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Bismuth accumulation and toxicity in freshwater biota: A study on the bioindicator species *Lemna minor* and *Echinogammarus veneris*

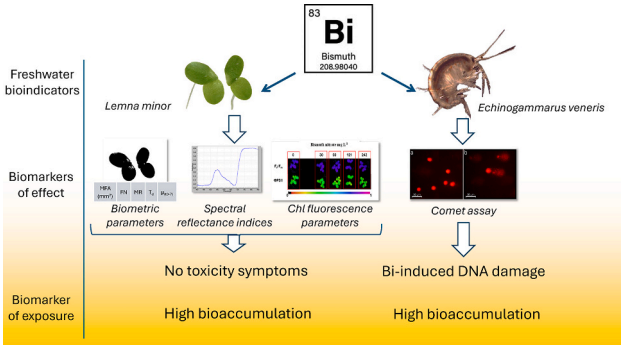
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HIGHLIGHTS

- *Lemna* plants and gammarid individuals exposed to various Bi concentrations in laboratory
- No effects of Bi on biometric and physiological parameters in *Lemna minor*
- Genotoxic effect of Bi in *Echinogammarus veneris* even at the lowest concentration tested
- Dose-dependent Bi accumulation in L. *minor* and *E. veneris* was detected
- First evidence of different sensitivity to Bi in the two freshwater bioindicator species

GRAPHICAL ABSTRACT



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ABSTRACT

The heavy metal bismuth (Bi) is attracting increasing interest for its wide range of applications, from industrial processes to medicine. Given the foreseeable increase in its use, the occurrence of Bi in the environment is expected to increase. There is a lack of information on the impact of this metal on biota, especially for the aquatic ecosystem. In this regard, an experimental study was performed under controlled conditions to assess the effects of Bi on two bioindicator species of the freshwater compartment, namely plants of *Lemna minor* L. (Lemnoideae) and individuals of *Echinogammarus veneris* (Heller, 1865) (Amphipoda, Gammaridae). A 7-day assay in *L. minor* fronds exposed to Bi nitrate in the range of 0–242 mg L^{-1} showed no effects of the metal on biometric and physiological endpoints (spectral reflectance indices and chlorophyll fluorescence parameters). In parallel, *E. veneris* individuals were treated with Bi nitrate (0–242 mg L^{-1}) for 24 h to assess genotoxicity by comet assay. The results showed significant Bi-induced DNA damage in gammarids even at the lowest Bi concentrations tested. The analysis of Bi content revealed the high capacity of both species to accumulate the metal in their tissues,

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demonstrating the ability of L. *minor* fronds to tolerate the presence of a relevant amount of Bi in solution, whereas *E. veneris* individuals showed a remarkable sensitivity to the presence of the metal. The effects of Bi observed in the two aquatic organisms represent the first evidence of a species-specific toxic action of this metal in the freshwater ecosystem.

1. Introduction

Ecosystem pollution due to heavy metals (HMs) released into the environment by anthropic activities has been far recognized as one of the main concerns for human and ecosystem health. A more sustainable management of the industrial processes has been targeted for the next decades addressing issues such as the green chemistry, the zero waste strategy, and the replacement of highly toxic chemical compounds with safer ones. In this latter case, as a non-toxic alternative to lead (Pb), the use of bismuth (Bi) has increased in many industrial applications such as ammunition formulations, hunting shot, fishing sinkers, plumbing fixtures and water pipes (Wang et al., 2019). Bi has been long known for its medical properties (Udalova et al., 2008) and widely used for treating stomach ulcers, burns, malignant tumours and, recently, also as an inhibitor for severe acute respiratory syndrome coronavirus (SARS-CoV) (Wang et al., 2019). Moreover, its use is rapidly increasing in diagnostic medicine as theragnostic agent to enhance image contrast (Badrigilan et al., 2020), as well as in cosmetics.

Given the growing interest in the utilisation of Bi in many different industrial processes and medical applications, it is reasonable to expect an increase in the occurrence of such HM in environmental compartments as a result of release to soil and wastewater. In this context, the occurrence of Bi in the alpine ice was reported by Legrand et al. (2023). Moreover, Amneklev et al. (2015, 2016) have highlighted a remarkable enhancement of the concentration of Bi in the sludge and wastewater of Swedish cities possibly linked to the increasing use of cosmetics and plastic products. This finding requires particular attention considering the interest in using sludge in the production of biofertilisers for agriculture. Moreover, dust falls originating from industrial and transport activities (Xiong et al., 2015), gunshot residue (Hallett et al., 2020) and particulate matter from fireworks (Massimi et al., 2021) have also been claimed as potential routes for Bi entrance into the environment.

The natural occurrence of Bi on the Earth is estimated as 0.025 mg Kg^{-1}, therefore it is classified as minor metal. Surveys on different environmental compartments have reported that in marine/river/lake water and sediments Bi concentrations range from 0.03 to 2.3 mg Kg^{-1} D.W. (Das et al., 2006), while in natural soil from 0.13 to 40 mg Kg^{-1} D. W. (Das et al., 2006; Fahey and Tsuji, 2006). Nevertheless, Bi concentration in soil may reach also impressive values such as those measured by Wei et al. (2011) in the proximity of an antimony mine and the related smelting area (up to 1672 mg Kg^{-1}). Similar results were reported by Li and Thornton (1993) in a study focused on historical metalliferous mining and smelting areas in England. Remarkably, a very high Bi concentration was found in forest soil close to a highway in Romania by Elekes and Busuioc (2010), with values up to 930–1891 mg Kg^{-1}.

Even though Bi is considered a "green metal", potentially being used as a substitute for more toxic HMs, the predictable increase of its presence in the different environmental compartments requires an advancing of the knowledge on the responses of biological organisms to its exposure (Zacchini, 2024). In fact, scarce information is present on the toxicity of Bi on human and animal cells (Badrigilan et al., 2020), earthworms (Omouri et al., 2018), microbes (Murata, 2006) and mushrooms (Elekes and Busuioc, 2010). Regarding plants, very few papers dealing with the phytotoxicity of Bi are reported in the literature (Zacchini, 2024). Recently, growth inhibition, alteration of the photosynthetic performances and ion accumulation and translocation, genotoxic effects, and dose-dependent Bi accumulation were reported in Bi-treated *Lepidium sativum* L. plants (Passatore et al., 2022; Pietrini et al., 2023a). Accordingly, Sudina et al. (2021) have observed that the germination of radish seeds and the root length were reduced in soil added with 30 and 300 mg Kg^{-1} Bi nitrate while a lower Bi concentration induced a stimulating effect. A similar result was reported by Nagata (2015) on *Arabidopsis* seed germination and root elongation. Higher phytotoxicity effects in association with increasing Bi nitrate concentrations were also observed by Nagata and Kimoto (2020) in *Solanum lycopersicum* L. by evaluating the seed germination and shoot and root biomass. Using two different Bi formulations, namely nitrate and citrate, Omouri et al. (2019) showed adverse effects at seed germination and root elongation level in *Lolium*, on filter paper and soil test, while a genotoxic effect of Bi oxide and Bi nanoparticles was highlighted by Liman (2013) in roots of *Allium cepa* L.

Regarding the aquatic ecosystem, even more scarce information on the toxicity of Bi on biological organisms is present in the literature (Huang et al., 2022). Specifically, to the best of our knowledge, no study on animal and plant species currently used as bioindicators of the freshwater ecosystem, such as amphipods and duckweeds, is reported. Therefore, in this study, experimental trials were performed to evaluate the effects of Bi exposure on *Lemna minor* L. fronds and *Echinogammarus veneris* (Heller, 1865) individuals (Amphipoda, Gammaridae) using biomarkers of exposure and effect. The parallel evaluation of the toxicity of this metal on the two bioindicators of the freshwater ecosystem, carried out in controlled conditions, could represent a valuable expansion of knowledge on the matter and a tool for predicting the impact on biota of the likely increase in Bi concentration in the aquatic compartment, as a possible scenario due to current anthropogenic activities.

Lemna was chosen as model plant for ecotoxicity studies regarding freshwater ecosystem (Baudo et al., 2015; Forni and Tommasi, 2015; Pietrini et al., 2015, 2016, 2019; Radić et al., 2011; Ziegler et al., 2019; Irfan et al., 2024), being officially utilized in an ecotoxicological assay (OECD/OCDE 221, 2006). To optimise the experimental approach, the Eco-Tox Photo system Tool (ETPT), recently set-up by Pietrini and Zacchini (2020), was used in order to evaluate, at the end of the 7-day assay, the growth parameters and the photosynthetic traits on the same plant material monitored along the trial in a non-destructive way.

Amphipods are frequently utilized as bioindicators in aquatic toxicity assessments due to their abundance in aquatic environments and their responsiveness to xenobiotic substances. They play a significant role in litter decomposition and nutrient cycling, moreover, representing a crucial food source for various predators such as birds, fish, or amphibians. Gammarids, and amphipods in general, bioaccumulate heavy metals, as frequently reported (Marsden and Rainbow, 2004; Redžović et al., 2023; Gestin et al., 2024) The gammarid *Echinogammarus veneris* is considered sensitive to environmental stress and in recent years it has been used for several ecotoxicological studies also to assess metal bioaccumulation (Marcoccia et al., 2017; Ronci et al., 2016) and genotoxic response to contaminants (Cosentino et al., 2022; Iannilli et al., 2019; Iannilli et al., 2023; Marcoccia et al., 2017).

Evaluating the effects of Bi exposure on L. *minor* and *E. veneris* represents a fundamental step to assess the potential toxicity of this metal on the freshwater ecosystem, considering the pivotal role of these organisms as primary producers and primary consumers, respectively, in the trophic chain.

2. Material and methods

2.1. Lemna minor - growth and experimental treatment

Lemna minor L. fronds were maintained as stock-culture in growth chamber and prepared for the experiments as in Pietrini et al. (2019). Covered 24-well sterile plates (TPP, Trasadingen, Switzerland) were filled with 0 (control), 30, 60, 121, 242 mg L^{-1} Bi nitrate ($Bi(NO_3)_3 \cdot 5H_2O$, Sigma-Aldrich, St. Louis, MO, USA), dissolved in half-strength Hoagland's nutrient solution, after sterilisation and correction for pH (5.5–6.0) under a flow cabinet. Two homogenous fronds of *Lemna* were gently placed in each well; plates were then randomly distributed on a rotary plate (50 rpm) in a growth chamber (25 °C, photoperiod 16 h light/8 h dark, irradiance of 60 μmol photons m^{-2} s^{-1}). The experiment was conducted without renewal (static) of the test solutions (OECD/OCDE 221, 2006) for 7 days, and the plates remained covered to minimise evaporation and accidental contamination.

2.2. Echinogammarus veneris – sampling, growth, and experimental treatment

The Circum-Mediterranean *Echinogammarus veneris* (Heller, 1865) is a gammarid typical of oligotrophic and oligo-mesotrophic waters, common in rivers and streams (Bazzanti et al., 2012). Specimens of *Echinogammarus veneris* were sampled with a hand net from the spring Fontana di Muro (Pontinia, Latium, Italy), transferred in laboratory in 10-L aerated glass aquarium tanks filled with water collected from the sampling sites and kept under controlled conditions in thermal cabinet: temperature 15 °C, photoperiod 12/12 light/dark. The gammarids were fed *ad libitum* with dry commercial fish food (Cosentino et al., 2022) After two weeks of laboratory acclimation period, *E. veneris* specimens were moved to glass beakers holding 150 mL of the test solution for the exposure experiments lasting 24 h. Small glass beads were also included to replicate the substrate in each beaker. Test solutions were obtained from the stock solution through serial dilutions, using dechlorinated tap water. We used six concentrations of Bi nitrate: 7.5, 15, 30, 60, 121, 242 mg L^{-1}. In selecting the concentrations, we complied with the range used for the plants, adding some intermediate concentrations to test whether the genotoxic response was more subtle.

2.3. Plant biometric and physiological analysis

Biometric and physiological endpoints were analysed out directly on covered plates by utilizing the Eco-Tox Photo system Tool (ETPT), an experimental device, set up in our laboratory (Pietrini and Zacchini, 2020), devoted to measure in real-time and in a non-destructive way the main growth and photosynthetic parameters on the same fronds of aquatic plants cultivated in multi-well plates (Pietrini et al., 2019).

At the start (t_0) and at the end of the experimental trial (7 days, 168 h, t_7), biometric parameters were analysed on *L. minor* fronds as reported in Pietrini et al. (2019).

At the end of the experimental trial (t_7), chlorophyll fluorescence parameters and their associated images were measured on plants exposed to different Bi nitrate concentrations to evaluate the performance of the photosynthetic apparatus (Maxwell1 and Johnson Maxwell and Johnson, 2000). Chlorophyll fluorescence parameters measured by the ETPT (see above) were maximal quantum efficiency of photosystem II (PSII) photochemistry (F_v/F_m) and quantum efficiency of PSII photochemistry (ΦPSII). They were measured in 30 min dark adapted fronds (F_v/F_m) and in fronds adapted to a light intensity of 60 μmol m^{-2} s^{-1} for at least 10 min to reach a steady-state condition (ΦPSII). The above-mentioned parameters were calculated as reported by Di Baccio et al. (2017).

2.4. Plant spectral reflectance measurements

At the end of the experimental trial (t_7), on the same plant material used for the biometric and chlorophyll fluorescence analyses, leaf reflectance spectra were acquired using an ASD Fieldspec-3 spectroradiometer (Analytical Spectral Devices Inc., Boulder, Colorado, USA) in the spectral range of 350 nm to 1025 nm. The reflectance spectra were measured following the methodology described in Iannilli et al. (2023). The mean of the eight spectra was then determined to provide a single spectral value. Five spectral reflectance indices, Photochemical Reflectance Index (PRI), Pigment Specific Simple Ratio (PSSR) for Chl*a* ($PSSR_a$), Chl*b* ($PSSR_b$), Carotenoids ($PSSR_c$) and Anthocyanin Reflectance Index (ARI) were derived from the collected data and calculated according to the following equations, where R is the reflectance value measured in each band expressed in nm that is indicated by the subscript number:

Photochemical Reflectance Index (PRI) = (R531 − R570) / (R531 + R570) (Gamon et al., 1997).

Pigment Specific Simple Ratio a ($PSSR_a$) = (R800) / (R680) (Blackburn, 1998).

Pigment Specific Simple Ratio b ($PSSR_b$) = (R800) / (R635) (Blackburn, 1998).

Pigment Specific Simple Ratio c ($PSSR_c$) = (R800) / (R470) (Blackburn, 1998).

Anthocyanin Reflectance Index (ARI) = (1 / R550) / (1 / R700) (Gitelson et al., 2001).

2.5. Genotoxicity test in E. veneris

To assess the genotoxic effect of Bi, the alkaline version of the Comet assay (Iannilli et al., 2023) was performed, allowing the visualization of DNA double and single-strand breaks through a gel electrophoresis-based method. Five specimens were exposed to each condition using 150 mL glass beakers. All the experiments were replicated 2 times. The comet test was conducted after 24 h exposure time on circulant hemolymph cells (haemocytes), extracted from 40 specimens in total, following the procedure described in Cosentino et al. (2022). The slides stained with ethidium bromide (20 μg/mL) were blind scored. DNA damage was determined by evaluation of 100 randomly selected nuclei each treatment, photographed at 40 × magnification by a Digital HD camera (Leica ICC50HD) and the software LAS V4.9. The images obtained were analysed by the software © 2017 TriTekCorp™ CometScore (Sumerduck, VA, USA), version 2.0 measuring the Tail Moment (TM), defined as the product of the tail length and the fraction of total DNA in the tail. This widely used parameter reflects the size of migrating DNA and the number of broken DNA fragments (Roudkenar et al., 2008).

2.6. Bismuth chemical analysis

L. minor fronds and *E. veneris* specimens exposed to different Bi nitrate concentrations (0–242 mg L^{-1}) were oven-dried at 70 °C for 48 h and then weighed. Afterwards, 0.01–0.02 g (D.W.) of each sample (two replicates per sample) was subjected to microwave-assisted acid digestion (Ethos Touch Control system with a Q20 rotor; Milestone, Bergamo, Italy) at 180 °C for 30 min, using a mixture of HNO_3/H_2O_2 (nitric acid 65 %, Carlo Erba; hydrogen peroxide Suprapur, Merck) in a 2:1 (*v*/v) ratio. Subsequently, the digested solution was diluted 1:100 with deionised water and filtered through cellulose nitrate syringe filters (25 mm diameter, 0.45 μm pore size, GVS Filter Technology). The concentration of Bi in each sample was determined by inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian Vista MPX CCD Simultaneous ICP-OES, with US nebulizer U 5000 AT+, Cetac Technologies). The calibration curve was generated by serially diluting 1000 ± 2 mg L^{-1} multi-standard stock solutions (Merck Millipore Ltd., Billerica, MA, USA). Yttrium was used as an internal standard for all measurements to control nebulizer efficiency. The standard deviations (SD)

of the replicates were all below 10 %. Further details on sample preparation and elemental analysis were reported by Passatore et al. (2022).

Bi concentration (mg Kg^{-1}) was calculated by dividing Bi content by the D.W. of each sample. As reported by Zacchini et al. (2009), the Bioconcentration factor (BCF) was calculated as the ratio between Bi concentration in the *L. minor* fronds or *E. veneris* specimens and the metal concentration in the nutrient solution or water, respectively.

2.7. Statistical analysis

The experimental trial was set up in duplicate following the OECD 221 guidelines for ecotoxicological test (7-day test, OECD/OCDE 221, 2006). Data reported in tables and figures refer to 8 replicates (each corresponding to a single well in the multi-well plates) for each treatment ($n = 8$), unless otherwise stated. Normally distributed data were processed by one-way ANOVA in order to evaluate the effects of the different Bi nitrate concentrations on L. *minor* fronds, using the SPSS (Chicago, IL, USA) software tool. The statistical significance of the mean data was assessed by the Tukey test ($P \leq 0.05$).

The results of comet assay on amphipods were presented as means ± SE and analysed using the statistical analysis program PAST (version 4.06b.). Since the data were not normally distributed (Shapiro Wilk test), we used a nonparametric test (Kruskal-Wallis) to compare each treatment with the relative control group and considered it significant for $P \leq 0.05$.

3. Results and discussion

L. minor plants exposed for 7 days to different Bi concentrations did not evidence any growth impairment (Table 1), as revealed by the calculation of biometric parameters usually utilized to evaluate the toxic effects of pollutants on duckweed plants (Iannilli et al., 2023; Pietrini et al., 2023b). This finding is not according with the few studies on terrestrial plant species (Nagata and Kimoto, 2020; Omouri et al., 2019; Passatore et al., 2022; Pietrini et al., 2023b) where toxic effects on seed germination, root elongation and shoot biomass resulting from exposure to similar Bi concentrations were reported. Therefore, besides being the first report on the effects of Bi on a plant species of the aquatic environment, this work provides the first evidence of the ability of a plant to tolerate high levels of Bi in the growth medium.

The analysis of the physiological parameters related to the leaf reflectance properties and the status of the photosynthetic apparatus has been highlighted as a valuable approach to study the toxicity of xenobiotics on duckweed plants (Iannilli et al., 2023; Oláh et al., 2021; Pietrini et al., 2019). Specifically, these measurements are characterised to be real-time, information-rich and non-destructive, evaluating non-standard endpoints such as spectral reflectance indices and chlorophyll fluorescence parameters (Pietrini et al., 2023a; Irfan et al., 2024). In fact, they are able to characterise the status of the plant primary process, such as photosynthesis, and therefore to evaluate the alteration of its physiological condition when plants are exposed to stressing agents (Alkimin et al., 2019; Dewez et al., 2018; Pietrini et al., 2016). In Table 2, the spectral reflectance indices, such as PRI, used as an index of photosynthetic performance, and specific pigment indices for chlorophyll *a* (PSSRa), chlorophyll *b* (PSSRb), carotenoids (PSSRc) and antocyanin (AR1) content are shown. As already observed for biometric parameters (Table 1), no alteration of the leaf reflectance properties was detected in *L. minor* fronds exposed to the various Bi concentrations. These data seem to confirm the ability of *L. minor* to tolerate the presence of a high content of Bi in the nutrient solution, already observed at the biometric level (Table 1), differently from what was found in a previous work using another model plant, *Lepidium sativum* L., which evidenced a decrease in all the spectral reflectance indices when exposed to similar Bi concentrations in soil (Pietrini et al., 2023b). The capability of *L. minor* plants to tolerate the presence of Bi in the growth solution was also confirmed by the analysis of the main chlorophyll fluorescence parameters (Table 3) and their associated images (Fig. 1). In fact, the maximum quantum efficiency of PSII photochemistry (F_v/F_m) and the quantum efficiency of PSII photochemistry (ΦPSII) were not altered by exposure to the metal. This feature highlights the inability of Bi to affect the photosynthetic process, in particular to damage or inactivate the PSII reaction centres, and therefore not inducing the photoinhibition of photosynthesis as observed by Pietrini et al. (2023b) in *L. sativum* plants treated with similar Bi concentrations.

The high level of tolerance to Bi exposure found in *L. minor* plants (Tables 1–3, Fig. 1) is not associated with a reduced metal accumulation in fronds, as shown in Fig. 2. In fact, a dose-dependent Bi accumulation in *L. minor* fronds to metal concentration in the nutrient solution was observed, with an extremely high value of Bi concentration (over 5000 mg Kg^{-1}) detected in plants treated with 242 mg L^{-1} of Bi nitrate. This trait confirmed previous investigations on *Lepidium sativum* plants (Passatore et al., 2022; Pietrini et al., 2023b) and on tomato shoots (Nagata and Kimoto, 2020), thus evidencing the notable ability of plants to uptake Bi. Remarkably, as already discussed about the plant tolerance, in the above reported works the increasing level of Bi accumulation in plants was coupled to a high level of damage at growth, physiological, and genomic levels, while in the present paper no toxic effect of Bi on plants was observed. Despite the notable capacity to accumulate Bi, the bioconcentration ability, measured by the bioconcentration factor (BCF = concentration in organisms/concentration in the medium) of *L. minor* plants increased up to 60 mg L^{-1} Bi nitrate (from a BCF value of 33 at 30 mg L^{-1} to a value of 58 at 60 mg L^{-1}) in the nutrient solution to slightly decrease at 121 and 242 mg L^{-1} (BCF values of 49 and 50, respectively). Thus, in accordance with results of Bi-treated *L. sativum* plants in soil (Pietrini et al., 2023b), where BCF values dropped in plants exposed to a Bi concentration higher than 30 mg Kg^{-1}, and given the lack of any toxic effect in *L. minor* fronds, this trait would suggest that a mechanism of metal uptake control is occurring, as also put in evidence for other metals and described as tolerance mechanism (Seregin and Kozhevnikova, 2006).

Table 1
Biometric parameters measured in plants of *Lemna minor* L. treated with different concentrations of Bi nitrate for 7 days: 0 (control, plants grown in Hoagland's nutrient solution without Bi nitrate); 30 (plants grown in Hoagland's nutrient solution with 30 mg L^{-1} Bi nitrate); 60 (plants grown in Hoagland's nutrient solution with 60 mg L^{-1} Bi nitrate); 121 (plants grown in Hoagland's nutrient solution with 121 mg L^{-1} Bi nitrate); 242 (plants grown in Hoagland's nutrient solution with 242 mg L^{-1} Bi nitrate). Data are the mean values of 8 replicates ± Standard Error (SE). One-way ANOVA was applied, and in columns, data followed by the same letters are not significantly different (Tukey test, $P \leq 0.05$).

Bi nitrate concentration (mg L^{-1})	Biometric parameters				
	MFA (mm^2)	FN	MR	T_d	$\mu_{(t0-7)}$
0	6.5 ± 0.2 a	13.7 ± 0.4 a	117.9 ± 1.7 a	2.00 ± 0.02 a	0.272 ± 0.004 a
30	6.6 ± 0.4 a	14.4 ± 0.5 a	121.2 ± 1.8 a	1.97 ± 0.01 a	0.279 ± 0.004 a
60	6.7 ± 0.2 a	13.2 ± 0.4 a	115.8 ± 2.4 a	2.02 ± 0.02 a	0.267 ± 0.005 a
121	6.9 ± 0.2 a	13.2 ± 0.3 a	115.7 ± 1.6 a	2.02 ± 0.01 a	0.266 ± 0.004 a
242	7.0 ± 0.2 a	13.7 ± 0.4 a	118.3 ± 1.8 a	1.99 ± 0.02 a	0.272 ± 0.004 a

MFA – mean frond area at the end of the experiment; FN –Total frond number; MR – Multiplication rate, calculated on the basis of changes in FN; T_d – Doubling time of frond number; $\mu_{(t0-7)}$ – Average specific growth rate, calculated on the basis of changes in FN.

Table 2
Spectral index values for *Lemna minor* L. fronds treated with different concentrations of Bi nitrate for 7 days: 0 (control, plants grown in Hoagland's nutrient solution without Bi nitrate); 30 (plants grown in Hoagland's nutrient solution with 30 mg L^{-1} Bi nitrate); 60 (plants grown in Hoagland's nutrient solution with 60 mg L^{-1} Bi nitrate); 121 (plants grown in Hoagland's nutrient solution with 121 mg L^{-1} Bi nitrate); 242 (plants grown in Hoagland's nutrient solution with 242 mg L^{-1} Bi nitrate). Data are the mean values of 8 replicates ± Standard Error (SE). One-way ANOVA was applied, and in columns, data followed by the same letters are not significantly different (Tukey test, $P \leq 0.05$).

Bi nitrate concentration (mg L^{-1})	Spectral index values				
	PRI	$PSSR_a$	$PSSR_b$	$PSSR_c$	AR1
0	0.040 ± 0.002 a	7.54 ± 1.21 a	3.87 ± 0.25 a	7.83 ± 1.29 a	−0.178 ± 0.020 a
30	0.043 ± 0.002 a	9.89 ± 1.16 a	4.41 ± 0.41 a	9.63 ± 1.48 a	−0.180 ± 0.018 a
60	0.039 ± 0.003 a	8.02 ± 1.05 a	3.92 ± 0.22 a	7.57 ± 0.79 a	−0.200 ± 0.050 a
121	0.039 ± 0.002 a	8.85 ± 1.15 a	3.85 ± 0.20 a	8.37 ± 0.97 a	−0.207 ± 0.012 a
242	0.040 ± 0.003 a	9.07 ± 1.37 a	3.95 ± 0.36 a	8.97 ± 1.62 a	−0.215 ± 0.027 a

PRI–Photochemical Reflectance Index; $PSSR_a$–Pigment Specific Simple Ratio for Chl*a*; $PSSR_b$–Pigment Specific Simple Ratio for Chl*b*; $PSSR_c$–Pigment Specific Simple Ratio for carotenoids; ARI–Anthocyanin Reflectance Index.

Table 3
Chlorophyll fluorescence parameters, maximal quantum efficiency (F_v/F_m) measured in dark adapted fronds and quantum efficiency of PSII photochemistry (ΦPSII) measured at steady state with light intensity of 60 μmol photons m^{-2} s^{-1} for at least 10 min to reach a steady-state condition, in fronds of *Lemna minor* L. treated with different concentrations of Bi nitrate for 7 days: 0 (control, plants grown in Hoagland's nutrient solution without Bi nitrate); 30 (plants grown in Hoagland's nutrient solution with 30 mg L^{-1} Bi nitrate); 60 (plants grown in Hoagland's nutrient solution with 60 mg L^{-1} Bi nitrate); 121 (plants grown in Hoagland's nutrient solution with 121 mg L^{-1} Bi nitrate); 242 (plants grown in Hoagland's nutrient solution with 242 mg L^{-1} Bi nitrate). Data are the mean values of 8 replicates ± Standard Error (SE). One-way ANOVA was applied, and in columns, data followed by the same letters are not significantly different (Tukey test, $P \leq 0.05$).

Bi nitrate concentration (mg L^{-1})	Chlorophyll fluorescence parameters	
	F_v/F_m	ΦPSII
0	0.814 ± 0.007 a	0.401 ± 0.014 a
30	0.807 ± 0.002 a	0.376 ± 0.020 a
60	0.811 ± 0.002 a	0.388 ± 0.014 a
121	0.812 ± 0.002 a	0.387 ± 0.011 a
242	0.807 ± 0.005 a	0.379 ± 0.015 a

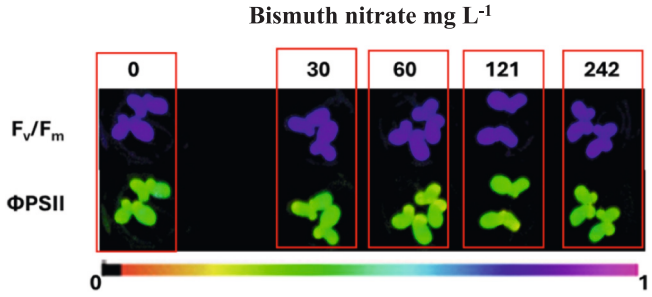


Fig. 1. Chlorophyll fluorescence images of maximum quantum efficiency of PSII photochemistry (F_v/F_m) in dark-adapted conditions and the quantum efficiency of PSII photochemistry (ΦPSII) at steady-state with actinic illumination of 60 μmol photons m^{-2} s^{-1} measured at the end of the experiment (7 days) in fronds of *Lemna minor* L. treated with different concentrations of Bi nitrate for 7 days: 0 (control, plants grown in Hoagland's nutrient solution without Bi nitrate); 30 (plants grown in Hoagland's nutrient solution with 30 mg L^{-1} Bi nitrate); 60 (plants grown in Hoagland's nutrient solution with 60 mg L^{-1} Bi nitrate); 121 (plants grown in Hoagland's nutrient solution with 121 mg L^{-1} Bi nitrate); 242 (plants grown in Hoagland's nutrient solution with 242 mg L^{-1} Bi nitrate). The false color code depicted at the bottom of the image ranges from 0.000 (black) to 1.000 (pink).

To evaluate the effects of Bi on *Echinogammarus veneris*, individuals were treated for 24 h with different Bi concentrations in water solution. At the end of the treatment, the alkaline comet assay (Cosentino et al., 2022) was performed on extracted circulant haemocytes. This cell type plays a crucial role in immune defence, phagocytosis, the transport and elimination of toxic substances, and the detoxification of xenobiotics, making it particularly vulnerable to exposure from environmental agents (Ronci et al., 2015). The comet assay, also known as the single-cell gel electrophoresis (SCGE) assay, is a widely used biomarker of effect, as it is a rapid and sensitive tool for detecting DNA damage at the single-cell level, which is indicative of genotoxic effects resulting from exposure to various agents. It can be performed on different tissues and cell types and has increasingly been used in genotoxicity testing. A representative image of the comet assay (Fig. 3) and the results expressed as Tail Moment (TM, Fig. 4) are reported. The genotoxicity assay showed significant DNA damage for *E. veneris* haemocytes at 15 mg L^{-1}, reaching the highest level of Tail Moment at 121 mg L^{-1} (TM = 34) before slightly decreasing at the highest concentration (242 mg L^{-1}). Although a direct comparison is challenging due to differences in methodology and the organisms analysed, a similar observation can be drawn from the study by Liman (2013), which investigated the effects of Bi nanoparticles on *Allium cepa* using the comet assay. At the highest concentration tested (100 mg L^{-1}), the author reported DNA damage approximately 3.5 times higher than the negative control, comparable to

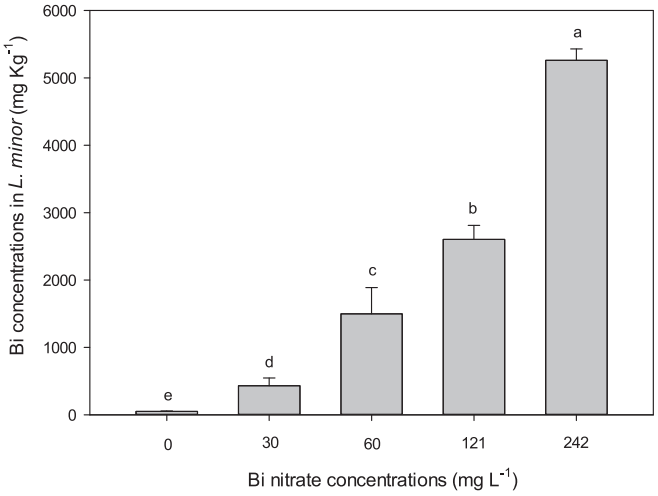


Fig. 2. Bismuth concentration measured at the end of the experiment (7 days) in fronds of *Lemna minor* L. treated with different concentrations of Bi nitrate for 7 days: 0 (control, plants grown in Hoagland's nutrient solution without Bi nitrate); 30 (plants grown in Hoagland's nutrient solution with 30 mg L^{-1} Bi nitrate); 60 (plants grown in Hoagland's nutrient solution with 60 mg L^{-1} Bi nitrate); 121 (plants grown in Hoagland's nutrient solution with 121 mg L^{-1} Bi nitrate); 242 (plants grown in Hoagland's nutrient solution with 242 mg L^{-1} Bi nitrate). In each bar, mean data (n = 3, ±S.E.) are shown. Different letters correspond to statistical different values (Tukey's test, $P \leq 0.05$).

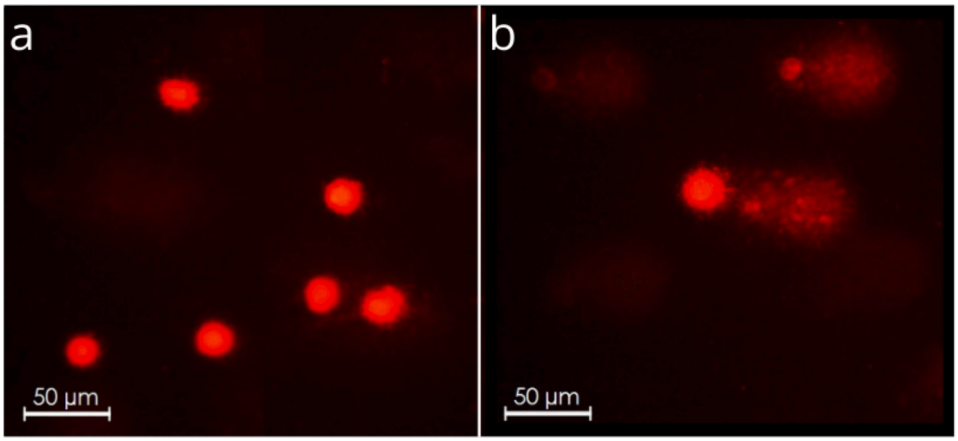


Fig. 3. Representative images of haemocytes nuclei from *E. veneris* observed after alkaline comet assay and staining with EtBr, exhibiting different DNA damage level: (a) untreated sample (dechlorinated tap water); (b) sample exposed for 24 h to 121 mg L^{-1} of Bi nitrate.

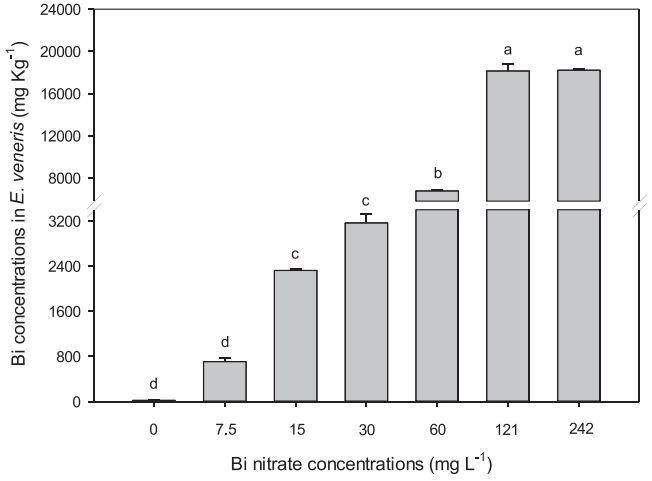


Fig. 4. DNA damage expressed as Tail Moment (TM), defined as the product of the tail length and the fraction of total DNA in the tail, in the haemocytes of *E. veneris* after 24 h of treatment with dechlorinated tap water supplied with 0 (control), 7.5, 15, 30, 60, 121, 242 mg L^{-1} Bi nitrate. Data are reported as mean ± S.E. Different letters correspond to statistical different values (Kruskal-Wallis test, $P \leq 0.05$).

the trend observed in our study.

When present in excessive amounts, metals can disrupt normal metabolic processes in amphipods, potentially leading to lethal effects. Metals are reported to have the ability to interfere with the gill function of crustaceans, causing hypoxia, enzyme inhibition, and mitochondrial dysfunction, which can have detrimental effects on their growth and reproduction (Redžović et al., 2023). Bismuth compounds, however, are generally regarded as relatively non-toxic, and this low toxicity is largely attributed to their insolubility in nearly neutral aqueous solutions, such as biological fluids. This characteristic indicates minimal potential for toxicity to aquatic organisms. In *Daphnia magna*, for instance, the 48-h exposure EC50 (mobility) is >100 mg L^{-1} of Bi Sigma-Aldrich, 2022).

However, our findings underscore the toxic effects of Bi that may manifest at lower levels of biological organization, particularly at the molecular level. This observation is consistent with the work of Ahamed et al. (2019), who demonstrated that Bi_2O_3 nanoparticles (NPs) induced changes in the mRNA expression levels of genes involved in the apoptotic pathway, including Bax, Bcl-2, and caspase-3, in human breast cancer (MCF-7) cells. Furthermore, Öztaş et al. (2019) evaluated the potential for oxidative damage caused by Bi_2O_3 nanoparticles (NPs) by measuring the levels of glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD) in human neuroblastoma SH-SY5Y cells, finding that Bi_2O_3-NPs induced dose-dependent oxidative damage. Various types of nanoparticles have been shown to stimulate the generation of reactive oxygen species (ROS), which are known to play a pivotal role in inducing DNA damage (Ahamed et al., 2019).

Our investigation into Bi body burdens revealed remarkably high uptake by *E. veneris* (Fig. 5), with Bi concentrations ranging from 21 mg Kg^{-1} in the control group to 18,050 mg Kg^{-1} until they reach a stationary state (exposure to 121 mg L^{-1}). This trend mirrors the DNA damage observed after exposure to the same concentrations. Our findings underscore the bioconcentration capacity of gammarids for Bi, as evidenced by consistently high BCF values exceeding 200, which decreased notably at the highest exposure concentration in water (242 mg L^{-1}), suggesting a potential control or excretion mechanism by *E. veneris*. Our results highlight that *E. veneris* can accumulate large amounts of Bi, far higher than that reported for other animal species in the little literature available. For instance, in earthworm tissue after 28 days of incubation, the highest Bi concentration detected was 21.26 mg Kg^{-1} following exposure to 1.864 mg Kg^{-1} of Bi available in dry soil (Omouri et al., 2018). Similarly, in a study assessing Bi concentration in invertebrates collected in the High Arctic, they ranged from 0.6 to 0.9 mg Kg^{-1} Bi (Singh et al., 2022).

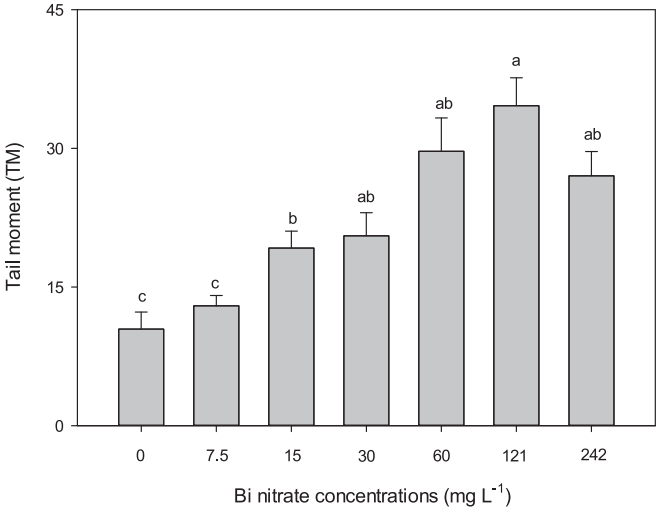


Fig. 5. Bismuth concentration in *E. veneris* (Heller, 1865) individuals after 24 h of treatment with dechlorinated tap water supplied with 0 (control), 7.5, 15, 30, 60, 121, 242 mg L^{-1} Bi nitrate. Data are reported as mean ± S.E. Different letters correspond to statistical different values (Kruskal-Wallis test, $P \leq 0.05$).

E. veneris has been previously used in other studies evaluating the bioaccumulation of metals, though not specifically for Bi. Notably, its ability to accumulate Fe, as well as other metals such as Al, Cu, Pb, Sr, Zn (Marcoccia et al., 2017), with BCF values exceeding 36,000 for Mn (Iannilli et al., 2016) was observed. However, it is essential to recognize that relying solely on conventional evaluations of overall body bioaccumulation rates, without considering the differential distribution of absorbed metals among specific organs, or the amount of metal integrated into and coating exoskeletons, may lead to inaccurate interpretations of dose-response relationships. This approach overlooks the fraction of sequestered metals biologically unavailable for metabolic activity, as emphasized by Pastorinho et al. (2009).

In addition, the greater biological effect and bioaccumulation by *E. veneris* could be attributed to its ecological characteristics as a benthic organism. Living in close interaction with sediments, where many chemicals, including heavy metals, tend to accumulate due to precipitation and adsorption processes, makes them more exposed to substances such as Bi. They can ingest Bi-containing particles during feeding and absorb the metal through their body surfaces, likely increase their exposure enhancing bioaccumulation.

4. Conclusion

The results obtained revealed a remarkable bioaccumulation of Bi in *L. minor* and an even higher uptake in *E. veneris*. Interestingly, a different behaviour was observed in the two bioindicators species of the freshwater compartment in terms of tolerance to the metal presence in the solution. While *L. minor* plants did not evidence any toxic symptoms, both at the biometric and physiological level, the individuals of *E. veneris* showed remarkable damage at genome level even at the lowest Bi concentration tested. Benthic invertebrates, such as *E. veneris*, are probably more at risk due to their ecological characteristics, diet, and biology. On the other hand, *Lemna* is known to resist metal stress through a variety of mechanisms, including enzymatic reactions, cell surface changes, and antioxidant enzyme activities, enhanced by its transgenerational plasticity.

Taken together, the findings of the present study, obtained from two organisms that play a fundamental role in the freshwater trophic chain, pose significant concerns about the potential impact of Bi on the aquatic environment and its possible consequences for human health.

CRediT authorship contribution statement

Valentina Iannilli: Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Laura Passatore:** Writing – review & editing, Visualization, Resources, Investigation, Data curation. **Serena Carloni:** Resources, Methodology, Investigation. **Lorenzo Massimi:** Writing – review & editing, Validation, Methodology, Formal analysis. **Chiara Giusto:** Investigation. **Massimo Zacchini:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Conceptualization. **Fabrizio Pietrini:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation.

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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.

Data availability

Data will be made available on request.

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