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NATÁLIA OLIVEIRA DE FARIAS

ECO/GENOTOXICOLOGICAL CHARACTERIZATION OF NATURAL DYES

CARACTERIZAÇÃO ECO/GENOTOXICOLÓGICA DE CORANTES NATURAIS

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Animal Biology, in the area of Anthropic Relations, Environment and Parasitology.

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“Se o ar não se movimenta, não tem vento; se a gente não se movimenta, não tem vida.”

Itamar Vieira Júnior,

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RESUMO

Diversos compostos naturais têm uma longa história como corantes, mas após a transição do século XX para os corantes sintéticos, a sua utilização na coloração têxtil tem sido mínima. Devido as preocupações ambientais que se fazem tão presentes atualmente, existe uma tendência mundial de produzir materiais mais sustentáveis. Nesse sentido, pigmentos naturais extraídos de plantas e fungos estão sendo amplamente difundidos como fontes para o desenvolvimento de corantes mais seguros e ecologicamente amigáveis como alternativa aos corantes sintéticos, uma vez que estes são geralmente precursores de compostos mutagênicos. Este fato motivou o projeto *BioColour* a conceber e desenvolver novos produtos utilizando corantes naturais e a fornecer uma caracterização toxicológica aprofundada dos corantes mais promissores e dos efluentes gerados do processo de tingimento simulado em laboratório. O objetivo deste estudo foi desenvolver uma avaliação eco/genotoxicológica de corantes naturais. Neste trabalho, ensaios ecotoxicológicos foram realizados com diferentes organismos aquáticos, (microalga *Raphidocelis subcapitata*, microcrustáceos *Daphnia similis* e *Ceriodaphnia dubia*, anfípode marinho *Parhyale hawaiensis* e peixe *Danio rerio*) para verificar a toxicidade aguda e crônica de três antraquinonas naturais (dermocybin, dermorubin e emodin). Além disso, avaliações de genotoxicidade *in vivo* (ensaio cometa e micronúcleo com *Parhyale hawaiensis*) e *in vitro* pelo teste do Ames (protocolo MPA) foram realizadas. O dermorubin foi o corante mais promissor, não apresentou toxicidade para nenhum dos organismos aquáticos nas condições testadas. O dermocybin foi tóxico para *D. similis* (CE_{50} = 0,51 mg L^{-1}), *C. dubia* (CI_{10} = 0,13 mg L^{-1}) e *D. rerio* (CL_{50} = 2,44 mg L^{-1}). O emodin foi o corante mais tóxico e o embrião de *D. rerio* foi o organismo mais sensível, CL_{50} = 0,025 mg L^{-1}. Além disso, o emodin foi mutagênico *in vivo* para a *P. hawaiensis* pelo teste do micronúcleo e *in vitro* pelo teste do Ames. Pensando na aplicação dos corantes pela indústria têxtil, foi feita uma simulação de tingimento em laboratório com extrato dos corantes *yellow* (YO) e *red onion* (RO) junto com mordentes metálicos (Al e Fe). Os corantes, mordentes e os efluentes gerados foram avaliados quanto a sua toxicidade para organismos aquáticos, *D. similis* e *P. hawaiensis*. Os corantes não foram tóxicos para nenhum dos organismos testados, mas foram mutagênicos para as linhagens de *Salmonella* TA98 e TA100. Os mordentes e os efluentes gerados foram tóxicos para a *D. similis* e *P. hawaiensis*. Esse trabalho forneceu informações relevantes para o entendimento do perigo e potencial risco de corantes naturais para o meio ambiente.

Palavras-chave: toxicidade aquática, genotoxicidade, ensaio cometa, corantes, sustentabilidade.

ABSTRACT

Several natural compounds have a long history as dyes, but following the 20th century transition to synthetic dyes, their use in textile dyeing has been minimal. Due to the environmental concerns that are so present today, there is a global tendency to produce more sustainable materials, and natural pigments extracted from plants and fungi are being widely disseminated as sources for the development of safer and more ecologically friendly dyes as an alternative to synthetic dyes, since these are generally precursors of mutagenic compounds. This fact motivated the BioColour project to design and develop new products using natural dyes and to provide an in-depth toxicological characterization of the most promising dyes and the effluents generated from the dyeing process simulated in the laboratory. The objective of this study was to develop an eco/genotoxicological assessment of natural dyes. In this work, ecotoxicological tests were carried out with different aquatic organisms, representatives of different trophic levels (microalgae *Raphidocelis subcapitata*, microcrustaceans *Daphnia similis* and *Ceriodaphnia dubia,* marine amphipod *Parhyale hawaiensis* and fish *Danio rerio*) to verify the acute and chronic toxicity of three natural anthraquinones (dermocybin, dermorubin and emodin). Furthermore, genotoxicity assessments *in vivo (*comet and micronucleus assay with *P. hawaiensis*) and *in vitro* using the Ames test (MPA protocol) were performed. Dermorubin was the most promising dye, showing no toxicity to any of the aquatic organisms. Dermocybin was toxic to *D. similis* (EC_{50} = 0.51 mg L^{-1}), *C. dubia* (IC_{10} = 0.13 mg L^{-1}) and *D. rerio* (LC_{50} = 2.44 mg L^{-1}). Emodin was the most toxic dye and the *D. rerio* embryo was the most sensitive organism with an LC_{50} = 0.025 mg L^{-1}. Emodin was mutagenic *in vivo* for *P. hawaiensis* by the micronucleus test and *in vitro* by the Ames test. Regarding the application of dyes in the textile industry, a dyeing simulation was carried out in the laboratory with the extracted of yellow onion (YO) and red onion (RO) dyes together with metallic mordants (Al and Fe). The dyes, mordants and generated effluents were evaluated for their toxicity to aquatic organisms. The two dyes were not toxic to *D. similis* and *P. hawaiensis*, but they were mutagenic to *Salmonella* strains TA98 and TA100. The mordants and effluents generated by the dyeing process were toxic to *D. similis* and *P. hawaiensis*. This work provided relevant information for understanding the potential risk of natural dyes to the environment.

Keywords: aquatic toxicity, genotoxicity, comet assay, colourant, sustainability.

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LIST OF ABBREVIATIONS AND ACRONYMS

PNEC – Predicted No-Effect Concentration

MPA – Miniaturized Plate Agar

LAEG – Laboratório de Ecotoxicologia e Genotoxicidade

LC – Lethal Concentration

EC – Effective Concentration

IC – Inhibition Concentration

CAS – Chemical Abstracts Service (database website)

DMSO – Dimethyl sulfoxide

ANOVA – Variance analysis

MN – Micronuclei

UNICAMP – Universidade de Campinas

DNA – Desoxyribonucleic acid

USD – United States Dollar

USA – United States of America

WP – Work Package

UEF – University of Eastern Finland

UH – University of Helsinki

NCSU – North Carolina State University

CO_2 – Carbon dioxide

NaOH - Sodium hydroxide

HNO_3 – Nitric acid

THP-1 – Human leukemia monocytic cell line

HepG2 – Human hepatoma

HPLC – High-performance liquid chromatography

NMR – Nuclear magnetic resonance spectroscopy

CI – Colour Index

OECD – Organization for Economic Co-operation and Development

FET – Fish Embryo Toxicity

ABNT – Agência Brasileira de Normas Técnicas

USEPA – United States Environmental Protection Agency

CRED – Criteria for Reporting and Evaluating Ecotoxicity Data

AF – Assessment Factor

GLP – Good Laboratory Practice

QSAR – Quantitative structure–activity relationship

YO – Yellow onion

RO – Red onion

LR – Liquid ratio

LD – Limit of detection

LQ – Limit of quantification

ICP – Inductively Coupled Plasma

MS – Synthetic medium

EMS – Ethyl methanesulfonate

MMS – Methyl methanesulfonate

ISO – International Organization for Standardization

CEUA – Comissão de Ética no Uso de Animais

SUMMARY

1. Introduction

Natural dyes and BioColour project

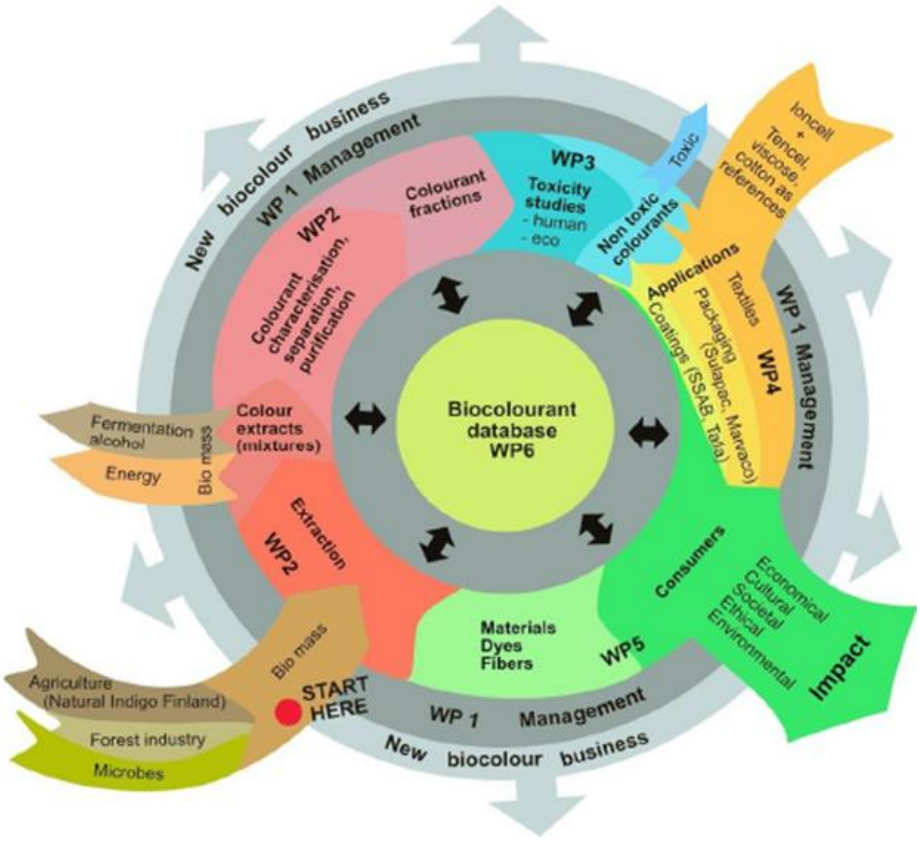
Synthetic dyes and pigments are produced in vast amounts, market value being 33.2 billion USD (2022) and growing by 6% from 2023 - 2028 (Textile Dyes Market Report, 2023). Textile dyes are considered emerging pollutants (Vilar et al., 2021). They are not included in monitoring programs and legislation relevant to environmental quality, but even at low concentrations they could have harmful effects on humans and aquatic organisms (Bafana et al. 2011) For example, some azo dyes are toxic, having carcinogenic and mutagenic potential (Freeman et al. 1990). Workers in the textile industry may encounter exposure to dyes, and the discharge of these dyes into nearby waterways can lead to adverse effects on the aquatic environment (Islam et al., 2023). The textile industry consumes a significant volume of water in its manufacturing processes, mainly including the dyeing and printing stages. The generated effluent by the textile industry is considered one of the biggest polluters of water bodies, due to the amount of water used and its chemical composition. It is estimated that during the dyeing process, approximately 12-50% of dyes are lost to the environment. (Croce et al., 2017; Shore, 1995). Synthetic dyes can negatively impact drinking water sources (Umbuzeiro et al. 2005) and also aquatic life (Vacchi et al., 2016).

Natural compounds have a long history as colourants but after the development of synthetic dyes having a high affinity for textiles, natural dye usage has been minimal (Cardon, 2010). The main reason is because synthetic colourants are designed to be stable under end-use conditions, which is an important characteristic for the efficiency of the dyeing process but sets challenges in biodegradability.

There is a worldwide trend to produce sustainable materials, but the possibility of using natural dyes had not been properly explored. This fact motivated the BioColour project (Bio-based Dyes and Pigments for Colour Palette, https://biocolour.fi) (2019 - 2025) to develop new products using environmentally sound colourants. The BioColour project is a consortium approved by the Strategic Research Council at the Research Council of Finland (https://www.aka.fi/en). The BioColour consortium comprises a multidisciplinary team of experts from Europe, the USA, and Brazil. This team integrates studies on new biocolourant production methods, structure-property relationships, toxicology, and dye-substrate interactions. The consortium is also engaged in scale-up efforts, co-creation, and investigations into the cultural, societal, and ethical aspects associated with producing and applying

biocolourants. The BioColour project addresses objectives to find solutions for transforming the unsustainable material foundation into more sustainable one (Figure 1).

Figure 1 - A schematic view of the BioColour project and its work packages (WPs)



Source: (https://biocolour.fi/)

The Brazilian group that is participating in the BioColour project has the leadership of Gisela de Aragão Umbuzeiro, coordinator of the Laboratory of Ecotoxicology and Genotoxicity (LAEG) from the School of Technology of UNICAMP. LAEG is participating in the Work Package WP3 (Toxicological Safety Assessment of Biocolourants) coordinated by Jaana Rysä from the School of Pharmacy of University of Eastern Finland (UEF). One of the tasks of the WP3 group (Figure 1) is assessing the toxicity of novel biocolourants to the environment to ensure the safety of the novel dyes. After chemical characterization and purification, toxicological tests are being conducted to assess toxicity to bacteria cell (Ames test) and aquatic biota (acute and chronic assays with organisms from different trophic levels, *e.g. Raphidocelis subcapitata, Daphnia similis, Ceriodaphnia dubia, Parhyale hawaiensis* and *Danio rerio*). It is also proposed toxicological analysis of simulated effluents of the dyeing process using natural dyes selected in the BioColour consortium, to have a more realistic evaluation of the hazard associated to the use of natural dyes. The dyes dermocybin, dermorubin, emodin, extract of

yellow and red onions, and fabrics wool and viscose samples used in this study were kindly provided by the research team that is part of the BioColour project and led by Prof. Dr. Riikka Räisänen from University of Helsinki (Finland). The dyes used in the project are mainly extracted from plants and fungi grown in Finnish territory and very common in agriculture and the forestry industry in this region. They are studied as raw material for the production of natural dyes, in a more sustainable way.

Hazard assessment of natural dyes

The hazard of a chemical depends on its intrinsic properties, *i.e.* its capacity to interfere with normal biological processes in living organisms. Hazard identification is the process of determining whether exposure to a substance can cause a specific adverse effect on human health or organisms in the environment (Swedish Chemical Agency, 2020). An adverse effect is described by the International Programme on Chemical Safety (IPCS) as: "*Change in morphology, physiology, growth, reproduction, development, or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increased susceptibility to the harmful effects of other environmental influences*."

The hazardous properties of chemicals are usually identified by testing and applying different internationally accepted methods. The objective of the environmental hazard assessment is to classify the substance regarding intrinsic hazardous properties for the environment and to determine a no-effect concentration below which adverse effects in the environmental spheres are not expected to occur (Swedish Chemical Agency, 2020). According to the Organisation for Economic Co-operation and Development (OECD) guidance document, to assess the environmental hazards for aquatic environment, aquatic toxicity of substances should be tested on organisms from different trophic levels such as algae, plants, invertebrates and vertebrates (OECD, 2001).

Toxicity testing with model organisms of different trophic levels offers a practical approach for characterizing the toxic effects of chemical compounds in biological systems (Schuijt et al., 2021). Organisms are chosen according to their ecological significance and simplicity of cultivation in laboratory. In acute toxicity tests, organisms are exposed to different concentrations of chemicals and the endpoint evaluated is mortality. In the end, a $L(E)C_{50}$ value is estimated, which represents the concentration of the dye that causes 50% effect in exposed organisms. Acute tests can also be used to establish concentrations to be used in subsequent

studies, such as chronic tests, for example. The chronic toxicity tests evaluate the effects of substances over extended periods of time and/or sequential generations, where the reproduction or growth of these exposed organisms is evaluated. At the end, NOEC (No-Observed-Effect-Concentration) or other equivalent L(E)Cx values are estimated (OECD, 2001).

All the organisms listed below exhibit sensitivity to chemical substances in aquatic environments and have well-established cultivation and testing protocols (OECD 201 – *Raphidocelis subcapitata*, OECD 202 – *Daphnia* sp., USEPA, 2002 – *Ceriodaphnia dubia*, Artal et al., 2018 – *Parhyale hawaiensis*, OECD 236 – *Danio rerio*). In this study, they were employed to evaluate the potential hazards associated with natural dyes.

Raphidocelis subcapitata is a species of unicellular green microalgae that generally forms dispersed colonies, belonging to the Selenastraceae family. It is a freshwater species, its reproduction occurs mainly by simple cell division (mitosis), where a mother cell divides into two genetically identical daughter cells. Under favorable conditions, this species can reproduce quickly, forming dense populations (Machado and Soares, 2024).

Daphnia similis is a species of small aquatic crustacean, 0.5 to 6 mm long, belonging to the cladocera group. They are predominantly found in freshwater environments. It is a filtering organism, feeding mainly on phytoplankton, bacteria and small debris present in the waterbodies. Its reproduction is predominantly asexual, through a process called parthenogenesis, in which females produce clones of themselves without the need for fertilization by male organisms (Bownik, 2017).

Ceriodaphnia dubia is a species of cladoceran crustacean, 0.5 to 2.0 mm long, found in freshwater environments. It is a filter-feeding organism, feeding mainly on phytoplankton, bacteria and debris present in the water. Like *D. similis*, *C. dubia* also reproduces asexually, through parthenogenesis, where females produce clones of themselves (Versteeg et al., 1997).

Parhyale hawaiensis is a species of amphipod crustacean belonging to the family Hyalidae. It is a marine organism, from circumtropical areas. They have segmented body with a distinct head, thorax and abdomen and are usually around 5 mm long as adults. They reproduce sexually and has a rapid life cycle (Dos Santos and Umbuzeiro, 2023).

Danio rerio (zebrafish), is a species of freshwater fish belonging to the Cyprinidae family. Originally from South Asia, zebrafish have gained prominence as a model organism in scientific research due to their rapid reproduction rate, embryonic transparency and well-

characterized genome. They usually reach 2.5 to 5 cm in length as adults, inhabit shallow waters of streams, rivers, lagoons and rice fields, preferring areas with dense vegetation. Zebrafish are oviparous and reproduce through external fertilization (Padilla and Glaberman, 2020).

To evaluate/monitor the quality of aquatic environments for the presence of contaminants it is necessary to determine the concentration of a substance below which an unacceptable effect will most likely not occur, named Predicted No Effect Concentrations (PNECs) (ECHA, 2008). According to the guidance published by ECHA, on information requirements and chemical safety assessment the greater the number of data on organisms from different trophic levels and belonging to the environment we are trying to protect (marine, estuarine, or freshwater), lower will be the assessment factor applied in the derivation of a PNEC (ECHA, 2008).

Genotoxicity tests are also an integral part of the safety assessment of chemical substances. Genotoxicity is an umbrella term that encompasses mutagenicity, which induces permanent, potentially transmitted genetic changes, and other types of genetic damage that may or may not be repaired by cellular machinery. The results obtained from genotoxicity tests are used in the risk assessment process and contribute to regulatory decisions on the registration and classification of substances (Kummrow and Umbuzeiro, 2021). Regardless of the purpose of use, chemical product registration procedures always require the assessment of their genotoxic potential, aiming to protect both human beings and the environment (Cartus and Schrenk, 2017). Several assays, both *in vitro* and *in vivo*, can be used to evaluate the genotoxic potential of substances. Generally, a tiered approach is adopted, starting with *in vitro* testing, such as the *Salmonella*/microsome test, and, depending on the results, proceeding with *in vivo* testing, such as the comet assay (Kummrow and Umbuzeiro, 2021).

2. Objective

The objective of this project was to provide a toxicological characterization of the natural dyes selected by the BioColour consortium and of dyeing effluents simulated in the laboratory, using different aquatic models to verify acute and chronic toxicity, including *in vivo* and *in vitro* genotoxicity assessment.

Specific objectives

The specific objectives of the work were:

- To evaluate the ecotoxicity of the natural anthraquinone dyes dermocybin (purity 99%) and dermorubin (>98%) and compare them with the ecotoxicities of other synthetic and natural anthraquinones from literature;

- To characterize the ecotoxicity and genotoxicity of the natural dye emodin (purity 99%) to aquatic organisms and mutagenicity using *Salmonella* bacteria.

- To evaluate the aquatic toxicity and mutagenicity of yellow and red onion dyes and effluents from simulated dyeing process.

3. Results presentation

The results of this Ph.D. thesis were organized into three chapters corresponding to the articles resulting from this work.

Chapter 01 – Is natural better? An ecotoxicity study of anthraquinone dyes: In this chapter, aquatic toxicity results were presented for two natural anthraquinone dyes, dermocybin and dermorubin, using different acute and chronic ecotoxicity tests, comparison with ecotoxicological data for other anthraquinones from natural and synthetic origin, and subsequent derivation of a Predicted No-Effect Concentration (PNEC) value for dermocybin to protect aquatic life.

Chapter 02 – The natural anthraquinone dye emodin: eco/genotoxicological characterization for aquatic organisms: In this chapter we present aquatic toxicity results for the anthraquinone dye emodin using different acute and chronic ecotoxicity assays and genotoxicity assessment using comet assay with *Parhyale hawaiensis* hemolymph and mutagenicity with Ames test (MPA protocol) and micronucleus with *P. hawaiensis* as well.

Chapter 03 – Yellow and red onion dyes and simulated dyeing effluents: In this chapter we present the results of ecotoxicity (*Daphnia similis* and *P. hawaiensis*) and mutagenicity (Ames test) generated from the simulation of dyeing effluents using yellow and red onions as natural dyes. In this part of the work, we counted on the visit of Prof. Dr. Riikka Räisänen (University of Helsinki, Finland) to the Ecotoxicology and Genotoxicity Laboratory (LAEG).

Chapter 01

Is natural better? An ecotoxicity study of anthraquinone dyes

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Abstract

The concept of sustainability has gained prominence in recent years, enhancing the need to develop products that are less harmful to the environment. Dyes are used by various industrial sectors and have a lot of market value; they are used on a large scale mainly by the textile industry that uses large volumes of water and is one of the main contributors to the contamination of water bodies. Some natural compounds, especially anthraquinones are re-emerging as possible alternatives to synthetic dyes, some of which are known for their toxic and/or mutagenic effects. The BioColour project (https://biocolour.fi/) which is interested in promoting the development of new alternative molecules to synthetic dyes, provided us highly purified anthraquinone dyes dermocybin and dermorubin (>98% purity) extracted from a specie of fungus *Cortinarius sanguineus*. Dyes were tested for their acute and chronic toxicity using different aquatic organisms. Dermorubin was not toxic to any of the organisms tested for the highest test concentration of 1 mg L^{-1} and it was the most promising dye. Dermocybin was toxic to *Daphnia similis* (EC_{50} = 0.51 mg L^{-1}), *Ceriodaphnia dubia* (IC_{10} = 0.13 mg L^{-1}) and *Danio rerio* embryos (extrapolated LC_{50} = 2.44 mg L^{-1}). Dermocybin was not toxic to *Parhyale hawaiensis* (LC_{50} = 0.7 mg L^{-1}) or *Raphidocelis subcapitata* (IC_{10} > 0.7 mg L^{-1}) at tested concentrations. A safety limit, *i.e*, predicted no-effect concentration (PNEC) of 0.0026 mg L^{-1} was derived based on the toxicity of dermocybin. The PNEC value can be used to provide hazard information for future application in commercial dyeing processes. Then, we compared the toxicity of dermocybin and dermorubin with ecotoxicity data available in the literature on other anthraquinone dyes of natural and synthetic origin. Some natural dyes can be as toxic as synthetic ones, or more toxic when chronic effects are considered. Despite natural dyes being used since centuries past, there are few ecotoxicological studies available. This study is designed to help develop a more comprehensive understanding of their toxicological properties.

Keywords: natural dyes, synthetic dyes, *Daphnia*, zebrafish, *Ceriodaphnia*, algae

4.1 Introduction

Commitments to sustainability have been standing out and gaining popularity in society, leading to a growing need to produce more sustainable materials to minimize negative environmental and social impacts. The definition of sustainability emerged in the World Commission on Environment and Development, created by the United Nations and is cantered on the fulfilment of 17 goals that propose a balance between economic development and environmental conservation (https://sdgs.un.org/goals). Different and important industrial sectors have sought to add new methodologies to their practices to generate less waste and preferably less toxic waste for human and environmental health (Che and Yang, 2022).

The textile industry is one of the sectors that contribute the most to environmental pollution, due to the extensive use of dyes and the large volume of water used in wet processing. Synthetic dyes are predominantly used, as they have low-cost, are easily accessible and have high affinity for different types of fabrics (Ardila-Leal et al., 2021; Freeman et al., 2021). However, large volumes of effluents contaminated with dyes are released into water bodies daily. In some cases, many of these effluents are not treated, or if treated, there is the potential for the formation of by-products that can be even more toxic than the original molecules (Vendemiatti et al., 2021).

Certain natural compounds have a long history as colourants. There is evidence in South America of the use of natural dyes for body and fabric painting by indigenous people (Polesna et al., 2011). In China, archaeological finds indicated the use of plant and animal materials for the extraction of natural dyes (Kramell et al., 2014). In Europe, dyes extracted from plants, *e.g*., *Isatis tinctoria* and *Rubia tinctorum*, have been widely used for dyeing fabrics (Mocquard et al., 2022). After the industrialization period, synthetic dyes replaced almost all natural dyes. Scientific and technological advances of the 19th century allowed the large-scale production of chemical structures having high stability to end-use conditions, making these synthetic dyes more advantageous in terms of handling, colour performance, reproducibility, low cost, and ease of obtaining compared to natural dyes (Ardila-Leal et al., 2021).

Due to the large-scale worldwide production of synthetic dyes and pigments and their widespread application areas, they permeate into different environmental compartments, especially in aquatic systems (Tkaczyk et al., 2020). Anthraquinones have been reported as the oldest known dyes, and nowadays, they represent the second most used group of dyes in the textile industry after the azo dyes group. They are chemically stable molecules derived from the anthracene containing quinone structure as the chromophore to which hydroxyl or amino groups can be attached (Berradi et al., 2019). We do not expect that anthraquinones (from

natural or synthetic origin) would be easily degraded in environmental conditions; therefore, we anticipate that aquatic organisms would be exposed after the release of effluents containing those dyes (Ismail and Sakai, 2022). The potential hazards of synthetic anthraquinone dyes have already been recognized. Some studies reported that synthetic anthraquinones are toxic to freshwater organisms (Croce et al., 2017; de Luna et al., 2014; Leme et al., 2015; Novotný et al., 2006) and can cause genetic damage, including mutagenicity and/or carcinogenicity (*e.g.*, emodin, aloe-emodin (Masuda and Ueno, 1984; Nesslany et al., 2009; Tanaka et al., 1987)).

Because of environmental concerns regarding certain synthetic dye use, the scientific community has been looking for greener and healthier alternatives for dyes known to pose environmental problems. Therefore, colourants extracted from natural sources have emerged as possible alternatives to problematic synthetic dyes, as some researchers tend to believe that they are less harmful to humans and the environment because they come from nature.

Although natural dyes from the class of anthraquinones have been used since ancient times, only three have been evaluated in aquatic toxicity tests, madder (Freeman et al., 2021), cochineal red E120 (Motta et al., 2019) and erythrostominone (Abe et al., 2017, 2019).

Recently, the BioColour project (https://biocolour.fi/), motivated by the desire to find molecules that are safer than and as effective as synthetic dyes, extracted two red dyes (dermocybin and dermorubin) from the blood red webcap *Cortinarius sanguineus*. Both dyes, besides their successful application in traditional water-based dyeing processes (Räisänen, 2019), were found suitable for the waterless dyeing of textile fibres in liquid CO_2, eliminating the production of wastewater and the need for a drying step following dyeing (Herrala et al., 2022). Subsequently, they have been tested for toxicity in cell viability tests using human HepG2 liver and THP-1 human monocyte cells, and mutagenicity in the *Salmonella*/microsome assay. Dermocybin and dermorubin were neither cytotoxic nor mutagenic (Herrala et al., 2022).

The aims of the present study were to evaluate acute and chronic toxicity of two natural anthraquinone dyes, dermocybin and dermorubin, using aquatic organisms from different trophic levels; and calculate their predicted no effect concentrations (PNECs), to provide benchmark values for risk assessments, in case they are applied in commercial dyeing processes. Also, we sought to compare their toxicity with data available in the literature with other anthraquinones dyes from natural and synthetic origins.

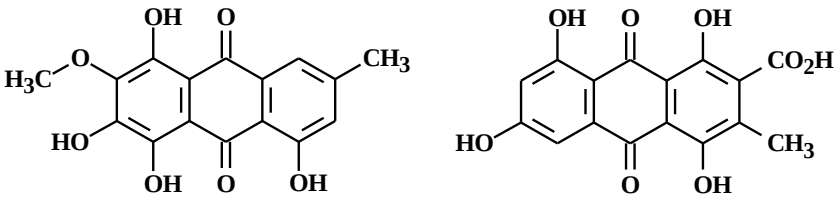
4.2. Material and Methods

4.2.1 Dyes

Dermocybin (purity 99.74%) and dermorubin (98.08% of dermorubin and 1.6% of 5-Cl-dermorubin) were supplied by the BioColour project. These dyes were extracted from the blood-red webcap fungal bodies from *Cortinarius sanguineus* as described by Hynninen et al., (2000). Anthraquinones were separated using multiple liquid-liquid partition as described by Hynninen and Räisänen (2001). Dyes' purity and their chemical structures were confirmed by HPLC-DAD-MS and NMR (13C and 1H) spectroscopy as described by Herrala et al., (2022) (Figure 1).

Synthetic dyes associated with this study included nine commercial anthraquinone compounds listed in the Colour Index (CI) and 4 experimental dyes (**5**, **42**, **145** and **183**) donated by Eastman Chemicals. The synthetic dyes were a combination of water soluble (*e.g.*, sulfonated) and hydrophobic dyes, the latter of which included anthraquinone disperse dyes that take their design from natural dye structures.

Figure 1 - Chemical structures of anthraquinone dyes dermocybin and dermorubin from *Cortinarius sanguineus*.



Dermocybin **Dermorubin**

4.2.2 Preparation of dye solutions for testing

Because both dyes were poorly soluble in water, dissolution was added with dimethyl sulfoxide, DMSO (Sigma Aldrich, purity ≥ 99.5%). DMSO was added gradually until the dye was visually solubilized (giving 10 g L^{-1} for dermorubin and 7 g L^{-1} for dermocybin). Then dilutions were made with testing media, and the maximum concentration tested was defined by the maximum allowed concentration of DMSO recommended in aquatic toxicity evaluations (0.01%) (OECD, 2019, 2013).

4.2.3 Ecotoxicity tests

Both dyes were tested with acute (*Daphnia similis, Parhyale hawaiensis,* and *Danio rerio*) and chronic (*Raphidocelis subcapitata* and *Ceriodaphnia dubia*) toxicity tests. The sensitivity of the cultures was monitored using reference compounds.

Only organisms from healthy cultures (*D. similis, C. dubia* and *R. subcapitata*) that showed acceptable EC/LC_{50} values according to the historical data from the laboratory were used. In the case of zebrafish, the sensitivity of the organisms was evaluated, by the inclusion of positive control group in each test. In this case, results were validated when ≥ 30% mortality of organisms were observed at the end of the test.

Tests were only considered valid when the negative and solvent controls (0.01% DMSO) showed mortality up to 10% for the acute tests and 20% for the chronic tests with *C. dubia*, and in the case of tests with *R. subcapitata*, the biomass in the control groups should have increased exponentially by a factor of at least 16 within the 72 h. Results were expressed in 50% effect or lethal concentration (EC/LC_{50}) values for the acute and in 10 or 50% inhibition concentration (IC_{10}/IC_{50}) for the chronic tests.

Acute

Daphnia similis

Acute toxicity tests were conducted with the microcrustacean *D. similis* following the OECD guideline n° 202 (OECD 202, 2004). *D. similis* was successfully cultivated in the Laboratory of Ecotoxicology and Genotoxicity (LAEG). Cultures were maintained in a climate chamber at 20 ± 2 °C, under photoperiod of 16:8 h (light/dark) (Eletrolab EL 2020/4LED), and daily fed with the green algae *R. subcapitata,* and total water exchange was performed three times per week. Cultures were maintained in 2 L glass vessels.

For the tests, twenty neonates (<24 h old) from 2- to 3-week-old mothers were placed in 4 replicates for each concentration (5 organisms/replicate). Solvent and negative controls were added to the tests. Glass tubes (10 mL) were used to prevent dye adsorption to the material. The temperature and photoperiod conditions were the same as in the cultivation. The organisms were not fed during the test. Organisms were exposed to concentrations of 0, 0.04, 0.09, 0.175, 0.35 and 0.7 mg L^{-1} for dermocybin and 0, 0.01, 0.03, 0.1, 0.3, 1.0 mg L^{-1} for dermorubin. After 48 h of exposure, immobilized organisms were counted. Tests were considered valid when immobility in the negative and solvent controls did not exceed 10%. Parameters such as conductivity, dissolved oxygen, and pH were determined at the beginning and at the end of the tests.

Parhyale hawaiensis

Parhyale hawaiensis cultures were maintained at 24 ± 2 °C, under 12 h photoperiod (light: dark), and kept in constant aeration. Food was provided daily from Monday to Friday with commercial brands of sinking fish food. Salt water was prepared by reconstituting commercial brands of sea salt in salinity 30 ± 2. Partial water exchange (50%) was conducted two times per week and total water exchange was performed once a month, when vessels were cleaned, and substrate renewed. Acute toxicity testing using the amphipod *P. hawaiensis* was conducted according to Artal et al., (2018). Test solutions were prepared by serial dilution using reconstituted seawater at salinity 30 (prepared using a combination of marine salt and deionized water). Reconstituted seawater was also used as a negative control and, DMSO at 0.01% was used as a solvent control. For each concentration, 32 neonates (≤ 7 days old) from 8 to 10 months old mothers were placed in 96 well plate (200 µL of solution and 1 organism per replicate). Tests were conducted in the same cultivation conditions. Organisms were exposed to concentrations of 0, 0.07, 0.13, 0.22, 0.4 and 0.7 mg L^{-1} for dermocybin and 0, 0.1, 0.2, 0.35, 0.6 and 1.0 mg L^{-1} for dermorubin. After 96 h, the number of dead organisms was recorded using a stereomicroscope (Stemi 2000-C, Carl Zeiss, Oberkochen, Germany). Tests were considered valid when mortality in the negative and solvent controls did not exceed 10% and, mortality above 10% indicated toxicity.

Danio rerio embryos

Before starting the tests with zebrafish embryos, this study was previously submitted and approved by the Ethics Committee of the University of Campinas (UNICAMP) (protocol n° 5645-1/2020), see Annex 1.

Zebrafish adults were kept in a recirculating system and maintained in aquariums with reconstituted water that consists of reverse osmosis water with a conductivity below 5 µS/s, plus sea salt. The temperature was maintained at 26 ± 1 °C, ammonia < 0.01 mg L^{-1}, conductivity at 750 ± 50 mS/cm, pH at 7.0 ± 0.5 and dissolved oxygen equal to or above 95% saturation. Fish were raised in a 12 h photoperiod (light: dark). Fish were daily fed with commercial food (GEMMA Micro). The fish embryotoxicity test was based on the OECD guideline n° 236 “Fish Embryo Toxicity” (FET) test (OECD, 2013) with few adaptations including the extension of time of exposure from 96 h to 168 h allowing a better evaluation of the locomotor activity (Farias et al., 2019). *D. rerio* embryos were exposed, immediately after fertilisation. A reference substance, 4 mg L^{-1} of 3,4-dichloroaniline (Sigma-Aldrich, 98% purity) was used as positive control and a solvent control were also included. The test was

performed in 24-well microplates, 20 wells were filled up with 2 mL of the test solution and four wells with reconstituted water (internal plate control, as required in the OECD guideline). A total of 20 eggs were used per treatment. Embryos were selected and distributed individually, one egg per well. The 24-well microplates with exposed embryos were kept in a climate chamber at the same conditions employed in the fish cultivation. Organisms were exposed to concentrations of 0, 0.007, 0.035, 0.07, 0.35, 0.7 mg L^{-1} for dermocybin and 0, 0.01, 0.03, 0.1, 0.3 and 1.0 mg L^{-1} for dermorubin. Embryos and larvae were observed daily under a stereomicroscope (Stemi 2000-C, Carl Zeiss, Oberkochen, Germany). Developmental parameters were monitored over the test period. Endpoints evaluated were lethality (coagulation, tail not detached, malformation of somite, no heartbeat), sub-lethality (development of eyes, inflated swimming bladder, loss of equilibrium – larvae side-lying in the bottom of the microplate well after mechanical stimulus, eye and body pigmentation, edemas, malformation of head, tail and otoliths, deformity of yolk sac and delay in development).

Chronic

Raphidocelis subcapitata

Raphidocelis subcapitata stock cultures were maintained in freshwater Oligo medium at 4 °C. The inoculum was composed of algae cells harvested from a liquid stock 3-days old algae culture and in logarithmic growth phase. The inoculum was incubated at 24 ± 2 °C under continuous fluorescent light (4,000 ± 400 lux). The algal biomass was adjusted to 1×10^4 cells mL^{-1} to allow exponential growth. Tests were performed with the freshwater algae *R. subcapitata* following OECD n° 201 (OECD 201, 2011) and ABNT/NBR 12648 (ABNT, 2018). Tests were performed in Erlenmeyer flasks filled with a final volume of 20 mL (dye, algae inoculum and enrichment medium), in triplicate, under continuous agitation (100-120 rpm) for 72 h without medium renewal, at 24 ± 2 °C under continuous fluorescent light (4,000 ± 400 lux). Organisms were exposed to concentrations of 0, 0.1, 0.17, 0.27, 0.44 and 0.7 mg L^{-1} for dermocybin and 0, 0.06, 0.12, 0.25, 0.5 and 1.0 mg L^{-1} for dermorubin. The growth inhibitory effect was measured in inhibition rate, based on the correlation of the number of algae cells with the spectrophotometric absorbance at 440 nm measured using a spectrophotometer (DR3900, Hach, Loveland, United States).

Ceriodaphnia dubia

Ceriodaphnia dubia culture was maintained in MS (synthetic medium) at 25 ± 2 °C, photoperiod of 16:8 h (light/dark), pH 7.0 - 7.6, and total hardness of 40 to 48 mg $CaCO_3$ L^{-1}.

Organisms were fed daily with *R. subcapitata* (2.0×10^6 organism cells), in addition to a nutraceutical supplement prepared from fermented trout feed, Tetramin® and yeast extract (*Saccharomyces cerevisiae*). Tests followed the recommendations of the U.S. protocol, EPA - Method 1002.0 (USEPA, 2002), with modifications according to the technical standard ABNT/NBR 13373 (ABNT, 2022). Mass cultures were used as reservoir of organisms and individual cultures (1 neonate/ 15 mL in glass beaker) were used as source of neonates for the test. The assay was performed with 10 replicates per concentration and one organism (neonates ≤ 24 h) per replicate. The organisms were individually placed in 15 mL of each test solution in a glass beaker and incubated for 7 days with renewal of the test solution every 48 h.

Ceriodaphnia dubia were fed daily with a suspension of the microalgae *R. subcapitata* (2.0×10^6 cells/mL per organism) and compost feed (50 μL of a combination of trout feed, Tetramin® with the addition of biological yeast). Tests were performed in the same cultivation conditions previously described. The glass beakers were covered with acrylic plates to prevent evaporation. Organisms were exposed to concentrations of 0, 0.04, 0.08, 0.175, 0.35 and 0.7 mg L^{-1} for dermocybin and 0, 0.0625, 0.125, 0.25, 0.5 and 1.0 mg L^{-1} for dermorubin. All organisms were monitored for survival, and neonates were counted three times, during the renewal of the test solutions. The endpoint evaluated was the survival and reproduction of the organisms. The tests were considered valid when 80% survival and at least 15 neonates/female on average for negative and solvent controls animals were found over the test period. The pH, conductivity, dissolved oxygen, and temperature were monitored at the beginning of the test and after each solution renewal.

4.2.4 Statistical analysis

Acute toxicity data from *D. similis*, zebrafish embryos and *P. hawaiensis* tests were modelled by generalized logistic model glmnet package (Friedman et al., 2010) to estimate the 50% effect and lethal concentrations (EC_{50} and LC_{50}) and their respective 95% confidence intervals (C.I.). It was selected the model that the concentration variable was statistically significant ($p \leq 0.05$). Analyses were conducted using R (RStudio Team, 2022).

For chronic toxicity tests with *R. subcapitata* and *C. dubia*, the normality of the data was analysed applying Kolmogorov-Smirnov test and it indicates nonparametric assumptions. One-way analysis of variance (Kruskal-Wallis), with Mann-Whitney post-hoc test, was used to identify significant differences ($p \leq 0.05$). When statistical differences between the solvent control (DMSO 0.01%) and the exposure concentrations were observed, the 50% inhibition

concentration (IC_{50}) and its respective 95% confidence interval (C.I.) were estimated using model fitting drm() function of the drc package (Ritz et al., 2015). Analyses were conducted using R (RStudio Team, 2022).

4.2.5 Derivation of Predicted No-Effect Concentrations (PNEC)

Predicted No-Effect Concentration (PNEC) values were derived using the deterministic approach according to European guidelines (European Commission, 2018). Essentially, the deterministic approach takes the most sensitive, reliable, and relevant toxicity endpoint of the available set of test results and applies an appropriate assessment factor (AF) to extrapolate to an environmentally protective concentration. The magnitude of the AF varies from 10 to 1000 depending on the uncertainties of the available ecotoxicity data. For the evaluation of the reliability and relevance of the ecotoxicity tests, we used the "Criteria for Reporting and Evaluating Ecotoxicity Data" (CRED) (Kase et al., 2016; Moermond et al., 2016) applies a set of 20 reliability and 13 relevance criteria (Supplementary Material I). CRED evaluates ecotoxicity tests according to transparent questions that allow the objective assessment of whether the test was conducted under appropriate conditions, as well as whether it is suitable for the given regulatory context.

4.3. Results and Discussion

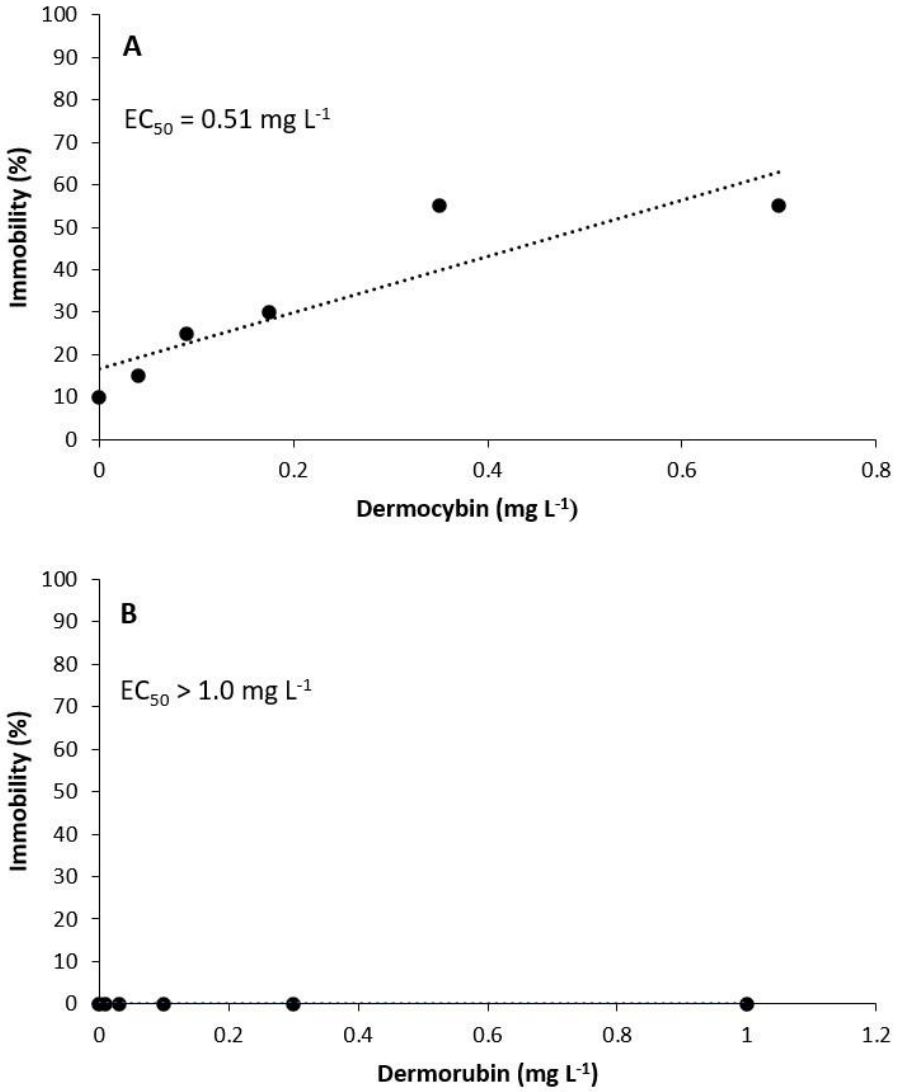
All physical-chemical parameters measured at the beginning and in the end of the tests were within recommended ranges for each test organism.

4.3.1 Acute toxicity

Daphnia similis

Acute toxicity tests with *D. similis* for dermocybin showed an EC_{50} = 0.51 mg L^{-1}, and for dermorubin, although we have observed the dye inside the gut, no toxic effects were observed (EC_{50} >1 mg L^{-1}) (Figure 2). Only two studies were found in the literature that reported toxicity of natural anthraquinones to *Daphnia* spp. Madder presented an EC_{50} of 4.4 mg L^{-1} for *D. similis* (Freeman et al., 2021) and erythrostominone an EC_{50} of 19.7 mg L^{-1} for *D. magna* (Abe et al., 2019) (Table 1). Dermocybin was the most toxic among the three natural dyes. For synthetic anthraquinones, dermocybin was more toxic than experimental dye **5** with EC_{50} of 0.94 mg L^{-1} and presented similar toxicity to experimental dye **145** with an EC_{50} of 0.57 mg L^{-1} (Umbuzeiro et al., 2019) (Table 1).

Figure 2 - Concentration × response curve and EC_{50} for dermocybin (A) and dermorubin (B) to *D. similis*



Parhyale hawaiensis

Dermocybin and dermorubin were not acutely toxic to *P. hawaiensis* at tested concentrations (LC_{50} > 0.7 and 1 mg L^{-1}, respectively), with mortality < 10 % (Table S4). However, orange-red colour was observed inside the gut as pictured in figures 3 and 4, proving that dyes reached the digestory system of the organisms. Motta et al., (2019) did not observe any effect on the survival of *Artemia salina* exposed to the natural anthraquinone cochineal red E120 (Table 1) (LC_{50} > 1,200 mg L^{-1}) although this result should be viewed with caution because a commercial product with undefined purity was used in the tests.

Figure 3 - Neonates of *P. hawaiensis* exposed to dermocybin. (A) Control and (B) 0.7 mg L^{-1}. The red arrow indicates dermocybin inside the gut.

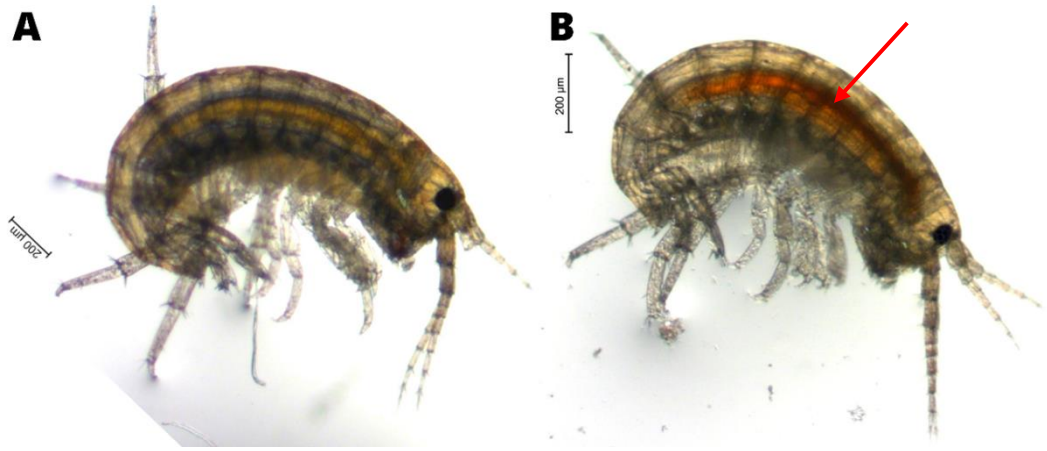
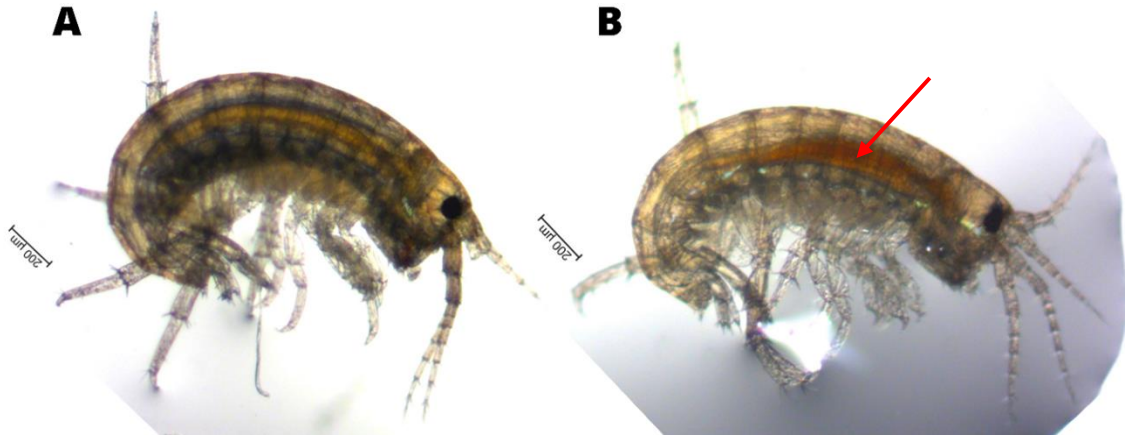


Figure 4 - Neonates of *P. hawaiensis* exposed to dermorubin. (A) Control and (B) 1.0 mg L^{-1}. The red arrow indicates dermorubin inside the gut.

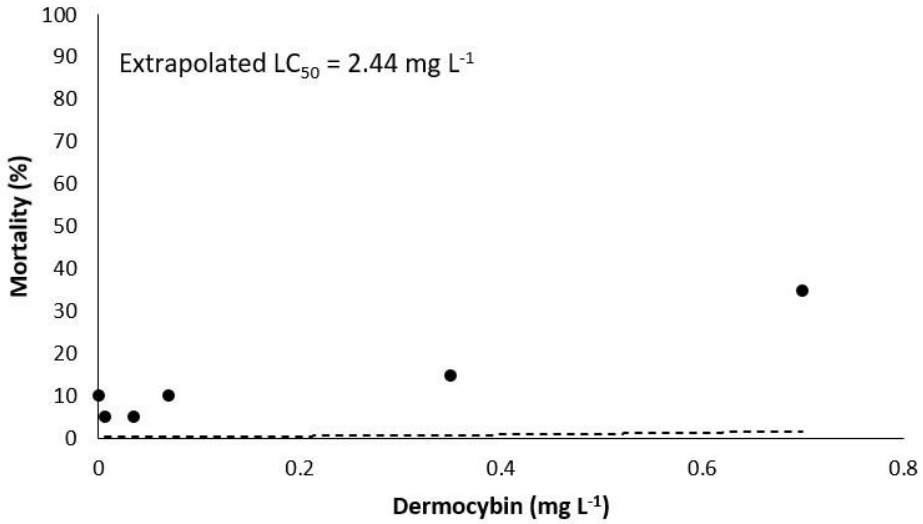


Danio rerio embryos

No mortality was observed in the negative and solvent controls, furthermore these organisms presented regular development with no detection of malformations, whereas the positive control (4 mg L^{-1} of 3,4-dichloroaniline) induced mortality higher than 30% at the end of the test as recommended by OECD guideline 236 (OECD, 2013).

Dermocybin was toxic to zebrafish embryos/larvae presenting 35% of mortality for the highest concentration tested (0.7 mg L^{-1}) (Figure 5). It was not possible to calculate LC_{50} value for dermocybin because mortality was observed in less than 50% of organisms. Although, we were able to extrapolate an LC_{50} of 2.44 mg L^{-1}. No adverse effect was observed when sub-lethal endpoints were evaluated (Table S5).

Figure 5 - Concentration × response curve and extrapolated LC_{50} for dermocybin to *D. rerio* embryos.



Dermorubin did not affect the survival of embryos/larvae after 168 h of exposure, LC_{50} >1 mg L^{-1}. Also, no adverse effect was observed when sub-lethal endpoints were evaluated (Table S5).

Motta et al., (2019) observed no effects on survival or morphological changes in zebrafish larvae exposed to cochineal red dye (LC_{50}/EC_{50} > 1,200 mg L^{-1}). The synthetic anthraquinone vat green 3 did not present lethal effects for zebrafish larvae (LC_{50} > 100 mg L^{-1}), but gas bladder inflation impairment and yolk sac edema were observed at 100 mg L^{-1} according to de Oliveira et al., (2016). Acid blue 80 and acid blue 129 also showed no toxic effects for the fish *Pimephales promelas* larvae with LC_{50} >10 mg L^{-1} (Parrott et al., 2016). Little and Lamb (1972) reported toxic effects of acid green 25 (LC_{50} = 6.2 mg L^{-1}), disperse blue 7 (LC_{50} = 12.0 mg L^{-1}) and acid blue 25 (LC_{50} = 52.0 mg L^{-1}) to *P. promelas* (Table 1).

4.3.2 Chronic toxicity

Raphidocelis subcapitata

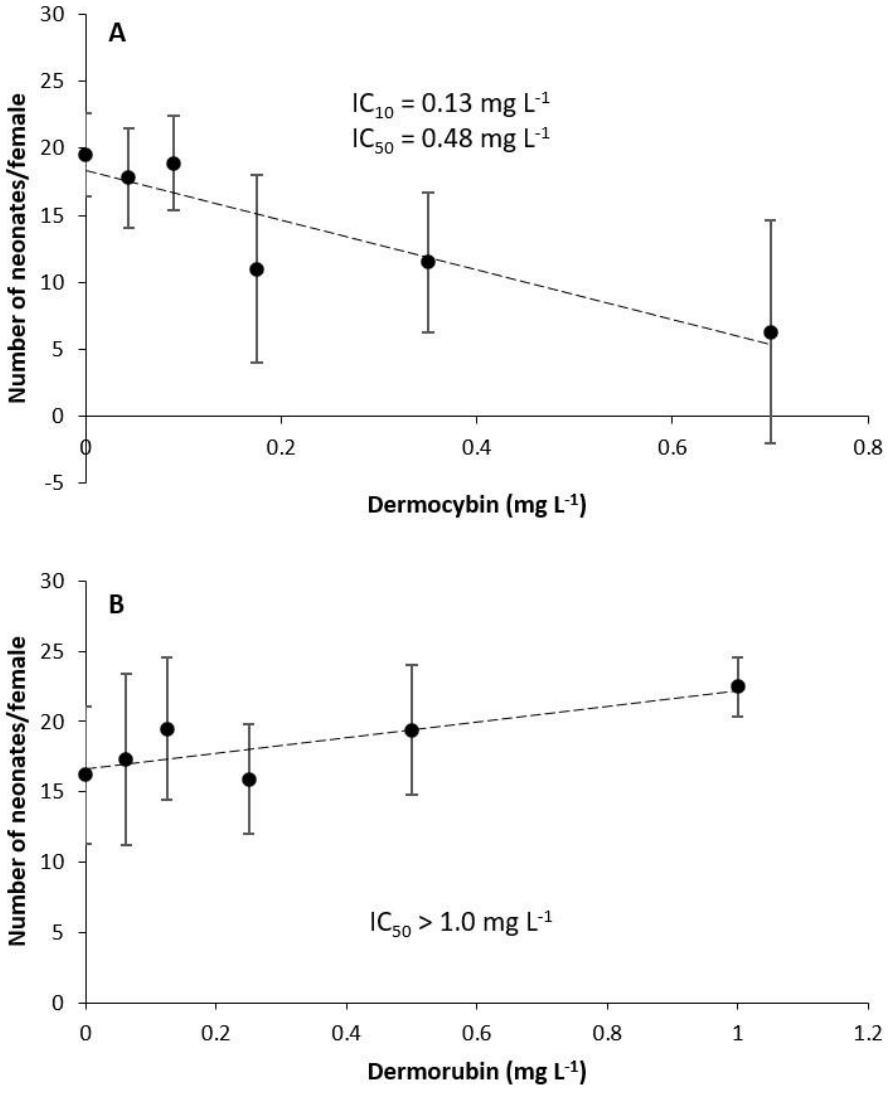
Both dyes did not show inhibition on the growth rate of *R. subcapitata* after 72 h of exposure (Table S6) with IC_{50} > 0.7 and > 1 mg L^{-1} for dermocybin and dermorubin, respectively. In a study conducted by Freeman et al., (2021), an IC_{50} of 8.9 mg L^{-1} was reported for madder with *P. subcapitata* (=*Raphidocelis subcapitata*). Four synthetic anthraquinones were tested using *P. subcapitata*, disperse blue 3 (IC_{50} of 0.5 mg L^{-1}) and remazol brilliant blue R (IC_{50} of 81.1 mg L^{-1}) (Novotný et al., 2006). de Luna et al., (2014) reported an IC_{50} of 5.6 mg L^{-1} for vat green 3 and Croce et al., (2017) reported an IC_{50} of 102.4 mg L^{-1} for acid violet 48

(Table 1). Those IC_{50} for synthetic anthraquinones should be viewed with caution because dye purity was less than 90% or not informed.

Ceriodaphnia dubia

This is the first report of chronic toxicity tests with *C. dubia* of natural anthraquinone dyes. Chronic exposure (7 d) to dermocybin impacted survival, with mortality of 70% of the organisms at 0.7 mg L^{-1} and reproduction, reducing the mean number of neonates, with an IC_{10} of 0.13 mg L^{-1} and IC_{50} of 0.48 mg L^{-1} (Figure 6A). de Luna et al., (2014) reported a similar IC_{50} of 0.5 mg L^{-1} for the synthetic anthraquinone vat green 3, but the purity was 55% (Table 1). Dermorubin did not inhibit the reproduction of *C. dubia* at tested concentrations, IC_{50} >1.0 mg L^{-1} (Figure 6B).

Figure 6 - Concentration × response curve for reproduction (mean of neonates/female) of *C. dubia* exposed to dermocybin (A) and dermorubin (B).



Besides the aquatic toxicity data presented in this work, it is worthwhile to state that both dyes were evaluated for mutagenicity using a miniaturized version of the *Salmonella*/microsome assay and provided negative results for the strains TA98, TA100, YG1041, TA97a and TA1537 in the presence and absence of metabolic activation (Herrala et al., 2022).

4.3.3 Quality assessment of ecotoxicity data and PNEC derivation

In dyeing processes, especially in textile production, large amounts of effluents are generated. They can contain residual dyes and consequently they can be released to the aquatic environment if the wastewater is not adequately pre-treated (Umbuzeiro et al., 2005; Vacchi et al., 2016). If these dyes are marketed, it is important to monitor them in the aquatic environment to protect the aquatic biota. For this, PNECs can be used as benchmarks in monitoring activities because they represent the concentration below which no toxic effect is expected to occur (Lepper, 2002).

With this in mind, a PNEC of 0.0026 mg L^{-1} was proposed for dermocybin, the only dye that presented toxicity in this study. To calculate the PNEC, we considered the results of the most sensitive organism obtained in the chronic tests (*C. dubia* IC_{10} = 0.13 mg L^{-1}) applying an assessment factor (AF) of 50 as we have results from chronic tests from two trophic levels (algae and crustacea) (Table 1). Confronting the calculated PNEC with the results obtained in the acute toxicity assessment, it seems protective as the dye was more toxic with crustacea (*D. similis*) than with fish (*D. rerio*) (Figures 2A, 5; Tables 1, S2).

The CRED evaluation method was applied to verify the reliability and relevance of the acute and chronic studies used in the PNEC derivation (Supplementary Material I – CRED guidance, Supplementary Material II – acute assay *Daphnia similis,* Supplementary Material III – chronic assay *Ceriodaphnia subcapitata,* and Supplemenetary Material IV – chronic assay *Raphidocelis subcapitata*). The studies fulfilled ≥ 17 of the 20 reliability criteria (*i.e.*, being strictly no GLP-certified laboratories study, using nominal concentrations and statistically significant response for *R. subcapitata*) and 11 of the 13 relevance criteria (the other two not being applicable). Therefore, dermocybin toxicity data was assigned to be reliable and relevant to derive a scientific sound PNEC.

As dermorubin did not show toxicity to any of the organisms evaluated in this study it was not possible to calculate a PNEC.

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued)

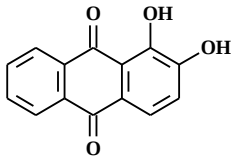
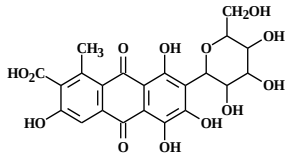
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Natural	Madder	Main component alizarin 89.8	12640-73-2		*D. similis*	4.4 (4.1 – 4.7)	-	Freeman et al., 2021
					P. subcapitata	-	8.9 (8.5 – 9.3)	
	Cochineal Red E120	Not informed	1343-78-8		*D. rerio*	Not toxic >1,200	-	Motta et al., 2019
					A. salina	Not toxic >1,200	-	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

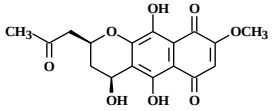
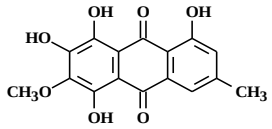
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Natural	Erythrostomin-one	88.5	26153-04-8		*D. rerio*	26.7 (22.7 – 31.5)	-	Abe et al., 2017
					D. magna	19.7 (15.7 – 24.9)	-	Abe et al., 2019
	Dermocybin	99.7	7229-69-8		*D. similis*	0.51 (0.20 – 1.55)	-	This report
					P. hawaiensis	> 0.7	-	
					D. rerio	2.44 (1.24 – 9.79)*	-	
					R. subcapitata	-	> 0.7	
					C. dubia	-	0.48 (0.15 – 0.81)	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

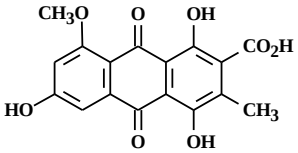
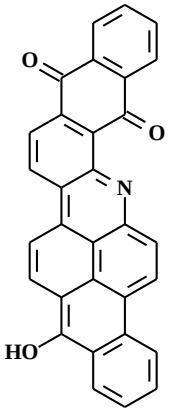
Origin	**Dye**	**Purity %**	**CAS Number**	**Chemical structure**	**Organism Test**	**Acute EC_{50} [mg L^{-1}] (C.I.)**	**Chronic IC_{50} [mg L^{-1}] (C.I.)**	**Reference**
Natural	Dermorubin	98.1	Not registered		*D. similis*	> 1.0	-	This report
					P. hawaiensis	> 1.0	-	
					D. rerio	> 1.0	-	
					R. subcapitata	-	> 1.0	
					C. dubia	-	> 1.0	
Synthetic	C.I. Vat Green 3	55	3271-76-9		*P. subcapitata*	-	5.6 (0.8 – 6.6)	de Luna et al., 2014
					D. similis	6.9 (6.3 – 7.6)	-	
					C. dubia	-	0.5 (0.15 – 0.86)	
		Not informed			*D. rerio*	Not toxic > 100.0	-	de Oliveira et al., 2016

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

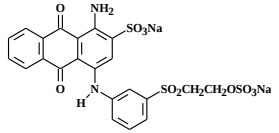
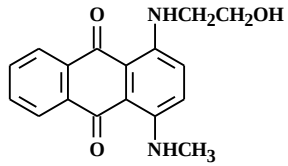
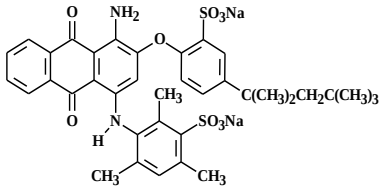
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Synthetic	Remazol Brilliant Blue R (C.I. Reactive Blue 19)	Not informed	2580-78-1		*P. subcapitata*	-	81.1 ± 3.5	Novotný et al., 2006
	C.I. Disperse Blue 3	Not informed	2475-46-9		*P. subcapitata*	-	0.5 ± 0.0	
		Not informed			*Pimephales promelas*	1.0	-	Little and Lamb, 1972
	C.I. Acid Violet 48	> 7 0	12220-51-8		*Daphnia magna*	32.8 (31.6 – 34.2)	-	Croce et al., 2017
					P. subcapitata	-	102.4 (96.7 – 113.4)	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

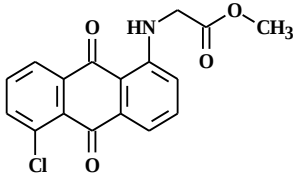
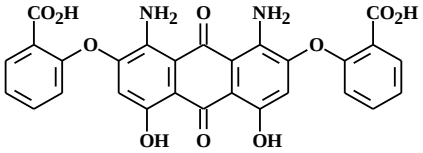
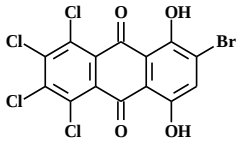
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Synthetic	Experimental dye 5	79	Not registered		*D. similis*	0.94 (0.63 – 0.99)	-	Umbuzeiro et al., 2019
	Experimental dye 42	100	208657-17-4			Not toxic > 6.4	-	
	Experimental dye 145	86	Not registered			0.57 (0.33 – 0.94)	-	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

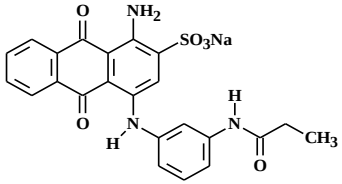
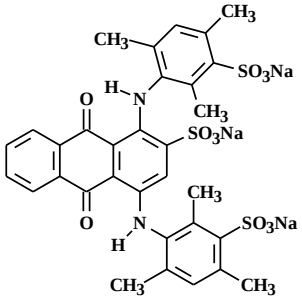
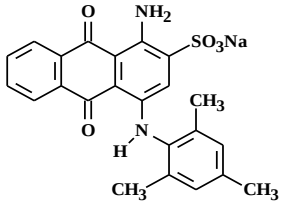
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Synthetic	Experimental dye 183	100	Not registered		*D. similis*	Not toxic > 8.3	-	Umbuzeiro et al., 2019
	C.I. Acid Blue 80	40	4474-24-2		*P. promelas*	Not toxic >10.0	-	Parrott et al., 2016
	C.I. Acid Blue 129	25	6397-02-0				-	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

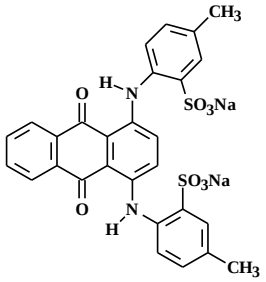
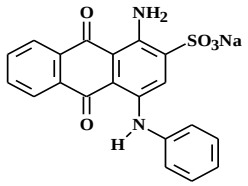
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Synthetic	C.I. Acid Green 25	Not informed	4403-90-1		*P. promelas*	6.2	-	Little and Lamb 1972
	C.I. Acid Blue 25	Not informed	6408-78-2			12	-	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (to be continued).

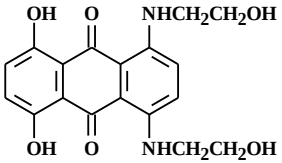
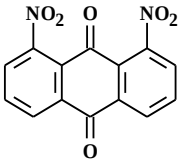
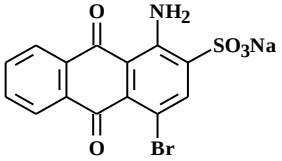
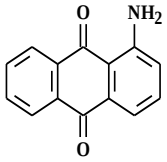
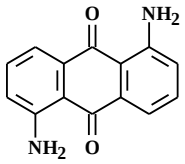
Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Synthetic	C.I. Disperse Blue 7	Not informed	3179-90-6		*P. promelas*	52	-	Little and Lamb 1972
	1,8-Dinitro-anthraquinone	97.6	129-39-5		*D. magna*	> 1.2	-	Wang et al., 2009
	Alizarine Cyanol Grey G (Bromamine acid)	95.3	116-81-4			0.27	-	

Table 1 - Comparison of the toxicity of dermorubin and dermocybin with other natural and synthetic anthraquinone dyes from studies found in the literature. The table presents dye's origin, name used by the authors, purity (%), CAS number, chemical structure, model organism used and toxicity expressed in EC_{50} (acute) and IC_{50} (chronic) (concluded).

Origin	Dye	Purity %	CAS Number	Chemical structure	Organism Test	Acute EC_{50} [mg L^{-1}] (C.I.)	Chronic IC_{50} [mg L^{-1}] (C.I.)	Reference
Synthetic	Fast Red AL (Smoke Orange G)	98.3	82-45-1		*D. magna*	0.14	-	Wang et al., 2009
	C.I. Disperse Red II (Smoke Red F)	95.1	129-44-2			0.034	-	

*Extrapolated EC_{50} for *D. rerio* embryos; - not tested

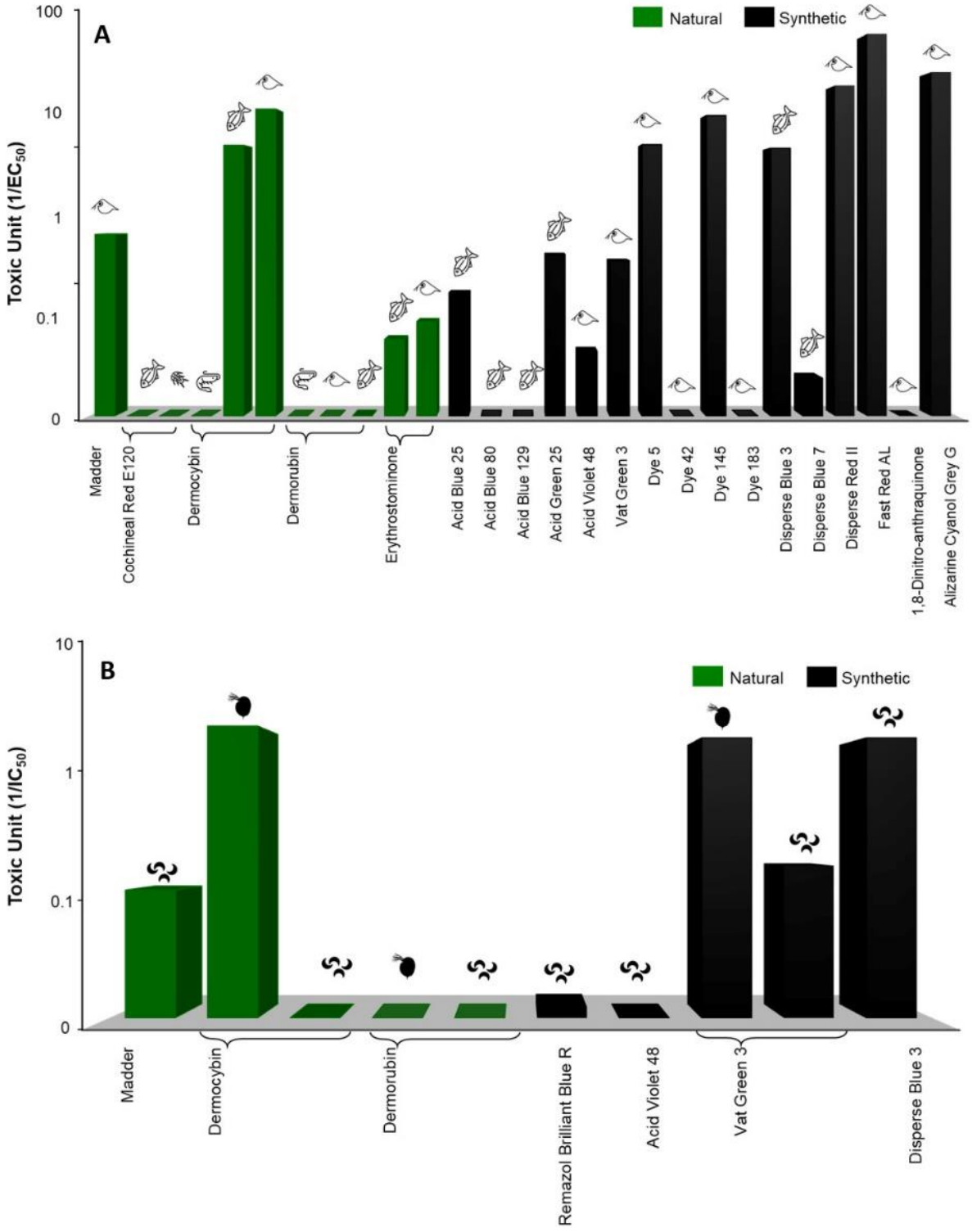
4.4. Final considerations

This work contributed data pertaining to the aquatic toxicity of two highly purified natural anthraquinone dyes (dermocybin and dermorubin). Dermocybin was toxic to freshwater microcrustacea *D. similis*/*C. dubia* and fish (*D. rerio*). But it was not toxic to the freshwater algae *R. subcapitata* or to the marine crustacean *P. hawaiensis*. A PNEC of 0.0026 mg L^{-1} was calculated using toxicity data that was assigned to be reliable and relevant to derive a scientifically sound PNEC according to CRED. Therefore, the proposed PNEC can be used for future risk assessment in case of dermocybin is selected to be used in commercial applications and eventually released to the aquatic environment.

Dermorubin seems to be the more promising natural anthraquinone dye as it was not toxic to any of the aquatic organisms included in this study, was not mutagenic and is suitable for use in waterless dyeing processes, such as supercritical CO_2 (Herrala et al., 2022).

Even with the limited number of anthraquinone dyes already tested for aquatic toxicity, we found that the dye's origin, natural or synthetic, is not directly related to its toxicity, because natural dyes can be as toxic as synthetic ones. For example, dermocybin, madder and erythrostominone presented similar acute toxicity as some synthetic dyes (Figure 7A). When chronic effects were considered, dermocybin was more toxic than any of the synthetic anthraquinones found in the literature (Figure 7B).

Figure 7 - Acute (7A) and chronic toxicity (7B) of natural (green) and synthetic (black) anthraquinone dyes expressed in toxic units ($1/EC_{50}$, for acute and $1/IC_{50}$ for chronic tests). Organisms are represented as (algae), (*Daphnia*/*Ceriodaphnia*), (*Parhyale*), (artemia) and (fish).



It seems that *Daphnia*/*Ceriodaphnia* are more sensitive to anthraquinones than other species but because data is so limited it is hard to find a general trend. As already indicated by Umbuzeiro et al., (2019), more data, of good quality (*e.g.*, highly purified dyes) on the toxicity of dyes are still needed. This would also allow the development of reliable QSAR (quantitative structure–activity relationships) predictions, for both acute and chronic effects that could be used in the prospection of new colourants, from natural or synthetic origin.

We conclude that natural is not always better in terms of aquatic toxicity and any dye, either natural or synthetic, should have their hazard fully assessed before going to the market. Furthermore, we highlight the need of more studies on anthraquinone dyes both from natural and synthetic origin.

Supplementary Material I

Criteria for Reporting and Evaluating ecotoxicity Data (CRED) - Explanatory guidance

Evaluated study (full reference):

Test substance:

Evaluated test:

Evaluated test species:

Evaluated test endpoint(s):

Evaluator (institution):

Please note that the questions and explanations / examples in the following are mainly based on the CRED system (Moermond et al. 2015). A weighting of criteria / questions has been included as suggested by Hobbs et al. (2005) and Breton et al. (2009). An overall score is derived for the evaluated data. Based on this score and, for reliability, the results of the evaluation of the data with regard to two cut-off criteria, a relevance and reliability class are proposed by the excel tool.

A. Relevance of the data

Remark: Relevance of a study mainly depends on the scope of the assessment / the regulatory framework, for which the study is evaluated. The following 12 questions should therefore be answered in the context of the overall assessment.

1 Is the tested species relevant for the compartment under evaluation?

Example: An aquatic species should be tested to evaluate risks for the aquatic environment.

2 Are the tested organisms relevant for the tested compound?

Example: In case of an ERA for an antibiotic, cyanobacteria should be used as test species instead of algae.

3 Are the reported endpoints appropriate for the regulatory purpose?

Example: Acute effects on aquatic organisms are not relevant for the environmental risk assessment of human pharmaceuticals.

4 Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?

Explanation: When a risk assessment is performed for a substance, for which information is available on a specific mode of action that is considered relevant for environmental organisms, studies including endpoints assessing this particular mode of action are most appropriate. For instance, if an API is known to affect reproduction of vertebrates, the endpoints of the fish early life stage test may not be appropriate. Instead, fish tests should include endpoints such as vitellgenin levels, secondary sex characteristics, sex ratio and reproduction depending on the specific mode of action of the substance (OECD 2012).

5 Is the effect relevant on a population level?

Explanation: Endpoints of the guideline studies, on which the ERA of human pharmaceuticals is based, are generally population relevant. For non-standard tests, population relevance has to be evaluated on a case-by-case basis.

6 Is the recorded effect statistically significant and biologically relevant?

Explanation: In the context of environmental risk assessment, a biologically relevant effect is an effect that is important and meaningful for environmental health (EFSA 2011). In a test system with relatively little control variation, minor changes may be statistically significant without necessarily being biologically relevant. To evaluate risks caused by chronic exposure, NOEC or EC_{10} values are used, while EC_{50} values are not appropriate. For the EC_{10}, it has to be evaluated on a case-by-case basis, if the effect is within biological variation of the control response. To evaluate risks caused by acute exposure (note that this is only relevant for some terrestrial tests with human pharmaceuticals), EC_{50} values are preferred.

7 Are appropriate life-stages studied?

Explanation/example: The tested life stage should be (a) appropriate for the selected test and test design and (b) relevant for the expected effect of the API. For instance, fish early life stages are not appropriate for studying effects on reproduction.

8 Are the test conditions appropriate for the tested species and relevant for the assessment?

Explanation/example: Test organisms should be tested under appropriate conditions. For instance, freshwater species should be tested in freshwater, and saltwater species in saltwater. If a test with freshwater or saltwater species is required depends on the scope of the assessment.

9 Is the timing and duration of exposure relevant and appropriate for the studied endpoints and species?

Explanation: The required exposure time should be appropriate for the test organism and the studied endpoint. Chronic studies should include sensitive life stages or cover the whole life cycle.

10 If recovery is studied, is this relevant for the framework for which the study is evaluated?

Explanation: In most regulatory frameworks (including the environmental risk assessment of human pharmaceuticals), recovery is not relevant (exception: authorisation of plant protection products).

11 Is the substance tested representative and relevant for the substance being assessed?

Explanation: Sufficient information should be provided to allow a clear identification of the test item. A substance may be tested as pure active substance or in a formulation. Tests performed with formulations are relevant for plant protection products, but less relevant within many other regulatory frameworks. Studies with mixtures of different substances are relevant for assessing toxicity of these mixtures, but not for assessing the individual substances contained in the mixture. For salts, the counter ion may influence toxicity. For pro-drugs, the active moiety and, if entering the environment in >10% of the administered does, the pro-drug need to be assessed (EMA/CHMP 2011). Depending on the regulatory framework, effects of transformation products may need to be considered. If the substance causing the effect is not the substance being assessed, expert judgement is needed to decide on how to deal with the results of the study and the resulting risk assessment.

12 Is the tested exposure route relevant for the assessment?

Explanation/example: The exposure route should be appropriate for the assessment. For instance, exposure by injection is generally not appropriate (Harris et al. 2014). For pharmaceuticals, exposure should be continuous. Intermittent exposure is generally not relevant. Exposure duration has to be sufficiently long. However, note that acute tests with some terrestrial organisms are also required in the environmental risk assessment of human pharmaceuticals.

B. Reliability of the data

	General information	
	Remark: Before evaluating the test, please check the physico-chemical characteristics of the test substance (what is the solubility, log K_{OW}, pK_a, is the compound volatile, does it hydrolyse, photolyse etc.?)	
1	**Use of a standard (e.g. OECD, ISO, US EPA) or modified standard method:**	
	Explanation: Please answer questions a-c with 'yes' or 'no'.	
	a **Is an unmodified standard method used?**	
	b **Is a slightly modified standard method used?**	
	c **Is a substantially modified standard method used?**	
2	**Is the test, including chemical analysis of the test substance where required, performed under GLP conditions?**	
3	**Validity criteria:**	
	a **Are all validity criteria fulfilled if applicable?**	
	Explanation: For standard tests, compliance with the validity criteria of the guideline is crucial for a study to be considered as reliable. Please check the corresponding test guideline where relevant. For non-guideline tests with standard species, validity criteria as described in a guideline for a similar test should be met if applicable.	
	b **Are validity criteria clearly failed?**	
	Explanation: If one or more validity criteria are clearly failed, a test is classified as '3' (not reliable).	
4	**Inclusion of appropriate controls:**	

Explanation: It depends on the test substance and test type which controls should be included; please check the corresponding test guideline where relevant. In addition to the negative control, a solvent control has to be included in all cases where a solvent is used. The concentration of solvent in the solvent control should correspond to the highest solvent concentration used in the test treatments. In some tests, a positive control with a reference substance is required. For standard tests, the corresponding guidelines provide information on how the controls should perform, e.g. with regard to survival, growth or reproduction. For non-standard tests and non-standard test organisms, expert judgement is needed to decide if performance of the controls is acceptable. Performance of the solvent control should preferably not differ significantly from performance of the negative control. If performance of one or several control(s) has already been covered in question 3a (validity criteria), question 4b, d and/or f should be answered with 'Not applicable' to avoid that this aspect is considered twice when deriving the overall quality score.

	a	**Was a negative control included?**
	b	**Was performance of the negative control acceptable?**
	c	**Was a positive control included, if required?**
	d	**Was performance of the positive control acceptable?**
	e	**Was a solvent control included, if required?**
	f	**Was performance of the solvent control acceptable?**
	Test substance	
5	**a**	**Is the test substance clearly identified with either name, CAS-number or SMILES code and, where relevant, information on stereochemistry?**

Explanation/example: If the salt of an API was tested, information on the type of salt should be provided. If the test substance is not clearly identified, a test is classified as '3' (not reliable).

	b	**Is it specified if test concentrations relate to free acid / free base or salt where relevant?**
6	**a**	**Is the purity of the test substance reported and in an acceptable range (>95%)?**
	b	**Is the source of the test substance reported and trustworthy?**
7	**If a formulation is used or if impurities are present:**	
	a	**Can it be excluded that other ingredients in the formulation or impurities exert an effect?**
	b	**Is the amount of test substance in the formulation indicated?**
	Test organism	
8	**Description of the test organisms:**	
	a	**Is the test species clearly identified?**

		Explanation: If the test species is not clearly identified, a test is classified as '3' (not reliable).
	b	**For algae: is mean cell density at the test start within an appropriate range? For other test organisms: Is mean body weight/length of the test organism in an appropriate range?**
		Explanation for 8 b-e: For standard tests, the corresponding guidelines provide information on required range of mean cell densities, age / life stage of the test organisms etc. at the test start.
	c	**Is age/life stage of the organisms at test start reported and in the required range, where appropriate (e.g. not for algae)?**
	d	**Is sex of the test organisms reported and is sex ratio appropriate, where relevant (e.g. when evaluating sexual-endocrine effects)?**
	e	**Is the species strain reported where required?**
9	**a**	**Are the test organisms from a reliable source?** **For field collected organisms: is the site of origin well-described?**
	b	**Have the organisms been acclimatized to test conditions (e.g. water type, temperature) before the start of exposure, where relevant? For tests with embryonic stages: have the parental organisms been held at appropriate conditions?**
	c	**Are the test organisms exempt from previous exposure or any other kind of stressor?**
	Experimental system and chemical analysis	
10	**Appropriateness of the experimental system for the test substance:**	
	a	**Is the type of exposure (e.g. static, semi-static, flow-through) appropriate for the test substance, taking its physico-chemical characteristics into account?**
		Explanation: Static systems are in most cases only appropriate for short-term tests (exception: water/sediment tests). Where appropriate, guideline requirements should be followed.
	b	**In case that the test substance is a difficult substance as defined in OECD (2000): is the selected test system appropriate for testing of this substance?**
		Explanation: Difficult test substances are substances which are e.g. poorly water soluble, volatile, photo-degradable, hydrolytically unstable, oxidizable, biodegradable, complexing or strongly adsorbing to surfaces of test vessels etc. In order to obtain reliable test results with such substances, test systems generally have to be adapted to take the difficult properties of the substance into account (e.g. by using a closed test system without headspace for volatile substances). For further details, please see OECD (2000). It has to be verified on a case-by-case basis, if the used test system is appropriate for the test substance.
	c	**For ionisable substances: has the test been performed in an appropriate pH-range?**

Explanation: Relatively small changes in pH can significantly alter the balance between dissociated and non-dissociated forms of some substances. An altered dissociation equilibrium may significantly affect the water solubility and the partition coefficient of the substance and hence, its bioavailability and toxicity.
For substances with a pKa that is in the normal pH range of the respective test, a preliminary test should be performed to evaluate if the two or more forms of the substance differ in toxicity. The definitive testshould be performed at a pH, within the pH range required for maintaining the health of the test organisms, at which the more toxic form of the test substance prevails. For further guidance, see OECD (2000), section 3.10.

11 Is the experimental system appropriate for the test organism (e.g. choice of medium / test water or soil, feeding, water or soil characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?

Explanation: The general requirements of the test species should be considered with regard to the characteristics of the selected test medium etc. Temperature, pH and oxygen content should be stable and within the appropriate range for the organism (where applicable, check the corresponding guideline). If control performance is not good (e.g. high mortality), this may indicate that test conditions were not appropriate. Where applicable, feeding should follow the guideline requirements, and all excess should be removed after feeding to avoid decreased bioavailability of the test substance.

12 a For aquatic tests: were exposure concentrations below the limit of water solubility?

b For aquatic tests: if a solvent was used, was solvent concentration within the appropriate range (i.e., not higher than 0.01%)?

13 Is a correct spacing between exposure concentrations applied?

Explanation: For standard tests, the corresponding guidelines provide information on the spacing factor. A factor of 3.2 is often recommended. As rule of thumb, the spacing factor should not be >10.

14 Is the exposure duration defined and appropriate?

15 Chemical analysis

a Are chemical analyses performed to a sufficient extent to verify test substance concentrations over the duration of the study where required, and is information on the results of these analyses presented?

Explanation: If required in the corresponding test guideline, nominal test substance concentrations should be verified by chemical analysis. Non-guideline test should be evaluated based on test guidelines for similar tests where appropriate. If it is stated that nominal substance concentrations were verified by chemical analysis, but no information is provided on the results of the chemical analysis, please answer with 'No' and add a comment. If question 15a is answered with 'No', questions 15b-e should be answered with 'Not applicable' to avoid that a test with insufficient chemical analysis receives a lower score than a test without chemical analysis.

b Is an appropriate analytical method used to measure test substance concentrations?

c Are the measured test substance concentrations within the calibration range of the analytical method?

d Are samples analysed from a sufficient number of treatments and controls, and from a sufficient number of time intervals?

Explanation: The frequency of chemical analyses should be evaluated based on the requirements of the corresponding test guideline or, for non-guideline studies, on a guideline for a similar test if appropriate.

	e	**Are test substance concentrations sufficiently stable during the course of the exposure?**

Explanation: Please evaluate according to the requirements of the corresponding test guideline or, for non-guideline studies, a test guideline for a similar test where appropriate.

16		**Is the biomass loading of the organisms in the test system within an appropriate range?**

Explanation: For standard tests, the corresponding guidelines provide information on maximum biomass loading. For non-standard tests / non-standard test species, expert knowledge is required to decide if the loading rate is appropriate.

	Statistical design	
17	**a**	**Is a sufficient number of replicates used for all controls and treatments?**
	b	**Is a sufficient number of organisms per replicate used for all controls and test concentrations?**

Explanation for 17 a and b: For standard tests, the guideline requirements should be followed. When a non-guideline study is evaluated, expert judgement is needed to assess if the study design is appropriate to obtain statistically reliable results.

18		**Are appropriate statistical methods used to derive the effect concentrations?**

Explanation: Generally, a description of the statistical methods is needed to assess the reliability of the test results. For standard tests, the corresponding guideline requirements should be followed. Further guidance is e.g. provided by OECD (2006). When a non-guideline study is evaluated, expert judgment may be needed. EC_X values should not be extrapolated considerably beyond the range of tested concentrations.

19	**a**	**Is a concentration-response curve observed?**

Explanation: The requirement for a concentration-response relationship depends on the objective of the study. If a limit test is performed at one (or two) concentration(s) to verify the lack of toxicity and no toxicity is recorded, a concentration-response relationship is obviously not needed to conclude that the LC_{50} or NOEC is above the highest tested concentration. However, if the intention of the study is to demonstrate an effect, reliability of the test results is higher, if (1) a sufficient number of concentrations have been tested and (2) the observed effect is regularly increasing (or regularly decreasing) with increasing test concentration (i.e., the concentration-response relationship is monotonous). Expert knowledge is needed, if an effect is only observed at the highest tested concentration. Expert knowledge is also needed in the case of non-monotonous concentration-response curves (e.g. U-, J- or inverted U-shaped curves). In such cases, the underlying mechanisms of effects and the reproducibility of the results should be considered (Harris et al. 2014).

	b	**Is the observed effect statistically significant?**

Explanation: The significance level and the statistical method used to evaluate the specific effect should be indicated.

20		**Are sufficient data available to check the calculation of endpoints and (if applicable) fulfilment of the validity criteria (e.g. control data, concentration-response curves)?**

Explanation: If enough data are presented, additional endpoints may be calculated by the assessor if not reported by the author of the study.

Supplementary Material II

CRED evaluation of acute assay with *Daphnia similis*

Acute assay evaluation

CAS Number	7229-69-8
Compound name	Dermocybin
Organism group	Crustaceans
Species	*Daphnia similis*
Marine/Freshwater	Freshwater
Effect Measured	EC_{50}
Relevant endpoint	Immobility
Endpoint concentration/dose	0.51
Endpoint measurement unit	mg L^{-1}
Test duration	48
Test duration unit	hours

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log Kow, pKa, is the compound volatile, does it hydrolyse, photolyse, etc.?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	**(X)**				Yes, the OECD guideline 202, with change in medium composition according Brazilian guideline ABNT/NBR 12713 (2022).

2	Is the test performed under GLP conditions?*		**(X)**			No, although without GLP/ISO certification, the laboratory quality management system follows the requirements of ISO/IEC 17025/2017.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, validity criteria are fulfilled. No mortality was observed in the negative control and no more than 10 per cent in the solvent control.
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. Dimethyl sulphoxide (DMSO) was used in the solvent control at 0.01% maximum concentration according OECD guideline 23 (2019). No toxicity was observed. Supporting Information Figure 2 and Table S2.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance name is Dermocybin, CAS number 7229-69-8. The chemical structure and its purity were confirmed by HPLC-DAD-MS and NMR (13C and 1H) as described by Herrala et al (2022).
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the purity was confirmed by MS in Herrala (2022).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	**X**				The purified dye (99.7%) was used.
	Test organism					

8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes, the specie scientific name is *Daphnia similis.* The choice of the test organism was justified in the paper published by Umbuzeiro et al. 2019. The age/life stage used was less than 24 h old neonates
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes, the organisms were donated by Dr. Francine Vacchi from Mérieux NutriScience, Brazil. Test organisms were exempt from previous stressors. They are held in our laboratory according to recommended culture conditions (ABNT/NBR 12713, 2022).
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	X				Yes, the test system was appropriate since the substance is not volatile. Moreover, the substance is a sparingly water-soluble dye, so a solvent was used to guarantee that the substance was dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	X				Yes, the experimental system was appropriate for the test organism, i.e. according to the OECD test guidance.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	X				Yes, exposure concentrations were below the limit of water solubility of 0.7 mg L^{-1}. A solvent control (DMSO) was performmed at 0.01%.
13	Is a correct spacing between exposure concentrations applied?	X				Yes, a dilution factor of 2 was used. The concentrations were 0.040, 0.090, 0.175, 0.350 and 0.700 mg L^{-1}.
14	Is the exposure duration defined?	X				Yes, the exposure duration was 48 hours under static conditions. The light: dark photoperiod of 16:8 hours, under controled temperature (20±2 ºC). No feeding during the test.

15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		X			No. Nominal concentrations were used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	X				Yes, in culture 30 organisms are maintained per liter. In the test the ratio is 1 organism to 2 mL. The limit of 20 organisms divided into 4 groups of 5 organisms was respected, according to OECD 202 (2004).
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	X				Yes, 4 replicates with 5 organism per replicate were used for the control and each concentration.
18	Are appropriate statistical methods used?	X				Yes, the EC50 was calculated by a logistic regression.
19	Is a dose-response curve observed? Is the response statistically significant?	(X)				Yes, a concentration-response curve was observed. The response was statistically significant ($p < 0.05$). Please see Supporting Information Figure 2.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	X				Yes, raw data is available. Please see Supporting Information Table S2.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				Despite that only nominal concentrations were used; the test has been conducted and recorded according to the OECD standard guideline.

CRED evaluation method for relevance to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments

	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	**X**				Yes, *Daphnia similis* is a primary consumer and a standard test organism in aquatic risk assessment.
2	Are the organisms tested relevant for the tested compound?	**X**				Yes, the species group could be exposed to the dye.
3	Are the reported endpoints appropriate for the regulatory purpose?	**X**				Yes, short term EC50 on immobility is commonly used to assess acute effects for Daphnids.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the endpoint used is representative for acute toxicity.
5	Is the effect relevant on a population level?	**X**				Yes, immobility is relevant for the population level.
6	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the short term EC50 represents the concentration at which effect at 50% of organisms is expected.
7	Are appropriate life-stages studied?	**X**				Yes, neonates are expected to be the most sensitive life stage.
8	Are the experimental conditions relevant for the tested species?	**X**				Yes, static exposure is a common exposure scenario in risk assessment.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	**X**				Yes, this is the standard time of exposure for acute tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			**X**		Not applicable.
	Exposure relevance					

11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			**X**		Not applicable. The purify substance was used.
12	Is the tested exposure scenario relevant for the substance?	**X**				Yes, acute toxicity can be expected from the continues input of this compound in water.
13	Is the tested exposure scenario relevant for the species?	**X**				Yes, the standard exposure scenario for this species was used.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				The test has been conducted and recorded according to the OECD standard guideline.

Supplementary Material III

CRED evaluation of chronic assay with *Ceriodaphnia dubia*

Chronic assay evaluation

CAS Number	7229-69-8
Compound name	Dermocybin
Organism group	Crustaceans
Species	*Ceriodaphnia dubia*
Marine/Freshwater	Freshwater
Effect Measured	IC_{10}
Relevant endpoint	Reproduction
Endpoint concentration/dose	0.1
Endpoint measurement unit	mg L^{-1}
Test duration	7
Test duration unit	Days

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log Kow, pKa, is the compound volatile, does it hydrolyse, photolyse, etc.?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	**(X)**				Yes, the USEPA Method 1002.0 (2002), with culture and test medium according ABNT/NBR 13373 (2017).

2	Is the test performed under GLP conditions?*		**(X)**			No, although without GLP/ISO certification, the laboratory quality management system follows the requirements of ISO/IEC 17025/2017.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, validity criteria are fulfilled. Survival of 80% and at least 15 neonates/female on average in the controls (negative and solvent).
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. Dimethyl sulphoxide (DMSO) was used in the solvent control at 0.01% maximum concentration according OECD guideline 23 (2019). No toxicity was observed. Supporting Information Figure 7 and Table S5.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance name is Dermocybin, CAS number 7229-69-8. The chemical structure and its purity were confirmed by HPLC-DAD-MS and NMR (13C and 1H) as described by Herrala et al (2022).
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the purity was confirmed by MS in Herrala (2022).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	**X**				The purified dye (99.7%) was used.
	Test organism					

8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes, the specie scientific name is *Ceriodaphnia dubia*. The age/life stage used was less than 24 h old neonates.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes, the organisms were donated by Dr. Francine Vacchi from Mérieux NutriScience, Brazil. Test organisms were exempt from previous stressors. They are held in our laboratory according to recommended culture conditions (ABNT/NBR 13373, 2017).
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	X				Yes, the test system was appropriate since the substance is not volatile. Moreover, the substance is a sparingly water-soluble dye, so a solvent was used to guarantee that the substance was dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	X				Yes, the experimental system was appropriate for the test organism, i.e. according to the USEPA test guidance.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	X				Yes, exposure concentrations were below the limit of water solubility of 0.7 mg L^{-1}. A solvent control (DMSO) was performed at 0.01%.
13	Is a correct spacing between exposure concentrations applied?	X				Yes, a dilution factor of 2 was used. The concentrations were 0.040, 0.090, 0.175, 0.350 and 0.700 mg L^{-1}.
14	Is the exposure duration defined?	X				Yes, the exposure duration was 7 days under semi-static conditions with test solutions renewed 3 times/week. The light: dark

						photoperiod of 16:8 hours, under controled temperature (25±2 ºC). Organisms were fed daily with *Raphidocelis subcapitata* and compound feed.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		**X**			No. Nominal concentrations were used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	**X**				Yes, mass cuture (30 organisms/1 L) maintained as a source of organism and individual cultures were used for toxicity tests (1 neonate/15 mL glass beaker), according USEPA Method 1002.0 (2002).
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	**X**				Yes, 10 replicates with 1 organism per replicate were used for the controls and for each treatment.
18	Are appropriate statistical methods used?	**X**				Yes, the IC10 was calculated by four parameters log logistic function
19	Is a dose-response curve observed? Is the response statistically significant?	**X**				Yes, a concentration-response curve was observed. The response was statistically significant ($p < 0.05$). Please see Results and Discussion section, Figure 6.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	**X**				Yes, raw data is available. Please see Supporting Information Table S7.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				Despite that only nominal concentrations were used; the test has been conducted and recorded according to the USEPA standard guideline.

CRED evaluation method for relevance to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	**X**				Yes, *Ceriodaphnia dubia* is a primary consumer and a standard test organism in aquatic risk assessment.
2	Are the organisms tested relevant for the tested compound?	**X**				Yes, the species group could be exposed to the dye.
3	Are the reported endpoints appropriate for the regulatory purpose?	**X**				Yes, long term IC10 on reproduction is commonly used to assess chronic effects for Daphnids.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the endpoint reproduction inhibition is representative for chronic toxicity.
5	Is the effect relevant on a population level?	**X**				Yes, reproduction is a relevant endpoint for the population level.
6	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the long term IC10 represents the concentration at which effect at 10% of organisms is expected.
7	Are appropriate life-stages studied?	**X**				Yes, neonates are expected to be the most sensitive life stage.

8	Are the experimental conditions relevant for the tested species?	X				Yes, semi static exposure is a common exposure scenario in risk assessment.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	X				Yes, this is the standard time of exposure for chronic tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			X		Not applicable.
	Exposure relevance					
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			X		Not applicable. The purify substance was used.
12	Is the tested exposure scenario relevant for the substance?	X				Yes, chronic toxicity can be expected from constant exposure to the compound throughout the organism's entire or partial life cycle
13	Is the tested exposure scenario relevant for the species?	X				Yes, the standard exposure scenario for this species was used.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				The test has been conducted and recorded according to the USEPA standard guideline.

Supplementary Material IV

CRED evaluation of chronic assay with *Raphidocelis subcapitata*

Chronic assay evaluation

CAS Number	7229-69-8
Compound name	Dermocybin
Organism group	Clorophyta
Species	*Raphidocelis subcapitata*
Marine/Freshwater	Freshwater
Effect Measured	IC_{10}
Relevant endpoint	Inhibition of growth
Endpoint concentration/dose	>0.7
Endpoint measurement unit	mg L^{-1}
Test duration	72
Test duration unit	hours

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log Kow, pKa, is the compound volatile, does it hydrolyse, photolyse, etc.?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	X				Yes, OECD n° 201 (OECD 201, 2011) was used for tests, with culture and test medium according

						ABNT/NBR 12648 (ABNT, 2018).
2	Is the test performed under GLP conditions?*		**(X)**			No, although without GLP/ISO certification, the laboratory quality management system follows the requirements of ISO/IEC 17025/2017.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, validity criteria are fulfilled. The biomass in the control increased exponentially a factor of at least 16 within the test finish.
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. Dimethyl sulphoxide (DMSO) was used in the solvent control at 0.01% maximum concentration according OECD guideline 23 (2019). No toxicity was observed. Supporting Information Table S6.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance name is Dermocybin, CAS number 7229-69-8. The chemical structure and its purity were confirmed by HPLC-DAD-MS and NMR (13C and 1H) as described by Herrala et al (2022).
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the purity was confirmed by MS in Herrala et al. (2022).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation	**X**				The purified dye (99.7%) was used.

	exert an effect? Is the amount of test substance in the formulation known?					
	Test organism					
8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	**X**				Yes, the specie scientific name is *Raphidocelis subcapiata*. The 3-days old algae culture in logarithmic growth phase was used in the tests.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	**X**				Yes, the algae were obtained by Prof. Armando A.H. Vieira from Physiology Laboratory, Department of Botany from University of São Carlos (UFSCAR), in 2004.
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	**X**				Yes, the test system was appropriate since the substance is not volatile. Moreover, the substance is a sparingly water-soluble dye, so a solvent was used to guarantee that the substance was dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	**X**				Yes, the experimental system was appropriate for the test organism, i.e. according to the OECD n° 201 test guidance.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	**X**				Yes, exposure concentrations were below the limit of water solubility of 0.7 mg L^{-1}. A solvent control (DMSO) was performed at 0.01%.
13	Is a correct spacing between exposure concentrations applied?	**X**				Yes, a dilution factor of ~1.6 was used. The concentrations were 0, 0.1, 0.17, 0.27, 0.44 and 0.7 mg L^{-1}.

14	Is the exposure duration defined?	**X**				Yes, the exposure duration was 72 hours in a static condition without medium renewed, under continuous fluorescent light (4,000 ± 400 lux) at 24±2 ºC.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		**X**			No. Nominal concentrations were used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	**X**				Yes, the initial algal biomass in the test was adjusted to 1×10^4 cels mL^{-1} to allow exponential growth, according OECD 201 (2011)
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	**X**				Yes, tests were performed in triplicate.
18	Are appropriate statistical methods used?	**X**				Yes, one-way analysis of variance with post-hoc test, was used to identify significant differences ($p \leq 0.05$).
19	Is a dose-response curve observed? Is the response statistically significant?			**(X)**		No, because dermocybin was not toxic at the maximun tested concentration (0.7 mg L^{-1}). Please see Supporting Information section, Table S6.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	**(X)**				Yes, validation criteria were met and controls were also tested, but it was not possible to obtain a concentration-response curve, as we did not observe effect of the dye on the tested concentrations.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				Despite that only nominal concentrations were used; the test has been conducted and recorded according to the OECD 201 standard guideline.

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	**X**				Yes, *Raphidocelis subcapitata* is a producer and a standard test organism in aquatic risk assessment.
2	Are the organisms tested relevant for the tested compound?	**X**				Yes, the species group could be exposed to the dye in the aquatic environment.
3	Are the reported endpoints appropriate for the regulatory purpose?	**X**				Yes, long term IC10 on growth is commonly used to assess chronic effects for algaes.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the endpoint growth inhibition is representative for chronic toxicity.
5	Is the effect relevant on a population level?	**X**				Yes, growth is a relevant endpoint for the population level.
6	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the long term IC10 represents the concentration at which effect at 10% of organisms is expected.

7	Are appropriate life-stages studied?	X				Yes, a 3-days old algae culture and in logarithmic growth phase was used.
8	Are the experimental conditions relevant for the tested species?	X				Yes, static exposure is a common exposure scenario in risk assessment.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	X				Yes, this is the standard time of exposure for chronic tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			X		Not applicable.
	Exposure relevance					
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			X		Not applicable. The purify substance was used.
12	Is the tested exposure scenario relevant for the substance?	X				Yes, chronic toxicity can be expected from constant exposure to the compound throughout the organism's entire or partial life cycle
13	Is the tested exposure scenario relevant for the species?	X				Yes, the standard exposure scenario for this species was used.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				The test has been conducted and recorded according to the OECD 201 standard guideline.

Supplementary Material V

Raw data

Table S1. Chemical analysis for purity determination. Peaks recognition in the HPLC.

Dye	Retention time [min]	Max absorption [nm]
Dermorubin + 5-chloro dermorubin	1.395 (dermorubin) 3.189 (5-chloro dermorubin)	480
Dermocybin	4.508	487

Table S2. Raw data for the acute test of dermocybin to *Daphnia similis* performed for 48 h.

Concentration	**Number of immobilized organisms**				**Total**	**Immobility (%)**
(mg L^{-1})	**1**	**2**	**3**	**4**		
DMSO (0.01%)	1/5	0/5	1/5	0/5	2/20	10
0.040	0/5	1/5	1/5	1/5	3/20	15
0.090	2/5	1/5	1/5	1/5	5/20	25
0.175	0/5	1/5	2/5	3/5	6/20	30
0.350	2/5	3/5	2/5	4/5	11/20	55
0.700	3/5	2/5	3/5	3/5	11/20	55

Table S3. Raw data for the acute test of dermorubin to *Daphnia similis* performed for 48 h

Concentration	**Number of immobilized organisms**				**Total**	**Immobility (%)**
(mg L^{-1})	**1**	**2**	**3**	**4**		
DMSO (0.01%)	0/5	0/5	0/5	0/5	0/20	0
0.01	0/5	0/5	0/5	0/5	0/20	0
0.03	0/5	0/5	0/5	0/5	0/20	0
0.1	0/5	0/5	0/5	0/5	0/20	0
0.3	0/5	0/5	0/5	0/5	0/20	0
1.0	0/5	0/5	0/5	0/5	0/20	0

Table S4. Data for the acute test of dermocybin and dermorubin to *Parhyale hawaiensis* performed for 96 h.

Concentration (mg L^{-1})	Number of dead organisms	Mortality (%)
Dermocybin		
DMSO (0.01%)	0/32	0
0.07	1/32	3.1
0.13	0/32	0
0.22	3/32	9.4
0.4	0/32	0
0.7	0/32	0
Dermorubin		
DMSO (0.01%)	0/32	0
0.10	0/32	0
0.20	1/32	3.1
0.35	0/32	0
0.60	0/32	0
1.00	0/32	0

Table S5. Summary of lethal and sub-lethal effects evaluated for zebrafish embryos/larvae to dermocybin and dermorubin during 168 h.

Endpoints Evaluated	**Time of exposure (168 h post-fertilization)**	
	Dermocybin	**Dermorubin**
Lethal		
Coagulation	**Observed**	
Tail detachment	**Not observed**	**Not observed**
No somite formation		
No heartbeat	**Observed**	
Sub-lethal		
Yolk sac absorption	Not observed	
Yolk sac and heart edema	Not observed	
General delay in development	Not observed	
Tail malformation	Not observed	**Not observed**
Inflated swimming bladder	Not observed	
Otolith formation	Not observed	
Body pigmentation	Not observed	
Loss of equilibrium [a]	Not observed	

[a] Loss of equilibrium = larvae side-lying in the bottom of the microplate well after mechanical stimulus.

Table S6. Data for the chronic test of dermocybin and dermorubin to *Raphidocelis subcapitata* performed for 72 h.

Concentration (mg L^{-1})	**Mean (SD) (Cells mL^{-1})**	**CV (%)**	**Growth inhibition (%)**	**Statistical significance**
Dermocybin				
DMSO (0.01%)	969 (56)	5.8	0.0	-
0.10	1121 (125)	11.2	-15.7	N. S.
0.17	1119 (81)	7.3	-15.4	N. S.
0.27	887 (28)	3.1	8.5	N. S.
0.44	1085 (152)	14.0	-12.0	N. S.
0.70	1060 (123)	11.6	-9.4	N. S.
Dermorubin				
DMSO (0.01%)	711.7 (127.2)	17.9	0.0	-
0.06	788.7 (39.5)	5.0	-10.8	N.S.
0.12	719.7 (26.3)	3.7	-1.1	N.S.
0.25	819.7 (32.0)	3.9	-15.2	N.S.
0.50	866.0 (61.5)	7.1	-21.7	N.S.
1.00	750.3 (57.1)	7.6	-5.4	N.S.

Mean = mean of algae cells/mL; SD = standard deviation; CV = coefficient of variation; N.S. = not statistically significant from control.

Table S7. Raw data for the chronic test of dermocybin to *Ceriodaphnia dubia* performed for 7 days.

Dermocybin (mg L^{-1})	**Survival of female (%)**	**Number of neonates/female**										**Total**	**Mean**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		
DMSO (0.01%)	100	18	20	12	19	11	26	19	16	19	9	169	16.9
0.044	90	19	11	19	12	21	19	16	21	*	22	160	17.8
0.08	100	17	20	20	20	16	15	19	16	18	28	189	18.9
0.175	70	*	*	20	4	12	*	13	8	0	20	77	11.0
0.35	80	*	*	15	15	3	5	8	16	18	12	92	11.5
0.7	30	18	0	*	*	*	*	1	*	*	*	19	6.3

*dead females

Table S8. Raw data for the chronic test of dermorubin to *Ceriodaphnia dubia* performed for 7 days.

Dermorubin (mg L^{-1})	**Survival of female (%)**	**Number of neonates/female**										**Total**	**Mean**
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		
DMSO (0.01%)	90	15	8	20	23	*	10	18	16	21	15	146	16.2
0.0625	100	2	16	21	15	18	18	21	23	23	16	173	17.3
0.125	100	23	21	19	23	27	14	19	14	24	11	195	19.5
0.25	90	12	18	10	18	21	11	*	18	19	16	143	15.9
0.5	100	25	12	15	25	21	18	15	19	19	25	194	19.4
1.0	100	23	25	19	19	22	22	23	24	24	24	225	22.5

*dead females

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Chapter 02

The natural anthraquinone dye emodin: eco/genotoxicological characterization for aquatic organisms

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Manuscript will be submitted in the future with corrections

Abstract

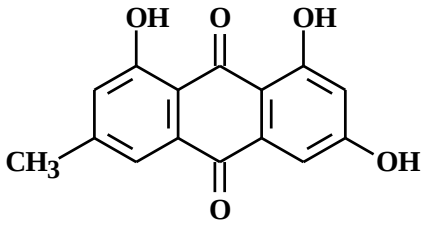
Emodin is a natural anthraquinone compound, and is a secondary metabolite of different species of plants and fungi. It is known for its pharmacological versatility, and in the textile industry for its good dyeing properties. Emodin application in the textile industry can involve the formation and disposal of large volumes of wastewater. Its mutagenic effect is known on bacteria and human cells, but little is known about its toxic and genotoxic/mutagenic effects in aquatic organisms. This work aims to evaluate the eco/genotoxicity of emodin to aquatic organisms. Emodin (99% purity) was extracted from the fungus *Cortinarius sanguineus* and supplied by the BioColour project. Emodin was toxic to *Daphnia similis* (EC_{50} = 0.13 mg L^{-1}) and zebrafish embryos (LC_{50} = 0.025 mg L^{-1}). No toxicity was observed for *Raphidocelis subcapitata*, *Ceriodaphnia dubia* and *Parhyale hawaiensis*. Additional biochemistry studies such as: enzyme activity is needed to elucidate the toxic/mutagenic pathways of emodin in aquatic organisms. In the present study, a PNEC value for emodin was calculated as 0.0005 mg L^{-1}. Besides mutagenic effects in the *Salmonella*/microsome assay, it was mutagenic in the micronuclei assay for the amphipod *P. hawaiensis*. Among the anthraquinone dyes tested to date, natural or synthetic, emodin was the most toxic dye ever found in the literature with low EC/LC_{50} values.

Keywords: natural dye, *Daphnia similis*; *Parhyale hawaiensis*; zebrafish, micronuclei

5.1. Introduction

The anthraquinone emodin is a secondary metabolite present widely in plants (*Rhamnus* spp.) (Abou-chaar and Shamlian, 1980) , terrestrial fungi (*Cortinarius* spp.) (Räisänen, 2019) and marine fungi (*Penicillium* spp.) (Eze et al., 2021). According to Gruber-Dorninger et al. (2017) emodin (Figure 1) is part of a list of emerging mycotoxins with potential health impact. It is a molecule used since ancient times in traditional Chinese medicine and currently by the pharmaceutical industry. It has diverse pharmacological activities, including antineoplastic, anti-inflammatory, anti-angiogenic, antidiabetic, and antimicrobial functions. (Dong et al., 2016; Duan et al., 2017). Emodin has also good dyeing properties and is used in textile coloration to obtain a variety of colors from yellow to red (Räisänen et al., 2021, 2001; Wu and Ke, 2011). Recent studies demonstrated that emodin can be successfully applied in a waterless dyeing medium such as supercritical carbon dioxide (sc-CO_2) (Herrala et al., 2022; Magalhães et al., 2023; Räisänen et al., 2021).

Figure 1 - Chemical structure of the anthraquinone dye emodin



Despite the application advantages of emodin, it is known since the mid-1980s that emodin is a mutagenic compound. In the *Salmonella*/microsome assay, emodin is a frameshift mutagen for TA1537 strain with metabolic activation, and the planar structure of its metabolites cause DNA intercalation (Masuda and Ueno, 1984). Müller et al. (1996) also showed that emodin induced micronucleus in mammalian cells (L5187Y cell line). Due to the mutagenic activities of emodin, which makes difficult the industrial use of this compound, Magalhães et al. (2023) proposed the development of non-mutagenic derivatives of emodin by incorporation of acryloyl groups, but those modified compounds were still mutagenic to the TA1537 strain of *Salmonella* bacteria (Ames test) and induced chromosome alterations (micronuclei) in the aquatic amphipod *Parhyale hawaiensis*.

Unlike waterless dyeing alternatives, conventional textile dyeing methods requires large amounts of water and consequently produces also large amounts of wastewater. If not properly treated, dyes and other harmful compounds can reach public receiving waters. Adverse effects of synthetic and natural dyes have been reported in different aquatic organisms (da Silva Leite

et al., 2016; Farias et al., 2023; Freeman et al., 2021; Vacchi et al., 2016a; Vendemiatti et al., 2021) Dyes were also considered as the main sources of mutagenic activity detected in the Cristais River (Umbuzeiro et al., 2005) and Piracicaba River at São Paulo, Brazil (Vacchi et al., 2016).

Few studies in the literature addressed the ecotoxicological effects of the natural dye, emodin (Chinnasamy et al., 2023; He et al., 2012; Nitulescu et al., 2017). Ecotoxicological data are mandatory to evaluate the environmental risk of chemicals, along with exposure data. They are generated with representative organisms from different trophic levels and the more quantity of data, to avoid the uncertainty associated with the calculation of safe concentrations in the water.

Although the mutagenicity of emodin has already been reported, especially in bacteria and in mammalian cells (Bösch et al., 1987; Brown and Brown, 1976; Masuda and Ueno, 1984; Morita et al., 1988; Müller et al., 1996; Sevcovicova et al., 2014; Tanaka et al., 1987), no information about the genotoxic/mutagenic effects on aquatic organisms was found in the literature. Mutagens in water have the potential to induce alterations at the molecular level, such as DNA base modifications, as well as at the chromosome level, including structural damage, micronuclei, or aneuploidy (Umbuzeiro, Heringa, Zeiger, 2016). Moreover, certain DNA mutations may be passed down through generations, resulting in population-level alterations across various organisms. Therefore, it is important to assess the mutagenicity of emodin for aquatic organisms.

This study aimed to characterize the ecotoxicity of emodin using acute, chronic and embryonic toxicity assays, and its genotoxic/mutagenic activity using comet and micronuclei tests in aquatic invertebrates. Also, we aim to generate a predicted no-effect concentration (PNEC) value for emodin, to assist future risk assessments in the case of its occurrence in water systems.

5.2. Material and Methods

5.2.1 Dyes and reagents

Emodin was supplied by BioColour project (biocolour.fi). It was obtained from the blood-red webcap *Cortinarius sanguineus*, as detailed by Hynninen et al. (2000), and subjected to purification through multiple liquid-liquid partitions as outlined by Hynninen and Räisänen (2001). The confirmation of dye purity and chemical structure was conducted using HPLC-DAD-MS and NMR (13C and 1H) spectroscopy, following the methodology described by Herrala et al. (2022).

Emodin (99%) was dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, purity ≥99.5%) at the limit of solubility (3.0 g L^{-1}). This solution was serially diluted in the appropriated media for each organism. For toxicity tests the maximum DMSO concentration in the test media used was 0.01% according to OECD 23 (2019). Testing concentrations ranged from 0 to 0.3 mg L^{-1}. For the geno/mutagenicity tests, sub-lethal concentrations were determined before testing, with a maximum of 1% DMSO concentration in the test media. DMSO concentrations from 0.01% to 1% (data not shown) revealed that an added volume by 1% was not cytotoxic to *P. hawaiensis* cells, so we could test higher concentrations ranged from 0 to 30 mg L^{-1}.

5.2.2 Acute toxicity tests

The experiments involved freshwater organisms, namely *Daphnia similis* (microcrustacean) and *Danio rerio* (fish embryos), as well as the marine amphipod *Parhyale hawaiensis*. The sensitivity of the cultures was assessed using reference compounds. Only organisms from healthy cultures exhibiting EC/LC_{50} values consistent with laboratory norms were utilized. In the case of zebrafish tests, a positive control group was introduced to validate organism sensitivity, with validation achieved when observing ≥ 30% mortality at the end of the tests. All tests were considered validated if the negative and solvent controls (0.01% DMSO) showed mortality rates up to 10% in acute toxicity tests. The results were expressed in terms of 50% effect or lethal concentration (EC/LC_{50}) values.

The acute toxicity tests were carried out with *D. similis*, adhering to OECD guideline n° 202 (OECD 202, 2004). The cultures were maintained at a temperature of 20 ± 2 °C, with a photoperiod of 16:8 h (light/dark) using Eletrolab EL 2020/4LED chamber. They were daily fed with the microalgae *Raphidocelis subcapitata*, and total water exchange occurred three times per week. The cultures were housed in 2 L glass vessels. For the tests, twenty neonates (<24 h old) from 2- to 3-week-old mothers were placed in four replicates for each concentration (5 organisms/replicate). Solvent and negative controls were included in the tests, utilizing 10 mL glass tubes to prevent dye adsorption to the material. The temperature and photoperiod conditions were consistent with those during cultivation. The organisms were not fed during the test. Emodin concentrations of 0, 0.019, 0.037, 0.075, 0.15, and 0.3 mg L^{-1} were administered, and after 48 h of exposure, immobilized organisms were counted. When the number of immobilized organisms in negative and solvent controls did not exceed 10%, tests were treated as valid. Conductivity, dissolved oxygen, and pH were measured at the beginning

and end of the tests. As a routine procedure in the laboratory, sodium chloride (NaCl, Sigma-Aldrich, ≥99% purity) was utilized as a reference toxicant, and a control chart presenting acute toxicity test results is provided in Supplementary Material I (Figure S1).

Prior to initiating tests with *D. rerio*, the study plans underwent submission and approval by the Ethics Committee of the University of Campinas (UNICAMP) (protocol n° 5645-1/2020).

Danio rerio adults were housed in a recirculating system and maintained in aquariums with reconstituted water composed of reverse osmosis water with a conductivity below 5 µS/s, supplemented with sea salt. The environmental conditions were carefully controlled, with a temperature maintained at 26 ± 1 °C, ammonia levels below 0.01 mg L^{-1}, conductivity at 750 ± 50 mS/cm, pH at 7.0 ± 0.5, and dissolved oxygen at or above 95% saturation. The fish were subjected to a 12-hour photoperiod (light: dark) and were fed daily with commercial food (GEMMA Micro). The fish embryotoxicity test followed the OECD guideline n° 236 "Fish Embryo Toxicity" (FET) test (OECD 236, 2013), with some modifications, including extending the exposure time from 96 h to 168 h to allow for a more comprehensive assessment of locomotor activity (Farias et al., 2019). *Danio rerio* embryos were exposed immediately after fertilization. For a positive control, a reference substance, 4 mg L^{-1} of 3,4-dichloroaniline (Sigma-Aldrich, 98% purity), was used, and a solvent control was also included. The test was conducted in 24-well microplates, with 20 wells filled with 2 mL of the test solution and four wells with reconstituted water (internal plate control, as required in the OECD guideline). Each treatment involved 20 eggs, individually distributed with one egg per well. During tests, microplates were maintained under the same conditions used during cultivation. Emodin concentrations of 0, 0.00375, 0.0075, 0.015, 0.03, and 0.06 mg L^{-1} were used, determined based on preliminary tests to establish a concentration-response curve. Embryos and larvae were observed daily under a stereomicroscope (Stemi 2000-C, Carl Zeiss, Oberkochen, Germany), and developmental parameters were monitored throughout the test period. Evaluated endpoints included lethality (coagulation, tail not detached, malformation of somite, no heartbeat) and sub-lethality (development of eyes, inflated swimming bladder, loss of equilibrium, eye and body pigmentation, edemas, malformation of head, tail and otoliths, deformity of yolk sac, and delay in development).

Cultures of *P. hawaiensis* were maintained at a temperature of 24 ± 2 °C, under a 12-h photoperiod (light: dark), with constant aeration. Daily feeding occurred five days a week using

commercial sinking fish food. Saltwater with a salinity of 30 ± 2 was prepared by reconstituting commercial sea salt brands. Partial water exchanges (50%) were conducted twice a week, while total water exchanges took place monthly during vessel cleaning and substrate renewal. Acute toxicity testing involving *P. hawaiensis* followed the methodology described by Artal et al. (2018). Test solutions were made through serial dilution using reconstituted seawater at a salinity of 30 (prepared with a combination of marine salt and deionized water). For a negative control Reconstituted seawater was used, and a solvent control (DMSO at 0.01%) was also included. For each concentration, neonates (32, ≤ 7 days old) from mothers aged 8 to 10 months were placed in 32 wells, with 200 µL of solution and one organism per replicate. Tests were conducted using the same conditions established for cultivation. Organisms were exposed to emodin concentrations of 0, 0.019, 0.037, 0.075, 0.15, and 0.3 mg L^{-1}. After 96 h, the number of dead organisms was recorded using a stereomicroscope (Stemi 2000-C, Carl Zeiss, Oberkochen, Germany). When mortality in negative and solvent controls did not exceed 10%, tests were treated as valid. Mortality rates higher than 10% were indicative of toxicity. Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$, Sigma-Aldrich, ≥99% purity) served as a reference toxicant, and a control chart featuring acute toxicity test results can be found in Supplementary Material I (Figure S2).

5.2.3 Chronic toxicity tests

Chronic tests were undertaken with freshwater organisms, specifically *Raphidocelis subcapitata* (green microalgae) and *Ceriodaphnia dubia* (microcrustacean). The sensitivity of the cultures employed in the chronic assays was continually assessed using reference compounds. Only organisms from robust cultures demonstrating IC_{50} values in line with laboratory norms were included in the tests. Validation criteria were set, requiring that both negative and solvent controls (0.01% DMSO) exhibit mortality rates up to 20% for the chronic toxicity tests with *C. dubia*. Additionally, in the chronic tests with *R. subcapitata*, the biomass in control groups needed to increase exponentially by a factor of at least 16 by the end of the test for validation. The results of these chronic tests were quantified in terms of 10 and 50% inhibition concentration (IC_{10}/IC_{50}).

Raphidocelis subcapitata stock cultures were preserved in freshwater Oligo medium at 4 °C. The inoculum comprised algae cells harvested from a liquid stock of a 3-day-old algae culture in logarithmic growth phase. The inoculum was incubated at 24 ± 2 °C under continuous fluorescent light (4,000 ± 400 lux), with the algal biomass adjusted to 1×10^4 cells mL^{-1} to

facilitate exponential growth. Tests with *R. subcapitata* were conducted using the same conditions established for cultivation in accordance with OECD n° 201 (OECD 201, 2006) and ABNT/NBR 12648 (ABNT, 2018) standards. The experiments were carried out in Erlenmeyer flasks, with final volume of 20 mL (algae inoculum, enrichment medium and dye), in triplicate. The flasks were subjected to continuous agitation (100-120 rpm) for 72 h without medium renewal. Organisms were exposed to emodin concentrations of 0, 0.019, 0.037, 0.075, 0.15, and 0.3 mg L^{-1}. The growth inhibitory effect was assessed by measuring the inhibition rate, correlating the number of algae cells with the spectrophotometric absorbance at 440 nm using a spectrophotometer (DR3900, Hach, Loveland, United States). Sodium chloride (NaCl, Sigma-Aldrich, ≥99% purity) was employed as a reference toxicant, and a control chart featuring chronic toxicity test results has been provided in Supplementary Material I (Figure S3).

Ceriodaphnia dubia culture was maintained in MS (synthetic medium) at 25 ± 2 °C, with a photoperiod of 16:8 h (light/dark), pH ranging from 7.0 to 7.6, and total hardness set at 40 to 48 mg $CaCO_3$ L^{-1}. Organisms were fed with algae (*Raphidocelis subcapitata*, 2.0×10^6 organism cells) and a nutraceutical supplement prepared from fermented trout feed, Tetramin®, and yeast extract (*Saccharomyces cerevisiae*) in a daily basis. The tests were conducted in accordance with recommendations of the U.S. protocol EPA - Method 1002.0 (USEPA, 2002), with adjustments following the technical standard ABNT/NBR 13373 (ABNT, 2022). Mass cultures served as a reservoir of organisms, and individual cultures (1 neonate/15 mL in a glass beaker) were used as the source of neonates for the test. The assay involved 10 replicates per concentration, with one neonate (≤ 24 h) per replicate. Organisms were individually placed in 15 mL of each test solution in a glass beaker and incubated for 7 days, with the renewal of the test solution every 48 h. The tests were conducted using the same conditions established for cultivation. Acrylic plates were used to cover the glass beakers to prevent evaporation. Organisms were exposed to emodin concentrations of 0, 0.009, 0.018, 0.037, 0.075, and 0.15 mg L^{-1}. Survival and reproduction of the organisms were evaluated, with all organisms monitored for survival and neonate counts performed three times during the renewal of the test solutions. The tests were considered valid when there was 80% survival and at least 12 neonates/female on average for negative and solvent control animals over the test period, following the criteria established by Moore et al. (2009). The pH, conductivity, dissolved oxygen, and temperature were monitored at the beginning of the test and after each solution renewal. Sodium chloride (NaCl, Sigma-Aldrich, ≥99% purity) was employed as a reference

toxicant, and a control chart featuring chronic toxicity test results has been provided in Supplementary Material I (Figure S4).

5.2.4 Derivation of Predicted No-Effect Concentrations (PNEC)

Predicted No-Effect Concentration (PNEC) values were determined through the deterministic approach following European guidelines (European Commission, 2018). Basically, this approach identifies the most sensitive, reliable, and relevant toxicity endpoint from the available set of test results and applies an appropriate assessment factor (AF) to extrapolate to an environmentally protective concentration. The AF magnitude can range from 10 to 1000, depending on the uncertainties associated with the available ecotoxicity data. To assess the reliability and relevance of ecotoxicity tests, the "Criteria for Reporting and Evaluating Ecotoxicity Data" (CRED) were applied (Kase et al., 2016; Moermond et al., 2016). CRED employs a set of 20 reliability and 13 relevance criteria, as detailed in Supplementary Material I, Chapter 01. The evaluation process involves transparent questions that enable an objective assessment of whether the test was conducted under appropriate conditions and is suitable for the given regulatory context.

5.2.5 Genotoxicity tests

Initially, we confirmed the mutagenic activity of our dye sample utilizing a miniaturized version of the *Salmonella*/microsome mutagenicity test in a Microplate format (MPA), as developed by Zwarg et al. (2018). Considering that emodin had previously exhibited mutagenicity, specifically with the TA1537 strain, only under certain conditions (in the presence of metabolic activation containing 10% of the S9 fraction in the mix, as indicated by Bösch et al., 1987), these specific test conditions were chosen for our investigations. The test was performed with negative and positive controls, to ensure the responsiveness of the strain and the effectiveness of the metabolic activation system (S9 mix). As a negative control we used DMSO. As positive controls, specific mutagenic compounds were used, without metabolic activation, acridine at 6 ng μL^{-1}, and with metabolic activation, 2-amino anthracene at 2 ng μL^{-1}.

Then we conducted a genotoxicity test (comet assay) and the micronuclei test with *P. hawaiensis*.

The standard alkaline comet assay (Singh et al., 1988) modified by Botelho et al. (2022) was performed with the hemocytes of exposed *P. hawaiensis* organisms. Eight organisms (four

males and four females) were individually exposed to 0.15 and 0.3 mg L^{-1} of emodin and the positive control ethyl methane sulfonate (EMS), 2mmol L^{-1} in 12-well microplates for 96 h. Solvent control (DMSO 0.01%) was also included in the experiment. The experimental conditions were carefully controlled, with a temperature maintained at 24 ± 0.5 °C and salinity set at 30 ± 1. Following the exposure period, hemolymph was extracted from two organisms of the same sex using a capillary needle inserted into the second dorsal segment. The extracted hemolymph was then transferred to a vial containing 20 µL of reconstituted seawater. Immediately after extraction, the cell suspension was mixed with 40 µL of low melting point agarose (1.0%) in Kenny's salt solution (0.4M NaCl, 9mM KCl, 0.7mM K_2HPO_4, 2mM $NaHCO_3$, pH 7.5). The mixture was spread on half of a glass slide surface that had been previously coated with normal melting point agarose (1.5%) in distilled water, and covered with a 20 x 20mm cover glass. This procedure was repeated twice, resulting in two gels per slide. Slides were then kept at 4 °C in the dark for 20 min to solidify the gel. Subsequently, the cover glass was removed, and the slides were transferred to a lysis solution (2.5M NaCl, 100M EDTA, 10mM Tris, 1% Triton X-100, 10% DMSO, pH 10) at 4 °C. After 2 h, the slides were rinsed with cold distilled water and transferred to the electrophoretic chamber filled with alkaline electrophoresis solution (Na_2EDTA 1mM, NaOH 300mM, pH > 13) for 20 min at 4 °C for DNA unwinding. Electrophoresis was then performed for 20 min at 20V (0.71V/cm) and 250mA. The slides were washed three times for 5 min with a neutralizing solution (Tris 0.4 M, pH 7.5) and dehydrated in ethanol for 15 min. Subsequently, the slides were left to dry at room temperature.

Hemolymph collected from two organisms under each condition was used to assess cell viability using the trypan blue exclusion method. Each gel was stained with ethidium bromide (4%), covered with a cover glass, and photographed under an epifluorescence microscope at 400x. The percentage of fragmented DNA (%DNA) in the comet tail was calculated for one hundred comets from each gel using CometScore™ Version 2.0. Cell viability was confirmed to be above 80% in all samples.

Micronucleus test, as detailed by Botelho et al. (2022), involved the preparation of emodin in DMSO, at the maximum solubility concentration, followed by dilution in reconstituted seawater, ensuring a maximum DMSO concentration of 0.01%. Individual exposure of organisms to concentrations of 0.15 and 0.3 mg L^{-1} of emodin and Zn^{2+} (1.5 mg L^{-1}) as a positive control occurred in 12-well microplates over a 96-h period. The experimental

setup also included negative and solvent controls (DMSO 0.01%). The temperature was maintained at 24 ± 0.5 °C, and salinity was set at 30 ± 1. Following the exposure period, hemolymph from each organism was collected using a capillary needle inserted into the second dorsal segment and placed in an Eppendorf tube with 10 µL of cold reconstituted seawater. The hemocyte suspension was spread on a glass slide and incubated inside a humid chamber for 15 min. Subsequently, slides were rinsed twice with reconstituted seawater, fixed in formaldehyde (1:10 in reconstituted seawater) for 10 min, and dried at room temperature. Further treatment involved immersing the slides in methanol for 10 min and staining with Giemsa (10%) for 20 min. Micronuclei counts were conducted on 500 cells under a microscope using 1000x magnification and immersion oil.

5.2.6 Statistical analysis

Acute toxicity data obtained from tests involving *D. similis*, *D. rerio* embryos, and *P. hawaiensis* were subjected to modeling using the generalized logistic model from the glmnet package (Friedman et al., 2010). This approach aimed to estimate the 50% effect and lethal concentrations (EC_{50} and LC_{50}) along with their corresponding 95% confidence intervals (C.I.). Model selection was based on the statistical significance of the concentration variable ($p \leq 0.05$). All analyses were performed using the R programming language (RStudio Team, 2022). For chronic toxicity tests involving *R. subcapitata* and *C. dubia*, data normality was assessed using the Kolmogorov-Smirnov test, indicating nonparametric assumptions. To identify significant differences ($p \leq 0.05$), a one-way analysis of variance (Kruskal-Wallis) was conducted, followed by the Mann-Whitney post-hoc test. In cases where statistical differences between the solvent control (DMSO 0.01%) and exposure concentrations were observed, the 10% inhibition concentration (IC_{10}) and its respective 95% confidence interval (C.I.) were estimated using the model fitting function drm() from the drc package (Ritz et al., 2015). Similar to acute toxicity analyses, these assessments were also performed using R (RStudio Team, 2022).

For the Ames test, the data underwent analysis using an ANOVA followed by a post-hoc Tukey test ($p < 0.05$) and subsequent regression analysis ($p < 0.05$) using the Bernstein model (Bernstein et al., 1982). Statistical analyses were conducted using the Salanal software, generously provided by Integrated Laboratory Systems from Research Triangle Institute, Research Triangle Park, NC, USA. The validity of the experiment was contingent on the appropriate response of cell viability and the positive control. In the comet and micronuclei assays, data were subjected to one-way ANOVA followed by Tukey's Test to assess differences

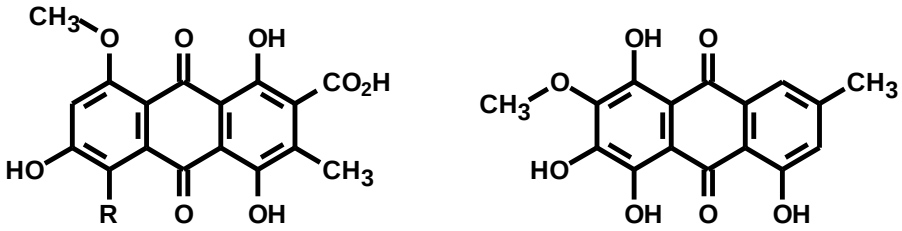
among the tested conditions. Residuals from the model were examined for normality and homoscedasticity using Shapiro-Wilk's Test and Levene's Test.

5.3. Results and Discussion

5.3.1 Ecotoxicity tests

In this study, acute and chronic assays using different aquatic model organisms were used to characterize emodin's ecotoxicological effects. We compared the obtained results with dermocybin and dermorubin (Figure 2), additional anthraquinones extracted from the same fungus (*C. sanguineus*), that have already been evaluated for aquatic toxicity (Farias et al., 2023).

Figure 2 - Molecular structures for anthraquinone dyes dermorubin (left) and dermocybin (right).

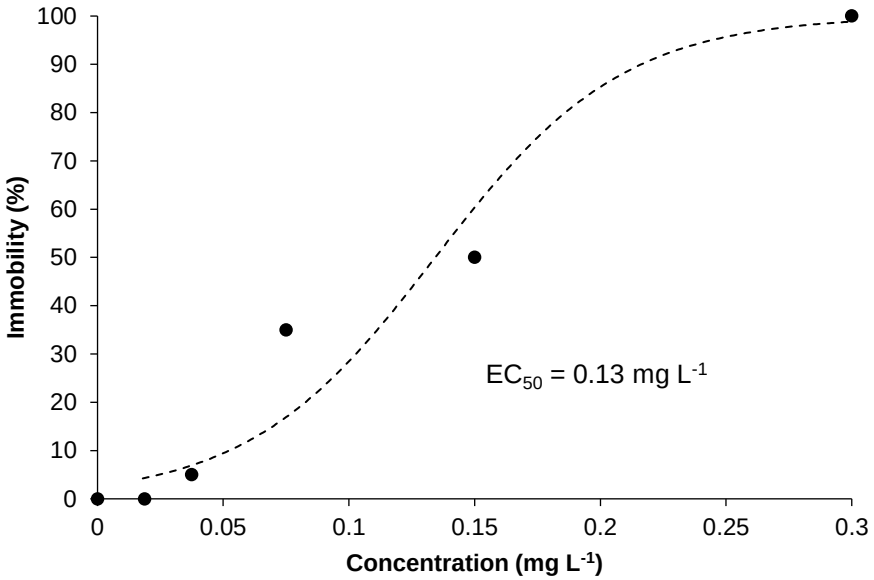


Acute toxicity tests

Daphnia similis

No immobility was observed in the negative and solvent controls. Emodin was toxic to *D. similis*, with an EC_{50} = 0.13 mg L^{-1} (Figure 3). Nitulescu et al. (2017) reported an EC_{50} of 6.53 mg L^{-1} for *Daphnia magna.* The final concentration of DMSO used was 1% and the purity of emodin was not mentioned. In a recent work, Farias et al. (2023) showed that other natural anthraquinone dermocybin was toxic to *D. similis* (EC_{50} = 0.51 mg L^{-1}) but dermorubin was not.

Figure 3 – Concentration × response (immobility) curve for emodin to *D. similis* (48 h).

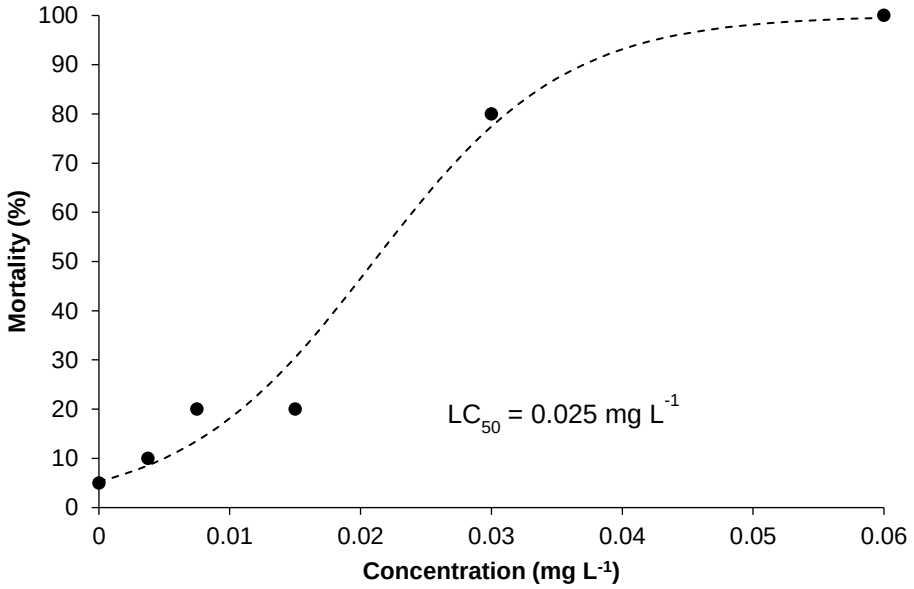


Zebrafish embryos

In both the negative and solvent controls, no mortality was observed. Additionally, the organisms exhibited regular development without any signs of malformations. In contrast, the positive control, consisting of 4 mg L^{-1} of 3,4-dichloroaniline, induced mortality exceeding 30% by the end of the test, aligning with outlined by OECD guideline 236 (OECD 236, 2013).

Emodin was toxic to *D. rerio* embryos with an LC_{50} = 0.025 mg L^{-1} (Figure 4). For the same organisms, He et al., (2012) reported an LC_{50} value 10 times higher (LC_{50} = 0.2 mg L^{-1}). ~~1~~. They also observed sub-lethal effects such as edema, malformation of the tail and abnormal morphogenesis of some organs at concentration of 0.1 mg L^{-1}. He et al., (2012) started the exposure of embryos to emodin after 7 h post fertilization, which could explain the difference in LC_{50} values obtained. OECD 236 (2013) recommends starting exposure in the first few hours (90 min post fertilization) to cover the most sensitive phase of embryonic development. Furthermore, the purity of the test compound was not reported. For dermocybin, Farias et al., (2023) reported an extrapolated LC_{50} of 2.44 mg L^{-1} and dermorubin was not toxic to zebrafish.

Figura 4 - Concentration × response (mortality) curve for emodin to zebrafish (*D. rerio*) embryos (168 h).



Marine amphipod, Parhyale hawaiensis

Emodin was not toxic for *P. hawaiensis* at the tested concentrations (0 – 0.30 mg L^{-1}) (Table 1). The only study found showing the effect of emodin in a marine crustacean was carried out by Chinnasamy et al., (2023) that evaluated the acute effect of emodin on *Artemia* nauplii. The tests were conducted using 10% DMSO, and emodin purity of 96%. They observed 54% of mortality at concentration of 8.0 mg L^{-1}. Dermocybin and dermorubin were not acutely toxic to *P. hawaiensis* (Farias et al., 2023).

Table 1 - Raw data for acute testing of emodin to *Parhyale hawaiensis* performed for 96 h at 24 °C.

Concentration (mg L^{-1})	**Number of dead organisms**	**Mortality (%)**
0	1/32	3.1
DMSO (0.01%)	0/32	0
0.019	0/32	0
0.037	0/32	0
0.075	0/32	0
0.15	1/32	3.1
0.30	1/32	3.1

Chronic toxicity tests

Raphidocelis subcapitata

Emodin was not toxic to *R. subcapitata* at tested concentration (0 – 0.30 mg L^{-1}) (Table 2). Farias et al., (2023) also showed that dermocybin and dermorubin were not toxic to microalgae *R. subcapitata*.

Table 2 - Data from chronic toxicity testing of emodin for *Raphidocelis subcapitata* performed for 72 h.

Concentration (mg L^{-1})	**Mean (SD)**	**CV (%)**	**Growth inhibition (%)**	**Statistical significance**
0	811 (51)	6.3	0.0	-
0.019	908 (75)	8.2	-12.0	N.S.
0.037	807 (66)	8.2	0.5	N.S.
0.075	895 (43)	4.8	-10.4	N.S.
0.15	840 (25)	3.0	-3.7	N.S.
0.30	865 (17)	1.9	-6.7	N.S.

Mean = mean of algal cells/mL; SD = standard deviation; CV = coefficient of variation; N.S. = not significant compared to the control

Ceriodaphnia dubia

Preliminary tests were performed to determine the best concentration range for the chronic assay, in which 0.15 mg L^{-1} was determined as the highest concentration. Chronic exposure (7 days) to emodin did not impact reproduction of *C. dubia* at tested concentrations (Table 3). Although a decrease in reproduction at 0.15 mg L^{-1} was observed, there was no significant difference in relation to the control group (ANOVA, $p > 0.05$). Dermocybin was toxic to *C. dubia* with a IC_{10} of 0.13 mg L^{-1} but dermorubin was not (Farias et al., 2023).

Table 3 - Raw data for the chronic testing of emodin to *Ceriodaphnia dubia* performed for 7 days.

Emodin (mg L^{-1})	Survival of female (%)	Number of neonates/female										Total	Mean
		1	2	3	4	5	6	7	8	9	10		
DMSO 0.01%	100	17	5	14	11	11	21	11	12	10	9	121	12.1
0.009375	90	19	12	*	14	4	12	2	7	14	3	87	9.7
0.01875	100	8	8	9	14	3	12	13	13	8	11	99	9.9
0.0375	100	8	13	12	10	16	7	10	12	11	18	117	11.7
0.075	100	8	12	16	9	11	19	7	12	13	9	116	11.6
0.15	70	*	8	3	*	10	*	11	9	7	6	54	7.7

*dead females

5.3.2 Quality assessment of ecotoxicity data and PNEC derivation

To calculate a Predicted No-Effect Concentration (PNEC) for emodin, we considered the results of the most sensitive organism obtained in the acute test, specifically *D. rerio* with an LC_{50} of 0.025 mg L^{-1}. Applying an assessment factor (AF) of 50, considering results from chronic tests from two trophic levels (algae and crustacea), a PNEC of 0.0005 mg L^{-1} was derived for emodin. The reliability and relevance of the acute and chronic studies used in the PNEC derivation were assessed using the CRED evaluation method. Supplementary Material II covers the acute assay with *Danio rerio*, Supplementary Material III includes the chronic assay with *Ceriodaphnia dubia*, and Supplementary Material IV relates to *Raphidocelis subcapitata* assay. All studies met 18 out of 20 reliability criteria (considering factors like non-GLP certification and nominal concentrations) and 11 out of 13 relevance criteria (with the remaining two criteria being inapplicable). Consequently, these studies have been deemed reliable and relevant for deriving a scientifically sound PNEC.

5.3.3 Genotoxicity

Salmonella/microsome mutagenicity test (MPA)

The mutagenicity of the emodin sample used in this study was confirmed using the miniaturized version of the *Salmonella*/microsome assay, named MPA protocol. As expected emodin was mutagenic only in the presence of metabolic activation (S9) with the strain (TA1537) (Table 4). We selected an S9 concentration of 10% in the mix because this was shown to be the best conditions for the detection of the mutagenic effect. Emodin and other hydroxyanthraquinones were previously described as mutagens detected by this strain (Bösch

et al., 1987). It is also known that emodin is bio-transformed by microsomal P450 enzymes mainly into the direct mutagen 2-hydroxyemodin (Brown and Brown, 1976; Masuda and Ueno, 1984; Tanaka et al., 1987).

Table 4 - Emodin mutagenicity data represented by mean ± standard deviation (SD) for *Salmonella enterica* serovar Typhimurium strain TA1537 in the absence (-S9) and presence (+S9) of metabolic activation using 10% of S9 in the mix.

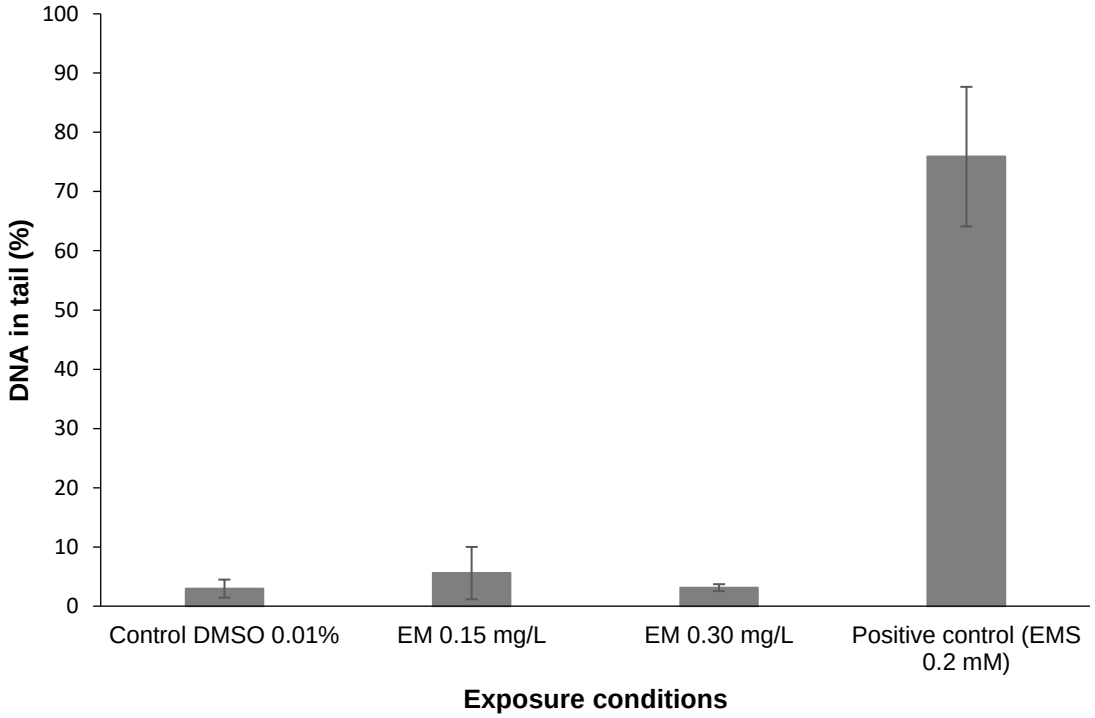
Emodin		
Concentration ($ng\ \mu L^{-1}/mg\ L^{-1}$)	-S9 Mean ± SD	+S9 Mean ± SD
DMSO[a]	1.5 ± 1.7	2.5 ± 1.0
1.9	4.2 ± 0.5	6.7 ± 2.1
3.7	1.5 ± 1.0	**8.5 ± 2.6**[*]
7.5	4.7 ± 1.0	**13.0 ± 1.4**[**]
15.0	5.7 ± 5.2	**13.7 ± 3.8**[*]
30.0	5.0 ± 1.4	**11.2 ± 2.1**[**]
60.0	4.7 ± 1.7	**16.7 ± 3.2**[**]
Positive control	>150	>150
Response	Negative	**Positive**

[a] negative control; [**]significant at 1%; [*]significant at 5%;

Comet assay

We did not observe genotoxic effects in the alkaline version of the comet assay up to the maximum concentration tested (0.30 mg L^{-1}) (Figure 5). Then, we decided to increase the DMSO concentration to 0.1 and 1% and thus increase the emodin concentration to 3 and 30 mg L^{-1}. After exposure to both concentrations, we observed that all organisms died before completing 96 h exposure. Therefore, it was not possible to carry out the comet assay at concentrations higher than 0.30 mg L^{-1}. Sevcovicova et al., (2014) showed that emodin exerted genotoxic activity in human lymphocytes at concentrations up to 2 mg L^{-1} using also the alkaline comet assay.

Figure 5 - DNA in tail (%) from comet assay in hemocytes of *Parhyale hawaiensis* exposed to emodin (EM).



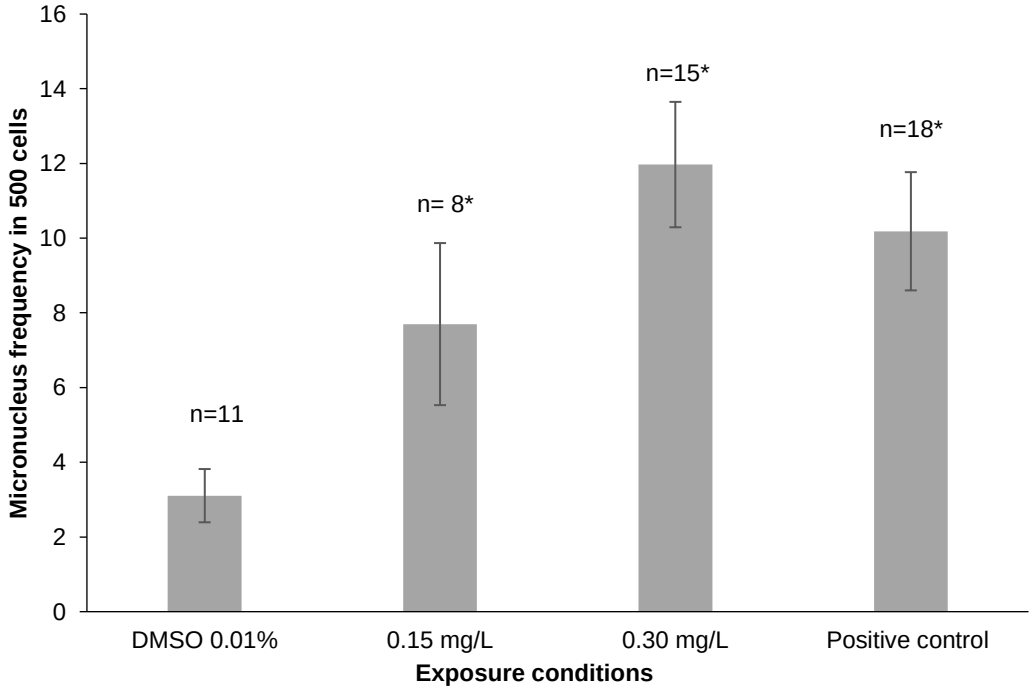
It is known that excessive reactive oxygen species (ROS) production may lead to oxidative stress. Emodin is capable of generating ROS in human (Qu et al., 2013), and in rat cells (Zhang et al., 2021). For aquatic organisms, only one study conducted by Cui et al., (2014) showed that ROS production increased with exposure to emodin in grass carp fish (*Ctenopharyngodon idellus*). ROS can cause breaks in DNA and the most specific indicator of oxidative attack is the presence of oxidized purines or pyrimidines (Collins, 2014). Several modifications of the alkaline standard comet assay were developed for detecting this specific type of lesions by adding endonucleases capable of recognizing oxidatively damaged bases and create additional breaks to aid in the detection of oxidative DNA damage (enzyme-modified comet assay) (Collins, 2014). Lesion-specific endonucleases, such as formamidopyrimidine DNA-glycosylase (FPG) or 8-oxoguanine DNA-glycosylase (hOGG1) can be used to recognize the number of oxidized purine bases.

We hypothesized that ROS could also be produced in the hemocytes of *P. hawaiensis*; therefore, more studies should be performed using enzyme modified comet assay to identify the genotoxicity of emodin in this aquatic organism.

Micronuclei test

Emodin significantly increased micronuclei frequency in *P. hawaiensis* hemocytes (Figure 6). Micronuclei frequency of emodin exposure was even higher than the positive control. To our knowledge there is limited information on the literature about the mutagenic effect of dyes in aquatic organisms. However, several dyes have already proved to be mutagenic by the Ames test (Ferraz et al., 2011; Leme et al., 2014; Novotný et al., 2006; Umbuzeiro et al., 2021; Vacchi et al., 2016; Venturini and Tamaro, 1979).

Figure 6 - Mean of micronuclei frequency in hemocytes of *Parhyale hawaiensis* exposed to emodin via water for 96 h. Zn^{2+} was used as positive control. Error bars represent the standard error and * results significantly different from control ($p<0.05$).



As stated previously, the mutagenic activity of emodin in the *Salmonella*/microsome assay is dependent on exogenous metabolism (CYP450s). The micronuclei data suggest that *P. hawaiensis* is able to metabolize emodin into a mutagen, but the metabolic pathways from this organism are not yet well described. In mammals, cytochrome P450s (CYP450s) are prominently expressed in the liver and play a crucial role as major contributors to phase I metabolism (Zhang et al., 2021). In aquatic invertebrates, CYP450s have been described for several species such as *Panulirus argus* (lobster) (James et al., 1996), *Homarus americanus* (lobster) (Snyder, 1998a), *Penaeus setiferus* (shrimp) (Snyder, 1998b), *Mytilus edulis* (mussel) (Zanette et al., 2013), *Crassostrea gigas* (oyster) (Zanette et al., 2010), *Diaphanosoma celebensis* (waterflea) (Han and Lee, 2021), *Brachionus rotundiformis* (marine rotifer) (Han et

al., 2019). It may be that CYP450s are involved in the metabolism of emodin in *P. hawaiensis*, but further molecular biology studies are needed.

Emodin was not toxic to *P. hawaiensis* but it was toxic to zebrafish. This may be due to lower basal expression of CYP450s in more sensitive species (Montgomery et al., 2023). In addition to mutagenic effects, CYP450s may also be involved in the mechanisms of emodin toxicity to aquatic organisms. The mortality observed for zebrafish embryos exposed to emodin may be mediated by hepatotoxicity effects. Li et al., (2020) showed that emodin was hepatotoxic to zebrafish embryos at concentrations ≥ 0.0625 mg L^{-1}. More recently, hepatotoxicity of human liver cells induced by the anthraquinone, aloe-emodin, was associated with the inhibition of some metabolic enzymes, such as CYP3A4 (Hu et al., 2022). He et al., (2012) also observed that CYP3A4 is involved in zebrafish embryo responding to emodin.

In humans, more than half of xenobiotics are metabolized by CYP3A4 (Anzenbacher and Anzenbacherová, 2001) Due to the genetic similarity between zebrafish and humans (Howe et al., 2013), CYP450s enzyme may be involved in the toxic activity performed by emodin in fish, but it is important to highlight the need for more molecular studies to advance in the topic of biotransformation of emodin in both aquatic vertebrates and invertebrates.

5.4. Conclusion

Emodin was toxic to aquatic organisms *D. similis* and *D. rerio* embryos. Zebrafish was the most sensitive organism but biochemistry/molecular studies are needed to comprehend the mechanisms underlying emodin´s toxicity to aquatic organisms. Compared to other anthraquinones, particularly those present in *Cortinarius sanguineus* fungus, emodin was a more toxic dye. In the alkaline version of the comet assay, emodin did not show any effect, but it caused micronuclei in the hemolymph of *P. hawaiensis* when organisms were exposed for 96 h. If emodin is applied as a dye on a large scale, a PNEC value of 0.0005 mg L^{-1} could be considered for aquatic risk assessments.

Acknowledgments

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Supplementary Material I

Control charts

Figure S1. Control-chart of *Daphnia similis* with acute toxicity tests using sodium chloride (NaCl) as reference toxicant. SD: standard deviation; EC_{50}: 50% effect concentration.

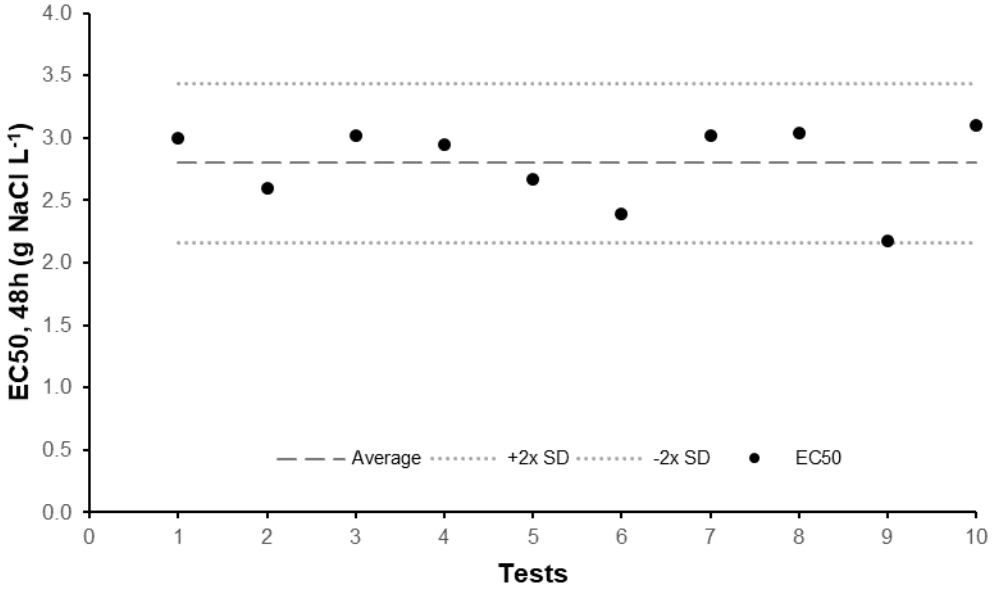


Figure S2. Control-chart of *Parhyale hawaiensis* with acute toxicity tests using zinc (Zn) as reference toxicant. SD: standard deviation; LC_{50}: 50% lethal concentration.

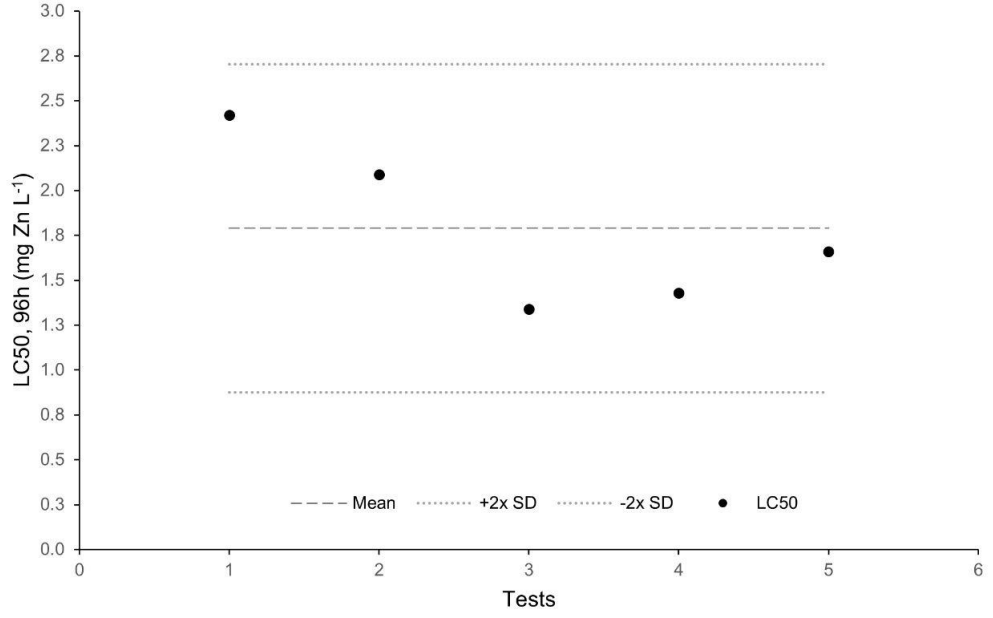


Figure S3. Control-chart of *Raphidocelis subcapitata* with chronic toxicity tests using sodium chloride (NaCl) as reference toxicant. SD: standard deviation; IC_{50}: 50% inhibition concentration.

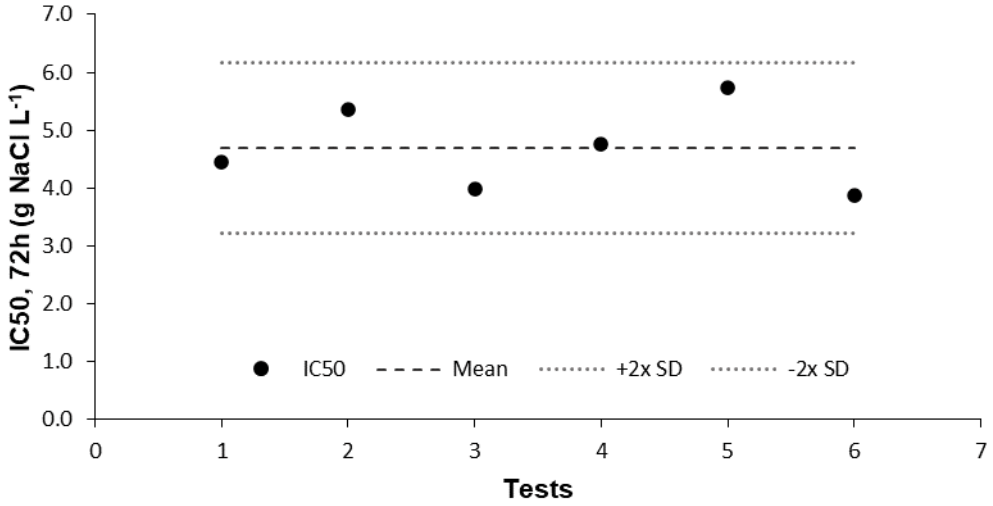
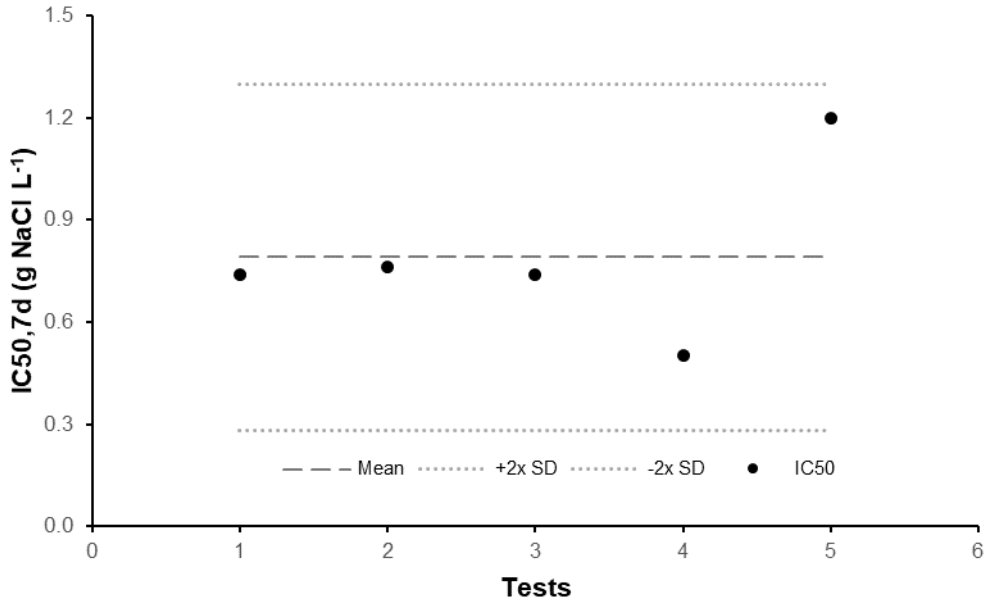


Figure S4. Control-chart of *Ceriodaphnia dubia* with chronic toxicity tests using sodium chloride (NaCl) as reference toxicant. SD: standard deviation; IC_{50}: 50% inhibition concentration.



Supplementary Material II

CRED evaluation acute assay with *Danio rerio* embryos

Acute assay evaluation

CAS Number	518-82-1
Compound name	Emodin
Organism group	Fish
Species	*Danio rerio*
Marine/Freshwater	Freshwater
Effect Measured	LC_{50}
Relevant endpoint	Mortality
Endpoint concentration/dose	0.025
Endpoint measurement unit	mg L^{-1}
Test duration	168
Test duration unit	hours

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log K_{OW}, pKa, is the compound volatile, does it hydrolyse, photolyse, etc.?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	**(X)**				Yes, the OECD guideline 236, with change in exposure time of 96 h to 168h according Farias et al. 2019.

2	Is the test performed under GLP conditions?*		**(X)**			No, despite the absence of GLP/ISO certification, the laboratory's quality management system adheres to the specifications outlined in ISO/IEC 17025/2017.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, the validity criteria have been met. No mortality was observed in the negative and solvent control groups, while greater than 30% mortality was noted in the positive control group treated with 3,4-dichloroaniline at the end of the test.
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. 3,4 DCA was used as reference substance in the positive control. Dimethyl sulphoxide (DMSO) was used in the solvent control at 0.01% maximum concentration according OECD guideline 23 (2019). No toxicity was observed. Supporting Information Figure 4.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance name is Emodin, CAS number 518-82-1. The chemical structure and its purity were confirmed by HPLC-DAD-MS and NMR (13C and 1H) as described by Herrala et al. (2022).
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the purity was confirmed by MS in Herrala et al. (2022).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an	**X**				The purified dye (99%) was used.

	effect? Is the amount of test substance in the formulation known?					
	Test organism					
8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	**X**				Yes, the specie scientific name is *Danio rerio.* Embryos with 90 min post-fertilization were used at the beginning of the test.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	**X**				Yes, the organisms were acquired by an aquarium store with veterinary inspection. The organisms were quarantined for a period of 14 days before being entered into the system. They are held in our laboratory according to recommended culture conditions (OECD 236, 2013).
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	**X**				Yes, the test system was appropriate since the substance is not volatile. Moreover, the substance is a sparingly water-soluble dye, so a solvent was used to guarantee that the substance was dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	**X**				Yes, the experimental system was appropriate for the test organism, i.e., according to the OECD test guidance.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	**X**				Yes, exposure concentrations were below the limit of water solubility of 0.3 mg L^{-1}. A solvent control (DMSO) was performmed at 0.01%.
13	Is a correct spacing between exposure concentrations applied?	**X**				Yes, a dilution factor of 2 was used. The concentrations were 0, 0.0037,

						0.0075, 0.015, 0.03 and 0.06 mg L^{-1}.
14	Is the exposure duration defined?	X				Yes, the exposure duration was 168 hours under static conditions. The light: dark photoperiod of 12:12 hours, under controlled temperature (26±1 ºC). No feeding during the test.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		X			No. Nominal concentrations were used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	X				Yes, it was added 1 embryo per well. Each well filled with 2 mL of the solution, according to OECD 236 (2013).
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	X				Yes, 20 replicates with 1 organism per replicate were used for the control and each concentration.
18	Are appropriate statistical methods used?	X				Yes, the LC50 was calculated by a logistic regression.
19	Is a dose-response curve observed? Is the response statistically significant?	X				Yes, a concentration-response curve was observed and the observed response achieved statistical significance ($p < 0.05$). Please see Supporting Information Figure 4.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	X				Yes, please see Supporting Information Figure 4.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				Despite that only nominal concentrations were used, the test has been conducted and recorded according to the OECD standard guideline.

CRED evaluation method for relevance to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	**X**				Yes, *Danio rerio* is a secondary consumer and a standard test organism in aquatic risk assessment.
2	Are the organisms tested relevant for the tested compound?	**X**				Yes, the species group could be exposed to the dye.
3	Are the reported endpoints appropriate for the regulatory purpose?	**X**				Yes, the short-term LC50 on mortality, is a common method for evaluating acute effects in fish.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the selected endpoint is indicative of acute toxicity.
5	Is the effect relevant on a population level?	**X**				Yes, mortality is relevant for the population level.
6	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the short term LC50 represents the concentration at which effect at 50% of organisms is expected.
7	Are appropriate life-stages studied?	**X**				Yes, embryos are expected to be the most sensitive life stage.
8	Are the experimental conditions relevant for the tested species?	**X**				Yes, static exposure is a common exposure scenario in risk assessment.

9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	X				Yes, the exposure time was extended for more 3 days to evaluate the toxic effects on the larval stage of the fish.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			X		Not applicable.
	Exposure relevance					
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			X		Not applicable. The purify substance was used.
12	Is the tested exposure scenario relevant for the substance?	X				Yes, acute toxicity can be expected from the continues input of this compound in water.
13	Is the tested exposure scenario relevant for the species?	X				Yes, the standard exposure scenario for this species was applied.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				The test has been conducted and recorded according to the OECD standard guideline.

Supplementary Material III

CRED evaluation chronic assay with *Ceriodaphnia dubia*

Chronic assay evaluation

CAS Number	518-82-1
Compound name	Emodin
Organism group	Crustaceans
Species	*Ceriodaphnia dubia*
Marine/Freshwater	Freshwater
Effect Measured	IC_{10}
Relevant endpoint	Reproduction
Endpoint concentration/dose	>0.15
Endpoint measurement unit	mg L^{-1}
Test duration	7
Test duration unit	days

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log K_{OW}, pKa, is the compound volatile, does it hydrolyse, photolyse, etc.?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	**(X)**				Yes, the USEPA Method 1002.0 (2002), with culture and test medium according ABNT/NBR 13373 (2017).

2	Is the test performed under GLP conditions?*		**(X)**			No, despite the absence of GLP/ISO certification, the laboratory's quality management system adheres to the specifications outlined in ISO/IEC 17025/2017.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, validity criteria are fulfilled. Survival ≥ 80% and at least 12 neonates/female on average in the controls (negative and solvent).
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. Dimethyl sulphoxide (DMSO) was used in the solvent control at 0.01% maximum concentration according OECD guideline 23 (2019). No toxicity was observed. Supporting Information Table 3, Figure S4.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance, Emodin (CAS number 518-82-1), underwent confirmation of its chemical structure and purity through HPLC-DAD-MS, as well as NMR (13C and 1H), as outlined in the methodology by Herrala et al. (2022).
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the purity was confirmed by MS in Herrala et al. (2022).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	**X**				The purified dye (99%) was used.
	Test organism					

8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes, the specie scientific name is *Ceriodaphnia dubia*. The age/life stage used was less than 24 h old neonates.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes, the organisms were donated by Dr. Francine Vacchi from Mérieux NutriScience, Brazil. Test organisms were exempt from previous stressors. They are held in our laboratory according to recommended culture conditions (ABNT/NBR 13373, 2017).
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	X				Yes, the test system was appropriate since the substance is not volatile. Moreover, the substance is a sparingly water-soluble dye, so a solvent was used to guarantee that the substance was dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	X				Yes, the experimental system was appropriate for the test organism, *i.e*. according to the USEPA test guidance.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	X				Yes, exposure concentrations were below the limit of water solubility of 0.3 mg L^{-1}. A solvent control (DMSO) was performed at 0.01%.
13	Is a correct spacing between exposure concentrations applied?	X				Yes, a dilution factor of 2 was used. The concentrations were 0.009, 0.019, 0.037, 0.075 and 0.15 mg L^{-1}.
14	Is the exposure duration defined?	X				Yes, the exposure duration was 7 days under semi-static conditions with test solutions renewed 3 times/week. The light:dark photoperiod of 16:8 hours, under controled temperature (25±2 ºC).

						Organisms were fed daily with *Raphidocelis subcapitata* and compound feed.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		**X**			No. Nominal concentrations were used.
16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	**X**				Yes, mass cuture (30 organisms/1 L) mainteneded as a source of organims and individual cultures were used for toxicity tests (1 neonate/15 mL glass beaker), according USEPA Method 1002.0 (2002).
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	**X**				Yes, 10 replicates with 1 organism per replicate were used for the controls and for each treatment.
18	Are appropriate statistical methods used?	**X**				Yes, one-way analysis of variance with post-hoc test, was used to identify significant differences ($p \leq 0.05$).
19	Is a dose-response curve observed? Is the response statistically significant?			**(X)**		No, because emodin was not toxic at the maximun tested concentration (0.15 mg L^{-1}). Please, see Table 3.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	**X**				Yes, raw data is available. Please, see Table 3.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				Despite that only nominal concentrations were used, the test has been conducted and recorded according to the USEPA standard guideline.

CRED evaluation method for relevance to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	**X**				Yes, *Ceriodaphnia dubia* is a primary consumer and a standard test organism in aquatic risk assessment.
2	Are the organisms tested relevant for the tested compound?	**X**				Yes, the species group could be exposed to the dye.
3	Are the reported endpoints appropriate for the regulatory purpose?	**X**				Yes, long term IC10 on reproduction is commonly used to assess chronic effects for Daphnids.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	**X**				Yes, the endpoint reproduction inhibition is representative for chronic toxicity.
5	Is the effect relevant on a population level?	**X**				Yes, reproduction is a relevant endpoint for the population level.
6	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC10, EC50)?	**X**				Yes, the long term IC10 represents the concentration at which effect at 10% of organisms is expected.
7	Are appropriate life-stages studied?	**X**				Yes, neonates are expected to be the most sensitive life stage.

8	Are the experimental conditions relevant for the tested species?	X				Yes, semi static exposure is a common exposure scenario in risk assessment.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	X				Yes, this is the standard time of exposure for chronic tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			X		Not applicable.
	Exposure relevance					
11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			X		Not applicable. The purify substance was used.
12	Is the tested exposure scenario relevant for the substance?	X				Yes, chronic toxicity can be expected from constant exposure to the compound throughout the organism's entire or partial life cycle
13	Is the tested exposure scenario relevant for the species?	X				Yes, the standard exposure scenario for this species was applied.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				The test has been conducted and recorded according to the USEPA standard guideline.

Supplementary Material IV

CRED evaluation chronic assay with *Raphidocelis subcapitata*

Chronic assay evaluation

CAS Number	518-82-1
Compound name	Emodin
Organism group	Clorophyta
Species	*Raphidocelis subcapitata*
Marine/Freshwater	Freshwater
Effect Measured	IC_{10}
Relevant endpoint	Inhibition of growth
Endpoint concentration/dose	>0.3
Endpoint measurement unit	mg L^{-1}
Test duration	72
Test duration unit	hours

CRED evaluation method for reliability to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test, please check the physico-chemical characteristics of your compound (handbooks/general sources). What is the solubility, log K_{OW}, pKa, is the compound volatile, does it hydrolyse, photolyse, etc.?	**If you have checked the physico-chemical parameters of the substance tested, please continue with the evaluation of reliability, using reliability criteria 1-20.**				
1	Is a standard method (e.g., OECD/ISO) or modified standard used?*	X				Yes, OECD n° 201 (OECD 201, 2011) was used for tests, with culture and test medium according ABNT/NBR 12648 (ABNT, 2018).

2	Is the test performed under GLP conditions?*		**(X)**			No, despite the absence of GLP/ISO certification, the laboratory's quality management system adheres to the specifications outlined in ISO/IEC 17025/2017.
3	If applicable, are validity criteria fulfilled (e.g. control survival, growth)?	**X**				Yes, the validity criteria have been met, with the biomass in the control increasing exponentially by a factor of at least 16 by the conclusion of the test.
4	Are appropriate controls performed (e.g. solvent control, negative and positive control)?	**X**				Yes, an appropriate negative and positive control were performed. NaCl was used as reference substance in the positive control. Dimethyl sulphoxide (DMSO) was used in the solvent control at 0.01% maximum concentration according OECD guideline 23 (2019). No toxicity was observed, please see Table 2.
	*These criteria are of minor importance for study reliability, but may support study evaluation					
	Test compound					
5	Is the test substance identified clearly with name or CAS-number? Are test results reported for the appropriate compound?	**X**				Yes, the substance, Emodin (CAS number 518-82-1), underwent confirmation of its chemical structure and purity through HPLC-DAD-MS, as well as NMR (13C and 1H), as outlined in the methodology by Herrala et al. (2022).
6	Is the purity of the test substance reported? Or, is the source of the test substance trustworthy?	**X**				Yes, the purity was confirmed by MS in Herrala et al. (2022).
7	If a formulation is used or if impurities are present: Do other ingredients in the formulation exert an effect? Is the amount of test substance in the formulation known?	**X**				The purified dye (99%) was used.
	Test organism					

8	Are the organisms well described (e.g. scientific name, weight, length, growth, age/life stage, strain/clone, gender if appropriate)?	X				Yes, the specie scientific name is *Raphidocelis subcapitata*. The 3-days old algae culture in logarithmic growth phase was used in the tests.
9	Are the test organisms from a trustworthy source and acclimatized to test conditions? Have the organisms not been pre-exposed to test compound or other unintended stressors?	X				Yes, the algae was obtained by Prof. Armando A.H. Vieira from Physiology Laboratory, Department of Botany from University of São Carlos (UFSCAR), in 2004.
	Exposure conditions					
10	Is the experimental system appropriate for the test substance, taking into account its physico-chemical characteristics?	X				Yes, the test system was appropriate since the substance is not volatile. Moreover, the substance is a sparingly water-soluble dye, so a solvent was used to guarantee that the substance was dissolved.
11	Is the experimental system appropriate for the test organism (e.g., choice of medium or test water, feeding, water characteristics, temperature, light/dark conditions, pH, oxygen content)? Have conditions been stable during the test?	X				Yes, the experimental system was suitable for the test organism, aligning with the guidelines outlined in OECD n°. 201.
12	Were exposure concentrations below the limit of water solubility (taking the use of a solvent into account)? If a solvent is used, is the solvent within the appropriate range and is a solvent control included?	X				Yes, exposure concentrations were below the limit of water solubility of 0.3 mg L^{-1}. A solvent control (DMSO) was performed at 0.01%.
13	Is a correct spacing between exposure concentrations applied?	X				Yes, a dilution factor of ~1.6 was used. The concentrations were 0, 0.019, 0.037, 0.075, 0.15 and 0.3 mg L^{-1}.
14	Is the exposure duration defined?	X				Yes, the exposure duration was 72 hours in a static condition without medium renewed, under continuous fluorescent light (4,000 ± 400 lux) at 24±2 ºC.
15	Are chemical analyses adequate to verify substance concentrations over the duration of the study?		X			No. Nominal concentrations were used.

16	Is the biomass loading of the organisms in the test system within the appropriate range (e.g. < 1 g/L)?	**X**				Yes, the initial algal biomass in the test was adjusted to 1×10^4 cels mL^{-1} to allow exponential growth, according OECD 201 (2011)
	Statistical Design and Biological Response					
17	Is a sufficient number of replicates used? Is a sufficient number of organisms per replicate used for all controls and test concentrations?	**X**				Yes, tests were performed in triplicate.
18	Are appropriate statistical methods used?	**X**				Yes, a one-way analysis of variance with a post-hoc test was employed to identify significant differences, with a significance level set at $p \leq 0.05$.
19	Is a dose-response curve observed? Is the response statistically significant?			**(X)**		No, because emodin was not toxic at the maximun tested concentration (0.3 mg L^{-1}). Please see Table 2.
20	Is sufficient data available to check the calculation of endpoints and (if applicable) validity criteria (e.g., control data, dose-response curves)?	**(X)**				Yes, validation criteria were met and controls were also tested, but it was not possible to obtain a concentration-response curve, as we did not observe effect of the dye on the tested concentrations.
	Final score (R1 = Reliable, R2 = Reliable with restrictions, R3 = Not reliable, R4 = Not assignable)	**R1**				Despite that only nominal concentrations were used; the test has been conducted and recorded according to the OECD 201 standard guideline.

CRED evaluation method for relevance to be used together with the accompanying guidance according to Kase et al. 2016, Moermond et al. 2016

Number	General information	Criterion fulfilled	Criterion not fulfilled	Criterion is not applicable	Criterion is not reported	Comments
	Before evaluating the test for relevance, indicate why you are evaluating this study. The relevance of the study might be different for different purposes (e.g., EQC derivation, PBT	**If you have indicated the purpose of the evaluation, please continue with the relevance criteria 1-13**				

	assessment, dossier evaluation for marketing authorisation), also depending on the framework for which the evaluation is requested.					
	Biological relevance					
1	Is the species tested relevant for the compartment under evaluation?	X				Yes, *Raphidocelis subcapitata* is a producer and a standard test organism in aquatic risk assessment.
2	Are the organisms tested relevant for the tested compound?	X				Yes, the species group has the potential to be exposed to the dye in the aquatic environment.
3	Are the reported endpoints appropriate for the regulatory purpose?	X				Yes, long term IC10 on growth is commonly used to assess chronic effects for algae.
4	Are the reported endpoints appropriate for the investigated effects or the mode of action of the test substance?	X				Yes, the endpoint growth inhibition is representative for chronic toxicity.
5	Is the effect relevant on a population level?	X				Yes, growth is a relevant endpoint for the population level.
6	Is the magnitude of effect statistically and biologically significant and relevant for the regulatory purpose (e.g. EC10, EC50)?	X				Yes, the long term IC10 represents the concentration at which effect at 10% of organisms is expected.
7	Are appropriate life-stages studied?	X				Yes, a 3-days old algae culture and in logarithmic growth phase was used.
8	Are the experimental conditions relevant for the tested species?	X				Yes, static exposure is a common exposure scenario in risk assessment.
9	Is the time of exposure relevant and appropriate for the studied endpoints and species?	X				Yes, this is the standard time of exposure for chronic tests with this species.
10	If recovery is studied, is this relevant for the framework for which the study is evaluated?			X		Not applicable.
	Exposure relevance					

11	In case of a formulation, other mixture, salts or transformation products: Is the substance tested representative and relevant for the substance being assessed?			X		Not applicable. The purify substance was used.
12	Is the tested exposure scenario relevant for the substance?	X				Yes, chronic toxicity can be expected from constant exposure to the compound throughout the organism's entire or partial life cycle
13	Is the tested exposure scenario relevant for the species?	X				Yes, the standard exposure scenario for this species was applied.
	Final score (C1 = Relevant, C2 = Relevant with restrictions, C3 = Not relevant, C4 = Not assignable)	**C1**				The test has been conducted and recorded according to the OECD 201 standard guideline.

Supplementary Material V

Raw data

Table S1. Raw data for acute test of emodin to *Daphnia similis* performed for 48 h at 21 °C

Concentration (mg L^{-1})	**Number of immobilized organisms**				**Total**	**%**
	1	**2**	**3**	**4**		
0	0/5	0/5	0/5	0/5	0/20	0
DMSO (0.01%)	0/5	0/5	0/5	0/5	0/20	0
0.019	0/5	0/5	0/5	0/5	0/20	0
0.037	0/5	0/5	0/5	1/5	1/20	5
0.075	1/5	1/5	2/5	3/5	7/20	35
0.15	3/5	3/5	1/5	3/5	10/20	50
0.30	5/5	5/5	5/5	5/5	20/20	100

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Chapter 03

Yellow and red onion dyes and simulated dyeing effluents

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Manuscript will be submitted in the future with corrections

Abstract

The textile industry has been the target of criticism and concern regarding social and environmental issues due to its contribution to air, water, and soil pollution. Its effluents contain potentially toxic substances such as dyes, salts, heavy metals, biocides, among other components that can cause adverse effects to the aquatic ecosystem. Studies in the literature on the aquatic toxicity of synthetic textile dyes for freshwater organisms show a high toxicity index. Because of this, the use of natural dyes in the textile industry has attracted interest as an ecological and sustainable alternative. This study aimed to evaluate the eco/genotoxicity of yellow and red onion dyes and their effluents from simulated dyeing baths. For the simulated dyeing process, we used yellow and red onion dyes and fabrics provided by the BioColour project. Furthermore, metallic mordants ($Al_2(SO_4)_3$ and $FeSO_4$) were added to the baths. Acute aquatic toxicity tests were performed with the dyes, mordants and generated effluents using a freshwater organism *Daphnia similis* and a marine organism *Parhyale hawaiensis.* Mutagenicity of the dyes were evaluated by the Ames test (MPA protocol). Dyes were not toxic to *D. similis* and *P. hawaiensis*. The mordants were toxic to *D. similis*, Al-EC_{50} = 28.7 mg L^{-1} and Fe-EC_{50} = 75.3 mg L^{-1}, and *P. hawaiensis* with Fe-EC_{50} = 157.8 mg L^{-1}, Al was not toxic. The effluents were toxic for both organisms, *D. similis* (EC_{50} ranging from 27.8 to 57.0%) and *P. hawaiensis* (LC_{50} ranging from 31.5 to 56.3%). Greater toxicity was observed in effluents with dye reuse, which had a higher concentration of metallic mordant. Yellow and red onion dyes were mutagenic in Ames test. The use of non-metal mordants, dyeing techniques without adding water (supercritical CO_2) and techniques for recovery of metals from effluents are some alternatives to be studied to obtain more satisfactory results in the toxicity of effluents that use natural dyes.

Keywords: aquatic toxicity; natural dyes; *Daphnia similis*; *Parhyale hawaiensis*; mordants.

6.1. Introduction

The textile industry is estimated to contribute to approximately 10% of global carbon dioxide (CO_2) emissions, exceeding 1.7 million tons annually. It stands as the second-largest consumer of water, utilizing 1.5 trillion liters per year, and concurrently generates substantial amounts of textile waste (over 92 million tons per year; Niinimaki et al., 2020).

In the textile manufacturing process, approximately 80% of the used water is released as wastewater (Vajnhandl; Valh, 2014). This procedure also contributes significantly to the consumption of various chemical products, including dyes, salts, heavy metals, and other components that can impact the aquatic ecosystem (Mani; Bharagava, 2018). Effluents from textile industries contain potentially toxic substances. A significant source of aquatic contamination stems from the discharge of dye remnants in textile industry effluents. The primary worry surrounding the introduction of coloured effluents into natural settings is their adverse aesthetic effects, which include altering water colour, impacting light penetration, and disturbing photosynthetic activity. (Lellis et al. 2019). In addition, many of the dyes and degradation products used in the textile industry are toxic and genotoxic to aquatic organisms (Azevedo et al. 2020; de Oliveira et al. 2016; Leme et al. 2015; Vacchi et al. 2016).

Synthetic dyes are extensively employed in the textile industry, encompassing approximately 8,000 different dyes. The annual global consumption of synthetic textile dyes is estimated to reach 800,000 tons (Patel; Bhatt, 2022). It is estimated that 15% of the world production of dyes reaches the environment during processing or application and the primary cause of this loss in the textile industry stems from the incomplete fixation of dyes during the dyeing stage of textile fibers (Guaratini; Zanoni, 2000).

In the fiber dyeing process, there are important steps such as dye penetration into fiber, dye fixing and washing as the after treatment to remove unfixed dye. The majority of natural dyes do not have much affinity to cellulosic textile fibers; hence, these are subjected to mordants which act in the fixation and enhance durability of the color (Samanta; Konar, 2011). The use of mordant for improving the quality of the dyeing process may release hazardous pollutants because some mordants, *e.g*. chrome, cooper, iron, and aluminum, which is commonly used, are considered as potentially toxic metals (İşmal; Yildirim, 2018). The textile industry is facing pressure to decrease the usage of harmful substances, particularly those with mutagenic, carcinogenic, and allergenic effects (Singh; Cadha, 2016). In response, researchers have

directed their efforts toward sustainable, cleaner, and cost-effective production methods and strategies.

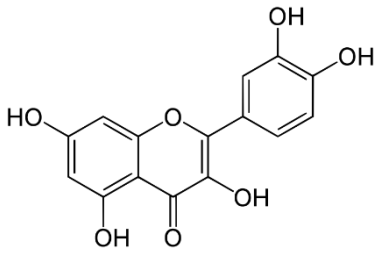
In this chapter, we propose a new dyeing method with dye reuse and evaluation of the toxicity of natural dyes (extracts of yellow and red onions) and their contribution to the toxicity of dyeing effluents to aquatic organisms, *D. similis*, and *P. hawaiensis*. The selection of these dyes was based on their availability in large quantities for the dyeing processes that requires dye concentrations in mg L^{-1}. The dyeing effluents were performed at LAEG with Dr. Riikka Räisänen who visited us in February/2022.

6.2. Material and Methods

6.2.1 Dyes, mordants and fabrics samples

Samples of two natural dyes called yellow onion (YO) and red onion (RO) skins were used. The dyes were extracted from the dry skins of onions (*Allium cepa*) in water, in a proportion of 1:10 (m/m). The solution was kept at 80 °C for 45 minutes, after being filtered through a 60 mesh (0.250 mm) and dried using a spray drying process.

Figure 1 - Quercetin, main colour compound of yellow and red onion samples.



Source: Grande et al., 2023; Räisänen et al., 2023

The fabrics used were of natural fibers: bamboo viscose and merino wool (Orneule; Orivesi, Finland). From the bamboo viscose piece (96% Bamboo viscose, 4% Lycra, single knit, fabric weight 250 g/m²) and merino wool (100% Merino wool, interlock, fabric weight 250 g/m²) pieces of 10 g were cut for the dyeing experiments.

Aluminum sulfate PA [$Al_2(SO_4)_3$.14-18H_2O, > 99%, Synth, Brazil] and iron sulfate II PA [$FeSO_4.7H_2O$, > 98%, Synth, Brazil] were used as mordants in the dye bath.

6.2.2 Simulation of dyeing effluents

Dyeing processes simulating an industrial batch dyeing technique was performed on a laboratory scale, according to Räisänen; Nousiainen; Hynninen (2001). The conditions for the

dye bath were mordant (10% by mass of fabric), dye (0.1% by mass of fabric) and fabric mass to liquid ratio (LR) was 1:20. Two processes were evaluated, a one-step viscose fabric dyeing and a two-step dyeing, where viscose was followed by wool with reuse of the dye bath and new addition of mordant (Figure 2). The order of fabric dyeing was carried out according to the chemical and physical properties of each fabric. Due to its porous structure, wool is known to absorb natural dyes very well, which is why it was added in the dye reuse stage (Sadik et al. 2023).

6.2.3 Dyeing bath

The dyeing bath with the viscose was carried out with the dye YO, with the mordants ($Al_2(SO_4)_3$ and $FeSO_4$) and, for the dye RO, only with $Al_2(SO_4)_3$. 10 g of fabric was soaked in deionized water, and the dyebath was prepared with deionized water, dye (0.2 g) and mordant (2 g). The soaked fabric was then added to the dyebath. Details of the dyeing bath are described below, and represented in the scheme of Figure 2:

Fabric preparation: two pieces of viscose fabric, 10 g each, were soaked into deionized water for 10 min and then the excess water was removed.

Pre-mordant: the bath was prepared in 200 mL of deionized water and 2 g of mordant. The bath was heated, under agitation, until it reached a temperature of 40 ºC. Upon reaching the temperature, the two pieces of fabric were added, the bath temperature was increased to 70 ºC and maintained for 30 min, under constant agitation. Then, the two pieces of fabric were removed and the dye bath prepared.

Dyeing: In the pre-mordant bath, 0.2 g of dye and 200 mL of deionized water were added, maintaining constant agitation until a temperature of 70 ºC was reached. Upon reaching the temperature, the fabrics were added to the bath and kept stirring for 60 min. Then, the viscose fabrics were removed, rinsed water and placed to dry at room temperature.

Dyeing bath with dye reuse

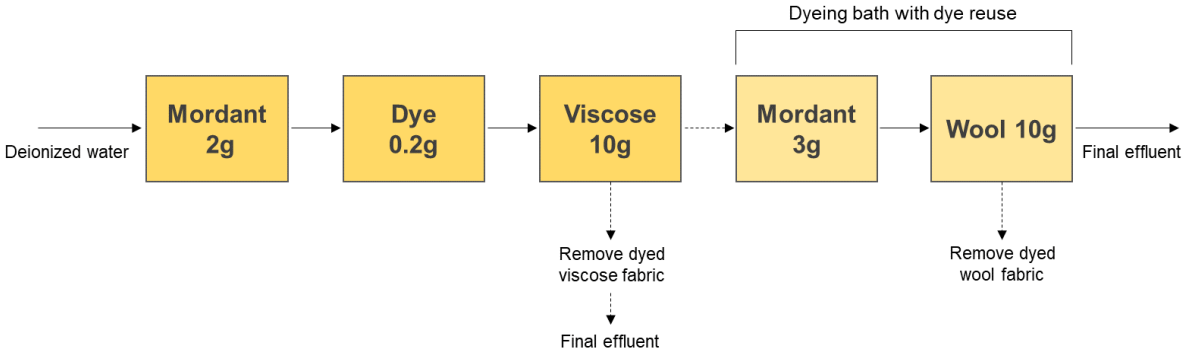
In order to reuse the dye bath after the viscose dyeing, a sample of wool was added to the sequence. This process was carried out with YO dye with the two mordants ($Al_2(SO_4)_3$ and $FeSO_4$) and, for RO dye, only with $Al_2(SO_4)_3$. In this case, altogether 3 g of mordant was added to the bath: 2 g in the first circle and 1 g in the second circle. Details are described below:

Fabric preparation: two pieces of each fabric, 10 g each, were soaked into deionized water for 10 min and then excess water was removed.

Pre-mordant: The bath was prepared in 200 mL of deionized water and 2 g of mordant. The bath was heated, under agitation, until it reached a temperature of 40 ºC. Once the temperature was reached, the two pieces of bamboo viscose were added. Then, the fabrics were removed. In sequence, 1 g of mordant was added and the two pieces of merino wool were added to the bath. The bath temperature was increased to 70 ºC and maintained for 30 min, under constant agitation. Then, the fabrics were removed and the dye bath prepared.

Dyeing: In the pre-mordant bath, 0.2 g of dye and 200 mL of deionized water were added, maintaining constant agitation until reaching temperature of 70 ºC. Upon reaching the temperature, the viscose fabrics were added to the bath and kept stirring for 60 min. Then, the fabrics were removed, rinsed water and placed to dry at room temperature. Next, the two fabrics of merino wool were added to the bath and kept in the bath at 70 ºC for 60 min. Then, the wool fabrics were removed, rinsed water and placed to dry at room temperature.

Figure 2 - A schematic diagram of the dyeing bath and dye reuse process.



6.2.4 Quantification of metals in dyeing effluents

The effluents obtained from the dyeing baths were acidified (pH < 2) by adding 1:1 v/v of nitric acid (HNO_3) solution. The determination of dissolved aluminum and iron concentrations in the effluents was carried out using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-OES) with a Perkin Elmer Optima 8300 model. The wavelengths used for the analysis were 283.204 nm for iron and 396.153 nm for aluminum. The plasma argon flow rate was 15 L min^{-1}, the auxiliary gas N2 was 0.5 L min^{-1} and the nebulizer was 0.75 L min^{-1} and the power was 1450 W. An analytical curve was used for quantification at concentrations of 0.1; 0.5; 1; 5; 10; 50 and 100 mg L^{-1}. The standard solutions of the metals were diluted in 1% nitric acid solution (HNO_3, Merck). The values of the limits of detection (LD) and quantification (LQ) of the methods for the metals, Al and Fe, were calculated as described in Miller and Miller (2005), through the analysis of the parameters of the analytical

curve. The limits of detection (LD) and quantification (LQ) for iron were 3.7 and 12.4 mg L^{-1}, respectively. For the aluminum method, they were 1.7 and 5.6 mg L^{-1}.

6.2.5 Ecotoxicity tests

Acute ecotoxicity tests were performed with *D. similis* and *P. hawaiensis*. The freshwater microcrustacean *D. similis* is widely used in the evaluation of the ecotoxicity of dyes and effluents (de Luna et al. 2014; Ferraz; Grando; Oliveira, 2011; Rosa et al. 2019; Vacchi et al. 2016a) as they are present in abundance in freshwater system and are an important link in the food chain. Due to the adjustment of the pH of the effluent samples for aquatic toxicity with *D. similis*, precipitation of metals was observed, so we also consider the use of the epibenthic organism, *P. hawaiensis*, for the evaluation of the aquatic toxicity of the samples.

Acute test with Daphnia similis

For natural dyes, toxicity tests involved the use of *D. similis*, following the OECD guideline n° 202 (OECD 202, 2004). Cultures of *D. similis* were maintained at 20 ± 2 °C, with a photoperiod of 16:8 h (light/dark) using an Eletrolab EL 2020/4LED system. The organisms were daily fed with the microalgae *Raphidocelis subcapitata*, and total water exchange was conducted three times per week. Cultures were housed in 2 L glass vessels. For the tests, twenty neonates (<24 h old) from 2- to 3-week-old mothers were placed in four replicates for each concentration, with five organisms per replicate. Negative and solvent controls were included in the tests, and glass tubes (10 mL) were used to prevent dye adsorption to the material. The temperature and photoperiod conditions during the tests were consistent with the cultivation conditions. The organisms were not fed during the 48-h exposure period. Immobilized organisms were counted after the exposure., When the number of immobilized organisms in negative and solvent controls did not exceed 10%, tests were treated as valid. Parameters such as conductivity, dissolved oxygen, and pH were determined at the beginning and end of the tests.

For metal and effluent samples, we performed the test according to the miniaturized protocol (Oliveira and Umbuzeiro, 2021) due to the restriction of the volume of simulated effluents. The preparation of metal and effluent samples for the toxicity test consisted of adjusting the pH (2.7 – 4.2) to the range of the MS medium (6.8 – 7.1), by adding 1N sodium hydroxide solution (NaOH, >98%, Sigma). For effluent samples, subsequent centrifugation at 4000 rpm for 20 min was an additional step. The supernatant was used in the test.

Acute test with marine amphipod Parhyale hawaiensis

Cultures of *P. hawaiensis* were maintained under controlled conditions at 24 ± 2 °C, with a 12-h photoperiod (light: dark), and constant aeration. Daily feeding occurred five days a week using commercial sinking fish food. Saltwater with a salinity of 30 ± 2 was prepared by reconstituting commercial sea salt, with partial water exchanges (50%) performed twice a week. Total water exchange took place once a month during vessel cleaning and substrate renewal.

For the acute toxicity testing of natural dyes using the amphipod *P. hawaiensis*, the methodology followed Artal et al., (2018). Test solutions were prepared through serial dilution using reconstituted seawater at salinity 30, achieved by combining marine salt and deionized water. Reconstituted seawater served as the negative control, and 0.01% DMSO functioned as the solvent control. Neonates (32, ≤ 7 days old) from 8 to 10 months old mothers were placed in 32 wells, with 200 μL of solution and one organism per replicate. The tests were conducted under the same cultivation conditions. After 96 h, the number of dead organisms was recorded using a stereomicroscope (Stemi 2000-C, Carl Zeiss, Oberkochen, Germany). When mortality in negative and solvent controls did not exceed 10%, tests were treated as valid. Mortality rates higher than 10% indicated toxicity.

For metal and effluents toxicity tests, instead of 32 organisms, we used 20 organisms for each concentration due to limited sample volume available for testing. In tests with effluent samples, salinity and pH were adjusted to acceptable ranges for the test organism, 30 ± 2 and 8 ± 1, respectively (Artal et al., 2018).

6.2.6 Mutagenicity test

For yellow and red onions dyes, the *Salmonella*/microsome mutagenicity test (Microplate format, MPA) developed by Zwarg et al., (2018) was performed with TA98 and TA100 strains with and without metabolic activation (S9 mix) at 5%. These strains were used because they are known for detecting most bacterial mutagens according to Williams et al. (2019). The test was performed with negative and positive controls, in order to ensure the responsiveness of the strains and the effectiveness of the metabolic activation system. As a negative control, the dimethyl sulfoxide (DMSO) solvent was used. As positive controls, specific mutagenic compounds for TA98 and TA100 strains were used: without metabolic activation, 4-nitroquinoline-1-oxide at 1.25 ng μL^{-1}, and with metabolic activation, 2-aminoanthracene at 5 ng μL^{-1}.

6.2.7 Colour fastness

To examine the dyeing result, the colour fastness properties of the dyed materials for rubbing and washing were measured according to ISO standards (ISO 105-X12:2016, ISO 105-C06:2010). The colour fastness to washing is tested by assessing the colour loss and staining on dyed fabric during the soap washing process. The colour change following the washing fastness test was assessed using a greyscale, ranging from grades 1 to 5. A rating of 5 indicates minimal change, while a rating of 1 indicates the maximum change (ISO 105-C06:2010). The colour fastness to rubbing test was used to determine the degree of colour which may be transferred from the surface of a coloured fabric to a specify test cloth for rubbing (which could be dry and wet) (Suganuma, 2013).

6.2.8 Statistical analysis

Acute toxicity data from *D. similis* and P. *hawaiensis* tests were subjected to modeling using the generalized logistic model from the glmnet package (Friedman et al., 2010). This approach was employed to estimate the 50% effect and lethal concentrations (EC_{50}) along with their corresponding 95% confidence intervals (C.I.). The model selected was based on the statistical significance of the concentration variable ($p \leq 0.05$). The analyses were carried out using R (RStudio Team, 2022).

For the Ames test, the data was analyzed using an ANOVA followed by a post hoc Tukey test ($p < 0.05$). Subsequently, regression analysis ($p < 0.05$) was performed using the Bernstein model (Bernstein et al., 1982). These statistical analyses were conducted with the assistance of the Salanal software, kindly provided by Integrated Laboratory Systems from Research Triangle Institute, Research Triangle Park, NC, USA. The experiment was considered valid only when cell viability and the positive control exhibited appropriate responses.

6.3. Results and Discussion

Six effluents were obtained from the dyeing baths. The identification of dyes, mordant and fabric used, as well as the name adopted for the effluents generated are presented in Table 1.

Table 1 - Identification of dyes, mordants, fabrics and effluents name

Dyes	Mordants	Fabrics	Effluents name
Yellow Onion (YO)	$Al_2(SO_4)_3$ (Al)	bamboo viscose	YO+Al (v)
		bamboo viscose + merino wool	YO+Al (v+w)
	$FeSO_4$ (Fe)	bamboo viscose	YO+Fe (v)
		bamboo viscose + merino wool	YO+Fe (v+w)
Red Onion (RO)	$Al_2(SO_4)_3$ (Al)	bamboo viscose	RO+Al (v)
		bamboo viscose + merino wool	RO+Al (v+w)

6.3.1 Quantification of metals

The concentrations of metals, total dissolved aluminum and iron were determined for the effluents of the dyeing baths. The values are shown in Table 2. The working range of the methods for iron and aluminum were from 0.1 to 100 mg L^{-1}. We observed that in baths where we added more metallic mordants (dye reuse step), the effluents generated, YO+Al (v+w); YO+Fe (v+w); RO+Al (v+w) contained higher concentrations of metals when compared to baths without dye reuse, YO+Al (v); YO+Fe (v); RO+Al (v).

Table 2 - Physicochemical parameters (pH and conductivity) of effluents and quantification of total dissolved Al and Fe

Effluents	pH	Conductivity (µS/cm)	Total dissolved Al (mg L^{-1})	Total dissolved Fe (mg L^{-1})	pH [a]	Conductivity (µS/cm) [b]
YO+Al (v)	2.94	3400	766	-	6.83	7140
YO+Al (v+w)	3.02	3400	1100	-	6.92	8920
YO+Fe (v)	4.17	134	-	21.5	6.85	156
YO+Fe (v+w)	3.52	273	-	42.9	6.92	326
RO+Al (v)	2.74	2579	556	-	6.93	5630
RO+Al (v+w)	3.44	3020	820	-	7.09	6000

[a] pH adjusted to a suitable value for acute testing with *D. similis*; [b] conductivity value after pH adjustment, for the highest tested concentration.

6.3.2 Ecotoxicity tests with yellow and red onion extracts, mordants and dyeing effluents

The wastewater generated from the dyeing using yellow and red onions was collected and tested using two organisms, *D. similis* (freshwater) and *P. hawaiensis* (salt water). We evaluated the toxicity of both dyes using the same organisms and the toxicity of the mordants in concentration-response experiments for comparisons with the toxicity of the effluents.

Dyes did not show toxicity to any of the organisms tested (Table 3). The two mordants used in the dye bath ($Al_2(SO_4)_3$ and $FeSO_4$) were evaluated for their toxicity as well. $Al_2(SO_4)_3$ concentrations ranged from 5.2 to 40 mg L^{-1} and 1 to 100 mg L^{-1} for *Daphnia* and *Parhyale* acute tests, respectively and $FeSO_4$ concentrations ranged from 3.12 to 100 mg L^{-1} and 10 to 1000 mg L^{-1}. According to İşmal; Yildirim (2018), the metallic mordants are ecologically hazardous. The amount of mordant fixed onto the textiles and left in wastewater have to be considered. The mordants showed acute toxicity to *D. similis* with EC_{50} of 28.7 mg L^{-1} for Al and 75.3 mg L^{-1} for Fe (Table 3). Fe was toxic to *P. hawaiensis* with EC_{50} of 157.8 mg L^{-1}, while Al was not toxic in tested concentrations (Table 3).

Table 3 - EC_{50}/LC_{50} values and confidence intervals obtained in the acute toxicity tests with *D. similis* and *P. hawaiensis* for the dyes and mordants.

Samples	**EC_{50}/LC_{50} (C.I.) mg L^{-1}**	
Dyes	*Daphnia similis*	*Parhyale hawaiensis*
Yellow onion	N.T.	N.T.
Red onion	N.T.	N.T.
Mordants		
$Al_2(SO_4)_3$	28.7 (9.8 – 47.5)	N.T.
$FeSO_4$	75.3	157.8 (64.4 – 359.1)

EC_{50}, effective concentration 50%; LC_{50}, lethal concentration 50% C.I., confidence interval; N.T., not toxic.

All effluents tested were toxic to *D. similis*, with immobility >10% (Table 4). Comparing the two types of dyeing, greater immobility was observed at effluents with dye reuse, of which more metallic mordant was added, therefore higher concentrations of metal were quantified. Due to high concentrations of metals added to the baths, their concentrations in the effluents are expected to be in the order of g L^{-1}.

Table 4 - Acute toxicity data and EC_{50} value for *D. similis* (48 h) exposed to effluent samples from yellow and red onion dyeing process

	Immobility (%)					
Concentration (%)	**YO+Al (v)**	**YO+Al (v+w)**	**YO+Fe (v)**	**YO+Fe (v+w)**	**RO+Al (v)**	**RO+Al (v+w)**
0	0	0	0	0	5	0
0.625	0	0				
1.25	0	0				
2.5	0	0				
3.125			0	0		
5	0	5				
6.25			0	0	10	15
10	0	0				
12.5			0	0	0	15
25	15	0	0	0	10	40
50	0	15	0	60	15	100
100	15	100	60	100	50	100
EC_{50} (C.I.)	N.E.	57	N.E.	48.5 (48.3 – 48.4)	N.E.	27.8 (12.1 – 43.6)

EC_{50}, effective concentration 50%; C.I., confidence interval; N.E. not estimated

All the effluents with dye reuse showed acute toxicity to *P. hawaiensis* (Table 5).

Probably the amount of metal present in the effluent samples may be the most responsible for the observed toxicity.

Table 5 - Acute toxicity data and LC_{50} value for *P. hawaiensis* (96 h) exposed to effluent samples from yellow and red onion dyeing process.

Concentration (%)	Mortality (%)					
	YO+Al (v)	YO+Al (v+w)	YO+Fe (v)	YO+Fe (v+w)	RO+Al (v)	RO+Al (v+w)
0	0	0	0	10	0	5
33	0	60	0	35	0	5
66	0	95	0	100	20	70
100	0	100	0	100	15	100
LC_{50} [a] (C.I.) [b]	N.T. [c]	31.5 (9.5 – 96.6)	N.T. [c]	34.5 (12.1 – 90.1)	N.E. [d]	56.3

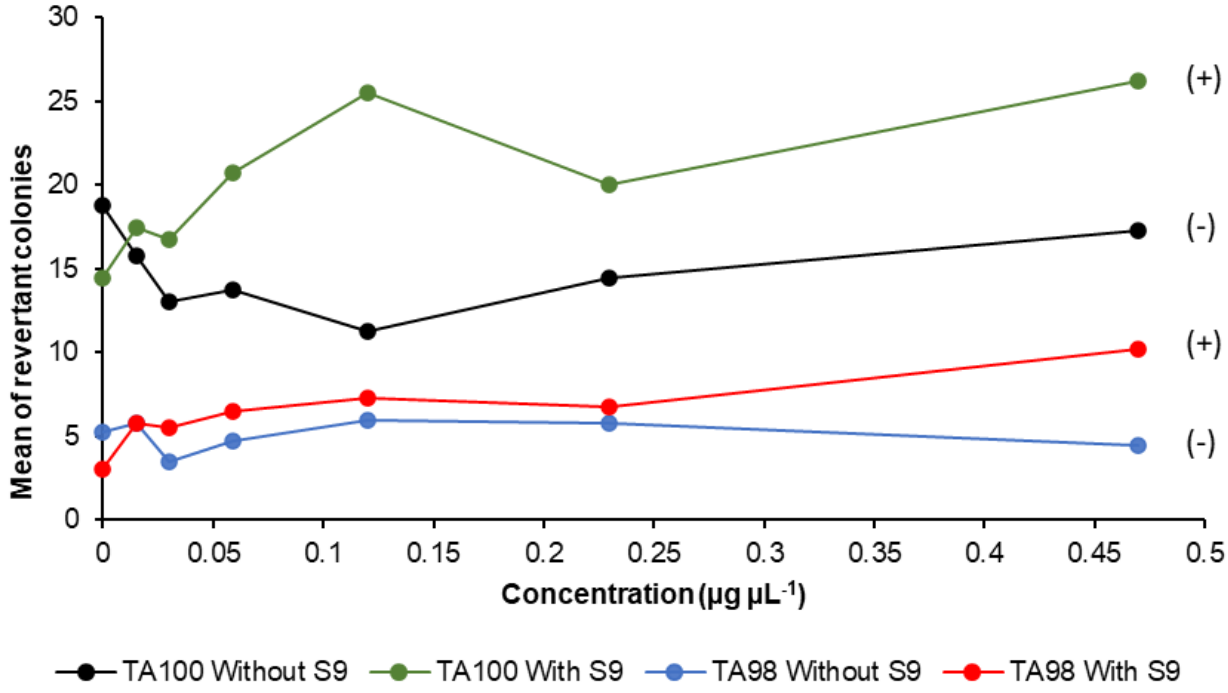
[a] LC_{50}, lethal concentration 50%; [b] C.I., confidence interval; [c] N.T., not toxic; [d] N.E., not estimated.
* pH values were within the range for the test-organism (8±1)

Although the yellow and red onions did not show acute toxicity to any of the organisms tested, their use is only efficient with the use of mordants, which in most cases are toxic metals for aquatic life. In order for the textile industry's dyeing process with natural dyes to be effectively sustainable and less aggressive to the environment, other ways of fixing the dye in the fabric must be considered. An alternative for replacing metallic salt mordants is the use of non-metallic mordents from natural source as tannins, chitosan, and barks which are already being studied for their effectiveness, considering the potential for color fixation, environmental aspects and biodegradation (Mathur; Gupta, 2003; Teli; Sheikh; Shastrakar, 2013).

6.3.3 Mutagenicity of yellow and red onion dyes

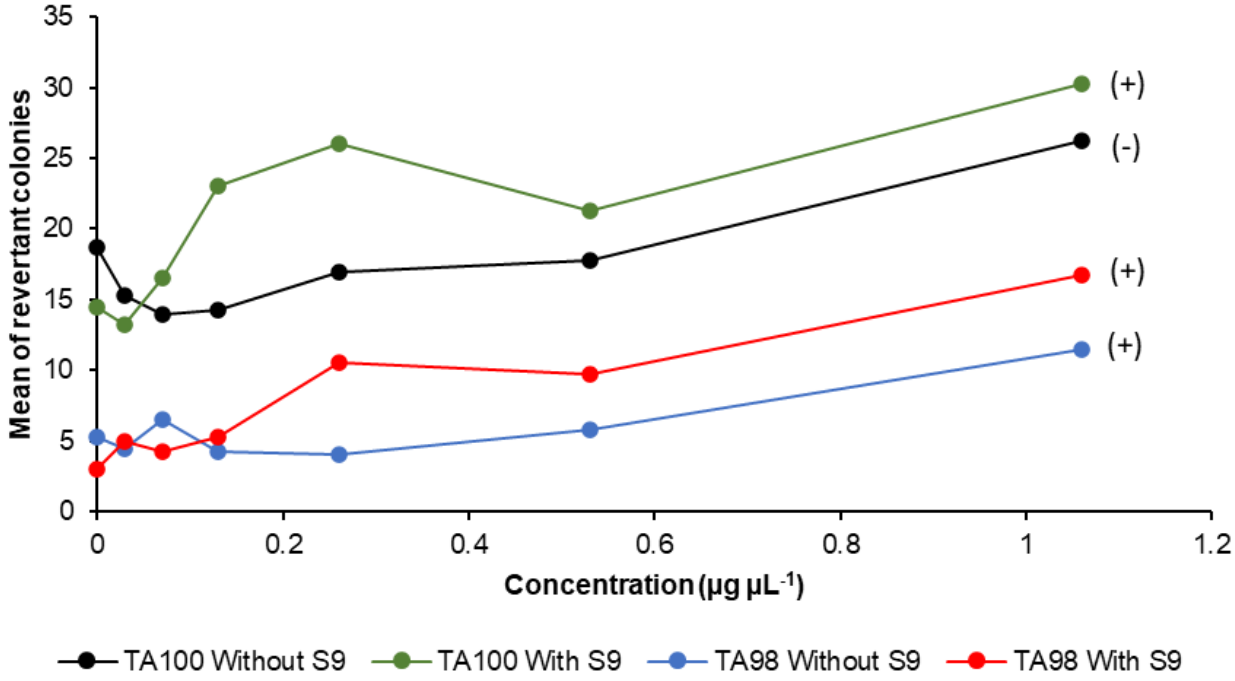
The hazard of the yellow and red onion dyes was tested for mutagenicity in the MPA test. The yellow onion samples were mutagenic for the TA98 and TA100 strains with S9 (Figure 3).

Figure 3 - Concentration-response curves for a yellow onion sample, using strains TA98 and TA100 in the *Salmonella*/microsome assay, miniaturized version (MPA). (+) indicates positive sample/condition and (-) negative response.



The red onion dye was mutagenic for the TA98 strain with and without S9 and for the TA100 strain with S9 (Figure 4). For the TA100 strain without S9, although no significant difference was observed, the effect of concentration response also seems evident. The observed mutagenicity may be related to the presence of a phenolic compound, quercetin (Gao et al., 2021).

Figure 4 - Concentration-response curves for a red onion sample, using strains TA98 and TA100 in the *Salmonella*/microsome assay, miniaturized version (MPA). (+) indicates positive sample/condition and (-) negative response.



6.3.4. Colour fastness

The fabrics, viscose and wool, dyed by the dyeing simulation with yellow and red onions were subsequently evaluated for dye fixation to the fiber at University of Helsinki, Finland (UH). Figure 5 illustrates the assessment of color-fastness properties for each fabric, while Table 6 provides a summary of the color-fastness properties of the dyed fabrics.

Figure 5 - Colour fastness to washing. (A) Original Hanau Linitest laboratory machine; (B) Vessel with colour fabrics after washing; (C) Colour-fastness assessment.

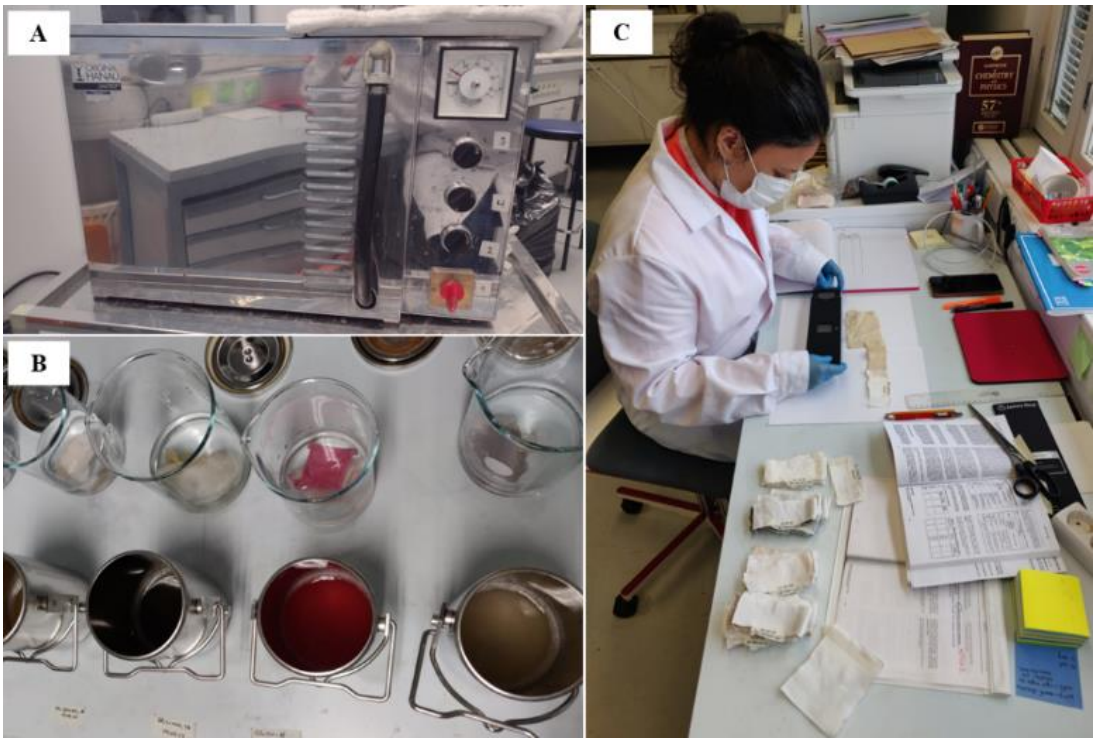


Table 6 - Color-fastness properties of dyed bamboo viscose and wool against washing and rubbing. The washing-fastness results in color change are given as visually assessed gray scale ratings

Fabric/dyeing bath	Washing fastness							Rubbing fastness	
	Color change (vis asst)	Staining						Dry staining (dm)	Wet staining (dm)
viscose		WO	PAN	PES	PA	CO	CA		
YO+Al (v)	1/2 w	5	5	5	5	5	5	4/5	4
YO+Al (v+w)	1/2 w	5	5	5	5	5	5	4/5	4
YO+Fe (v)	2 beige	5	5	5	5	5	5	5	4/5
YO+Fe (v+w)	2 beige	5	5	5	5	5	5	5	4/5
RO+Al (v)	1 w	4/5	5	5	5	5	5	4/5	4
RO+Al (v+w)	1 w	4/5	5	5	5	5	5	4/5	4
wool									
YO+Al (v+w)	4/5 y str	5	5	5	5	5	5	4/5	4
YO+Fe (v+w)	3 brown	5	5	5	5	5	5	4/5	4
RO+Al (v+w)	3 yellow	5	5	5	5	5	5	4/5	4

Vis asst = visual assessment, WO = wool, PAN = acrylic, PES = polyester, PA = polyamide, CO = bleached cotton, CA = diacetate, y = yellower, w = weaker, str = stronger, dm = direction of manufacture

From the perspective of dyes application, the dyed fabrics exhibit satisfactory resistance to washing and rubbing fastness, with values ranging from 4 to 5, indicating the effective role of metals like aluminum (Al) and iron (Fe) as mordants. This contribution aids in the successful binding of natural dyes to wool and viscose fibers during colour-fastness evaluation.

6.4. Conclusion

Yellow and red onion dyes were not acutely toxic to *Daphnia similis* and *Parhyale hawaiensis*, but they were mutagenic in the miniaturized version of the *Salmonella*/microsome assay (MPA) with TA98 and TA100 strains. The simulated effluents' toxicity can be explained by the metal (Al and Fe) concentrations used as mordants. The use of mordants from non-metallic sources, dyeing techniques without adding water (supercritical CO_2), and techniques for recovering metals from effluents are some alternatives to be explored in the future to reduce the toxicity of the generated wastewaters.

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7. Final considerations

Through the development of eco/genotoxicological characterization of natural dyes, it was possible to verify that their natural origin does not necessarily mean their environmental and human safety. Natural dyes need to be evaluated for their ecotoxicity and genotoxicity before being considered for use, and possible replacement of synthetic dyes on the market. The use of toxicity tests, acute and chronic, with a variety of aquatic organisms is also important for understanding the effects of dyes on aquatic environment since they can be released into the water bodies by effluents, especially from the textile industry. Dermorubin was the only dye that did not present toxicity to any of the aquatic organisms and it was not mutagenic.

Emodin was the most toxic dye among all the dyes tested, with the lowest PNEC value of 0.0005 mg L^{-1}. Zebrafish embryos (*D. rerio*) was the most sensitive organism. Furthermore, emodin was mutagenic to the aquatic organism *P. hawaiensis*. More studies on the possible mutagenic effects on germ cells of aquatic organisms are important to understand how this compound can affect the number of offspring and future generations.

In the context of utilizing natural dyes in the textile industry, their application typically involves the use of a mordant, which aids in fixing the dye within the fibers. The metallic mordants commonly used for this purpose can be more toxic than the dyes used. Therefore, not only the dyes used, but the entire dyeing process must be reevaluated in order to minimize environmental impacts. New dyeing methodologies that use less water and non-metallic mordants are encouraged to make the textile industry process less harmful to the environment and human health.

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8. Annex

Annex 1. Declaration of the Animal Use Ethics Committee (CEUA) of the State University of Campinas (UNICAMP), protocol nº 5645-1/2020

UNICAMP

CEUA/UNICAMP

C E R T I F I C A D O

Certificamos que a proposta intitulada **Caracterização eco/genotoxicológica de corantes naturais**, registrada com o nº **5645-1/2020**, sob a responsabilidade de **Prof. Dr. Gisela de Aragão Umbuzeiro e Natália Oliveira de Farias**, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo *Chordata*, subfilo *Vertebrata* (exceto o homem) para fins de pesquisa científica (ou ensino), encontra-se de acordo com os preceitos da **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, do **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**, e com as normas editadas pelo **Conselho Nacional de Controle da Experimentação Animal** (**CONCEA**), **tendo sido** aprovada pela **Comissão de Ética no Uso de Animais** da **Universidade Estadual de Campinas** - **CEUA/UNICAMP,** em reunião de **15/10/2020**.

Finalidade:	**( ) Ensino (X) Pesquisa Científica**
Vigência do projeto:	15/11/2020 a 15/12/2023
Vigência da autorização para manipulação animal:	15/10/2020 a 15/12/2023
Espécie / linhagem/ raça:	Peixe** / Danio rerio
No. de animais:	1440
Idade/Peso:	1.00 Dias / 0.00 Gramas
Sexo:	1440 Machos
Espécie / linhagem/ raça:	Peixe** / Danio rerio
No. de animais:	1440
Idade/Peso:	1.00 Dias / 0.00 Gramas
Sexo:	1440 Machos
Espécie / linhagem/ raça:	Peixe** / Danio rerio
No. de animais:	1440
Idade/Peso:	1.00 Dias / 0.00 Gramas
Sexo:	1440 Machos
Espécie / linhagem/ raça:	Peixe** / Danio rerio
No. de animais:	1800
Idade/Peso:	1.00 Dias / 0.00 Gramas
Sexo:	1800 Machos
Espécie / linhagem/ raça:	Peixe** / Danio rerio
No. de animais:	1800
Idade/Peso:	1.00 Dias / 0.00 Gramas
Sexo:	1800 Machos
Espécie / linhagem/ raça:	Peixe** / Danio rerio
No. de animais:	3600
Idade/Peso:	1.00 Dias / 0.00 Gramas

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Sexo:	3600 Machos
Origem:	Biotério de Cultivo de Peixe-zebra, Laboratório de Ecotoxicologia e Genotoxicidade - FT/UNICAMP
Biotério onde serão mantidos os animais:	Biotério de Cultivo de Peixe-zebra, Laboratório de Ecotoxicologia e Genotoxicidade, FT/UNICAMP

A aprovação pela CEUA/UNICAMP não dispensa autorização a junto ao **IBAMA**,**SISBIO** ou **CIBio** e é **restrita** a protocolos desenvolvidos em biotérios e laboratórios da Universidade Estadual de Campinas.

Campinas, **28 de outubro de 2020**.

Prof. Dr. Wagner José Fávaro	Rosangela dos Santos
Presidente	Secretária Executiva

IMPORTANTE: Pedimos atenção ao prazo para envio do relatório final de atividades referente a este protocolo: até 30 dias após o encerramento de sua vigência. O formulário encontra-se disponível na página da CEUA/UNICAMP, área do pesquisador responsável. A não apresentação de relatório no prazo estabelecido impedirá que novos protocolos sejam submetidos.

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