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2025-01-15
Academic press
http://hdl.handle.net/10138/592642
Oliveira de Farias, N, Siviero Guilherme Pires, M, de Jesus Moreira, B, dos Santos, A, Freeman, H S, Toukola, P P, Fernandes de Albuquerque, A, Räisänen, R & de Aragão Umbuzeiro, G 2025, 'Natural indigo toxicity for aquatic and terrestrial organisms', Ecotoxicology and Environmental Safety, vol. 290, 117606. https://doi.org/10.1016/j.ecoenv.2024.117606

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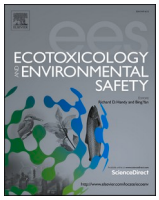
Ecotoxicology and Environmental Safety 290 (2025) 117606



Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv





Natural indigo toxicity for aquatic and terrestrial organisms

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ARTICLE INFO

Keywords:
Natural indigo
Leuco dye
Chemical characterization
Parhyale hawaiensis
Enchytraeus crypticus

ABSTRACT

Indigo is a widely used colorant available from natural and synthetic origin. It is practically insoluble in water. Indigo can reach aquatic sediments through wastewater discharges from dyeing processes, terrestrial compartments from the treatment sludges used as biosolids and dyed textiles disposed in landfills. The aim of this work was to chemically characterize a commercial natural indigo dye from *Isatis tinctoria* (woad) and, evaluate its toxicity using a sediment organism (*Parhyale hawaiensis*) in an acute test (96 h) and the soil dwelling invertebrate *Enchytraeus crypticus* in a chronic assay (21 days). These organisms are model organisms and representative of the environmental compartments where dye's destination is expected. Also, the toxicity of natural indigo was evaluated under the conditions in which it is applied to textiles. Specifically, water column invertebrate *Daphnia similis* was used to test indigo in its leuco form along with the salts used for its generation. The composition of the test sample was 91 % indigo, 4 % indirubin and 5 % of other components including flavonoids. The sample was toxic to *P. hawaienis* (LC_{50} 309 g kg^{-1}) and inhibited the reproduction of *E. crypticus* at concentrations 5.06 and 7.59 g kg^{-1} in dry soil. The leuco form of indigo was acutely toxic to *Daphnia similis* at concentrations 0.2 and 1 g L^{-1}. The data of this study can be used to guide other indigo toxicity studies and provide information that can be used in preliminary risk assessment evaluations of environmental compartments, such as aquatic sediments and indigo contaminated soils.

1. Introduction

Today, indigo is the largest volume dye produced in the world in amounts over 50 k tons per year (Market Size and Trends, 2024) and its production is estimated to grow nearly 4 % by 2031. Indigo is used for dyeing, especially jeans and other denim textiles (Market Size and Trends, 2024; Rai et al., 2021). Indigo dye can be obtained from natural and synthetic sources, and although the chemical structure of indigo from both sources is the same, commercial formulations can have different compositions and impurities (Periyasamy and Periyasami, 2023).

Natural indigo is obtained from the leaves of different plants, such as *Indigofera tinctoria*, *Polygonum tinctorium,* and *Isatis tinctoria* (woad). In the plants' leaves the dye is in its colorless precursors' form, which for *I. tinctoria* is a mixture of indican and isatan (Fig. 1) (Cardon, 2007). In aqueous media both precursors are converted into indigo (also called indigotin) and into indirubin, by an enzyme found in the plants' leaves, followed by air oxidation (Puntener and Schlesinger, 2000). Despite its relatively small molecular size (MW 262.2), indigo is practically insoluble in water and organic solvents (Periyasamy and Periyasami, 2023; Lohtander et al., 2021; Mocquard et al., 2022; Papanastasiou et al., 2012; Yuk et al., 2023), having a closely packed crystal structure as demonstrated by its melting point of 390 °C. As a water-insoluble dye at ambient temperature, its application to cotton textiles involves a vat dye process, which consists of the dye reduction to the water-soluble leuco-form using a combination of a reducing agent (sodium hydrosulfite, $Na_2S_2O_4$) and alkali (sodium carbonate, Na_2CO_3) (Periyasamy and Periyasami, 2023; Mocquard et al., 2022; Aspland, 1997). Once inside

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https://doi.org/10.1016/j.ecoenv.2024.117606
Received 18 September 2024; Received in revised form 12 December 2024; Accepted 20 December 2024
Available online 28 December 2024
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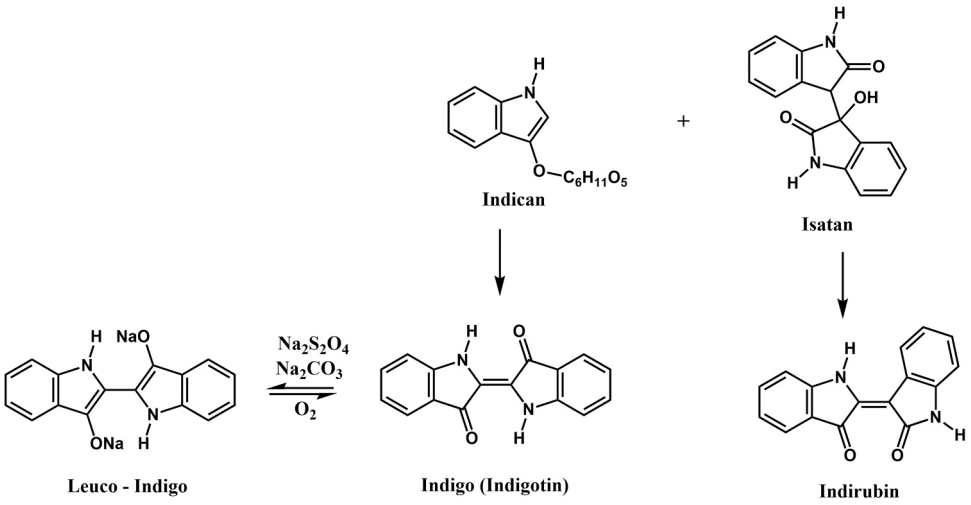


Fig. 1. Chemistry of natural indigo dye, involving enzyme mediated formation of indigo and indirubin from colorless precursors, and vat dyeing process involving conversion of indigo into leuco-indigo.

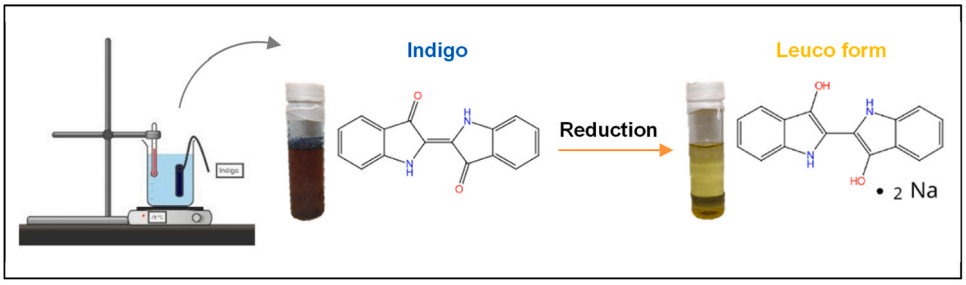


Fig. 2. Scheme of the method used for preparing leuco-indigo for toxicity testing.

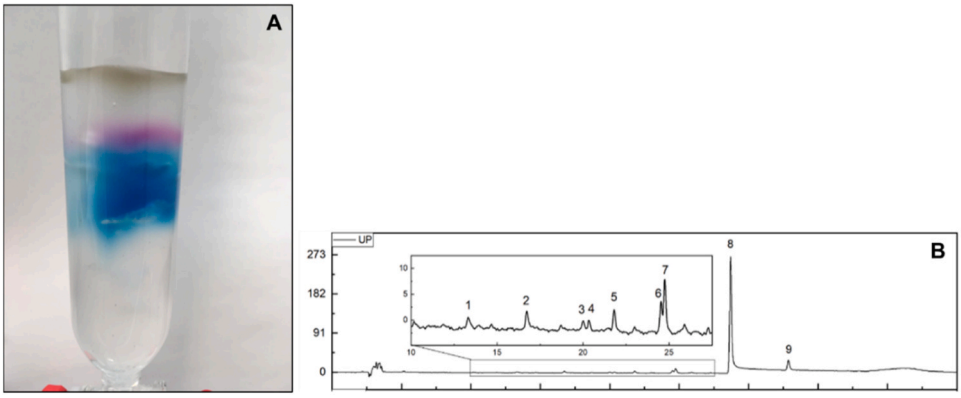


Fig. 3. (A) Chromatographic separation of components in the natural indigo dye evaluated in this study, showing a blue component (indigo) and a pink component (indirubin) as well as dark minor impurities on the top of the silica gel. (B) The HPLC-DAD-chromatogram of the sample at 280 nm with peak numbers explained in Table 1.

the textile fiber, the water-soluble form is converted back to the oxidized form using molecular oxygen from the atmosphere or a mild oxidizing agent.

The textile industry generates substantial amounts of wastewater and in general residual dyes are not easily removed during standard wastewater treatment (Akter et al., 2023; Panhwar et al., 2024). Effluents contaminated with dyes can be released into the aquatic environment and can be transferred to sludge (Carneiro et al., 2010). Indigo, as a water insoluble dye, is expected to have the sediment as its final destination in the aquatic environment. It is also expected to achieve the terrestrial environment if the sludge containing indigo is used as soil amendment or disposed in landfills. Another important possible source of indigo soil contamination is the disposal of unwanted indigo dyed textiles in landfills or open fields.

Based on the high quantities used around the world we would expect indigo to be ubiquitous in the environment, but there is no information in the literature regarding the its occurrence in the aquatic and terrestrial environment.

As a potential environmental contaminant, it is important to study indigo's toxicity. There are studies that evaluated indigo toxicity using aquatic organisms that inhabit the water column. Porkodi and coworkers assessed the toxicity of natural indigo (CAS number 482–89–3)

Table 1
The chromatographic (LC retention time, t_R), UV-Vis (λ_{max}) and mass spectroscopy (ESI-MS, MS/MS) characteristics of the compounds detected in the commercial natural indigo dye.

			Positive ionization (base peak in bold)		*Negative ionization (base peak in bold)*		
No.	**t_R (min)**	**λ_{max} (nm)**	**MS (*m/z*)**	**MS/MS (*m/z*)**	**MS (*m/z*)**	**MS/MS (*m/z*)**	**Tentative idenification**
1	13.3	270, 346	625 $[M+H]^+$, 579, **463**	463 > 445, 427, 409, **397**, 391, 379, 367, 343, 313, 301	623 $[M-H]^-$, 577, **461**	623 > 533, 443, **353**, 338, 297, 295, 268 461 > 371, **341**, 299	Chrysoeriol di-hexoside or Diosmetin di-hexoside
2	16.7	266, 294, 554	463, **279**		**439**	439 > 289, 278, **277**, 276	—[a]
3	20.0	262, 338, 448, 596	427 $[M+Na]^+$, **405** $[M+H]^+$	405 > **387**	**403** $[M-H]^-$	403 > **343**, 285	—[a]
4	20.4	262, 366	**303** $[M+H]^+$		**301** $[M-H]^-$	301 > 273, 257, 233, **179**, 151, 121, 107	Quercetin
5	21.8	266, 348	**287** $[M+H]^+$		**285** $[M-H]^-$	285 > 267, 257, 243, 241, 217, 213, 201, **199**, 176, 175, 151	Luteolin
6	24.5	268, 336	439 $[M+Na]^+$, 417 $[M+H]^+$, **271**	417 > 399, **381**, 364, 337, 313	**415** $[M-H]^-$, 269	415 > 341, 323, **311**, 283, 269 269 > 226, **225**, 201, 187, 183, 181, 151, 150, 149, 107	Apigenin deoxyhexoside
7	24.7	268, 348	323 $[M+Na]^+$, **301** $[M+H]^+$		**299** $[M-H]^-$	299 > **284**	Chrysoeriol or Diosmetin
8	28.7	246, 286, 336, 616	285 $[M+Na]^+$, **263** $[M+H]^+$	263 > 235, 234, 233, **219**	**545** $[2M-2H+Na]^-$, 261 $[M-H]^-$	545 > **261**, 217 261 > 263, 232, 218, **217**	Indigo
9	32.9	288, 364, 552	285 $[M+Na]^+$, **263** $[M+H]^+$	263 > 235, **219**, 217, 183	**261** $[M-H]^-$	261 > 218, **217**, 192, 141	Indirubin

—[a] Not established

Table 2
Data from acute toxicity testing of indigo mixed with sand to *Parhyale hawaiensis* (96 h).

Concentration (g kg^{-1})	Number of immobilized organisms	Mortality (%)
0 (Control)	0/12	0
34.5	0/12	0
61.5	3/12	25
125	3/12	25
250	5/12	42
500	8/12	67

and compared with a synthetic indigo (Porkodi et al., 2024). As synthetic, they selected the water-soluble sulfonated derivative of synthetic indigo, known as indigo carmine (CAS number 860–22–0) with distinct physicochemical properties. Both dyes had ≥ 98 % purity and were evaluated in the zebrafish (*Danio rerio*) embryo test. They obtained 96 h LC_{50} values of 350 and 300 mg L^{-1}, respectively. Fracacio et al. observed effects on the reproduction of *D. rerio* fish exposed to synthetic indigo (95 % purity) and natural indigo (unknown purity) at a single concentration (100 mg L^{-1}) (Fracacio et al., 2023). Both dyes reduced the reproductive rates of *D. rerio*, affecting the numbers of eggs laid, the proportions of viable eggs, and survival of hatching embryos. In a related study, Ferreira and Fracacio observed degeneration of the *D. rerio* fish's nervous tissue exposed to natural and synthetic indigo at the same single concentration (Ferreira and Fracacio, 2023).

OECD SIDS (1994) reported the hazard assessment of indigo exposed to aquatic organisms at three different trophic levels, the algae *Selenastrum capricornutum* (72 h EC_{50} = 6.5 mg L^{-1} (acute); 72 h NOEC = 3.1 mg L^{-1} (chronic), the microcrustacean *Daphnia magna* (24 h EC_{50} = 250 mg L^{-1} (acute); 21 d-NOEC = 0.78 mg L^{-1} (chronic) (OECD SIDS, 1994). For the fish *Oryzias latipes* results showed 96 h LC_{50} > 1000 mg L^{-1} (acute). No information on the type of indigo tested (natural or synthetic), sample purity, or product origin was presented. Also, no information on how the samples were prepared/diluted to be tested at the stated concentrations was presented.

No toxicity studies were found for sediment or terrestrial organisms, despite the fact that those are the expected compartments of indigo's final destination. Therefore, the aim of this work was to evaluate the toxicity of a chemically characterized commercial natural indigo dye obtained from *Isatis tinctoria* (woad) using a sediment organism (*Parhyale hawaiensis*) in an acute test (96 h) and the soil dwelling invertebrate (*Enchytraeus crypticus)* in a chronic assay (21 d). We also simulated the formation of the leuco-form of indigo during commercial dyeing and evaluated the obtained solution for toxicity with a water column organism, *Daphnia similis*, in a 48 h experiment.

2. Materials and methods

2.1. Indigo sample and reagents

The indigo sample was obtained from *Isatis tinctoria* (woad) grown in Nivala, Finland (63.8524° N, 24.9828° E) and acquired from Natural Indigo Finland Oy. It was a commercial powder formulation aimed for dyeing applications. For the chemical analysis, analytical reagent grade dimethyl sulfoxide (DMSO) (Fisher Scientific, UK) was used as solvent. Formic acid (HCO_2H) (≥ 99 %) (VWR international, USA), HPLC grade methanol (MeOH) (Honeywell Riedel-de Häen, USA) and ultrapure type I water were used as eluents. For the ecotoxicological test reagents ACS grade solvents were employed.

2.2. Chemical analysis

The chemical analysis of the indigo sample was conducted with high performance liquid chromatography (HPLC) coupled to a diode array detector (DAD) and electrospray ionization tandem mass spectrometer (ESI-MS/MS). Indigo dye solutions were prepared by agitating 5 mg sample in DMSO (4.5 mL) at 80 °C for 10 min in polypropylene centrifuge tubes in a water bath. These conditions were chosen as optimal for indigo extractions (Mantzouris et al., 2014). The samples were vortexed before and after dissolution and filtered with 0.45 μm PTFE syringe filters prior to analysis. An HP Agilent 1100 HPLC system (Agilent Technologies, USA) equipped with a degasser, a binary pump, an autosampler and a DAD detector was used. The instrument was coupled to a Bruker Esquire 3000 plus (Bruker, USA) quadrupole ion trap mass spectrometer equipped with an ESI ion source for the MS/MS analysis. Analyte separation was achieved with a Gemini C18 column (15 mm × 4.6 mm, 3 μm) (Phenomenex, Torrance, USA) fitted with a Security Guard C18 precolumn. HPLC grade MeOH (solvent A) and 1 % HCO_2H

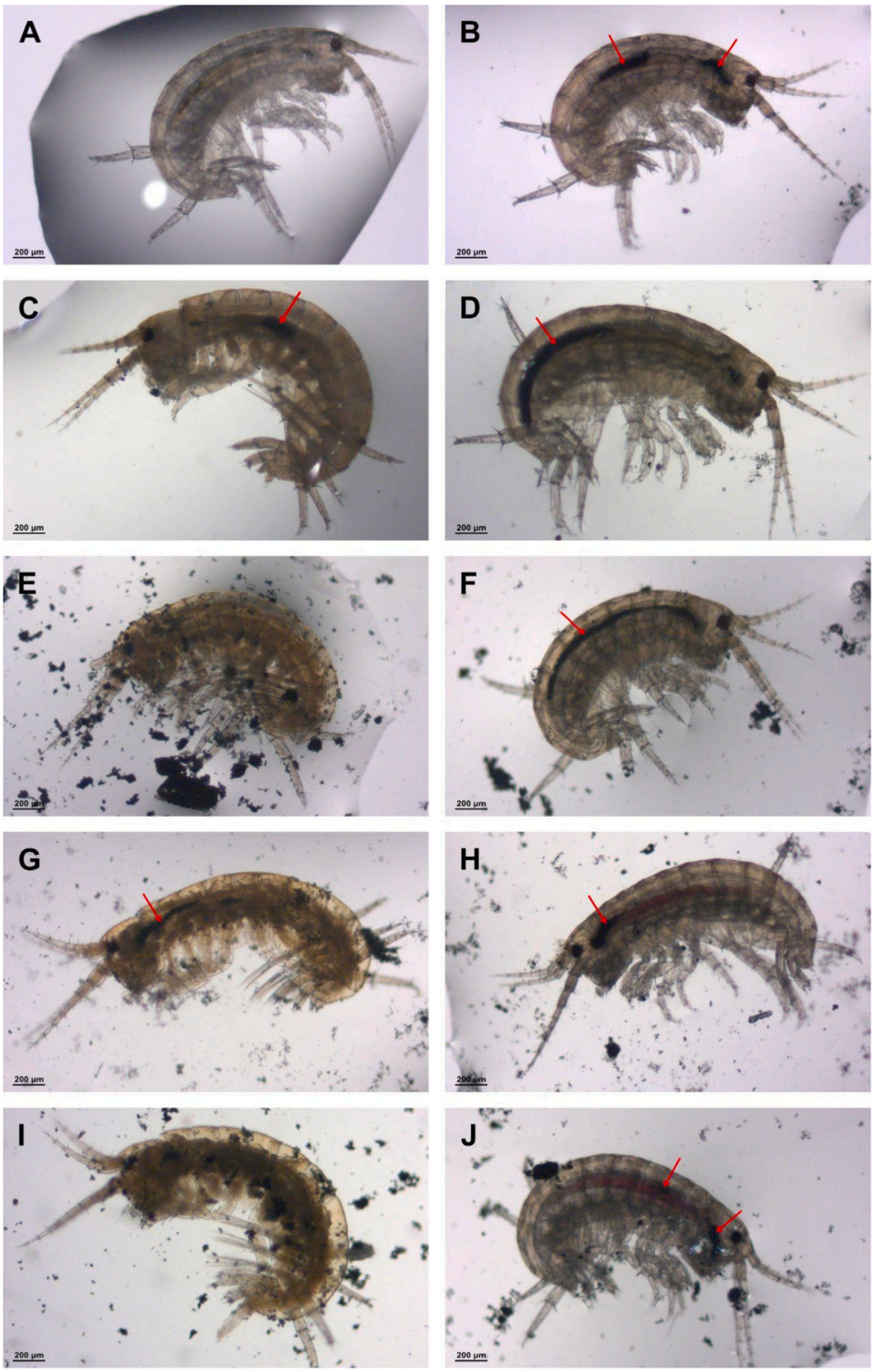


Fig. 4. Toxicity test with *Parhyale hawaiensis* and indigo mixed with sand after 96 h. (A): Control group, (B): A surviving neonate at the end of exposure from indigo concentration 34.5 g kg^{-1}; and (C-J): Neonates at the end of exposure to indigo concentrations 61.5, 125, 250, and 500 g kg^{-1}, including a dead (E, G, I) and a surviving organism (F, H, J) from each concentration. The red arrows indicate the presence of indigo particles inside the gut of the organisms.

in ultrapure water (v/v) (solvent B) were used as eluents. The following gradient was applied: 0–2 min 70 % B, 4 min 60 % B, 24 min 30 % B, 35 min 20 % B, 38 min 0 % B. The flow rate was 0.7 mL min^{-1} and the injection volume was 20 µL. Wavelengths of 280, 300, 360, 540 and 606 nm were monitored.

For MS/MS data acquisition, 0.5 mL of MeOH was added to the dye extracts to reduce the viscosity prior to HPLC-DAD-ESI-MS/MS analysis. For ESI-MS/MS analysis both positive and negative ion modes were employed. The following ESI conditions were used: drying gas flow 12 L min^{-1}, nebulizer pressure 50 psi, capillary voltage 4.2 kV and capillary temperature 300 °C and 340 °C for positive and negative ionization respectively. Nitrogen was used as nebulizer and as drying gas. Full scan mass spectra were measured over the range *m/z* 100–900.

Manual background subtraction was conducted on the MS spectra

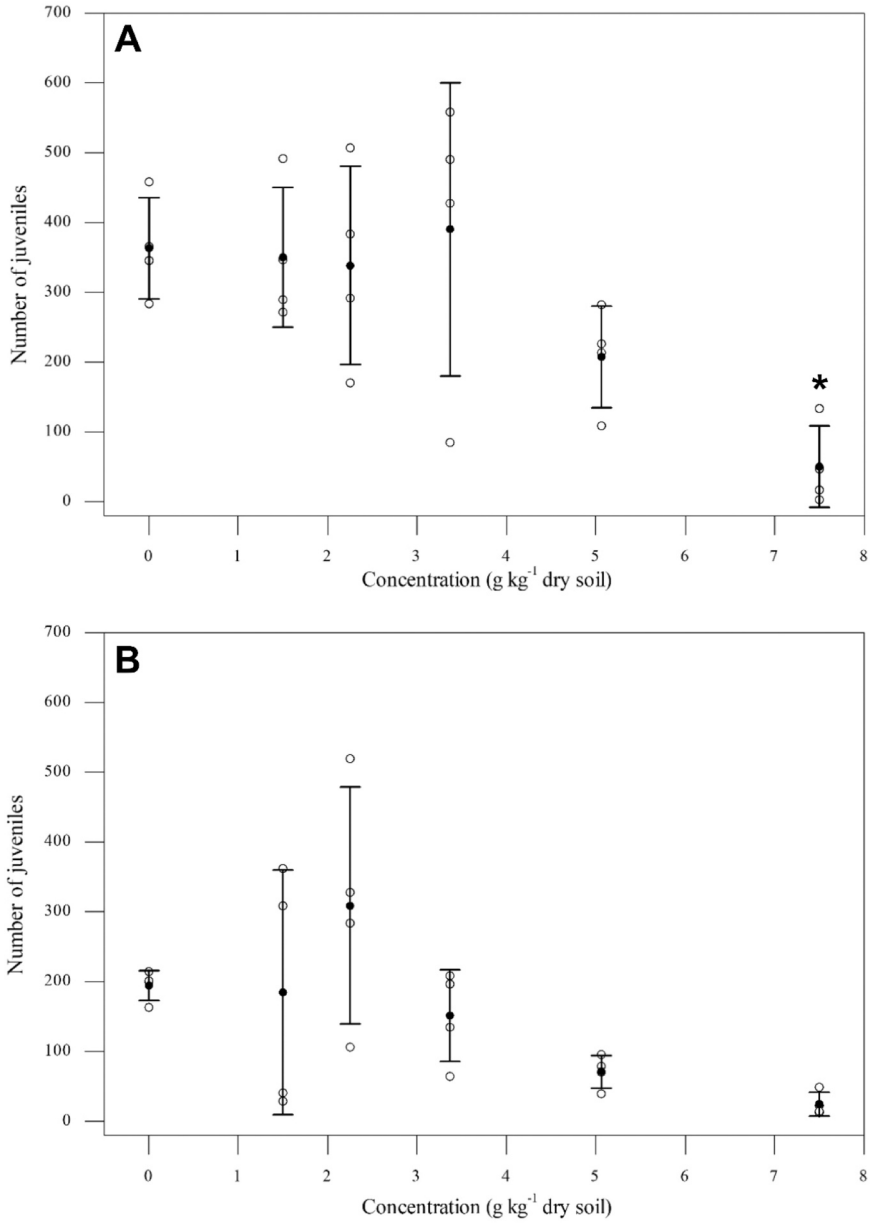


Fig. 5. Effects of indigo in tests (A) and (B) on the reproduction of *E. crypticus* after 21 days of exposure. Open circles are raw data, solid circles represent the mean, and the vertical lines represent the standard deviation. *Indicates significant differences ($p \leq 0.05$) between the concentration groups and the negative control.

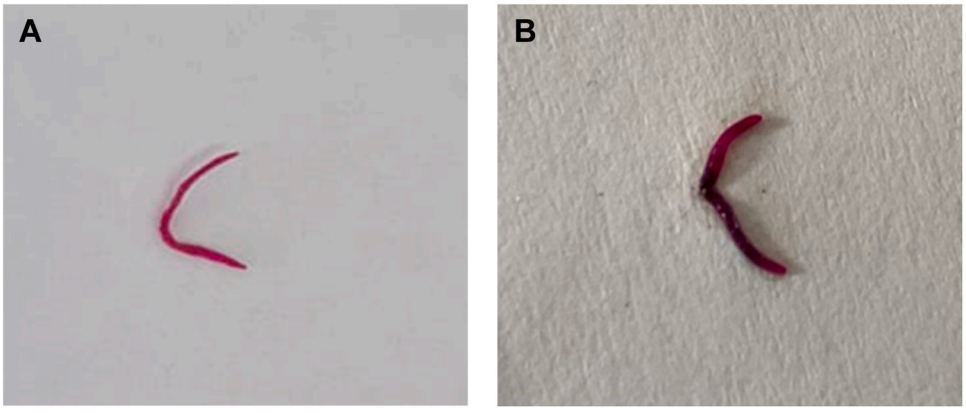


Fig. 6. *E. crypticus* stained with rose bengal at the end of test (A) from negative control and (B) from exposure to the dye concentration of 7.59 g kg^{-1} dry soil.

Table 3
Data from the acute toxicity testing leuco-indigo with *Daphnia similis*.

Dye concentration (g L^{-1})[a]	Number of immobilized organisms and Immobility (%)				pH			Conductivity (µS/cm)		
	24 h		48 h		0 h	24 h	48 h	0 h	24 h	48 h
0 (Control)	0/5	(0)	0/5	(0)	6.44	6.64	6.59	239	239.6	244.8
0.002	0/5	(0)	0/5	(0)	7.05	6.79	6.5	246.2	243	246.8
0.02	0/5	(0)	0/5	(0)	6.62	6.49	5.69	312	311	320
0.2	5/5	(100)	5/5	(100)	6.35	5.91	5.22	957	954	952
1	5/5	(100)	5/5	(100)	6.34	5.77	3.91	3440	3330	3360

[a] Equivalent to g of indigo L^{-1}

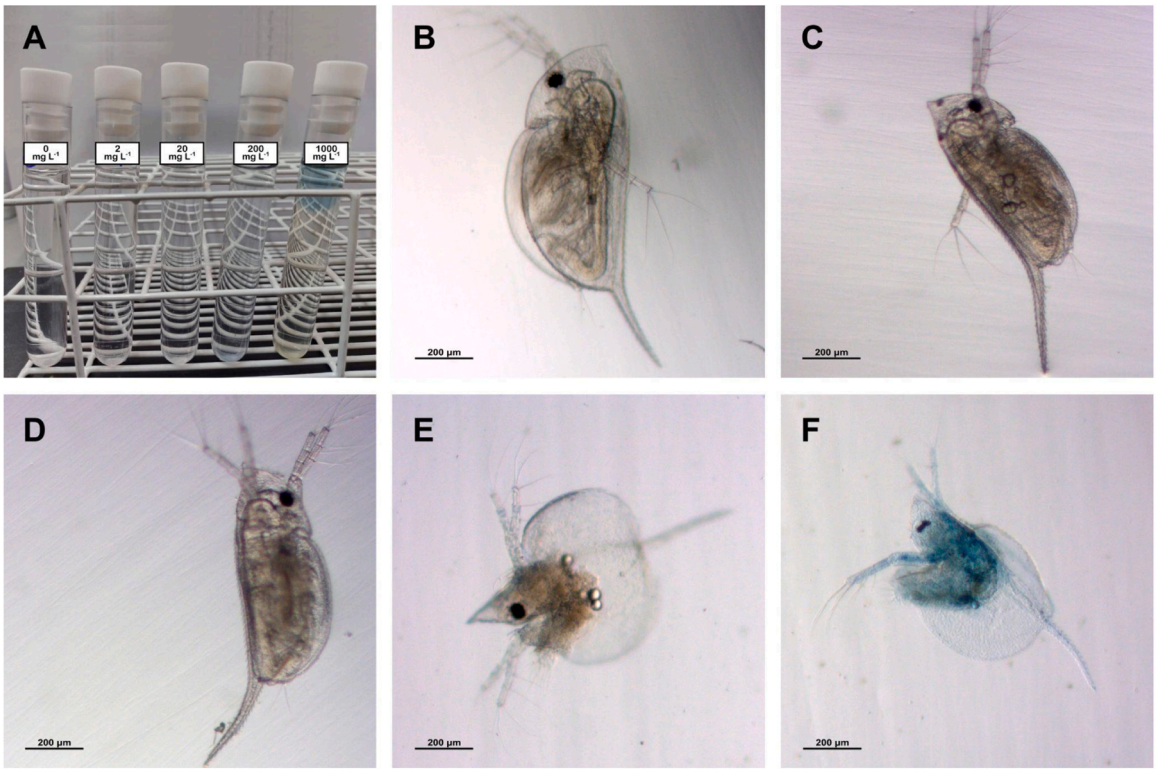


Fig. 7. Toxicity testing with leuco-indigo and *D. similis*. (A): Change in color of the leuco-indigo solution, from yellow to blue at the top of the water column after 24 h; and (B-F): Changes to *D. similis* following exposures at leuco-indigo concentrations of 0 (B); 0.002 (C); 0.02 (D); 0.2 (E), and 1 (F) g L^{-1}

acquired in positive ion mode to improve the detection of low intensity sample ions by reducing background noise. A sum spectrum from a chromatogram section with no analyte compound elution (5–10 min) was selected as the background spectrum and was subtracted from the MS spectra.

2.3. Indigo toxicity

2.3.1. Parhyale hawaiensis test

The culture of *Parhyale hawaiensis* was established in 2010 from organisms collected in the coast of São Paulo state, Brazil. It has been maintained in the laboratory at 24 ± 2 °C, under a photoperiod of 12:12 h light/dark, with reconstituted seawater prepared using commercial brands of sea salt and deionized water at salinity of 30 ± 2, pH 8 ± 1 and dissolved oxygen of 6 ± 2 mg L^{-1}. Organisms were fed daily with a commercial sinking fish food pellet (e.g., JBL® tabs). Partial water exchanges were performed twice a week and total water exchanges were performed once a month (Artal et al., 2018). The sensitivity of the *P. hawaiensis* culture was monitored with zinc sulfate as a reference substance, and only batches within acceptable sensitivities were used in the tests.

We assessed the toxicity of the indigo mixed with standard sand (Sigma Aldrich, CAS number 14808–60–10, 50–70 mesh particle size) according to the protocol described by Vacchi et al (Vacchi et al., 2019). Indigo and sand were mixed in the proportions 1:2, 1:4, 1:8, 1:16 and 1:32 w/w. The indigo concentrations represented 500, 250, 125, 62.5 and 34.5 g kg^{-1}. The test was conducted using neonates (≤ 7 days old), in 24-well microplates, containing 100 mg of mix, 2 mL of reconstituted seawater and 1 organism per well. Twelve organisms were used for each concentration. The negative control was carried out under the same conditions, using only sand and reconstituted seawater. After 96 h of exposure, neonates were observed using a stereomicroscope (Zeiss®, Stemi, 2000-C) and the dead neonates were recorded. The test was considered valid when mortality in the negative control did not exceed 10 %. Results were expressed in % mortality. Data were statistically analyzed using the 'drm' function in the 'drc' package (Ritz et al., 2015) within RStudio (RStudio Team, 2023) to estimate the 50 % Lethal Concentration (LC_{50}) with 95 % confidence interval (CI).

2.3.2. Enchytraeus crypticus test

Cultures were maintained at 20 ± 2 °C, in a photoperiod of 12:12 h (light/dark), fed twice a week with commercial oats, and supplemented with water. To set up the test, 30 g of moist natural or contaminated soil was placed in glass containers and 10 adult clitellate organisms were

added (4 replicates per concentration).

Tests were carried out with indigo mixed with a tropical soil collected in non-anthropized areas, geographic coordinates (-22.0041° S, −47.8984° W), classified as Oxisol. This soil is representative from Brazil, constituting approximately 39 % of the national territory, distributed across all regions of the country (Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA, 2018) containing 39 % clay, 11 % silt, 50 % sand, pH of 5.0 using $CaCl_2$, and 29 g L^{-1} of organic matter content.

After collection, the soil was dried in the oven at 60 °C for 48 h and then sieved through a 2-mm mesh screen. The dye sample was previously autoclaved and crushed manually with a mortar and pestle to reduce particle size. Then the sample was mixed with the soil in plastic bags and homogenized. The volume of water necessary to reach the soil's field capacity was also added. Then the soil (negative control) and soil mixed with indigo at concentrations of 1.5, 2.25, 3.37, 5.06 and 7.59 g kg^{-1} was left to rest for a period of 48 h.

Two independent tests (A and B) were carried out with *E. crypticus* to evaluate reproduction rate, in accordance with ISO 16387:2023 (International Organization for Standardization, 2023) with modifications. Tests were conducted for 21 days, under the same conditions as the cultivation of the organisms, and at the end of the period to each container 70 % alcohol and 6 drops of bengal rose solution (1 %) were added and left for 24 h for staining of the organisms. The soil in each container was sieved (0.053 mm) and washed with tap water. The sieved portion was then transferred to an appropriate tray in which manual counting was performed using a stereoscope microscope. Results were expressed in % of reproduction inhibition. Data was tested for normality using the 'shapiro.test' function in the 'RVAideMemoire' package (Hervé, 2022) and for variance analysis using the 'leveneTest' function in the 'car package' (Fox and Weisberg, 2019). This was followed by a one-way ANOVA implemented in the 'aov' function from the 'stats' package (Core Team, 2023), with statistical significance set at $p \leq 0.05$. Post-hoc comparisons were conducted using the Tukey test, implemented via the 'PostHocTest' function in the 'DescTools' package (Signorell et al., 2023), to identify significant differences between concentration groups and the negative control ($p \leq 0.05$).

2.4. Toxicity of indigo leuco-form

For the commercial vat dyeing process, indigo needs to be converted to its leuco-form using reducing agents and alkali. We simulated its leuco dye formation in the laboratory (Fig. 2) and the resulting solution was tested for toxicity. As testing organism, we selected the water column model invertebrate *Daphnia similis* exposed for 48 h.

To produce the leuco-form, a solution containing 2 g L^{-1} of indigo, 0.5 g L^{-1} of sodium carbonate (Na_2CO_3), and 5 g L^{-1} of sodium hydrosulfite ($Na_2S_2O_4$) was prepared in a glass vial (Baig, 2012). The vial, containing 15 mL of the solution was sealed with Teflon tape to prevent premature reversion of leuco-indigo to indigo. The solution was incubated in a water bath at 75 °C for 1 h and was mixed every 15 min (Fig. 2). We diluted the solution to 50 % with SM media (1 equivalent g indigo L^{-1}) and also prepared an additional 3 dilutions corresponding 0.002, 0.02, 0.2 equivalent g indigo L^{-1} and tested for toxicity.

D. similis culture was maintained in a synthetic medium (SM) at 20 ± 2 °C, pH 7–7.6, hardness level of 40–48 mg L^{-1} as $CaCO_3$, under the photoperiod of 16:8 h light/dark as recommended by ABNT NBR 12713 (ABNT, 2022). Culture was fed daily with algae *Raphidocelis subcapitata* (10^6 cell/org). The sensitivity of *D. similis* culture was monitored with sodium chloride as a reference substance. Only cultures within acceptable sensitivities were used in the tests.

A simplified toxicity test, using only 5 neonates (≤ 24 h old) instead of 20 was performed with *D. similis* following the OECD guidelines N° 202 (OECD, 2004) and ABNT NBR 12713 (ABNT, 2022). We tested different concentrations of each sample, including the negative control (only SM). Tests were performed in glass tubes containing 10 mL of each sample. Temperature was 20 ± 2 °C, under a photoperiod of 16:8 h light/dark exposure for 48 h, without feeding. Conductivity and pH were monitored. Results were expressed in % of immobilized organisms. Samples were considered toxic when mortality was higher than 10 %.

3. Results and discussion

3.1. Chemical analysis

The HPLC-DAD and HPLC-DAD-ESI-MS/MS analyses revealed that the commercial natural indigo dye was a mixture of indigo, as the main compound (peak 8) (91 %), and indirubin (peak 9) (4 %) as well as other impurities such as flavonoids (peaks 1–7) in minor amounts (Fig. 3; Table 1). Silica gel 60 was used as a stationary phase and chloroform as eluent in the chromatographic separation shown in Fig. 3 A which clearly reveals the two main compounds, the blue indigo and the red indirubin together with the impurities on the top of the silica column. HPLC-DAD and ESI-MS/MS results of the peaks in Fig. 3B are compiled and explained in detail in Table 1. Small amounts of flavonoids such as luteolin, apigenin and quercetin in the dye stem from the leaves of woad. Flavonoids are water-soluble and remain in the precipitating slurry in the indigo dye production.

3.2. Indigo toxicity

3.2.1. Parhyale hawaiensis test

Indigo mixed with sand presented acute toxicity for neonates of *P. hawaiensis* (Table 2). The LC_{50} obtained was 309 g kg^{-1} (95 % CI, 191–426).

The presence of indigo was noted both within and in the medium around the organisms (Fig. 4). Nicholson and John (Nicholson and John, 2009) introduced indigo in a bacterial liquid culture at 0.1 mg L^{-1} and observed particles with a size distribution from 1 to 55 μm confirming that this dye is not soluble in water at least at this concentration. In our experiments with *Parhyale* we suspect that the lethality was caused by the indigo particles present in the media and in the gut of the organisms. (Fig. 4B-J).

3.2.2. Enchytraeus crypticus test

After 21 days of exposure, in the two independent tests (Fig. 5A and B), indigo dye decreased the organisms' reproduction at the last two highest concentrations (5.06 and 7.59 g kg^{-1} dry soil), but only at the highest tested concentration in test A, the reproduction was significantly inhibited ($p \leq 0.05$) (Fig. 5). The high variability in the number of juveniles among replicates at the lowest concentrations (1.5, 2.25 and 3.37 g kg^{-1} dry soil) in both tests may be attributed to an uneven distribution of indigo particles within the soil.

The main route of exposure of *E. crypticus* of contaminants occurs through ingestion or absorption of the contaminant through the skin (Alves et al., 2015; Buch et al., 2017). We observed the dye inside the organisms, indicating ingestion, which could be the cause of the reproduction impairment (Fig. 6).

3.3. Toxicity of indigo leuco-form

At the two lowest concentrations tested, no mortality was observed after 48 h (Table 3, Fig. 7B–D). A 100 % of mortality was observed for the two highest concentrations (0.2 and 1 g L^{-1} after 24 h of exposure (Table 3, Fig. 7E–F). The color changed from yellow to blue at the highest concentration after 24 h, at the surface of the solution (Fig. 7A), indicating the oxidation of the leuco-form to indigo due to the contact with oxygen at the surface. After 48 h, solutions at the equivalent concentrations of 0.2 and 1 g L^{-1} the solution became blue and, the carapace of the dead organisms were also stained in blue (Fig. 7F). The pH of the test solution became more acidic over time, probably due to hydrosulfite decomposition to bisulfite. It was also found that

conductivity of the test medium increased with time, especially at the two highest concentrations. We suspect that the toxicity was caused by the high conductivity combined with low pH. Dead organisms seem to have their osmoregulation affected as shown in Fig. 7E–F. As an example of the quantities of those salts used in the commercial process of converting leuco to indigo every year, a total of 84,500 ton of sodium hydrosulfite and 53,500 ton of sodium carbonate are employed (Yuk et al., 2023).

4. Conclusions

Indigo is used in kilo tons every year, but only a few ecotoxicity studies have been published. Also, no information on occurrence of this dye in the aquatic or terrestrial environmental was found.

Testing water insoluble dyes such as indigo for ecotoxicity must consider not only water column organisms but also sediment and terrestrial species, as those compartments seem to be its final destination. In the present study, indigo, when mixed with sand, was acutely toxic to sediment representative organism, *Parhyale hawaiensis*, with an LC_{50} of 309 g kg^{-1}. Indigo mixed with soil impaired the reproduction of *Enchytraeus crypticus* at the highest concentrations tested (5.06 and 7.59 g kg^{-1} dry soil). This is the first report on the toxicity of indigo to sediment and terrestrial organism. More tests with other organisms from the same environmental compartments should be done to complement its hazard evaluation.

As a vat dye, indigo is applied to textiles in its water-soluble leuco-form. Indigo was tested under this condition, and the solution containing its leuco-form and the salts used for its generation was acutely toxic to *Daphnia similis* at the highest concentrations tested (0.2 and 1 g L^{-1}.)

The data generated in this study can be used to guide other indigo toxicity studies and provide toxicity information that can be used in preliminary risk assessment evaluations of environmental compartments as aquatic sediments and soils, contaminated with indigo dye.

Declarations

None

Ethical approval

Not applicable.

Funding

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance code 001; Strategic Research Council at the Research Council of Finland, BioColour Project no 327178, 327213, 352460 and Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP 2020/04628–8.

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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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