = 9$ per treatment. Fish were euthanized according to schedule 1 approved methods using an overdose of anaesthesia (MS222), followed by pithing, causing the cessation of brain activity. The whole fish weight was recorded and then the mid- and hind intestine, liver, gallbladder, kidney, and gills were dissected, with the carcass remaining. The mid- and hind- intestines were washed with high-purity water, and then blotted to remove any residual food. For the carcass, the remainder of the gastrointestinal tract was removed to ensure that there was no undigested food and Pd-content in this compartment. The remaining tissue (muscle, bone and sinew) is termed as the “carcass”. Each tissue was weighed (wet weight) and then stored in at −20 °C. The tissues were then freeze dried using a Lablyo freeze dryer for 24 h and then weighed again to record the dry weight. The carcass was crushed into a powder and a sub-sample was taken for subsequent analysis of total Pd content. The tissue weights are shown in Table 1.

2.6. Total palladium analysis

Tissue digestion was conducted according to the validated method by Clark et al. (2022) for the PS-Pd NPs. Each tissue was added to a glass vial, and nitric acid and hydrochloric acid were added in a ratio of 3:1. The volumes added depending on the size of the tissue, with final acid volumes ranging from 1 to 5 mL (0.75–3.75 mL nitric acid and 0.25–1.25 mL hydrochloric acid). The samples were allowed to digest at room temperature for 1 h, and then heat was slowly applied on a hot plate to simmering (~100 °C). The samples were left to digest until the reaction was complete

Table 1
The internal organs, carcass and whole fish dry weights (g).

Time (days)	Treatment	Mid intestine	Hind intestine	Liver	Gall-bladder	Kidney	Gill	Carcass	Whole fish
3	Control	0.012 ± 0.002	0.019 ± 0.006	0.073 ± 0.007	0.002 ± 0.001	0.017 ± 0.003	0.081 ± 0.007	0.041 ± 0.009	2.780 ± 0.276
3	PS-Pd NPs	0.013 ± 0.006	0.021 ± 0.010	0.090 ± 0.028	0.003 ± 0.001	0.021 ± 0.006	0.081 ± 0.018	0.046 ± 0.005	3.142 ± 0.937
7	Control	0.017 ± 0.003	0.030 ± 0.009	0.110 ± 0.018	0.003 ± 0.001	0.021 ± 0.003	0.095 ± 0.024	0.049 ± 0.002	3.186 ± 0.356
7	PS-Pd NPs	0.014 ± 0.005	0.019 ± 0.008	0.112 ± 0.038	0.005 ± 0.002	0.020 ± 0.003	0.069 ± 0.014	0.047 ± 0.009	2.968 ± 0.690
14	Control	0.018 ± 0.006	0.024 ± 0.012	0.076 ± 0.012	0.003 ± 0.001	0.016 ± 0.005	0.060 ± 0.037	0.049 ± 0.003	2.280 ± 0.767
14	PS-Pd NPs	0.017 ± 0.007	0.024 ± 0.008	0.092 ± 0.025	0.006 ± 0.002	0.020 ± 0.004	0.076 ± 0.015	0.047 ± 0.006	2.870 ± 0.456

Data are mean ± standard deviation (n = 8–9).

(i.e., production of a colourless liquid and absence of fumes). Samples were then allowed to cool to room temperature, diluted with high-purity water, and spiked to give a final concentration of 10 μg L^{-1} indium and iridium for use as internal standards to monitor instrument drift during analysis by ICP-MS. Each set of tissue samples was compared to a series of standards that were matrix matched. During the analysis, the standards were used as a check standard and analysed every 10–15 samples.

2.7. In chemico digestibility assay

The *in chemico* digestibility assay can be used to understand the behaviour of particles in synthetic stomach fluids. The concern for the PS-Pd NPs was the acidic conditions could degrade the particles and release ionic Pd, with resultant tissue concentrations coming from the ion rather than the particle, and was conducted according to Clark et al. (2019). A total of 1 g of each diet (control and PS-Pd NPs) was added into 15 cm sections of dialysis tubing and sealed at both ends. An additional treatment was included whereby the dialysis tubing alone was used as a control. Each treatment (n = 4 per treatment, total 12 tubes) was placed in a 50 mL falcon tube containing 20 mL of synthetic stomach fluid (0.9 % NaCl, 0.1 M HCl, adjusted to pH 2). Samples (0.5 mL) of the stomach fluid were taken at 0, 1, 2 and 4 h, and acidified with 0.75 mL nitric acid and 0.25 mL hydrochloric acid and diluted to 4 mL. The instrument limit of detection was 0.06 μg L^{-1}.

2.8. Calculations and statistical analysis

All data are presented as mean ± SEM. The total Pd concentrations were used to convert the Pd signal into the number of particles in a tissue as derived by single particle ICP-MS (see Clark et al., 2022). Statistical analysis and graphs were conducted in SigmaPlot 14.5. Data sets with suspected outliers were checked for normality (Shapiro-Wilk, $P > 0.05$) and, having passed this, were analysed for outliers using a Grubbs test ($P > 0.05$). Subsequently, all results were then checked for normality and equal variance (Brown-Forsythe test). Data was analysed for time and treatment related effects using a two-way ANOVA (total Pd concentration, body distribution and total number of particles). Data that was not normal, or could not be log transformed to pass the check for normality, were analysed using a Kruskal-Wallis test. Following each test, a post hoc test of Tukey's or Dunn's ($P < 0.05$) was conducted. The P values from the Post hoc tests are reported in the text.

3. Results

3.1. Quality control checks and fish health

The water quality was checked daily for pH, dissolved oxygen and temperature. In the control tanks, the pH, dissolved oxygen and temperature was 7.32 ± 0.07, 9.39 ± 0.13 mg L^{-1} and 16.81 ± 0.78 °C, respectively (mean ± S.D., n = 42). Tanks that received PS-Pd NPs had comparable values of 7.33 ± 0.07, 9.37 ± 0.13 mg L^{-1} and 16.79 ± 0.80 °C, respectively. When the water was measured for total Pd, there was no detectable total Pd, with the limit of detection at 0.07 μg L^{-1} (3 × S.D. + blank), ensuring the husbandry plan for removing ensured the Pd exposure was from the diet only.

Trace amounts of total Pd was found in the control diet (3.9 ± 0.1 μg kg^{-1}, n = 5). However, the PS-Pd NP diet contained significantly higher amounts of total Pd from PS-Pd NP addition, giving a final concentration of 7.3 ± 0.2 mg kg^{1}. The *in chemico* digestibility assay showed there was no detectable total Pd leaching from the trace amounts present in the control diet (no added PS-Pd NPs) during the 4 h. The limit of detection was 0.06 ng mL^{-1}, which equates to 0.25 ng mL^{-1} in the gut saline, and 4.82 ± 0.12 ng g^{-1} in the diet. Equally, there was no detectable total Pd from the PS-Pd NP diet, indicating the Pd remained inside the nanoplastics.

During the study, there was no mortality recorded, with only 1 fish removed for euthanasia at Day 2 due to health concerns. A visual post-mortem examination of the internal tissues revealed the decline in health of the fish was unrelated to the exposure.

3.2. Systemic uptake of NPs into the fish organs and carcass with subsequent depuration

There was no detectable Pd in the control fish at any time point during the exposure (Day 3 and 7). For fish exposed to PS-Pd NPs, Pd was readily detectable in all tissues except the gallbladder after 3 and 7 days, showing some tissue related differences (Table 2). For instance, there was ng Pd g^{-1} dw accumulation in the mid intestine (32.5 ± 8.3), hind intestine (42.3 ± 8.2), liver (1.3 ± 0.4), kidney (15.9 ± 5.2), gill (6.1 ± 1.5) and carcass (3.4 ± 1.1). Both of the intestinal regions were significantly higher in total Pd concentration compared to the liver (1.3 ± 0.4, $P < 0.001$ for both) and carcass (3.4 ± 1.1, P = 0.012 and 0.004, respectively). The overall Day 3 tissue total Pd concentration ranking was hind intestine = mid intestine > kidney > gill > carcass > liver. By Day 7, ng g^{-1} dw concentrations of total

Table 2
The concentration of total Pd (ng g^{-1} dw) in the tissues following 3 or 7 day exposure to a control diet or 10 mg Pg kg^{-1} as PS-Pd NPs, and after a week-long depuration on control diet only (Day 14).

Time (days)	Treatment	Mid intestine	Hind intestine	Liver	Gall-bladder	Kidney	Gill	Carcass
3	Control	2.1 ± 0.9*	6.4 ± 1.8*	0.6 ± 0.2*	6.8 ± 2.6*	3.0 ± 0.5*	2.0 ± 0.4*	1.3 ± 0.5*
3	PS-Pd NPs	**32.5 ± 8.3** ^{A}	**42.3 ± 8.2** ^{A}	**1.3 ± 0.4** ^{B}	6.8 ± 2.6*	**15.9 ± 5.2** ^{AB}	**6.1 ± 1.5** ^{AB}	**3.4 ± 1.1** ^{B}
7	Control	2.1 ± 0.9*	6.4 ± 1.8*	0.6 ± 0.2*	6.8 ± 2.6*	3.0 ± 0.5*	2.0 ± 0.4*	1.3 ± 0.5*
7	PS-Pd NPs	**10.6 ± 1.7** ^{AB}	**26.5 ± 4.4** ^{A}	**1.1 ± 0.1** ^{B}	6.8 ± 2.6*	**65.6 ± 25.4** ^{A}	**4.6 ± 1.2** ^{AB}	**2.2 ± 0.4** ^{B}
14	Control	2.1 ± 0.9*	6.4 ± 1.8*	0.6 ± 0.2	6.8 ± 2.6*	3.0 ± 0.5*	2.0 ± 0.4*	1.3 ± 0.5*
14	PS-Pd NPs	2.1 ± 0.9*	6.4 ± 1.8*	0.6 ± 0.2*	6.8 ± 2.6*	3.0 ± 0.5*	2.0 ± 0.4*	1.3 ± 0.5*

Different upper-case letters denote significant difference between organs or carcass (rows). There were no time related effects during the exposure (3 to 7 days), and day 14 is 7 days post exposure. Note values in bold are above the limit of detection. (*) denotes the limit of detection for Pd in the organs or carcass at each time point. Due to tissue size, each tissue has its own limit of detection. Also note, 1 ng of Pd as PS-Pd NPs equates to 10.3 ± 0.5 × 10^{6} particles (Clark et al., 2022). Data are mean ± SEM (n = 8–9).

Pd was still detectable in the mid intestine (10.6 ± 1.7), hind intestine (26.5 ± 4.4), liver (1.1 ± 0.1), kidney (65.6 ± 25.4), gill (4.6 ± 1.2) and carcass (2.2 ± 0.4, Table 2), whereby only the hind intestine was significantly elevated compared to the liver and carcass. Overall, the Day 7 tissue total Pd concentration ranking was similar to Day 3, with only the kidney changing its relative position; kidney > hind intestine > mid intestine > gill > carcass > liver. Between the time points, there were no significant differences, despite some tissues showing apparent changes, such as the kidney, which increased over 4-fold between the time points (Table 2).

Following the exposure period, the fish underwent a depuration period whereby all treatments were placed on the control diet for a further 7 days. In the control fish, similarly to the exposure period, there was no detectable total Pd in any organ or carcass. Equally, all tissues (mid intestine, hind intestine, liver, kidney, gill and the carcass) from the PS-Pd NP treatment had no detectable Pd (Table 2) by Day 14.

3.3. The highest percent body distribution was the carcass

Whilst tissue total Pd concentrations inform on the highest load per gram of dry tissue, it does not account for the relative contribution of plastic contamination in each organ or the carcass to the total weight of the fish. Thus, it is informative to standardise the data to account for this through percent body burden. There were some tissue related differences in the body distribution of total Pd (Fig. 2) following exposure to PS-Pd NPs. After 3 days of exposure, of the total Pd found in each tissue, the carcass had the highest amount of body burden (68.7 ± 6.5 %), which was typically 4.8 to 52.8-fold higher than the internal organs, reflecting its larger size (Table 1). Even so, the carcass was significantly higher than the liver ($P < 0.001$) and kidney ($P = 0.030$), which had 1.3 ± 0.4 and 3.2 ± 0.6 % of the body burden, respectively. However, there were no significant differences between any other organs, which contained 5.4 ± 1.4, 14.3 ± 5.2, 7.1 ± 3.0 % in the mid intestine, hind intestine and gill, respectively (Fig. 2). The gallbladder was the only organ to show no detectable Pd (i.e., <LOD). Therefore, the order of tissue total Pd body burden based on mean values was carcass (68.7 ± 6.5 %) > hind intestine (14.3 ± 5.2 %) > gill (7.1 ± 3.0 %) > mid intestine (5.4 ± 1.4 %) > kidney (3.2 ± 0.6 %) > liver (1.3 ± 0.4 %) > gallbladder (0 %).

Similar trends were observed at day 7, where the carcass was the tissue which had the highest percent body burden with 72.5 ± 5.2 % (Fig. 2). This was typically 4.8 to 48.3-fold higher than the internal organs, but had a significantly higher body burden compared to only the liver ($P < 0.001$), mid intestine ($P < 0.001$) and gill ($P = 0.015$). There was no significant difference between any other organs, with the hind intestine and kidney containing 6.0 ± 1.2 and 15.0 ± 5.5 % of the body burden, respectively (Fig. 2). Similar to the gallbladder of day 3 fish, there was no detectable total Pd (i.e., 0 %). Therefore, the order of ranked tissue body burden was carcass (72.5 ± 5.2 %) > kidney (15.0 ± 5.5 %) > hind intestine (6.0 ± 1.2 %) > gill (3.3 ± 0.6 %) > mid intestine (1.6 ± 0.3 %) > liver (1.5 ± 0.2 %) > gallbladder (0 %). There were no time-related differences in organ or carcass body burden (Fig. 2).

3.4. Millions of NPs accumulated in the fish organs and carcass

The total number of particles in each tissue showed some significant differences between tissues (Fig. 3). After 3 days of exposure, the highest number of particles were found in the carcass, reaching 121.9 ± 54.9 × 10^6 particles. This was significantly elevated compared to the liver ($P < 0.001$) and kidney ($P = 0.036$), which contained 1.0 ± 0.4 and 3.4 ± 1.4 × 10^6 particles, respectively. There were no other significant differences between the total numbers of particles in any other organs, which ranged from 3.4 to 10.6 × 10^6 particles (Fig. 3). A similar pattern was observed after 7 days exposure, whereby the carcass had the highest number of particles present reaching 68.2 ± 11.8 × 10^6 particles. Whilst this was approximately half the particles found in the carcass at day 3, there was no significant difference between the two time points ($P > 0.05$). Similarly, the kidney increased ~4.5-fold to contain 15.2 ± 6.6 × 10^6 particles, but this was not significantly different compared to day 3. However, the carcass was significantly elevated compared to the mid intestine ($P < 0.001$), liver ($P < 0.001$) and gill ($P = 0.014$).

Combined, the total number of particles taken into the fish at day 3 and 7 were 145.2 ± 57.0 and 94.5 ± 13.5 × 10^6 particles, respectively (Fig. 3). There was no significant difference in the number of particles in the fish during the exposure period (Kruskal-Wallis, $P = 0.564$). Through a mass balance of the amount of Pd in the tissues and the known 2 % feeding ration, it is possible to calculate the percent transfer of the diet into the tissue. After 3 and 7 days, this equated to 0.24 ± 0.10 and 0.07 ± 0.01 %, respectively, but there was no significant difference between the two sampling periods (one-way ANOVA, $P = 0.052$).

4. Discussion

This is the first study to determine the *in vivo* rapid uptake of NPs into the internal organs and carcass (along with particle numbers) of a salmonid fish from the gastrointestinal tract, and their subsequent excretion during a depuration period. The highest percentage of particles was found in the carcass, owing to its large size. Over the 7 day exposure period, the fish accumulated nearly 100 million PS-Pd NPs, with these spread throughout the intestine, liver, kidney, gills and carcass. Whilst a large number of particles

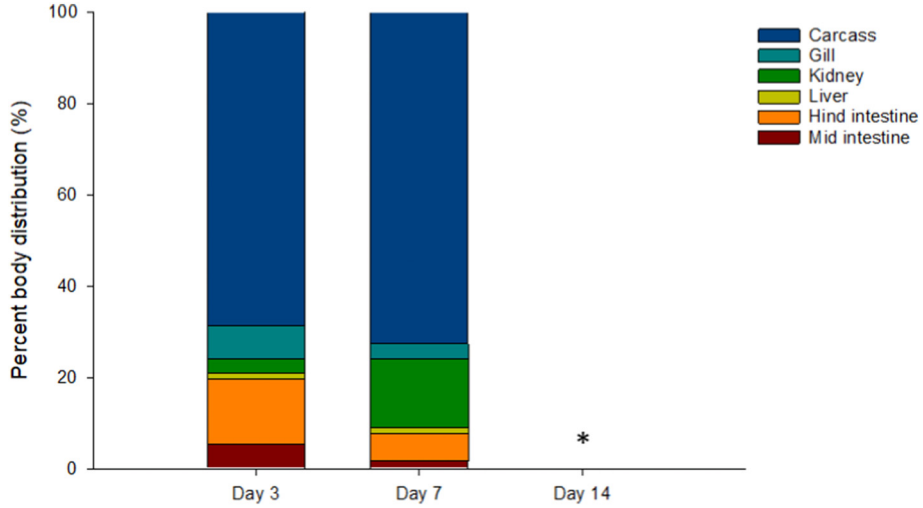

Fig. 2. The percent body distribution of total Pd following 3 or 7 day exposure to a diet containing 10 mg kg Pd NPs. After the depuration period, there was no detectable Pd. (*) denotes no detectable Pd in any organ or carcass at day 14. Data are mean ± SEM (n = 8–9).

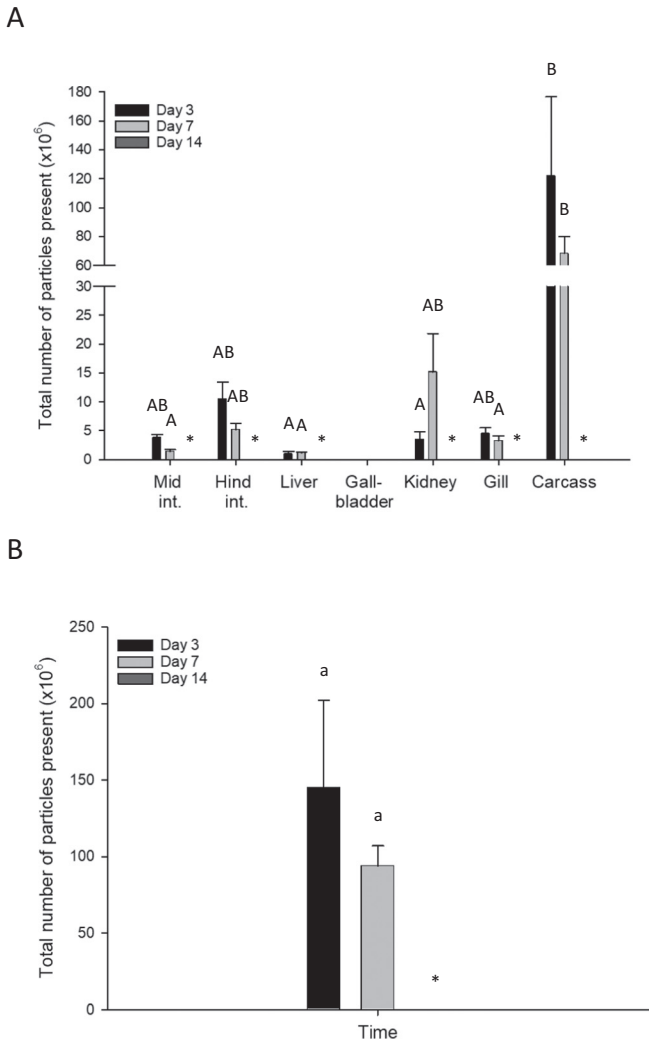

Fig. 3. The total number of particles found in (A) each organ or carcass or (B) the whole fish, following 3 or 7 day exposure to a diet containing 10 mg kg Pd NPs. Different upper case letters denote significant difference between tissues from the same time point (panel A). Different lower case letters denote significant difference between time points (panel B). Note, there was no total Pd found in the gallbladder. There were no time related effects during the exposure (3 to 7 days in panel A and B). After the depuration period, there was no detectable Pd. (*) denotes no detectable Pd after the depuration period. Data are mean ± SEM (n = 8–9).

entered the fish, they represented a modest amount of transfer from the diet which contained NP concentrations that likely exceed those in the environment, typically <1 %. When the exposure to PS-Pd NPs was ceased and fish allowed to depurate on a clean diet, there was complete clearance of the NPs from all organs and the carcass. Although the exposure concentration used here are likely higher than those found in the fishes gastrointestinal tract in the wild based upon the environmental measurements available (Materić et al., 2022b), the exposure concentration was chosen to ensure adequate detection of any Pd taken into the tissue. Based upon the established proportional relationship between uptake and exposure concentration (Kamunde and Wood, 2003), proportional levels of uptake at more environmentally realistic levels would be expected.

For dietary exposures, chemicals must enter the lumen of the gastrointestinal tract and travel to the site of uptake, typically the intestine. For carnivorous fish, such as rainbow trout, the stomach contains a low pH (Bucking and Wood, 2009) that could degrade the PS-Pd NPs to release Pd before reaching the tissues. However, the *in chemico* digestibility assay suggested this was no occurring and that the Pd in the tissues is from intact NPs. The first 3 days of the exposure resulted in measurable PS-Pd NPs in the internal organs of fish. Quantitative assessments for the uptake of NPs into fish are rare, and comparable data are not readily found in the literature. Indeed, NP uptake via the gastrointestinal tract is known to occur in a matter of hours. We previously demonstrated, using an *ex vivo* gut sac technique that 700,000 PS-Pd NPs (of the same materials used here) were able to translocate across the anterior intestine (with pyloric caeca) barrier and enter the blood space within 4 h (Clark et al., 2022). It was reasoned this fraction could distribute around the body and permeate the internal organs and carcass, resulting in systemic exposure (Clark et al., 2022). This *ex vivo* model was accurate in being able to demonstrate the bioavailability of the PS-Pd NPs *in vivo*, but may have underestimated the number of particles that could enter the body. If the total number of particles in the organs and carcass are expressed as a rate per 4 h (i.e., to match the gut sac experiment) from the first 3 days of exposure, it indicates that 7,200,000 NPs can translocate the gut epithelium every 4 h. Of course, there are several reasons for this discrepancy, notably the dosimetry between the *ex vivo* (1 mg L^{-1}) and *in vivo* (10 mg kg^{-1}) exposure concentrations. Additionally, there would be a time delay in PS-Pd NP passage into the serosal saline from the tissue of the gut sac (i.e., the precise time of appearance was not measured, but one time point after 4 h), making time-based comparisons difficult. Regardless, whilst tracking the plastic part of the nanoplastic remains difficult, by tracking the Pd as a proxy, it suggests <1 % of this enters the fish from the gastrointestinal tract.

The current data show that exposure to PS-Pd NPs via the diet resulted in systemic distribution as total Pd was found in all tissues sampled, with the exception of the gallbladder. Interestingly, the present methods revealed no time-related changes in tissue total Pd concentrations. The processes that control tissue concentration are adsorption, distribution, metabolism and excretion (ADME), and with no significant changes in tissue total Pd concentrations, it suggests there is a balance between these processes where uptake is equal to excretion. For inorganic nanoparticles, this process can take a few weeks to reach saturation (e.g., Boyle et al., 2021; Clark et al., 2019). However, these cited studies were conducted with higher exposure concentrations than those used here (100 mg kg^{-1}, Clark et al., 2019; 750 mg kg^{-1}, Boyle et al., 2021). Regardless, when the data is standardised to the percent of material taken in via the diet, it suggests the PS-Pd NPs have a lower bioavailability. For instance, silver nitrate and silver nanoparticle exposures resulted in approximately 3 % uptake from the diet, and for silver sulphide nanoparticles it was lower, at approximately 0.3 % uptake (Clark et al., 2019). For the PS-Pd NPs, this value was <0.1 % at day 7.

When a chemical exposure ceases (i.e., the depuration period), the accumulated material in the tissues can start to decrease, and this has been shown for NPs. Marine scallops exposed to 15 μg L^{-1} of ^{14}C-labelled nanoplastics of 250 nm (i.e., similar size to those used here) for 6 h and then transferred into clean waters still contained NPs within the tissues 48 days later, despite most (68 %) being removed from the tissues within the first 3 days (Al-Sid-Cheikh et al., 2018). Similarly, earthworms exposed to PS-Pd NPs at a concentration of 464 mg kg^{-1} for 21 days reached mean concentrations of approximately 100 ng g^{-1} (Lahive et al., 2021). During the depuration period of a further 21 days where earthworms were transferred to clean soil (i.e., no added PS-Pd NPs), the tissue concentrations reduced by 75 %, but contained detectable total Pd (Lahive et al., 2021). Similar observations have been made with *Gammarus pulex*. Following 28 days exposure to 3 % PS-Pd NPs in the sediment, whole body concentrations reached 1600 μg g^{-1}. Following a short, 1 day depuration period, the concentrations dropped to around 600 μg g^{-1} (Redondo-Hasselerharm et al., 2021). The present study in fish showed consistency with the previous invertebrate studies, but that within a week, total clearance from all tissues was achieved, suggesting fish may be able to better regulate the excretion of NPs, potentially due to their closed circulatory system and physiology. To compare how different organisms respond to the depuration period following NP exposure, greater harmonization of exposures and a systematic approach to data collecting for different polymer types is required.

The gills contained a small concentration of Pd in them at Day 3 and 7 (6.1 ± 1.5 and 4.6 ± 1.2 ng Pd g^{-1}, respectively). This is consistent with

the idea that the gill is an excretory organ for metabolic waste products, and following dietary exposures to chemicals, low concentrations can be found, consistent with the gill being continuously perfused with blood containing these chemicals (Clark et al., 2019). When expressed as a body burden, rather than tissue concentration, these are often low (Clark et al., 2019). The data here is consistent with this idea that the gills have a low percentage of the total body burden, and so are unlikely to be the main route of excretion. In fish, there are two predominant excretory pathways, the kidney and the liver. The kidney, however, is unlikely to be a main contributor to the excretion of the PS-Pd NPs. The pore size of the glomerulus is too small to allow a particle to pass into the urine (Handy et al., 2008). The pH of fish blood is around 7.8, and even within conditions as low as pH 2 in the *in chemico* digestibility assay, there was no release of Pd, which would have been indicative of complete particle breakdown, suggesting breakdown in the circulatory system to be unlikely. However, this does not rule out surface modification/degradation of the NP that does not contain the Pd. The alternative route is via the liver, which can transfer chemicals to the gallbladder followed by excretion into the gut lumen (and via the faeces). Dietary silver and copper nanoparticle exposures result in the highest total metal concentrations within the liver compared to other tissues (Boyle et al., 2021; Clark et al., 2019), with measureable excretion into the gallbladder (e.g., Clark et al., 2019). The PS-Pd NP exposure resulted in the inverse of this; relatively low concentrations of PS-Pd NPs in the liver, and no detectable Pd in the gallbladder. One explanation for the present data is that the liver concentrations are low because they are efficiently removed to the gallbladder and excreted into the gut lumen. Alternatively, NPs could be handled by the fish in a different manner compared to other inorganic nanoparticles, whereby they are not accumulated in the liver, and therefore result in systemic exposure to other organs and the carcass. The latter idea is based on the one particle type used here where its physico-chemical properties may differ compared to those found in the environment and would therefore need support from NP exposures of different polymer types and/or states of weathering.

Following the depuration period, the number of PS-Pd NPs reduced to below the limit of detection in the tissues, including the carcass, which had the highest total number of particles. Complete removal from the carcass has been observed following silver sulphide ENM (Ag_2S ENM) exposure. Following a 4 week dietary exposure to Ag_2S ENMs, the mean particle number concentration in the carcass was $160 \pm 50 \times 10^6$ particles g^{-1} dry weight. However, after a 2 week depuration where fish were fed the control diet (with no added Ag_2S ENMs), they were removed from the carcass to below the limit of detection (Clark et al., 2021). This coincided with consistent kidney and liver concentrations, suggested mobilisation to these organs for excretion. Similar observations have been made with copper oxide ENM (CuO ENM) exposures, with clearance from the carcass of total copper from the exposures to near-background concentration following the depuration (Boyle et al., 2021). However, in the present study of PS-Pd NPs, there was no transfer to the liver or kidney following the depuration, or it had occurred within this time frame but was eliminated before the end of the depuration phase. However, both of the Ag_2S and CuO ENM studies used a design that had an unequal depuration phase compared to the uptake, and so clearance from the kidney and liver may have occurred within a further two weeks. In addition to experimental difference, comparing the differences between nano-sized particulates again highlights that NPs are dissimilar to ENMs in terms of behaviour *in vivo*.

The focus of the present study was on the uptake and depuration of NPs where the latter occurred rapidly after the fish was removed from the NP exposure. In some instances, such studies are conducted as determining a complete mass-balance, but this is made easier when using a closed experimental system (e.g., a gut sac model (Clark et al., 2022)). With whole organism studies, mass-balance requires the collection of faecal matter as it is produced throughout the exposure and depuration periods. As fish waste quickly dissipates into the water, there is no routine sampling method for collecting waste, particularly when using a flow through system where some waste is removed from the tank, and the requirement for this material to be removed from the bottom of the tank to ensure high water quality parameters (i.e., fish welfare). As a result, faeces were not sampled as a mass-balance was not the aim of the study, and the focus, as such with the regulatory tests for fish studies (e.g., the Organisation for Economic Cooperation and Development (OECD) test guidelines (TG) 305), was to evaluate transfer of material into/out of the tissues. Regardless, within this depuration period, there is evidence of rapid excretion, which is assumed in the faeces. Similarly, assessments of toxicity were also not part of this current study, but several investigations have shown that ingested microplastics can cause tissue damage in within the digestive tracts of fish (Barboza et al., 2018; Jabeen et al., 2018; Qiao et al., 2019). Furthermore, it was recently shown that following the chronic feeding of polyethylene microplastics to Wami Tilapia (*Oreochromis urolepis*), there were significant degenerative changes in the small intestine, including villi height, epithelial cell height and leucocytic infiltration. Some of the observed changes remained even after MPs were excreted during a 60 day depuration period demonstrating that damage persists even in absence of the particulate stressor (Mbugani et al., 2022). Similar studies are yet to be conducted with NPs, but a common explanation of intestinal damage from microplastics is physical injury (Guerrera et al., 2021) whereas nanoplastic evidently cross the gut lining which may elicit further deleterious impacts. Again, this highlights that nanoplastics may be dissimilar in toxicological mode of action to larger MPs. However, if commonalities do exist then the clearance of the NPs, as we quantify here, may not be indicative of a full physiological recovery.

The largest compartment for PS-Pd NP accumulation was the carcass. It is this compartment that is of concern from a human health risk due to consumption of the fillet in commercially important species. Indeed, MPs have been found in the muscle of *Serranus scriba* found in the Mediterranean Sea, reaching concentrations of 2 to 6 pieces of plastic per gram of tissue (Zitouni et al., 2020). The present study suggests NPs could be a greater concern for plastic contamination into the food chain, as the carcass concentrations reached up to 100×10^6 particles per gram of tissue following the exposure to concentrations that likely exceed those in the environment. At least for MPs, their presence in food is widely documented including in condiments, alcohol and seafood (Cox et al., 2019). Recently, it was shown that plastic particles larger than 700 nm can enter the blood stream of humans (Leslie et al., 2022), although their pathway (e.g., from the gastrointestinal tract or through the lungs) was not be determined as it was beyond the scope of that study. Therefore, this present study highlights that produce that is fed contaminated foods could enter the human food chain.

5. Conclusions

In summary, our study provides the first quantitative *in vivo* assessment of the uptake of NPs into fish across the gastrointestinal tract. The exposure to nanoplastics resulted in measureable amounts in multiple organs within the fish, with the largest fraction found in the carcass, raising questions over human exposure via trophic transfer. Despite NPs accumulating into the internal organs and the carcass during the exposure phase, the depuration phase on clean diets (i.e., no added PS-Pd NPs) resulted in complete clearance of the particles. Further investigation should include longer term exposures to determine the effects of chronic exposures on the fishes ability to remove the NPs from tissues, as well as detailing the toxicokinetics of the uptake and depuration to determine nanoplastic behaviour and any residual indirect effects following the removal of the exposure (e.g., impairment of intestinal functioning).

CRediT authorship contribution statement

Nathaniel J. Clark: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Visualization, Project administration. **Farhan R. Khan:** Methodology, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Charlotte Crowther:** Methodology, Writing – review & editing. **Denise M. Mitrano:** Resources, Resources, Writing – review & editing. **Richard C. Thompson:** Resources, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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