Bioavailability of cobalt and iron from citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles in the terrestrial isopod *Porcellio scaber*

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Abstract

The aim of this study was to determine whether citric acid adsorbed onto cobalt ferrite (CoFe$_2$O$_4$) nanoparticles (NPs) influences the bioavailability of their constituent Co and Fe. Dissolution of Co and Fe was assessed by two measures: (i) in aqueous suspension using chemical analysis, prior to application onto the food of test organisms; and (ii) in vivo, measuring the bioavailability in the model terrestrial invertebrate (Porcellio scaber, Isopoda, Crustacea). The isopods were exposed to citric-acid-adsorbed CoFe$_2$O$_4$ NPs for 2 weeks, and tissue accumulation of Co and Fe was assessed. This was compared to pristine CoFe$_2$O$_4$ NPs, and CoCl$_2$ and Fe(III) salts as positive controls. The combined data shows that citric acid enhances free metal ion concentration from CoFe$_2$O$_4$ NPs in aqueous suspension, although in vivo, very similar amounts of assimilated Co were found in isopods exposed to both types of NPs. Therefore, evaluation of the dissolution in suspension by chemical means is not a good predictor of metal assimilation of this model organism; body assimilation of Co and Fe is rather governed by the physiological capacity of P. scaber for the uptake of these metals. Moreover, we propose that citric acid, due to its chelating properties, may hinder the uptake of Co that dissolves from citric-acid-adsorbed CoFe$_2$O$_4$ NPs, if citric acid is present in sufficient quantity.

Keywords: bioavailability, cobalt ferrite, nanoparticles, dissolution, citric acid, voltammetry
Introduction

Cobalt ferrite (CoFe$_2$O$_4$) nanoparticles (NPs) are one of the most extensively developed magnetic NPs for medical purposes (Baldi et al., 2007; Mohapatra et al., 2011) and are also promising candidates for many applications in commercial electronics, such as video and audio tapes, high-density digital recording media, and magnetic fluids (Zi et al., 2009). Their widespread use would thus indicate increasing exposure to Co in everyday life, and its presence in the environment when the consumer products are discarded.

The toxic effects of Co are well known, and its absorption, distribution, metabolism and excretion have been thoroughly reviewed by the Agency for Toxic Substances and Disease Registry (ASTDR, 2004) and the World Health Organisation (WHO, 2006). The newest findings on Co toxicity reported that Co$^{2+}$ ions are the primary toxic form of Co (reviewed in Simonsen et al., 2012). Therefore, Co dissolution is an important issue that needs to be discussed in depth when considering the applications for Co-containing NPs.

It has been shown that suspensions of pristine CoFe$_2$O$_4$ NPs have a high dissolution rate of Co under acidic conditions. Soler et al. (2007) reported that at pH ~1, after 14 days, up to 22% of the Co can be lost from CoFe$_2$O$_4$ NPs, or after 60 days, up to 30%, depending on the type of acid. A high dissolution rate was also reported for Co NPs after incubation in cell culture medium with pH ~7.4 and at 37 °C, where the dissolution was from 20% for citrate-stabilised Co NPs after 72 h, 34% for bare Co NPs after 48 h, and up to 90% for cysteine-stabilised Co NPs after 72 h (Horev-Azaria et al., 2011; Hahn et al., 2012). However, Papis et al. (2009) and Sabbioni et al. (2012) noted that the dissolution of Co$_3$O$_4$ NPs (at 37 °C) and Co NPs (temperature not reported), respectively, was negligible in deionised water, at less than 1% in both cases. Moreover, an unequal dissolution rate of the constituent metals is characteristic for mixed metal oxides; at pH ~1, the dissolution of Co from CoFe$_2$O$_4$ NPs has been reported to be ~2 orders of magnitude greater than that for Fe (Soler et al., 2007). These
data suggest that the dissolution of Co-containing NPs is a complex process and depends on many factors, such as crystal structure, temperature, pH, complexing agents, and ionic strength of the medium.

Organic ligands are widely used surface modifiers in nanoparticle preparations, to stabilise them against agglomeration, to render them compatible with another phase (i.e., metal particles can be made water-soluble when appropriate groups are attached), to promote their self-organisation, or to allow deliberate interactions of NPs with other molecules, NPs, surfaces, or solids (Neouze & Schubert, 2008). Citric acid is one such widely used substance for the coating of NPs, as it gives them a negative charge, the electrostatic forces of which prevent agglomeration of the NPs in aqueous suspension (Čampelj et al., 2008; Huynh et al., 2011; Tejamaya et al., 2012). It has also been reported that citric acid coating on NPs reduces their toxicity, in comparison to pristine NPs with the same chemical composition (El Badawy et al., 2011; Hong et al., 2011; Nguyen et al., 2013). These observations can be explained in terms of strong metal ion chelating ability of citric acid, which possesses three carboxylate groups and one hydroxyl group as potential ligands. Chelation alters the solubility of metals and significantly influences their mobilisation and bioavailability in biological media (Matzapetakis et al., 2000).

Although dissolution of Co-containing NPs has been shown in aqueous suspensions and in cell culture media (Soler et al., 2007; Horev-Azaria et al., 2011; Hahn et al., 2012), there are very few data on in vivo dissolution of CoFe₂O₄ NPs (i.e. inside the bodies of living organisms). In our previous study (Novak et al., 2013), we showed that pristine CoFe₂O₄ NPs do not enter the digestive glands of model organisms, terrestrial isopods Porcellio scaber (Isopoda, Crustacea), and that these isopods assimilate the dissolved Co, but not the Fe. This was our motivation to continue our studies on the dissolution of Co and Fe from CoFe₂O₄ NPs, both in suspension and in vivo.
The main goal of the present study was to establish whether citric acid adsorbed onto CoFe$_2$O$_4$ NPs can influence the nanoparticle dissolution, and how this is reflected in the metal assimilation of the isopods. The bioavailable share (sensu Riding et al., 2013) prior to feeding of the isopods on citric-acid-adsorbed CoFe$_2$O$_4$ NPs was defined as the amount of dissolved Co and Fe that was possible to be quantified by chemical means. We hypothesised that this amount would be elevated due the presence of citric acid (Matzapetakis et al., 2000). The actual bioavailability (Meyer, 2000) was estimated on the basis of the accumulated Co and Fe in the digestive glands of the isopods after dietary exposure. Both of these were compared to evaluate the potential additional dissolution in vivo and the impact of the citric acid on the metal bioavailability. Co(II) and Fe(III) salts at metal concentrations the same as those in the CoFe$_2$O$_4$ NPs were used as positive controls, providing information on the physiological tendencies for the assimilation of Co$^{2+}$ and Fe$^{3+}$.

1. Materials and methods

1.1 Preparation and characterisation of nanoparticle suspensions

The CoFe$_2$O$_4$ NPs were synthesised by co-precipitation from aqueous solutions of Co$^{2+}$ and Fe$^{3+}$ ions, as described by Gyergyek et al. (2012). Citric acid was adsorbed onto the surface of CoFe$_2$O$_4$ NPs following the protocol of Čampelj et al. (2008) in order to provide the NPs with a strong negative zeta ($\zeta$)-potential. The pristine (‘as synthesised’) and citric-acid-adsorbed CoFe$_2$O$_4$ NPs were characterised using transmission electron microscopy in combination with energy-dispersive X-ray spectroscopy, dynamic light scattering and $\zeta$-potential measurements. Transmission electron microscopy (TEM) images were obtained using a JEOL 2100 microscope (JEOL Ltd, Tokyo, Japan), operated at 200 kV. The specimens for TEM were prepared by drying the aqueous suspension of NPs (pH 7) at room temperature on a transparent carbon foil supported on a copper grid. Dynamic light scattering
measurements of the hydrodynamic size of the particles were performed in suspensions with concentration 0.1 mg of particles per mL using an Analysette 12 DynaSizer (Fritsch GmbH, Idar-Oberstein, Germany). The zeta potentials of the pristine and citric-acid-adsorbed CoFe$_2$O$_4$ NPs suspended in deionized water were measured with a ZetaPALS (Brookhaven Instruments Corp, Holtsville, NY, USA).

1.2 Chemical analysis of the dissolution of CoFe$_2$O$_4$ nanoparticles

Currently, the most common methods to measure dissolution are separation by dialysis or centrifugal ultracentrifugation combined with metal analysis techniques, which are mainly spectroscopic (e.g., atomic absorption spectrometry, inductively coupled plasma mass spectroscopy), and also others, such as ion-selective electrodes (reviewed in Misra et al., 2012). As effective separation of particles from the dissolved species remains a challenge, other approaches are preferential, where no separation between the particles and ions is mandatory (Misra et al., 2012). One of these approaches is voltammetry, which has proven to be successful in a number of aquatic toxicity studies, where electrode-reactive metal species have been good predictors of true bioavailability (Tubbing et al., 1994; Huang et al., 2002; Huang and Wang, 2003). Electrochemical methods also enable minimal perturbation of the sample, in contrast to filtration, as the possibility of ion adsorption to the filter is avoided. In the present study, we tested both the spectroscopic and voltammetric approaches for their accuracy, and further compared them with the true bioavailability in the test with living organisms.

The suspensions of citric-acid-adsorbed CoFe$_2$O$_4$ and pristine CoFe$_2$O$_4$ nanoparticles (NPs) in deionised water were prepared in the same way as for the in vivo experiments, to obtain the final concentration of 2000 µg/mL Co or 5000 µg/mL Co and 3800 µg/mL Fe, or 9500 µg/mL Fe (the concentrations were the same as in Novak et al., 2013). Five milliliters of
the dispersions were ultracentrifuged at 100,000× g for 30 min at 20 °C (Beckman Coulter L8-70M class H preparative ultracentrifuge; SW 65 Ti rotor; 5 mL thinwalled polyallomer tubes). Also, the solutions of CoCl$_2$·6H$_2$O and C$_6$H$_8$O$_7$·xFe$^{3+}$·yNH$_3$ in the same Co or Fe concentrations were centrifuged to determine whether any ions were lost during this step; i.e., due to binding to the walls of the ultracentrifuge tubes.

The supernatant was separated from the pellet formed by the NPs, and divided into three 1.5 mL aliquots. The first aliquot was diluted with an equal volume of 1 M HCl (*pro analysi*; Merck, Darmstadt, Germany), the second one with an equal volume of deionised water (Supplementary Data, Figure S1). The first two aliquots were then analysed by flame atomic absorption spectrometry (Perkin Elmer AAnalyst 100; Waltham, Massachusetts, USA) to determine the differences in Co and Fe ion contents between the acidified and non-acidified supernatants. To check whether particles were still present in the acidified and non-acidified aliquots, the supernatants were inspected by DLS using a 3D-DLS-SLS spectrometer (LS Instruments GmbH, Fribourg, Switzerland). The details of the DLS instrument operating parameters and data analysis are presented in the Supplementary Data.

We presumed that if the metal content quantified by flame atomic absorption spectrometry for the non-acidified aliquots was lower than that in the acidified ones, this would be an additional proof that the sedimentation was incomplete, as acidification would cause dissolution of the remaining NPs, and therefore enable complete atomisation in the flame. On the contrary, if the metal contents quantified by flame atomic absorption spectrometry for the acidified and non-acidified aliquots were comparable, then this conclusion could not be drawn. Such a result would mean either that the sedimentation was complete, and therefore ions were the only metal species present in the supernatants, or that there was relatively complete atomisation of the unsedimented NPs in the flame.
The original suspensions of pristine CoFe$_2$O$_4$ NPs and citric-acid-adsorbed CoFe$_2$O$_4$
NPs were also analysed by flame atomic absorption spectrometry for their actual Co and Fe
content. Prior to the analysis, the suspensions were diluted (1:1000 and 1:2500, respectively,
to the final concentration of 2 µg/mL Co) with 1 M HCl, and incubated in acid for 3 days for
complete dissolution.

The third aliquot of the supernatants was analysed by square-wave cathodic adsorptive
stripping voltammetry (SW-CAdSV) (Mirčeski et al., 2007), to determine the best possible
approximation to the free Co$^{2+}$ ion content (Pesavento et al., 2009). SW-CAdSV was applied
using an EG&G Princeton Applied Research Model 303A stationary mercury-drop electrode
assembly coupled to an Autolab PGSTAT 101 potentiostat, via an IME303 interface. The
experimental approach was adapted after Pihlar et al. (1981). The working electrode was a
hanging mercury-drop electrode, the auxiliary electrode was a platinum wire, and the
reference electrode was a Ag/AgCl/3 mol/L KCl electrode (i.e., silver/silver chloride
electrode, SSCE). In the electrolytic cell, 20 µL supernatant was added to a mixture of 5 mL
deionised water, 0.5 mL 2 M NH$_4$Cl/NH$_3$ buffer, and 5 µL 0.1 M dimethylglyoxime, for a
5.525 mL total volume. Before any measurements were taken, the solution was purged with
N$_2$ for 4 min, and at all times, the headspace of voltammetric cell was continuously flushed
with N$_2$ to avoid O$_2$ interference. The background Co$^{2+}$ concentration was measured before
each sample by substituting the supernatant with deionised water. SW-CAdSV was performed
first by adsorption step of 1 minute duration at −0.7 V vs. SSCE, with the magnetic stirrer
switched on, and followed by a square wave scan from −0.7 V to −1.3 V vs. SSCE with 25
mV amplitude at 50 Hz and scan rate of 50 mV/s. The method of sequential standard
additions was then used, adding 20 µL 0.2 µg/mL Co$^{2+}$ standard in each step. The Co
concentrations were calculated by linear regression using the ChemCal package (Ranke,
2013) for the R statistical software (R Core Team, 2013).
The dissolved Co\textsuperscript{2+} share was calculated as the ratio between the SW-CAdSV-
determined Co concentration in the NP supernatant and its total content in the original NP
suspensions (Tables 1, 2). The dissolved Fe concentrations were not measured by SW-
CAdSV, as no assimilation of Fe in the isopods digestive glands was detected (Figure 3) and
no further calculations could be performed.

1.3 Test organisms

Terrestrial isopods (P. scaber, Latreille 1804) were collected in July, 2012, from a compost
heap in a non-polluted location near Ljubljana, Slovenia. The isopods were kept in a terrarium
filled with a layer of moistened soil and a thick layer of partly decomposed hazelnut tree
leaves (Corylus avellana), alder (Alnus glutinosa) and birch (Betula pendula) leaves, and their
surrounding medium was maintained constantly moist. The terrarium was kept in a controlled
chamber at constant temperature (20 ±2 °C) and light (16 h light, 8 h darkness) regimes.

1.4 Experimental set-up

Two separate experiments were carried out, one with the citric-acid-adsorbed CoFe\textsubscript{2}O\textsubscript{4} NPs,
and the other with the Fe(III) salt. The latter served as the positive control, to determine
whether the Fe\textsuperscript{3+} ions influenced the food consumption of the isopods, and whether they are
assimilated into their bodies when provided in the form of salt. The citric-acid-adsorbed
CoFe\textsubscript{2}O\textsubscript{4} NPs were initially suspended in deionised water (MilliQ, Millipore, Billerica,
Massachusetts, USA [pH 5.7, \(\rho 18.5\;\text{M}\Omega\cdot\text{cm}\)]) to obtain concentrations of 2000 \(\mu\text{g}\) Co/mL
and 5000 \(\mu\text{g}\) Co/mL (Novak et al., 2013). Ammonium iron(III) citrate (\(\text{C}_6\text{H}_8\text{O}_7 \cdot x\text{Fe}^{3+} \cdot y\text{NH}_3\),
16.5%–18.5% Fe content, reagent grade) was purchased from Sigma-Aldrich (St. Louis,
Missouri, USA). The \(\text{C}_6\text{H}_8\text{O}_7 \cdot x\text{Fe}^{3+} \cdot y\text{NH}_3\) was chosen as the source of free Fe\textsuperscript{3+} ions because
other soluble iron salts are known to be highly acidic and corrosive, such as Fe(NO\textsubscript{3})\textsubscript{3} \cdot 9\text{H}_2\text{O}
FeCl₃ (Sigma Aldrich MSDS, 2011, 2013), as was especially relevant at the high concentrations used in this study. The C₆H₈O₇·xFe³⁺·yNH₃ was dissolved in deionised water at 3800 mg Fe/L and 9500 mg Fe/L, which corresponded to the Fe content in the nanoparticle suspensions. In the negative control groups, the food for the isopods (leaves) was spiked with deionised water.

The experimental setup for both experiments (with the citric-acid-adsorbed CoFe₂O₄ NPs and with the Fe(III) salt) was the same as in our previous study (Novak et al., 2013). During the experiments, the isopods were fed with hazelnut tree leaves (C. avellana) on which suspensions of test chemicals were applied. Hazelnut leaves were collected in an uncontaminated area near the Department of Biology, Ljubljana, Slovenia, and dried at room temperature. The dried leaves were cut into pieces of 100 ±10 mg. Then, 100 µL of test chemicals were applied per 100 mg of leaf, to obtain the final nominal concentrations of 2000 µg Co and 5000 µg Co per g leaf dry mass, or 3800 µg Fe and 9500 µg Fe per g leaf dry mass. The test chemicals were applied evenly onto the abaxial leaf surfaces with a paintbrush (Bruynzeel Holland, size 4). The leaves were left to dry at room temperature for 24 h.

Adult isopods of both sexes at the intermoult stages (according to Zidar et al., 1998) and of >25 mg were used. The average fresh body weight of the isopods was 46 ±14 mg (mean ±SD; n = 72). Both experiments consisted of feeding the isopods on metal-spiked food (citric-acid-adsorbed CoFe₂O₄ NPs; Fe(III) salt) for 14 days, followed by 1 day depuration to remove the metal-spiked food from the digestive system. Each isopod was placed individually into a 9 cm plastic Petri dish to which individual pieces of chemical-spiked dry leaves were added. No substrate was used. All of the Petri dishes were kept in a large glass container under controlled conditions, in terms of the air humidity (≥80%), temperature (21 ±1 °C) and light regime (16:8 h light:dark photoperiod). The food was not replaced during the exposure period, and fecal pellets were collected weekly. At the end of the experimental period, the
remnants of the leaves were collected, air dried, and weighed. Fecal pellets were also weighed after drying in a desiccator for 24 h. The isopods were decapitated and the digestive glands were isolated with tweezers. The glands were placed on separate small pieces of filter paper (approximately 4 mm × 7 mm in size) and stored in plastic tubes until analysis by flame atomic absorption spectrometry.

1.5 Flame atomic absorption spectrometry of Co and Fe content in organic material

The Co and Fe contents were measured in the isopod digestive glands (hepatopancreas) and in the remnants of leaves after the experiments. Prior to analysis, samples were digested in a heating block with a mixture of concentrated nitric (65% HNO₃, pro analysi; Merck; Darmstadt, Germany), and perchloric acid (70% HClO₄, pro analysi; Merck; Darmstadt, Germany) (HNO₃:HClO₄ = 7:1, v/v). After evaporation of the acid, the residue was dissolved in 0.2% HNO₃. The total Co and Fe concentrations in the digestive glands were analysed with a flame atomic absorption spectrometer (Perkin Elmer AAnalyst 100; Waltham, Massachusetts, USA). Within each measurement, a certified reference material (TORT-2, National Research Council of Canada) was used to check the accuracy and precision of the analytical procedures. Along with the samples, 20 replicates of a known amount of certified reference material were also acid digested, and each sample was measured in triplicate. The calculations followed the approach of Jorhem (2004) and Phillips et al. (2007). The certified concentration of Co in the reference material was 0.51 ±0.09 mg/kg; our measurement was 0.64 ±0.14 mg/kg (mean±SD, n = 60), Z’ = 2.62. For Fe, the certified concentration in the reference material was 105 ±13 mg/kg; our measurement was 101 ±14 mg/kg (mean ±SD, n = 60), Z’ = -0.63.
1.6 Data analysis

In both experiments, 12 isopods per test regimen were exposed to the citric-acid-adsorbed CoFe$_2$O$_4$ NPs or the Fe(III) salt, although the numbers of isopods in the final analyses were lower due to the mortality caused by moulting, and due to the development of marsupia in the females. All of these isopods were excluded from further data processing (total lost from the analysis, n = 9), and the numbers of the analysed animals are presented as part of the Figures.

The formulae for all of the calculations that were used in the present study (feeding parameters of isopods, metal assimilation and the share of ions that dissolved from the nanoparticles) are provided in the Supplementary Data, as Equations (S6) to (S9). The data are presented as means, and the uncertainties are expressed as ±standard deviations (±SD). Statistically significant differences between the control and the exposed groups of isopods were subjected to Mann-Whitney U-tests (*, p<0.05; **, p<0.01; ***, p<0.001) using the Statgraphics software (Statgraphics Plus for Windows 4.0, Statistical Graphics, Herndon, VA, USA).

For the purpose of the comparisons and the discussion, the present study includes also the (adapted) data from the pristine CoFe$_2$O$_4$ nanoparticles and CoCl$_2$ experimental systems of our previous study of Novak et al. (2013), with permission from ACS Publications (Novak et al., 2013. Cellular internalisation of dissolved cobalt ions from ingested CoFe$_2$O$_4$ nanoparticles: in vivo experimental evidence. Environmental Science and Technology, 47 (10), 5400–5408; Copyright, American Chemical Society, 2014). We have here reused the data and adapted the Figures for the feeding rates (Supplementary Data, Figure S3) and the Co and Fe assimilation (Figure 3), and we have included these data in the calculations of the metal bioavailability (Table 2).
2. Results

2.1 Characteristics of the pristine and citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles

Transmission electron microscopy showed a size distribution of the pristine CoFe$_2$O$_4$ nanoparticles from 5 nm to >15 nm with the presence of larger agglomerates (Figure 1a), whereas citric-acid-adsorbed CoFe$_2$O$_4$ NPs were present as individual NPs or formed smaller agglomerates, <50 nm in size (Figure 1b). The energy-dispersive X-ray spectroscopy analysis showed a composition that matched the stoichiometry of CoFe$_2$O$_4$.

Figure 1. Representative transmission electron microscopy images of the pristine CoFe$_2$O$_4$ nanoparticles (a) and the citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles (b).

As the pristine CoFe$_2$O$_4$ NPs in the aqueous suspension agglomerated strongly, the DLS measurements were not reliable. Suspension of the citric-acid-adsorbed CoFe$_2$O$_4$ NPs in deionised water formed agglomerates with around 210 nm in size, although agglomerates >1 μm were also present. The ζ-potentials of both the pristine CoFe$_2$O$_4$ NPs and the citric-acid-adsorbed CoFe$_2$O$_4$ NPs were measured across the complete pH range. The aqueous suspension of the pristine CoFe$_2$O$_4$ NPs had an isoelectric point at pH 7. The citric-acid-adsorbed CoFe$_2$O$_4$ NPs had an isoelectric point at pH ~3 and a strong negative ζ-potential at
neutral pH (between -35 mV and -40 mV), due to the citric acid ions on their surface (Figure 2).

**Figure 2.** ζ-Potential of the pristine (▼) and citric-acid-adsorbed (▲) CoFe$_2$O$_4$ nanoparticles dispersed in deionised water, as a function of the pH of the suspensions.

**2.2 Dissolution of Co and Fe in suspensions of the pristine and citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles**

The total concentrations of Co and Fe in NP suspensions and salt solutions used for the feeding experiments were generally in good agreement with the nominal values (less than 10% deviation), with the exception of the highest concentration of Co in the case of the pristine CoFe$_2$O$_4$ NPs, which was higher by 30% (Table 1).

Centrifugation did not remove any Co$^{2+}$ or Fe$^{3+}$ from the supernatants, as shown by the data for CoCl$_2$ and C$_6$H$_8$O$_7$·xFe$^{3+}$·yNH$_3$ (Supplementary Data, Table S1). The DLS measurements of the non-acidified supernatants of both the pristine and citric-acid-adsorbed CoFe$_2$O$_4$ NPs showed that NP were still present (Supplementary Data, Figure S2). For the supernatant of the pristine CoFe$_2$O$_4$ NPs, the scattering intensity at the detector was very low...
approximately 15-25 kHz at the maximum incident laser intensity), which indicates that the unsedimented particle share was very small. For the citric-acid adsorbed CoFe$_2$O$_4$ NPs, much larger share of particles remained in the supernatant as the scattering intensity was much higher (>100 kHz at the maximum incident laser intensity). In the acid-diluted samples, the scattering intensity at the detector was the same as that of the pure solvent (approximately 3-4 kHz at the maximum incident laser intensity). Moreover, in the limit of the sensitivity of the DLS technique, the measurement did not indicate any large particles ($R_h \approx 100$ nm) in solution. This demonstrates that the particles completely dissolved in the acid. For this reason, we cannot provide any graphs of the particle size distributions.

However, the atomic absorption spectrometry measurements did not yield a significant difference between the metal concentrations of each of the acidified and non-acidified supernatant aliquots (Supplementary Data, Table S1), therefore we concluded that unsedimented NPs were atomised in the flame and the atomic absorption spectrometry technique must have overestimated the free ion content. Accordingly with the DLS data, the concentrations of Co and Fe in the supernatants as measured by AAS were significantly higher for the citric-acid-adsorbed CoFe$_2$O$_4$ NPs compared to the pristine CoFe$_2$O$_4$ NPs (Table 1). The estimations of Co$^{2+}$ content in NP supernatants obtained by SW-CAdSV were lower than the ones by AAS, which confirmed that SW-CAdSV enables better quantification of the dissolved Co$^{2+}$ for both the pristine and the citric-acid-adsorbed CoFe$_2$O$_4$ NPs (Table 1).

Please insert Table 1 here

We made no attempt to determine the concentrations of Fe ion species in the supernatants by electrochemical means, because the primary focus was on Co, which showed
a tendency to accumulate in the digestive glands of the isopods when they were fed with both the pristine CoFe$_2$O$_4$ NPs (Novak et al. 2013) and the citric-acid-adsorbed CoFe$_2$O$_4$ NPs (Figure 3a). In contrast, no assimilation of Fe was detected in the digestive glands (Figure 3b). The values determined for the flame atomic absorption spectrometry of Fe in the supernatants of both the pristine and the citric-acid-adsorbed CoFe$_2$O$_4$ NPs correspond to those obtained for Co (Supplementary Data, Table S1), and are therefore likely to denote the total value of unsedimented NPs, and not ions.

2.3 Metal content in the digestive gland tissue

After the 14-day exposure of the isopods to the citric-acid-adsorbed CoFe$_2$O$_4$ NPs, the Co contents in the digestive glands of both treated groups rose significantly, in comparison to the controls (Figure 3a). There were no significant differences (p >0.05; not marked on Figure 3a) between the isopods exposed to the different concentrations of the citric-acid-adsorbed CoFe$_2$O$_4$ NPs. For clarity, we also compared our results with the ones from Novak et al. (2013). The isopods exposed to the citric-acid-adsorbed CoFe$_2$O$_4$ NPs accumulated less Co than the CoCl$_2$-exposed ones (Novak et al., 2013). At the lower exposure concentration (2000 µg Co/g dry leaf), the isopods exposed to the citric-acid-adsorbed CoFe$_2$O$_4$ NPs accumulated significantly more Co (p <0.05; not marked on Figure 3a) than the isopods exposed to the pristine CoFe$_2$O$_4$ NPs (Novak et al., 2013), while at the higher exposure concentration (5000 µg Co/g dry leaf), the situation was reversed (p <0.05; not marked on Figure 3a).

The data shown in Figure 3b were obtained according to the two separate experiments, with the first set of isopods exposed to citric-acid-adsorbed CoFe$_2$O$_4$ NPs, and the second set exposed to the Fe(III) salt. The corresponding control groups are shown as Control 1 and Control 2, respectively, and there were no statistical differences between these controls (Figure 3b). The hepatopancreatic Fe content increased significantly in comparison
to the controls only in the groups exposed to Fe(III) salt ($p < 0.01$), and hence not for the isopods exposed to the citric-acid-adsorbed CoFe$_2$O$_4$ NPs (Figure 3b). Significant differences in the Fe concentrations were seen between the two exposure concentrations of Fe$^{3+}$ ($p < 0.01$; not marked on Figure 3b).
Figure 3. Concentration of Co (Fig. 1a) or Fe (Fig. 1b) in hepatopancreas of P. scaber fed for 14 days on food dosed with CoCl$_2$ (Co$^{2+}$), Fe(III) salt (Fe$^{3+}$), pristine CoFe$_2$O$_4$ nanoparticles (CF) or citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles (CF-CA). Symbols on the box plot represent minimum and maximum data values (whiskers), mean value (□), 75$^{th}$ percentile (upper edge of the box), 25$^{th}$ percentile (lower edge of the box), median (line in the box), max and min value (┴), and outliers ( - ). Statistically significant differences between exposed and
control isopods (C1, C2; both controls were generated in this study) are indicated by *** ($p < 0.001$). Data for groups of isopods exposed to CoCl$_2$ and both nanoparticles were compared to Control 1, while isopods exposed to the Fe(III) salt were compared to Control 2. There were no significant differences in the amounts of Fe between the isopods in the control groups from the two experiments. Nominal exposure concentrations (2000 µg or 5000 µg Co/g of leaf, or 3800 µg or 9500 µg Fe/g of leaf; originated from Co or Fe salts or from suspensions of pristine CoFe$_2$O$_4$ or citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles) are provided on the x-axes. N, number of isopods that were analysed in each group (from 15 per group exposed at the beginning of the feeding). For the purposes of comparison, the unshaded box plots are reprinted (adapted) with permission from Novak et al. 2013 (Cellular internalisation of dissolved cobalt ions from ingested CoFe$_2$O$_4$ NPs: in-vivo experimental evidence. Environmental Science and Technology, 47 (10), 5400–5408). Copyright (2014) American Chemical Society.

2.4 The role of in vivo dissolution of pristine and citric-acid-adsorbed CoFe$_2$O$_4$ nanoparticles on the assimilation of Co into the hepatopancreas

As only Co showed a tendency to assimilate into the hepatopancreas when the isopods were exposed to the CoFe$_2$O$_4$ NPs (Figure 3), we did not further analyse the bioavailability of Fe. On the basis of the measured concentrations of dissolved Co$^{2+}$ for both the pristine and the citric-acid-adsorbed CoFe$_2$O$_4$ NPs in suspension and before application to the isopod food (Table 1), and considering the amount of consumed leaves during the two experiments, we calculated the hypothetical concentrations of Co in the hepatopancreas in case only the free ions were assimilated and no additional dissolution occurred in the isopod digestive system (Table 2). We compared these concentrations to those measured for Co in the hepatopancreas (Table 2). For the pristine CoFe$_2$O$_4$ NPs (data adapted from Novak et al., 2013), the actual assimilation was significantly higher than the calculated one (Table 2; $p<0.001$, for both exposure concentrations). However, with the citric-acid-adsorbed CoFe$_2$O$_4$ NPs, the difference was present only at the lower exposure concentration (Table 2; $p<0.001$ for 2000 µg Co/g dry food, and $p>0.05$ for 5000 µg Co/g dry food). The formulae (Equations (S6)-(S9)) used are provided in the Supplementary Data.
3. Discussion

The results of this study show that citric acid enhances free Co$^{2+}$ concentration from the citric-acid-adsorbed CoFe$_2$O$_4$ NPs in comparison to the pristine CoFe$_2$O$_4$ NPs in aqueous suspension. Additional dissolution of Co from both the pristine and the citric-acid-adsorbed CoFe$_2$O$_4$ NPs occurred also in the isopod digestive system, independently of the presence of citric acid. However, in the feeding experiments very similar amounts of assimilated Co were found regardless of the citrate modification of NPs. We argue that citric acid may hinder the uptake of Co by the isopods due to its chelating properties, which are favoured by the near-neutral pH of their gut. Furthermore, the assimilation of the Fe dissolved from both types of CoFe$_2$O$_4$ NPs was negligible due to the low physiological capacity of P. scaber for the uptake of Fe into their digestive glands.

If the concentration of the dissolved metal ion species is to be accurately determined, reliable separation of the dissolved fraction from remaining NPs needs to be ensured, or methods that are sensitive only to ions need to be employed. As several papers have pointed out, the combination of ultracentrifugation and spectroscopic methods often overestimates the free ion share (David et al., 2012; Misra et al., 2012; Xu et al., 2013), therefore voltammetry is a better choice for the dissolution studies (David et al., 2012; Jiang and Hsu-Kim, 2014). The free ion shares obtained by the two methods correlate closely only when the centrifugation is performed at very high speeds and long durations (Jiang and Hsu-Kim, 2014). This was also confirmed in our study, where we showed by both flame AAS and DLS assessment of the supernatants of CoFe$_2$O$_4$ NPs that centrifugation at 100000 g for 30 min did not suffice to sediment the NPs, neither pristine nor the citric-acid-adsorbed ones (Supplementary Data, Figure S2). The flame AAS therefore necessarily overestimated the
dissolved metal share (Table 1), even if the samples were not acidified (Supplementary Data, Table 1). We propose voltammetry as one of the method of choice for the accurate and direct quantification of the dissolved metal species, especially for the metals for which the commercially-available ion-selective electrodes do not exist (Pesavento et al., 2009).

In suspensions of citric-acid-adsorbed CoFe₂O₄ NPs, a fraction of the citric acid is not adsorbed and remains in solution and in equilibrium with the absorbed citric acid (Čampelj et al., 2008). Citric acid is known to mediate chelation-induced dissolution (Matzapetakis et al., 2000; Hajdú et al., 2009), which was reflected in the voltammetrically determined Co²⁺ concentration in the supernatants of CoFe₂O₄ NPs. The supernatants of the pristine CoFe₂O₄ NPs contained some µg/L of the dissolved Co²⁺, while the dissolved Co²⁺ content reached some tens of mg/L of the citric-acid-adsorbed CoFe₂O₄ NPs (Table 1). However, we cannot exclude the possibility that the citrate-Co²⁺ complexes reacted indiscriminately at the electrode, so the term “dissolved” in this context should encompass all the Co²⁺ that was not bound to nanoparticles, regardless whether it was present in the form of chelates with the citric acid or free in the solution.

The difference in the dissolved metal content in the NP suspensions used for the in vivo experiments was not reflected in the uptake of metals by isopods, where the citric acid adsorbed onto the CoFe₂O₄ NPs did not affect the assimilation of Co or Fe in the digestive glands (Figure 3, Table 2). This indicates that the evaluation of dissolution in suspension by chemical means is not a good predictor of actual metal assimilation in the digestive system of isopods P. scaber. Instead, distinct metal dissolution and assimilation processes must have taken place inside the test organisms. This finding has implications for in silico approaches to predict metal bioavailability, where the discrepancy between the chemical estimates and in vivo approaches should be taken into consideration.
As in our previous study (Novak et al., 2013), Co and Fe showed different tendencies for assimilation into the digestive glands of the model organisms in both experiments presented here (Figure 3). The presence of Co in the digestive glands was observed after the isopod feeding on the citric-acid-adsorbed CoFe$_2$O$_4$ NPs (Figure 3a), although the amount of Co was lower in comparison to the animals fed on the CoCl$_2$ at the same concentration (Figure 3a; Novak et al., 2013), regardless of the lowered feeding rate of the CoCl$_2$-exposed isopods (Supplementary Data, Figure S3). Fe was accumulated when the isopods were exposed to both concentrations of the Fe(III) salt, but not when they were fed on either the pristine (Figure 3b; Novak et al., 2013) or the citric-acid-adsorbed CoFe$_2$O$_4$ NPs containing the same concentrations of Fe (Figure 3b). The data from the Fe(III) salt shows that the amount of free Fe$^{3+}$ ions must be very high for even a small amount of Fe$^{3+}$ to be assimilated (Figure 3b). The same observation was noted already by Hopkin (1990a), who discovered that the majority of Fe from the metal-polluted leaves was retained in the gut of the P. scaber and later excreted, while some other metals, such as Cd, Cu, Pb and Zn, became assimilated into the hepatopancreas in large quantities. The difference in the assimilation tendencies for different metals is a consequence of the different pathways of their uptake and storage in the hepatopancreatic cells of P. scaber (Hopkin, 1990b). While Co follows the Type B pathway, which is the same as for Cu and other metals with the affinity for sulphur-bearing ligands (Hopkin, 1990b; Novak et al., 2013), Fe follows a specific pathway designated Type C, and binds to haemosiderin, a breakdown product of ferritin (Hopkin, 1990b).

As the physiological capacity of the isopods to assimilate Fe$^{3+}$ was confirmed to be extremely low per se, despite its bioavailability (sensu Riding et al., 2013), we concentrated exclusively on the in vivo dissolution of Co$^{2+}$. To evaluate the role of the isopods in the dissolution of the CoFe$_2$O$_4$ NPs, we compared (i) the hypothetical concentrations of Co in the
hepatopancreas (on the basis of the voltammetric determination), to (ii) the actual measured concentrations of Co in the hepatopancreas.

For the pristine CoFe₂O₄ NPs (Novak et al., 2013), the measured hepatopancreas concentrations of Co were 4-5 orders of magnitude higher than those calculated (Table 2). Therefore, a large proportion of the Co²⁺ must have dissolved in the digestive system during feeding. This was confirmed also by the absence of Co and Fe co-localisation in the cells, as shown by the low-energy X-ray fluorescence microscopy, which indicates that only Co²⁺, and not the whole NPs, entered the cells (Novak et al., 2013). However, in the case of the citric-acid-adsorbed CoFe₂O₄ NPs, the measured concentrations of Co were higher than calculated at the lower exposure of 2000 µg Co/g dry leaf mass, but the same at the higher exposure of 5000 µg Co/g dry leaf mass (Table 2). We can therefore confirm that there was additional dissolution of Co from the citric-acid-adsorbed CoFe₂O₄ NPs after consumption only for the lower exposure (2000 µg Co/g dry leaf mass). At the higher exposure (5000 µg Co/g dry leaf mass), the dissolved (voltammetrically determined) Co was the same as the bioavailable Co share (p>0.05; Table 2). However, the data from CoCl₂ shows that the isopods would be able to assimilate even more Co²⁺ from CoFe₂O₄ NPs if more was bioavailable; it is expected that the dissolution of particulate matter is slower than that of the soluble salts, so the dissolution of CoFe₂O₄ NPs in the isopod digestive system was probably limited in the given exposure period.

A closer look at the experimental results for the higher exposure concentrations also reveals that the isopods fed on the citric-acid-adsorbed CoFe₂O₄ NPs assimilated less Co compared to those fed on the pristine CoFe₂O₄ NPs (Figure 3a; p <0.05). A possible explanation for this might lie in the chelation of Co²⁺ by the citric acid. Namely, suspensions with higher concentrations of citric-acid-adsorbed CoFe₂O₄ NPs contain a larger amount of citric acid that is not bound to particles (Čampelj et al., 2008) and that is therefore free to
chelate metal ions that are released from the NPs (Matzapetakis et al., 2000). The stability constant of the Co$^{2+}$-citrate complex is 4.4 (Furia, 1972), which means that the dissociation to Co$^{2+}$ from the citric acid is not favoured in non-acidic environments (Furia, 1972), as is the case in the isopod digestive tract (Zimmer & Topp, 1997; Zimmer & Brune, 2005).

It has been shown that a large proportion of the Co dissolves from the pristine CoFe$_2$O$_4$ NPs under acidic conditions, at pH ~1 (Soler et al., 2007), which is comparable to the conditions in the vertebrate stomach. However, measurements with a pH microelectrode in the gut of *P. scaber* showed pH 5.5 to pH 6.0 in the anterior hindgut, and pH 6.0 to pH 6.5 in the posterior hindgut (Zimmer & Topp, 1997). In the hepatopancreas, the pH is 5.8 to 6.4 in the proximal region, and 5.8 to 6.1 in the distal region (Zimmer & Brune, 2005). Therefore, the dissolution of Co from the CoFe$_2$O$_4$ NPs inside the digestive tract is more likely to be of a chelating/ligand-promoted type than protonation-induced. It is known that very high concentrations of surfactant lipids are present in the gut fluid of isopods, to reduce the potential impact of ingested tannins via their food (Zimmer, 1997). These substances, along with other constituents of the isopod gut content, are likely to be the cause of enhanced dissolution of CoFe$_2$O$_4$ NPs inside the digestive tract, despite it being only a slightly acidic environment. Also, studies of metal solubilisation in polluted marine sediments with digestive juices extracted from representatives of different marine invertebrate taxa have showed that the digestive juices can significantly mobilise sediment-bound metals, even though their pH was close to 7 (Lawrence et al., 1999; Mayer et al., 2001; Weston and Maruya, 2002).

4. Conclusions

We propose that the assimilation of Co dissolved from consumed CoFe$_2$O$_4$ NPs is very complex and depends on several factors inside the isopod digestive system. Dissolution of NPs in suspension and additional dissolution *in vivo* due to the specific digestive juice
composition make the most important contributions here, while citric acid plays a dual role if it is present in sufficient amounts: it enhances the NP dissolution in suspension, but hinders the metal assimilation in vivo. However, citric acid does not entirely prevent the assimilation of Co into the digestive glands, which shows that citric-acid-adsorbed CoFe₂O₄ NPs are not chemically inert in contact with living organisms. Finally, the metal assimilation in vivo is critically controlled also by the propensity of the isopods for the uptake of the metal in question, which is evident from the difference between Co and Fe. This shows that chemical methods alone are not sufficient for the proper evaluation of the bioavailability of NPs; it is crucial that they are combined with in vivo experiments with such model organisms which enable precise quantification of the NP uptake.

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Appendix. Supplementary Data

Supplementary material associated with this article is provided in the separate document that accompanies the manuscript.
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