Biodiversity restoration and conservation of inland water ecosystems for environmental and human well-being

BioReset

BiodivRestore-406

2020 - 2021 Joint Call
Joint COFUND Call on “Conservation and restoration of degraded ecosystems and their biodiversity, including a focus on aquatic systems”

Deliverable 3.1

Resilience of diatom biofilms to climate changes and micropollutants

Lead Beneficiary	Work package	Delivery month
CIIMAR	**3**	**44**

1. Executive Summary

Diatoms are unicellular microalgae with a porous siliceous cell wall. They can be found in practically all aquatic environments and are well-recognised as bioindicators of ecological water quality. They respond to abiotic stress in short time scales, showing differentiated responses to a variety of aquatic contaminants. Diatoms are the main components of biofilms. These are aggregates of various microorganisms (diatoms and other microalgae, protozoa, fungi and bacteria) embedded in a polymeric matrix. The biofilms are visible macroscopically as greenish to brownish layers colonising different substrates (rocks, pebbles, blocks, shells, clay...).

Diatom sampling is the basis for the evaluation of the ecological water quality with taxonomic quality indices as recommended in the Water Framework Directive. This is a suitable and widely used method, but it is cumbersome and expensive, requiring sample preparation and identification knowledge and experience, among others (Pinto et al., 2021). Various methods have thus been considered for its suitable replacement, though the proposals available are also expensive and require sample preparation significant technical resources and expertise (Pinto et al., 2021). **BioReset** proposes the development of an innovative approach based on Raman Spectroscopy (RS), which does not require sample preparation, can be employed with a minimum number of measurements done by non-specialised personnel, with results analysed using pre-established chemometrics routines. For this, a relevant step on this pathway is to better understand the predictive ability of RS under different laboratory-controlled conditions, as well as the sensitivity of the approach relative to other relevant test organisms and sensitive endpoints. This is core to future implementation of the developed method in regular monitoring for adaptive management. This deliverable thus reports on works that were started within the scope of the previous REWATER project (WaterWorks 2015, Water JPI, WaterJPI/0008/2016) where the working hypothesis of **BioReset** was forged and works further developed within **BioReset**. Within the scope of the work developed, one MSc dissertation, three PhD theses and six international publications were done. They served the planning and comparative purposes for the biofilm experiments carried out. The following of the deliverable reports on the main contributions in these indicators, their input to the experiments and the biofilm experiments carried out. Future work will be focused on expanding the experiments to improve understanding on the field results and the gradients of ecosystem recovery.

2. Task description

The work was started within the scope of project REWATER (WaterWorks 2015, Water JPI, WaterJPI/0008/2016) and continued or concluded within the scope of WP3 (Evaluation of ecosystem conservation and restoration: diatom biofilms), coordinated by CIIMAR. Within the work developed, to better understand the sensitivity of the RS approach, various experiments were conducted with emerging contaminants of concern, some including mixtures of pharmaceuticals, and various species and endpoints, namely chironomidea, triops, freshwater snails and zebrafish embryos em larvae and diatom biofilms. The animal experiments were always done with species or life stages not covered by the EU and PT Animal Welfare Act protecting experimental animals, to develop suitable alternatives to animal testing. For improved comparison, the RS application was done to diatom biofilms, but also extended to freshwater snails and zebrafish embryos and larvae.

3. WP3 – Team members involved in the work development

The following researchers contributed to the samplings and development of the work presented herein:

Name	Organization	Role
Laura Guimarães	CIIMAR	Institutional leader and WP coordinator
António Paulo Carvalho	CIIMAR	Task coordinator
Luís Oliva-Teles	CIIMAR	Task coordinator
Raquel Pinto	CIIMAR	PhD student allocated to the project (FCT grant)
Pedro Rodrigues	CIIMAR	PhD student
Isabel Abreu	CIIMAR	PhD student
André Pereira	CIIMAR	PhD student
João Amorim	CIIMAR	PhD student

4. Developed activities

The activities were developed to fulfil knowledge gaps related to sensitive species and endpoints, and the ability of diatoms to anticipate results that may occur later in time on species from communities taking longer time to respond to environmental pollution and climate stress, for their longer generation time (e.g. macroinvertebrates, vertebrates). The experiments varied in experimental design, duration, factors tested. An account of the main contributes generated is provided in the following.

5. Controlled lab experiments

5.1. Toxicants, species and endpoints

Part of the work was related to investigating which transformation products of pharmaceuticals and pesticides were in urgent need for more information about their potential toxicological burden in aquatic systems. Pharmaceutical and pesticides are know to enter into aquatic systems, through sources that include wastewater treatment plants (WWTP). However, parental compounds are not the only substances of concern. Their metabolites and degradation products, i.e. transformation products, are increasingly detected in freshwater samples and WWTP effluents. To systematise the information available and contribute with empirical data for regulatory objectives and experimental planning, an in-depth review work was carried out. Overall, concentrations for 190 transformation products (92 from pesticides and 98 from pharmaceuticals) in water bodies and WWTP effluents were gathered. A concentration heatmap (Figure 1) was produced to easily spot the substances found at higher levels and plan future research. The very limited available toxicological data linked exposure to transformation products to adverse outcomes in humans (genotoxicity and alteration in detoxification processes) and aquatic species (mostly related to apical endpoints). The results were the subject of a publication (Rodrigues et al., 2022) in a highly considered international scientific journal and were used in subsequent work (Oberdorfer, 2022; Rodrigues, 2023; Rodrigues et al., 2023; Amorim, 2024; Pereira, 2024; Pereira et al., 20224; Abreu et al., 2024).

Rodrigues et al. (2023) tested six transformation products reported in WWTP effluents, belonging to three psychoactive drugs (Figure 2); carbamazepine-10,11-epoxide, 10,11-dihydrocarbamazepine, O-desmethylvenlafaxine, N-desmethylvenlafaxine, O-desmethyltramadol, N-desmethyltramadol). The exposures were done with zebrafish up to 168 h post-fertilisation to levels ranging from 0.1 to 100 µg/L. A concentration-response relationship was found for the effects of some embryonic malformations. Carbamazepine-10,11-epoxide, O-desmethylvenlafaxine and tramadol elicited the highest malformation rates. All compounds significantly decreased larvae responses on a sensorimotor assay compared to controls. Altered expression was found for most of 32 tested genes. In particular, abcc1, abcc2, abcg2a, nrf2, pparg and raraa were found to be affected by all three drug groups. For each group, the modelled expression patterns showed differences in expression between parental compounds and metabolites. Potential biomarkers of exposure were identified for the venlafaxine and carbamazepine groups. The results were part of a PhD thesis (Rodrigues, 2023) and very relevant for the planning of experiments employing RS, namely with zebrafish embryos and larvae (Abreu et al., 2024) and diatom biofilm experiments (see next section).

The works of Guimarães et al. (2024), Pereira (2024), Pereira et al. (2024) and Amorim (2024) were related to the sensitivity of behaviour assessments in invertebrate species (Figure 3) and comparison of it with RS. Despite advances in treatment technologies, WWTP effluents still contain a myriad of pollutants, including legacy and emerging contaminants of concern that need to be assessed regularly for potential toxicity and negative impact in aquatic systems. Behavioural responses (e.g. locomotor behaviour) have been found to be extremely sensitive to contaminants. They are the integrated result of vital biochemical and physiological processes within animals and have a reflex at the population level. The short-term and long-term locomotor responses of juvenile and adult/mature *Triops longicaudatus* (Figure 3) were first investigated. *Triops longicaudatus* is a crustacean typically inhabiting temporary freshwater bodies in regions with a Mediterranean climate. These crustaceans are easily maintained in the laboratory and show a set of biological features that make them suitable for diagnosing environmental quality and health. This also led to the full development of the setup to be used in the subsequent research, a video-tracking biological early warning system (BEWS). Short-term responses were assessed upon exposure to low concentrations of five model toxicants upon a very short 1.5 h exposure. The compounds tested were tributyltin, mercury, lindane, sodium hypochlorite and formaldehyde. The long-term responses were assessed upon exposure to reconstituted complex mixture of pharmaceuticals representing combinations found in rural, urban and hospital WWTPs. The pharmaceuticals in the mixtures were Atenolol, Carbamazepine, Diclofenac, Naproxen, Sulfamethoxazole, and Trimethoprim. The data were analysed with an artificial neural network to identify distinct behaviours, followed by Chi-square and Correspondence analysis to characterise the response to each treatment. The results showed that triops behaviour is sensitive to aquatic contamination, particularly sodium hypochlorite in an exposure as short as 1.5h duration. Evaluation of locomotor behaviour also proved to be the most sensitive indicator in long-term exposures to simulated WWTP effluents. The results were part of a PhD thesis (Pereira et al., 2024).

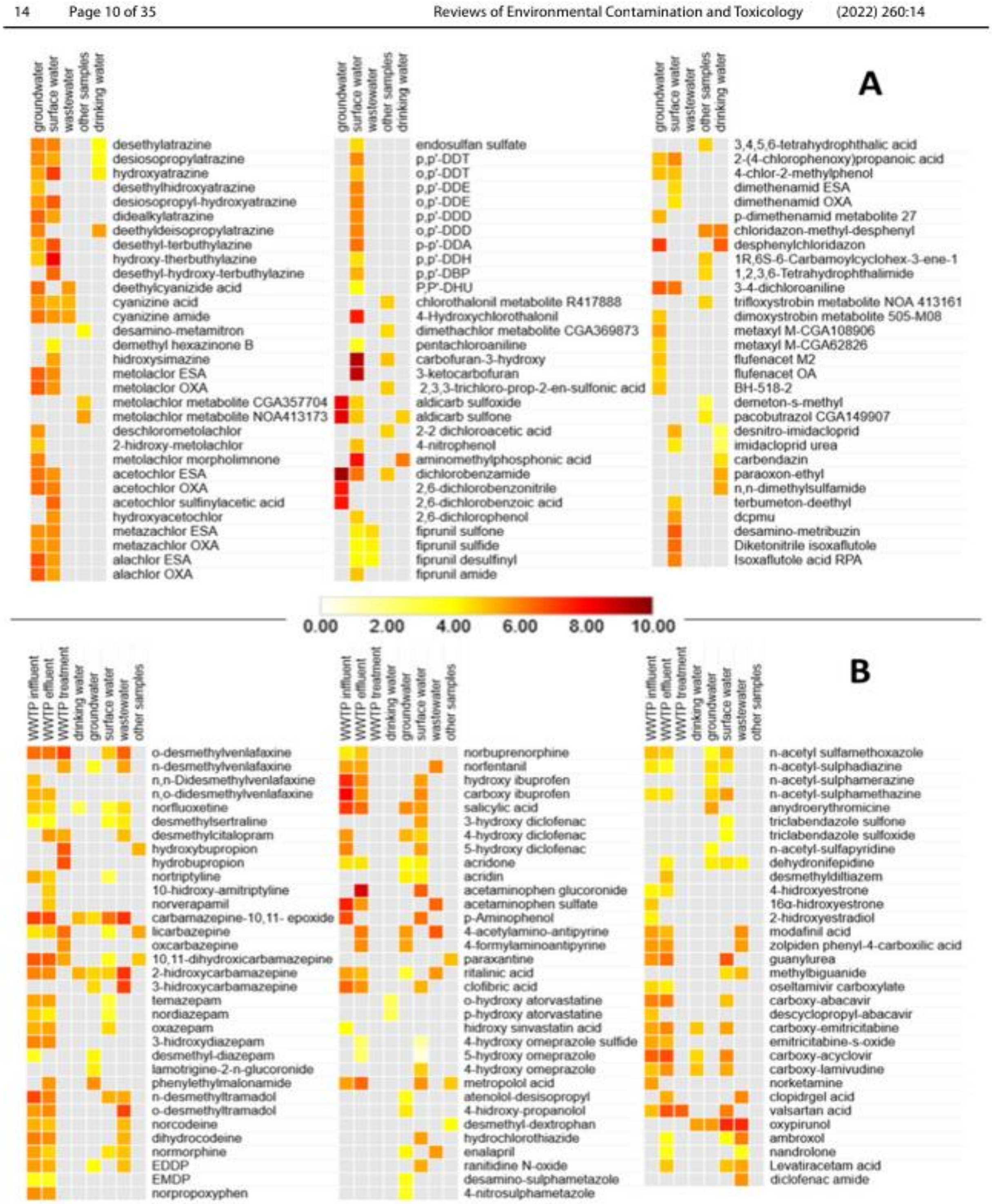

Figure 1. Maximum concentrations of pesticide (A) and pharmaceutical (B) transformation products found in different water compartments. The heatmaps were done with the log-transformed values (pg/L). Grey squares represent cases for which no information could be found (Rodrigues et al., 2022).

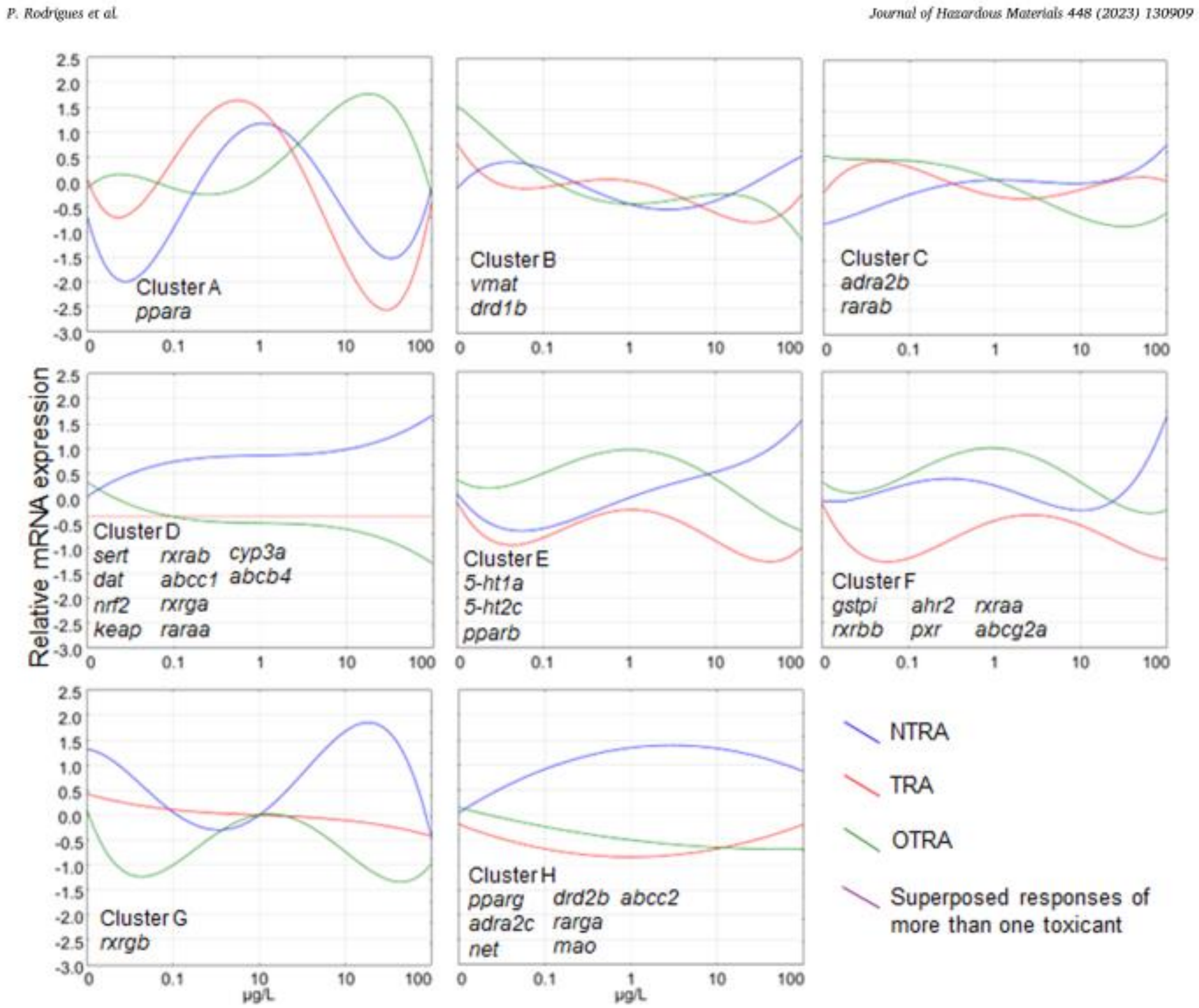

Figure 2. Model expression responses derived for gene clusters identified by a Cluster Analysis. Gene expression was determined in zebrafish larvae exposed for 168 hpf to tramadol, O-desmethyltramadol (OTRA) or N-desmethyltramadol (NTRA). The model responses were derived using the equation of the saturated orthogonal multiple linear regression (Rodrigues et al., 2023).

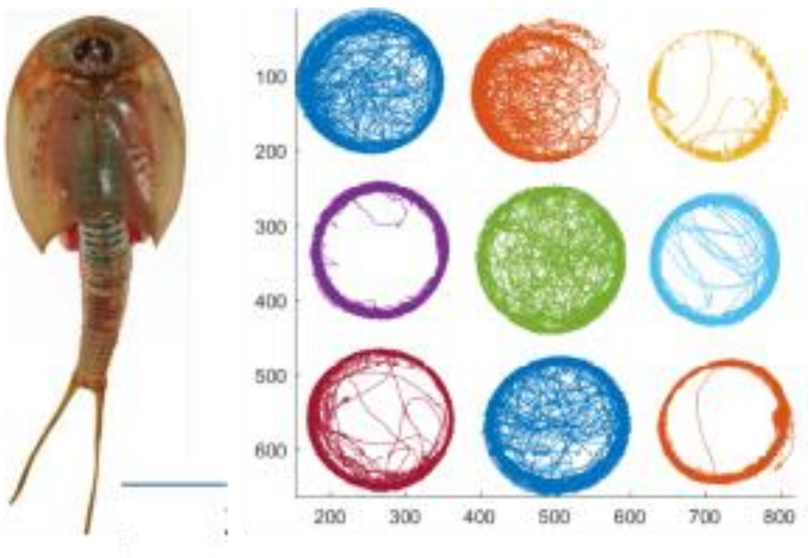

Figure 3. *Triops longicaudatus* (left, scale bar = 10 mm) and an example of its recorded locomotor behaviour (right) (Pereira, 2024).

A similar experiment that was also started in REWATER was done with *Lymnaea stagnalis*, but pairing both the behavioural video-tracking system and RS assessments. *Lymnaea stagnalis* is a freshwater snail used as experimental model in ecotoxicology. The exposure experiments lasted two weeks and toxicants investigated were aminomethylphosphonic acid (AMPA, metabolite of glyphosate), bisphenol A (BPA), cadmium (Cd), perfluorooctanesulfonic acid (PFOS) and venlafaxine. The video-tracking system was the one developed in the work with triops and the test concentrations were planned based on the results of the works described above. The test concentrations were 1.8 µg Cd/L, 3.3 µg AMPA/L, 0.4 µg BPA/L, 0.1 µg PFOS/L and 1.3 µg venlafaxine/L. Venlafaxine and AMPA were found to elicit slight significant increases in the snail's feeding behaviour, in relation to the control. Conversely, BPA, Cd and PFOS were found to reduce feeding intake. AMPA stimulated egg laying, while BPA caused a reduction in oviposition, compared to the control. All treatments altered the locomotor behaviour of the exposed snails, compared to the controls, leading to opposite behaviour. The strongest alterations were found for cadmium, AMPA and BPA. Raman spectroscopy was used to investigate spectra acquired from the ovotestis, ganglia, digestive gland, mantle and foot. Alterations elicited by the different treatments were found for bands 868, 952, 963, 1005, 1023, 1137, 1157, 1191, 1210, 1269, 1308, 1376, 1450, 1520, 1591, 1629, 1637, 1629 and 1637 cm^{-1}. The results were presented in a PhD thesis (Amorim, 2024) and further investigations are on course to better understand the relation between the alterations found by RS to the locomotory performance detected. This work was particularly useful to better understand how to apply RS to organs and tissues. Despite the available literature, it was not clear whether any sample treatment would be need or, for example, if changes elicited by toxicant exposure would be more efficiently assessed in fresh tissues or in homogenates. The results thus brought important insight that allowed to extend RS application to zebrafish embryos em larvae.

The available literature was already pointing out the potential of RS for early diagnosis of detrimental effects elicited by toxicant exposure during zebrafish developmental stages. No baseline spectra were, however, available that could allow for suitable planning of toxicological assessments, comparison with toxicant-elicited spectra or mechanistic understanding of biochemical and physiological responses to the exposures. With this in mind, the work of Abreu et al. (2024) carried out a baseline characterisation of Raman spectra of zebrafish embryos and larvae throughout early development. Raman spectra were recorded from the iris, forebrain, melanocytes, heart, muscle and swim bladder between 24 and 168 h post-fertilisation (Figure 4). A chemometrics approach, based on partial least-squares discriminant analysis (PLS-DA), was used to obtain a Raman characterisation of each tissue or organ. In total, 117 Raman bands were identified, of which 24 were well represented and, thus, retained in the data analysed. Only three bands were found to be common to all organs and tissues. The PLS-DA provided a tentative Raman spectral fingerprint typical of each tissue or organ, reflecting the ongoing developmental dynamics. The bands showed frequencies previously assigned to collagen, cholesterol, various essential amino acids, carbohydrates and nucleic acids. The results were published in a scientific journal and as an open access dataset (Abreu et al., 2024). The work then proceeded with the exposure of embryos and larvae to a dichloroaniline (positive control in zebrafish assays for which much information is available) and to tramadol (previously investigated by the team). The experimental design closely follows that of Rodrigues et al. (2023) and the results are now under replication for confirmation and relation to the gene expression and proteomic alterations already found.

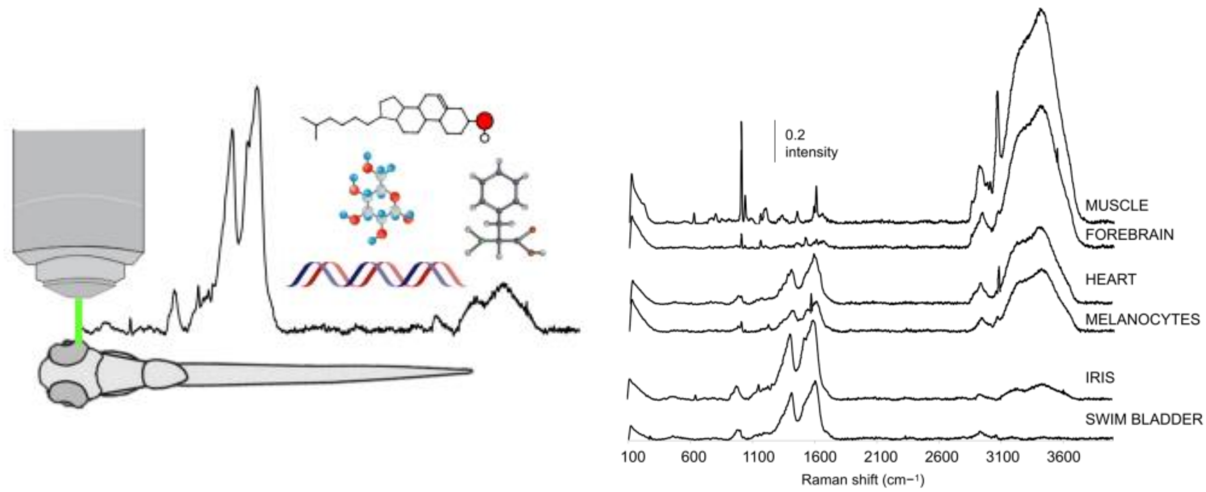

Figure 4. Raman spectra recorded from 100 to 4000 cm^{-1} from zebrafish larvae at 144 hpf (Abreu et al., 2024).

5.2. Diatom biofilms

The biofilm experiments started early in the project development. They proved, however, very challenging in various aspects. The first of them was the biofilm harvesting for laboratory maintenance. Various substrates were tested for local incubation in the water courses, namely glass slides, ceramic surfaces, pebbles. They were, however, easily lost for various reasons, including adverse weather conditions or unplanned disturbance. In alternative, sampling techniques were tested to harvest the biofilms and bring them to the lab. The maintenance under controlled laboratory conditions was another significant challenge. Several approaches had to be refined, including the medium supplementation with base nutrients. Different nutrient rates had to be tested, as depending on the original biofilm, a natural shift towards the growth of green microalgae and cyanobacteria in detriment of diatoms occurs very easily. Discussions were held with colleagues from the Helmholtz Environmental Centre UFZ Leipzig, with experience in biofilm culture. They confirmed the various challenges and time-consuming work necessary to develop their own biofilm experiments and provided relevant clues on how to address the problems.

Meanwhile, cultures with *Phaeodactylum tricornutum* were carried out to investigate toxicant concentrations of copper and other compounds. After several biofilm stabilisation attempts, the first biofilm experiments could be done. The biofilms require at least three weeks after harvesting in the lab to mature into a stage appropriate for the exposure experiments. They are grown in glass slides incubated in 10-20 L recipients with a low aerification level. Over the incubation, medium renewal is done every two days and after the second week supplementation with nutrients is done to avoid growth arrest due to silica limitation. The first biofilm experiments were carried out with copper to establish a positive control. The results of the experiments with *P. triocornutum* were used to determine exposure concentrations and all the test logistic and assessments required were refined on these experiments. Given the high variability of biofilms in composition, species ratios, and local conditions in the harvesting sites, including a positive control in the experiments is crucial, particularly in cases of negative results, i.e. the test toxicants elicit no changes in the biofilm diatom communities.

Desvenlafaxine was then tested in the biofilm experiments. The exposure lasted for eight days and included five treatments; three venlafaxine treatments and negative and positive controls. Assessments of the diatom communities were done on days before exposure and on exposure days 0, 2, 4, 6 and 8 on the glass slides. Exposure to desvenlafaxine showed the drug can negatively affect diatom growth, compared to the negative control, at concentrations as low as 1 mg/L (40-50% biomass decrease) and 0.1 mg/L (30-40% decrease) (Figure 5).

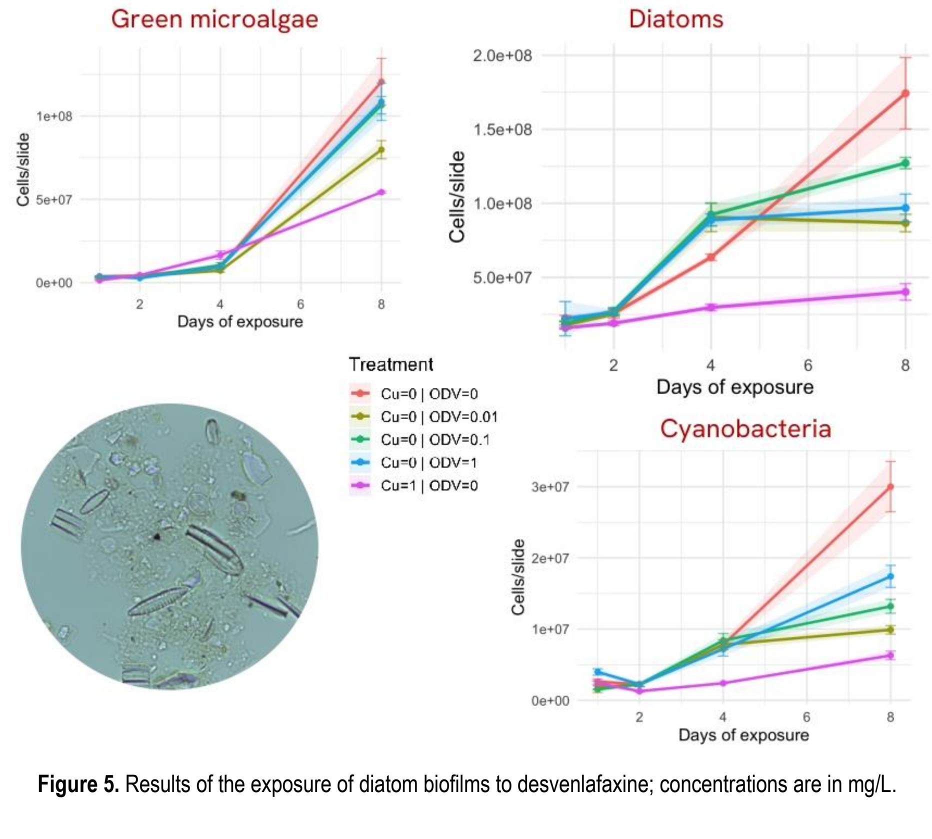

Figure 5. Results of the exposure of diatom biofilms to desvenlafaxine; concentrations are in mg/L.

Significant growth inhibitory effects, compared to the negative control, were also found at 1 mg/L desvenlafaxine, though this is a concentration already above levels commonly found in environmental waters. Nevertheless, the strongest inhibition appears to be elicited by the lowest exposure concentration. This comes to reinforce the well acknowledge fact that many organisms show non-monotonic dose-response curves upon exposure to antidepressants or their transformation products (Rodrigues, 2023, Rodrigues et al., 2023). The same appears to occur with diatoms. Diatoms were the group most affected by desvenlafaxine and copper exposure (Figure 6). Similar results were, however, found for the microalgae and cyanobacteria, though the inhibition rate was lower. In all groups, copper exposure was found to cause more severe impacts than desvenlafaxine. Recovery after exposure depends highly on the original biofilm and the effects size. There is a natural very high biofilm variability, which highly impacted by the harvesting season. For example, collection during the summer brings higher differences in the biofilms and their responses to contamination, compared to winter collected biofilms. This requires a much higher replication effort of the experiments to ensure reproducibility and deep understanding of and confidence on the results obtained and conclusions drawn. Part of the experiments are thus still on course to increase the number of toxicants assessed and model the recovery conditions. The work is also done within the scope of the PhD thesis of student Raquel Pinto, with scholarship from the Foundation for Science and Technology allocated to the project. The results obtained will be confronted with those reported in the previous section and the field studies.

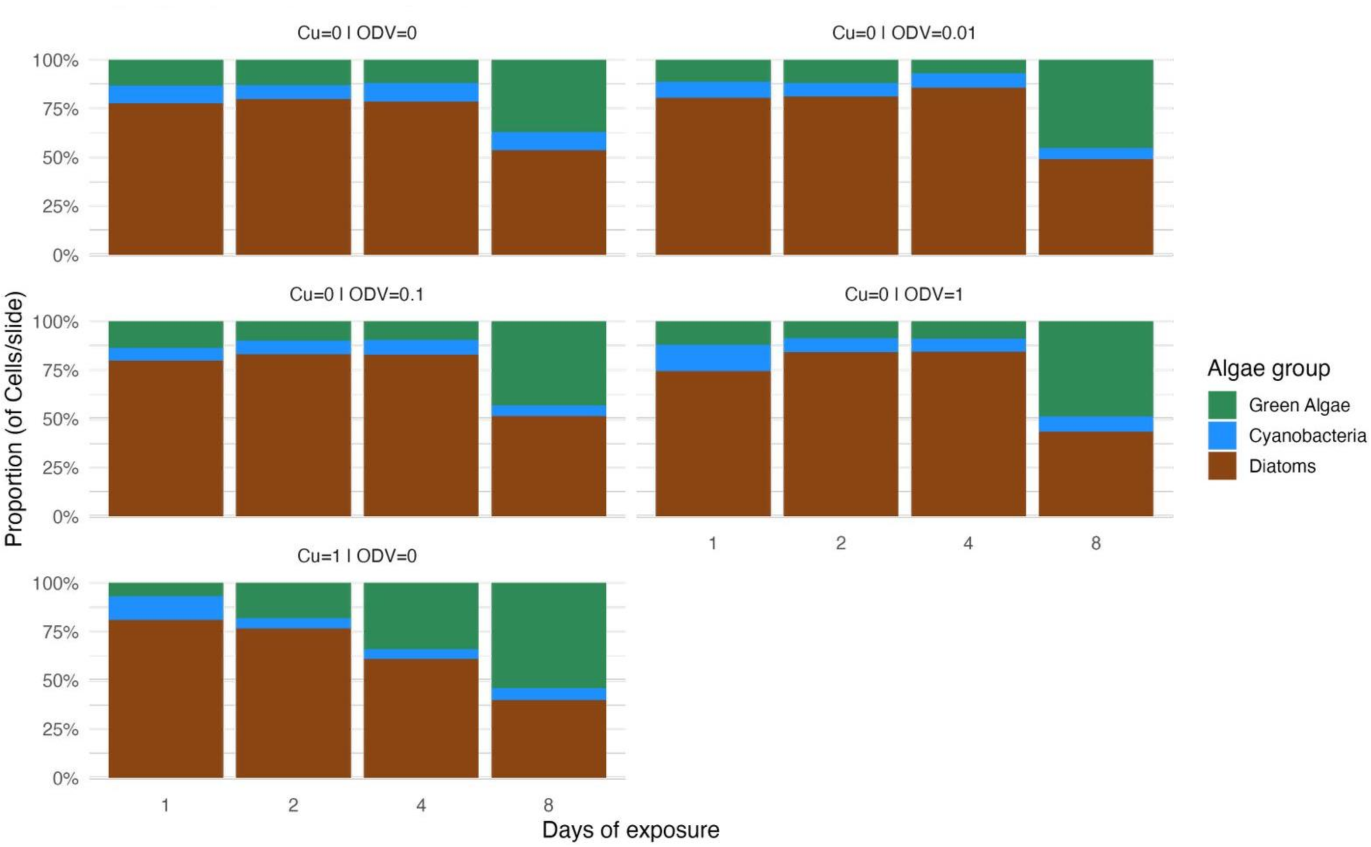

Figure 6. Relative composition in microalgae groups of the biofilms exposed to to desvenlafaxine for eight days; concentrations are in mg/L.

6. Associated indicators

The indicators which led to the development of this work that made or will make use of it are indicated below.

Publications

1. Rodrigues, P., Oliva-Teles, L., Guimarães, L., Carvalho, A.P. (2022). Occurrence of Pharmaceutical and Pesticide Transformation Products in Freshwater: Update on Environmental Levels, Toxicological Information and Future Challenges. Reviews of Environmental Contamination and Toxicology, 260: 14. DOI: 10.1007/s44169-022-00014-w
2. Rodrigues, P., Guimarães, L., Carvalho, A.P., Oliva-Teles, L. (2023). Carbamazepine, venlafaxine, tramadol, and their main metabolites: Toxicological effects on zebrafish embryos and larvae. Journal of Hazardous Materials, 448: 130909. DOI: 10.1016/j.jhazmat.2023.130909
3. Pereira, A.C., Saraiva, A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P. (2024). Ecotoxicological Effects of Potassium Dichromate on the Tadpole Shrimp *Triops longicaudatus*. Animals, 14, 3: 358. DOI: 10.3390/ani14030358
4. Guimarães, L., Carvalho, A.P., Ribeiro, P., Teixeira, C., Silva, N., Pereira, A., Amorim, J., Oliva-Teles, L. (2024). Sensitivity of *Triops longicaudatus* Locomotor Behaviour to Detect Short Low-Level Exposure to Pollutants. Water, 16, 1:126. DOI: 10.3390/w16010126
5. Abreu, I.O., Teixeira, C., Vilarinho, R., Rocha, A.C.S., Moreira, J.A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P. (2024). Baseline Raman Spectral Fingerprints of Zebrafish Embryos and Larvae. Biosensors, 14, 538: 41640. DOI: 10.3390/bios14110538
6. Abreu, I.O., Teixeira, C., Vilarinho, R., Rocha, A.C., Moreira, J.A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P. (2024). Dataset for baseline Raman spectral fingerprints of zebrafish embryos and larvae. Zenodo. DOI: 10.5281/ZENODO.13937098, https://zenodo.org/records/13937098

Communications

1. Abreu, I.O., Teixeira, C., Vilarinho, R., Rocha, A.C.S., Moreira, J.A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P., Deciphering Zebrafish Spectral Signatures: Insights from Raman Spectroscopy, III 1-H TOXRUN International Congress, Porto, Portugal, May 2-3, 2024, Oral presentation.
2. Rodrigues, P., Oliva-Teles, L., Carvalho, A.P., Guimarães, L., Tramadol and its main metabolites: toxicological effects on zebrafish embryos and larvae, III 1-H TOXRUN International Congress, Porto, Portugal, May 2-3, 2024, Oral presentation.
3. Guimarães, L., Impacto da Poluição e das Alterações Climáticas na Saúde Ambiental: Monitorização e Perspetivas Futuras, IX Jornadas do Mar e da Atmosfera, Universidade de Aveiro, Aveiro, Portugal, February 12-14, 2025, Oral presentation.
4. Guimarães, L., Reutilização segura de água residual, como assegurar?, 9.ª Conferência de Professores do Mar, Ciência Viva - Agência Nacional para a Cultura Científica e Tecnológica, Pavilhão do Conhecimento, Lisboa, Portugal, May 10-11, 2024, Oral presentation.
5. Surra, E., Guimarães, L., Delerue-Matos, C., New Environmental Risk Thresholds for 36 Pharmaceuticals in Life Cycle Assessment, SETAC Europe 34th Annual Meeting, Seville, Spain, May 5-9, 2024, Oral presentation.
6. Guimarães, L., Monitoring aquatic pollution using Raman Spectroscopy, International Conference Biodiversity restoration and conservation of inland water ecosystems, Theme III – Wastewater treatment evaluation, ISEP, Porto, Portugal, April 18-19, 2024, Oral presentation.
7. Guimarães, L., Avaliação e monitorização de contaminação ambiental para a sustentabilidade dos ecossistemas aquáticos, I Workshop in Environmental Management, Universidade Positivo, Curitiba, Brazil, Online, November 16, 2023, Oral presentation.
8. Abreu, I.O., Teixeira, C., Vilarinho, R., Rocha, A.C.S., Moreira, J.A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P. Raman spectral signatures of zebrafish organs, Blue Think Conference, CIIMAR, Matosinhos, Portugal, September 13, 2023, Oral presentation.
9. Guimarães, L., Assessment of water treatments and conservation of aquatic ecosystems for environmental and human well-being, II TOXRUN International Congress 2023, Porto, Portugal, April 27-28, 2023, Keynote Speaker.
10. Guimarães, L., BioReset - Biodiversity restoration and conservation of inland water ecosystems for environmental and human well-being. BioDivRestore, CIIMAR Annual Meeting 2022, CIIMAR, Matosinhos, Portugal, September 26, 2022, Oral presentation.
11. Guimarães, L., Aproveitamento e reciclagem de água: onde entra a toxicologia ambiental?, Conversas com Ciência em Serralves, Fundação de Serralves, Porto, Portugal, March 27, 2022, Oral presentation.
12. Abreu, I.O., Teixeira, C., Vilarinho, R., Rocha, A.C.S., Moreira, J.A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P., Comparative Spectral Signatures of Zebrafish Organs, Encontro Ciência 2023: Ciência e o Oceano Para Além do Horizonte, University of Aveiro, Portugal, July 5-7, 2023, Poster presentation.

13. Rodrigues, P., Guimarães, L., Carvalho, A.P., Oliva-Teles, L., Evaluation of the hazardous effects of pharmaceuticals impacting the neuroendocrine system, Encontro Ciência 2023: Ciência e o Oceano Para Além do Horizonte, University of Aveiro, Portugal, July 5-7, 2023, Poster presentation.
14. Abreu, I., Teixeira, C., Vilarinho, R., Rocha, A.C.S., Moreira, J.A., Oliva-Teles, L., Guimarães, L., Carvalho, A.P., Baseline Spectral fingerprints of zebrafish organs, Ocean3R Final Workshop and Meeting, Matosinhos, Portugal, June 27, 2023, Poster presentation.
15. Teixeira, C., Oberforder, L., Silva, A.P., Gravato, C., Oliva-Teles, L., Carvalho, A.P., Hornek-Gausterer, R., Guimarães, L., Effects of bis (4-chlorophenyl) sulfone (BCPS) on Chironomus riparius at low environmental levels, Ocean3R Final Workshop and Meeting, Matosinhos, Portugal, June 27, 2023, Poster presentation.

PhD thesis

1. Pedro Rodrigues (2023). Transformation products of pharmaceuticals occurring in wastewater effluents: molecular to population level toxicity for zebrafish embryos and larvae. PhD in Environmental Toxicology and Contamination, University of Porto, https://hdl.handle.net/10216/147965
2. André Pereira (2024). Potential of *Triops longicaudatus* to assess wastewater contamination. PhD in Environmental Toxicology and Contamination, University of Porto, https://repositorio-aberto.up.pt/bitstream/10216/164718/2/703643.pdf
3. João Amorim (2024). Developing new biomonitoring systems to assess water quality and diagnose environmental contamination using *Lymnaea stagnalis*. PhD in Environmental Toxicology and Contamination, University of Porto, https://repositorio-aberto.up.pt/bitstream/10216/164090/2/700041.pdf

Master dissertation

1. Lena Oberdorfer (2022). Ecotoxicological effects of Bis (4-chlorophenyl) sulfone exposure on *Chironomus riparius*. Master degree in Engineering, Technical Environmental Management and Ecotoxicology, FH University of Applied Sciences Technikum Wien, Austria (Erasmus Student).