UNIVERSIDADE FEDERAL DE JUIZ DE FORA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE E CONSERVAÇÃO DA NATUREZA

Jéssica Andrade Vilas Boas

The use of ciliated microeukaryotes and zooplankton in ecotoxicological studies: a look into the future

Juiz de Fora

2021

Jéssica Andrade Vilas Boas

The use of ciliated microeukaryotes and zooplankton in ecotoxicological studies: a look into the future

Tese de doutorado apresentada ao Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Instituto de Ciências Biológicas da Universidade Federal de Juiz de Fora, como requisito parcial para obtenção do título de Doutor.

Orientador: Dr. Roberto Júnio Pedroso Dias
Co-orientador: Dr. Andreu Rico Artero
Colaborador: Dr. Marcus Vinicius Xavier Senra

Juiz de Fora
2021

Ficha catalográfica elaborada através do programa de geração
automática da Biblioteca Universitária da UFJF,
com os dados fornecidos pelo(a) autor(a)

Vilas Boas, Jéssica Andrade.
The use of ciliated microeukaryotes and zooplankton in ecotoxicological studies: a look into the future / Jéssica Andrade Vilas Boas. -- 2021.
121 p.

Orientador: Roberto Júnio Pedroso Dias
Coorientador: Andreu Rico Artero
Tese (doutorado) - Universidade Federal de Juiz de Fora, Instituto de Ciências Biológicas. Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, 2021.

1. Microeucariotos ciliados. 2. Ecotoxicologia aquática. 3. Ecossistemas aquáticos. I. Pedroso Dias, Roberto Júnio, orient. II. Rico Artero, Andreu, coorient. III. Título.

Jéssica Andrade Vilas Boas

The use of ciliated microeukaryotes and zooplankton in ecotoxicological studies: a look into the future

Tese de doutorado apresentada ao Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Instituto de Ciências Biológicas da Universidade Federal de Juiz de Fora, como requisito parcial para obtenção do título de Doutor.

BANCA EXAMINADORA

_____

Dr. Roberto Júnio Pedroso Dias – Orientador. Universidade Federal de Juiz de Fora

_____

Dr. Andreu Rico Artero – Co-Orientador. Instituto Madrilenho de Estudos Avançados

_____

Dr. Reinaldo Luiz Bozelli – Titular Externo. Universidade Federal do Rio de Janeiro

_____

Dr. André Megali Amado – Titular Interno. Universidade Federal de Juiz de Fora

_____

Dr. Luiz Felipe Machado Velho – Titular Externo. Universidade Estadual de Maringá

_____

Dra. Ana Lúcia Fonseca – Titular Externa. Universidade Federal de Itajubá

Dedico este trabalho à **minha família**: meus pais *Silvete* e *Célio*, minha irmã *Tatiane* e meus sobrinhos *Lívya*, *Pietro* e *Lorenzo*: o combustível que me sustentou durante todo o meu caminho.

"*Não há um caminho certo na arte ou na vida. Às vezes, não há nenhum caminho e você deve demolir paredes e esculpir o seu próprio caminho através das madeiras para chegar onde precisa*".
(Anne with an E)

Agradecimentos

À ***Deus***, a energia maior que rege todas as coisas, que conduz a minha vida e me deu forças para chegar até aqui.

À minha ***família***, meus pais, ***Célio*** e ***Silvete***, meus maiores exemplos de honestidade e força. Obrigada por acreditarem em mim, me incentivarem e por viverem meus sonhos comigo. À minha irmã ***Tatiane*** e meu cunhado ***Fernando*** por serem meus exemplos de dedicação e persistência. Aos meus sobrinhos ***Lívya***, ***Pietro*** e ***Lorenzo***, sinal de luz e pureza em meu caminho. Em tantos momentos, olhar para cada um de vocês me trouxe a força necessária para perseverar. Meu incondicional amor e gratidão por tudo!

Ao meu querido orientador, amigo e conselheiro ***Roberto Dias***. Exemplo de humildade, dedicação e amor pelo que faz. Obrigada por acreditar em mim, pela paciência, por cada palavra e pelas lições na vida profissional e pessoal. Tê-lo como orientador foi um privilégio!

Ao meu colaborador e querido amigo ***Marcus Senra***. Obrigada pela paciência, carinho e zelo. Foram muitas as lições e os momentos compartilhados e sem você essa jornada não seria a mesma.

À minha amiga, parceira e conselheira ***Simone Cardoso***. Sua presença em minha jornada me trouxe mais certeza da fé, me fazendo crer ainda mais que tudo tem o seu tempo e que aquilo que nos pertence chega até nós. Você é luz!

Aos pesquisadores ***Andreu Rico*** e ***Marco Vighi*** pela gentileza, recepção e por ter me dado a oportunidade de ter trabalhado no Laboratório de Ecotoxicologia no Instituto Madrileno de Estudos Avançados (IMDEA-Agua). Agradeço, ainda, à ***Alba Arenas-Sánchez*** pelo carinho, paciência, amizade e por ter dividido comigo dias de muito trabalho. A todos os outros amigos do Lab Ecotox ***Raquel Dafouz***, ***Theresa Schell***, ***Francesco Pollazo*** pela paciência, gentileza e ensinamentos durante todo meu período na Espanha. De longe, foi a melhor e a mais transformadora experiência da minha vida, o meu coração transborda gratidão pela oportunidade e privilégio de ter vivido este período com vocês!

Aos meus companheiros (as) do LabProto, ***Bianca*** (Morena), ***Rafaela*** (ZN), ***Fabíola*** (ZN2), ***Glauber*** (Graube), ***Priscila*** (Creide), ***Talys*** (Haemoproteus), ***Vitor*** (Gente grande), ***Zacarias*** (Moçambique), ***Saulo*** (Saulin), ***Yasmine*** (Menina Cross Fit) e ***Roberto*** (Marchesi). Foram muitos os cafés, idas e vindas ao RU regados a risadas, lágrimas e trocas de experiências. Levo um pedacinho de cada um em meu coração!

Aos queridos alunos de Iniciação Científica ***Pedro Mendes*** e ***Luana Carvalho*** por terem abraçado o trabalho comigo, pela paciência, carinho e amizade. Vocês me ensinaram o verdadeiro sentido de aprender!

Aos meus amigos "bocós" ***Elen Furtado***, ***Lucas Lima***, ***Fran Cedrola*** e ***Marcus Senra***. A minha primeira família em Juiz de Fora. Obrigada por me acolherem em suas vidas e em suas casas. Foram muitas as trocas de experiências e as lições que me trouxeram força e leveza. Rimos, rezamos, choramos, brigamos, comemos (e muito) e eu não poderia desejar que fosse diferente. Eu amo vocês!

Aos meus amigos da "Fenda do Biquíni" e companheiros do LEA, ***Gabrielle Quadra***, ***José Reinaldo***, ***Iollanda Ivanov*** e ***Joyce Santos***. Obrigada por compartilharem a vida e a ciência. Ter convivido com vocês me trouxe a alegria, leveza e a luz necessária para caminhar. Meu carinho e gratidão!

As minhas "Power girls", ***Gabrielle Quadra*** e ***Iollanda Ivanov***, ter cruzado com vocês em meu caminho fez ainda mais minha jornada valer a pena. Vocês foram um dos (re)-encontros mais lindos que a vida me deu nesses últimos anos (irmãs de alma). Obrigada por cada momento compartilhado, pelas infinitas risadas, lições valiosas, puxões de orelha, incentivos e por me mostrarem que não importa onde e nem quando, nós sempre estaremos juntas no coração e no pensamento. Amo vocês!

A minha melhor amiga ***Carol Lanza*** por tantos anos de convivência e por ser aquela que, mesmo distante se faz presente. Você é peça fundamental em meu caminho: "*definir o amor não dá, então direi apenas 'Obrigado' e sei que entenderás*" (Anjos de Resgate). Amo você!

A minha amiga e companheira de casa, ***Nathália Resende***. Obrigada por ter me acolhido na sua vida e por ter dividido comigo muito mais do que só um lugar para morar. Foram muitos os momentos compartilhados (e põe momentos nisso), altos e baixos, sorrisos e lágrimas, conselhos e silêncio. Eu amo você!

Ao meu amigo ***Rafael Albo***, pelo carinho e por compartilhar as filosofias da vida comigo. Sua existência é um presente em minha vida!

Aos "amigos de Alcalá de Henares" ***Jessica Paião***, ***Uanderson Holsback***, ***Audrey Matteucci***, ***Rafael Maroto***, ***Noa Romero*** e ***David Rodriguez*** pela amizade, carinho e por terem dividido comigo dias incríveis. Vocês foram os presentes mais lindos que a Espanha me deu!

Aos queridos amigos ***Cláudia Menzer***, ***José Said Menzer***, ***Ricardo Menzer***, ***Flávio Menzer*** e ***Heleninha Menzer*** pela amizade, carinho e apoio.

Aos meus pais emprestados, ***Tio Camilo*** e ***Tia Nanci*** pelo carinho, amizade, orações, por celebrarem e viverem comigo cada passo desse caminho. Eu amo vocês!

Aos meus Tios ***Janete*** e ***Marcos*** e primos, ***Ana Paula*** e ***Alexandre*** pela presença carinhosa e por sempre torcerem por mim. Muito amor por vocês!

Ao meu casal de amigos ***Maria Fernanda*** e ***Diego Oliveira***, pela amizade, por rezarem por mim e se fazerem presente nas pequenas coisas. Amo vocês!

Ao meu companheiro, amor e amigo, ***Tiago Ferreira*** pela doçura e paciência nas palavras e atitudes em meio a pandemia e reta final para entrega da tese. Sua presença trouxe leveza e a força necessária para prosseguir. Te amo!

Aos meus companheiros do LAZ, ***Antônio Toledo***, ***Thiago Novato*** e ***Luana Caiafa***. Compartilhamos o café, a vida, os sonhos e inseguranças, construímos uma amizade e parceria que vai além das paredes do laboratório.

As minhas companheiras do Lab Plâncton, ***Ana Luísa***, ***Emília***, ***Gabizinha*** e ***Thai*** por transmitirem alegria e doçura, compartilhando o trabalho e a vida.

Ao querido parceiro ***Haroldo Lobo***, pela paciência, incentivo e disponibilidade. Sua experiência contribuiu significativamente para minha formação e me fez ver a ecotoxicologia com outros olhos.

As secretárias do PPG em Biodivesidade, ***Rosi***, ***Priscila***, ***Dayane*** e ***Marlú***, por toda paciência e carinho nos assuntos burocráticos.

Ao professor ***Nathan Barros*** por acreditar em mim e ter me dado à oportunidade de crescer profissionalmente por meio da convivência de laboratório.

À todos os demais ***amigos*** e ***pessoas*** que cruzaram meu caminho e que de alguma forma contribuíram fazendo parte deste jornada.

À ***Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)*** pela bolsa concedida.

À ***Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)*** pelo financiamento da pesquisa (FAPEMIG CRA 003648/16).

Preamble

The present thesis was based on two important paradigms to aquatic ecotoxicology: the first is related to the importance of studying different groups of organisms, their ecological representativeness, and the intrinsic characteristics that classify them as potential models for ecotoxicological studies, taking into account the detailed review of the applied methodologies, the main problems and possible solutions for perfect and/or develop reproducible test protocols for conducting laboratory bioassays; the second one corresponds to the importance of studying not only the individual effects of environmental stressors but also their combination in a climate change scenario, in order to know what are the responses of aquatic organisms at the level of population and community and the consequences of these effects for ecosystem processes and services. Therefore, this thesis was elaborated and organized in four chapters, divided into two sections: Section 1 included reviews chapters with the history of ecotoxicological studies using ciliated microeukaryotes and, a meta-analysis showing the sensitivity of ciliate species to different heavy metals, discussing the main problems, gaps and future perspectives for bioassays. Section 2 included two experimental studies, the first one is a study with ciliate species (e.g., *Paramecium caudatum*) as a model organism exposed to caffeine, presenting a more refined methodology compared to what was found in literature and discussions regarding the ecological implications, the imminent risk that this substance has for aquatic ecosystems, and the other one an experimental study evaluating the effect of multiple stressors on the zooplankton community in a climate change scenario, which was carried out during the Doctorate Sandwich period in Spain.

Abstract

Aquatic ecosystems have been affected by different human activities (e.g., urbanization, industrial and agricultural activities) and hence, changing the water quality, the process, and function exercised by these environments. The evaluation of water quality through ecotoxicological studies has become an effective tool for understanding the effects of pollutants on biological communities, allowing the measurement of anthropogenic effects on aquatic systems. The use of microeukaryotes ciliates as bioindicators has been identified as a great potential for assessing water quality, however, these organisms are still neglected in ecotoxicological fields being necessary to enlarge the studies with these organisms. Moreover, the interaction effect among more than one environmental stressor (e.g., chemical contaminant, temperature) also needs to be more study due to the complexity of the aquatic ecosystems functioning. In the present study, we demonstrated through three chapters that the microeukaryotes ciliates can be included in the ecotoxicological studies since as long as they are expanded the classical ecotoxicological studies where it is possible to develop standard methods and deepen knowledge. Furthermore, a fourth chapter showed the interaction between more than one environmental stressor could contribute to a biodiversity decline and even, future research should be dedicated to assessing different interactions in the Mediterranean aquatic ecosystems. (Chapter 1) By means of a mini review, and discussed a brief history, the current scenario and pointing out their methodological approaches gaps of the ecotoxicological studies with ciliates. (Chapter 2) Performing a meta-analysis, we assessed the available toxicity data of heavy metals and ciliates. The results showed the tolerance of ciliates to heavy metals varies notably being partly influenced by differences in methodological conditions across studies. Moreover, most ciliates are tolerant to heavy metal pollution than the standard test species used in ecotoxicological risk assessments, i.e., *Raphidocelis subcapitata*, *Daphnia magna*, and *Onchornyncus mykiss*. Finally, this study highlighted the importance of developing standard toxicity test protocols for ciliates, which could lead to a better comprehension of the toxicological impact of heavy metals and other contaminants on ciliate species. (Chapter 3) By means of ecotoxicological tests, this study demonstrated the tolerance of *Paramecium caudatum* to caffeine. The results showed that this species had higher resistance in the environment. Even, we observed a moderate risk for *P. caudatum* regarding maximum environmental concentrations of caffeine in surface freshwater being that the global distribution of caffeine and the probability of increasing environmental concentrations highlight the need for more studies to better understand caffeine in aquatic ecosystems and the associated risks. (Chapter 4) Finally, by means of an indoor microcosm experiment, it was demonstrated that the temperature can influence the direct and indirect effects of salinity and pesticides on zooplankton communities in Mediterranean coastal wetlands and highlights vulnerable taxa and ecological responses that are expected to dominate under future global change scenarios.

Keywords: aquatic ecosystems, ciliates, ecotoxicology, multiple stressors, zooplankton.

Resumo

Os ecossistemas aquáticos vêm sendo afetados por diferentes atividades humanas (e.g., urbanização, atividades industriais e agrícolas) e, portanto, alterando a qualidade da água, os processos e as funções exercidas por esses ambientes. A avaliação da qualidade da água por meio de estudos ecotoxicológicos tem se tornado uma ferramenta eficaz para o entendimento dos efeitos dos poluentes nas comunidades biológicas, permitindo a mensuração dos efeitos antrópicos nos sistemas aquáticos. O uso de microeucariotos ciliados como bioindicadores tem sido apontado como um grande potencial para avaliação da qualidade da água, entretanto, esses organismos ainda são negligenciados no campo da ecotoxicologia sendo necessário ampliar os estudos com esses organismos. Além disso, o efeito de interação entre mais de um estressor ambiental (e.g., contaminantes químico, temperatura) também precisa ser mais estudado devido à complexidade do funcionamento dos ecossistemas aquáticos. No presente estudo, foi demonstrado por meio de três capítulos que os microeucariotos ciliados podem ser incluídos nos estudos ecotoxicológicos desde que sejam expandidos os estudos ecotoxicológicos clássicos onde é possível desenvolver métodos padronizados e aprofundar os conhecimentos. Além disso, um quarto capítulo mostrou que a interação entre mais de um estressor ambiental poderia contribuir para o declínio da biodiversidade e, mesmo, pesquisas futuras devem ser dedicadas a avaliar as diferentes interações nos ecossistemas aquáticos mediterrâneos. (Capítulo 1) Por meio de uma mini revisão, discutiu-se um breve histórico, o cenário atual e apontou-se as lacunas de suas abordagens metodológicas dos estudos ecotoxicológicos com ciliados. (Capítulo 2) Realizando uma meta-análise, avaliou-se os dados de toxicidade disponíveis de metais pesados e ciliados. Os resultados mostraram que a tolerância dos ciliados aos metais pesados varia notavelmente sendo parcialmente influenciada por diferenças nas condições metodológicas entre os estudos. Além disso, a maioria dos ciliados é mais tolerante à poluição por metais pesados do que as espécies de teste padrão usadas em avaliações de risco ecotoxicológico, ou seja, *Raphidocelis subcapitata*, *Daphnia magna* e *Onchornyncus mykiss*. Finalmente, este estudo destacou a importância do desenvolvimento de protocolos de teste de toxicidade padrão para ciliados, o que poderia levar a uma melhor compreensão do impacto toxicológico de metais pesados e outros contaminantes em espécies de ciliados. (Capítulo 3) Por meio de testes ecotoxicológicos, este estudo demonstrou a tolerância do *Paramecium caudatum* à cafeína. Os resultados demostraram que esta espécie apresenta maior resistência no ambiente. Ainda, observamos um risco moderado para *P. caudatum* em relação às concentrações ambientais máximas de cafeína na água doce superficial, sendo que a distribuição global da cafeína e a probabilidade de aumentar as concentrações ambientais destacam a necessidade de mais estudos para melhor compreender a cafeína em ecossistemas aquáticos e os associados riscos. (Capítulo 4) Finalmente, por meio de um experimento de microcosmo interno, foi demonstrado que a temperatura pode influenciar os efeitos diretos e indiretos da salinidade e dos pesticidas nas comunidades zooplanctônicas nas terras úmidas costeiras do Mediterrâneo, e destaca táxons vulneráveis e respostas ecológicas que devem dominar sob futuros cenários de mudança global.

Palavras-chave: ecossistemas aquáticos, ciliados, ecotoxicologia, múltiplos estressores, zooplâncton.

List of figures

General introduction

Chapter I

Chapter II

Chapter III

Chapter IV

List of tables

Chapter II

Chapter IV

Summary

1 General Introduction

Over the last few hundreds of years, our society has entered the 'Anthropocene', a period characterized by the overexploitation of natural resources. Freshwater ecosystems have been severely affected by chemical pollution, flow modification, and habitat degradation (Dudgeon et al., 2006). Especially chemical pollution by industrial activities, agriculture and urban wastewaters have contributed to a loss of biodiversity, and changes in the structure and composition of aquatic ecosystems, hence, changing their ecological dynamics and the services they provide (Reid et al., 2018).

Currently, emerging contaminants (ECs) (e.g., pharmaceuticals, personal care products, pesticides) have been a major concern among environmental researchers since they have been monitored in a wide range of freshwater ecosystems worldwide (Fekadu et al., 2019; Valdez-Carrillo et al., 2020). ECs can produce neurotoxicity and endocrine disruption even at environmentally relevant concentrations (e.g., nanogram to microgram per liter; Sumpter and Johnson, 2005, 2008). Especially in Brazil, as an example of a country with an increasing per capita consumption and a lack of sanitation systems, these compounds have been often reported in water matrices since they are only partially removed during conventional treatment or are discharged into the environment without any treatment (Valdez-Carrillo et al., 2020). Furthermore, many of these substances lack any formal legislation that evaluates their effects and risks to aquatic and human life in Brazil (Montagner et al., 2017). As an example, caffeine is a psychoactive substance that has been reported in Brazilian freshwater ecosystems in concentrations of μg L^{-1} to mg L^{-1} (Montagner et al., 2014; Rodríguez-Gil et al., 2018) and, although these concentrations are not enough to cause acute toxic effects (Selderslaghs et al., 2013; Zarrelli et al., 2014), the consumption of this compound is increasing mainly due to population growth and, in the future, it may exceed tolerance limits in the environment, affecting species previously described as resistant (Quadra et al., 2020).

One of the major tasks of aquatic ecotoxicologists is to define acceptable limits of environmental contamination (Moiseenko et al., 2008). Such limits are used for the evaluation and registration of chemical products used in different commercial sectors (e.g., pharmaceutical industry and agriculture) in Europe and in North America and allow an evaluation of their potential side-effects on non-target organisms (Lombardi, 2004). Ecotoxicologists usually work in close cooperation with scientists working in different areas (e.g., chemistry, biology, toxicology, environmental conservation), and attempt to

understand how chemical effects are propagated across populations, communities, ecosystems and, even, on humans (Zagatto and Bertoletti, 2008; Releya and Hoverman, 2006).

During the last Brazilian Ecotoxicology Congress (Ecotox, 2018), it was discussed the main areas of aquatic ecotoxicology that still need to be advanced, especially in Brazil. Sub-areas such as the assessment of the effects of emerging contaminants (using microcosms, mesocosms, and *in situ* experiments), the use of predictive modeling, or the evaluation of ecosystem services and multiple stressor effects (including chemical mixtures) were highlighted as needing further development in the nearby future. Moreover, it is required to study species that are beyond the classical model organism groups (e.g., algae, microcrustaceans, and fish) such as microeukaryotes ciliates, which actively participate in the mediation of important ecosystem processes. In this sense, based on the issues discussed above, the diagram below (Figure 1A) presents the important topics that still need to be more explored in order to improve our understanding on the effects of chemicals on freshwater ecosystems, some of which will be investigated as part of this thesis.

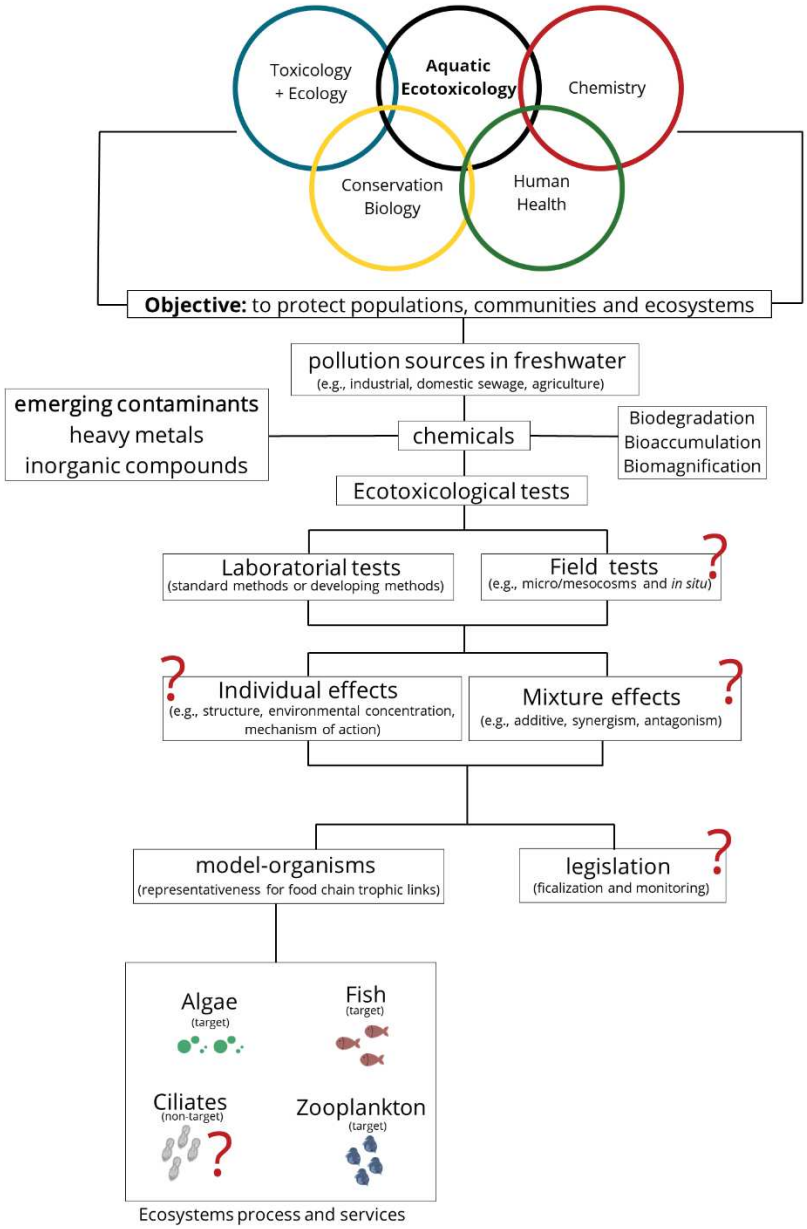


Figure 1A. Aquatic ecotoxicological diagram based on their classical concepts and principles. The question marks represent the subjects in aquatic ecotoxicology that still needs to be more investigating in the coming future, particularly in Brazil.

Up to date, the ecotoxicological risk of chemicals has been based on the extrapolation of the toxicological effects measured in one single or few model organisms to complex communities and ecosystems (Chapman, 2002; Baird et al., 2007). In general, the choice of such model organisms is based on their sensitivity to selected chemicals, their wide geographical distribution, ecological representativeness, short life cycle, ease of cultivation and maintenance in the laboratory and, if possible, their commercial value (Niemeyer et al., 2010). The use of model organisms contributes to the identification of

compounds that affect particular biological systems and, hence, allow classification of compounds based on their general toxicological mode of action (Zagatto and Bertoletti, 2008; Krull and Barros, 2012).

Since the 1960s, the organisms most commonly used in ecotoxicological studies are selected species of algae, microcrustaceans and fish (Zagatto and Bertoletti, 2008). Currently, there are standardized protocols and regulations that are used to perform tests worldwide, aiming at their comparison and reproducibility (e.g., OECD, 2011, 2019). However, there are still some groups of organisms, which contribute notably to the maintenance of ecosystem processes that are still neglected in ecotoxicological studies, and that need to be considered to improve our understanding of the effects of chemicals at the ecosystem level (Gutiérrez et al., 2015; Mansano et al., 2016; Segovia et al., 2016). Among them, ciliated microeukaryotes are good examples. Although they have already been used in ecotoxicological studies, they are not taken into account in risk assessment studies with enough frequency (Mansano et al., 2016; Vilas-Boas et al., 2020b).

Ciliates are abundant in aquatic ecosystems and can be used as bioindicators of water quality in biomonitoring programs in order to assess the degree of contamination of aquatic ecosystems (Fenchel, 1987; Sherr and Sherr, 1994; Gerhardt et al., 2010). They are preyed upon by some zooplankton species and can consume algae, bacteria and even other smaller protozoa, and are therefore an important link between primary producers and decomposers with the higher trophic levels (Fenchel, 1987; Madoni, 2005; Gomiero et al., 2013; Debastiani et al., 2016). Few researchers have been dedicated to their study and to develop toxicity protocols for ciliates, so that our knowledge on their sensitivity to chemical pollution is limited (Mansano et al., 2016; Vilas-Boas et al., 2020 a, b).

Moreover, the need to improve our understanding on the response of aquatic ecosystems to chemicals and additional stressors (e.g., temperature, pH, turbidity), requires the evaluation of species interactions using more complex experimental designs and systems (e.g. micro and mesocosms). This is a more realistic approach as compared to testing only the effects of one single chemical compound in one organism, and allows the identification of complex interactions (e.g., synergism and antagonism) between stressors on individuals and populations (Jackson et al., 2016; Jackson, 2018). However, this does not mean that tests performed in the laboratory lose their importance and, therefore, should still be considered since they allow deciphering chemical effects at the individual level (Relyea and Hoverman, 2006), and constitute a more rapid solution for

testing the number of compounds that is produced on a large scale worldwide (Bernhardt et al., 2017).

This thesis makes an attempt to include new potential organisms in ecotoxicological studies (e.g., ciliates) making use of single species tests, and to improve our understanding on the direct and indirect impacts of chemicals (and additional stressors) on aquatic populations and communities, thus contributing to tackle some of the important challenges that the risk assessment of chemicals will face in the nearby future.

References

Baird, D. J., Brown, S. S., Lagadic, L., Liess, M., Maltby, L., Moreira-Santos, M., Schulz, R., Scott, G. I. (2007). In situ-based effects measures: determining the ecological relevance of measured responses. Integrated Environmental Assessment and Management. 3, 259-267.

Bernhardt, E.S., Rosi-Marshall, E.J., Gessner, M.O. (2017). Synthetic chemicals as agents of global change. Frontiers in Ecology and the Environment. 5, 84-89.

Chapman, P. M. (2002), Integrating toxicology and ecology: putting the ‘‘eco’’ into ecotoxicology. Marine Pollution Bulletin. 44: 7-15.

Debastiani, C., Meira, B.R., Lansac-Tôha, F.M., Velho, L.F.M., Lansac-Tôha, F.A. (2016). Protozoa ciliates community structure in urban streams and their environmental use as indicators. Brazilian Journal of Biology. 76, 1043-1053.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z., Knowler, D., Lévêque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L.J., Sullivan, C.A. (2006). Freshwater biodiversity: importance, threats, status and conservation challenges. Biologcal Reviews. 81, 163-82.

Fenchel, T. (1987). The Ecology of the Protozoa. in: The Biology of Free-living Phagotrophic Protists. Springer Verlag: Berlin and Tokyo, pp.197.

Fekadu, S., Alemayehu, E., Dewil, R., Van der Bruggen, B. (2019). Pharmaceuticals in freshwater aquatic environments: A comparison of the African and European challenge. Science of the Total Environment. 654, 324-337.

Gerhardt, A., Ud-Daula, A., Schramm, K.W. (2010). *Tetrahymena* spp. (Protista, Ciliophora) as test species in rapid multilevel ecotoxicity tests. Acta Protozoologica. 49, 271-280.

Gomiero, A., Dagnino, A., Nasci, C., Viarengo, A. (2013). The use of protozoa in ecotoxicology: Application of multiple endpoint tests of the ciliate *E. crassus* for the evaluation of sediment quality in coastal marine ecosystems. Science of the Total Environment. 442, 534-544.

Guetiérrez, J.C., Amaro, F., Martín-González, A. (2015). Heavy metal whole-cell biosensors using eukaryotic microorganisms: an updated critical review. Frontiers in Microbiology. 6, 1-8.

Jackson, M.C. (2018). Multiple stressors in freshwater ecosystems: biocides and climate change. Freshwater Biology. 27, 2-10.

Jackson, M.C., Loewen, C.J.G., Vinebrooke, R.D., Chimimba, C.T. (2016). Net effects of multiple stressors in freshwater ecosystems: a meta−analysis. Global Change Biology. 22, 180-189.

Krull, M., Barros, F. (2012). Key Issues in Aquatic Ecotoxicology in Brazil: A Critical Review. Journal of the Brazilian Society of Ecotoxicology. 7, 57-66.

Lombardi, J.V. Fundamentos de toxicologia aquática. In: M.J.T. Ranzani-Paiva, R.M. Takemoto and M.A.P. Lizama, eds. Sanidade de organismos aquáticos. São Paulo: Varela, 2004, pp. 263-272.

Madoni, P. (2005). Ciliated protozoans communities and saprobic evaluation of water quality in the hilly zone of some tributaries of the Po River (northern Italy). Hydrobiologia. 541, 55-69.

Mansano, A.S., Moreira, R.A., Pierozzi, M., Oliveira, T.M.A., Vieira, E.M., Rocha, O., Regali-Seleghim, M.H. (2016). Effects of diuron and carbofuran pesticides in their pure and commercial forms on *Paramecium caudatum*: The use of protozoan in ecotoxicology. Environmental Pollution. 213, 160-172.

Moiseenko, T.I. (2008). Aquatic Ecotoxicology: Theoretical Principles and Practical Application. Water Research. 35, 530-541.

Montagner, C.C., Vidal, C., Sodré, F.F., Pescara, I.C., Jardim, W.F. (2014). A cafeína no ambiente. IN: Canela MC, Jardim WF, Sodré FF, Grassi MT (eds), Cafeína em águas de abastecimento pública no Brasil. Intituto Nacional de Ciências e Tecnologias Analíticas Avançadas.

Niemeyer, J.C., Moreira-Santos, M., Nogueira, M.A., Carvalho, G.M., Ribeiro, R., DA Silva, E.M., Sousa, J.P. (2010). Environmental risk assessment of a metal contaminated area in the tropics. Tier I: screening phase. Journal of Soils and Sediments. 10, 1557-1571.

OECD (2011), Guidelines for the testing of chemicals, Freshwater Alga and Cyanobacteria, Growth Inhibition, Test, nº 201.

OECD (2019), Guidelines for the testing of chemicals, Fish Acute Toxicity Test, nº 203.

Quadra, G. R., Paranaíba, J. R., Vilas-Boas, J., Roland, F., Amado, A. M., Barros, N., Dias, R.J.P., Cardoso, S.J. (2020). A global trend of caffeine consumption over time and related-environmental impacts. Environmental Pollution. 256, 113343.

Reid, A., Carlson, A.K., Creed, I.F., Cooke, S. (2018). Emerging threats and persistent conservation challenges for freshwater biodiversity. Biological Reviews. 1-25.

Releya, R., Hoverman, J. (2006). Assessing the ecology in ecotoxicology: a review and synthesis in freshwater systems. Ecology Letters. 9, 1157-1171.

Rodríguez-Gil, J.L., Cáceres, N., Dafouz, R., Valcárcel, Y. (2018). Caffeine and paraxanthine in aquatic systems: Global exposure distributions and probabilistic risk assessment. Science of the Total Environment. 612, 1058-1071.

Segovia, B.T., Lansac-Toha, F.M., Meira, B.R., Cabral, A.F., Lansac-Toha, F.A., Velho, L.F.M. (2016). Anthropogenic disturbances influencing ciliate functional feeding groups in impacted tropical streams. Environmental Science and Pollution Research. 23.

Selderslaghs, I.W.T., Hooyberghs, J., Blust, R., Witters, H.E. (2013). Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. Neurotoxicology and Teratology. 37, 44-56.

Sherr, E.B., Sherr, B.F. (1994). Bacterivory and herbivory: key roles of phagotrophic protists in pelagic food webs. Microbiology and Ecology. 28, 223-235.

Sumpter, J.P. (2005). Endocrine disrupters in the aquatic environment: an overview. Acta Hydrochimica et Hydrobiologica. 33, 9-16.

Sumpter, J.P., Johnson, A.C. (2008). 10th anniversary perspective: reflections on endocrine disruption in the aquatic environment: from known knowns to unknown unknowns (and many things in between). Journal of Environment Monitoring. 10:1476-1485.

Valdez-Carrillo, M., Abrell, L., Ramírez-Hernández, J., Reyes-López, J. A., Carreón-Diazconti, C. (2020). Pharmaceuticals as emerging contaminants in the aquatic environment of Latin America: a review. Environmental Science and Pollution Research. 27, 44863-44891.

Vilas-Boas., J.A., Senra, M.V.X., Dias, R.J.P. (2020a). Ciliates in ecotoxicological studies: A minireview. Acta Limnologica. 32, e202.

Vilas-Boas., J.A., Cardoso, S.J., Senra, M.V.X., Rico, A., Dias, R.J.P. (2020b). Ciliates as model organisms for the ecotoxicological risk assessment of heavy metals: A meta–analysis. Ecotoxicology and Environmental Safety. 199, 110669.

Zagatto, P.A; Bertoletti, E. Ecotoxicologia. In: Zagatto, P.A.; Bertoletti, E. (Eds.). Ecotoxicologia Aquática: Princípios e Aplicações. 2008.

Zarrelli, A., DellaGreca, M., Iesce, M. R., Lavorgna, M., Temussi, F., Schiavone, L., Isidori, M. (2014). Ecotoxicological evaluation of caffeine and its derivatives from a simulated chlorination step. Science of the Total Environment. 470, 453-458.

2 Objectives

2.1 Main objective

The main objectives of this thesis were to investigate if the microeukaryotes ciliates could be a potential group of model organisms for ecotoxicological studies being important trophic links for the maintenance of ecosystem processes and to assess the resilience of zooplankton communities to multiple stressors in freshwater ecosystems.

2.2 Specific objectives

- to assess the sensitivity of ciliates to chemicals and their potential as model organisms in ecotoxicological studies through a bibliographic review and a meta-analytical study;
- to study their life cycle by constructing the growth curve, as well as establishing optimal conditions for their cultivation in the laboratory;
- to evaluate the sensitivity of model organisms (e.g., *Paramecium caudataum*) to an emerging contaminant (e.g., caffeine) such as caffeine and to propose a refined methodology for conducting toxicity tests using reference substances;
- to evaluate the effects of multiple stressors related to global change (salinity intrusion, insecticide pollution and temperature regimes) on zooplankton communities under Mediterranean conditions.

3 Chapter I

(Chapter published in the journal *Acta Limologica Brasiliensia*, B3, Qualis Capes 2013-2016)
https://doi.org/10.1590/s2179-975x6719

Ciliates in ecotoxicological studies: A minireview

Jéssica Andrade Vilas-Boas[1,2], Marcus Vinicius Xavier Senra[3], Roberto Júnio Pedroso Dias[1,2]

[1] Departamento de Zoologia, Laboratório de Protozoologia, Universidade Federal de Juiz de Fora 36036-900, Juiz de Fora, Minas Gerais, Brazil

[2] Programa de Pós-graduação em Ecologia (PGECOL), Universidade Federal de Juiz de Fora 36036-900, Juiz de Fora, Minas Gerais, Brazil

[3] Instituto de Recursos Naturais, Pós-graduação em Meio Ambiente e Recursos Hídricos, Universidade Federal de Itajubá 37500-903, Itajubá, Minas Gerais, Brazil

Corresponding author: jessica_biol@outlook.com

Abstract: The present study has conducted a minireview of ecotoxicological studies using ciliated microeukaryotes, presenting a brief history, describing the current scenario and pointing out their methodological approaches gaps. We highlight in a clear and objective way the ecological importance of ciliates for ecosystems, their usefulness and the inherent characteristics that classify them as a good model organism. Finally, we discuss some modern tools that can be added to studies with ciliates in the near future.

Keywords: Ciliophora, ecotoxicology; model organism.

Resumo: O presente estudo realizou uma pequena revisão de estudos ecotoxicológicos utilizando microeucariotos ciliados, apresentando um histórico, descrevendo o cenário atual e apontando suas lacunas nas abordagens metodológicas. Destacamos de forma clara e objetiva a importância ecológica dos ciliados para os ecossistemas, sua utilidade e as características inerentes que os classificam como bons organismos modelo. Finalmente, discutimos algumas ferramentas modernas que podem ser adicionadas aos estudos com os ciliados em um futuro próximo.

Palavras-chave: Ciliophora, ecotoxicologia; organismo modelo.

1. Introduction

Despite all the importance of water for mankind, the acelerated deterioration of aquatic systems has been observed all over the world, affecting human health and the environment (Schwarzenbach et al., 2010). The population increase, occupation of irregular areas, agriculture, discard of industrial and urban effluents, mining and landfill activities, fragmentation of the landscape, deviation of rivers and the load of contaminants from different sources has been causing, unfortunately, huge and unprecedented impacts on aquatic ecosystems causing changes in the chemical profile of waterbodies (Molden et al., 2007). According to the Chemical Abstracts Registry database (www.cas.org) there are more than 100 million chemical substances and less than 0.36% are regularized. These chemicals can reach both surface and subterranean water bodies even those in far remote areas (Bernhardt et al., 2017). This represents a human health concerned, because many of these substances can be toxic, reducing the availability of potable water (Oki & Kanae, 2006; UNESCO, 2009). In fact, it is estimated that by 2025, 50% of the population will live in countries with water shortages (Qureshi & Hanjra, 2010).

Accordingly, the best way to ensure the quality of water resources is through the establishment of accurate standard protocols, management methods and environmental legislation, which would provide also the guidelines for proper environmental monitoring (Schwarzenbach et al., 2006; Schwarzenbach et al., 2010). The degree of contamination of aquatic environments is evaluated mainly through chemical analysis and biomonitoring programs (USEPA, 1992; Silveira, 2004). However, these approaches are not enough to water quality control and must be complemented by ecotoxicological studies, which are useful for assessing the ability of a toxic agent to produce effects on organisms (Forget et al., 2000).

Ecotoxicology is used to integrate and understand the harmful effects of chemical substances on populations, communities, and ecosystems (Walker et al., 1996; Forbes & Forbes, 1994), and this knowledge can be used to complement environmental impact studies and on risk assessment programs (Silva et al., 2015). One of the most important steps for ecotoxicological studies is to find a model organism which is ideal and able to provide reproducible information on the acute and chronic toxicity of pollutants in aquatic environments. Some good examples of model organisms (*i.e. Pseudokiriella subcaptata*, *Daphnia similis*, *Ceriodaphnia* spp. and *Danio rerio*) are representatives of different trophic levels and are useful for answering different questions. This representativeness

allows a better understanding of the effects caused by toxic agents and contributes to the establishment of toxicity threshold values (Grolière et al., 1990; Lombardi, 2004).
The choice of a model organism should be based on previous knowledge of their biology, physiology, behavior, and food habits; they need to be abundant and available; have ecological representativeness within the ecosystem; have constant and accurate sensitivity; short life cycle; commercial importance and; be easily cultivated and maintained in the laboratory (Niemeyer et al., 2010).

Ciliated microeukaryotes have been used in ecotoxicological studies since the 1950s (Grebecki & Kuznicki, 1956). However, these organisms have been historically neglected in detriment of other model organisms (Figure 1). Many ciliate species, such as from genera *Paramecium* and *Tetrahymena* have many biological features that makes them appropriate to toxicity bioassays, such as the presence of a delicate cell membrane, small size (from ~ 10 μm to 4,500 μm), which allows the use of reduced volumes for maintenance and experimentation, short life cycle and high reproductive rate, allowing for long-term transgenerational assays in relatively short time periods. Also, encystement stages allow greater resistance to environmental stressors and allow the measuring the possible effects of toxic substances (Soldo & Van Wagtendonk, 1969; Lee & Soldo, 1992; Nerad & Daggett, 1992; Madoni et al., 1996; Gilron & Lynn, 1998; Madoni, 2000, 2003, 2011; Gutierrez et al., 2003; Delmonte Corrado et al., 2005; Kchaou et al., 2009; Gomiero et al., 2013).

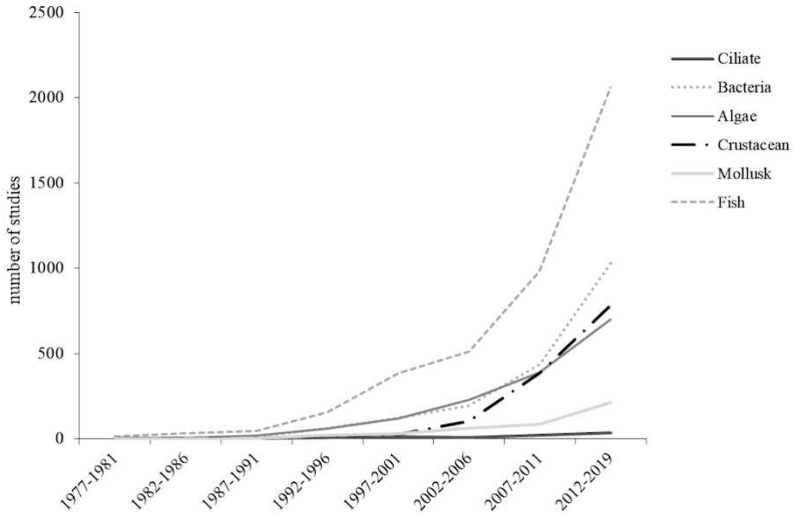


Figure 1. Number of ecotoxicological studies per model organism. Data available in the Scopus database from 1977 to 2019 (survey conducted 06/06/2019). Codes used to search: Ecotoxicol * AND ciliate *, Ecotoxicol * AND algae *, Ecotoxicol * AND bacteria *, Ecotoxicol * AND mollusk *, Ecotoxicol * AND crustacean * AND Ecotoxicol * AND fish *.

The phylum Ciliophora represents a basic component of microplankton and microbenthic within environments (Finlay & Fenchel, 1996; Madoni, 2000). They have a wide geographical distribution and can be found in freshwater, brackish, salty, bromeliads and edaphic environments, such as superficial soils, mosses and lichens (Puytorac, 1994; Corliss, 1979; Joppert et al., 1995; Foissner et al., 2002; Foissner, 2003; Lynn, 2008). Ciliates are mostly heterotrophic organisms, predators of bacteria and other protozoa (Fenchel, 1987; Beaver & Crisman, 1989; Müller et al., 1991), and are preyed by different species from the zooplankton. Therefore, representing an essential link for the flow of carbon and energy to higher trophic levels (Fenchel, 1987; Sherr & Sherr, 1994; Madoni, 2000).

The presence of ciliates in several habitats is influenced by their tolerance and adaptability to the different physical and chemical conditions of the environments (Noland, 1925; Sleigh, 1988). When under unfavorable conditions, they form resistance cysts, which can be dispersed and transported by wind, water, insects, among other animals (Finlay & Fenchel, 1996; Finlay, 2002; Esteban & Finlay, 2003; Fenchel & Finlay, 2004). This adaptive changes may be linked to the specificity of the recurrent changes in the physical-chemical factors of the water, such as temperature, amount of dissolved organic matter, pH, conductivity and oxygen concentration (Noland, 1925; Kudo, 1966; Sleigh, 1988; Madoni, 2005; Madoni & Barghiroli, 2007). Seasonal, vertical, trophic and flood pulse variations can also be determining factors in the patterns of distribution, composition and abundance of ciliates in aquatic systems (Madoni, 2003, 2005; Velho et al., 2005, 2013; Gomiero et al., 2013; Debastiani et al., 2016; Pauleto et al., 2009).

Ciliates can be used as bioindicators of water quality in biomonitoring studies (Madoni, 1994, 2003, 2005; Dias et al., 2008; Bagantini et al., 2013; Debastiani et al., 2016). They play an important role in the purification process in water treatment plants, especially in the activated sludge system, where it makes up more than 9% of the biomass of microorganisms (Madoni, 1994, 2003, 2011).

The use of ciliates as model organisms had its beginning with the work of Gause (1934) in population ecology. The Russian ecologist used the ciliate species *Paramecium caudatum* and *Paramecium aurelia* to test the hypothesis of competitive exclusion. The results showed that both species of ciliates occupy the same ecological niche and can not coexist. Since then, the use of these organisms as models for applied studies has been through the knowledge of the species through the use of techniques of optical microscopy

and appropriate techniques (Klein, 1958; Foissner, 1994, 1999; Dieckmann, 1995; Kapuscinski, 1995; Foissner & Berger, 1996; Finlay & Fenchel, 1999; Lynn, 2008; Mitchell & Meisterfeld, 2005). Biology information (Lynn, 2008; Lynn & Small, 2002; Pratt & Cairns Júnior, 1985; Weisse, 2017), evolution and taxonomy (Foissner & Berger, 1996; Lynn, 2008; Gao et al., 2016), behavioral traits (Nishigami et al., 2018; Ohmura et al., 2018; Ishikawa, 2019), physiology, histology and even the genome description of the species, *i.e.*, *Tetrahymena thermophila*, *Paramecium tetraurelia*, *Euplotes* crassus, *Oxytricha trifallax* and *Stentor coeruleus* (Aury et al., 2006; Eisen et al., 2006; Vinogradov et al., 2012; Swart et al., 2013; Slabodnick et al., 2017) may be useful for answering different questions within ecotoxicology. Therefore, the present study presents a minireview of the ecotoxicological studies with ciliates, the importance of these organisms for the aquatic ecosystems, the approaches and gaps found, besides presenting some perspectives for the advancement of the studies in the near future.

2. Ciliates as Models in Ecotoxicological Studies

2.1. Brief history and approaches

The first ecotoxicological stuty using ciliates was published in the 1950s (Grebecki & Kuznicki, 1956). *Paramecium caudatum* species were exposed to different concentrations of Copper, Mercury, Cadmium, Zinc, Nickel, Cobalt, and Chromium. Posteriorly, some studies were done using different ciliate species and approach (Figure 2A, Supplementary Material Table S1). The main ecotoxicological approaches using ciliated organisms are those related to the determination of lethal concentrations, changes in behavioral, substances accumulation and morphological changes (Figure 2B). Acute and chronic tests have been used since the first works to identify the maximum concentration ranges that cause population to decline and/or mortality (mean lethal concentration - LC_{50}), to check cell deformities, heavy metal accumulation and/or behavioral changes. Mortality and/or survive states are commonly established through optical microscope (Madoni, 1994; Madoni et al., 1992, 1996; Madoni & Romeo, 2006; Mansano et al., 2016). Moreover, microscopy can also be used to evaluate other features such as behavior, morphological deformities, changes in generation time, abundance and cell shape. Rao et al. (2006) observed changes in cell shape, developing irregular membrane bubbles promoting cell lysis. In addition, a tracking system with optical microscope was used to observe changes in the locomotion of *Paramecium caudatum*.

An initial increase and subsequent decrease in swimming speed were observed when exposed to concentrations of an acephate insecticide. The number of generations decreased, and the generation time increased significantly in a manner dependent on the sublethal concentrations used. Changes in the number and ultrastructure of cilia were observed in the study by Li et al. (2016) in the exposure of an emerging compound (Tris (1,3-dichloro-2-propyl) phosphate (TDCIPP). The authors conclude by highlighting that the results suggest that chemical exposure in organisms of the early trophic levels can cause long-term damage. The resistance of ciliates to metal ions shows the importance of these organisms for bioremediation in industrial wastewater (Madoni, 1994; Martin-Gonzalez et al., 2006; Rehman et al., 2009; Chaudhry & Shakoori, 2011; Elguero et al., 2019).

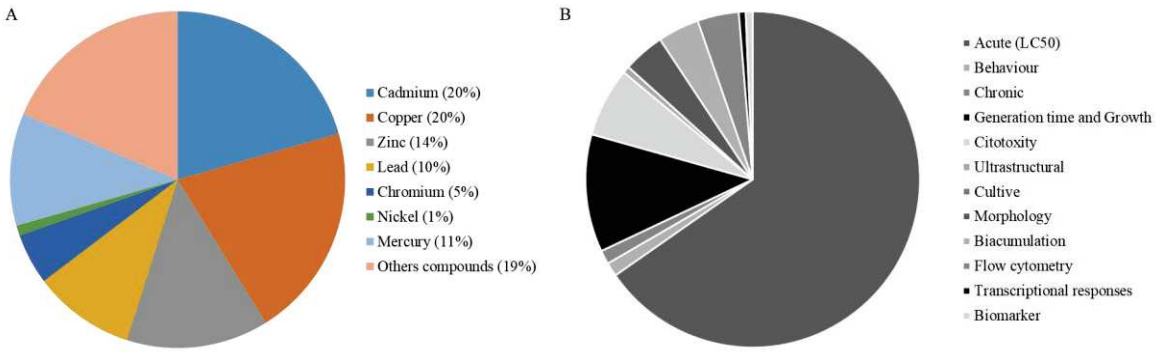


Figure 2. A=Percentage of ecotoxicological studies per compound; B= Endpoints distributions.

Rico et al. (2009) evaluated the induction of reactive oxygen species (ROS) after exposure to heavy metals in ciliates. Benbouzid et al. (2012) observed reduced growth, increased generation time, respiratory metabolism disturbance (significant increase in oxygen uptake), and high percentage response in a ciliate species in a short-term (24h) experiment. Kim et al. (2014) measured intracellular oxygen levels (ROS) and total glutathione content (GSH) using gene expression by means of RT-PCR. Wu et al. (2015) used UV spectrophotometry to visualize changes in enzymatic antioxidants in a ciliate species. The application of modern techniques may be useful for ecotoxicological studies with ciliates, such as the use of flow cytometry by Liu et al. (2017) to evaluate the Mercury accumulation properties in species of the genus *Tetrahymena*.

The use of the 'Omics' in ecological studies has allowed advances and brought the possibility of evaluating changes in different levels of cellular organization, such as individual, tissue, cell and molecule (Zhang et al., 2018). The union of these sciences allows the uncovering of cellular processes details of many organisms, such as the screening of genes whose loss of function may confer resistance or sensitivity to phenotypic toxicity, such as programmed cell death (Jo et al., 2009). In the study by Kim et al. (2018) it was possible to evaluate the transcriptional response of the antioxidant defense system and heat shock protein of the *Euplotes crassus* species. The production of proteins or metabolites for the formulation of vaccines and drugs has also been performed from the use of ciliates (Putten et al., 2006, Hartmann et al., 2000). The successes of studies like these require the use of organisms that are culturable under laboratory/industrial conditions such as ciliates. In this sense, there are a lot of tools that can be used to evaluate environmental alteration using ciliates in different approaches contributing to the understanding of how environmental changes can affect the role of these organisms in the ecosystem processes.

2.2. Species

In regards to ciliates species, more than 50 have been used in ecotoxicological studies to evaluate the impact of different compounds (Table S1). In this sense, given its wide geographic distribution and short cell cycles, *Paramecium caudatum* could be considered a good model for behavioral and population reproductive rate studies allowing evaluation of a large number of generations in short time spans (Rao et al., 2006; Mansano et al., 2016). While *T. thermophila, Paramecium tetraurelia* (and also *P. caudatum*), *Euplotes crassus, Oxytricha trifallax and Stentor coeruleus* can be considered are potential model species in molecular (ecotoxicogenomics) approaches, given the availability of their genome in sequence databases (Aury et al., 2006; Eisen et al., 2006; Vinogradov et al., 2012; Swart et al., 2013; Slabodnick et al., 2017).

The intensification in the use of ciliated protists will be of great value to the field of ecotoxicological. They present several characteristics that make them model organisms (Giovanni Junior & Carvalho, 2017) and since many species are aquatic, their use can contribute to more accurately evaluate the changes these environments have been facing, as consequence of anthropic activities.

2.3. Substances

As available within the AQUIRE database (USEPA, 2019), the heavy metals were the first and the most often chemicals used in ecotoxicological studies using ciliates as model organisms (Figures 2A, 3 and 4). However, over the time, the environmental impact of contaminants of emerging concerns, such as inorganic, organic, β-blockers, herbicides, insecticides and pesticides started to have their toxicity evaluated also within ciliate species (Figures 2A, 3 and 4). Among heavy metals, Copper, Cadmium, Zinc, Lead and Mercury were the most frequently used in ciliates and within these, the assays directed toward their effects over populations (growth rate), mortality (lethal concentration) and accumulation were the most often used (Figures 2A, 3 and 4). With regards to contaminants of emergent concern, the personal pharmaceutical care products (PPCPs) were the most often used compounds against ciliates, followed by polycyclic aromatic hydrocarbons, in mortality and populational alteration assays (Figures 2A, 3 and 4).

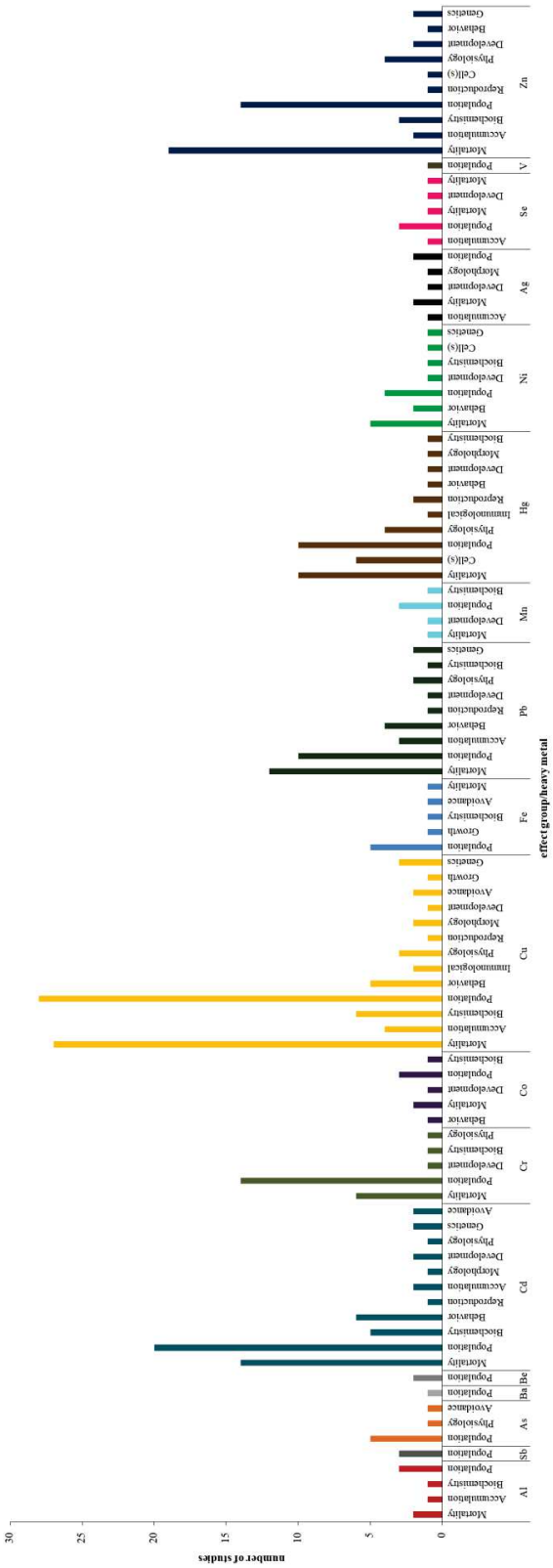

number of studies
30
25
20
15
10
5
0
effect group:heavy metal
Al
As
Ba
Be
Cd
Co
Cr
Cu
Fe
Hg
Mn
Ni
Pb
Sb
Se
Ag
V
Zn
Mortality
Accumulation
Biochemistry
Population
Behavior
Reproduction
Cell(s)
Physiology
Development
Genetics
Avoidance
Growth
Histopathology
Immunological

Figure 3. Number of studies with heavy metals and their respective approaches according to the Ecotox knowledgebase database (USEPA, 2019) (survey conducted on 05/28/2019); Number of studies with heavy metals and their respective approaches according to the Ecotox knowledgebase database (USEPA, 2019) (survey conducted on 05/28/2019).

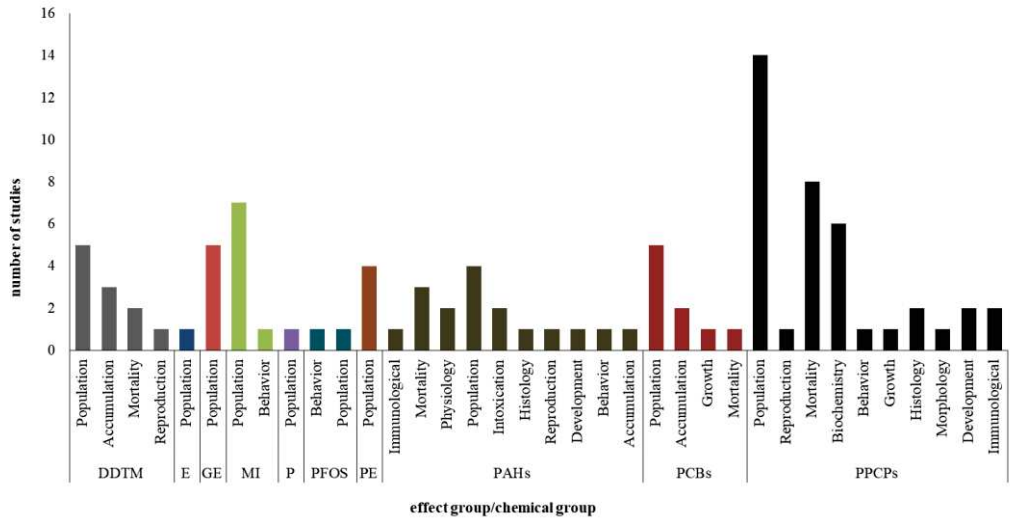


Figure 4. Number of studies with organic compounds and their respective approaches according to the Ecotox knowledgebase database (USEPA, 2019) (survey conducted on 05/28/2019); DDTM: DDT and metabolites, E: Explosives; GE: Glycol ethers, MI: Major ions, P: Perchlorates, PFOS: Perfluorooctane sulfonates and acids, PE: Phthalate Esters, PAHs: Polyaromatic Hydrocarbons, PBCs: Polychlorinated Biphenyls, PPCPs: Pharmaceutical Personal Care Products.

2.4. Methodological approaches gaps

There are numerous growth media and conditions available in the literature for *in vitro* maintenance of ciliates in laboratory. For example, species of the genus *Tetrahymena* can be cultivated in axenic cultures based on protease (Carter & Cameron, 1973; Schlenk & Moore, 1994; Gallego et al., 2007; Zhang et al., 2013, Liu et al., 2017). *Paramecium* and many others can grow using the Cerophyl culture medium proposed by Sonnebobn (1970) was used by Joshi & Misra (1986), Juchelka & Snell (1994), Pratt et al. (1997) and Salvadó et al. (1997). Mineral water and bark rice, is a common general used media used to promote the growth of bacteria that are, actualy the sources of food for ciliates employed by Ruthven & Cairns Junior (1973), Dive et al. (1980), Madoni et al.(1992), Madoni (1994, 2000), Madoni & Romeo (2006), Wanick et al. (2008) and Bitencourt et al. (2016). Water from the original sample sites are used in association with a variety of cereals as in the studies of Nalecz-Jawecki et al. (1993), Madoni et al. (1996), Rehman et

al. (2005, 2006, 2007, 2007a, 2008, 2008a, 2009, 2010, 2010a) and Shakoori et al. (2011). There are also culture media available for marine ciliates, such as proposed by Yoo & Hur (2002) and used by Kim et al. (2011, 2014, 2018). On the other hand, Madoni et al. (1996) did not use methods of cultivation in laboratory. The species used in the bioassays were directly from activated sludge. Twagilimana et al. (1998) tested different culture conditions for the *Spirostomum ambiguum* species. The authors conclude by highlighting the need for reproducibility of the assays in order to allow comparison between laboratories.

The major challenge today limiting the use of ciliates in ecotoxicological tests is the creation of a standard methods. Thus, it is necessary to develop specific methods to each potential species leading to efficient, replicable and comparable bioassays. The standardization of procedures in toxicity tests is paramount for the reproducibility and comparability of results comparability of results from laboratories in different geographical regions (Soares & Calow, 1993). Furthermore, they can help environmental managers since one of the first approaches in environmental studies is precisely the ecotoxicological risk assessment, which is responsible for showing the fate and effects of chemicals in the environment (Vindimian, 2001).

3. Final Considerations

Here, we highlighted the advantages of using ciliates in ecotoxicological studies. They have many characteristics that make them good model organisms for environmental toxicity evaluation. Therefore, they can potentially contribute to the establishment of more accurate guidelines and risk management programs, and also represent a valuable system to study how environmental contaminants may impacts normal cell biological functions. For these reasons, we hope that in a near future more ecotoxicological works will be using ciliates as model organisms.

Acknowledgements

The authors acknowledge the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES) for the post-doctoral (PNPD) and PhD fellowship confered to MS and JAVB, respectively. This work was granted by the “Fundação de Amparo à Pesquisa do Estado de Minas Gerais” (FAPEMIG, CRA 03648/16) and “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq, Bolsa de Produtividade to RJPD).

References

ALMEIDA, V.L.S., DANTAS, E.W., MELO-JÚNIOR, M., BITTENCOURT-OLIVEIRA, M.C. and MOURA, A.N. Zooplanktonic community of six reservoirs in northeast Brazil. *Brazilian Journal of Biology,* 2009, 69 (1), 57-65.

AURY, J.M., JAILLON, O., DURET, L., NOEL, B., JUBIN, C., PORCEL, B.M., SÉGURENS, B., DAUBIN, V., ANTHOUARD, V., AIACH, N., ARNAIZ, O., BILLAUT, A., BEISSON, J., BLANC, I., BOUHOUCHE, K., CÂMARA, F., DUHARCOURT, S., GUIGO, R., GOGENDEAU, D., KATINKA, M., KELLER, A.M., KISSMEHL, R., KLOTZ, C., KOLL, F., LE MOUËL, A., LEPÈRE, G., MALINSKY, S., NOWACKI, M., NOWAK, J.K., PLATTNER, H., POULAIN, J., RUIZ, F., SERRANO, V., ZAGULSKI, M., DESSEN, P., BÉTERMIER, M., WEISSENBACH, J., SCARPELLI, C., SCHÄCHTER, V., SPERLING, L., MEYER, E., COHEN, J. and WINCKER, P. Global trends of whole-genome duplications revealed by the ciliate *Paramecium tetraurelia*. *Nature,* 2006, 444, 171-178.

BAGANTINI, I.L., SPÍNOLA, A.L.G., PERES, B.M., MANSANO, A.S., RODRIGUES, M.A.A., BATALHA, M.A.P., LUCCA, J.V., GODINHO, M.J.L., TUNDISI, T.M. and REGALI-SELEGHIM, M.H. Protozooplankton and its relationship with environmental conditions in 13 water bodies of the Mogi-Guaçu basin - SP, Brazil. *Biota Neotropica,* 13, 2013, 1-12.

BEAVER J.R and CRISMAN T.L. The Role of Ciliated Protozoa in Pelagic Freshwater Ecosystems. *Microbiol Ecology,* 1989, 17, 111-136.

BENBOUZID, H., BERREBBAH, H., BERREDJEM, M. and DJEBAR, M.R. Toxic effects of phosphoramidate on *Paramecium* sp. with special emphasis on respiratory metabolism, growth, and generation time. *Toxicological and Environmental Chemistry,* 2012, 94(3), 557-565.

BERNHARDT, E.S., ROSI-MARSHALL, E.J. and GESSNER M.O. Synthetic chemicals as agents of global change. *Frontiers in Ecology and the Environment,* 2017, 5(2), 84-89.

BITENCOURT, J.A.P., PEREIRA, D.C., SILVA-NETO, I.D., CRAPES, M.C.C. and NETO, J.A.B. Evaluation of sensitivity to zinc and copper of *Diophrys appendiculata* (Protozoa, Ciliophora) and their associated bacteria, both isolated from a tropical polluted bay. *Revista Brasileira de Zoociências,* 2016, 17(1), 112-123.

CARTER, J.W. and CAMERON.I.L. Toxicity bioassay of heavy metals in water using *Tetrahymena pyriformis*. *Water Research,* 1973, 7, 951.

CHAUDHRY, R. and SHAKOORI, A.R. Characterization of copper resistant ciliates: Potential candidates for consortia of organisms used in bioremediation of wastewater. *African Journal of Biotechnology,* 2011, 10(45), 9101-9113.

CORLISS, J.O. The ciliated protozoa: Pergamon Press, London, 1979, pp.455.

DEBASTIANI, C., MEIRA, B.R., LANSAC-TÔHA, F.M., VELHO, L.F.M. and LANSAC-TÔHA, F.A., Protozoa ciliates community structure in urban streams and their environmental use as indicators. *Brazilian Journal of Biology,* 2016, 76(4), 1043-1053.

DELMONTE CORRADO, M.U., TRIELLI, F., AMAROLI, A., OGNIBENE, M. and FALUGI, C. Protists as tools for environmental biomonitoring: importance of cholinesterase enzyme activities. In: Burk, A.R. ed. *Water Pollution*. Nova Iorque: Nova Science Publishers, 2005, pp. 181-200.

DIAS, R.J.P., WIELOCH, A.H.B. and D'AGOSTO, M.A., 2008. The influence of environmental characteristics on the distribution of ciliates (Protozoa, Ciliophora) in an urban stream of southeast. *Brazilian Journal of Biology*, 2008, 68(2), 287-295.

DIECKMANN, J. An improved protargol impregnation for ciliates yielding reproducible results). *European Journal of Protistology,* 1995, 31, 372-382.

DIVE, D., LECLERC, H. and PERSOONE, G. Pesticide Toxicity on the Ciliate Protozoan *Colpidium campylum*: Possible Consequences of the Effect of Pesticides in the Aquatic Environment. *Ecotoxicology and Environmental Safety,* 1980, 4, 129-133.

DINI, F. Relationship between breeding systems and resistance to mercury in *Euplotes crassus* (Ciliophora: Hypotrichida). *Marine Ecology Progress Series,* 1981, 41, 195-202.

ESTEBAN, G.F. and FINLAY, B.J. Cryptic freshwater ciliates in a hypersaline lagoon. *Protistology,* 2003, 154, 411-418.

EISEN, J.A., COYNE, R.S., WU, M., WU, D., THIAGARAJAN, M., WORTMAN, J.R., BADGER, J.H., REN, Q., AMEDEO, P., JONES, K.M., TALLON, L.J., DELCHER, A.L., SALZBERG, S.L., SILVA, J.C., HAAS, B.J., MAJOROS, W.H., FARZAD, M., CARLTON, J.M., SMITH, R.K.J.R., GARG, J., PEARLMAN, R.E., KARRER, K.M., SUN, L., MANNING, G., ELDE, N.C., TURKEWITZ, A.P., ASAI, D.J., WILKES, D.E., WANG, Y., CAI, H., COLLINS, K., STEWART, B.A., LEE, S.R., WILAMOWSKA, K., WEINBERG, Z., RUZZO, W.L., WLOGA, D., GAERTIG, J., FRANKEL, J., TSAO, C.C., GOROVSKY, M.A., KEELING, P.J., WALLER, R.F., PATRON, N.J., CHERRY, J.M., STOVER, N.A., KRIEGER, C.J., DEL TORO, C., RYDER, H.F., WILLIAMSON, S.C., BARBEAU, R.A., HAMILTON, E.P. and ORIAS E. Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote. *Plos Biology,* 2006, 4(9), e286.

ELGUERO, M.E., NUDEL, C.B. and NUSBLAT, A.D. Biotechnology in ciliates: an overview. *Critical reviews in biotechnology,* 2019, 39(2), 220-234.

FOISSNER, W. Morphology and morphogenesis of *Circinella arenicola*nov. gen., nov. spec., a cephalized hypotrich (Ciliophora, Hypotrichida) from sand dunes in Utah, USA. *European Journal of Protistology,* 1994, 30(2), 156-170.

FOISSNER, W. Protist diversity: estimates of the near-imponderable. *Protistology,* 1999, 150, 363-368.

FOISSNER, W., AGATHA, S. and BERGER, H. Soil ciliates (Protozoa,Ciliophora) from Namibia (Southwest Africa), with emphasis on two contrasting environments, the Etosha Region and the Namib Desert. *Denisia,* 2002, 5, 1-1459.

FOISSNER, W. and BERGER, H. A user-friendly guide to ciliates (Protozoa, Ciliophora) commonly used by hydrobiologists as bioindicators in rivers, lakes, and waste waters, with notes on their ecology. *Freshwater Biology,* 1996, 35, 375-498.

FOISSNER, W. Morphology and ontogenesis of *Bromeliophyra brasiliensis* gen. n., sp. n., a new tetrahymenid ciliate (Protozoa: Ciliophora) from Brazilian tank bromeliads (Bromeliaceae). *Acta Protozoologica,* 2003, 42, 55-70.

FORBES, V.E. and FORBES, T.L. *Ecotoxicology in Theory and Practice.* Chapman and Hall Ecotoxicology: Series 2: London, 1994, 1st ed.

FORGET, G., GAGNON, P., SANCHEZ, W.A. and DUTKA, B.J. Overview of methods and results of the eight countries international development research centre (IDRC) water tox project. *Environmental Toxicology,* 2000, 15, 264-276.

FENCHEL, T. The Ecology of the Protozoa: The Biology of Free-living Phagotrophic Protists. *Springer Verlag,* 1987.

FENCHEL, T. and FINLAY, B.J. The ubiquity of small species: patterns of local and global diversity. *Bio Science,* 2004, 54, 777-784.

FINLAY, B.J. Outing the Researcher: The Provenance, Principles and Practice of Reflexivity. *Qualitative Health Research*, 2002, 12(4), 531-45.

FINLAY, B.J. and FENCHEL, T. Ecology: role of ciliates in the natural environment, In: HAUSMANN, K. and P.C. BRADBURY, ed. *Ciliates: cells as organisms*. Stuttgart, 1996, pp. 417- 440.

FINLAY, B.J and FENCHEL, T. Divergent perspectives on protist species richness. *Protistology,* 1999, 150, 229-233.

GALLEGO, A., MARTÍN-GONZÁLEZ, A., ORTEGA, R. and GUTIÉRREZ, J. Flow cytometry assessment of cytotoxicity and reactive oxygen species generation by single and binary mixtures of cadmium, zinc and copper on populations of the ciliated protozoan *Tetrahymena thermophila*. *Chemosphere,* 2007, 68, 647-661.

GAO, F., WARREN, A., ZHANG, Q., GONG, J., MIAO, M., SUN, P., XU, D., HUANG, J., YI, Z. and SONG, W. The All-Data-Based Evolutionary Hypothesis of Ciliated Protists with a Revised Classification of the Phylum Ciliophora (Eukaryota, Alveolata). *Nature/Scientific Reports,* 2016, 29(6), 1-14.

GREBECKI, A. and KUZNICKI, L. Autoprotection in *Paramecium caudatum* by influencing the chemical properties of its medium. *Acta Biological Experimentalis,* 1956, 17, 71-107.

GROLIÈRE, C.A., CHAKLI, R., SPARAGANO, O. and PEPIN, D. Application de la colonisation d'un substrat artificiel par les ciliés à l'étude de la qualité des eaux d'une riviére. *European Journal of Protistology,* 1990, 25(4), 381-390.

GILRON, D.L. and LYNN, D.H. Ciliated protozoa as test organism in toxicity assessment. P.G. Wells, K. Lee, C. Blaise. ed. *Microscale Testing in Aquatic Toxicology,* 1998, pp. 323-336.

GOMIERO, A., DAGNINO, A., NASCI, C. and VIARENGO, A. The use of protozoa in ecotoxicology: Application of multiple endpoint tests of the ciliate *E. crassus* for the

evaluation of sediment quality in coastal marine ecosystems. *Science of the Total Environment,* 2013, 442, 534-544.

GERHARDT, A., UD-DAULA, A. and SCHRAMM, K.W. *Tetrahymena* spp. (Protista, Ciliophora) as test species in rapid multilevel ecotoxicity tests. *Acta Protozoologica*, 2010, 49, 271-280.

GONG, Z., CHEN, Y., YAN, Y., PEI, S., WU, D., ZHANG, M. and WANG, Q. Toxicity of Three Heavy Metal Pollutants of the Pharmaceutical Wastewater to *Paramecium caudatum*. *Advanced Materials Research*, 2014, 937, 571-577.

GUARINELLO, M.L., SHUMCHENIA, E.J. and KING, J.W. Marine Habitat Classification for Ecosystem-Based Management: A Proposed Hierarchical Framework. *Environmental Management,* 2010, 45(4), 793-806.

GUTIÉRREZ, J.C., MARTÍN-GONZÁLEZ, A., DÍAZ, S. and ORTEGA, R. Ciliates as a potential source of cellular and molecular biomarkers/biosensors for heavy metal pollution. *European Journal of Protistology,* 2003, 39, 461-467.

HARTMANN, M., GUBERMAN, A., FLORIN-CHRISTENSEN, M. and TIEDTKE, A. Screening for and characterization of phospholipase A1 hypersecretory mutants of *Tetrahymena thermophila. Applied Microbiology and Biotechnology,* 2000, 54, 390-396.

ISHIKAWA, T. Swimming of ciliates under geometric constraints. *Journal of Applied Physics,* 2019, 125, 1-10.

JAYARAM, J., PAPOYAN, A., BISHARYAN, Y., CASSIDY-HANLEY, D., ZHANG, X., COLUSSI, P., APPLETON, J.A., GAGLIARDO, L. and CLARK, T.G. An alternative platform for rapid production of effective subunit vaccines. *BioPharm International,* 2010, 23, 6-13.

JIANG, Y., XU, H., HU, X., WARREN, A. and SONG, W. Functional groups of marine ciliated protozoa and their relationships to water quality. *Environmental Science and Pollution Research,* 2013, 20(8), 5272-5280.

JO, W. J., LOGUINOV, A., WINTZ, H., CHANG, M., SMITH, A. H., KALMAN, D., ZHANG, L., SMITH, M. T. and VULPE, C.D. Comparative functional genomic analysis identifies distinct and overlapping sets of genes required for resistance to monomethylarsonous acid (MMAIII) and arsenite (AsIII) in yeast. *Toxicological Sciences*, 2009*,* 111 (2), 424-36.

JOPPERT, F., GUEDES, L.H.C. and SILVA-NETO, I.D. Qualitative study of the ciliated protozoa in tanks of *Aechmea nudicalis* (Bromeliaceae) at Barra de Maricá, Rio de Janeiro. *Memórias do Instituto Oswaldo Cruz,* 1995, 90, 251.

JUCHELKA, C.M. and SNELL, T.W. Rapid Toxicity Assessment Using Ingestion Rate of Cladocerans and Ciliates. *Archives of Environmental Contamination and Toxicology,* 1994, 28, 508-512.

JUNIOR, N.G. and CARVALHO, M.B. A utilização de protozoários como organismos bioindicadores em ensaios ecotoxicológicos e suas respostas à exposição a fármacos e

outros compostos. *Periódico Eletrônico Fórum Ambiental da Alta Paulista,* 2017, 13(4), 24-37.

KAPUSCINSKI, J. DAPI: a DNA-Specific Fluorescent Probe. *Journal Biotechnic & Histochemistry,* 1995, 70, 220-233.

KCHAOU, N., ELLOUMI, J., DRIRA, Z., HAMZA, A., AYADI, H., BOUAIN, A. and ALEYA, L. Distribution of ciliates in relation to environmental factors along the coastline of the Gulf of Gabes, Tunisia. *Estuarine, Coastal and Shelf Science,* 2009, 83, 414-424.

KIM, S.H., JUNG, M.Y. and LEE, Y.M. Effect of Heavy Metals on the Antioxidant Enzymes in the Marine Ciliate *Euplotes crassus. Toxicology,* 2011, 3, 213-219.

KIM, S.H., KIM, J.S., LEE, J.S. and LEE, Y.M. Acute effects of heavy metals on the expression of glutathione-related antioxidant genes in the marine ciliate *Euplotes crassus. Marine Pollution Bulletin,* 2014, 85, 455-462.

KIM, B.M., RHEE, J.S., CHOI, I.Y. and LEE, M.Y. Transcriptional profiling of antioxidant defense system and heat shock protein (*Hsp*) families in the cadmium- and copper-exposed marine ciliate *Euplotes crassus. Genes and Genomics,* 2018, 40, 85-98.

KLEIN, B.N. The "dry" silver method and its proper and use. *The Journal of Protozoology,* 1958, 5(2), 99-103.

KUDO, R.R. Protozoology. Illinois, Charles C. Thomas.1966, pp.786.

LOMBARDI, J.V. Fundamentos de toxicologia aquática. In: Ranzani-Paiva, M.J.T. Takemoto, R.M. and Lizama, M.A.P. ed. *Sanidade de organismos aquáticos*. São Paulo: Varela, 2004, pp. 263-272.

LI, JING., MA, X., SU, G., GIESY, J.P., XIAO, Y., ZHOU, B., LETCHER, R.J. and LIU, C. Multigenerational effects of tris(1,3-dichloro-2-propyl) phosphate on the free-living ciliate protozoa Tetrahymena thermophila exposed to environmentally relevant concentrations and after subsequente recovery. *Environmental Pollution,* 2016, 218, 50-58.

LEE J.J, Soldo A.T. Protocols in protozoology: United States of America, 1992, pp. 655.

LIU, C.B., QU, G.B., CAO, M.X., LIANG, Y., HU, L.G., SHI, J.B., CAI, Y. and JIANG, G.B. Distinct toxicological characteristics and mechanisms of Hg^{2+} and MeHg in *Tetrahymena* under low concentration exposure. *Aquatic Toxicology,* 2017, 193, 152-159.

LYNN, D.H. The Ciliated Protozoa: Characterization, Classification and Guide to the Literature. Springer Press: New York, 2008,. 3ed.

LYNN. D.H. and SMALL, E.B. An illustred Guide to the Protozoa. In: LEE, J.J., P.C. BRADBURY and G.F. LEEDALE., ed. *Phylum Ciliophora*. Society of Protozoologists, Lawrence: Kansas, 2002, pp. 371-656.

MADONI, P. The acute toxicity of nickel to freshwater ciliates. *Environmental Pollution,* 2000, 109(1), 53-59.

MADONI, P. Protozoa as indicators of wastewater treatment efficiency. In: Mara, D., Horan, N., ed. *The Handbook of Water and Wastewater Microbiology*. Academic Press, 2003, 361-371.

MADONI, P. Ciliated protozoans communities and saprobic evaluation of water quality in the hilly zone of some tributaries of the Po River (northern Italy). *Hydrobiologia,* 2005, 541(1), 55-69.

MADONI, P. Protozoa in wastewater treatment processes: A minireview. *Italian Journal of Zoology,* 2011, 78(1), 3-11.

MADONI, P. A sludge biotic index (SBI) for the evaluation of the biological performance of activated sludge plants based on the microfauna analysis. *Water Research,* 1994, 28(1), 67-75.

MADONI, P. and BARGHIROLI, S. Changes in the ciliate assemblage along a fluvial system related to physical, chemical and geomorphologic characteristics. *European Journal of Protistology,* 2007, 43, 67-75.

MADONI, P., DAVOLI, D., GORBI, G. and VESCOVI, L. Toxic effect of heavy metals on the activated sludge protozoan community. *Water Research,* 1996, 30(1), 135-141.

MADONI, P., ESTEBAN, G. and GORBI, G. Acute Toxicity of Cadmium, Copper, Mercury, and Zinc to Ciliates from Activated Sludge Plants. *Bulletin of Environmental Contamination and Toxicology,* 1992, 49, 900-905.

MADONI, P. and ROMEO, M.G. Acute toxicity of heavy metals towards freshwater ciliated protists. *Environmental Pollution,* 2006, 141, 1-7.

MARTÍN-GONZÁLEZ, A., DÍAZ, S., BORNIQUEL, S., GALLEGO, A. and GUTIÉRREZ, J.C. Cytotoxicity and bioaccumulation of heavy metals by ciliated protozoa isolated from urban wastewater treatment plants. *Research in Microbiology,* 2006, 157, 108-118.

MITCHELL, E.A.D. and MEISTERFELD, R. Taxonomic confusion blurs the debate on cosmopolitanism versus local endemism of free living protists. *Protistology,* 2005, 156, 263-267.

MOLDEN, D., FRENKEN, K., BARKER, R., DE FRAITURE, C., MATI, B., SVENDSEN, M., SADOFF, C. and FINLAYSON, C.M. Trends in water and agricultural development. In: Molden, D. ed. *Water for Food, Water for Life: A Comprehensive Assessment of Water Management in Agriculture*. Earthscan and International Water Management Institute: London and Colombo, 2007.

MORTIMER, M., KASEMETS, K. and KAHRU, A. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila. Toxicology,* 2010, 269, 182-189.

MÜLLER, H., SCHÖNE, A., PINTO-COELHO, RM., SCWEIZER, A. and WEISSE, T. Seasonal succession of ciliates in Lake Constance. *Microbial Ecology,* 1991, 21(1), 119-138.

NALECZ-JAWECKI, G., Demkowicz-Dobrzahski, K. and SAWICKI, J. Protozoan *Spirostomum ambiguum* as a highly sensitive bioindicator for rapid and easy determination of water quality. *The Science of the Total Environment,* 1993, 1227-1234.

NERAD, T.A. and DAGGETT, P.M. Cultivation of selected colorless crypromonads. In. Soldo, A.T. ed. *Protocols of protozoology*. Society of Protozoologists: Lawrence, 1992, pp. A71-A73.

NIEMEYER, J.C., MOREIRA-SANTOS, M., RIBEIRO, R., DA SILVA, E.M. and SOUSA, J.P. Environmental risk assessment of a metal contaminated area in the tropics. Tier I: screening phase. *Journal of Soils Sediments,* 10, 2010, 1557-1571.

NISHIGAMI, Y., OHMURA, T., TANIGUCHI, A., NONAKA, S., MANABE, J. and ISHIKAWA, T., ICHIKAWA, M. Influence of cellular shape on sliding behavior of ciliates. *Communicative and Integrative Biology,* 2018, 11(4), e1506666.

NOLAND, L.E. Factors influencing the distribution of frech water ciliates. *Ecology*, 1925, 6(4), 437-452.

OHMURAA, T., NISHIGAMIA, Y., TANIGUCHIB, A., NONAKAB, S., MANABEC, J., ISHIKAWAC, T. and ICHIKAWAA, A. Simple mechanosense and response of cilia motion reveal the intrinsic habits of ciliates. *PNAS,* 2018, 115(13), 3231-3236.

OKI, T. and KANAE, S. Global hydrological cycles and world water resources. *Science* 313:1068-72 4. Fenwick A. Waterborne infectious diseases-Could they be consigned to history?. *Science,* 2006, 313, 1077-1081.

PAULETO, G.M., VELHO, L.F.M., BUOSI, P.R.B., BRÃO, A.F.S., LANSAC-TÔHA, F.A. and BONECKER, C.C. Spatial and temporal patterns of ciliate species composition (Protozoa: Ciliophora) in the plankton of the Upper Paraná River floodplain. *Brazilian Journal of Biology,* 2009, 69(2), 517-527.

PINHEIRO, M.D.O., POWER, M.E., BUTTLER, B., DAYEH, V.R., SLAWSON, R., LEE, L.E.J., LYNN, D.H. and BOLS, N.C. Use of Tetrahymena thermophila to study the role of protozoa in inactivation of viruses in water. *Applied and Environmental Microbiology,* 2007, 73(2), 643-649.

PRATT, J.R. and J. JR. CAIRNS. Functional groups in the Protozoa: roles in differing ecosystems. *Journal of Protozoology,* 1985, 32, 415-422.

PRATT, J.R., MOCHAN, D. and. XU, Z. Rapid Toxicity Estimation Using Soil Ciliates: and Bioavailability. *Bulletin of Environmental Contamination and Toxicology,* 1997, 58, 387-393.

PUTTEN, A., KONIG, T., FABRITUS, D., KIY, T., LESKE, A. and PLOTE, J. Method for producing gamma-linolenic acids from a ciliate culture by adding suitable precursor molecules to said culture medium, US20060205047A1, 2006.

PUYTORAC, P. Phylum Ciliophora Doflein, 1901. In: Traité de zoologie, tome ii, infusoires ciliés, fasc. 2, systématoque. ed. *P. de Puytorac*. Masson: Paris, 1994, pp.1-15.

QURESHI, M.E. and HANJRA, M.A. Global water crisis and future security in an era of climate change. *Food Policy,* 2010, 35(5), 365-377.

RAO, J.V., SRIKANTH, K., AREPALLI, S.K. and GUNDA, V.G. Toxic effects of acephate on *Paramecium caudatum* withspecial emphasis on morphology, behaviour, and generation time. *Pesticide Biochemistry and Physiology,* 2006, 86, 131-137.

RICO, D., MARTÍN-GONZÀLEZ, A., DÍAZ, S., LUCAS, P. and GUTIÉRREZ, J.C. Heavy metals generate reactive oxygen species in terrestrial and aquatic ciliated protozoa. *Comparative Biochemistry and Physiology,* Part C, 2009, 149, 90-96.

REHMAN, A., ASHRAF, S., QAZI, J.I. and SHAKOORI, A.R. Uptake of Lead by a Ciliate, *Stylonychia mytilus*, Isolated from Industrial Effluents: Potential Use in Bioremediation of Wastewater. *Bulletin of Environmental Contaminaton and Toxicology,* 2005, 75, 290-296.

REHMAN, A., SHAKOORI, F.R. and SHAKOORI, A.R. Heavy metal resistant ciliate, *Euplotes mutabilis,* isolated from industrial effluents can decontaminate wastewater of heavy metals. *Bulletin of Environmental Contamination and Toxicology,* 2006, 76, 907-913.

REHMAN, A., SHAKOORI, F.R. AND SHAKOORI, A.R. Multiple metal resistance and uptake by a ciliate, *Stylonychia mytilus*, isolated from industrial effluents and its possible use in wastewater treatment. *Bulletin of Environmental Contamination and Toxicology,* 2007, 79, 410-414.

REHMAN, A., SHAKOORI, F.R. and SHAKOORI, A.R. Potential Use of a Ciliate, *Vorticella microstoma,* Surviving in Lead Containing Industrial Effluents in Waste Water Treatment. *Pakistan Journal of Zoology,* 2007a, 39(4), 259-264.

REHMAN, A., SHAKOORI, F.R. AND SHAKOORI, A.R. Heavy metal resistant freshwater ciliate, *Euplotes mutabilis*,isolated from industrial effluents has potential to decontaminate wastewater of toxic metals. *Bioresource Technology,* 2008, 99, 3890-3895.

REHMAN, A., SHAKOORI, F.R. AND SHAKOORI, A.R. Uptake of heavy metals by *Stylonychia mytilus* and its possible use in decontamination industrial wastewater. *World Journal Microbioogy and Biotechnology,* 2008a, 24, 47-53.

REHMAN, A., SHAKOORI, F.R. and SHAKOORI, A.R. Heavy Metal Uptake by *Euplotes mutabilis* and its Possible Use in Bioremediation of Industrial Wastewater. *Bulletin of Environmental and Contamination Toxicology,* 2009, 83, 130-135.

REHMAN, A., SHAKOORI, F.R. and SHAKOORI, A.R. Resistance and Uptake of Heavy Metals by *Vorticella microstoma* and Its Potential Use in Industrial Wastewater Treatment. *Environmental Progress and Sustainable Energy,* 2010, 29(4), 481-486.

REHMAN, A., SHAKOORI, F.R. and SHAKOORI, A.R. Multiple Heavy Metal Tolerant Ciliates, *Oxytricha fallax* and *Paramecium caudatum*, Isolated From Industrial Effluents and Their Potential Use in Wastewater Treatment. *Pakistan Journal of Zoology,* 2010a, 42(3), 301-309.

RUTHVEN, J. and CAIRNS, J.J. Response of Fresh-Water Protozoan Artificial Communities to Metals. *Journal of Protozoology,* 1973, 20(1), 127-135.

SALVADO, H., GRACIA, M.P., AMIGÓ, J.M. and RIUS, M. Effects of Cadmium on Growth and Motility in *Euplotes aediculatus* Isolated from Activated Sludge. *Bulletin of Environmental and Contamination Toxicology,* 1997, 58, 838-844.

SCHWARZENBACH, R.P., THOMAS, E.G.L.I., THOMAS, B., HOFSTETTER, U.R.S., VON, G. and BERNHARD, W. Global water pollution and human health. *Annual Review of Environment and Resources,* 2010, 35, 109-136.

SCHWARZENBACH, R.P., ESCHER, B.I., FENNER, K., HOFSTETTER, T.B., JOHNSON, C.A., VON, G. and WEHRLI, B. The challenge of micropollutants in aquatic systems. *Science,* 2006, 313,1072-77.

SCHLENK, D., and MOORE, C. T. Effect of pH and time on the acute toxicity of copper sulfate to the ciliate protozoan *Tetrahymena thermophila*. *Bulletin of Environmental Contamination and Toxicology,* 1994, 53(6), 800.

SILVEIRA, M.P. Aplicação do Biomonitoramento para Avaliação da Qualidade da Água em Rios. *Embrapa Meio Ambiente,* 2004, 68.

SILVA, D.C.V.R., POMPÊO, M. and PAIVA, T.C.B. A ecotoxicologia no contexto atual no Brasil. Ecologia de reservatórios e interfaces, São Paulo: Instituto de Biociencias da Universidade de São Paulo, 2015.

SHAKOORI, F.R., ZAFAR, M.F., FATEHULLAH, A., REHMAN, A. and SHAKOORI, A.R. Response of Glutathione Level in a Protozoan Ciliate, Stylonychia mytilus, to Increasing Uptake of and Tolerance to Nickel and Zinc in the Medium. *Pakistan Journal of Zoology,* 2011, 43(3), 569-574.

SIQUEIRA-CASTRO, I.C.V., GREINERT-GOULART, J.A., ROSSETTO, R., GUIMARÃES, J.R. and FRANCO, R.M.B. Ciliated protozoa community of a combined UASB-activated sludge system in southeastern Brazil. *Environmental Science and Pollution Research,* 2016, 23(23), 23804-23814.

SLABODNICK, M. M., RUBY, G., REIFF, S.B., SWART, C.E., GOSAI, S., PRABAKARAN, S., WITKOWSKA, E., LARUE, G.E., FISHER, S., FREEMAN, R.M., GUWARDENA, J., CHU, W., STOVER, N.A., GREGORY, B.D., NOWACKI, M., DERISI, J., ROY, S.W., WALLACE, F.M., SOOD, P. The Macronuclear Genome of *Stentor coeruleus* Reveals Tiny Introns in a Giant Cell. *Current Biology*, 2017, 27, R146-R148.

SLEIGH, M. Protozoa and other Protists. Chapman and Hall, New York, 1988, pp.399.

SOARES, A. M. V. M. and CALOW, P. Seeking standardization in ecotoxicology. In: SOARES, A. M. V. M. and CALOW, P. ed. *Progress in standardization of aquatic toxicity test*. Boca Raton: Lewis Publishers, 1993.

SOLDO, A. T. and VAN WAGTENDONK, W. J. The nutrition of *Paramecium aurelia*, stock 299. *Journal of Protozoology,* 1969, 16, 500-506.

SONNEBOBN, T.M. Methods in Paramecium research. In: Methods in Cell Physiology, New York: Academic Press, 1970, pp. 241-339.

SWART, E., BRACHT J.R., MAGRINI, V., MINX, P., CHEN, X., ZHOU, Y., KHURANA, J.S., GOLDMAN, A.D., NOWACKI, M., SCHOTANUS, K., JUNG, S., FULTON, R.S., LY, A., McGRATH, S., HAUB, K., WIGGINS, J.L., STORTON, D., MATESE, J.C., PARSONS, L., CHANG, W.J., BOWEN, M.S., STOVER, N.A., JONES, T.A., EDDY, S.R., HERRICK, G.A., DOAK, T.G., WILSON, R.K., MARDIS, E.R. and LANDWEBER, L.F. The *Oxytricha trifallax* macronuclear genome: a complex eukaryotic genome with 16,000 tiny chromosomes. *PLoS Biol*. 2013;11, e1001473.

TWAGILIMANA, L., BOHATIER, J., GROLIÈRE, C.A., BONNEMOY, F. and SARGOS, D. New Low-Cost Microbiotest with the Protozoan *Spirostomum teres*: Culture Conditions and Assessment of Sensitivity of the Ciliate to 14 Pure Chemicals. *Ecotoxicology and Environmental Safety,* 1998, 41, 231- 244.

UNESCO - Organização das Nações Unidas. *The United Nations World Water Development Report 3: Water in a Changing World*. Paris/New York: UNESCO/Berghahn Books 2. Huntington TG. 2006. Evidence for intensification of the global water cycle: review and synthesis. *Journal of Hydrology, 2009,* 319:83-95.

US EPA - United States Environmental Protection Agency. Methods for Chemical Analysis of Water and Wastes. EPA-600/4-79- 020, Environmental Monitoring and Support Laboratory, Cincinnati OH, 1992

VELHO, L.F.M., PEREIRA., D.G., PAGIORO, T.A., SANTOS, V.D., PERENHA, M.C.Z. and LANSAC-TÖHA, F.A. Abundance, biomass and size structure of planktonic ciliates in reservoirs with distinct trophic states. *Acta Limnologica Brasiliensia,* 2005, 17(4), 361-371.

VELHO, L.F.M., LANSAC-TÔHA, F.M., BUOSI, P.R.B., MEIRA, B.R., CABRAL., A.F. and LANSAC-TÔHA, F.A. Structure of planktonic ciliates community (Protist, Ciliophora) from an urban lake of southern Brazil. *Acta Scientiarum Biological Sciences,* 2013, 35(4), 531-539.

VINDIMIAN, E. *The role of ecotoxicology for monitoring ecosystem health. Freshwater Forum,* 2001, 16, 91-97.

VINOGRADOV, D.V., TSOI, O.V., ZAIKA, A.V., LOBANOV, A.V., TURANOV, A.A., GLADISHEV, V.N. and GEL'FAND, MS. Draft macronucleus genome of *Euplotes crassus* ciliate. *Molecular Biology*, 2012; 46, 328–333.

XU, Y., STOECK, T., FORSTER, D., MA, Z., ZHANG, LL and FAN, X. ,Environmental status assessment using biological traits analyses and functional diversity indices of benthic ciliate communities. *Marine Pollution Bulletin,* 2018, 131, 646-654.

XU, H., JIANG, Y. and XU, G. Identifying functional species pool of planktonic protozoa for discriminating water quality status in marine ecosystems. *Ecological Indicators,* 2016, 62, 306-311.

WANICK, R.C., PAIVA, T.S., DE CARVALHO, C.N. and SILVA-NETO, I.D. Acute Toxicity of Cadmium to Freshwater Ciliate *Paramecium bursaria. Biociências,* 2008, 16(2), 104-109.

WALKER, C.H, HOPKIN, S.P, SIBLY, R.M and PEAKALL, D.B *Principles of Ecotoxicology.* Taylor and Francis: London, 1996.

WEISSE, T. Functional diversity of aquatic ciliates. *European Journal of Protistology,* 2017, 61, 331-358.

WWAP - World Water Assessment Programme. The United Nations World Water Development Report 2017. Wastewater: The Untapped Resource, 2017.

WU, D., YAN, T., PEI, S., ZHANG, M., WANG, Q., GONG, Z. and CHEN, Y. Inhibitory Effect of Three Heavy Mental Pollutants on Antioxidant Enzymes of *Paramecium caudatum*. *Advanced Materials Researc,* 2015, 1065, 3071-3076.

YOO, J.Y. and HUR, S.B. Evaluation of six species ciliates as a live food and culture enviroment for *Euplotes* sp. *Journal of the Korea Fish Society,* 2002, 35, 342-347.

ZHANG, P., XU, J., LU, J., LIANG, A. and WANG, W. Different response of Two metallothionein subfamilies exposed To chromium (vi) in *Tetrahymena thermophila. Fresenius Environmental Bulletin,* 2013, 22(7), 1924-1928.

ZHANG, X., XIA, P., WANG, P., YANG, J. and BAIRD, D.J. Omics Advances in Ecotoxicology. *Environmental Science and Technology,* 2018, 527, 3842-3851.

4 Chapter II

(Chapter published in the journal *Ecotoxicology and Environmental Safety*, A2, Qualis Capes 2013-2016, FI: 4.872)
https://doi.org/10.1016/j.ecoenv.2020.110669

Ciliates as model organisms for the ecotoxicological risk assessment of heavy metals: a meta–analysis

Jéssica Andrade Vilas–Boas[a,b]*, Simone Jaqueline Cardoso[b,c]*, Marcus Vinicius Xavier Senra[a,d], Andreu Rico[e], Roberto Júnio Pedroso Dias[a,b,c]

[a] Laboratório de Protozoologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[b] Programa de Pós–graduação em Biodiversidade e Conservação da Natureza, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[c] Departamento de Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036–900, Juiz de Fora, Minas Gerais, Brazil

[d] Instituto de Recursos Naturais, Pós–graduação em Meio Ambiente e Recursos Hídricos, Universidade Federal de Itajubá 37500–903, Itajubá, Minas Gerais, Brazil

[e] IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Avenida Punto Com 2, 28805, Alcalá de Henares, Madrid, Spain

*These authors contributed equally to this work

E–mail address: jessica.andrade@ecologia.ufjf.br

Abstract: Ciliates are key components of aquatic ecosystems, significantly contributing to the decomposition of organic matter and energy transfer to higher trophic levels. They are considered good biological indicators of chemical pollution and relatively sensitive to heavy metal contamination. In this study, we performed a meta–analysis of the available toxicity data of heavy metals and ciliates to assess: (1) the sensitivity of freshwater ciliates to different heavy metals, (2) the relative sensitivity of ciliates in comparison to the standard test species used in ecotoxicological risk assessment, and (3) the difference in sensitivity across ciliate taxa. Our study shows that the tolerance of ciliates to heavy metals varies notably, which is partly influenced by differences in methodological conditions across studies. Ciliates are, in general, sensitive to Mercury > Cadmium > Copper > Zinc > Lead > Chromium. Also, this study shows that most ciliates are more

tolerant to heavy metal pollution than the standard test species used in ecotoxicological risk assessments, i.e., *Raphidocelis subcapitata, Daphnia magna*, and *Onchornyncus mykiss*. Threshold concentrations derived from toxicity data for these species is expected to confer sufficient protection for the vast majority of ciliate species. Our data analysis also shows that the most commonly tested ciliate species, *Paramecium caudatum* and *Tetrahymena thermophila,* are not necessarily the most sensitive ones to heavy metal pollution. Finally, this study stresses the importance of developing standard toxicity test protocols for ciliates, which could lead to a better comprehension of the toxicological impact of heavy metals and other contaminants to ciliate species.

1. Introduction

Heavy metal contamination in aquatic ecosystems is a pressing and recurrent issue for biodiversity preservation and for human health. Heavy metal contamination is primarily associated to residues from mining and industrial activities, and environmental discharges of treated and untreated domestic sewage sludge (Green et al., 2010; Diaz–de Alba et al., 2011). A large number of studies show that heavy metals may exhert toxicological effects to aquatic organisms at concentrations monitored in surface waters with notable antrophogenic pressure (Lopa and Adhikari, 2006; Arenas-Sanchez et al., 2019). The toxicological effect of heavy metals to aquatic organisms largely depends on the exposure concentration, their speciation under environmental conditions, and their nature *i.e.*, essential *vs* non–essential metals (Bolan and Duraisamy, 2003; Green et al., 2008; Liu et al., 2017; Meng et al., 2016). For example, essential metals such as Zinc or Cadmium are key elements for the growth of living organisms. However, at large doses, these metals can inhibit growth, reduce endocytosis and food absorption rates, and concentrate on the cell membrane, leading to cell disruption and lysis (Xin et al., 2015; Schuler and Relyea, 2018). On the other hand, non–essential metals such as Lead and Mercury bind to thiol–containing groups and oxygen sites, cause alterations in the configurational structure of nucleic acids and proteins, and interfere with the oxidative phosphorylation and osmotic balance of cells and organisms (Hughes and Poole, 1989; Poole and Gadd, 1989).

The ecotoxicological risk assessment of heavy metals generally involves the development of threshold concentrations based on toxicity data for a limited number of standard test species (*i.e*., algae, *Daphnia* sp., fish) and the application of assessment factors (Water Framework Directive, 2006). Assessment factors are expected to take into

account inter–species sensitivity differences, so that the derived threshold concentrations are able to also protect non–standard test organisms. The protectiveness of these assessment factors for non–standard test species of primary producers, invertebrates and fish has been well documented (Moreira et al., 2016; Montassir et al., 2017; Santos–Medrano and Rico–Martínez, 2018; Belangera and Carr, 2019). However, the sensitivity of these standard test species as regards to that of microorganisms such as bacteria, fungi or ciliates has not been properly evaluated.

Ciliates are considered important components of aquatic ecosystems as they significantly contribute to organic matter decomposition and to the transfer of energy to higher trophic levels (Fenchel, 1987; Sherr and Sherr, 1994; Jiang et al., 2007; Weisse, 2017). Laboratory toxicity tests show that ciliates can be severely affected by heavy metal pollution. These studies describe, for example, impacts on feeding capacity and growth rates, and variations in respiration and oxidative stress through the generation of increased levels of ROS (Oxigen Reactive Species), which in many cases leads to high individual mortalities (Gutiérrez et al., 2003; Liu et al., 2017). Furthermore, it has been suggested that those impacts are generally a consequence of ultrastructural changes, membrane alterations, and inhibition of the organization of main cytoskeleton components (Makhija et al., 2015; Pudpong and Chantangsi, 2015). Ciliates are on the first level of the aquatic food chain and, therefore, have the capacity to transfer contaminants such as heavy metals to higher trophic levels (Mortuza et al., 2005; Gerhardt et al., 2010). However, the impact of heavy metals on the structure and functioning of ciliate communities, and their potensial side-effects for the whole aquatic ecosystem, have not been sufficiently investigated (Trielli et al., 2007; Velho et al., 2013; Segovia et al., 2016).

Despite their relevance for ecosystem integrity, ciliates are usually not considered in prospective risk assessments (Gomiero et al., 2013; Mansano et al., 2016). One probable reason for this is the lack of standard protocols for testing the toxicity of chemicals. As documented by some authors, many ciliate species do not adapt well to laboratory conditions and are, therefore, not suitable for *in vitro* testing (Delmonte Conrrado et al., 2006), while some others (*e.g.*, *Paramecium caudatum, Tetrahymena thermophila*) have been used in sensitivity and resilience tests against a variety contaminants (Madoni et al., 1992, 1994; Madoni and Romeo, 2006; Gomiero et al., 2012; Mansano et al., 2016). Morevoer, given some particular characteristics of ciliates (*i.e.*, small size, short time generations, quick response to disturbances and easy sampling), many authors have advocated for their inclusion as whole-cell biosensors or water quality

indicators for chemical risk monitoring (Cairns et al., 1993; Trielli et al., 2007; Tan et al., 2010).

In this study we performed a meta–analysis of the available toxicity data for heavy metals and ciliates. The objectives of this study were to provide an overview on the sensitivity of ciliates to heavy metals, and to test the capacity of standard test species used in ecotoxicology to protect ciliate species assemblages. For this, we first comparatively assessed the sensitivity of ciliates to different heavy metals. Then, we evaluated the sensitivity of ciliates as regards to that of the standard test species commonly used in ecotoxicological risk assessments (*i.e*., algae, *Daphnia*, fish). And, finally, we compared the sensitivity of the most commonly tested ciliate species with that of the other, less investigated, ciliate taxa. Furthermore, in this study we discuss the need to include ciliates in future heavy metal risk assessments, and provide recommendations for the selection of model organisms and the development of standard toxicity test methods.

2. Materials and methods

2.1. Toxicity data mining

We carried out an extensive search for studies that assessed the toxicity of heavy metals to ciliates. Toxicity data were primarily retrieved from the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/index.cfm; 24th May 2019). Furthermore, additional studies were searched in Scopus, Web of Science, PubMed and Google Scholar databases using the code: *ecotoxicolog** OR "*heavy metal**" AND *ciliate*. Additionally, we also searched for references cited by these articles to ensure a greater volume of studies and to identify additional information related to the topic. Only data for the most frequently tested (≥ 5 %) heavy metals (*i.e.*, Zinc, Lead, Mercury, Copper, Chromium, Cadmium) were considered for the analysis in order to provide a robust sensitivity comparison. In this way, Nickel and Cobalt were not included in our analysis due to the small number of studies investigating them. In our study, we focused on LC_{50} or EC_{50} (growth) values for freshwater ciliate taxa obtained under laboratory conditions after an exposure period of 24 h. The toxicity values were converted to a standard unit (mg L^{-1}) for the analysis.

Toxicity data for standard test species of algae, invertebrates and fish commonly used in ecotoxicological risk assessments were also retrieved from the US EPA ECOTOX database (26th Sep 2019). The scientific name of the standard test species, the exposure duration and the endpoints selected for each were: *Raphidocelis subcapitata* (EC_{50} 72 h

– 96 h, growth inhibition), *Daphnia magna* (EC_{50} 48 h, immobility) and *Oncorhynchus mykiss* (LC_{50} 96 h, mortality).

2.2. Relative sensitivity of ciliates to the different heavy metals

To compare the relative sensitivity of ciliates to the different heavy metals included in this study, we calculated the standardized sensitivity z–score according to the following equation:

$$z = \frac{x-\mu}{\sigma} \qquad \text{(Equation 1)}$$

where x is the raw lethal concentration data described in the studies with ciliates, μ is the overall mean of all data per heavy metal, and σ is the standard deviation of all data for all heavy metals included in this study.

The z–score is a widely used meta–analitical approach to assess differences in the effect sizes in ecological and toxicological studies (Adams et al., 1997; Gurevitch and Hedges, 1999; Melvin and Wilson, 2013; Melvin and Leusch, 2016). Calculations were performed in MS Excel by firstly calculating the standardized and partial z–score values, standard deviation, confidence interval, overall effect size, and later calculating the sum of the squared deviations. Figures were performed in SigmaPlot (version 12.0).

2.3. Sensitivity comparison with standard test species

The sensitivity of ciliates to heavy metals was compared with that of the standard test species by using the Relative Tolerance (Trel) approach. Trel values were calculated by diving the toxicity values of the ciliate species by the toxicity values of the standard test species using Microsoft Excel. A Trel of one indicates a tolerance equal to that of the standard test species. A Trel value lower than one indicate that the ciliate species is more sensitive than the standard test species, while a Trel value larger than one indicates that the ciliate species is more tolerant than the standard test species to the evaluated heavy metal. Prior to the Trel calculation, the geometric mean was calculated when there were more than one toxicity value available for each combination of heavy metal and species, both for ciliates toxicity data and for the standard test species. The Trel values were used to calculate cumulative distribution functions, so that the relative percentage of ciliate taxa with Trel above or below one can be easily visualized.

2.4. Sensitivity comparisons across ciliates taxa

Species Sensitivity Distributions (SSDs) were constructed for ciliates in order to visualize the relative sensitivity of *Paramecium caudatum* and *Tetrahymena thermophila* as regards to that of the other tested ciliate species. The SSDs were calculated on the basis of log-normal distributions by using the ETX computer program version 2.2 (Van Vlaardingen et al., 2004). Log–normality of the fitted distributions was evaluated by using the Kolmogorov–Smirnov test, using a significance level of 0.05.

3. Results

3.1. Data availability

Our article search yielded a total of 27,328 studies: 47 from the ECOTOX database, 184 from Scopus, 21,878 from Web of Science, 87 from PubMed, 5,090 from Google Scholar, and 6 additional ones taken from references cited in these studies. After screening all studies, only 66 provided toxicity data that fulfilled the inclusion criteria and were selected for the meta–analysis (Figure 1). Cadmium (22%), Copper (26%), Zinc (19%), Chromium (12%), Lead (12%) and Mercury (9%) were the most frequently tested heavy metals in the included studies.

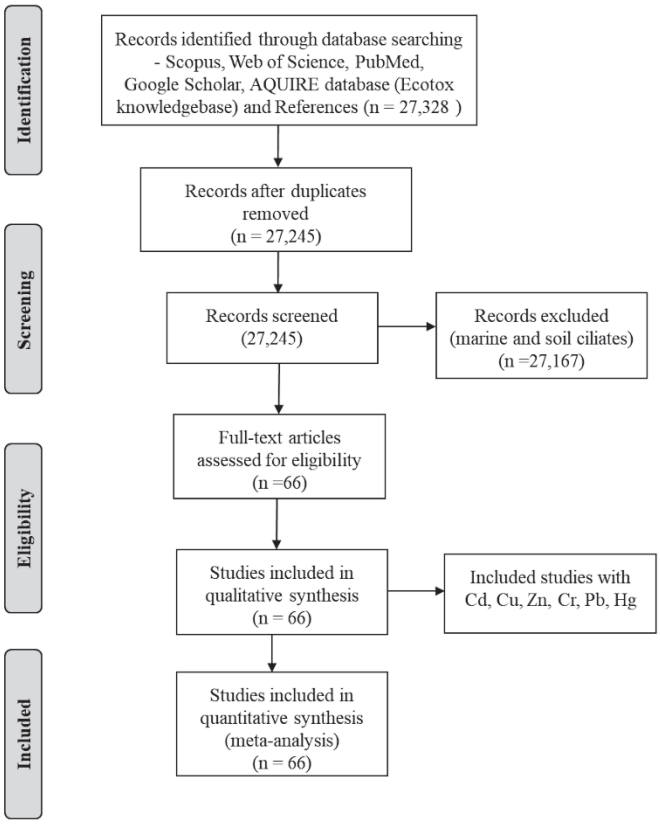


Fig. 1. Flow diagram of the literature searched in Scopus, Google Scholar, ACQUIRE Database and references within articles found and included in our study. Figure adapted from PRISMA (Moher et al., 2009); Cd: Cadmium, Cu: Copper, Zn: Zinc, Cr: Chromium, Pb: Lead and Hg: Mercury.

The selected studies provided 427 toxicity values for 46 species of ciliates (see e.g. Figure 2), belonging to 8 classes (Appendix A. Table S1). The number of studies found per species ranged from 1 to 41 (Figure 3). Our study showed that *P. caudatum* was the most tested species, followed by *Tetrahymena thermophila*, *Colpidium campylum* and *Spirostomum ambiguum* (Figure 3). The number of studies per ciliate species for each of the selected heavy metals are presented in Figure 4.

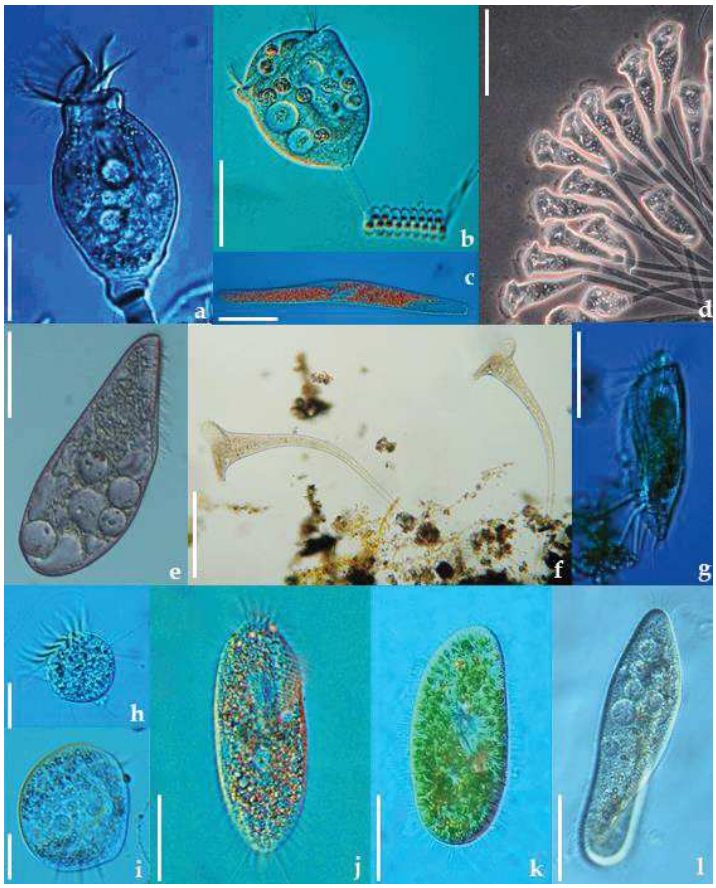


Fig. 2. Ciliates used in ecotoxicological studies. a. *Opercularia*. b. *Vorticella*. c. *Spirostomum*. d. *Epistylis*. e. *Blepharisma*. f. *Stentor.* g. *Euplotes.* h. *Halteria.* i. *Aspidisca.* j. *Tetmemena.* k. *Paramecium bursaria*. l. *Paramecium caudatum*. Bars: a-e, g, j–l: 50 µm; f: 100 µm; h: 30 µm; i: 20 µm.

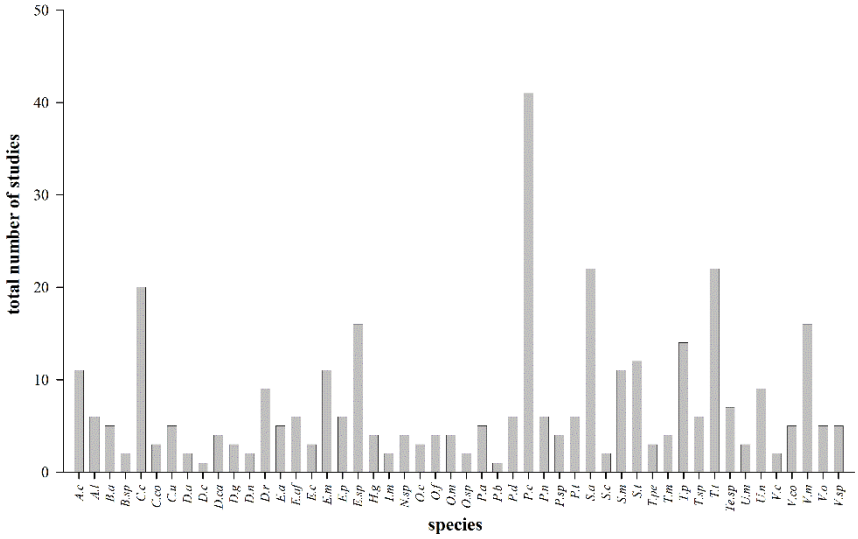


Fig. 3. Total number of L(E)C_{50} values per ciliate species. Species abreviations are: *A.c: Aspidisca cicada, A.l: Aspidisca lynceus, B.a: Blepharisma americanum, B.sp: Blepharisma* sp.*, C.u: Chilodonella uncinata, C.e:Cyrtolophosis elongata, C.c: Colpidium campylum, C.co:Colpidium colpoda, C.cu: Chinodella uncinada, D.a:*

Diophrys appendiculata, D.ca: Dexiostoma campyla, D.c: Dexiostoma campylum, D.gr: Dexiostoma granulosa, D.g:Dexiotricha granulosa, D.n: Didinium nastum, D.r: Drepanomonas revoluta, E.a: Euplotes aediculatus, E.af: Euplotes affinis, E.c: Euplotes crassus, E.m: Euplotes mutabilis, E.p: Euplotes patella, E.sp: Euplotes sp.*, H.g: Halteria grandinella, I.m: Ichthyophthirius multifilis, N.sp: Notohymena* sp.*, O.c: Opercularia coarctata, O.f:Oxytricha fallax, O.m: Opercularia minima, O.sp: Opercularia* sp.*, P.a: Paramecium aurelia, P.b: Paramecium bursaria, P.c:Paramecium caudatum, P.d: Paramecium dubosqui, P.n: Paramecium nephridiatum, P.sp: Pseudourostyla* sp.*, P.t: Paramecium trichium, S.a: Spirostomum ambiguum, S.c: Stentor coeruleus, , S.m: Stylonychis mytilus, S.t: Spirostomum teres, T.p: Tachysoma pellionella, T.m: Trochilia minuta, T.py: Tetrahymena pyriformis, T.sp: Tetmemena* sp.*, T.t: Tetrahymena thermophila, Te.sp: Tetrahymena* sp.*, U.m: Uronema marinum, U.n: Uronema nigricans, V.c: Vorticella campanula, V.c: Vorticella convallaria, V.m: Vorticella microstoma, V.o: Vorticella octava, V.sp: Vorticella* sp.

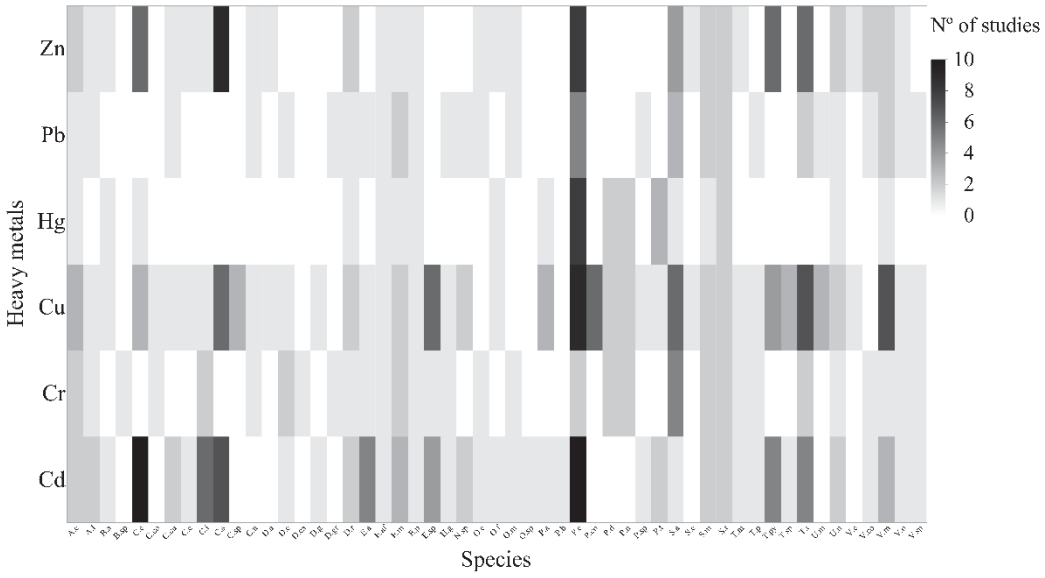


Fig. 4. Total number of L(E)C50 values per ciliate species and heavy metal (1956–2017). Zn=Zinc; Pb=Lead; Hg=Mercury; Cu=Copper; Cr=Chromium; Cd= Cadmium. Species abreviations are the same as in Fig. 3.

3.2. Relative sensitivity of ciliates to different heavy metals

The overall effect size for all heavy metals was 4.31 (confidence interval = 2.29–6.34) (Figure 5). Overall, our study shows that ciliates are, in general, more sensitive to Mercury>Cadmium>Copper>Zinc>Lead>Chromium. Copper, Lead, Chromium, and to a lower extent Zn, showed large confidence intervals, indicating a high variability in the available toxicity data. The L(E)C_{50} values for *P. caudatum,* the most commonly

evaluated species, varied greatly within and across metals, with lower toxicity values found for Copper, Mercury and Cadmium (Appendix A. Table S2).

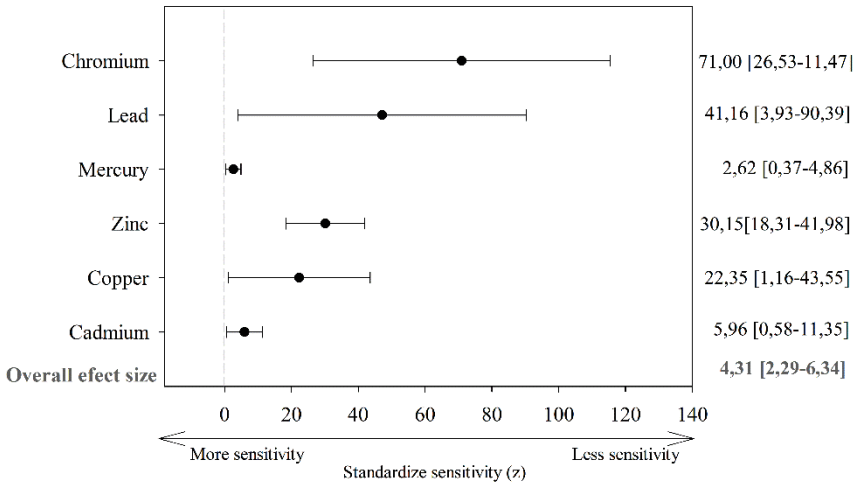


Fig. 5. Average and confidence interval of the L(E)C_{50} z–score for ciliate species in the ecotoxicological tests performed with heavy metals. The overall effect size unit–less was included for a representation purpose. The number of samples L(E)C50 values per heavy metal was: Chromium = 47, Lead = 46, Mercury = 35, Zinc = 81, Copper = 115, Cadmium = 103, Overall = 427.

3.3. Sensitivity comparison with standard test species

Raphidocelis subcapitata was in general more sensitive than the majority of ciliate species, particularly for Zinc and Cadmium (Figure 6). Ciliate species were considerably more sensitive than *Daphnia magna* to Lead, and overly more tolerant to Cadmium, Copper, Mercury and Chromium. For Zinc, *D. magna* was at the center of the sensitivity distribution (Figure 6). Trel values for *Onchornyncus mykiss* were in general higher than one, indicating a higher tolerance of ciliates to heavy metals than the fish standard test species, except for Chromium and Mercury. For these two metals *O. mykiss* was also placed in the midle of the sensitivity distribution (Figure 6).

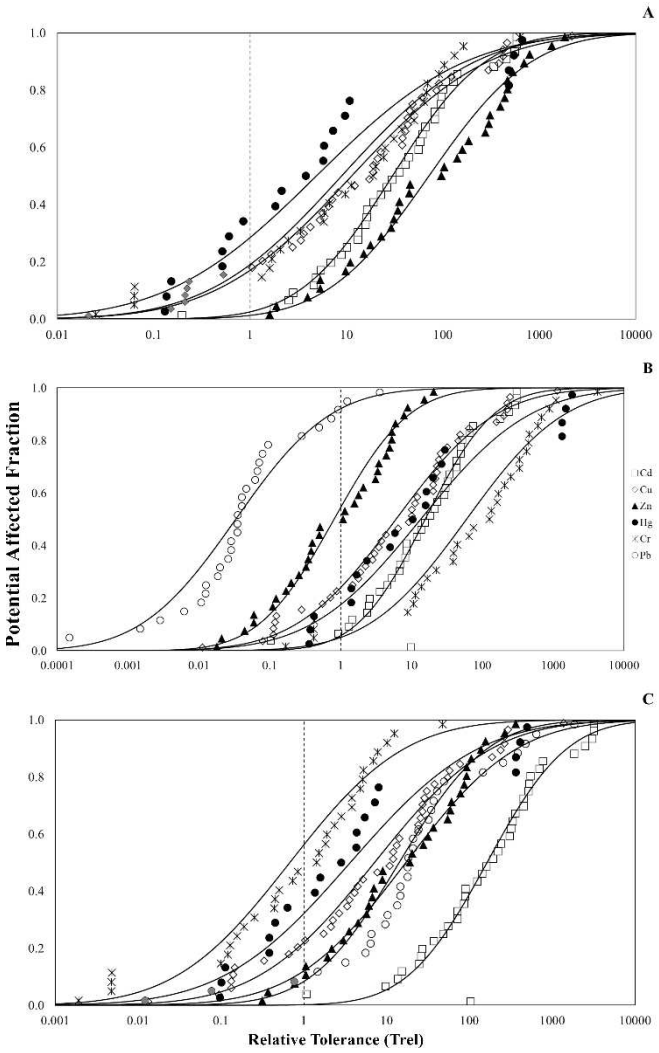


Fig. 6. Cumulative distribution function of calculated Trel values for A=*Raphidocelis subcapitata*, B=*Daphnia magna* and C=*Oncorhynchus mykiss*. Dashed line (Trel = 1) indicates the sensitivity of the standard test species. Trel<1 and Trel>1 indicate a greater and lower sensitivity of the ciliate species as regards to the standard test species, respectively. Trel values for Lead and *R. subcapitata* are not available due to the absence of a toxicity value for that species.

Table 1 shows the quantity of Trel values that are higher or lower than one, and the number of Trel values that are below or above one or more orders of magnitude as regards to the three standard test species (Appendix A. Table S3). Table 1 also indicates the standard test species that shows the lowest toxicity value and that will, therefore, trigger the development of metals threshold concentrations (*i.e.*, *R. subcapitata* for Copper and Zinc, *D. magna* for Mercury and Chrormium, and *O. mykiss* for Cadmium and Lead). The results of this evaluation show that for these metals and standard test species combinations, the quantity of ciliate species below the Trel value of one is generally low. Furthermore, the application of an assessment factor of 10 (corresponding to a Trel of 0.1), which is usually applied to account for interspecies sensitivity differences, will result in a sufficient protection level for most ciliate taxa. The exceptions

are Copper and Lead, for which the application of an assessment factor of 10 to the toxicity value of the most sensitive standard test species will leave 1 and 2 of the ciliate species unprotected, respectively. The species that are not protected are *Blepharisma americanum* (Copper), *Dexiostoma campylum* (Lead) and *Aspidisca cicada* (Lead).

Table 1: Number of Trel values within each specific range. Trel values were calculated by dividing the toxicity value of each ciliate species by the toxicity value of *Raphidocelis subcapitata*, *Daphnia magna* or *Oncorhynchus mykiss* (Appendix A. Table S3). Trel < 1 indicate a higher sensitivity of the ciliate as regards to that of the standard test species, and Trel > 1 indicate a lower sensitivity. Bold numbers indicate the most sensitive standard test species for each heavy metal. Trel values for lead and *R. subcapitata* are not available due to the absence of a toxicity value for that species. The number of samples (L(E)C_{50} values) per metal was: Cadmium = 38, Copper = 42, Zinc = 33, Mercury = 19, Lead = 30 and Chromium = 31.

R. subcapitata								
Heavy metals	<0.01	0.01–0.1	0.1–1	1–10	10–100	>100	<1	>1
Cadmium	0	0	1	8	21	8	1	37
Copper	**0**	**1**	**0**	**12**	**16**	**7**	**1**	**35**
Zinc	**0**	**0**	**0**	**6**	**11**	**16**	**0**	**33**
Mercury	0	0	7	7	1	4	7	12
Lead	–	–	–	–	–	–	–	–
Chromium	0	4	0	10	13	4	4	27

D. magna								
Heavy metals	<0.01	0.01–0.1	0.1–1	1–10	10–100	>100	<1	>1
Cadmium	0	0	0	14	17	5	0	36
Copper	0	2	8	11	15	6	10	32
Zinc	0	5	11	15	3	0	16	17
Mercury	**0**	**0**	**3**	**6**	**6**	**4**	**3**	**16**
Lead	5	19	4	2	0	0	28	2
Chromium	**0**	**0**	**4**	**1**	**10**	**16**	**4**	**27**

O. mykiss								
Heavy metals	<0.01	0.01–0.1	0.1–1	1–10	10–100	>100	<1	>1
Cadmium	**0**	**0**	**0**	**2**	**13**	**23**	**0**	**38**
Copper	0	2	7	11	16	6	9	33
Zinc	0	0	6	8	12	5	6	25
Mercury	0	1	6	6	0	4	7	12
Lead	**0**	**2**	**1**	**6**	**15**	**6**	**3**	**27**
Chromium	4	0	11	13	3	0	15	16

Note: –: no data for Lead.

3.4. Sensitivity comparisons across ciliate taxa

Our study shows that *P. caudatum* was more sensitive to Cu > Hg > Cd > Cr > Zn > Pb and, *T. thermophila* was more sensivitve to Cd > Cu > Pb > Zn > Cr (based on the geometric mean of the available toxicity values). The relative sensitivity of *P. caudatum* and *T. thermophila* as regards to the other ciliate species can be observed in the SSDs shown in Figure 7. *P. caudatum* was found to be positioned in the lower tail of the SSD for Cadmium, Copper and Chromium, and showed a midle-to-high tolerance for Zinc, Mercury and Chromium as compared to other ciliates. *T. thermophila* was positioned in the lower SSD tail for Cadmim and Lead, and was found to be relatively tolerant to Copper, Zinc, and Chromium (note that there was not toxicity data available for this species and Mercury). The results of this analysis show that, altough these two species are relatively sensitive for some compounds, none of them is systematically positioned in the lower tail of all SSDs, indicating that both species may be important for the risk assessment of metals to ciliates. Moreover, the application of an interspecies assessment factor of 10 to the most sensitive of the two, will result in a protection level of 7%, 26%, 78%, 43%, 67%, 84% of the species for Cadmium, Copper, Zinc, Mercury, Lead and Chromium, respectively.

Figure 7 also displays the relative position of the different ciliate classes within the SSD. It shows that the Classes Oligohymenophorea and Spirotrichea were distributed all along the SSD curve (Figure 7), demonstrating variable sensitivity. In general, the class Heterotrichia showed a higher sensitivity to Copper, Mercury and Zinc and high–to–median tolerance to Cadmium, Lead and Chromium. The class Phyllopharyngea was found to be highly sensitive to Zinc, and Nassophorea to Chromium, although the amount of toxicity data for these classes is limited.

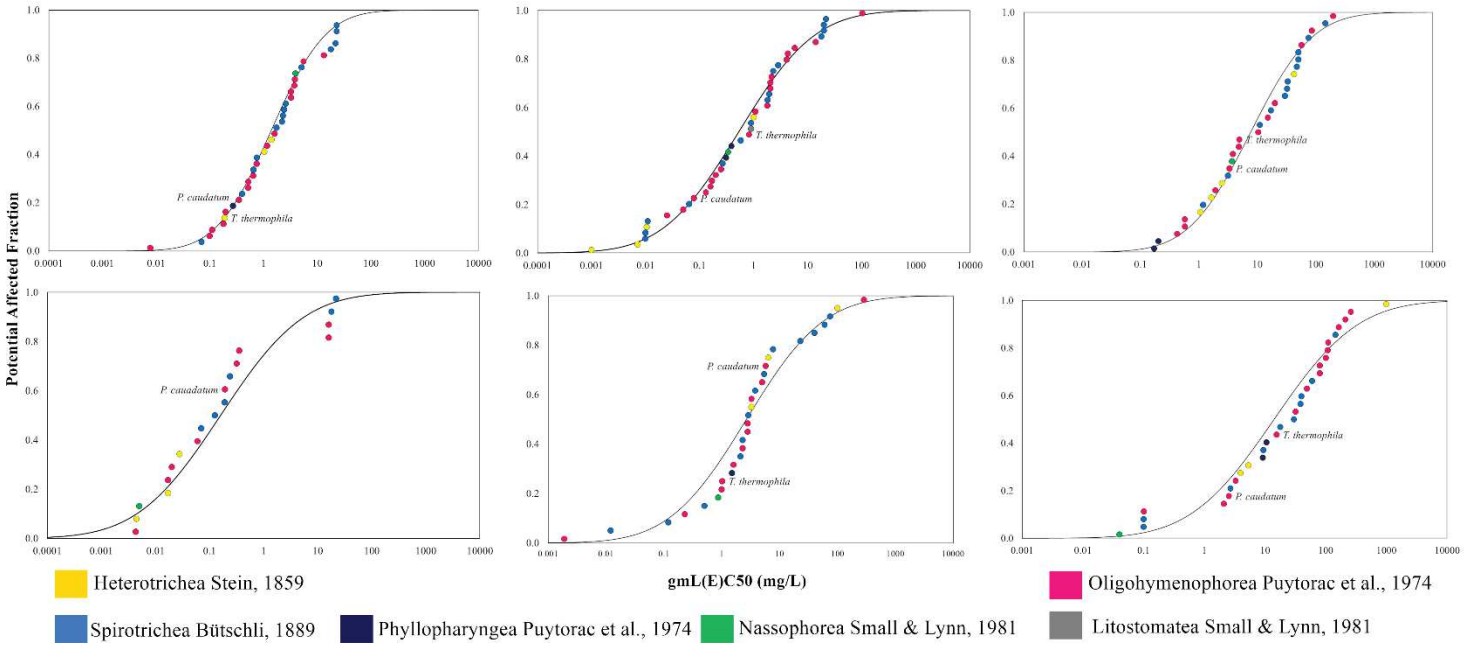


Fig. 7. Species Sensitivity Distributions (SSDs) for heavy metals and ciliates indicating the relative sensitivity of *Paramecium caudatum* and *Tetrahymena thermophila* as regards to the other ciliate taxa. All SSDs passed the Kolmogorov–Smirnov test.

4. Discussion

4.1. Ciliates sensitivity to heavy metals

In general, Chromium, Lead, Zinc, Copper were overly less toxic to ciliates but showed large confidence intervals, which indicates that there is a large variability in the toxicity data for these metals. Such large toxicity data variation may partly be explained by the diversity of experimental conditions and test methods used in the different studies (Appendix A. Table S2). For example, axenic cultures based on protease peptone culture medium (Carter and Cameron, 1973; Gallego et al., 2007; Zhang et al., 2013; Liu et al., 2017), as proposed by Sonneborn (1970), have been extensively used in toxicity tests; but also mineral water and bark rice (Ruthven and Cairns, 1973; Dive et al., 1989; Madoni et al., 1992, 1994; Madoni, 2000; Madoni and Romeo, 2006; Wanick et al., 2008; Bitencourt et al., 2016), and natural waters (Nalecz–Jawecki et al., 1993; Madoni et al., 1996; Rehman et al., 2005, 2006, 2007, 2007a, 2008, 2008a, 2009, 2010, 2010a). When exposed to heavy metals in grain rice medium and boiled wheat (considered as the closest to the natural condition as possible; see Appendix A. Table S2), L(E)C_{50} values ranged from 0.001 mg L^{-1} to 211 mg $L^{-1}$for all metals. It is important to mention that the studies that show lower L(E)C_{50} variation across species and metals were all conducted within the

same laboratory. This suggests that inter-laboratory variability may be large, supporting the need to establish standardized test conditions.

Mercury and Cadmium were found to be the most toxic heavy metals and showed smaller confidence intervals. Mercury is a highly toxic element in the environment and has large potential to be transported through trophic chains (Ehrlich and Newman, 2008). The Mercury uptake by ciliates situated at the bottom of aquatic food chains can be a gateway for Mercury accumulation in upper trophic levels, and therefore deserves further attention (Lin et al., 2013). Cadmium is commonly found in aquatic ecosystems (Filho et al., 2000). The effect of Cadmium and the other heavy metals in freshwater organisms depends on their form (i.e., ion, organic and inorganic complexes, or insoluble complexes with hydroxide or sulfide ions), which is influenced by environmental conditions such as pH, alkalinity, hardness, and organic carbon content (USEPA, 1980; Suedel et al., 1997). Cadmium can cause mitochondrial degeneration and cristae disintegration in ciliates, both leading to serious modifications in vegetative cells (Iftode et al., 1985).

The sensitivity comparison of ciliates with *R. subcapitata*, *D. magna* and *O.mykiss* shows that ciliates are overly more tolerant than the standard test species commonly used in ecotoxicological risk assessments. Also, this study shows that, in general, the application of an assessment factor of 10 to the most sensitive of the three standard test species can result in a sufficient protection level for most ciliates. This suggests that current heavy metal threshold values and common prospective risk assessments, although disregard microorganisms in the data evaluation, are in principle protecting ciliates. The species that were found to be highly sensitive to heavy metals and that are apparently not protected by current threshold concentrations are *Blepharisma americanum*, *Dexiostoma campylum* and *Aspidisca cicada*. For these species, further toxicity testing is recommended.

Our study shows that the application of an assessment factor of 10 to the lowest toxicity value of the two most tested ciliate species (*P. caudatum* and *T. thermophila*) leaves a large number of ciliate taxa unprotected, particularly for Zinc, Lead and Chromium. This suggests that further toxicity studies should consider additional sensitive species; and that the development of heavy metal threshold concentrations for ciliates on the basis of these two common test species requires the application of a larger assessment factor.

4.2. Ciliates diversity and their use as toxicity models

The Phylum Ciliophora includes a wide diversity of species, ~ 8,000 (Gao et al., 2016). These species are ubiquitous in different types of environments, including marine, fresh and brackish waters, and edaphic systems such as soils, mosses, and lichens (Puytorac et al., 1994; Foissner et al., 2002; Foissner, 2003; Lynn, 2008). This versatility allows species of ciliates of distinct habitats to express different levels of sensitivity to heavy metal exposure (Gutiérrez et al., 2003, 2015).

According to the literature (Noland, 1925; Sleigh, 1988), the presence of ciliates in different habitats is influenced by their capacity to adapt to the different physical and chemical conditions. They can form resistance cysts under unfavorable conditions, being dispersed and transported by wind, water, and insects or other animals (Finlay et al., 1996; Finlay, 2002; Esteban and Finlay, 2003; Fenchel and Finlay, 2004). The encystment ability of ciliates is significant in their life history, being an important trait to survive adverse environmental conditions, including pulsed contaminant exposure (Weisse, 2017). Environmental conditions of the water (*e.g.,* temperature, amount of dissolved organic matter, pH, conductivity, and oxygen concentration) may be linked to the specificity of ciliates and recurrent changes in community structure (Noland, 1925; Kudo, 1966; Sleigh, 1988; Madoni, 2005; Madoni and Braghiroli, 2007). Furthermore, other factors such as seasonal, vertical, trophic and flood availability may also be determining the patterns of distribution, richness and, abundance of ciliates in aquatic systems (Velho et al., 2005, 2013; Pauleto et al., 2009; Segovia et al., 2016).

The aforementioned ecological elements can be an explanation to the variability in data referring to ciliates´ chemical sensitivity. The unavailability of a standardized protocol, with particular grow medium and environmental conditions, is the main factor influencing the L(E)C_{50} data variability. However, other factors that may explain the intrinsic species variability in sensitivity are the diverse ciliate ecological origin, morphology, behaviour and ecological niche.

P. caudatum and *T. thermophila* are the species that are most commonly used in toxicity tests performed with heavy metals (Grebecki et al., 1956; Nush, 1982; Madoni et al., 1992,1994; Miranda and Martins, 2013; Gong et al., 2014) and other chemical compounds (Miyoshi et al., 2003; Rao et al., 2006; Alves, 2010; Amanchie and Hussain, 2010; Mansano et al., 2016). Both species have a large ammount of published information regarding their biology, morphology, and behavior as compared to other ciliate species

(Rao et al., 2006; Gerhardt et al., 2010), and have a widespread geographic distribution (Sauvant et al., 1999). *P. caudatum* is mostly found in freshwater environments, in the hypolimnion or associated to macrophytes *(e.g.*, lentic and lotic environments, but also sewage–treatment facilities). *P. caudatum* feeds on bacteria and have high tolerance to the presence of organic matter and, therefore, high saprobic index (Foissner and Berger, 1996; Miyoshi et al., 2003; Gerhardt et al., 2010). Moreover, as demonstrated by several studies, they grow well under *in vitro* conditions (Rao et al., 2006; Alves et al., 2016). As shown in this study, *P. caudatum,* but also *T. thermophila,* are sensitive to some heavy metals. However, none of them is the most stensitive species for all heavy metals, neither rank in the lowest sensitivity range for all metals, indicating that their suitability as model test organisms for heavy metal contamination may be questionable. Further research should also focus in the selection of other potential candidate model organisms. Among the potential candidates, we propose *Blepharisma americanum*, *Dexiostoma campylum* and *Aspidisca cicada* as these were the most sensitive species for at least two of the six metals analyzed.

4.3. Future perspectives

Ciliates are unicellular eukaryotes and have been used as whole–cell ecotoxicological models (Puytorac, 1994; Gutiérrez et al., 2003). Due to their high growth rates, they are able to double their individual numbers in a population in a short time span (Fenchel and Finlay, 1983). Ciliates reproduce both asexually and sexually, through binary fission or conjugation, respectively (Grell, 1973; Corliss, 1979). They do not have cell wall. This allows an easy uptake of chemicals resulting in modifications in the membrane structure, inhibition of the organization of cytoskeletons components, changes in the morphological integrity of organisms, and movement alterations (Olabarrieta et al., 2001; Pudpong and Chantangsi, 2015). These characteristics allow them to easily reflect changes due to exposure to heavy metals and other compounds in the field. Furthermore, there is a growing ciliate molecular database at GenBank (Banson et al., 2013), which can provide advancement for ecotoxicological studies by allowing the evaluation of genetic responses to toxicity in these organisms. Molecular tools may be applied to understand the mechanism of action of pollutants, to measure gene expression, and to develop indicators of toxic pressure and cellular damage, also helping to understand adaptation to changes in the external environment (Hamadeh et al., 2002; Calzolai et al., 2007).

Moreover, the advent of "omics" allow studies on genetic diversity in temporal and spatial scales across populations of ciliates, contributing to several interpretations regarding gene flow and the origin of ciliate populations (Tarcz et al., 2012; Zhao et al., 2013; Lu et al., 2019). The genome of some ciliates species (*T. thermophila, Paramecium tetraurelia, Euplotes crassus*, *Oxytricha trifallax* and *Stentor coeruleus*), has already been sequenced (Aury et al., 2006; Eisen et al., 2006; Vinogradov et al., 2012; Swart et al., 2013; Slabodnick et al., 2017). These species share a greater degree of functional conservation with human genes than with other eukaryotic models, and therefore are also suitable for human toxicological studies. Pan et al. (2018) described the sequencing of transcriptome and microRNAomic of *Euplotes vannus* exposed to nanoparticles, evidencing high expression of various antioxidant genes to prevent oxidative stress, and studying impacts over the TCA (tricarboxylic acid cycle) cycle and celular repair mechanisms. We expect that the application of these novel methodologies with ciliates will allow the development of ecotoxicological assays for heavy metals and other contaminants.

5. Conclusions

In this study we comparatively assessed the sensitivity of freshwater ciliates to six metals, their relative sensitivity as compared to the standard test species commonly used in ecotoxicology (*Raphidocelis subcapitata, Daphnia magna and Onchornyncus mykiss*), and their inter–taxa sensitivity differences. The study shows that ciliates are more sensitive to Mercury, Cadmium and Copper, as compared to Chromium, Zinc and Lead. The sensitivity comparison with standad test species used in ecotoxicology shows that ciliates are generally less sensitive to heavy metal contamination. Also, this study suggest that threshold concentrations based on standard test species are expected to result in a sufficient protection level for the vast majority of ciliate taxa. Finally, this study shows that some ciliate classes show a higher sensitivity to some metals (Phyllopharyngea and Nassophorea), but the ciliate taxa that have been most commonly tested (*P. caudatum* and *T. thermophila*) are not necessarily within the most sensitive taxa. These findings, and the great variability in the avilable toxicity data observed in this study, support the need for further studies aimed at testing the sensitivity of other ciliate taxa and the development of standard toxicity test protocols.

CRediT authorship contribution statement

Jéssica Andrade Vilas–Boas: Conceptualization, Methodology,Validation, Formal analysis, Data curation, Writing - original draft. **Simone Jaqueline Cardoso:** Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Marcus Vinicius Xavier Senra:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision. **Andreu Rico:** Writing - original draft, Writing - review & editing, Supervision. **Roberto Júnio Pedroso Dias:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

This study was sponsored by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) (fellowship to JAVB and MVXS), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG, CRA 03648/16) and, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (fellowships to RJPD, Bolsa de Produtividade). A. Rico is supported by a postdoctoral grant provided by the Spanish Ministry of Science, Innovation and University (IJCI–2017–33465). We are very grateful José Reinaldo Paranaíba for his help with grid plot graph.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.110669.

References

Adams, D.C., Gurevitch, J., Rosenberg, M.S., 1997. Resampling tests for meta–analysis of ecological data. Ecology. 78, 1277–1283. https://doi.org/10.2307/2265879.

Alves, H.C., 2010. Estudo biológico de linhagens do protozoário ciliado *Paramecium caudatum* Ehrenberg, 1833 e avaliação experimental do efeito tóxico do agrotóxico Fipronil. Dissertation, Universidade Federal de São Carlos.

Amanchi, R.N., Hussain, M.M., 2010. Cytotoxicity assessment of monocrotophos in *Paramecium caudatum* and *Oxytricha fallax.* J. Environ. Biol. 31, 603–607.

Aury, J. M., Jaillon, O., Duret, L., Noel, B., Jubin, C., Porcel, B.M., Ségurens, B., Daubin, V., Anthouard, V., Aiach, N., Arnaiz, O., Billaut, A., Beisson, J., Blanc, I., Bouhouche, K., Câmara, F., Duharcourt, S., Guigo, R., Gogendeau, D., Katinka, M., Keller, A.M., Kissmehl, R., Klotz, C., Koll, F., Le, Mouël, A., Lepère, G., Malinsky, S., Nowacki, M., Nowak, J.K., Plattner, H., Poulain, J., Ruiz, F., Serrano, V., Zagulski, M., Dessen, P., Bétermier, M., Weissenbach, J., Scarpelli, C., Schächter, V., Sperling, L., Meyer, E., Cohen, J., Wincker, P., 2006. Global trends of whole–genome duplications revealed by the ciliate *Paramecium tetraurelia*. Nature 9, 171–178. https://doi.org/10.1038/nature05230.

Arenas-Sánchez, A., Rico, A., Rivas-Tabares, D., Blanco, A., Garcia-Doncel, P., Romero-Salas, A., Nozal, L., Vighi, M., 2019. Identification of contaminants of concern in the upper Tagus river basin (central Spain). Part 2: Spatio-temporal analysis and ecological risk assessment. Sci. Total Environ. 667, pp.222-233. https://doi.org/10.1016/j.scitotenv.2019.02.286.

Belangera, S.E., Carr., G.J., 2019. SSDs Revisited: Part II—Practical Considerations in the Development and Use of Application Factors Applied to Species Sensitivity Distributions. Environ. Toxicol. Chem. 38, 1526–1541. https://doi.org/10.1002/etc.4444.

Belkin, S., 2003. Microbial whole–cell sensing systems of environmental pollutants. Curr. Opin. Microbiol. 4, 939–952. https://doi.org/10.1016/S1369–5274(03)00059–6.

Bitencourt, J.A.P., Pereira, D.C., Silva–Neto, I.D., Crapes, M.C.C., Neto, J.A.B., 2016. Evaluation of sensitivity to zinc and copper of *Diophrys appendiculata* (Protozoa, Ciliophora) and their associated bacteria, both isolated from a tropical polluted bay. Rev. Bras. Zoo. 17, 112–123.

Bolan, N.S., Duraisamy, V.P., 2003. Role of inorganic and organic soil amendments on immobilisation and phytoavailability of heavy metals: A review involving specific case studies. Soil Res. 41, 533–555. https://doi.org/10.1071/SR02122.

Cairns, Jr., McCormick, P.V., Niederlehner, B.R., 1993. A proposal framework for developing indicators of ecosystem health. Hydrobiologia 263, 1–44.
Calzolai, L., Ansorge, W., Calabrese, E., Denslow, N., Part, P., Lettieri, T., 2007. Transcriptomics and proteomics. Applications to ecotoxicology. Comp. Biochem. Physiol. 2, 245–249. https://doi.org/10.1016/j.cbd.2007.04.007.

Carter, J.W., Cameron, I.L., 1973.Toxicity bioassay of heavy metals in water using *Tetrahymena pyriformis*. Water Res. 7, 951–961. https://doi.org/10.1016/0043–1354(73)90177–2.

Corliss, J.O., 1979. The ciliated protozoa. Pergamon Press, London, pp. 455.

Delmonte Conrrado, M.U., Amaroli, A., Trielli, F., Falugi, C., 2006. Cholinesterase enzyme activity in protists and environmental biomonitoring. Curr. Trends Microbiol. 2, 123–136.

Díaz–de Alba, M., Galindo–Rianõ, M.D., Casanueva–Marenco, M.J., Garcia–Vargas., M., Kosore, C.M., 2011. Assessment of the metal pollution, potential toxicity and speciation of sediment from Algeciras Bay (South of Spain) using chemometric tools. J. Hazard. Mater. 190, 177–187. https://doi.org/10.1016/j.jhazmat.2011.03.020.

Dive, D., Leclerc, H., Persoone, G., 1980. Pesticide Toxicity on the Ciliate Protozoan *Colpidium campylum*: Possible Consequences of the Effect of Pesticides in the Aquatic Environment. Ecotox. Environ. Safe. 4, 129–33. https://doi.org/10.1016/0147–6513(80)90014–7.

Ehrlich, H.L., Newman, D.K., 2008. Geomicrobiology of mercury, *in:* Geomicrobiology, (Eds), C.R.C Press, Boca Raton, F.L. pp.65–278.

Eisen, J.A., Coyne, R.S., Wu, M., Wu, D., Thiaragarajan, M., Wortman, J.R., Badger, J.H., Ren, Q., Amedeo, P., Jones, K.M., Tallon, L.J., Delcher, A.L., Salzberg, S.L., Silva, J.C., Haas, B.J., Majoros, W.H., Farzad, M., Carlton, J.M., Smith, R.K. Jr., Garg, J., Pearlman, R.E., Karrer, K.M., Sun, L., Manning, G., Elde, N.C., Turkewitz, A.P., Asai, D.J., Wilkes, D.E., Wang, Y., Cai, H., Collins, K., Stewart, B.A., Lee, S.R., Wilamowska, K., Weinberg, Z., Ruzzo, W.L., Wloga, D., Gaertig, J., Frankel, J., Tsao, C.C., Gorovsky, M.A., Keeling, P.J., Waller, R.F., Patron, N.J., Cherry, J.M., Stover, N.A., Krieger, C.J., del, Toro, C., Ryder, H.F., Williamson, S.C., Barbeau, R.A., Hamilton, E.P., Orias, E., 2006. Macronuclear genome sequence of the ciliate *Tetrahymena thermophila*, a model eukaryote. Plos Biol. 4, e286. http://doi.org/10.1371/journal.pbio.0040286.

European Commission (2003) Common implementation strategy for the Water Framework Directive (2000/60/EC). Guidance Document Nº. 8. Public Participation in Relation to the Water Framework Directive. Office for Official Publications of the European Communities, Luxembourg.

Fenchel, T., Finaly, B.J., 1983. Respiration rates in heterotrophic, free–living protozoa. Microb. Ecol. 9, 99–122. http://doi.org/10.1007/BF02015125.

Fenchel, T., 1987. The Ecology of the Protozoa. in: The Biology of Free–living Phagotrophic Protists. Springer Verlag: Berlin and Tokyo, pp.197.

Filho, J.L.V.B.N., Novelli, E.L.B., Manzano, M.A., Lopes, A.M., Cataneo, A.C., Barbosa, L.L., Ribas, B.O., 2000. Effect of alpha–tocopherol on superoxide radical and toxicity of cadmium exposure. Int. J. Environ. Health R. 10, 125–134. https://doi.org/10.1080/09603120050021128.

Foissner, W., 2003. Morphology and ontogenesis of *Bromeliophyra brasiliensis* gen. n., sp. n., a new tetrahymenid ciliate (Protozoa: Ciliophora) from Brazilian tank bromeliads (Bromeliaceae). Acta Protozool. 39, 63–82. http://doi.org/0932–4739/03/39/04–365 $ 15.00/0.

Foissner, W., Agatha, S., Berger, H., 2002. Soil ciliates (Protozoa,Ciliophora) from Namibia (Southwest Africa), with emphasis on two contrasting environments, the Etosha Region and the Namib Desert. Denisia 5, 1–1459.

Foissner, W., Berger, H., 1996. A user–friendly guide to ciliates (Protozoa, Ciliophora) commonly used by hydrobiologists as bioindicators in rivers, lakes, and waste waters, with notes on their ecology. Freshw. Biol. 35, 375–482. https://doi.org/10.1111/j.1365–2427.1996.tb01775.x.

Gallego, A., Martín–González, A., Ortega, R., Gutiérrez, J., 2007. Flow cytometry assessment of cytotoxicity and reactive oxygen species generation by single and binary mixtures of cadmium, zinc and copper on populations of the ciliated protozoan *Tetrahymena thermophila*. Chemosphere 68, 647–61. http://doi.org/10.1016/j.chemosphere.2007.02.031.

Gao, F., Warren, A., Zhang, Q., Gong, J., Miao, M., Sun, P., Xu, D., Huang, J., Yi, Z., Song, W., 2016. The all–data–based evolutionary hypothesis of ciliated protists with a revised classification of the Phylum Ciliophora (Eukaryota, Alveolata). Sci. Rep. 6, 24874. http://doi.org/10.1038/srep24874.

Gerhardt, A., Ud–Daula, A., Schramm, K.W., 2010. *Tetrahymena* spp. (Protista, Ciliophora) as test species in rapid multilevel ecotoxicity tests. Acta Protozool. 49, 271–280.

Gomiero, A., Sforzini, S., Dagnino, A., Nasci, C., Viarengo, A., 2012. The use of multiple endpoints to assess cellular responses to environmental contaminants in the interstitial marine ciliate *Euplotes crassus.* Aquat. Toxicol. 114, 206–216. http://doi.org/10.1016/j.aquatox.2012.02.030.

Gomiero, A., Dagnino, A., Nasci, C., Viarengo, A., 2013. The use of protozoa in ecotoxicology: Application of multiple endpoint tests of the ciliate *E. crassus* for the evaluation of sediment quality in coastal marine ecosystems. Sci. Total Environ. 442, 534–544. http://doi.org/10.1016/j.scitotenv.2012.10.023.

Gong, Z., Chen, Y., Yan, Y., Pei, S., Wu, D., Zhang, M., Wang, Q., 2014. Toxicity of Three Heavy Metal Pollutants of the Pharmaceutical Wastewater to *Paramecium caudatum*. Adv. Mater. Res. 571–577. https://doi.org/10.4028/www.scientific.net/AMR.937.571.

Grell, K.G., 1973. Protozoology. Springer Verlag: Berlin, pp.554.

Green, S.M., Machin, R., Cresser, M.S., 2008. Effect of long–term changes in soil chemistry induced by road salt applications on N–transformations in roadside soils. Environ. Pollut. 152, 20–31. https://doi.org/10.1016/j.envpol.2007.06.005.

Green, I.D., Diaz, A., Tibbett, M., 2010. Factors affecting the concentration in seven–spotted ladybirds (*Coccinella septempunctata* L.) of Cd and Zn transferred through the food chain. Environ. Pollut. 158, 135–141. https://doi.org/10.1016/j.envpol.2009.07.032.

Gurevitch, J., Hedges, L.V., 1999. Statistical issues in ecological meta–analyses. Ecol. 80, 1141–1149. https://doi.org/10.1890/0012–9658(1999)080[1142:SIIEMA]2.0.CO;2.

Gurevitch, J., Koricheva, J., Nakagawa, S., Stewart, G., 2018. Meta–analysis and the science of research synthesis. Nature 555, 175–182. http://doi.org/10.1038/nature25753.

Gutiérrez, J.C., Martín–González, A., Díaz, S., Ortega, R., 2003. Ciliates as a potential source of cellular and molecular biomarkers/biosensors for heavy metal pollution. Europ. J. Protistol. 39, 461–467. https://doi.org/10.1078/0932–4739–00021.

Guetiérrez, J.C., Amaro, F., Martín–González, A., 2015. Heavy metal whole–cell biosensors using eukaryotic microorganisms: an updated critical review. Front. Microbiol. 6, 1–8. http://doi.org/10.3389/fmicb.2015.00048.

Grebecki, A., Kuznicki, L., 1956. Autoprotection in *Paramecium caudatum* by influencing the chemical properties of its medium. Acta Biol. Exp. 17, 71–107.

Hamadeh, H.K., Bushel, P.R., Jayadev, S., Martin, K., Di Sorbo, O., Sieber, S., Bennett, L., Stoll, R., Barrett, J.C., Blanchard, K., Paules, R.S., Afshari, C.A., 2002. Gene expression analysis reveals chemical–specific profiles. Toxicol. Sci. 67, 219–31. http://doi.org/10.1093/toxsci/67.2.219.

Hughes, M.N., Poole, R.K., 1989. Metals and Microorganisms. Chapman and Hall: New York, pp.83–110.

Iftode, F.J.J, Fleury, C.A., Fryd–Versavel, G., 1985. Action of a heavy ion Cd2+, and the antagnostic effect $Ca^{2+,}$ on two ciliates *Tetrahymena pyriformis* and *Euplotes vannus*. Acta Protozool. 24, 273–279.

Jiang, G.J., Wu, S.G., Shen, Y.F., 2007. Effects of seasonal succession and water pollution on the protozoan community structure in an eutrophic lake. Chemosphere 66, 523–532. http://doi.org/10.1016/j.chemosphere.2006.05.042

Lin, T.Y., Kampalath, R.A., Lin, C.C., Zhang, M., Chavarria, K., Lacson, J., Jay, J.A., 2013. Investigation of mercury methylation pathways in biofilm versus planktonic cultures of Desulfovibrio desulfuricans. Environ. Sci. Technol. 47, 5695–5702. http://doi.org/10.1021/es400079n.

Liu, C.B., Qu, G.B., Cao, M.X., Liang, Y., Hu, L.G., Shi, J.B., Cai, Y., Jiang, G.B., 2017. Distinct toxicological characteristics and mechanisms of Hg^{2+} and MeHg in *Tetrahymena* under low concentration exposure. Aquat. Toxicol. 193, 152–159. http://doi.org/10.1016/j.aquatox.2017.10.014.

Lopa, G., Adhikari, S., 2006. Accumulation of heavy metals in freshwater fish – an assessment of toxic interactions with calcium. Am. J. Food Technol. 1, 139–148. http://doi.org/10.3923/ajft.2006.139.148.

Lu, X., Gentekakib, E., Xua, Y., Huanga, L., Lia, Y., Lua, X., Zhaoc, Y., Lina, X., Yia, Z., 2019. Intra–population genetic diversity and its effects on outlining geneticdiversity of ciliate populations: Using *Paramecium multimicronucleatum* as an example. Eur. J. Protistol. 67, 142–150. http://doi.org/10.1016/j.ejop.2018.12.005.

Lynn, D.H., 2008. The Ciliated Protozoa: Characterization, Classification and Guide to the Literature. Springer Press: New York, pp. 1–628.

Madoni, P., 2000. The acute toxicity of nickel to freshwater ciliates. Environ. Pollut. 109, 53–59. https://doi.org/10.1016/S0269–7491(99)00226–2.

Madoni, P., Esteban, G., Gorbi, G., 1992. Acute toxicity of cadmium, copper, mercury, and zinc to ciliates from activated sludge plants. Bull. Environ. Contam. Toxicol. 49, 900–905. http://doi.org/10.1007/bf00203165.

Madoni, P., 2005. Ciliated protozoans communities and saprobic evaluation of water quality in the hilly zone of some tributaries of the Po River (northern Italy). Hydrobiologia 541, 55–69. http://doi.org/0.1007/s10750–004–4667–8.

Madoni, P., Davoli, D., Gorbi, G., 1994. Acute Toxicity of Lead, Chromium, and Other Heavy Metals to Ciliates from Activated Sludge Plants. Bull. Environ. Contam. Toxicol. 53, 420–425. http://doi.org/10.1007/bf00197235.

Madoni, P., Davoli, D., Gorbi, G., Vescovi, L., 1996. Toxic effect of heavy metals on the activated sludge protozoan community. Water Res. 30, 135–141. https://doi.org/10.1016/0043–1354(95)00124–4.

Madoni, P., Romeo, M.G., 2006. Acute toxicity of heavy metals towards freshwater ciliated protists. Environ. Pollut. 141, 1–7. http://doi.org/10.1016/j.envpol.2005.08.025.

Mansano, A.S., Moreira, R.A., Pierozzi, M., Oliveira, T.M.A., Vieira, E.M., Rocha, O., Regali–Seleghim, M.H., 2016. Effects of diuron and carbofuran pesticides in their pure and commercial forms on *Paramecium caudatum*: The use of protozoan in ecotoxicology. Environ. Pollut. 213, 160–172. https://doi.org/10.1016/j.envpol.2015.11.054.

Makhija, S., Gupta, R., Toteja, R., Abraham, J.S., Sripoorna, S., 2015. Cadmium induced ultrastructural changes in the ciliate, *Stylonychia mytilus* (Ciliophora, Hypotrichida). J. Cell. Tissue Res. 15, 5151–5157.

Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: A meta–analysis. Chemosphere 93, 2217–2223. https://doi.org/10.1016/j.chemosphere.2013.07.036.

Melvin, S.D., Leusch, F.D.L., 2016. Removal of trace organic contaminants from domestic wastewater: A meta–analysis comparison of sewage treatment Technologies. Environ. Int. 92, 183–188. https://doi.org/10.1016/j.chemosphere.2013.07.036.

Meng, Q., Li, X., Feng, W., Cao, Z., 2008. The Acute and Chronic Toxicity of Five Heavy Metals on the *Daphnia Magna*. 2nd International Conference on Bioinformatics and Biomedical Engineering. https://doi.org/10.1109/ICBBE.2008.298.

Miranda, M.M.P., Martins, N.F., 2013. Avaliação experimental do efeito tóxico de metais em *Paramecium caudatum* Ehrenberg, 1833. Rev. Ver. 8, 247–262.

Miyoshi, N., Kawano, T., Tanaka, M., Kadono, T., Kosaka, T., Kunimoto, M., Takahashi, T., Hosoya, H., 2003. Use of *Paramecium* species in bioassays for environmental risk management: determination of IC50 values for water pollutants. J. Health Sci. 49, 429–435. http://doi.org/10.1248/jhs.49.429.

Moher, D., Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., The PRISMA Group (2009) Preferred reporting items for systematic reviews and meta–analyses: the PRISMA statement. J. Clin. Epidemiol. 62, 1006–1012. http://doi.org/10.1371/journal.pmed.1000097.

Montassir, L., Berrebaan, I., Melloki, F., Zkhiri, F., Boughribil, S., Ennaji, M.M., Bessi, H., 2016. Ecotoxicity assessment of aqueous extract of *Tetraclinis articulata* on green alga *Pseudokirchneriella subcapitata*. Comparison with toxicity on *Daphnia magna*. J. Mater. Environ. Sci. 8, 4824–4829.

Moreira, R.A., da Silva, M.A., Rocha, A., Daam, M.A., 2016. The use of rotifers as test species in the aquatic effect assessment of pesticides in the tropics. Hydrobiologia 773, 1–9. http://doi.org/10.1007/s10750-016-2675-0.

Mortuza, M.G., Takahashi, T., Ueki, T., Kosaka, T., Michibata, H., Hosoya, H., 2005. Toxicity and bioaccumulation of hexavalent chromium in green paramecium, *Parmecium bursaria*. J. Health. Sci. 51, 676–682. http://doi.org/10.1080/10807039.2017.1336425.

Nalecz–Jawecki, G., Demkowicz–Dobrzahski, K., Sawicki, J., 1993. Protozoan *Spirostomum ambiguum* as a highly sensitive bioindicator for rapid and easy determination of water quality. Sci. Total Environ. 134, 1227–1234. https://doi.org/10.1016/S0048–9697(05)80128–7.

Nusch, E.N., 1982. Prufung der biologischen Schadwirkungen von Wasserinhaltsstoffen mit Hilfe von Protozoentests. Decheniana Beih. 26, 87–98.

Olabarrieta, I., L'Azou, B., Yuric, J., Cambar, J., Cajaraville, M.P., 2001. In vitro effects of cadmium on two different animal cell models. Toxicol. in vitro. 15, 511–517. https://doi.org/10.1016/S0887–2333(01)00056–X.

Pan, Y., Zhang, W., Lin, S., 2018. Transcriptomic and microRNAomic profiling reveals molecular mechanisms to cope with silver nanoparticle exposure in the ciliate *Euplotes vannus.* Environ. Sci. Nano. 5, 1–16. http://doi.org/10.1039/C8EN00924D.

Poole, R.K., Gadd, G.M. 1989. Metal–microbe Interactions, IRL Press, Oxford, pp. 19–38.

Pudpong, S., Chantangsi, C., 2015. Effects of four heavy metals on cell morphology and survival rate of the ciliate *Bresslauides* sp. Trop. Nat. Hist. 15, 117–125.

Puytorac, P., 1994. Phylum Ciliophora Doflein, 1901, in: Puytorac P (ed). Traité de Zoologie, Tome II, Infusoires Ciliés. Masson, Paris, pp. 1–15.

Rao, J.V., Srikanth, K., Arepalli, S.K., Gunda, V.G., 2006. Toxic effects of acephate on *Paramecium caudatum* withspecial emphasis on morphology, behaviour, and generation time. Pest. Biochem. Physiol. 86, 131–137. https://doi.org/10.1016/j.pestbp.2006.02.005.

Rehman, A., Ashraf, S., Qazi, J.I., Shakoori, A.R., 2005. Uptake of Lead by a Ciliate, *Stylonychia mytilus*, Isolated from Industrial Effluents: Potential Use in Bioremediation of Wastewater. Bull. Environ. Contam. Toxicol. 75, 290–296. http://doi.org/10.1007/s00128–005–0751–7.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2006. Heavy metal resistant ciliate, *Euplotes mutabilis,* isolated from industrial effluents can decontaminate wastewater of heavy metals. Bull. Environ. Contam. Toxicol. 76, 907–913. http://doi.org/10.1007/s00128–006–1004–0.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2007. Multiple metal resistance and uptake by a ciliate, *Stylonychia mytilus*, isolated from industrial effluents and its possible use in wastewater treatment. Bull. Environ. Contam. Toxicol. 79, 410–414. http://doi.org/ 10.1007/s00128–007–9270–z.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2007a. Potential Use of a Ciliate, *Vorticella microstoma,* Surviving in Lead Containing Industrial Effluents in Waste Water Treatment. Pak. J. Zool. 39, 259–264.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2008. Heavy metal resistant freshwater ciliate, *Euplotes mutabilis*, isolated from industrial effluents has potential to

decontaminate wastewater of toxic metals. Bioresource Technol. 99, 3890–3895. http://doi.org/10.1016/j.biortech.2007.08.007.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2008a. Uptake of heavy metals by *Stylonychia mytilus* and its possible use in decontamination industrial wastewater. World J. Microb. Biot. 24, 47–53. http://doi.org/10.1007/s11274–007–8436–1.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2009. Heavy Metal Uptake by *Euplotes mutabilis* and its Possible Use in Bioremediation of Industrial Wastewater. Bull. Environ. Contam. Toxicol. 83, 130–135. http://doi.org/10.1007/s00128–009–9725–5.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2010. Resistance and Uptake of Heavy Metals by *Vorticella microstoma* and Its Potential Use in Industrial Wastewater Treatment. Environ. Prog. Sustain. 29, 481–486. http://doi.org/10.1002/ep.10450.

Rehman, A., Shakoori, F.R., Shakoori, A.R., 2010a. Multiple Heavy Metal Tolerant Ciliates, *Oxytricha fallax* and *Paramecium caudatum*, Isolated From Industrial Effluents and Their Potential Use in Wastewater Treatment. Pak. J. Zool. 42, 301–309.

Ruthven, J., Cairns, J.J., 1973. Response of Fresh–Water Protozoan Artificial Communities to Metals. J. Protozool. 20, 127–135. https://doi.org/10.1111/j.1550–7408.1973.tb06015.x.

Santos–Medrano, G.M., Rico–Martínez, R., 2018. Acute Sensitivity Comparison among *Daphnia magna* Straus, 1820 *Daphnia pulex* Leydig, 1860 and *Simocephalus vetulus* Müller, 1776, Exposed to Nine Toxicants. Turk. J. Fish. and Aquat. Sci. 19, 615–623. https://doi.org/10.4194/1303-2712-v19_7_08.

Sauvant, M.P., Pepin, D., Piccini, E., 1999. *Tetrahymena pyriformis*: A tool for toxicological studies. A review. Chemosphere 38, 1631–1699. https://doi.org/10.1016/s0045-6535(98)00381-6.

Schuler, M.S., Relyea, R., 2018. A Review of the Combined Threats of Road Salts and Heavy Metals to Freshwater Systems. Bio Sci. 68, 327–335. https://doi.org/10.1093/biosci/biy018.

Sherr, E.B., Sherr, B.F., 1994. Bacterivory and herbivory: key roles of phagotrophic protists in pelagic food webs. Microb. Ecol. 28, 223–235. http://doi.org/10.1007/BF00166812.

Sonnebobn, T.M., 1970. Methods in *Paramecium* research, in: Methods in Cell Physiology. Academic Press, New York, pp. 241–339.

Slabodnick, M. M., Ruby, G., Reiff, S.B., Swart, C.E., Gosai, S., PRABAKARAN, S., Witkowska, E., Larue, G.E., Fisher, S., Freeman, R.M., Guwardena, J., CHU, W., Stover, N.A., Gregory, B.D., Nowacki, M., Derisi, J., Roy, S.W., Wallace, F.M., SOOD, P., 2017.

The Macronuclear Genome of *Stentor coeruleus* Reveals Tiny Introns in a Giant Cell. Curr. Biol. 27, 569–557. https://doi.org/10.1016/j.cub.2016.12.057.

Suedel, B.C., Rodgers, Jr.J.H., Deaver, E., 1997. Experimental that may affect toxicity of cadmium to freshwater organisms. Environ. Contam. Toxicol. 33, 188–193. http://doi.org/10.1007/s002449900241.

Swart, E., Bracht J.R., Magrini, V., Minx, P., Chen, X., Zhou, Y., Khurana, J.S., Goldman, A.D., Nowacki, M., SChotanus, K., Jung, S., Fulton, R.S., Ly, A., McGrath, S., HAUB, K., Wiggins, J.L., Storton, D., Matese, J.C., Parsons, L., Chang, W.J., Bowen, M.S., Stover, N.A., Jones, T.A., Eddy, S.R., Herrick, G.A., Doak, T.G., WIlson, R.K., Mardis, E.R., Landweber, L.F., 2013. The *Oxytricha trifallax* macronuclear genome: a complex eukaryotic genome with 16,000 tiny chromosomes. PLoS Biol. 11, e1001473. https://doi.org/10.1371/journal.pbio.1001473.

Tan, X., Shi, X., Liu, G., Xu, H., Nie, P., 2010. An approach to analyzing taxonomic patterns of protozoan communities for monitoring water quality in Songhua River, northeast China. Hydrobiologia 638, 193–201. http://doi.org/10.1007/s10750-009-0040-2.

Tarcz, S., Potekhin, A., Rautian, M., Przybos, E., 2012. Variation in ribosomal and mitochondrial DNA sequences demonstrates the existence of intraspecific groups in *Paramecium multimicronucleatum* (Ciliophora, Oligohymenophorea). Mol. Phylogenet. Evol. 63, 500–509. http://doi.org/10.1016/j.ympev.2012.01.024.

Trielli, F., Amaroli, A., Sifredi, F., Marchi, B., Falugi, C., Corrado, M.U.D., 2007. Effects of xenobiotic compounds on the cell activities of *Euplotes crassus*, a single–cell eukaryotic test organism for the study of the pollution of marine sediments. Aquat. Toxicol. 83, 272–283. http://doi.org/10.1016/j.aquatox.2007.05.002.

USEPA (1980) Ambient water quality criteria for pentachlorophenol. Criteria and Standards Division, U. S. Environmental Protection Agency, Washington (DC), National Technical Information Service.

Velho, L.F.M., Lansac–Tôha, S.M., Buosi, P.R.B., Meira, B.R., Cabral, A.F., Lansac–Tôha, F.A., 2013. Structure of planktonic ciliates community (Protist, Ciliophora) from an urban lake of southern Brazil. Acta Scientiarum. Biol. Sci. 35, 531–539. http://doi.org/10.4025/actascibiolsci.v35i4.18579.

Vinogradov, D.V., Tsoi, O.V., Zaika, A.V., Lobanov, A.V., Turanov, A.A., Gladishev, V.N., Gel'fand, MS., 2012. Draft macronucleus genome of *Euplotes crassus* ciliate. Mol. Biol. 46, 328–333. http://doi.org/10.1134/S0026893312020197.

Xin, Z., Wenchao, Z., Zhenguang, Y., Yiguo, H., Zhengtao, L., Xianliang, Y., Xiaonan, W., Tingting, L., Liming, Z., 2015. Species sensitivity analysis of heavy metals to

freshwater organisms. Ecotoxicology 24, 1621–1631. http://doi.org/10.1007/s10646-015-1500-2.

Zhang, P., Xu, J., Lu, J., Liang, A., 2013. Wang, W. Different response of Two metallothionein subfamilies exposed to chromium (vi) in *Tetrahymena thermophila.* Fresen. Environ. Bull. 22, 1924–1928.

Zhao, Y., Gentekak, E., Yi, Z., Lin, X., 2013. Genetic Differentiation of the Mitochondrial Cytochrome Oxidase c Subunit I Gene in Genus *Paramecium* (Protista,Ciliophora). Plos One 8:e77044. https://doi.org/10.1371/journal.pone.0077044.

Wanick, R.C., Paiva, T.S., De Carvalho, C.N., Silva–Neto, I.D., 2008. Acute Toxicity of Cadmium to Freshwater Ciliate *Paramecium bursaria.* Biociências 16, 104–109.

Weisse, T., 2017. Functional diversity of aquatic ciliates. Eur. J. Protistol. 61, 331–358. https://doi.org/10.1016/j.ejop.2017.04.001.

5 Chapter III

(In progress, *Archives of Environmental Contamination and Toxicology*, B1, Qualis Capes 2013-2016, FI: 2.400)

Investigating the aquatic risks of caffeine using *Paramecium caudatum* (Alveolata, Ciliophora) as a model organism

Jéssica Andrade Vilas-Boas[1,2]*, Gabrielle Rabelo Quadra[2,3], Marcus Vinicius Xavier Senra[1,5], Pedro Mendes de Souza[1], Aline Corrêa Ribeiro[4], Jorge Willian Leandro Nascimento[4], Andreu Rico[6], Roberto Júnio Pedroso Dias[1,2]

[1]Departamento de Zoologia, Laboratório de Protozoologia (LAZ), Universidade Federal de Juiz de Fora 36036-900, Juiz de Fora, Minas Gerais, Brazil

[2]Pós-graduação em Biodiversidade e Conservação da Natureza, Universidade Federal de Juiz de Fora 36036-900, Juiz de Fora, Minas Gerais, Brazil

[3]Laboratório de Ecologia Aquática, Universidade Federal de Juiz de Fora 36036-900, Juiz de Fora, Minas Gerais, Brazil

[4]Departamento de Farmacologia, Laboratório de Farmacologia Clínica e Experimental, Universidade Federal de Juiz de Fora 36036-900, Juiz de Fora, Minas Gerais, Brazil

[5]Instituto de Recursos Naturais, Pós-graduação em Meio Ambiente e Recursos Hídricos, Universidade Federal de Itajubá 37500-903, Itajubá, Minas Gerais, Brazil

[6]IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Avenida Punto Com 2, 28805, Alcalá de Henares, Madrid, Spain

*Corresponding author: jessica.andrade@ecologia.ufjf.br

Abstract

Caffeine is a widely consumed psychoactive substance, which is continuously disposed into the environment with the potential to affect aquatic ecosystems. Ciliates play a key role in aquatic ecosystems and are considered good biological models to evaluate the impacts of chemical pollution. This study aimed to assess the sensitivity of *Paramecium caudatum* to caffeine, and to evaluate its relative sensitivity as compared to other aquatic organisms. Finally, we evaluated the risks of caffeine to ciliates and to other aquatic taxonomic organism groups. The LC_{10} and LC_{50} of caffeine for *P. caudatum* were 190 and 710 mg L^{-1}, respectively. This study demonstrates that *P. caudatum* is relatively tolerant as compared to other aquatic organisms. Further ecotoxicological studies with

other ciliate species are recommended to compare their chemical sensitivity, and to understand the ecological implications of their population decline for ecosystem functioning.

Keywords: aquatic ecosystems, ciliates, emerging contaminants, model organisms, risk assessment

Introduction

Caffeine is probably the most consumed psychoactive substance globally since coffee and many other food products and medicines contain caffeine (Gardinali and Zhao 2002; Diogo et al. 2013). Humans excrete part of the ingested caffeine (1% to 5% is not metabolized), and a considerable amount is discarded down-the-drain (e.g. by washing coffe machines and disposal of coffee grounds). Caffeine is not completely removed by wastewater treatment plants (Montagner et al. 2014a; Quadra et al. 2017), so a significant amount of caffeine reaches the environment (Tokimoto et al. 2005; Montagner et al. 2014a). Caffeine has been found in a wide range of aquatic systems, including oceans, surface waters, groundwater, and even drinking water (Barnes et al. 2008; Bruton et al. 2010; Machado et al. 2016). Moreover, caffeine may work as an indicator of the presence of other organic pollutants in the environment related to human presence, such as illicit drugs, pharmaceuticals and personal care products (Buerge et al. 2003; Ferreira et al. 2005; Montagner et al. 2014a,b).

Caffeine is generally recognized as an unhazardous compound for humans, but may pose toxicity to aquatic organisms depending on the exposure regime and the environmental conditions (Price et al. 1990, Hollingsworth et al. 2003, Montagner et al. 2014b). Caffeine has been found in concentrations up to μg L^{-1} and mg L^{-1} in aquatic ecosystems (Spongberg et al. 2011; Tran et al. 2014; Rodríguez-Gil et al. 2018; Quadra et al. 2020). Some toxicity studies show that these concentrations are not expected to cause acute toxicity to aquatic organisms (Calleja et al. 1994; Moore et al. 2008; Selderslaghs et al. 2013; Zarrelli et al. 2014). However, caffeine is continuously disposed into the environment and, according to Rodríguez-Gil et al. (2018), chronic effects are expected to occur in surface waters and estuary waters. Indeed, previous studies have shown that concentrations of few mg L^{-1} of caffeine affect the development of amphibians and fish (Sakamoto et al. 1993; Chen et al. 2008). Also, Rosi-Marshall et al. (2013) demonstrated the influence of caffeine on algal and bacterial biomass and respiration at

environmentally relevant concentrations. Furthermore, a previous investigation with coffee waste demonstrated that this residue induces mutagenicity and toxic effects in crustaceans and bacteria (Moore et al. 2008; Rosi-Marshall et al. 2013; Fernandes et al. 2017). Despite some laboratory studies indicate a potentical chronic risk of caffeine to aquatic ecosystems, the long-term effects at the ecosystem level have not been evalauted (Aguirre-Martínez et al. 2015). This is particularly important as a recent study suggests that caffeine concentrations in the aquatic environment might reach higher levels in the near future, especially in countries with high per capita consumption and/or a lack of sanitation system (Quadra et al. 2020).

Ciliates are considered an essential component of aquatic ecosystems. They are predators of bacteria and other protozoa and can be preyed by zooplankton species, mediating the energy flow from one trophic level to another (Sherr et al. 1988; Sherr and Sherr 1994; Madoni 2000; Mansano et al. 2014). Toxicity studies with ciliates are relatively scarce when compared to other microorganisms such as bacteria and algae. However, their small size, short life cycle, high reproduction rate and easy sampling allow the evaluation of environmental impacts in a short timescale (Groliére et al. 1990; Tan et al. 2010). Ciliates are considered good biological models, and have been proposed as model organisms for ecotoxicological studies (Gutiérrez et al. 2003, 2015, Gomiero et al. 2013). The sub-phylum Ciliophora is constituted by organisms with different physiological and morphological characteristics, which confer a wide range of sensitivities to pollutants in the aquatic environment (Madoni and Romeo 2006). Therefore, it is necessary to evaluate different species to find which one may be the most suitable model organism for different toxicological modes of action.

The aim of this study was to assess the sensitivity of *Paramecium caudatum* (Alveolata, Ciliophora) to caffeine and its usefulness as an indicator of caffeine pollution in aquatic ecosystems. Furthermore, we evaluated the relative sensitivity of *P. caudatum* as compared to other aquatic organisms, and discuss the potential risks of caffeine to ciliates making use of a global exposure dataset.

Materials and Methods

Toxicity tests

P. caudatum (Ehrenberg, 1833) individuals were isolated from the São Pedro stream, Juiz de Fora, Minas Gerais, Brazil (-43.378154, -21.762157). The stock cultures

were maintained for more than one year in the Protozoology Laboratory at Federal University of Juiz de Fora, Minas Gerais, Brazil. The culture medium used for maintenance was Cerophyl (Sonneborn 1957) with *Escherichia coli* (see Supplementary Material SI1). Cerophyl was added weekly to the culture and kept under room temperature. The characteristics of the culture medium were: temperature 23 ºC, dissolved oxygen 85% (7.27 mg L^{-1}), turbidity 6.3 Nephelometric Turbidity Units, total dissolved solids 395 mg L^{-1}, salinity 0.24 ‰, and pH 8.76.

Five ciliates were introduced into each well in the 24 wells of the culture plate with 300 µl of culture medium Cerophyl (Sonneborn 1957), with three replicates during the growth phase (see Supplementary Material SI2, Table 1 and 2). The samples were maintained in an incubator at controlled temperature (25 ºC) with a light:dark regime of 12 h / 12 h during 120 h. At each 24 h interval, the samples were fixed in Bouin for cell counts using a Sedgewick-Rafter chamber (Velho et al. 2013). The growth curve was made in MS Excel using the arithmetic mean and standard deviation of the cell counts. Only individuals from populations at logarithmic growth phase were used for the tests. The sensitivity range (positive control) of *P. caudatum* was assessed using sodium chloride acute toxicity tests, which were repeated 20 times. The control chart showing the sensitive range was calculated in MS Excel, where the sodium chloride LC_{50} is displayed.

The organisms used in the experiments were removed from the stock culture using a micropipette two hours before the test to acclimatize in mineral water. The chronic toxicity test was carried using a control and six concentrations: 50 mg L^{-1}, 100 mg L^{-1}, 200 mg L^{-1}, 400 mg L^{-1}, 800 mg L^{-1} and 1,600 mg L^{-1} (300 µl per concentration). The tests were developed in culture plates with 24 wells. For each treatment level, 10 organisms were used per replicate (three for each concentration and control). The mortality was assessed by counting the number of cells (movement cell was considered alive). The LC_{50} and LC_{10} for Caffeine was calculated using R Version 3.6.0. (see Supplementary Material SI3), using a nonlinear regression model. Firstly, we checked the data normality using the Shapiro-Wilks Test and then subjected to a one-way analysis of variance (ANOVA). A non-parametric Kruskal-Wallis test was applied for the non-normal data. We considered significant when p value < 0.05.

Relative toxicity

We used the toxicity data (see Supplementary Material SI4) from the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/index.cfm; 10th July 2020) to calculate

the relative toxicity (Trel) of *P. caudatum* as compared to other aquatic organisms. The toxicity data for other aquatic microorganisms included population effects based on growth, mortality, reproduction and development (EC_{50}/LC_{50}; 1-6h), and population, reproduction and mortality (NOEC/LOEC; > 6h) as used by Rodríguez-Gil et al. (2018). The Trel was calculated by dividing the toxicity values for the other species by the mean value LC_{50} to *P. caudatum* using Microsoft Excel (Daam and Rico 2018). The values lower than one indicate that species are more sensitive than *P. caudatum*, while Trel values larger than one indicates that the standard species are larger sensitivity to caffeine. All calculated Trel values were used to build a cumulative distribution function that helps to visualize their higher or lower sensitivity as regards to that of *P. caudatum*.

Risk assessment

We performed a risk assessment of caffeine considering the maximum measured environmental concentrations (MEC) reported by Rodriguez-Gil et al. (2018), Quadra et al. (2020) and updated literature. Risks were calculated for ciliates, using the toxicity value for *P. caudatum* (NOEC = 100 mg L^{-1}) derived as part of this study, but also toxicity data representative of other taxonomic groups (algae, crustaceans and fish). For algae, the NOEC was 0.1 mg L^{-1} derived from an *in situ* test checking biofilms' population biomass (Lawrence et al. 2005). For crustaceans, it was based on toxicity data for *D. magna* (NOEC of 0.12 mg L^{-1}) (Lu et al. 2013). For fish, it was based on a toxicity value for *Carassius auratus,* with a NOEC of 0.0032 mg L^{-1} reported by observing an enzymatic effect (Li et al. 2012). The assessment factor (AF) of 100, which is commonly used for chronic toxicity data (Mansano et al. 2016), was applied to obtain the PNEC values (NOEC/AF). Therefore, the risk quotient (RQ) was obtained by dividing MEC by PNEC, and results above 1 were considered as high risk, while below 0.1 low risk and values in between as moderate risk.

Results and Discussion

Sensitivity of *P. caudatum* to caffeine

Chemical analysis showed that the actual exposure concentrations in the experiments with caffeine differed by less than 8 % from the nominal concentrations and so the LC_{10} and LC_{50} concentrations were calculated based on the nominal concentrations (see Supplementary Material SI5). The calculated LC_{10} for *P. caudatum* was 190 mg L^{-1}

ranging from 150 to 240 mg L^{-1} and, the LC_{50} was 710 mg L^{-1} ranging from 600 to 810 mg L^{-1} (Fig. 1). The caffeine affected *P. caudatum* after chronic exposure but the LC_{10} (190 mg L^{-1}) and LC_{50} (710 mg L^{-1}) values were bigger than values detected in the environment. According to Harris and Sumpter (2015), an important issue about ecotoxicological studies is the lack of key information that enables quality evaluation, allowing comparisons and reproducibility. Hanson et al. (2017) proposed nine requirements to follow and evaluate ecotoxicological studies in order to improve the quality and reach environmental protection. We met all the requirements, although we did not develop an hypotheses test. However, especially to ciliates bioassays, most of the studies do not describe a considerable part of these requirements, such as exposure confirmation and presentation of all raw data, inducing problems of reliability, reproducibility, and comparison. According to Vilas-Boas et al. (2020a,b) standard toxicity test protocols are necessary to improve the quality of the toxicological studies with ciliates.

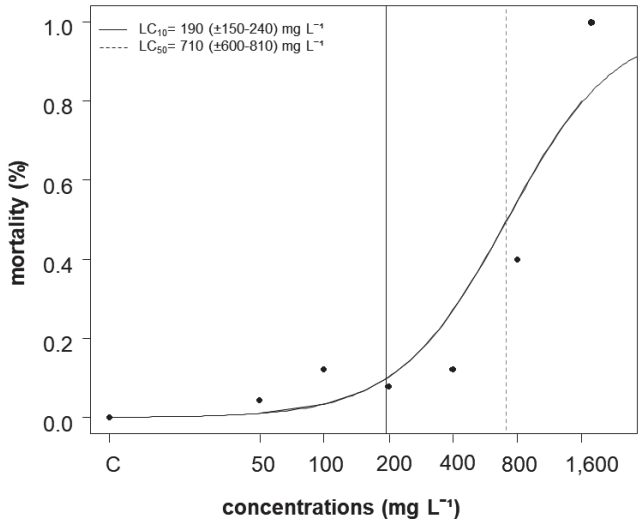


Fig 1. LC_{50} and LC_{10} values of *Paramecium caudatum* after 24 h exposure of caffeine.

Relative sensitivity of *P. caudatum* to caffeine

Some data reported caffeine toxicity above 100 mg L^{-1}. The rotifer species *Plationus patulus* presented two different LC_{50} values of 350 and 419 mg L^{-1} (Martinez Gomez et al. 2015) and, *Photobacterium phosphorusphosporeum* species showed a LC_{50} equal 671.8 mg L^{-1}. These LC_{50} values are the closest to *P. caudatum* showing perhaps a

similar sensitivity. Caffeine concentrations in water samples in the order of mg L^{-1} may cause adverse effects on amphibians, fish and microorganisms. Sakamoto et al. (1993) observed changes in the development of *Xenopus leavis* after exposed to caffeine (100 mg L^{-1}). The *Danio rerio* embryos demonstrated that caffeine in concentrations higher than 300 mg L^{-1} caused mortality (Chen et al. 2008). Another study showed that embryos exposed to 150 mg L^{-1} exhibited changes in the formation and hence, affecting development and decreased ability to locomotion (Yeh et al. 2012). Chronic effects have also been reported affecting the reproduction of *Ceriodaphnia dubia* and growth inhibition of *Pimephales promelas* exposed to caffeine and the LC_{50} values were 44 mg L^{-1} and 71 mg L^{-1}, respectively (Moore et al. 2008). In addition, caffeine may reduce respiration and biomass of the bacteria and algae (Rosi-Marshall et al. 2013). Moreover, coffee residues proved to induce mutagenicity effects by affecting the DNA (Fernandes et al. 2017).

Based on Trel values (Fig. 2), *Daphnia magna* was more sensitive than the other organisms. In the distribution, *P. caudatum* demonstrated not be a good model to protect the other species.

The application of an assessment factor of 10 – usually applied to account for interspecies sensitivity differences – is not be enough, except for *Brachionus calyciflorus*.

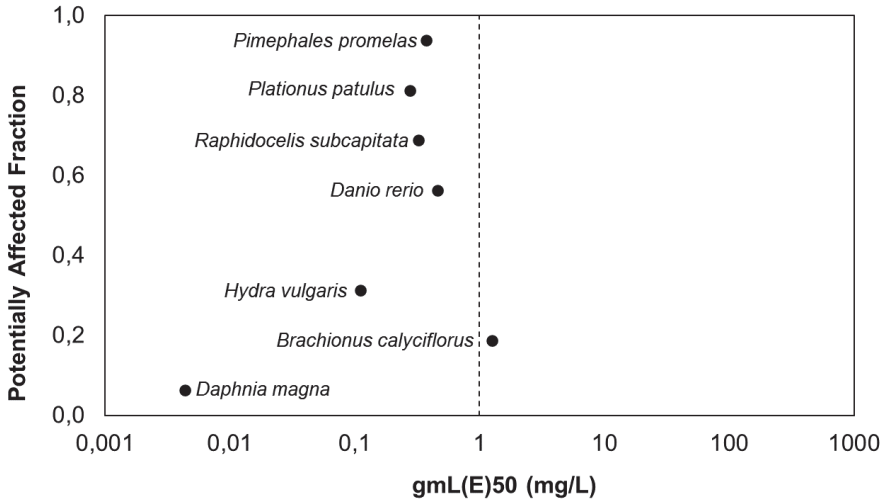


Fig 2. Species Sensitive distributions (SSD): Potentially Affect Fraction based on relative toxicity (Trel) values to *P. caudatum* and other model organisms to caffeine rank from higher sensitivity to lower sensitivity. The dashed line corresponds to Trel =1 indicating the sensitivity of *P. caudatum* being Trel <1 and Trel >1 showing a greater and lower sensitivity relative of aquatic organisms.

Risk assessment

The maximum environmental concentrations of caffeine in surface water ecosystems of different countries varied from 0.02 µg L^{-1} (Estonia) to 1121.5 µg L^{-1} (Costa Rica), with an overall mean of 57 ± 202 µg L^{-1} (mean ± standard deviation) and median of 2.1 µg L^{-1} (Fig. 3). The global map shows a similar trend of caffeine consumption presented by Quadra et al. (2020), then high consumption is probably correlated to the environmental concentrations. However, some European countries showed a high consumption (Quadra et al. 2020) but lower environmental concentrations. Important factors to take into account are the number of investigations within the country, patterns of consumption, and the sewage network and wastewater treatment plant technology. The United States, for example, has many monitoring programs and the number of samples is high (Rodriguez-Gil et al. 2018), which increases the probability of finding higher concentrations. Therefore, besides those countries with more studies, the countries with a lack of sanitation show high environmental levels.

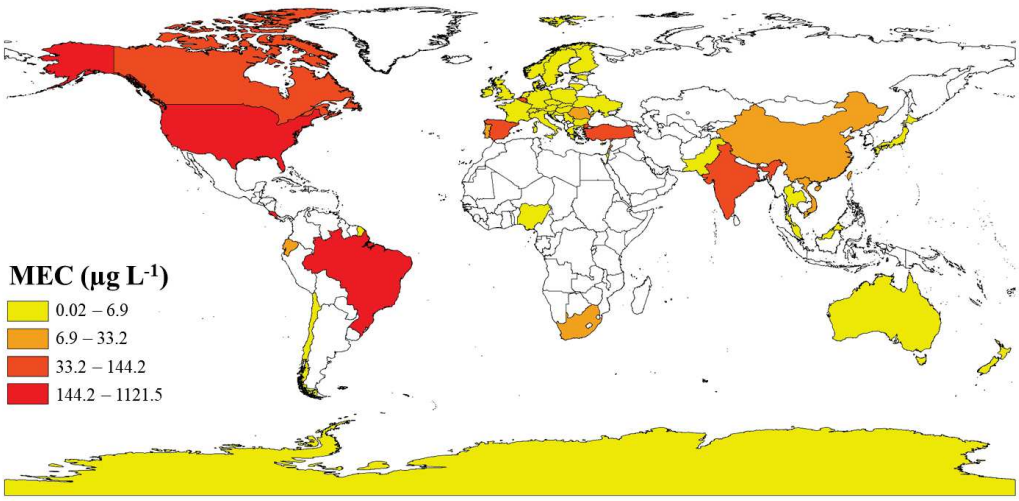


Fig 3. Maximum environmental concentration (MEC) of caffeine (µg L^{-1}) in surface freshwater worldwide. Literature data is available on Supplementary Material SI3.

The risk assessment showed that the ciliate *P. caudatum* was tolerant comparing to others (Fig. 4). However, important to note that although all the studies investigated chronic toxicity, the observed effects differ by sensibility. Mortality is normally an observed effect less sensitive than reproduction and enzymatic effects. Moreover, the algae test with biomass was developed in field studies, which is usually more sensitive as well. Although it is not common to use different observed effects than the standard ones (growth, reproduction, mortality) to perform the risk assessment, we believe it is

necessary to develop tools to make the comparisons more robust considering different observed effects and *in situ* studies. However, the techniques to observe sublethal effects and *in situ* studies with ciliates are not entirely dominated and understudied, representing time and resource consuming.

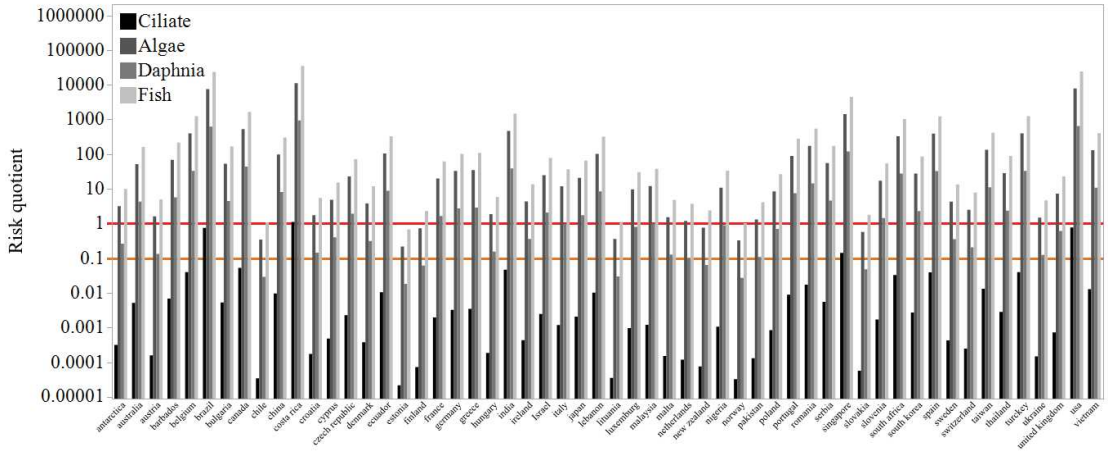


Fig 4. Risk assessment of ciliate, algae, daphnia, and fish worldwide considering the MEC and PNEC values. The orange line represents a moderate risk, while the red line high risk.

The risk assessment performed here is not only to compare different species but to show that the environmental concentrations of caffeine are posing a high risk to aquatic ecosystems considering the enzymatic test using fish. Considering the ciliate, the maximum environmental concentrations of Brazil, Singapore and the United States would pose a moderate risk to the species. In contrast, Costa Rica concentration would pose a high risk. Important to mention that finding a tolerant species does not mean that the contaminants do not represent a threat to the aquatic ecosystems. It is also crucial to consider potential mixtures effects and behavioral toxicity since caffeine already showed synergetic effects with other chemicals and some species are sensitive to low environmental concentrations using behavior as an observed effect (Dafouz et al. 2018; Rodríguez-Gil et al. 2018; Steele et al. 2018). Besides considering other observed effects and mixture toxicity, the tolerant species may also change the community composition and, consequently, the ecosystem processes and services.

Conclusion

Our study is the first to evaluate the sensitivity of ciliates (*P. caudatum*) to caffeine. The ciliate demonstrated tolerance to caffeine, which may imply a higher

resistance in the environment. Moreover, we observed a moderate risk for *P. caudatum* regarding maximum environmental concentrations of caffeine in surface freshwater. Their relative sensitivity comparison showed that *P. caudatum* used as standard test species would not have sufficient protection for most freshwater species. Even the global distribution of caffeine and the probability of increasing environmental concentrations highlight the need for more studies to better understand caffeine in aquatic ecosystems and the associated risks.

Acknowledgments

This study is part of the thesis of Jéssica Andrade Vilas−Boas, whitch was sponsored by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001 (fellowship to JAVB, GRQ), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) – CRA 03648/16 (fellowship to PMS) and, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (fellowships to RJPD, PQ 310237/2018-1). The authors also acknowledge CAPES for the post-doctoral fellowship (PNPD) (88882.317976/2019-01) conferred to MS. Andreu Rico is supported by a postdoctoral grant provided by the Spanish Ministry of Science, Innovation and University (IJCI–2017–33465). We are grateful to José Reinaldo Paranaíba for his help with the global map.

Declaration of competing interest

The authors declare no conflicts of interest.

References

Aguirre-Martínez, G. V., DelValls, A. T., & Martín-Díaz, M. L. (2015). Yes, caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen have an effect on Corbicula fluminea (Müller, 1774). Ecotoxicology and environmental safety, 120, 142-154.

Barnes, K. K., Kolpin, D. W., Furlong, E. T., Zaugg, S. D., Meyer, M. T., & Barber, L. B. (2008). A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States—I) Groundwater. Science of the Total Environment, 402(2-3), 192-200.

Bezirci, G., Akkas, S. B., Rinke, K., Yildirim, F., Kalaylioglu, Z., Severcan, F., & Beklioglu, M. (2012). Impacts of salinity and fish-exuded kairomone on the survival and macromolecular profile of Daphnia pulex. Ecotoxicology, 21(2), 601-614.

Bruton, T., Alboloushi, A., De La Garza, B., Kim, B. O., & Halden, R. U. (2010). Fate of caffeine in the environment and ecotoxicological considerations. In Contaminants of Emerging Concern in the Environment: Ecological and Human Health Considerations (pp. 257-273). American Chemical Society.

Buerge, I. J., Poiger, T., Müller, M. D., & Buser, H. R. (2003). Caffeine, an anthropogenic marker for wastewater contamination of surface waters. Environmental science & technology, 37(4), 691-700.

Calleja MC, Personne G, Geladi P. (1994). Comparative acute toxicity of the first 50 multicentre evaluation of in vitro cytotoxicity chemicals to aquatic non-vertebrates. Arch Environ Contam Toxicol 1:69–78.

Chen, Y. H., Huang, Y. H., Wen, C. C., Wang, Y. H., Chen, W. L., Chen, L. C., & Tsay, H. J. (2008). Movement disorder and neuromuscular change in zebrafish embryos after exposure to caffeine. Neurotoxicology and teratology, 30(5), 440-447.

Daam, M. A., & Rico, A. (2018). Freshwater shrimps as sensitive test species for the risk assessment of pesticides in the tropics. Environmental Science and Pollution Research, 25(14), 13235-13243.

Dafouz, R., Cáceres, N., Rodríguez-Gil, J. L., Mastroianni, N., de Alda, M. L., Barceló, D., ... & Valcárcel, Y. (2018). Does the presence of caffeine in the marine environment represent an environmental risk? A regional and global study. Science of the Total Environment, 615, 632-642.

Diogo, J. S., Silva, L. S., Pena, A., & Lino, C. M. (2013). Risk assessment of additives through soft drinks and nectars consumption on Portuguese population: A 2010 survey. Food and chemical toxicology, 62, 548-553.

Elphick, J. R., Bergh, K. D., & Bailey, H. C. (2011). Chronic toxicity of chloride to freshwater species: effects of hardness and implications for water quality guidelines. Environmental Toxicology and Chemistry, 30(1), 239-246.

Ferreira, A. P. (2005). Caffeine as an environmental indicator for assessing urban aquatic ecosystems. Cadernos de Saúde Pública, 21(6), 1884-1892.

Fernandes, A. S., Mello, F. V. C., Thode Filho, S., Carpes, R. M., Honório, J. G., Marques, M. R. C., Felzenszwalb, I., & Ferraz, E. R. A. (2017). Impacts of discarded coffee waste on human and environmental health. Ecotoxicology and environmental safety, 141, 30-36.

Gardinali, P. R., & Zhao, X. (2002). Trace determination of caffeine in surface water samples by liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC–APCI–MS). Environment International, 28(6), 521-528.

Gomiero, A., Dagnino, A., Nasci, C., & Viarengo, A. (2013). The use of protozoa in ecotoxicology: application of multiple endpoint tests of the ciliate E. crassus for the evaluation of sediment quality in coastal marine ecosystems. Science of the total environment, 442, 534-544.

Gomiero, A., & Viarengo, A. (2014). Effects of elevated temperature on the toxicity of copper and oxytetracycline in the marine model, Euplotes crassus: A climate change perspective. Environmental pollution, 194, 262-271.

Grolière, C.A., Chakli, R., Sparagano, O. and Pepin, D. (1990). Application de la colonisation d'un substratartificiel par les Ciliés à l'Étude de la qualité des eauxd'unerivière. European Journal of Protistology, 25(4), 381-390.

Gutiérrez, J. C., Amaro, F., & Martín-González, A. (2015). Heavy metal whole-cell biosensors using eukaryotic microorganisms: an updated critical review. Frontiers in microbiology, 6, 48.

Gutiérrez, J. C., Martín-González, A., Díaz, S., & Ortega, R. (2003). Ciliates as a potential source of cellular and molecular biomarkers/biosensors for heavy metal pollution. European journal of protistology, 39(4), 461-467.

Hanson, M. L., Wolff, B. A., Green, J. W., Kivi, M., Panter, G. H., Warne, M. S. J., ... & Sumpter, J. P. (2017). How we can make ecotoxicology more valuable to environmental protection. Science of The Total Environment, 578, 228-235.

Harris, C. A., & Sumpter, J. P. (2015). Could the quality of published ecotoxicological research be better?.

Hollingsworth, R. G.; Armstrong, J. W.; Campbell, E. (2003) Caffeine as a novel toxicantforslugsandsnails. Annals of Applied Biology,, 142, 91–97. 18.

Lawrence, J. R., Swerhone, G. D., Wassenaar, L. I., & Neu, T. R. (2005). Effects of selected pharmaceuticals on riverine biofilm communities. Canadian Journal of Microbiology, 51(8), 655-669.

Li, Z., Lu, G., Yang, X., & Wang, C. (2012). Single and combined effects of selected pharmaceuticals at sublethal concentrations on multiple biomarkers in Carassius auratus. Ecotoxicology, 21(2), 353-361.

Lu, G., Li, Z., & Liu, J. (2013). Effects of selected pharmaceuticals on growth, reproduction and feeding of Daphnia magna. Fresenius Environmental Bulletin, 22(9), 2583-2589.

Machado, K. C., Grassi, M. T., Vidal, C., Pescara, I. C., Jardim, W. F., Fernandes, A. N., Sodré FF, Almeida FV, Santana JS, Canela MC, Nunes CRO, Bichinho KM, Severo FJR. (2016). A preliminary nationwide survey of the presence of emerging contaminants in drinking and source waters in Brazil. Science of the Total Environment, 572, 138-146.

Madoni, P; Romeo, M. G. (2006). Acute toxicity of heavy metals towards freshwater ciliated protists. Environmental Pollution, Londres, 141(1), p.1-7.

Mansano, A.S; Moreira, R.A; Pierozzi, M; Oliveira, T.M.A; Vieira, E.M; Rocha, O; Regali-Seleghim, M.H. (2016). Effects of diuron and carbofuran pesticides in their pure and commercial forms on Paramecium caudatum: The use of protozoan in ecotoxicology. Environmental Pollution, Reino Unido, 213,160-172.

Mansano, A.S., Hisatugo, K.F., Hayashi, L.H., Regali-Seleghim, M.H. (2014). The importance of protozoan bacterivory in a subtropical environment (Lobo-Broa Reservoir, SP, Brazil). Brazilian Journal of Biololy, 74 (3), 569-578.

Martinez Gomez, D. A., Baca, S., & Walsh, E. J. (2015). Lethal and sublethal effects of selected PPCPs on the freshwater rotifer, Plationus patulus. Environmental toxicology and chemistry, 34(4), 913-922.

Montagner, C. C., Vidal, C., Sodré, F. F., Pescara IC, Jardim WF (2014a). A cafeína no ambiente. IN: Canela MC, Jardim WF, Sodré FF, Grassi MT (eds), Cafeína em águas de abastecimento pública no Brasil. Intituto Nacional de Ciências e Tecnologias Analíticas Avançadas.

Montagner, C. C., Umbuzeiro, G. A., Pasquini, C., Jardim, W. F., (2014b). Caffeine as an 395 indicator of estrogenic activity in source water. Environmental Science: Processes and 396 Impacts 16, 1866-1869.

Moore, M. T., Greenway, S. L., Farris, J. L., & Guerra, B. (2008). Assessing caffeine as an emerging environmental concern using conventional approaches. Archives of environmental contamination and toxicology, 54(1), 31-35.

Moser, C., & Bell, G. (2011). Genetic correlation in relation to differences in dosage of a stressor. Journal of evolutionary biology, 24(1), 219-223.

Price, K. R., & Fligner, D. J. (1990). Treatment of caffeine toxicity with esmolol. Annals of emergency medicine, 19(1), 44-46.

Quadra, G. R., De Souza, H. O., dos Santos Costa, R., & dos Santos Fernandez, M. A. (2017). Do pharmaceuticals reach and affect the aquatic ecosystems in Brazil? A critical review of current studies in a developing country. Environmental Science and Pollution Research, 24(2), 1200-1218.

Quadra, G. R., Paranaíba, J. R., Vilas-Boas, J., Roland, F., Amado, A. M., Barros, N., Dias, R.J.P., & Cardoso, S. J. (2020). A global trend of caffeine consumption over time and related-environmental impacts. Environmental Pollution, 256, 113343.

Rodríguez-Gil, J. L., Cáceres, N., Dafouz, R., & Valcárcel, Y. (2018). Caffeine and paraxanthine in aquatic systems: Global exposure distributions and probabilistic risk assessment. Science of The Total Environment, 612, 1058-1071.

Rosi-Marshall, E. J., Kincaid, D. W., Bechtold, H. A., Royer, T. V., Rojas, M., & Kelly, J. J. (2013). Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms. Ecological Applications, 23(3), 583-593.

Sakamoto, M. K., Mima, S., Kihara, T., Matsuo, T., Yasuda, Y., & Tanimura, T. (1993). Developmental toxicity of caffeine in the larvae of Xenopus laevis. Teratology, 47(3), 189-201.

Selderslaghs IWT, Hooyberghs J, Blust R, Witters HE. (2013). Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. Neurotoxicol Teratol 37:44–56

Sherr, E. B., & Sherr, B. F. (1994). Bacterivory and herbivory: key roles of phagotrophic protists in pelagic food webs. Microbial Ecology, 28(2), 223-235.

Sherr, B.F., Sherr, E.B., Hopkinson, C.S. (1988). Trophic interactions within pelagic microbial communities: interactions of feedback regulation of carbono flow. Hydrobiologia 159, 19-26.

Sonneborn, T.M. (1957). Breeding systems, reproductive methods and species problems in Protozoa. In The Species Problem (E. Mayr, ed.) Amer. Association for the Advancement of Science, Washington, 155-324.

Sornom, P., Felten, V., Médoc, V., Sroda, S., Rousselle, P., & Beisel, J. N. (2010). Effect of gender on physiological and behavioural responses of Gammarus roeseli (Crustacea Amphipoda) to salinity and temperature. Environmental pollution, 158(5), 1288-1295.

Spongberg, A.L., Witter, J.D., Acuna, J., Vargas, J., Murillo, M., Umana, G., Gomez, E., Perez, G., 2011. Reconnaissance of selected PPCP compounds in Costa Rican surface waters. Water research 45, 6709-6717.

Steele, W. B., Mole, R. A., & Brooks, B. W. (2018). Experimental Protocol for Examining Behavioral Response Profiles in Larval Fish: Application to the Neuro-stimulant Caffeine. Journal of Visualized Experiments, (137), e57938.

Tan, X., Shi, X., Liu, G., Xu, H., Nie, P. (2010). An approach to analyzing taxonomic patterns of protozoan communities for monitoring water quality in Songhua River, northeast China. Hydrobiology 638, 193-201.

Tokimoto, T., Kawasaki, N., Nakamura, T., Akutagawa, J., Tanada, S. (2005). Removal of lead ions in drinking water by coffee grounds as vegetable biomass. J. Colloid Interface Sci. 281, 56–61.

Tran, N. H., Li, J., Hu, J., & Ong, S. L. (2014). Occurrence and suitability of pharmaceuticals and personal care products as molecular markers for raw wastewater contamination in surface water and groundwater. Environmental Science and Pollution Research, 21(6), 4727-4740.

Velho, L.F.M, Brão, A.F.S, Lansac-Tôha, F.M, Buosi, P.R.B, Meira, B.R, Cabral, A.F, Lansac-Tôha, F.A. 2013. Structure of planktonic ciliates community (Protist, Ciliophora) from an urban lake of southern Brazil. Acta Scientiarum. Biological Sciences Maringá, 35(4), 531-539.

Vilas–Boas, J. A., Cardoso, S. J., Senra, M. V. X., Rico, A., & Dias, R. J. P. (2020). Ciliates as model organisms for the ecotoxicological risk assessment of heavy metals: A meta–analysis. Ecotoxicology and Environmental Safety, 199, 110669.

Vilas-Boas, J. A., Senra, M. V. X., & Dias, R. J. P. (2020). Ciliates in ecotoxicological studies: A minireview. Acta Limnologica Brasiliensia, 32.

Yeh, C. H., Liao, Y. F., Chang, C. Y., Tsai, J. N., Wang, Y. H., Cheng, C. C., ... & Chen, Y. H. (2012). Caffeine treatment disturbs the angiogenesis of zebrafish embryos. Drug and chemical toxicology, 35(4), 361-365.

Zarrelli, A., DellaGreca, M., Iesce, M. R., Lavorgna, M., Temussi, F., Schiavone, L., ... & Isidori, M. (2014). Ecotoxicological evaluation of caffeine and its derivatives from a simulated chlorination step. Science of the total environment, 470, 453-458.

6 Chapter IV

(Chapter published in the journal *Chemosphere*, A2, Qualis Capes 2013-2016, FI: 5.778)
https://doi.org/10.1016/j.chemosphere.2020.129381

Multiple stressors in Mediterranean coastal wetland ecosystems: influence of salinity and an insecticide on zooplankton communities under different temperature conditions

Jéssica Andrade Vilas−Boas[a,b*], Alba Arenas−Sánchez[c*], Marco Vighi[c], Susana Romo[d], Paul van den Brink[e,f], Roberto Júnio Pedroso Dias[a,b] and Andreu Rico[c]

[a] Laboratório de Protozoologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora 36036−900, Juiz de Fora, Minas Gerais, Brazil

[b] Programa de Pós−graduação Biodiversidade e Conversação da Natureza, Universidade Federal de Juiz de Fora 36036−900, Juiz de Fora, Minas Gerais, Brazil

[c] IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Avenida Punto Com 2, 28805, Alcalá de Henares, Madrid, Spain

[d] Departamento de Ecologia, Faculdade de Biologia, Campus Burjasot, Universidade de Valencia, Valencia, Burjastot, Valencia, Spain

[e] Aquatic Ecology and Water Quality Management group, Wageningen University, P.O. Box 47, 6700 AA Wageningen, the Netherlands

[f] Wageningen Environmental Research, Wageningen University & Research, P.O. Box 47, 6700 AA Wageningen, the Netherlands

[*]These authors contributed equally to this work

Corresponding author:

Andreu Rico

Tel.: +34 91 830 59 62

E−mail address: andreu.rico@imdea.org

Abstract

Temperature increase, salinity intrusion and pesticide pollution have been suggested to be among the main stressors affecting the biodiversity of coastal wetland ecosystems. Here we assessed the single and combined effects of these stressors on zooplankton communities collected from a Mediterranean coastal lagoon. An indoor microcosm experiment was designed with temperature variation (20 °C and 30 °C), salinity (no addition, 2.5 g/L NaCl) and the insecticide chlorpyrifos (no addition, 1 µg/L) as treatments. The impact of these stressors was evaluated on water quality variables and on the zooplankton community (structure, diversity, abundance and taxa responses) for 28 days. This study shows that temperature is the main driver for zooplankton community change, followed by salinity and chlorpyrifos. The three stressors contributed to a decrease on zooplankton diversity. The increase of temperature contributed to an increase of zooplankton abundance. Salinity generally affected Cladocera, which resulted in a Copepoda increase at 20 °C, and a reduction in the abundance of all major zooplankton groups at 30 °C. The insecticide chlorpyrifos affected primarily Cladocera, altough the magnitude and duration of the direct and indirect effects caused by the insecticide substantially differed between the two temperature scenarios. Chlorpyrifos and salinity resulted in antagonistic effects on sensitive taxa (Cladocera) at 20 °C and 30 °C. This study shows that temperature can influence the direct and indirect effects of salinity and pesticides on zooplankton communities in Mediterranean coastal wetlands, and highlights vulnerable taxa and ecological responses that are expected to dominate under future global change scenarios.

Keywords: climate change, multiple stressors, pesticides, salinization, coastal lagoons.

Highlights

- Multiple stressor effects investigated on Mediterranean zooplankton communities
- Temperature (T) > salinity (S) > chlorpyrifos (CPF) affected community structure
- Community effects of S and CPF were influenced by T regime
- S and CPF resulted in antagonistic effects on sensitive taxa (Cladocera).
- There was no significant interaction between the three stressors (T, S, CPF)

Introduction

Coastal wetland ecosystems support a large share of the global aquatic biodiversity and provide important ecological services, such as nutrient cycling, food provision or erosion control (Pérez−Ruzafa et al., 2010; Barbier, 2011). Several authors have identified wetland ecosystems in the Mediterranean region as particularly vulnerable to climate change and anthropogenic pressures (Romo et al. 2005; 2016; Pérez−Ruzafa et al., 2011; Osland et al., 2016; Gabler et al., 2017). Low precipitation and increasing evaporation associated to climate change, and the increasing substraction of freshwater resources for agricultural irrigation and urban expansion modify significantly their water balance (Navarro−Ortega et al., 2012). The reduction of freshwater inputs generally results in an increase of salinity, mainly due to seawater intrusion (Millán et al., 2011; Cañedo−Argüelles et al., 2016, 2019), which modifies physicochemical ecosystem variables and changes the metabolism of aquatic organisms, interfering with their life−history traits and fitness (Rokneddine and Chentoufi, 2004; Jeppesen et al., 2015). For instance, Nielsen et al. (2003) described decreased diapausing eggs' emergence of zooplankton and richness related to slight increases in water salinity. According to Coldsnow et al. (2017), increasing salinity generally results in a decrease of cladocerans and copepods, and an increase of rotifers, which facilitates the increase of phytoplankton blooms and the alteration of functional ecosystem parameters. Furthermore, salinity can induce changes in species competition patterns and alter predation regimes, which affect population dynamics and the structure of freshwater communities (Hintz et al., 2018; Bracewell et al., 2019).

Chemical emissions from agricultural, industrial and urban activities are also important drivers of biodiversity loss in coastal wetlands (Liu and Diamond, 2005; Navarro−Ortega et al., 2012; Bassi et al., 2014). Pesticides, which are commonly used in rice, vegetable and fruit production surrounding coastal wetlands, have been identified as one of the chemical groups of highest environmental concern (Picó et al., 2019). Particularly, organophosphorus insecticides, and some carbamate and azole fungicides, have been identified as a potential threat to freshwater biodiversity in Mediterranean river estuaries and wetlands (Ccanccapa−Cartagena et al., 2019). Aquatic ecosystems in Mediterranean regions are naturally influenced by seasonal temperature variations, reaching water temperatures of nearly 30 °C during the summer period. According to the 5th report from the Intergovernmental Panel for Climate Change (IPCC, 2014), the

prevalence of summer peak temperatures, heatwaves, and periods dominated by high water temperature is expected to increase markedly in the Mediterranean region in the coming years. Increasing temperatures may influence the metabolic rate of aquatic organisms, leading to different reproductive patterns and species interactions, which affect the structure of communities (Gillody et al., 2001; Devreker et al., 2009) and their vulnerability to additional stressors (Klausmeyer and Shaw, 2009; Mantyka−Pringle et al., 2012; Rojo et al., 2017; Arenas−Sánchez et al., 2019).

Despite the elevated number of studies describing the influence of temperature, salinity and chemical pollution in freshwater ecosystems, evidence on the interactive effects of these stressors remains limited (Mantyka−Pringle et al., 2014; Jackson, 2018; Arenas−Sánchez et al., 2019). Heugens et al. (2001) and Bracewell et al. (2019) published literature reviews discussing the influence of temperature, nutritional state and salinity on the sensitivity of different groups of aquatic organisms to various classes of chemicals. Moreover, previous review papers have considered the interaction between contaminants and temperature (Cairns et al., 1975), contaminants and salinity (Hall and Anderson, 1995) and the combination of these three factors (McLusky et al., 1986). These studies show that temperature generally tends to increase metabolic rates, leading to an increase in water exchange that results in a higher uptake and toxicity of chemicals to some aquatic organisms. No clear trend was observed for the combined effects of organic chemicals and salinity, except for organophosphate insecticides, for which a synergistic effect has been described (Heugens et al., 2001). This finding was based on laboratory experiments performed with mosquito larvae (*Aedes taeniorhynchus)* and brine shrimps (*Artemia* sp.) exposed to aldicarb and dimethoate under isosmotic and hyperosmotic conditions (Song and Brown, 1998). Particularly, for mosquito larvae, the short term LC_{50} for dimethoate was an order of magnitude lower under hyperosmotic conditions, suggesting that further studies are needed to assess the interactive effects of organophosphate insecticides and salinity in freshwater organisms.

The aim of the present study was to assess the effects of salinity, insecticide pollution and temperature regimes on zooplankton communities characteristic of a Mediterranean coastal wetland ecosystem. The main hypotheses were that different temperatures will influence community structure, and affect the single and combined effects of both chemical stressors. Also, as previously described, we expected that an increase in salinity enhances the uptake and sensitivity of some freshwater taxa to insecticide pollution. In our study, these hypotheses were tested by performing an indoor

microcosm experiment with planktonic organisms collected from the Albufera lagoon (Valencia, Spain). The Albufera lagoon is a shallow (mean depth 1.2 m), oligohaline (salinity 1−2‰), polymictic and eutrophic−to−hypereutrophic lagoon surrounded by intensive agriculture and urbanization (Romo et al. 2005). In our study, zooplankton was selected as focal taxonomic group due to their high sensitivity and rapid response to the selected stressors, and due to their relevant contribution as a food resource for fish, amphibians and birds in aquatic ecosystems. As test compound, we selected the organophosphate insecticide chlorpyrifos (CPF) [O, O−diethyl o−(3, 5, 6−trichloro−2−pyridyl) phosphorothioate]. CPF is a broad−spectrum insecticide that is widely used throughout the world in several agricultural crops (Cortina−Puig et al., 2010). This compound has been detected in a wide range of coastal wetland ecosystems and has been identified as highly toxic to aquatic non−target invertebrates and fish in the Mediterranean as well as in other regions (Moreno−González and León, 2017; Huang et al., 2020; Rico et al. In press). Since January 2020, CPF has been banned in the European Union due to its possible genotoxic and neurological effects in children (EC 2020). However it is considered a very good model compound given the amount of published data regarding its environmental exposure and effects (Huang et al. 2020). Moreover, it is expected to be replaced by surrogate pesticides with the same toxicological mode of action in European agriculture. The salinity and thermal regimes included in this study were based on monitoring data from the regional agricultural ministry (Valencian Agricultural Ministry, 2019) and represent realistic worst−case scenarios for the coastal aquatic system from which the zooplankton was collected. Overall, this study contributes to improve our understanding on the resilience of zooplankton communities to multiple stressors in Mediterranean coastal wetlands, and to identify vulnerable taxa and ecological responses that are expected to dominate under future global change scenarios.

Materials and methods

Experimental design

Thirty two microcosms (diameter 20.5 cm, total height 37 cm) were set−up in the ecotoxicology laboratory of the IMDEA Water Institute. The microcosms were filled with 12 L of water and plankton collected from the Albufera lagoon (39°20N, 0°21W). Water was collected on the 1st of October of 2019 from the inner part of the lagoon and transported to the laboratory. Zooplankton was collected on the same date and

concentrated *in−situ* with a plankton net (mesh size: 55 µm). Afterwards, it was distributed homogeneously over the test microcosms.

The experiment followed a factorial design with three treatments and two different levels each, and was run with four replicates (n=4). The treatments were: temperature (20 °C and 30 °C), salinity (no salt addition, 2.5 g/L salt addition in form of NaCl) and CPF exposure (i.e., control, 1 µg/L). The temperature of the 30 °C microcosms was increased slowly during the first 7 days using a temperature−controlled water bath. The salinity (NaCl) was added at a rate of 0.5 g/L per day during 5 days (until reaching 2.5 g/L) using a saline stock solution of 100 g/L and adding 60 mL per day to each exposed microcosm. CPF was added as a single dose 14 days after the set−up of the experiment. The choice of the temperature and salinity were done according to available monitoring data from the Albufera lagoon (see Supporting Information, Figures S1 and S2), and represent maximum temperature and salinity values of late summer periods.

The microcosms were subjected to a light/dark regime of 16:8 h. The light intensity was 3112 lux, which was considered powerful enough to maintain the phytoplankton community in the microcosms according to the OECD protocol for toxicity testing with algae (OECD 2006). Aeration was set inside of each microcosm with a low air flow to prevent extreme anoxic conditions, and to simulate mild water movement from air characteristic of natural ecosystems. The whole experiment had a duration of 6 weeks, with an aclimatization/adaptation period of 2 weeks and a post−insecticide exposure period of 4 weeks. The microcosms were checked weekly and refilled with demineralised water when excessive evaporation occurred.

Chlorpyrifos dosing, sampling, and analysis

A concentrated CPF stock solution of 12 mg/L in methanol was prepared. Aliquots (1 mL) of this stock solution were evenly distributed over the water surface of the corresponding microcosms and stirred with a glass pipette to reach a CPF concentration of 1 µg/L. Methanol (1 mL) was added to each chemical control according to the requirements specified by the OECD (2000) guidelines. Nominal concentrations were calculated on the basis of concentrations in the stock solution.

Water samples (100 mL) were taken from the microcosms by means of a glass pipette and transferred into glass flasks to measure CPF concentrations. Water samples were collected 2 h, and 1, 3, 7 and 10 days after the application in the microcosms that received the CPF application. Samples in the microcoms that did not receive CPF were

also collected on days 1 and 10 relative to the CPF application in order to assess any potential cross contamination. In general, samples were analyzed within 48 h after collection. When this was not possible, they were frozen and stored at −20 °C until further analysis.

CPF concentrations in the microcosm water were analyzed using a gas chromatograph (GC) system (Agilent 7890A) coupled to a mass spectrometer (MS) with a triple quadrupole analyzer (Agilent 7000 GC/MS Triple Quad). The GC column used was a HP-5ms (Agilent) capillary column. Chlorpyrifos was extracted using the Stir Bar Sorptive Extraction (or Twister) technique. The Twister was placed in an Erlenmeyer flask with 100 mL of water sample containing the internal standard (chlorpyrifos-d10, 20 µL of a standard solution of 250 ng/mL) and stirred at 850 rpm overnight. After extraction, the stir bar was introduced in a glass thermal desorption tube, placed in the thermal desorption unit of the GC-MS. The limit of quantification (LOQ) and the limit of detection (LOD) of the method were 30 ng/L and 3 ng/L, respectively. The mean recovery of the method was: 98% (Relative Standard Deviation, RSD: 2%) at 30 ng/L, and 105% (RSD: 5%) at 100 ng/L (n=3).

Water quality measurements

Water quality variables (i.e., temperature, electric conductivity, pH, dissolved oxygen and total dissolved solids) were measured with a multi−meter probe (model HI98194; HANNA Instrumenst) at 10 cm depth on days −14, −7, −4, 7, 14 and 28 relative to the CPF application. Water samples (1 L) were collected on days −3 and 14 relative to the CPF application for assessing ammonia, nitrate, orthophosphate and total P (TP) concentrations. Nitrate and ammonia concentrations were used to calculate the dissolved inorganic nitrogen (DIN). Chlorophyll−*a* concentrations were measured on days −3, 7, 14 and 28 relative to the CPF application. Nutrient and chlorphyll−*a* samples were analyzed following the methods described in APHA (2005). The results of the water chlorophyll−*a* and nutrient concentrations were used to characterize the systems and to evaluate the possible influence of the tested stressors on ecosystem functional parameters.

Zooplankton sampling and determination

Zooplankton samples were taken on days −4, 7, 14 and 28 relative to the CPF application. The samples (1.5 L) were collected using a suction pump and filtered through a zooplankton net (mesh size: 55 µm). Subsequently, the filtered water was returned to

the original microcosm. The concentrated zooplankton samples (maximum volume 100 mL) were preserved with Lugol's iodine solution (approximately 4% v/v) and let to sediment for 24 h. Afterwards, the supernatant was carefully removed to obtain a concentrated sample. Macrozooplankton (Cladocera, Copepoda, Ostracoda) were identified and counted in the entire zooplankton sample using an Olympus SZx2−TR30 stereomicroscope (magnification 20x). Microzooplankton (Rotifera) and Copepoda nauplii were assessed in 1 mL sub-samples, which were analyzed with a binocular Olympus UCTR30−2 microscope (magnification 100x). Macrozooplankton and microzooplankton taxa were identified to the lowest practical taxonomic resolution level. Abundance of each taxon was re-calculated to number of individuals per litre of the microcosm water.

Data analyses

The half−life of CPF (DT50) in the different temperature and salinity treatments was calculated by dividing Ln(2) by the dissipation coefficient. The dissipation coefficients were calculated by means of linear regression of the ln−transformed measured concentrations with the software Microsoft Excel version 2010 assuming first−order kinetics.

The influence of temperature, salinity and CPF on the zooplankton community was first evaluated with a Permutation Multivariate Analysis of Variance (PERMANOVA) test based on Euclidean distances with 999 Monte Carlo permutations (for further details see Anderson, 2001). A Principal Response Curve (PRC) analysis was performed in order to evaluate the responses associated to the different temperature scenarios, the responses associated to salinity and CPF separately in each temperature scenario, and the interaction between salinity and CPF in each of the two temperature scenarios. In each PRC analysis, the overall significance of the treatments on the dataset was tested by 999 Monte Carlo permutations under the Redundancy Analysis (RDA) option (for further details see Van den Brink and Ter Braak, 1999). The first four PRCs were inspected since the different stressors may result in different effects on the evaluated datasets, so it was expected that more than one PRC is needed to display the different community responses. In addition, a variation partitioning analysis was performed to assess the relative influence of each stressor on the zooplankton community using the RDA option (for rationale, see Peres−Neto et al. 2006). The PERMANOVA analyses were performed using the SIMPER version 7 Software (Clarke and Gorley, 2015). The

PRC and the variation partitioning analyses were performed using the CANOCO Software, version 5 (Ter Braak and Šmilauer, 2012). In all cases data were log (x+1) transformed prior to the statistical analyses, and a significance level of 0.05 was used to distinguish significant responses associated to each treatment and their interaction.

Population-level analyses were performed only with selected taxa: *Diaphanosoma* sp*., Moina* sp*., Anuraeopsis* sp. and *Lecane lunari.* These taxa were selected based on their relative large positive or negative response to the evaluated stressors as result of the PRC analysis (i.e., high or low b_k values). Population-level responses were evaluated by performing a three−way ANOVA for each sampling date, and including temperature, salinity and CPF as independent variables. Prior to any analysis, abundance data were log(x+1), exponential, log10, and square root transformed, and the best transformation was chosen for each sampling date based on the results of the Levene's variance homogeneity test and the Shapiro−Wilk normality test. When one of the two assumptions were not met, homogeneity of variances was prevailing over normality. The same analysis was performed for the calculated diversity (Simpson´s index, Simpson 1949) and total zooplankton abundance, as well as for the total abundance of Cladocera, Copepoda and Rotifera, and for the water quality parameters (including chlorophyll-*a*). In this case, a first assessment of homogeneity of variances and normality was performed with non−transformed data, and only when data was not complying with the assumptions, transformed data was used based on the same criteria as described above for the population-level analyses. Ostracoda were not further evaluated since their abundances were extremely low during the course of the experiment in all treatments. The three−way ANOVAs and the variance homogeneity and normality tests were performed with the Software Jamovi 1.2.2.0 (Şahin and Aybek, 2019). The significant influence of the single stressors or their interactions on the response variables were established when the calculated p-value was lower than 0.05, although marginally significant values (0.05<p-value<0.1) are also indicated in the text.

Results

Chlorpyrifos concentrations

CPF dosing was properly done based on the concentrations measured in the stock solution. After application, CPF dissipated relatively fast from the water column. The calculated DT50 at 20 °C was 3.10 d in the microcosms that did not receive salt addition, and 4.40 d in the microcosms that received salt addition. At 30 °C, the DT50s were 1.61 and 1.85 d in the microcosms without salt and with salt addition, respectively. Therefore,

the dissipation of CPF at 30 °C was generally twice more rapid than at 20 °C, and salinization resulted in a slight delay on the dissipation of CPF.

Water quality parameters

The mean and standard deviation of the water quality variables measured in the microcosms are shown in Table 1. The measured temperatures in the 20 °C treatment were slightly above 20 °C, but in the 30 °C treatment they were very close to the intended temperature. Electric conductivity (µS/cm) was more than two times higher in the microcosms that received salt addition, which is consistent with the expected salinity increase (Table 1).

Table 1. Water quality parameters (mean ± standard deviation) measured in the different treatments during the experiment. The raw data are provided in Appendix I.

Parameters	T20	T20−S	T20−CPF	T20−S−CPF	T30	T30−S	T30−CPF	T30−S−CPF
Temperature (˚C)	21.5 ± 0.3	21.5 ± 0.3	21.5 ± 0.3	21.9 ± 1.7	29.9 ± 1.5	29.4 ±1.4	29.8 ± 1.5	29.5 ±1 .4
EC (µS/cm)	1918 ± 206	5540 ± 1722	2053 ± 454	5315 ± 2097	2021 ± 222	5787 ± 1793	1943 ± 462	5720 ± 1768
pH	**8.2 ± 0.21**	8.3 ± 0.23	8.3 ± 0.11	8.3 ± 0.19	8.2 ± 0.22	8.1 ± 0.15	8.1 ± 0.33	8.1 ± 0.21
DO (%)	94.3 ± 19.1	103 ± 11.7	137 ± 187	133 ± 183.8	85.7 ± 8.9	87.0 ± 7.3	83.0 ± 9.3	87.0 ± 8.6
DO (mg/L)	7.8 ± 0.9	8.1 ± 0.8	8.1 ± 0.6	7.7 ± 1.3	5.9 ± 0.7	5.9 ± 0.5	5.6 ± 0.7	5.9 ± 0.6
TDS (mg/L)	964 ±117	2770 ± 861	1053 ± 203	2658 ± 919	1014 ± 110	2713 ± 1018	1008 ± 356	2848 ± 883
Ammonia (mg/L)	0.01 ± 0.02	<0.01	0.01 ± 0.01	0.20 ± 0.3	0.19 ± 0.2	0.3 ± 0.4	0.39 ± 0.35	0.38 ± 0.33
N-NO3 (mg−/L)	1.23 ± 0.21	1.29 ± 0.13	1.22 ± 0.17	1.19 ± 0.16	1.13 ± 0.10	1.72 ± 0.53	1.28 ± 0.25	1.82 ± 0.57
DIN (µg/L)	0.29 ± 0.06	0.29 ± 0.03	0.28 ± 0.05	0.37 ± 0.26	0.35 ± 0.19	0.72 ± 0.46	0.67 ± 0.33	0.65 ± 0.34
Ortophosphate (mg/L)	19.8 ± 9.4	22.9 ± 7.4	23.7 ± 8.85	15.4 ± 21.2	11.7 ± 6.4	6.7 ± 4.6	7.4 ± 4.1	9.5 ± 3.7
TP (mg/L)	96.7 ± 31.9	94.9 ± 16.4	99.4 ± 17.2	67.6 ± 21.3	50.6 ± 11.9	52.2 ± 24.5	42.2 ± 14.9	59.7 ± 15.7
Chlorophyll−a (µg/l)	125 ± 167	81.9 ± 110	103 ± 138	69.1 ± 90.3	110 ± 146	70.3 ± 90.3	86.9 ± 116.1	57.7 ± 59.2

EC: electric conductivity; DO: dissolved oxygen; TDS: total dissolved solids; T20: temperature at 20 ºC; T30: temperature at 30 ºC.

The three-way ANOVA indicates that all water quality variables were affected by temperature conditions (Table S1). Water pH ranged between 7.7 and 9.4, was found to be significantly lower in the 30 °C treatment and decreased after the salt and CPF addition (Table S1). As expected, dissolved oxygen concentrations were significantly higher in the 20 °C microcosms (7.7−8.1 mg/L) than in the 30 °C ones (5.6−5.9 mg/L). Oxygen levels significantly increased after the CPF addition, and in the treatments affected by CPF and salinity. TDS values were generally higher in the microcosms that had higher temperatures and received salt addition. Ammonia concentrations significantly increased in the 30 °C microcosms, and in the ones exposed to CPF and salinity (Table S1), up to

1.7 mg/L in the 30 °C microcosms that received salt on day -4. The interaction between temperature and salinity, and temperature and CPF also resulted in significantly higher values on day 14 (Table S1). DIN levels were generally similar across the different treatments, with slightly higher values in the 30 °C treatment and in the salinity ones. Conversely, TP was significantly lower in the treatment at 30 °C, with no consistent differences in the microcosms that received salinity or CPF. Mean chlorophyll-*a* concentrations ranged between 58-125 µg/L, which are representative of eutrophic freshwater ecosystems (Table 1). Chlorophyll-*a* concentrations were significantly lower in the microcosms at 30 °C (Table S1) and were notably reduced by the addition of salt in both temperature scenarios (Table 1).

Zooplankton responses

Eigtheen zooplankton taxa were identified, most of them belonging to the Rotifera phylum (12 taxa) followed by the Cladocera order (3 taxa: *Diaphanosoma* sp*., Alona* sp., *Moina* sp.). Copepoda were subdivided into Copepoda nauplii and Cyclopoida (including adults and copepodites), and Ostracoda were not further identified.

The variation partitioning analysis perfomed to asses the relative influence of each stressor on the zooplankton community showed that temperature was the main stressor accounting for the zooplankton community variation (45% of explained variation), followed by salinity (32%) and CPF (24%; Table S2). The PERMANOVA analysis indicated that the different temperature regime significantly influenced the zooplankton community during the whole experimental period (Table 2). At 30 °C, the zooplankton diversity was signficantly reduced, while the total abundance was higher, particularly at the beginning of the experiment (Table 3; Figure 1).

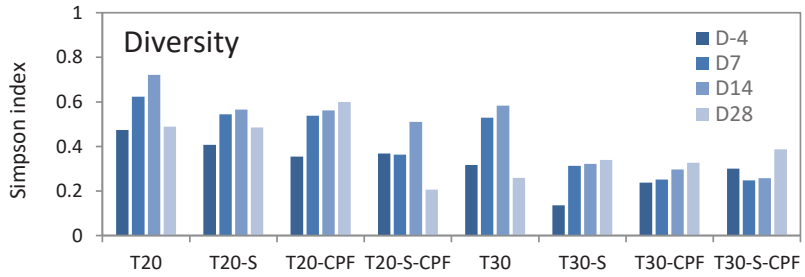
Table 2. Results of the PERMANOVA analysis performed with the zooplankton dataset. Bold values indicate significant effects (p-value<0.05) of the single stressors or their interactions. T: temperature, CPF: chlorpyrifos, S: salinity, D: days relative to the chlorpyrifos application.

Day	T	S	CPF	TxS	TxCPF	SxCPF	TxSxCPF
D−4	**<0.001**	0.152	0.181	**0.002**	0.066	0.512	0.182
D7	**<0.001**	**<0.001**	**<0.001**	**0.005**	**0.008**	0.365	0.639
D14	**<0.001**	**<0.001**	**<0.001**	**0.003**	0.303	0.296	0.710
D28	**<0.001**	**<0.001**	**0.005**	0.121	**0.002**	**0.04**	0.118

Table 3. Results of the three-way ANOVA (p-value) performed with the diversity index (Simpson), total abundance, and Cladocera, Copepoda and Rotifera abundances. Bold values indicate significant effects (p-value<0.05). T: temperature, CPF: chlorpyrifos, S: salinity, D: days relative to the chlorpyrifos application.

	T	S	CPF	TxS	TxCPF	SxCPF	TxSxCPF
Diversity							
D−4	**<0.001**	0.584	0.212	0.080	0.622	**0.023**	0.230
D7	**0.008**	**0.024**	0.073	0.761	0.891	0.651	0.237
D14	**<0.001**	**0.004**	**0.008**	0.450	0.599	0.077	0.511
D28	0.066	0.823	0.304	0.253	0.036	0.104	0.142
Total abundance							
D−4	**<0.001**	0.565	0.766	**0.022**	**0.023**	0.866	0.448
D7	0.642	0.454	**0.011**	**0.022**	**0.005**	0.375	0.652
D14	0.059	0.899	0.183	0.294	0.266	0.681	0.842
D28	0.625	0.146	0.052	0.838	0.763	**0.026**	0.118
Cladocera							
D−4	**0.003**	0.216	0.283	0.095	**0.001**	0.800	0.724
D7	**0.009**	**0.002**	**0.006**	**0.014**	0.578	**0.012**	0.056
D14	0.438	0.050	**0.003**	**0.045**	0.152	0.445	0.934
D28	0.068	0.486	0.117	0.451	0.154	0.787	0.937
Copepoda							
D−4	**<0.001**	0.611	0.895	**0.028**	**0.042**	0.767	0.723
D7	**0.037**	0.532	0.240	0.360	**0.001**	0.901	0.286
D14	0.059	0.899	0.183	0.294	0.266	0.681	0.842
D28	0.081	0.213	0.955	0.673	0.250	0.106	0.269
Rotifera							
D−4	**0.038**	0.449	0.471	0.105	0.139	**0.048**	**0.006**
D7	0.068	0.669	**<0.001**	**0.005**	0.713	0.905	0.676
D14	0.080	0.052	0.540	0.357	0.134	0.617	0.919
D28	**0.008**	0.462	**0.002**	0.431	**0.020**	0.901	0.126

The three−way ANOVA indicated a significant, or marginally significant, increase of Cladocera, Copepoda and Rotifera at 30 °C for the majority of sampling dates (Table 3; Figure 1).



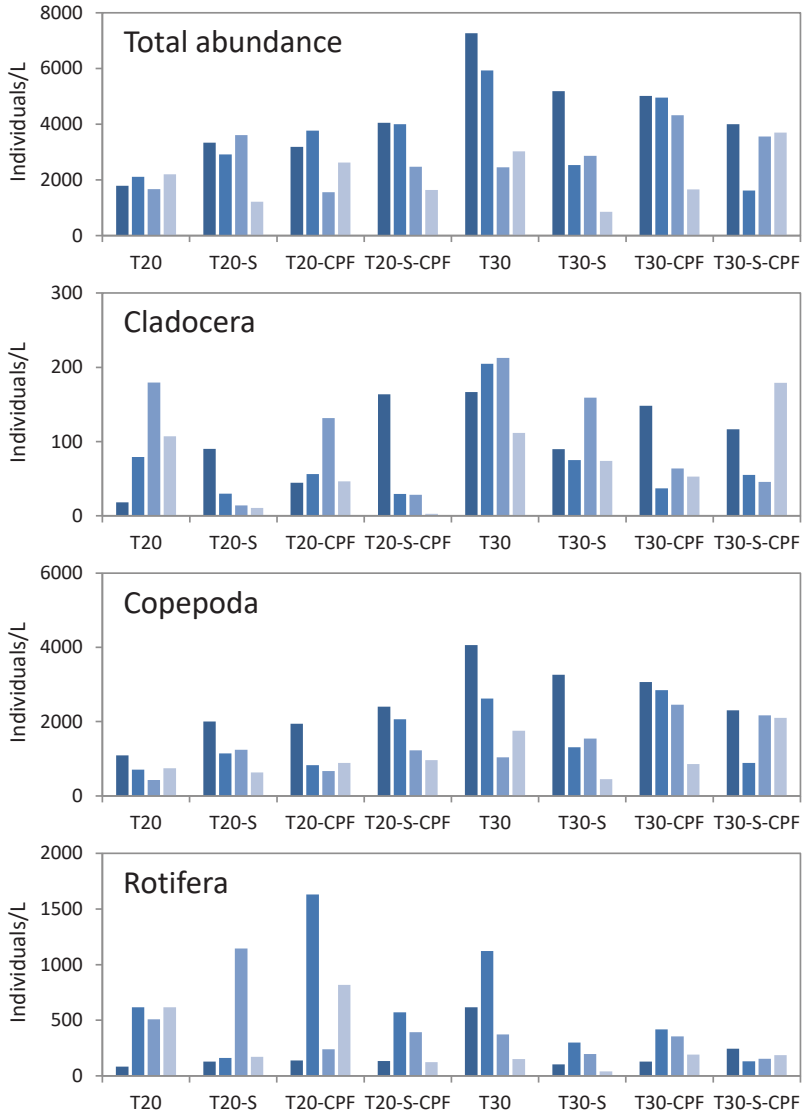


Figure 1. Average diversity, total abundance, and Cladocera, Copepoda and Rotifera abundance in the different treatments. T: temperature, CPF: chlorpyrifos, S: salinity, D: days relative to the chlorpyrifos application.

The PRC analysis performed with the temperature controls shows that temperature had a significant influence on the community structure (Table 4).

Table 4. Percentages of total variance attributed to time, differences between replicates and treatment regime in all PRC analyses performed in this study. The table also shows the fraction of treatment variance captured by the significant PRCs and the results of the Monte Carlo permutations (p−value). NS: not significant. T: temperature, CPF: chlorpyrifos, S: salinity.

Dataset	% of variance accounted for			% of variance explained by treatment regime captured by the different PRCs (p−value)		
	Time	Differences between replicates	Treatment	First PRC	Second PRC	Third PRC
T	46.0	52.4	1.6	60.6 (p=0.02)[a]	NS	NS
S at T20	21.2	57.4	21.4	64.8 (p=0.02)[b]	NS	NS
S at T30	19.7	46.5	33.8	63.5 (p=0.02)[b]	17.9 (p=0.04)[d]	NS
CPF at T20	33.5	51.6	14.9	59.8 (p=0.05)[b]	NS	NS
CPF at T30	29.6	48.9	21.5	66.9 (p=0.02)[b]	NS	NS
CPF and S at T20	43.5	49.9	6.7	36.5 (p<0.01)[c]	23.9 (p=0.03)[c]	NS
CPF and S at T30	45.7	45.3	9.0	45.7 (p<0.01)[c]	39.3 (p<0.01)[c]	12.9 (p=0.02)[d]

[a] Figure 2, [b] Figure 4, [c] Figure S3, [d] Not shown.

The taxa that showed a marked increase in abundance at 30 °C were *Brachionus falcatus*, *Diaphanosoma* sp. and Copepoda nauplii, while *Ascomorpha* sp. and *Moina* sp. showed a slight decrease as compared to the 20 °C treatment (Figures 2 and 3).

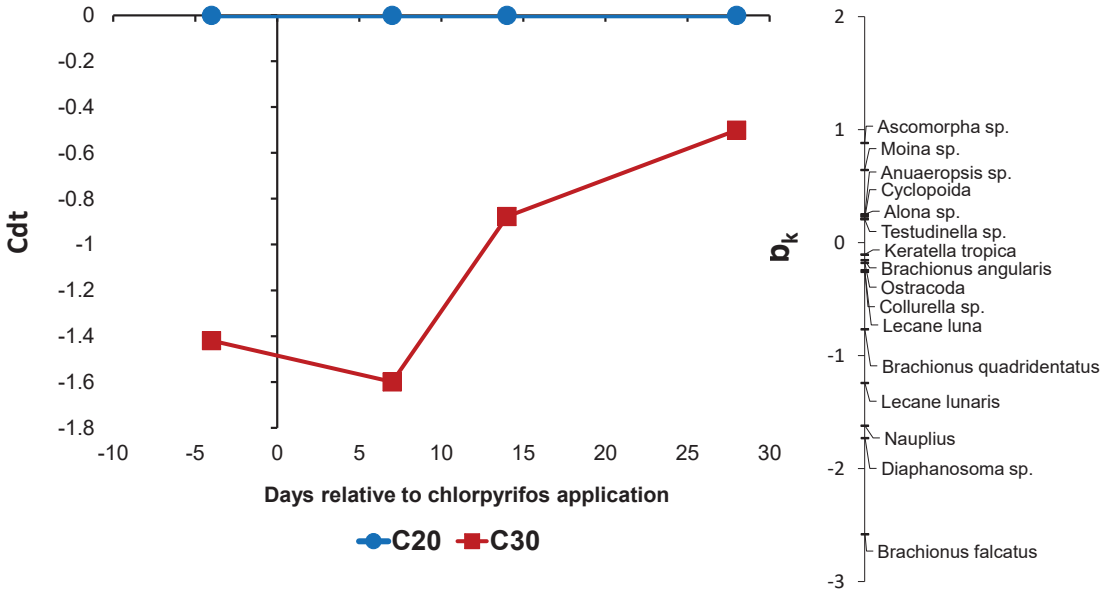


Figure 2. PRC calculated with the 20 °C and 30 °C replicates not receiving any chlorpyrifos or salinization treatment. The percentages of explained variance by time, differences between replicates and treatment regime are shown in Table 4. The raw data on taxa counts is provided in Appendix I.

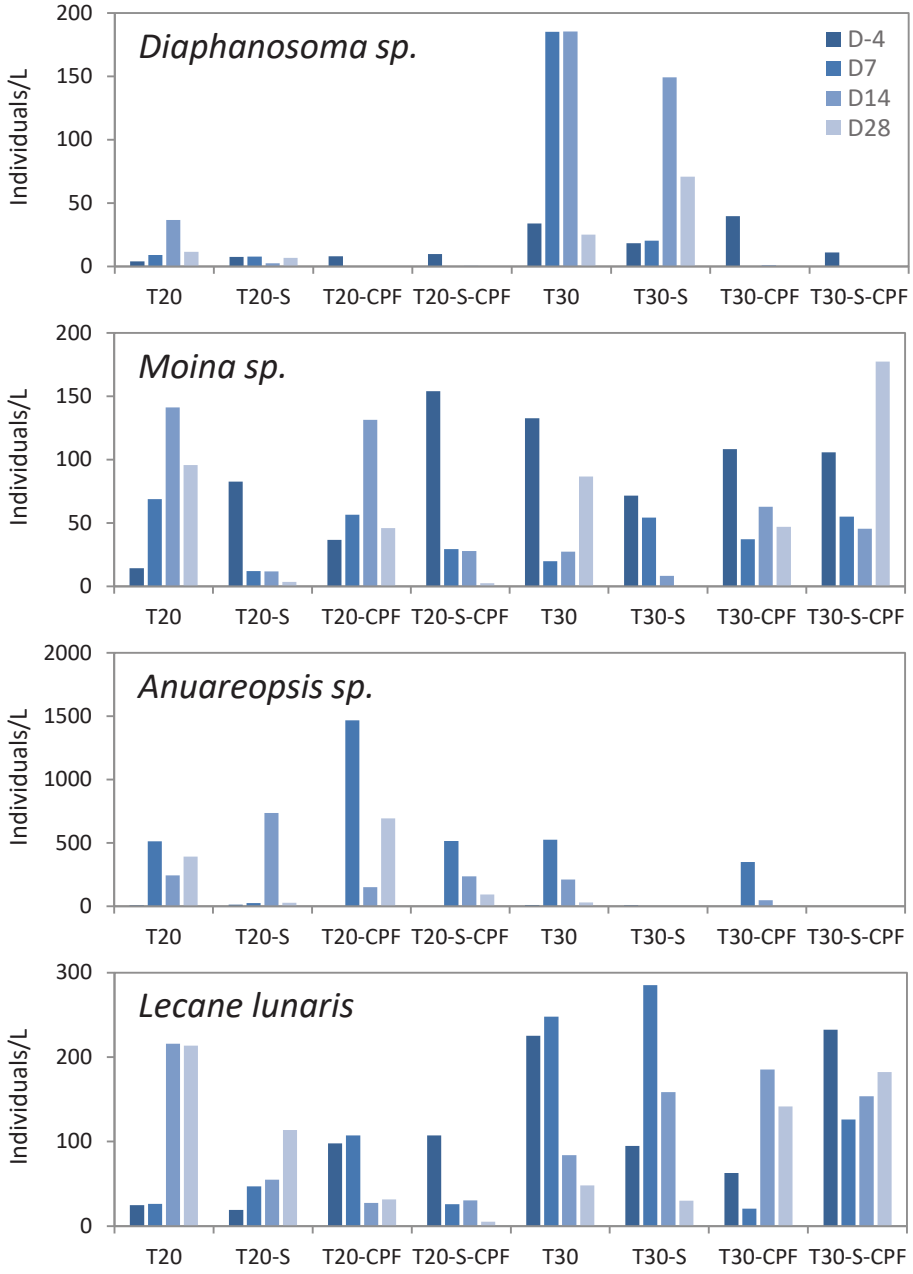


Figure 3. Abundance of *Diaphanosoma* sp., *Moina* sp., *Anuraeopsis* sp. and *Lecane lunaris* in the different treatments. T: temperature, CPF: chlorpyrifos, S: salinity, D: days relative to the chlorpyrifos application.

The PERMANOVA analysis showed that salinity significantly influenced the zooplankton community from day 7 relative to the CPF application until the end of the experiment (Table 2). The salinity treatment significantly reduced the zooplankton diversity and Cladocera abundance (Table 3, Figure 1). The interaction between temperature and salinity was significant during day −4 to day 14, indicating that the community effects of salinity were different at the different temperature regimes (Table 2). The temperature−salinity interaction was significant for total zooplankton abundance

at the beginning of the experimental period (day −4 and 7), and for Cladocera (day 7 and 14), Copepoda (day -4) and Rotifera (day −7; Table 3). At 20 °C, salinity resulted in a decrease on Cladocera and Rotifera and an increase of Copepoda, which resulted in an increase of total abundance. At 30 °C, salinity resulted in a decrease of Cladocera, Rotifera and Copepoda, which contributed to a significant abundance decline (Figure 1). The PRC analysis showed that, at 20 °C, salinity explained 21% of the variation (only the first PRC is significant), while at 30 °C it explained 34% (with the first and second PRCs being significant), suggesting that the impacts of salinity on zooplankton community changes at 30 °C were slightly larger than at 20 °C (Table 4). This is also observed in Figure 4, which shows that salinity effects persisted during the whole experimental period, but started earlier (some days before the CPF application) and were larger (particularly on days −4 and 7) at 30 °C than at 20 °C. At 20 °C, the PRC shows that *Anuraeopsis* sp. and *Moina* sp. decreased due to salt addition, while there was an increase of *Brachionis quadridentatus* and Copepoda nauplii. At 30 °C, the first PRC indicates a higher treatment-related decrease of *Anuraeopsis* sp. (Figure 3) and *Brachionus falcatus*, while there were no apparent population increases (all b_k values > -0.5). The second PRC shows a slight decrease of *Brachionus falcatus* and *Moina* sp. at the beginning and at the end of the experimental period (data not shown). The different response of *Moina* sp. and *Anuraeopsis* sp. to salinity in the different temperature treatments is also indicated by the significance of the three−way ANOVA interaction at the beginning and at the end of the experimental period, respectively (Table S3), confirming that *Moina* sp. had a larger population abundance decrease at 20 °C and *Anuraeopsis* sp. at 30 °C (Figure 3).

The PERMANOVA analysis showed that CPF affected the zooplankton community significantly from day 7 until the end of the experiment (Table 2), producing a (marginally) significant decrease of diversity and Cladocera abundance on day 7 and 14 (Table 3, Figure 1). The interaction between temperature and CPF was significant after the application (day 7) and at the end of the experiment (day 28; Table 2). The PRC analysis showed that CPF explained 15% of variability at 20 °C, and 21% at 30 °C (Table 4). At 20 °C, the maximum CPF effects were reached at end of the experimental period, while at 30 °C the maximum effects were already reached on day 7 and shows smaller differences towards the end of the experiment, indicating partial community recovery. At 20 °C, the CPF application resulted in a decline of *Lecane lunaris* and *Diaphanosoma* sp., and a slight increase of Copepoda nauplii and *Anuraeopsis* sp. However, at 30 °C, there was a decline of *Diaphanosoma* sp. and *Anuraeopsis* sp., and no clear treatment-

related increases (Figure 4). The different responses to the CPF treatment of *Diaphanosoma* sp., *Anuraeopsis* sp. and *Lecane lunaris* are also displayed in Figure 2, and the significant interactions are provided in Table S3.

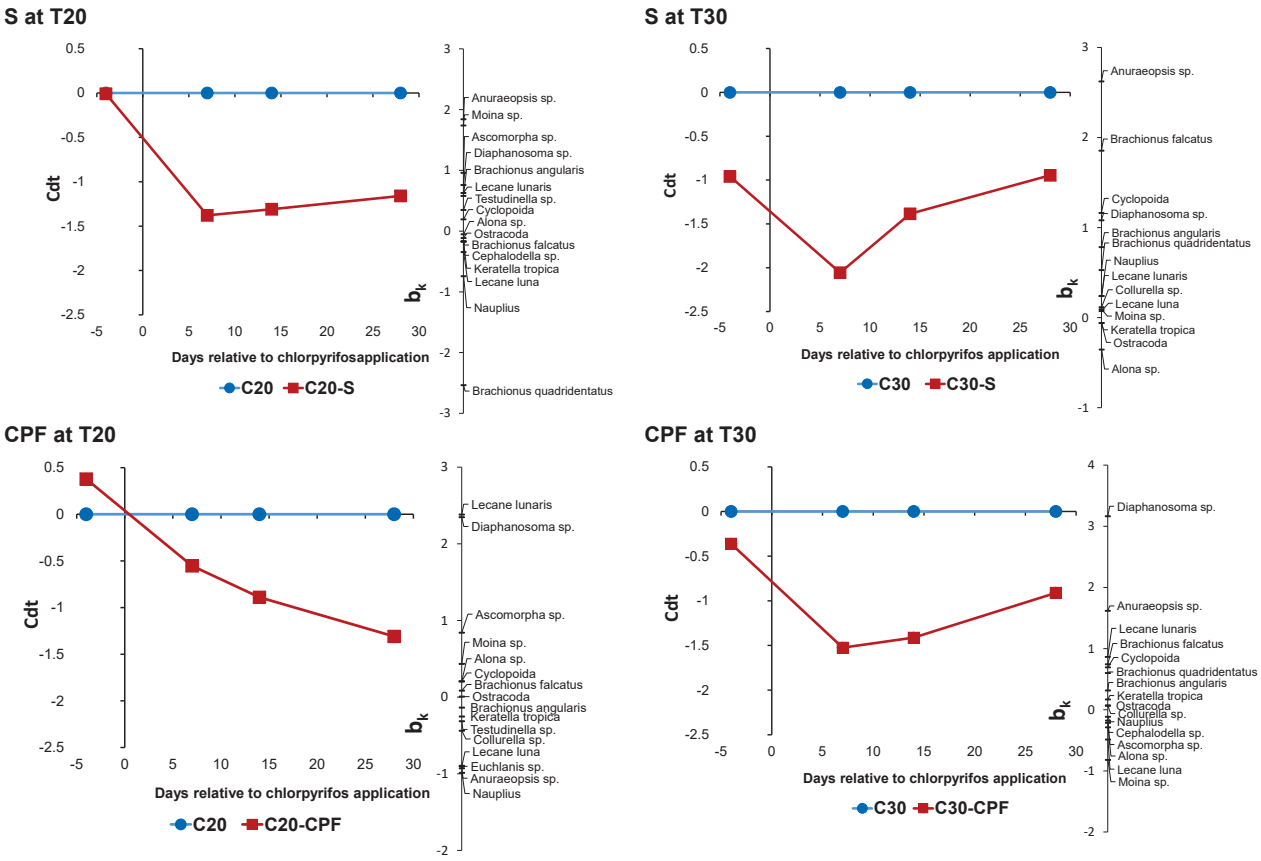


Figure 4. PRCs calculated with the chemical controls and the microcosms exposed to salinity (S) or chlorpyrifos (CPF) at 20 °C and 30 °C. The percentages of explained variance are shown in Table 4. The raw data on taxa counts is provided in Appendix I.

The PERMANOVA analysis showed that the interaction between salinity and CPF was generally not significant, except for the last sampling date, and the interaction between the three stressors was not significant at any sampling date (Table 2). However, the interaction between salinity and CPF was significant for Cladocera (Table 3) on day 7 after the CPF application (Table 3). The abundance of that taxonomic group, which was clearly dominated by *Diaphanosoma* sp., was somewhere in between the effects caused by salinity and CPF alone (Figure 1), so that it can be classified as antagonistic. *Diaphanosoma* sp. also showed significant interactive effects on that sampling date (Table S3), however the abundances were so low (Figure 3) that it was impossible to evaluate the response type.

The PRC analysis including the salinity and the CPF treatments at 20 °C and 30 °C showed that more than one PRC was significant (Table 4). As expected, by inspecting the first and second PRC it can be concluded that the majority of species responded similarly to the combination of both stressors at 20 °C and 30 °C, except for *Moina* sp. (Figure S3), which showed a clear decline at 20 °C and a notable increase at 30 °C when both stressors (i.e., salinity and CPF) were present (Figure 3). This is also supported by the marginally significant p-value calculated by the three-way ANOVA for the salinity and CPF interaction, although the triple interaction was not significant (Table S3). Overall, the variation partitioning analysis indicated that the influence of salinity was larger than the influence of CPF on the zooplankton community at 20 °C, while the contribution of each stressors was similar at 30 °C (Table S2). Moreover, the first PRC of the combination of both stressors suggests that the community effects of salinity and CPF are more strongly correlated at 30 °C than at 20 °C (Figure S3).

Discussion

CPF dissipation and water quality variables

In this study, temperature notably enhanced CPF dissipation, while salinity contributed to a slightly reduced dissipation rate. Microbial activity associated to temperature variation has been discussed to be one the main factors that affects the dissipation of CPF from the water column (Racke, 1993), although other factors such as turbidity, organic matter (including algae) and sediment characteristics can also play an important role (Daam and Van den Brink, 2009). In an outdoor mesocosm performed under Mediterranean conditions, López-Mancisidor et al. (2008) calculated an average DT50 of 2.2 days (average water temperature was 20 °C), which is similar to the results of our study. The slight differences related to salinity may be explained by decreased hydrolysis rates (Wang and Hoffman 1991), and by the lower algae density in the water column observed in the salinity treatment (Table 1).

The assessment of the water quality variables indicated a decrease of chlorophyll-*a* at 30 °C and dissolved oxygen. The increase of temperatures resulted in an increase of zooplankton density, which could have contributed to an increasing grazing pressure on the phytoplankton community. Furthermore, salinity seemed to contribute to a reduction of phytoplankton. In our study, the levels of ammonia were increased by temperature and salinity, probably due to accelerated organic matter decomposition of decaying planktonic organisms. In fact, we observed a flocculation and sedimentation of algae in

these microcosms, which has been described as a response to biotic and abiotic stress (Sukenik and Shelef, 1984; Brady et al., 2014). The levels of unionized ammonia reached in the 30 °C microcosms, and particularly in those also treated with salt and CPF, are expected to result in toxic effects on sensitive aquatic organisms (Arthur et al., 1987) and may thus have contributed to the changes observed in the zooplankton community. It should also be noted that the water sampled for nutrient analysis was relatively high as compared to the total water volume of the microcosms (8%), so that there could be a slight impact created by this on later sampling days (e.g. day 28) which may have affected the natural dynamics of the planktonic communities in all microcosms.

Zooplankton responses to varying temperatures

Our experiment indicated that temperature was the most important factor influencing the zooplankton community, followed by salinity and CPF. Changes in water temperature, related to seasonality or climate change, have been reported as an important factor modifying the composition, structure and dynamics of zooplankton communities in wetland ecosystems, and hence, on ecosystem processes and services (Thompson et al., 2012; Gabler et al., 2017). In the present study, we show that at 30 °C there was an abundance increase of all zooplankton groups (Cladocera, Copepoda and Rotifera). For many zooplankton taxa, temperature is considered an important factor that affects their metabolic rate and the amount of energy allocated for egg production, hence, changing their life−history traits and population growth (Gillooly et al., 2001; Devreker et al., 2009). The taxa *Brachionus falcatus*, *Diaphanosoma* sp. and copepoda nauplii had an increased abundance at the beginning of the experiment at 30 °C, indicating a preference for high water temperatures. On the other hand, *Ascomorpha* sp. and *Moina* sp. abundance slightly decreased as response to the temperature increase. We also observed that community structure differences between the 20 °C and 30 °C controls were larger at the beginning of the experiment than at the end (Figure 2), indicating a potential adaptation of some taxa to the new thermal scenario. According to the existing literature (Beaugrand et al., 2002; Arenas−Sánchez et al., 2019), higher temperatures favor the dominance of smaller organisms, but may also result in a favorable condition for competition and predation among smaller zooplankton groups.

Zooplankton responses to salinity at different temperatures

At 20 °C, salinity increase induced a decrease in zooplankton diversity and total abundance. Salinity also resulted in a significant decrease of Cladocera individuals and produced an increase of Copepoda (at Nauplius stages). This is in good agreement with the mesocosm study peformed by Thompson and Shurin (2012), who observed a significant increase of cyclopoid copepods after a slight salinity increase (0.3 ppt). Some authors have argued that such structural changes in the zooplankton community are characteristic of brackish lagoons during high osmotic pressure periods, and are a consequence of reduced fitness of Cladocera and competitive exclusion by well adapted copepods (Brucet et al., 2009; Jensen et al., 2010). Other studies have demonstrated that short rises of salinity can result in the dominance of few Rotifera taxa (Schallenberg et al., 2003; Anton−Pardo and Armengol, 2012; Coldsnow et al., 2017). Our experiment shows that at 20 °C, salinity resulted in a notable increase of *Brachionus quadridentatus* and a decrease of *Anuraeopsis* sp. (Figure 4). This can be explained by the low tolerance of *Anuraeopsis* taxa to osmotic pressure as compared to *Brachionus*, as demonstrated by the growth curves calculated by Sarma et al. (2006) for these two genera under laboratory conditions. In addition, Anton−Pardo and Armengol (2012) found a high prevalence of *Brachionus* species in brackish coastal Mediterranean ponds, while *Anuraeopsis fissa* was one of the few Rotifera taxa that were distributed among ponds irrespective of salinity levels.

Our experiment shows a significant interaction between temperature and salinity on the zooplankton community. In fact, under warmer conditions (30 °C), salinity had a higher impact on the zooplankton community, reducing the abundance of all the taxonomic groups. It should be noted, however, that zooplankton abundances were in the majority of the cases still above the ones in the salinity treatments at 20 °C because, as described above, temperature resulted in high zooplankton densities. For some particular taxa (e.g., *Moina* sp*., Anuraeopsis* sp.) salinity-related declines showed considerable differences between the two tested temperature conditions, which may be related to different metabolic rates and tolerances to osmotic pressure (Miracle and Serra, 1989), as well as the availability of phytoplankton resources and the presumable toxicity caused by unionized ammonia. Therefore, this study confirms that temperature influences the direct and indirect effects of salinity to the zooplankton community, so that seasonal temperature variation and climate change may significantly interact with the ecological

effects caused by salinity. In addition, some studies have reported that the shift in the structure of zooplankton communities caused by salinity intrusion in coastal lagoons, leading towards a lower zooplankton abundance or the dominance of smaller and less efficient zooplanktonic grazers, may result in a functional diversity loss and increasing eutrophication risks (Jeppesen et al., 2015; Gutierrez et al., 2018).

Zooplankton responses to CPF at different temperatures

In the present study, CPF reduced zooplankton diversity, mainly affecting Cladocera. Similar results were observed in other model ecosystem experiments performed under Mediterranean-like conditions (Van Wijngaarden et al., 2005; López−Mancisidor et al., 2008; Pereira et al. 2017). At 30 °C, we found larger community effects in the first week after the insecticide application, but also partial recovery as compared to the 20 °C treatment. This is in line with previous studies, which demonstrated a large influence of temperature in the response of aquatic organisms to pesticide stress (Patra et al., 2015; Rocha et al., 2016; Pawar et al., 2020), which affects community structure dynamics. For example, Arenas-Sánchez et al. (2019) found that the time to onset of effects caused by the insecticide lufenuron and the post-exposure recovery of zooplankton populations and communities was considerably faster at 28 °C as compared to 20 °C.

Our study also shows that the indirect effects caused by CPF were influenced by temperature. At 20 °C, the reduction of Cladocera (mainly *Diaphanosoma* sp.) resulted in an increase of some Rotifera and Copepoda taxa. This caused a temporary increase of total abundance after the insecticide application at 20 °C and indirect negative effects on chlorophyll-*a*. At 30 °C, such indirect increases in abundance were not observed, potentially due to the algae flocculation described above. In our study, the observed indirect effects were relatively mild, however they should not be neglected as these can result in long-term changes on the structure of aquatic communities and potentially affect predator-prey relationships at higher trophic levels (e.g., macroinvertebrates, fish; Thompson et al., 2015).

Zooplankton responses to salinity and CPF under different temperatures

Contrary to previous studies indicating synergistic effects of salinity and CPF on freshwater invertebrates (Song and Brown, 1998), our study shows that the interaction between these two stressors was generally not significant during the period of maximum toxicant effects (first week). Neither it was the triple interaction including temperature. The joint effects of salinity and CPF on the most sensitive taxonomic group (Cladocera)

were found to be antagonistic, possibly due to the complex bound formation of CPF with salts and the resulting slightly reduced bioavailability (Maryoung et al., 2014). In line with this, Pawar et al. (2020) observed a reduction in the toxicity of CPF to the euryhaline white leg shrimp under higher salinity, and other studies report similar findings testing these stressor combinations on fish survival (Lavado et al., 2009; Maryoung et al., 2014) or assessing biochemical responses (Durieux et al., 2011).

Overall, our study shows that, at the tested stressor levels, salinity is a more severe stressor at 20 °C than CPF; while at 30 °C, both had similar impacts and more correlated responses. This has implications for ecosystem management and suggests that under future climate change scenarios the influence of salinity on zooplankton communities can be confounded with that caused by temperature increase. Moreover, this study shows that the interaction between increasing temperatures, salinity and insecticide stress could contribute to a biodiversity decline in an additive manner. Further research should be dedicated to assess the interaction between other stressor combinations related to global change on Mediterranean wetlands and to explore their consequences for ecosystem service provision and management.

Acknowledgments

This study is part of the CICLIC project (Smart tools and technologies to assess the environmental fate and risks of Contaminants under Climate Change), funded by the Spanish Ministry of Science, Innovation and Universities (RTI2018−097158−A−C32). The PhD of Jéssica Andrade Vilas−Boas was sponsored by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior − Brasil (CAPES) − Finance Code 001 (fellowship to JAVB) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Bolsa de Produtividade 2018) of Brazil (fellowships to RJPD). Andreu Rico is supported by a postdoctoral grant provided by the Spanish Ministry of Science, Innovation and University (IJCI−2017−33465). We thank the Valencian Agricultural Ministry for providing salinity and temperature data in the Albufera lagoon, and the Albufera´s Natural Park Office for allowing zooplankton collection and for providing the corresponding permits.

References

American Public Health Association, 2005. Standard Methods for the Examination of Water and Wastewater. American Water Works Association/American Public Works

Association/Water Environment Federation. https://doi.org/10.2105/AJPH.51.6.940−a

Anderson, M.J., 2001. A new method for non−parametric multivariate analysis of variance. Austral Ecol. 26, 32−46. https://doi.org/10.1111/j.1442−9993.2001.01070.pp.x

Anton−Pardo, M., Armengol, X., 2012. Effects of salinity and water temporality on zooplankton community in coastal Mediterranean ponds. Estuar. Coast. Shelf Sci.114, 93−99. https://doi.org/10.1016/j.ecss.2011.08.018

Arenas-Sánchez, A., López-Heras, I., Nozal, L., Vighi, M. and Rico, A., 2019. Effects of increased temperature, drought, and an insecticide on freshwater zooplankton communities. Environ. Toxicol. Chem. 38, 396−411. https://doi.org/10.1002/etc.4304

Arthur, J.W., West, C.W., Allen, K.N., Hedtke, S.F., 1987. Seasonal toxicity of ammoniato five fish and nine invertebrate species. Bulletin of Environmental Contamina-tion and Toxicology 38, 324–331.

Barbier, E.B., 2011. Wetlands as natural assets. Hydrolog. Sci. J. 56, 1360−1373. https://doi.org/10.1080/02626667.2011.629787

Bassi, N., Dinesh Kumar, M., Sharma, A., Pardha−Saradhi, P., 2014. Status of wetlands in India: A review of extent, ecosystem benefits, threats and management strategies. J. Hydrol. Reg. Stud. 2, 1−19. https://doi.org/10.1016/J.EJRH.2014.07.001

Beaugrand, G., Reid, P.C., Ibañez, F., Lindley, A., Edwards, M., 2002. Reorganization of North Atlantic Marine Copepod Biodiversity and Climate. Science. 296, 1692-1694. https://doi.org/10.1126/science.1071329

Bracewell, S., Verdonschot, R.C.M., Rafl, C.M., Schäfer, R.B., Bush, A., Lapen, D.R., Van den Brink, P.J., 2019. Qualifying the effects of single and multiple stressors on the food web structure of Dutch drainage ditches using a literature review and conceptual models. Sci. Total. Environ. 684, 727-740. https://doi.org/10.1016/j.scitotenv.2019.03.497

Brady, P.V., Phillip, I., Hewson, J.C., 2014. A coordination chemistry model of algal autoflocculation. Algal Res. 5, 226−230. https://doi.org/10.1016/j.algal.2014.02.004

Brucet, S., Boix, D., Gascón, S., Sala, J., Quintana, X.D., Badosa, A., 2009. Species richness of crustacean zooplankton and trophic structure of brackish lagoons in contrasting climate zones: north temperate Denmark and Mediterranean Catalonia (Spain). Ecography, 32, 692−702. https://doi.org/10.1111/j.1600−0587.2009.05823.x

Cairns, J., Jr., Heath, A.G., Parker, B.C., 1975. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia. 47, 135−171.

Cañedo−Argüelles, M., Hawkins, C.P., Kefford, B.J., Schafer, R.B., Dyack, B.J., Crucet, S., Buchwalter, D., Dunlop, J., Fror, O., Lazorchak, J., Coring, E., Fernadez, H.R., Goodfellow, W., González Achem, A.L., Hatfield−Dodds, S., Karimov, B.K., Mensah, P., Olson, J.R., Piscart, C., Prat, N., Ponsá, S., 2016. Saving freshwater from salts. Science. 351, 914−916. https://doi.org/10.1126/science.aad3488

Cañedo−Argüelles, M., Schafer, R.B., Kefford, B., 2019. Salt in freshwaters: Causes, effects and prospects − Introduction to the theme issue. Philos T. R. Soc. 374, 1−6. https://doi.org/10.1098/rstb.2018.0002

Ccanccapa−Cartagena, A., Picó, Y., Ortiz, X., Reiner, E.J., 2019. Suspect, non−target and target screening of emerging pollutants using data independent acquisition: Assessment of a Mediterranean River basin. Sci. Total Environ. 687, 355−368. https://doi.org/10.1016/j.scitotenv.2019.06.057

Clarke, K.R., Gorley, R.N., 2015. Getting started with PRIMER v7. Primer−E. Plymouth, Plymouth Marine Laboratory, p.20.

Coldsnow, K.D., Mattes, B.M., Hintz, W.D., Relvea, R. A., 2017. Rapid evolution of tolerance to road salt in zooplankton. Environ. Pollut. 222, 1−7. https://doi.org/10.1016/j.envpol.2016.12.024

Cortina−Puig, M., Istamboulie, G., Marty, J.L., Noguer, T., 2010. Analysis of pesticide mixtures using intelligent biosensors. INTECH Open Access Publisher. https://doi.org/10.5772/7157

Daam, M.A., Van den Brink, P.J., 2009. Implications of differences between temperate and tropical freshwater ecosystems for the ecological risk assessment of pesticies. Ecotoxicology, 19, 21−37. https://doi.org/10.1007/10.1007/s10646-009-0402-6

Devreker, D., Souissi, S., Winkler, G., Forget−Leray, J., Loboulenger, F., 2009. Effects of salinity, temperature and individual variability on the reproduction of *Eurytemora affinis* (Copepoda; Calanoida) from the Seine estuary: A laboratory study. J. Exp. Mar. Biol. Ecol. 368, 113−123. https://doi.org/10.1016/j.jembe.2008.10.015

Durieux, E.D.H., Farver, T.B., Fitzgerald, P.S., Eder, K.J., Ostrach, D.J., 2011. Natural factors to consider when using acetylcholinesterase activity as neurotoxicity biomarker in Young-Of-Year striped bass (*Morone saxatilis*). Fish Physiol. Biochem. 37, 21-29. https://doi.org/10.1007/s10695-010-9412-9

EC, European Commission 2020. Commission Implementing Regulation (EU) 2020/18 of 10 January 2020 concerning the non-renewal of the approval of the active substance chlorpyrifos, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market, and amending the Annex to Commission Implementing Regulation (EU) No 540/2011.

Gabler, A.C., Osland, M.J., Grace, J.B., Stagg, C.L., Day, R.H., Stephen, B.H., Enwright, N.M., From, A.S., McCoy, M.L., McLeod, J.L., 2017. Macroclimatic change expected to transform coastal wetland ecosystems this century. Nat. Clim. Change. 7, 142−417. https://doi.org/10.1038/nclimate3203

Gillooly, J.F., Brown, J.H., West, G.B., Savage, V.M., Charnov, E.L., 2001. Effects of size and temperature on metabolic rate. Science. 293, 2248−2251. https://doi.org/10.1126/science.1061967

Gutierrez, M.F., Tavsanoglu, U.N., Vidal, N., Yu, J., Mello, F.T., Çakiroglu, A., He, H., Liu, Z., Jeppesen, E., 2018. Salinity shapes zooplankton communities and functional diversity and has complex effects on size structure in lakes. Hydrobiologia. 813, 237−255. https://doi.org/10.1007/s10750−018−3529−8

Hall, L.W., Jr., Anderson, R.D., 1995. The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. Crit. Rev. Toxicol. 25, 281−346. https://doi.org/10.3109/10408449509021613

Heugens, E.H.W., Hendriks, A.J., Dekker, T., van Straalen, N.M., Admiraal, W., 2001. A Review of the Effects of Multiple Stressors on Aquatic Organisms and Analysis of Uncertainty Factors for Use in Risk Assessment. Crit. Revi.Toxicol. 31, 247−284. https://doi.org/10.1080/20014091111695

Hintz, W.D., Jones, D.,K., Relyea, R., 2018. Evolved tolerance to freshwater salinization in zooplankton: life−history trade−offs, cross−tolerance and reducing cascading effects. Philos. T. R. Soc. B. 374, 20180012. https://doi.org/10.1098/rstb.2018.0012

Huang, X., Cui, H., Duan, W., 2020. Ecotoxicity of chlorpyrifos to aquatic organisms: A review. Ecotox. Environ. Safe. 200, 110731. https://doi.org/10.1016/j.ecoenv.2020.110731

Intergovernmental Panel on Climate Change. 2014. Climate change 2014: Synthesis report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Geneva, Switzerland.

Jackson, M.C. 2018. Multiple stressors in freshwater ecosystems: biocides and climate change. Freshw. Biol. 27, 2−10. https://doi.org/10.1111/j.1365−2427.2009.02395.x

Jeppesen, E., Brucet, S., Naselli−Flores, L., Papastergiadou, E., Stefanidis, K., Noges,T., Noges, P., Attayde, J.L., Zohary, T., Coppens, J., Bucak, T., Fernandes Menezes,R., Sousa Freitas, F.R., Kernan, M., Sondergaard, M., Beklioglu, M., 2015. Ecological impacts of global warming and water abstraction on lakes and reservoirs due to changes in water level and related changes in salinity. Hydrobiologia, 750, 201−227. https://doi.org/10.1007/s10750−014−2169−x

Jensen, E., Brucet, S., Meerhoff, M., Nathansen, L., Jeppesen, E., 2010. Community structure and diel migration of zooplankton in shallow brackish lakes: role of salinity and predators. Hydrobiologia, 646, 215−229.

Klausmeyer KR, Shaw MR., 2009. Climate change, habitat loss, protected areas and the climate adaptation potential of species in Mediterranean ecosystems worldwide. PloS One. 4, e6392. https://doi.org/10.1371/journal.pone.0006392

Lavado, R., Rimoldi, J.M., Schlenk, D., 2009. Mechanisms of fenthion activation in rainbow trout (*Oncorhynchus mykiss*) acclimated to hypersaline environments. Toxicol. Appl. Pharmacol. 235, 143-152. https:// 10.1016/j.taap.2008.11.017

Liu, J.G., Diamond, J., 2005. China's environment in a globalizing world. Nature. 435, 1179−1186. https://doi.org/10.1038/4351179a

López−Mancisidor, P., Carbonell, G., Marina, A., Fernández, C., Tarazona, J.V., 2008. Zooplankton community responses to chlorpyrifos in mesocosms under Mediterranean conditions. Ecotoxicol. Environ. Safe. 71, 16−25. https://doi.org/10.1016/j.ecoenv.2007.06.006

Mantyka−Pringl, C.S., Martin, T.G., Rhodes, J.R., 2012. Interactions between climate and habitat loss effects on biodiversity: a systematic review and meta−analysis. Glob. Change Biol. 18, 1239−1125. https://doi.org/10.1111/j.1365−2486.2011.02593.x

Mantyka−Pringl, C.S., Martin, T.G., Moffatt, D.B., Linke, S., Rhodes, J.R., 2014. Understanding and predicting the combined effects of climate change and land−use change on freshwater macroinvertebrates and fish. J. Appl. Ecol. 51, 572−581. https://doi.org/0.1111/1365−2664.12236

Maryoung, L.A., Lavado, R., Schlenk, D., 2014. Impacts of hypersaline acclimation on the acute toxicity of the organophosphate chlorpyrifos to salmonids. Aquat. Toxicol. 152, 284-290. https://doi.org/10.1016/j.aquatox.2014.04.017

McLusky, D.S., Bryant, V., Campbell, R., 1986. The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. Oceanogr. Mar. Biol. 24, 481−520.

Millán, A., Velasco, J., Gutiérrez−Cánovas, C., Arribas, P., Picazo, F., Sánchez−Fernández, D., Abellán, P., 2011. Mediterranean saline streams in southeast Spain: What do we know? J. Arid Environ. 75, 1352−1359. https://doi.org/10.1016/j.jaridenv.2010.12.010

Miracle, M.R., Serra, M., 1989. Salinty and temperature influence in rotifer life history characteristics. In Rotifer Symposium V (pp. 81-102). Springer, Dordrecht.

Moreno−González, R., León, V.M., 2017. Presence and distribution of current−use pesticides in surface marine sediments from a Mediterranean coastal lagoon (SE Spain). Environ. Sci. Pollut. Res. 24, 8033−8048. https://doi.org/10.1007/s11356−017−8456−0

Navarro−Ortega, A., Acuña, V., Batalla, R.J., Blasco, J., Conde, C., Elorza, F.J., Elosegi, A., Francés, F., La−Roca, F., Muñoz, I. and Petrovic, M., 2012. Assessing and forecasting the impacts of global change on Mediterranean rivers. Environ. Sci. Pollut. Res. 19, 918−933. https://doi.org/10.1007/s11356−011−0566−5

Nielsen, D.L., Brock, M.A., Rees, G.N., Baldwin, D.S., 2003. Effects of increasing salinity on freshwater ecosystem in Australia. Aust. J. Bot. 51, 655−665. https://doi.org/10.1071/BT02115

OECD, Organisation for Economic Co−operation and Development, 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. Series on Testing and Assessment, No. 23. ENV/JM/MONO (2000)6. Paris, France.

OECD, 2006. Organisation for Economic Co-operation and Development. Freshwater alga and cyanobacteria growth inhibition test. Guideline 201. Paris, France.

Osland, M.J., Enwright, N.M., Day, R.H., Gabler, C.A., Stagg, C.L., Grace, J.B., 2016. Beyond just sea−level rise: considering macroclimatic drivers within coastal wetland vulnerability assessments to climate change. Glo. Change Biol. 22, 1−11. https://doi.org/10.1111/gcb.13084

Patra, R.W., Chapman, J., Lim, R.P., Gehrke, P.C., Sunderam, R.M., 2015. Interactions between water temperature and contaminant toxicity to freshwater fish. Environ. Toxicol. Chem. 34, 18091817. https://doi.org/10.1002/etc.2990

Pawar, A.P., Sanaye, S.V., Shyama, S., Sreepada, R.A., Dake, A.S., 2020. Effects of Salinity and Temperature on the Acute Toxicity of the Pesticides, Dimethoate and Chlorpyrifos in Post−larvae and Juveniles of the Whiteleg Shrimp. Aquacult. Rep. 16, 100240. https://doi.org/10.1016/j.aqrep.2019.100240

Pereira, A.S., Cerejeira, M.J., Daam, M.A., 2017. Toxicity of environmentally realistic concentrations of chlorpyrifos and terbuthylazine in indoor microcosms. Chemosphere, 128, 348−355. https://doi.org/10.1016/j.chemosphere.2017.05.032

Pérez−Ruzafa, A., Marcos, C., Pérez−Ruzafa, I., Pérez−Marcos, M., 2010. Coastal lagoons: ‘‘transitional ecosystems” between transitional and coastal waters. J. Coast. Conserv. 15, 369−392. https://doi.org/10.1007/S11852−010−0095−2

Pérez−Ruzafa, A., Marcos, C., Pérez−Ruzafa, I.M., 2011. Mediterranean coastal lagoons in an ecosystem and aquatic resources management context. Phys. Chem. Earth. 36, 160−166. https://doi.org/10.1016/j.pce.2010.04.013

Peres−Neto, P.R., Legendre, P., Dray, S., Borcard, D., 2006. Variation partitioning of species data matrices: estimation and comparison of fractions. Ecology. 87, 2614−2625. https://doi.org/10.1890/0012−9658(2006)87[2614:VPOSDM]2.0.CO;2

Picó, Y., Alvarez−Ruiz, R., Alfarhan, A.H., El−Sheikh, M.A., Alshahrani, H.O., Barceló, D., 2019. Pharmaceuticals, pesticides, personal care products and microplastics contamination assessment of Al−Hassa irrigation network (Saudi Arabia) and its shallow lakes. Sci.Total Environ. 701, 135021. https://doi.org/10.1016/j.scitotenv.2019.135021

Racke, K.D., 1993. Environmental fate of chlorpyrifos. Rev. Environ. Contam. Toxicol, 131, 1−154.

Rico, A., Dafouz, R., Vighi, M., Rodríguez-Gil, J.L. and Daam, M.A., In press. Use of post-registration monitoring data to evaluate the ecotoxicological risks of pesticides to surface waters: a case study with chlorpyrifos in the Iberian Peninsula. Environmental Toxicology and Chemistry. https://doi.org/10.1002/etc.4927

Rocha, A.C.S., Reis−Henriques, M.A., Galhano, V., Ferreira, M., Guimaraes, L., 2016. Toxicity of seven priority hazardous and noxious substances (HNSs) to marine organisms: current status, knowledge gaps and recommendations for future research. Sci. Total Environ. 542, 728−749. https://doi.org/10.1016/j.scitotenv.2015.10.049

Rojo, C., Carramiñana, M., Cócera, D., Roberts, G.P., Puche, E., Calero, S., Rodrigo, M.A. 2017. Different responses of coexisting Chara species to foreseeable Mediterranean temperature and salinity increases. Aquat. Bot. 138, 53−63. https://doi.org/10.1016/j.aquabot.2017.01.003

Rokneddine, A., Chentoufi, M., 2004. Study of salinity and temperature tolerance limits regarding four crustacean species in a temporary salt water swamp (Lake Zima, Morocco). Anim. Biol. 54, 237−253. https://doi.org/10.1016/10.1163/1570756042484719

Romo, S., Villena, M.., Sahuwuillo, M., Soria, J.M., Giménez, M., Alfonso, T., Vicente, E., Miracle, M.R., 2005. Response of a shallow Mediterranean lake to nutriente diversion: does it follow similar patterns as in northern shallow lakes? Freshw. Biol. 50, 1706−1717. https://doi.org/10.1111/j.1365−2427.2005.01432.x

Romo S., Soria J., Olmo C., Flor J., Calvo S., Ortells R., 2016. Nutrients and carbon in some Mediterranean dune ponds. Hydrobiologia (2016) 782:97–109. https://DOI 10.1007/s10750-016-2692-z

Simpson, E.H., 1949. Measurement of diversity. Nature, 163(4148): 688-688.

Sarma S. S. S., Nandini S., Morales−Ventura J., Delgado−Martinez I., Gonzalez−Velverde, L., 2006. Effects of NaCl salinity on the population dynamics of freshwater zooplankton (rotifers and cladocerans). Aquatic Ecol. 40, 349, 2006. https://doi.org/10.1007/s10452−006−9039−1

Şahin, M.D., Aybek, E.C., 2019. Jamovi: An Easy to Use Statistical Software for the Social Scientists. International Journal of Assessment Tools in Education, 6, 670−692.

Schallenberg M., Hall C.J., Burns C.W., 2003. Consequences of climate−induced salinity increases on zooplankton abundance and diversity in coastal lakes. Mar. Ecol. Prog. Ser. 251, 181−189. https://doi.org/10.3354/meps251181

Song, M.Y. and J.J. Brown., 1998. Osmotic effects as a factor modifying insecticide toxicity on Aedes and Artemia. Ecotoxicol. Environ. Safe. 41, 195−202. https://doi.org/10.1006/eesa.1998.1693

Sukenik, A., Shelef, G., 1984. Algal Autof Iocculation-Verif ication and Proposed Mechanism. Biotechnol. Bioeng. 26, 142–147. https://doi.org/doi:10.1002/bit.260260206

Ter Braak, C.J.F., Šmilauer, P., 2012. Canoco Reference Manual and User's Guide: Software for Ordination (Version 5.0). Microcomputer Power, Ithaca, NY, USA, 496pp.

Thompson, P.L., Shurin, J., 2012. Regional zooplankton biodiversity provides limited buffering of pond ecosystems against climate change. J. Anim. Ecol. 81, 251−259. https://doi.org/10.1111/j.1365−2656.2011.01908.x

Thompson, M.S.A., Bankier, C., Bell, T., Dumbrell, A.J., Gray, C., Ledger, M.E., Lehmann, K., McKew, B.A., Sayer, C.D., Shelley, F., Trimmer, M., Warren, S.L., Woodward, G., 2015. Gene−to−ecosystem impacts of a catastrophic pesticide spill: testing a multilevel bioassessment approach in a river ecostystem. Freshw. Biol. 61, 2037−2050. https://doi.org/10.1111/fwb.12676

Valencian Agricultural Ministry, 2020. Coselleria d'Agricultura, Desenvolupament Rural, Emergència Climàtica i Transició Ecològica. Monitoring program of wetland ecosystems. Available at: http://www.agroambient.gva.es/es/web/espacios-naturales-protegidos/programa-de-seguimiento-de-zonas-humedas (Accessed on the 4th of September of 2019).

Van den Brink, P.J., Ter Braak, C.J.F., 1999. Principal response curves: analysis of time dependent multivariate responses of biological community to stress. Environ. Toxicol.and Chem. 18, 138−148. https://doi.org/10.1002/etc.5620180207

Van Wijngaarden, R.P.A., Brock, T.C.M., Douglas, M.T., 2005. Effects of chlorpyrifos in freshwater model ecosystems: the influence of experimental conditions on ecotoxicological thresholds. Pest. Manage. Sci. 61, 923−935. https://doi.org/10.1002/ps.1084

Wang, T.C., Hoffman, M., 1991. Degradation of Organophosphorus Pesticides in Coastal Water. J. AOAC Int., 74, 883-886. https://doi.org/10.1093/jaoac/74.5.883

7 Final Considerations

The present study contributed to expanding knowledge regards to the use of microeukaryotes ciliates as a model organism in ecotoxicological studies allowed to identify several gaps still existing in the study of ciliates and even directing the next steps to the future. The wide systematic review along with a meta-analysis contributed to improving the understanding of the ecotoxicological studies with ciliates being a group much neglected in studies of this nature. The caffeine bioassays added information about their chemical sensitivity on *P. caudatum* species. This species demonstrated resistance to caffeine, however, can be considered a good model inside in a tolerant class of aquatic organisms (e.g., Chironomidae). Moreover, it was added information about the effects of multiple stressors interactions on the zooplankton community in the aquatic ecosystem as a consequence of a partnership through internationalization networks. The increase in temperature, salinity, and pesticide (i.e., chlorpyrifos) affected the zooplankton community, but these three stressors have only an additive effect on the community. Based on the aquatic ecotoxicology scenario, the available data in this thesis will be used to develop more studies in this field with both ciliates and zooplankton in order to provide more details about the emerging contaminant (e.g., individual and interaction) effects and hence, the consequences to aquatic ecosystems process and services.

APPENDIX OF THESIS

Science of the Total Environment 671 (2019) 505–509

Contents lists available at ScienceDirect

Science of the Total Environment

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

Short Communication

Investigation of medicines consumption and disposal in Brazil: A study case in a developing country

Gabrielle R. Quadra [a,*], Pâmela S.A. Silva [b], José R. Paranaíba [a], Iollanda I.P. Josué [c], Helena Souza [d], Rafaela Costa [e], Marcos Fernandez [f], Jéssica Vilas-Boas [g], Fábio Roland [a]

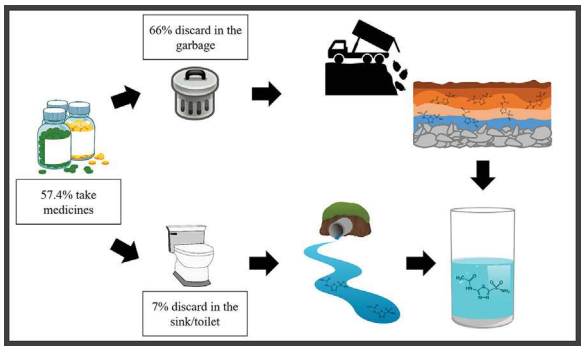
[a] Laboratório de Ecologia Aquática, Programa de Pós-Graduação em Ecologia, Universidade Federal de Juiz de Fora, 36036-900, Brazil
[b] Departamento de Farmacologia, Universidade Federal de Juiz de Fora, 36036-900, Brazil
[c] Laboratório de Limnologia, Programa de Pós-Graduação em Ecologia, Universidade Federal do Rio de Janeiro, 21941-590, Brazil
[d] Programa de Pós-Graduação em Meio Ambiente, Universidade Estadual do Rio de Janeiro, 20550-900, Brazil
[e] Programa de Pós-Graduaçãoem Desenvolvimento e Meiio Ambiente, Universidade Federal do Rio Grande do Norte, 59064-741, Brazil.
[f] Laboratório de Ecotoxicologia Marinha, Departamento de Oceanografia Química, Universidade Estadual do Rio de Janeiro, 20550-900, Brazil
[g] Laboratório de Protozoologia, Programa de Pós-Graduação em Ecologia, Universidade Federal de Juiz de Fora, 36036-900, Brazil

HIGHLIGHTS

- The incorrect disposal of medicines may represent a risk to the environment.
- Investigate how people are disposing unused medicines is crucial.
- Online questionnaires were spread out in Brazil.
- 66% of the respondents discard unused or expired medicines in common garbage.
- Environmental education may help to mitigate pharmaceuticals pollution.

GRAPHICAL ABSTRACT

The most important pathways for pharmaceuticals to the environment considering human disposal in the present study case.



ARTICLE INFO

Article history:
Received 14 January 2019
Received in revised form 28 February 2019
Accepted 22 March 2019
Available online 24 March 2019

Editor: Damia Barcelo

Keywords:
Environment
Environmental education
Garbage
Pharmaceuticals
Self-medication

ABSTRACT

The incorrect disposal of medicines can be harmful to the environment. Here, we aim to understand the consumption and disposal of medicines in Brazil using online forms. 64% of the respondents have the habit to self-medicate. 66% of respondents dispose the disused or expired medicines in the garbage. 71.9% of respondents never receive any information about correct disposal of medicines. 95.2% of respondents believe that residues of medicines can be harmful to the environment. Environmental education can provide information to the population and help to mitigate pharmaceuticals pollution.

* Corresponding author.
E-mail address: gabrielle.quadra@ecologia.ufjf.br (G.R. Quadra).

https://doi.org/10.1016/j.scitotenv.2019.03.334
0048-9697/

Environmental Pollution 256 (2020) 113343

Contents lists available at ScienceDirect

Environmental Pollution

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

A global trend of caffeine consumption over time and related-environmental impacts☆

Gabrielle R. Quadra [a, *], José R. Paranaíba [a, 1], Jéssica Vilas-Boas [b, 1], Fábio Roland [a], André M. Amado [a], Nathan Barros [a], Roberto Júnio P. Dias [b, c], Simone J. Cardoso [a, c]

[a] Laboratório de Ecologia Aquática, Programa de Pós-Graduação em Ecologia, Universidade Federal de Juiz de Fora, Juiz de Fora, 36036-900, Brazil
[b] Laboratório de Protozoologia, Programa de Pós-Graduação em Ecologia, Universidade Federal de Juiz de Fora, Juiz de Fora, 36036-900, Brazil
[c] Departamento de Zoologia, Universidade Federal de Juiz de Fora, Juiz de Fora, 36036-900, Brazil

ARTICLE INFO

Article history:
Received 3 July 2019
Received in revised form
30 August 2019
Accepted 2 October 2019
Available online 10 October 2019

Keywords:
Coffee
GDP
HDI
Surface water
Wastewater

ABSTRACT

Caffeine is one of the most consumed substances, and it has been largely detected in aquatic ecosystems. We investigated the trends in caffeine consumption over three decades and its relationships with gross domestic product (GDP) and human development index (HDI) to understand global patterns and to identify potential hotspots of contamination. The total caffeine consumption is increasing mainly due to population growth. Moreover, caffeine consumption per capita is also increasing in some countries, such as Brazil, Italy, and Ethiopia. A high positive correlation between caffeine consumption per capita with HDI and GDP was found for coffee-importing countries in Europe, while a high negative correlation was found for coffee-exporting countries in Africa. The literature review showed that the highest caffeine concentrations coincide with countries that present an increasing caffeine consumption per capita. Also, approximately 35% of the caffeine concentrations reported in the literature were above the predicted no-effect concentration in the environment and, again, overlaps with countries with increasing per capita consumption. Despite the high degradation rate, caffeine consumption tends to increase in a near future, which may also increase the overall amount of caffeine that comes into the environment, possibly exceeding the thresholds of several species described as tolerant to the current environmental concentrations. Therefore, it is essential to prevent caffeine from reaching aquatic ecosystems, implementing sewage treatment systems, and improving their efficiency.

1. Introduction

Nowadays, caffeine has become one of the most consumed psychoactive substances in the world (Diogo et al., 2013). Caffeine is part of the methylxanthines – a group of central nervous system stimulants commonly present in the daily life of humans (Diogo et al., 2013). Caffeine consumption mostly occurs through food products, such as tea, coffee, energy drinks, products containing cocoa or chocolate, but can also be found in cold medicines, painkillers, appetite suppressants, and stimulants (Buerge et al., 2003). The caffeine concentration in the products vary greatly, but coffee presents one of the highest amounts of caffeine compared to other beverage categories (Mitchell et al., 2014). Moreover, coffee is highly consumed worldwide. As an example, only in 2014, more than 8 million tons of coffee were consumed around the world (International Coffee Organization, 2015).

A small fraction of the total caffeine consumed is excreted in its original form (2–3%; Tang-Liu et al., 1983). However, a considerable amount can be discarded without being consumed, especially by cafeterias (Montagner et al., 2014a; Tokimoto et al., 2005). According to World Water Assessment Programme (WWAP-World Water Assessment Programme, 2017), around 80% of the total volume of wastewater is discharged without prior treatment in the environment. Thus, significant concentrations of caffeine may be found in sewage and it has been used as a domestic sewage tracer (Strauch et al., 2008; Tokimoto et al., 2005). Once in the sewers, caffeine can reach water bodies, and spread in surface waters, groundwater, and also in drinking waters around the world (Bruton et al., 2010; Machado et al., 2016). Wastewater treatment plants

☆ This paper has been recommended for acceptance by Dr. Da Chen.
* Corresponding author.
E-mail address: gabrielle.quadra@ecologia.ufjf.br (G.R. Quadra).
[1] These authors contribute equally for this study.

https://doi.org/10.1016/j.envpol.2019.113343
0269-7491/

Inventário de microeucariotos ciliados (Alveolata: Ciliophora) em ecossistemas límnicos no município de Juiz de Fora (MG)[1]

Checklist of ciliated microeukaryotes (Alveolata: Ciliophora) in limnic ecosystems in the city of Juiz de Fora (MG)

Pedro Mendes de Souza[2]

Fabíola da Silva Costa[3]

Jéssica Andrade Vilas-Boas[2]

Roberto Júnio Pedroso Dias[3]

DOI: https://doi.org/10.34019/2179-3700.2019.v19.29919

Resumo

Neste trabalho foi realizado um inventário de ciliados bentônicos em ecossistemas límnicos, localizados no município de Juiz de Fora (MG), com amostragens mensais, no período entre março e agosto de 2018. Os ciliados foram identificados com base em observações *in vivo* e, quando necessário, foi realizada impregnação pela prata e pela coloração por DAPI. Vinte e uma morfoespécies, distribuídas em cinco classes de Ciliophora, foram identificadas. Destas, *Euplotes* sp. 2, *Euplotes eurystomus*, *Paramecium bursaria* e *Paramecium caudatum* foram as espécies encontradas que poderiam ser facilmente mantidas sob condições *in vitro*.
Palavras-chave: Ciliados. Checklist. Cultivo. Bioindicadores. Córregos.

Abstract

In this work, an inventory of benthic ciliates in liminetic ecosystems located in the municipality of Juiz de Fora, Minas Gerais, Brazil, with monthly samplings was performed from March 2018 to August 2018. Ciliates were identified based on their *in viv*o data, and when necessary, silver impregnation and DAPI staining were also carried out. 21 morphospecies distributed over five Ciliophora classes were identified. Of these, *Euplotes*sp. 2*, Euploteseurystomus, Paramecium bursaria*and *Paramecium caudatum* were among the found species that could be easily maintained under *in vitro* conditions.
Keywords: Ciliates.Checklist. Culture. Bioindicators. Streams.

[1] Trabalho premiado no Seminário de Iniciação Científica da UFJF em 2018.

[2] Bolsista dos Programas de iniciação científica da Universidade Federal de Juiz de Fora (PROBIC/FAPEMIG e BIC/UFJF)

[3]Programa de Pós-graduação em Ecologia da UFJF (bolsista CAPES)

[3]Departamento de Zoologia, Labotatório de Protozoologia (LAZ), Universidade Federal de Juiz de Fora, 36036-900, Juiz de Fora, Minas Gerais

Science of the Total Environment 771 (2021) 144754

Contents lists available at ScienceDirect

Science of the Total Environment

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

Three-bestseller pesticides in Brazil: Freshwater concentrations and potential environmental risks

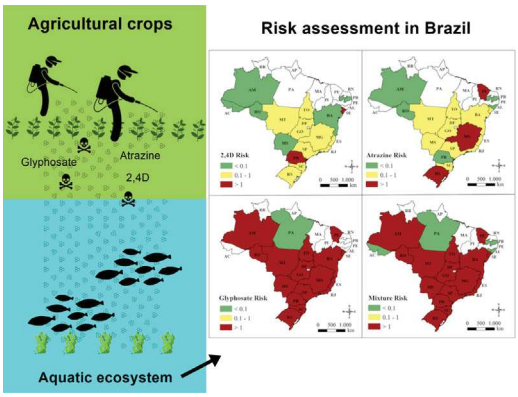
Emília Marques Brovini [a,b,*], Beatriz Corrêa Thomé de Deus [d], Jéssica Andrade Vilas-Boas [b,c], Gabrielle Rabelo Quadra [a,b], Luana Carvalho [c], Raquel Fernandes Mendonça [a,b], Renata de Oliveira Pereira [d], Simone Jaqueline Cardoso [a,b]

[a] Laboratório de Ecologia Aquática, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, 36036–900 Juiz de Fora, Minas Gerais, Brazil
[b] Programa de Pós-Graduação em Biodiversidade e Conservação da Natureza, Universidade Federal de Juiz de Fora, 36036-900, Brazil
[c] Laboratório de Protozoologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, 36036–900 Juiz de Fora, Minas Gerais, Brazil
[d] Programa de Pós-Graduação em Engenharia Civil, Universidade Federal de Juiz de Fora, Juiz de Fora, MG 36036-900, Brazil

HIGHLIGHTS

- 2,4D, atrazine and glyphosate are the three-bestseller pesticides in Brazil.
- The knowledge on pesticides in Brazilian freshwaters is incipient.
- Monitoring programs at natural and contaminated areas are suggested.
- Most Brazilian states are at high environmental risk considering pesticides.
- A review of the Brazilian freshwater legislation is suggested.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 15 October 2020
Received in revised form 11 December 2020
Accepted 22 December 2020
Available online 26 January 2021

Editor: Kevin V. Thomas

Keywords:
2,4D
Atrazine
Glyphosate
Legislation
Systematic review

ABSTRACT

Agricultural production in Brazil is favored by weather conditions and by the large amount of available land. Therefore, currently, Brazil is the second largest exporter of agricultural products globally. Pesticides are widely used in Brazilian crops due to their high efficiency, their low cost, and permissive legislation. However, pesticides tend to reach water resources threatening organisms and the water quality. Thereby, we aimed to review the surface freshwater concentrations of the three-bestseller pesticides in Brazil (glyphosate, 2,4D, and atrazine), and discuss the results with sales, legislation, toxicity and potential risks. For that, we performed a systematic review of quantitative studies of glyphosate, atrazine, and 2,4D in Brazilian freshwater and included monitoring data provided by the Brazilian Ministry of Health in our analysis. Finally, we calculated the risk assessment for the three pesticides. Only a few scientific studies reported concentrations of either of the three-bestseller pesticides in Brazilian freshwaters. Between 2009 and 2018, an increase in the sales of 2,4D, atrazine, and glyphosate was observed. It was not possible to evaluate the relation between concentrations and sales, due to limited number of studies, lack of standard criteria for sampling, individual environmental properties, and type of pesticide. Atrazine showed a higher toxicity compared to 2,4D and glyphosate. Regarding the environmental risks, 65%, 72%, and 94% of the Brazilian states had a medium to high risk to 2,4D, atrazine, and glyphosate, respectively. Finally, 80% of the Brazilian states evaluated showed a high environmental risk considering a mixture of the

* Corresponding author at: Laboratório de Ecologia Aquática, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, 36036–900 Juiz de Fora, Minas Gerais, Brazil.
E-mail address: emilia.brovini@engenharia.ufjf.br (E.M. Brovini).

https://doi.org/10.1016/j.scitotenv.2020.144754