



Universidade Federal do Rio Grande
Instituto de Ciências Biológicas
Pós-graduação em Biologia de
Ambientes Aquáticos Continentais



**Uso de modelos matemáticos para estimar a
toxicidade dos PPCPs metilparabeno e diclofenaco
para diferentes espécies de microalgas verdes**

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Dissertação apresentada ao Programa de Pós-graduação em Biologia de Ambientes Aquáticos Continentais como requisito parcial para a obtenção do título de Mestre em Biologia de Ambientes Aquáticos Continentais.

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DEDICATÓRIA

*Dedico esta dissertação para:
minha mãe Patricia, meu pai Fermin e Leidy minha querida e nova irmã.*

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RESUMO

Este trabalho teve como objetivo a avaliação da toxicidade dos contaminantes metilparabeno (MeP) e diclofenaco (DFC) (*PPCPs – Pharmaceutical and Personal Care Products*) para microalgas verdes usando diversas respostas e modelos matemáticos. Foi avaliada a toxicidade dos contaminantes puros por meio de testes clássicos de toxicidade padronizados (72 h de exposição - microalgas verdes). Foram utilizados como *endpoints*: a taxa de crescimento, teor de clorofila-a e a viabilidade celular. Para o cálculo dos parâmetros de toxicidade usaram-se modelos e abordagens estatísticas diferentes para a obtenção da concentração de efeito para x% dos organismos (CE_x). Para o MeP, composto com menos resultados disponíveis na literatura, foi derivado um *Predict not Effect Concentration value* (PNEC) com duas abordagens determinística e probabilística. Dentro da abordagem probabilística foi construído a curva de sensibilidade das espécies (SSD), utilizando dados confiáveis e relevantes, de acordo com a ferramenta web de SciRAP. Para a microalga *Desmodesmus communis* utilizando a taxa de crescimento como *endpoint* foram obtidos diferentes valores de CE₅₀ e desenvolvidos passo a passo em 3 modelos estatísticos: Weibull e Logístico da abordagem frequentista, e Beta da abordagem bayesiana. Os resultados de CE₅₀ para *D. communis* variaram entre 82,36 mg/L de DFC para Logístico/frequentista; 89,47 mg/L de DFC para Weibull/frequentista; e 101,70 mg/L de DFC para Beta/Bayesiana. Os três modelos foram adequados para o conjunto de dados (*D. communis* - DFC), mas o modelo Weibull foi escolhido por apresentar o valor mais baixo de EC₁₀ e ser aceitável em um contexto Bayesiano. No caso do MeP foi testada a toxicidade em três microalgas *Pediastrum boryanum*, *D. communis* e *Raphidocelis subcapitata*. Os valores de toxicidade CE₅₀ utilizando modelos Weibull e logístico de 3 parâmetros, e regressão assintótica de 2 parâmetros foram, respectivamente, 81,18; 124, 88; 18,27 mg/L de MeP, sendo que o *endpoint* utilizado foi o teor de clorofila-a. Com estes dados e os disponíveis na literatura (avaliados pelo ScRIP) foram utilizados na construção da SSD e derivação do PNEC probabilístico em 65 µg/L de MeP. De acordo com isto, as espécies mais suscetíveis ao MeP foram a *Daphnia magna* e o *Vibrio fischeri*, e entre as microalgas, *R. subcapitata* foi a mais suscetível e *D. communis* a mais tolerante. No entanto, na abordagem determinística foi estimado um PNEC de 5,7 µg/L de MeP. Assim, foi possível concluir que a CE₅₀ é um valor que pode variar para o mesmo conjunto de dados (organismo e composto teste) de acordo com o *endpoint* escolhido e o modelo de regressão usado para calcular a toxicidade.

Palavras-chave: algas, toxicidade, PNEC, modelagem, *endpoint*.

ABSTRACT

This work aims to evaluate the toxicity of methylparaben (MeP) and diclofenac (DFC) contaminants (PPCPs) for green microalgae using different endpoints and mathematical models. The toxicity of pure contaminants was assessed by standardized toxicity tests (72 h of exposure - green microalgae). The endpoints were: growth rate, chlorophyll-a content and cell viability. For the calculation of toxicity, different statistical models and approaches were used to obtain the effect concentration for x% of organisms (EC_x). For the MeP, compound with fewer results available in the literature, a Predict Non-Effect Concentration value (PNEC) was derived with two deterministic and probabilistic approaches. Within the probabilistic approach, the species sensitivity curve (SSD) was constructed using reliable and relevant data, according to the SciRAP web tool. For *Desmodesmus communis* different values of EC₅₀ were obtained based on growth rate and they were developed step by step in three statistical models: Weibull and Logistic of the frequentist approach, and Beta of the Bayesian approach. The results of EC₅₀ for *D. communis* ranged from 82.36 mg/L of DFC to Logistic / frequentist; 89.47 mg/L DFC for Weibull / frequentist; and 101.70 mg/L DFC to Beta / Bayesian. The three models were suitable for the data set (*D. communis* - DFC), but the Weibull model was chosen because it presented the lowest value of EC₁₀ and was acceptable in a Bayesian context. In the case of MeP toxicity was tested in three microalgae *Pediastrum boryanum*, *D. communis* and *Raphidocelis subcapitata*. The EC₅₀ toxicity values using Weibull and logistic models of 3 parameters, and asymptotic regression of 2 parameters were, respectively, 81.18; 124, 88; 18.27 mg/L of MeP, and the endpoint used was the chlorophyll-a content. With these data and those available in the literature (evaluated by ScRIP) were used to the construction of the SSD and derivation of the probabilistic PNEC at 65 µg/L of MeP. Accordingly, the most susceptible species to MeP were *Daphnia magna* and *Vibrio fischeri*, and between microalgae, *R. subcapitata* was the most sensitive and *D. communis* more tolerant. However, in the deterministic approach a PNEC of 5.7 µg/L of MeP was estimated. Thus, it was possible to conclude that EC₅₀ is a value that can vary for the same data set (organism and test compound) according to the chosen endpoint and the regression model used to calculate the toxicity.

Key-words: algae, toxicity, PNEC, modeling, endpoint.

APRESENTAÇÃO

O trabalho apresentado aqui está dividido em 4 seções, sendo a primeira uma introdução geral, seguida de objetivos e referências bibliográficas no formato alfabético, após, vem dois capítulos em formato de artigo científico e escritos de acordo com as normas das revistas às quais serão submetidos, e, por fim, considerações finais. O 1º capítulo intitulado “**Toxicity of diclofenac to the green algae *Desmodesmus communis* by different approaches and using Classical and Bayesian models**” será submetido à revista *Ecotoxicology and Environmental safety*. O 2º capítulo intitulado “**Toxicity of Methylparaben to green microalgae species and derivation of a predicted no-effect concentration (PNEC) in freshwater ecosystems**” submetido à revista *Chemosphere*.

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LISTA DE SIGLAS

ABNT - Associação Brasileira de Normas Técnicas
CE – Concentração de efeito
CENO – Concentração de efeito não observado
CEO – Concentração de efeito observado
Chl-a – Chlorophyll-a
EC_x – Effect concentration for x % of organisms
ECHA – European Chemicals Agency
EPA – Environmental Protection Agency
EQS – Environmental quality standards
ERA – Ecological risk assessment
ETEs – Estações de tratamento de efluentes
DEA – Drug Enforcement Administration
DFC – Diclofenaco
Gr – Growth rate
I – Inhibition vector
IC_x – Median concentration that inhibited x %
ISO – International Organization for Standardization
MCMC – Markov chain Monte Carlo
MeP – Metilparabeno
NR – Neutral Red
NSAID – Non-steroidal anti-inflammatory drugs
OD – Optical density
OECD – Organisation for Economic Co-operation and Development
PNEC – Predicted non-effect concentration
PNECd – Deterministic PNEC
PNECp – Probabilistic PNEC
PPCPs – Pharmaceutical and Personal Care Products
SSD – Species sensitivity curve
USEPA – Environmental Protection Agency
V – cellular viability
WWTPs – wastewater treatment plants

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1. INTRODUÇÃO

1.1 Perspectiva geral dos PPCPs

Os corpos d'água superficiais vêm sendo afetados pelo uso de produtos químicos globalmente conhecidos como Contaminantes Emergentes. Nesta classificação entram os Produtos Farmacêuticos e de Higiene Pessoal, também conhecidos como PPCPs (*Pharmaceutical and Personal Care Products*). Eles têm como principais rotas de entrada no ambiente aquático as descargas de efluentes domésticos direta, ou através das estações de tratamento de efluentes (ETEs) (Bartrons and Peñuelas, 2017; Ebele et al., 2017; Haman et al., 2015; Kleywegt et al., 2007; Norvill et al., 2016). Esta contaminação ocorre, portanto, pelo consumo de produtos que providenciam algum benefício particular à humanos e animais (estéticos ou medicinais) como fármacos, conservantes e cosméticos (Chopra and Kumar, 2018). No caso do efluente tratado, dependendo do composto entrante (molécula orgânica) e do tratamento empregado (primário, secundário, avançado) na ETE, a eficiência pode ser total, parcial ou nenhuma (Haman et al., 2015; Kasprzyk-Hordern et al., 2009; Norvill et al., 2016; Tarpani and Azapagic, 2018). Assim, os PPCPs podem alcançar os ecossistemas aquáticos, os quais são objeto de diversos usos, incluindo o abastecimento de água. O fluxo de PPCPs está resumido na Figura 1.

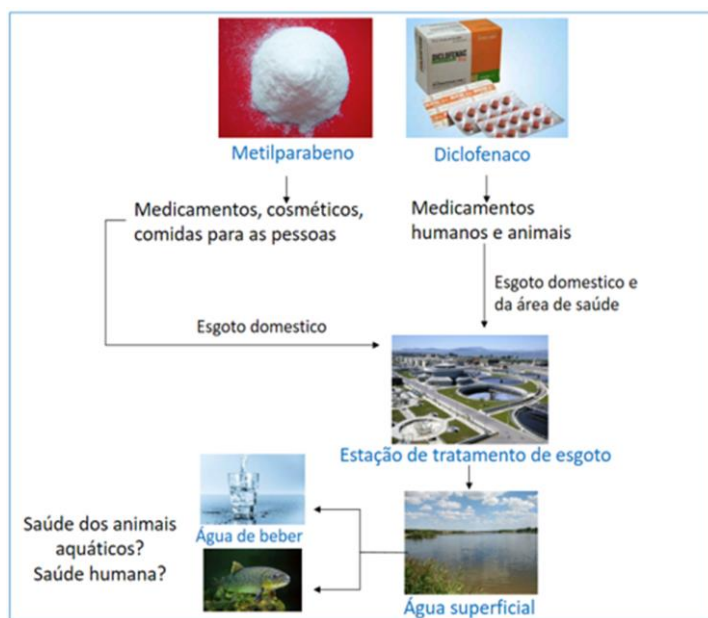


Figura 1. Principias rotas de entrada de dois exemplos de PPCPS (Metilparebeno e Diclofenaco) no ambiente aquático.

Apesar do ciclo dos PPCPs estar bem identificado, não é fácil resolver o problema. Estudos sobre os PPCPs com diferentes abordagens como identificação e concentração, biodegradabilidade e metabólitos, toxicidade, riscos ambientais, riscos humanos, interações químicas e biológicas e regulação, vêm crescendo substancialmente (Daughton, 2016; Gao et al., 2014; Gredelj et al., 2018; Minguez et al., 2016; Montes-Grajales et al., 2017; Norvill et al., 2016; Peng et al., 2014; Stuart et al., 2012; Tarpani and Azapagic, 2018; Wu et al., 2017; Zhao et al., 2017). Os PPCPs podem ser identificados como milhares de substâncias químicas existentes, por exemplo mais de 4000 fármacos em uso atual, e alguns estudos ainda indicam que são os compostos-traço detectados com maior frequência no ambiente aquático natural (Bartrons and Peñuelas, 2017; Gibson, 2010). Fatores que contribuem para este quadro podem ser resumidos como falta de cuidado no descarte de medicamentos, número de remédios vendidos sem prescrição médica, falta de saneamento básico, bem como, falta de regulamentação ambiental.

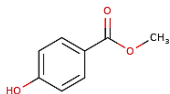
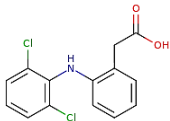
O uso intensivo e extensivo juntamente com a ineficiência nos processos de tratamento de efluentes, está levando a liberação contínua dos PPCPs nos ecossistemas aquáticos, sem que se saiba com clareza dos efeitos que isto pode ter a longo prazo. De maneira geral, os dados que se têm até agora mostram que muitos PPCPs representam um risco para a qualidade da água e saúde dos ecossistemas (Kasprzyk-Hordern et al., 2009; Silva et al., 2009; Zhao et al., 2017). Os efeitos podem variar de espécie em espécie; entre grupos taxonômicos, condições ambientais, tempo de exposição, concentrações presentes e propriedades físico-químicas. Inclusive, algumas destas moléculas possuem estabilidade química o que facilita a incorporação e biocumulação nas cadeias tróficas (Kleywegt et al., 2007).

No sentido da regulamentação, os PPCPs têm sido ponto focal de Agências como CONAMA – Conselho Nacional do Meio Ambiente (Brasil), EPA - *Environmental Protection Agency* (USA), ECHA – *European Chemicals Agency* (EU), DEA - *Drug Enforcement Administration* (USA), mas ainda carecem de informações para que estes produtos sejam controlados do ponto de vista ambiental (Sophia A. & Lima, 2018). Com ênfase nos ecossistemas aquáticos, estes compostos têm sido encontrados na água superficial, subterrânea, e até mesmo na água de abastecimento, em muitos países (UK, USA, Espanha, China, Canada, Brasil). As concentrações geralmente variam de ng/L e

µg/L (Dai et al., 2014; Montagner et al., 2017; Norvill et al., 2016). O risco associado a estes compostos é ainda pouco conhecido, porém, existem trabalhos que evidenciam o potencial dos PPCPs como desreguladores endócrinos, agentes indutores de estresse oxidativo, citotoxicidade, genotoxicidade, sendo que tais efeitos podem ser vistos agudamente ou cronicamente. Os grupos taxonômicos mais usados nos estudos de toxicidade com PPCPs são peixes, microcrustáceos e algas (Ebele et al., 2017; Kleywegt et al., 2007; Ortiz de García et al., 2014).

Para a presente dissertação foram escolhidos os PPCPs diclofenaco (DCF) e metilparabeno (MeP), representados na Tabela 1. Enquanto o DCF é um fármaco analgésico muito utilizado pela população humana e *pets*, o MeP é um conservante com ação anti-microbiana. Estes químicos foram selecionados seguindo um amplo levantamento bibliográfico sobre a presença de PPCPs em corpos d'água e efluentes. Por exemplo, estudos reportam a presença do DCF em efluentes de águas na Suécia (120 ng/L), no Reino Unido (6– 500 ng/L), nos Estados Unidos (8-177 ng/L), no Canadá (190 ng/L), na Espanha (6-430 ng/L), na China (345 ng/L), com baixas taxas de remoção nas ETEs (Dai et al., 2014). Carmona et al. (2014), reportaram concentrações do DFC e MeP na água do Rio Turia na Espanha em 49 e 119 ng/L e na água mineral em 25 e 40 ng/L, respectivamente. Este mesmo estudo reportou uma baixa e alta eficiência de remoção do DCF e MeP, respectivamente. Stuart et al. (2012) reportaram uma concentração de MeP de 5 µg/L na água subterrânea em uma cidade do Reino Unido; e Peng et al. (2014) reportaram uma média aproximada de 0,3 a 9 ng/L no delta do rio Pearl na China. No Brasil, Ghiselli (2006) apresentou dados da ETE de Samambaia em Campinas / SP e, mostrou que nesta só se consegue remover 38% do DFC presente no esgoto bruto, evidenciando como os processos de tratamento convencional de esgoto com lodo ativado têm baixo potencial para remover o fármaco. Américo-Pinheiro et al. (2017) detectaram DCF em uma concentração de 0,120 a 8,250 µg/L ao longo do ano, isto à jusante da ETE Três Lagoas no Mato Grosso do Sul. Em outro estudo, Caldas et al. (2013) analisaram 10 amostras da ETE CORSAN- Companhia Riograndense de Saneamento em Morro Redondo / RS, em 9 delas o DCF ficou abaixo do limite de quantificação (8 ng/L de DFC).

Tabela 1. Propriedades físico-químicas dos compostos Metilparabeno e Diclofenaco.

Nome comum	Formulações	No CAS	Fórmula química	Massa Molecular	Log (Kow)	Constante de dissociação (pKa)	Solubilidade (mg L ⁻¹) a 25°C	Constante da Lei de Henry atm-cu m/mol a 25 °C	Estrutura Molecular
Metilparabeno	éster metílico do ácido 4-hidroxibenzóico que contém, 98% até 102% de C ₈ H ₈ O ₃	99-76-3	C ₈ H ₈ O ₃	152,15	1,96	8,5	2,5x10 ³	2,23X10-9	
Diclofenaco	Diclofenaco sódico 100 mg Excipientes	153007-86-5	C ₁₄ H ₁₁ Cl ₂ NO ₂	296,15	4,51	4,15	2,37	4,73X10-12	

1.1.1 Metilparabeno (MeP)

O MeP é um antimicrobiano pertencente ao grupo dos parabenos, de cadeia curta, amplamente utilizado na indústria, em diferentes produtos de cuidado pessoal como shampoo, desodorantes, cremes, além de alimentos. Em produtos alimentícios o MeP pode ser usado em concentrações de até 0,1%, em cosméticos até 1%, e em produtos farmacêuticos até 20%. Também está na formulação de produtos como papel, jornais e toalhas higiênicas. Naturalmente, ele pode ser encontrado no mirtilo ou *blueberry* (Dambal et al., 2017). De maneira geral, os parabenos são considerados conservantes ideais pelo amplo espectro de atividade contra os micróbios (bactérias gram-positivas, mofo, leveduras), estabilidade no pH e baixo custo (Martín-Villamil et al., 2016). A fórmula química do MeP e suas propriedades estão detalhadas na Tabela 1. Por ser orgânico, o MeP é solúvel em acetona (39%), álcool (32%), ésteres cosméticos, glicerina (3,3%) e outros (Hallstar, 2017).

Entre os parabenos, o MeP é um dos mais detectados em efluentes bruto, tratado e corpos d'água superficiais (Dambal et al., 2017; Haman et al., 2015), entrando em contato com organismos não-alvos presentes nos ecossistemas aquáticos. Na Tabela 2 são indicadas as concentrações de efeito expressas como CE₅₀ para diferentes organismos e grupos ecológicos, para o MeP. Estes dados além de indicar o potencial tóxico do MeP, também mostram que dependendo do *endpoint* medido e do método ou modelo estatístico usado para calcular a concentração de efeito (CE_x), o valor de toxicidade pode variar, mesmo entre espécies e condições de testes iguais (exemplo ver Tabela 2, dados de *Daphnia magna*). Adicionalmente aos dados de toxicidade, existem evidências de que o MeP em concentrações subletais é um desregulador endócrino (Alnuqaydan and Sanderson, 2016). O MeP não está presente em nenhuma legislação ambiental nacional ou internacional para o ambiente, apenas é um dos parabenos controlado pela *European chemicals legislations – REACH (Registration, Evaluation, Authorization and Restriction of Chemicals)* para consumo humano, por apresentar baixo potencial de bioacumulação (Haman et al., 2015) e meia-vida curta que pode variar de minutos há alguns dias (ECHA [echa.europa.eu/registration-dossier]).

Tabela 2. Toxicidade do MeP em CE₅₀ ou CL₅₀ para diferentes espécies e grupos ecológicos.

Organismo	Gênero e espécie	Tempo	Resultado (mg/L)	Endpoint	Método o modelo estatístico de calculo	Referência
Protozoário	<i>Tetrahymena thermophila</i>	24h	54	Crescimento	Protoxkit	Bazin et al., 2010
		28h	58	Crescimento	Protoxkit	
Bactéria	<i>Photobacterium leiognathi</i>	15 min	31	Luminescência	ToxScreen	Adame and Rocha, 2014
		30 min	35	Luminescência	ToxScreen	
	<i>Vibrio fischeri</i>	15 min	9.6	Luminescência	Microtox	
		30 min	10	Luminescência	Microtox	
	<i>Vibrio fischeri</i>	5min	6.8	Luminescência	Sistema MPFS - <i>Multi-pumping flow systems</i> Bioensaio baseado em fluxo. Equação Linear	
Alga	<i>Vibrio fischeri</i>	5min	6.2	Luminescência	<i>Reference batch procedure</i>	Yamamoto et al., 2011
	^a <i>Pseudokirchneriella subcapitata</i>	72h	80	Crescimento	Correlação linear usando um plot log-normalizado	
	^a <i>Pseudokirchneriella subcapitata</i>	72h	91	Crescimento	Statistical parameters calculated from continuous responses based on continuous mean.	
					Toxedo' (VKI 1992).	
Crustáceo	^a <i>Pseudokirchneriella subcapitata</i>	72h	35.25	Crescimento-fluorescência de clorofila	Equação de Hill. Macro REGTOX do Excel TM	Di Poi et al., 2018
	<i>Daphnia magna</i>	48h	15.67	Valor predito por modelagem	T.E.S.T - <i>Toxicity Estimation Software Tool</i> - do modelo de relação estrutura-atividade quantitativa (QSAR)	
	<i>Daphnia magna</i>	48h	71.43	Valor predito por modelagem	ECOSAR - <i>Ecological Structure Activity Relationships</i> - do modelo de relação estrutura-atividade quantitativa (QSAR)	

	<i>Daphnia magna</i>	48h	24.6	Imobilização	Daphtoxkit	Bazin et al., 2010
	<i>Daphnia magna</i>	48h	34	Imobilização	probit or logit conversion analysis using Ecotox- Statistics softwar	Yamamoto et al., 2011
	<i>Daphnia magna</i>	48h	11.2	Imobilização	Probit	Madsen et al., 2001
	<i>Daphnia magna</i>	48h	41.1	Imobilização	Probit	Kamaya et al., 2005
	<i>Daphnia magna</i>	48h	62	Imobilização	Probit	Terasaki et al., 2009
	<i>Daphnia magna</i>	48h	24.6	Mortality	Probit o TSK - <i>Trimmed Spearman Karber</i>	Dobbins et al., 2009
	<i>Daphnia magna</i>	48h	41.23	Imobilização	Equação de Hill	Di Poi et al., 2018
	<i>Daphnia magna</i>	48h	5.7	Imobilização ao ar livre (<i>sunlight</i>)	Probit	Lee et al., 2017
	<i>Daphnia magna</i>	48h	36.73	Imobilização	Probit	
	<i>Ceriodaphnia dubia</i>	7d	7.3	Reprodução	Probit	Terasaki et al., 2013
Peixe	<i>Oryzias latipes</i> - 10d	96h	63	Mortalidade	Ecotox-Statistics software ver. 2.6	Yamamoto et al., 2011
	<i>Danio rerio</i> - Embrião	120h	50	Desenvolvimento	Não reportado	Ate,s et al., 2018
	<i>Oreochromis niloticus</i> - 3 a 5 meses	48h	67.11	Mortalidade	Probit	Silva et al., 2018
	<i>Oryzias latipes</i>	96h	59.5	Mortalidade		ECHA [https://echa.europa.eu/ registration-dossier/- /registered- dossier/14310/6/2/2]
Amphibia	<i>Xenopus laevis</i> - Embrião	96h	178.14	Mortalidade	TSK - Trimmed Spearman Karber	San Segundo et al., 2013
Platelmintos	<i>Dugesia japonica</i>	96h	77	Mortalidade	TSK - Trimmed Spearman Karber	Li, 2012

^amesma espécie que *Raphidocelis subcapitata*

1.1.2 Diclofenaco (DCF)

O DCF é um fármaco de ação analgésica e anti-inflamatória comumente utilizado para o tratamento de dores em humanos ou animais domésticos, uma vez que reduz a inflamação em condições de doença. Faz parte do grupo dos anti-inflamatórios não esteroides (ANE) (Jr. Salvador Gamarra et al., 2015; Lonappan et al., 2016). Não necessita de prescrição médica para ser vendido e sua administração pode ser tópica ou oral. A fórmula química e demais propriedades são detalhadas na Tabela 1. Apesar de possuir meia vida bastante curta (horas a poucos dias), o DCF é continuamente encontrado nos efluentes domésticos, bem como, em ambientes naturais, principalmente pela dificuldade de remoção do composto pelos sistemas e de tratamento de esgoto e pelo uso contínuo (Carmona et al., 2014; Dai et al., 2014). Com relação à toxicidade do DCF, na Tabela 3 se apresenta dados de CE50 ou CL50 para diversas espécies grupos ecológicos, tirados da literatura.

O DCF não está presente em nenhuma legislação ambiental nacional ou internacional, mas parece que há firmes intenções para, em breve, regular este composto. A União Europeia na diretiva 2008/105/EC sob o marco da política da água incluiu o DCF como um dos compostos que fazem parte da lista de substâncias para monitoramento. A finalidade é determinar medidas adequadas do químico para lidar com o risco associado a ele (EU, 2015).

Tabela 3. Toxicidade do DCF em CE₅₀ ou CL₅₀ para diferentes espécies e grupos ecológicos.

Organismo	Gênero e espécie	Tempo	Resultado (mg/L)	Endpoint	Método o modelo estatístico de calculo	Referência
Bactéria	<i>Vibrio fischeri</i>	30min	11.45	Luminescência	Log Logística (modelo Hill)	Ferrari et al., 2004
		5min	3.8	Luminescência	Sistema MPFS - <i>Multi-pumping flow systems</i> Bioensaio baseado em fluxo. Equação Linear	Adame and Rocha, 2014
		5min	3.4	Luminescência	<i>Reference batch procedure</i>	
Alga	<i>Desmodesmus subspicatus</i>	72h	71.9	Crescimento - Clorofila	Função logística de quatro parâmetros - SIGMA PLOT 2000	Cleuvers, 2003
		96h	16.3	Crescimento -Clorofila	log-logistic model (Hill model)	Ferrari et al., 2004
		72h	21.3	Taxa de crescimento - fluorescência de clorofila	Modelos de relação estrutura-atividade quantitativa (QSAR)	Villain et al., 2016
		72h	64.8	Crescimento	Log-linear	Quinn et al., 2011
		96h	257.1	Crescimento - densidade celular	Modelo Sigmoide (Logístico)	Zhou et al., 2016
	<i>Scenedesmus vacuolatus</i>	24h	48.1	Crescimento - fluorescência de clorofila - densidade celular	Log logístico (modelo Hill)	Schulze et al., 2010
Alga (Diatomácea)	<i>Cyclotella meneghiniana</i>	96h	19.24	Crescimento	Log logístico (modelo Hill)	Ferrari et al., 2004
Alga (Cianobactéria)	<i>Synechococcus leopolensis</i>	96h	14.5	Crescimento	Log logístico (modelo Hill)	
Crustáceo	<i>Daphnia magna</i>	48h	123.3	Imobilização	Regressão não linear-Técnica de mínimos quadrados. Software Statística 11.0 (Tulsa, OK)	Oliveira et al., 2016
	<i>Daphnia magna</i>	48h	22.43	Mortalidade	Log logístico (modelo Hill)	Ferrari et al., 2004
	<i>Daphnia magna</i>	48h	68	Imobilização	Função logística de quatro	Cleuvers, 2003

	<i>Daphnia magna</i>	48h	67	Imobilização	parâmetros - SIGMA PLOT 2000	
	<i>Daphnia magna</i>	21d	28.1	Imobilização	Binomial	Quinn et al., 2011
	<i>Daphnia magna</i>	48h	39.9 e 44.7	Mortalidade	Método de <i>Spearman-Karber</i>	
	<i>Daphnia magna</i>	48h	60.7	Imobilização	Regressão não linear para resposta contínua	Happ et al., 2008
	<i>Daphnia similis</i>	48h	46	Imobilização	Probit - ToxStat version 3.5	Lee et al., 2011
	<i>Moina macrocopa</i>	48h	142.6	Imobilização	Método de <i>Spearman-Karber</i> ajustado, usando concentrações nominais.	Castro et al., 2014
	<i>Ceriodaphnia dubia</i>	48h	22.7	Mortalidade	Probit - ToxStat version 3.5	Lee et al., 2011
Peixe	<i>Danio rerio Juvenil</i>	96h	166.6	Mortalidade	Log logístico (modelo Hill)	Ferrari et al, 2004
	<i>Danio rerio embrião</i>	144h	6.11	Mortalidade	Analises probit EKO-TOX 5.2	Praskova et al., 2011
	<i>Danio rerio embrião</i>	72h	5.3	Efeitos múltiplos	Analises probit EKO-TOX 5.2	van den Brandhof and Montforts, 2010
	<i>Danio rerio embrião</i>	72h	7.8	Mortalidade	Modelo <i>trimmed Spearman-Karber</i>	
	<i>Cyprinus carpio</i>	96h	70.98	Mortalidade	Modelo <i>trimmed Spearman-Karber</i>	Islas-Flores et al., 2013
	<i>Poeciliopsis lucida</i>	24h	5.6	Citotoxicidade	Probit - <i>EPA Analysis Program v1.5</i>	
Macrofita	<i>Lemna minor</i>	7d	7.5	Crescimento	Microsoft Excell Regtox 6.3 macro	EPA [https://cfpub.epa.gov/ecotox/ecotox_home.cfm]
Platelmintos	<i>Dugesia japonica</i>	96h	4.7	Mortalidade	Função logística de quatro parâmetros - SIGMA PLOT 2000	Cleuvers, 2003
					TSK - <i>Trimmed Spearman Karber</i>	Li, 2013

^amesma espécie que *Raphidocelis subcapitata*

1.2 Ensaios ecotoxicológicos

Os ensaios ecotoxicológicos são ferramentas que indicam toxicidade a curto (agudo) e a longo prazo (crônico) com probabilidade de ocorrer aos organismos presentes no ambiente (OECD, 2002). São ensaios desenvolvidos sob condições controladas, procedimentos padronizados que permitem concluir sobre a dose e o efeito de agentes físicos ou químicos sobre os organismos-teste (Castillo Morales, 2004). Um ensaio de toxicidade trata-se da exposição de organismos-teste a diferentes concentrações da amostra a ser testada em soluções contidas nos frascos-teste, mantidos sob condições ambientais constantes por determinado período de tempo. Os ensaios podem ser normatizados por órgãos que atestam padrões de qualidade como a ABNT - Associação Brasileira de Normas Técnicas, a OECD - *Organisation for Economic Co-operation and Development* e ISO - *International Organization for Standardization*. As respostas ou *endpoints* podem variar de fecundidade, desenvolvimento, crescimento à mortalidade. Os resultados podem ser expressos de diferentes formas, mas sempre com base numa relação dose-resposta clara. Geralmente para ensaios agudos, deriva-se valores de CL_{50} ou CE_{50} que correspondem à concentração mediana que causa letalidade ou efeito em 50% da população do organismo teste, respectivamente. Ensaios crônicos geralmente expressam os resultados como CENO (maior concentração de efeito não observado) e CEO (menor concentração de efeito observado), ou tóxica e não tóxica após aplicação de estatística. Os valores de toxicidade trazem informação importante para uma avaliação preliminar, rápida e pratica sobre o efluente ou contaminante, e servem de base para estudos mais complexos (Castillo Morales, 2004; Klaassen and Watkins III, 2012). Os órgãos de fiscalização ambiental tanto no Brasil quanto fora do país exigem em suas normativas e resoluções testes de toxicidade para aprovar a qualidade de um efluente e avaliar / monitorar a qualidade de ambientes aquáticos naturais. Dependendo do composto ou efluente, as regulamentações pedem ser ensaios de toxicidade com diferentes níveis tróficos, isto inclui testes com algas, que representam o nível basal da cadeia trófica.

1.2.1 Microalgas como modelos biológicos para medidas de toxicidade

Microalgas são seres unicelulares autotróficos que apresentam-se como bons modelos biológicos na área da ecotoxicologia, devido à características básicas como elevada sensibilidade, fácil cultivo, e baixo custo para manutenção e ensaios (ABNT NBR 12648:2011, 2011; OECD, 2002). Dentre as microalgas-verdes de água doce pertencentes ao grupo das Chlorophytas e consideradas como modelos para os ensaios de toxicidade estão as espécies *Pseudopediastrum boryanum* (Turpin) Meneghini (1840), *Desmodesmus communis* (E. Hegewald) E. Hegewald (2000), e *Raphidocelis subcapitata* (Korshikov) classificada anteriormente como *Pseudokirchneriella subcapitata* (Korshikov) Nygaard, et al. (1987), ou *Selenastrum capricornutum* (Printz) (Guiry, M. D, 2019).

As microalgas do gênero *Pseudopediastrum* são estreladas e se agrupam em colônias, são cosmopolitas e habitam lagos rasos, principalmente, sob condições eutrofizadas e, preferencialmente em regiões tropicais (Weckström et al., 2010). As algas do gênero *Desmodesmus* são células alongadas que também se dispõem em colônias. Elas são encontradas em corpos d'água lânticos, desde ambientes mesotróficos a eutrofizados (Viruela, 2014). *Raphidocelis* possui forma de ferradura e não vive em colônias. Também é encontrada em diversos tipos de ambientes de água doce, é recomendada por todos os protocolos de toxicidade de microalgas por sua alta taxa de crescimento, sensibilidade aos tóxicos, fácil reprodutibilidade e manutenção do cultivo (Yamagishi et al., 2017).

As cepas utilizadas nos testes estão registradas no Banco de Cultivo de Microalgas Dulcícolas da Universidade Federal do Rio Grande (FURG), são cultivadas em meio WC (Guillard and Lorenzen, 1972) à temperatura de 23 ± 2 °C, intensidade luminosa de ~7000 lux e fotoperíodo de 12 horas claro e 12 horas escuro. *P. boryanum* foi isolada da Lagoa Mirim (Brasil – RS), enquanto *D. communis* foi isolada de um lago do Campus carreiro da FURG. *R. subcapitata*, por sua vez, foi doada pela CORSAN ao Banco de Cultivo da Empresa.

A Tabela 4 resume as principais características das espécies de microalgas usadas na presente dissertação.

1.2.2 *Endpoints* em ensaios com microalgas

Como mencionado acima, existem várias possíveis formas de se quantificar a toxicidade de um composto, tanto no que diz respeito a resposta observada, quanto com relação ao método matemático / estatístico para se avaliar a resposta e inferir sobre a toxicidade. No caso das microalgas, o *endpoint* mais comumente utilizado nos ensaios de toxicidade é a análise da biomassa da cultura algácea, seja por taxa de crescimento (Equação 1) ou a densidade celular (que é obtida através de absorbância a 750 nm) (ABNT NBR 12648:2011, 2011; Fu et al., 2015; Nyholm, 1985; OECD 2002). Geralmente, calcula-se a CE₅₀ com base nestes *endpoints*, seguindo exposição de 72 h a um determinado composto, mistura ou efluente.

Equação 1

$$r = [\ln(df) - \ln(di)] / (tf - ti),$$

Onde, r é a taxa de crescimento; $d(f,i)$ é a densidade inicial i e a final f ; $t(f,i)$ é o tempo inicial i e final f .

Outros *endpoints* também utilizados com microalgas são: o teor de pigmentos, principalmente, a clorofila-a, fotossíntese e viabilidade celular (Repetto et al., 2008; da Luz et al., 2016). A clorofila é um pigmento com importante papel na captura de energia e no processo da fotossíntese. O teor de clorofila e sua localização podem ser chaves para quantificar uma resposta frente a uma situação de estresse, como exposição à produtos químicos (Cleuvers et al., 2004; Di Poi et al., 2018; Liu et al., 2011; Villain et al., 2016).

Por outro lado, a viabilidade tem se mostrado uma medida sensível não apenas para detectar efeitos de compostos nocivos, como para detectar a saúde de uma cultura. Ela pode ser estimada por diferentes técnicas, como, por exemplo, a técnica do Metil-tiazolil-tetrazólico (MTT) que analisa a atividade mitocondrial da célula algal; Azul de Tripan (AT) ou Vermelho Neutro (VN) que estimam a viabilidade com base na integridade da membrana celular (Figura 2).

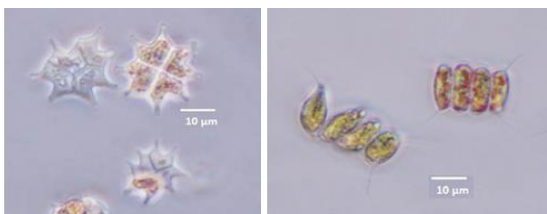


Figura 2. *Pseudopediastrum boryanum* (esquerda) e *Desmodesmus communis* (direita). Viabilidade celular pelo método de VN. Fonte: Saul da Luz et al., 2016. Células coradas em vermelho são viáveis.

Independentemente da metodologia para quantificar a toxicidade, a resposta tem que ser sempre em função da concentração de exposição. Neste sentido, diversos modelos estatísticos calculam a toxicidade com base na construção de uma curva doses-resposta, expressa como uma função não linear f (Equação 2) (Ritz and Streibig, 2005).

Equação 2

$$Y_{ij} = f(x_{ij}, \beta_i) + \varepsilon_{ij}$$

Onde, Y_{ij} é a resposta no tratamento i , correspondente a réplica j (exemplo: inibição do crescimento); x_{ij} é dose ou concentração do composto; β_i é o parâmetro desconhecido; e ε_{ij} é o erro associado com o ajuste da curva.

Os modelos comumente usados são as curvas sigmoide como o logístico ou probito, da abordagem clássica ou frequentista; no entanto, os resultados podem ser abordados pela estatística bayesiana. A partir da curva ou da relação dose-resposta é possível obter parâmetros de importância como a concentração de efeito observado para 50% da população (CE_{50}), ou a concentração de efeito não observado e observado (CENO e CEO, respectivamente). Como esses valores não são medidos, mas são calculados matematicamente, é importante entender que o resultado do parâmetro é obtido em função da relação matemática usada (modelos e abordagem). Portanto, o valor da CE_{50} , por exemplo, pode variar para o mesmo conjunto de dados dependendo do modelo e abordagem usada para calcular a toxicidade (Huang et al., 2018; Nyholm, 1985; Ritz et al., 2015; Ritz and Streibig, 2008).

Uma abordagem alternativa à frequentista ou clássica na construção da relação dose-resposta, é a abordagem bayesiana, ainda com poucos trabalhos na área da ecotoxicologia (Billoir et al., 2008; Feckler et al., 2018; Fox, 2010). Usando a estatística bayesiana, é possível ajustar as curvas dentro de padrões e escolher a mais representativa de uma relação dose-resposta. O cálculo do parâmetro CE_{50} estará acompanhado por intervalos de credibilidade que constituem uma faixa de valores possíveis com uma probabilidade conhecida (Feckler et al., 2018). Por exemplo, um intervalo de credibilidade de 95% define um intervalo dos 95% mais plausíveis da CE_{50} (Kinas e Andrade, 2010), diferente aos intervalos de confiança entregues na abordagem frequentista (Feckler et al., 2018). Neste sentido, na presente dissertação foram desenvolvidos e ajustados modelos frequentistas e bayesianos para o mesmo conjunto de dados e os resultados foram comparados na forma de CE_{50} .

Os dados de toxicidade podem ser usados para o delineamento de uma curva de sensibilidade de espécies (SSD – *Species sensitivity distribution*). A curva compila resultados experimentais de toxicidade que quando modelados matematicamente permitem prever a probabilidade de resposta de um ecossistema (composto por muitos organismos) frente a presença de uma substância ou composto. Em outras palavras, a partir de uma curva de sensibilidade podemos prever a capacidade suporte do ambiente para o composto representado na curva. Esta capacidade suporte representa a concentração máxima que um ecossistema pode receber daquele contaminante de maneira ainda considerada como pouco “comprometedora”, e é representada por um valor de concentração conhecido como PNEC (*Predicted non-effect concentration*), que por sua vez é uma concentração que espera-se que não cause nenhum efeito adverso a qualquer população natural num ambiente que esteja exposto a uma determinada substância que representa um risco (Del Signore et al., 2016; Huang et al., 2018; Zhao et al., 2017). Numa curva de sensibilidade é possível identificar as espécies mais sensíveis e mais tolerantes e a finalidade é a proteção de um percentual das espécies testadas (exemplo 95%). A curva de sensibilidade e os PNECs são ferramentas de utilidade para o desenvolvimento de políticas de regulação ambiental, como os critérios de qualidade da água (Martins, 2008; Zhao et al., 2017).

Tabela 4. Características gerais das microalgas *P. boryanum*, *D. communis* e *R. subcapitata*.

Divisão	Família	Nome científico	Características	Dimensões [µm]	Habitat	Estrutura	Referência
Chlorophyta Algas verdes	Hydrodictyaceae	<i>Pseudopediastrum boryanum</i>	Unicelular, colônias apresentadas em múltiplos de 8 (8, 16, 32, raramente acima de 128 per colônia), comumente em organismos unicelulares, das algas verdes mais comuns, em colônias abundantes podem-se ver a simples vista. Uso potencial entre outros biocombustíveis e tratamento de águas de esgoto.	Altura: 10 Diâmetro: em redor de 10	Lagos, lagoas, mar, rios, riachos		Szubryt, 2014
	Scenedesmaceae	<i>Desmodesmus communis</i>	Cenóbio plano, 4 células dispostas linearmente; células internas oblongas, polos arredondados, células externas oblongas a trapeziformes, 1 espinho longo em cada polo, espinhos do cenóbio com arranjo quadricaudato; parede celular lisa, sem costelas; cloroplastídeo 1, parietal, 1 pirenoide	Comprimento: 12-18,8 Largura: 2,5-6,5 Comprimento espinho: 10-17	Perifiton, água fresca principalmente com temperatura moderada, ligeiramente eutrofizados		Ramos, et al., 2015
	Selenastraceae	<i>Raphidocelis subcapitata</i>	Unicelular, em forma solitária. Células são liberadas por meios da ruptura da parede celular parental, sem formar colônia. Sua aparência é curva e torcida como uma foice. Espécie bioindicadora.	Comprimento: 7 - 10 Largura: 1-2	Lagos, lagoas, riachos		Eol [https://eol.org/pages/913704/articles] Yamagishi et al., 2017; Rodrigues, 2002

Em resumo, para esta dissertação foram escolhidos os PPCPs MeP e DCF pois, segundo a literatura, são compostos amplamente detectados em efluentes domésticos, em ETEs, em ambientes aquáticos naturais (águas superficiais e subterrâneas) a até na água de abastecimento. Os efeitos deste compostos foram determinados em três cepas de microalgas da classe Cloroficea, *D. communis*, *P. boryanum*, *R. subcapitata*, que são representativas de ambientes naturais e também sugeridas pelos guias técnicos para ensaios ecotoxicológicos. Os resultados de toxicidade dos compostos-foco deste trabalho para as espécies de microalgas foram abordados sob a ótica de diferentes modelagens estatísticas com o objetivo de obter resultados mais confiáveis.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a toxicidade dos PPCPs Metilparabeno (MeP) e o Diclofenaco (DCF) para microalgas de água doce usando diversas *endpoints* e modelos matemáticos.

2.2 Objetivos específicos

- Estimar concentrações de efeito de MeP e DCF para diferentes espécies de microalgas verdes, usando distintos modelos estatísticos;
- Indicar formas mais adequadas para expressar os valores de toxicidade com base em diferentes *endpoints* (crescimento, concentração de clorofila e viabilidade) e em diferentes abordagens estatísticas (frequentista e bayesiana);
- Construir uma curva de distribuição de sensibilidade das espécies (SSD) para o MeP e calcular valores de PNEC para o químico, por meio de abordagem determinística e probabilística;
- Contribuir para o conhecimento acerca de dois contaminantes emergente (MeP e DCF) com foco na regulamentação destes compostos.

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4. CAPITULO 1

Toxicity of diclofenac to the green algae *Desmodesmus communis* by different endpoints and using Classical and Bayesian models

(a ser submetido para a revista *Ecotoxicology and Environmental Safety*)

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Abstract

In this study, toxicity of diclofenac to the microalgae *Desmodesmus communis* was assessed by EC₁₀, EC₅₀, EC₉₀ (concentration that affects 10, 50 and 90 % of the algal population, respectively) based on culture growth rate that presented the best dose-response curve. They ranged between 38 to 138 mg/L of diclofenac from EC₁₀ to EC₉₀. To obtain the EC_x values, different statistical approaches were used, such as the classical frequentist, and the Bayesian which is less used. Other endpoints as chlorophyll-a and cell viability were also checked. Chl-a did not show a monotonic profile and cell viability as sensitive but not applicable for toxicity tests because of the decreasing in the number of the cells with the increasing drug concentration (there is no cell to estimate viability). The three models used in this study to estimate toxicity (frequentist and Bayesian) were adequate for the dataset, but the Weibull is suggested for the lowest value of EC₁₀. The Bayesian analysis provided a statistical framework more intuitive, producing results in form of probability distribution. Free software like R-Studio 3.5.1 is a tool that permits to develop the own model, and/or implement the different approaches, frequentist (e.g. minpack.lm) and Bayesian (e.g. rjags) to provide the most representative data of toxicity.

Keywords: microalga, viability, chlorophyll, Logistic, Weibull, Beta regression.

1. Introduction

Thousands of emerging compounds are continually released in the environment. According to the US Environmental Protection Agency – USEPA, the emerging contaminants can be defined as “a chemical or nonchemical materials that have a reasonably possible pathway to enter the environment; present a potential unacceptable human health or environmental risk; and either do not have federal regulatory standards based on peer-reviewed science, or the regulatory standards are evolving due to the new science, detection capabilities, or pathways” (USEPA, 2018). Generally, emerging compounds achieve the environment through domestic sewage without treatment. In addition, lots of them are not efficiently removed from the effluent in the wastewater treatment plants, reaching the aquatic systems (Sophia A. and Lima, 2018; Yap et al., 2018; Zhou et al., 2016). The anti-inflammatory diclofenac is an example of emerging contaminant (Haap et al., 2008; Lonappan et al., 2016; Zhang et al., 2008). It is a weak organic acid that belongs to the group of non-steroidal anti-inflammatory drugs – NSAID (Madikizela and Chimuka, 2017), soluble in water (2.37 mg/L at 25 °C) and with short half-life (hours to few days) (Paje et al., 2002; Lawrence et al., 2007). Diclofenac has been detected in the domestic wastewater before and after treatment, and also in aquatic ecosystems (lentic and lotic) (Kasprzyk-Hordern et al., 2009; Madikizela and Chimuka, 2017; Montagner et al., 2017; Yang et al., 2017).

Ecotoxicity tests are useful tools to evaluate the potential risk of a chemical or effluent to the ecosystems (OECD, 2006). In this sense, the effect or lethal concentrations of a compound (e.g. EC₅₀ or LC₅₀) have been used to assess ecological risks, and to derive regulation framework, for the management and control of pollutants in the environment (ECHA, 2008; Kleywegt et al., 2007; Kusk et al., 2018). Standard protocols with different organisms have been developed and used to determine the chemicals toxic potential. They include, procedures of organism cultivation, experimental design, methods for test validation and manners to express the results of the assay with statistical analysis. For the algae, majority of protocols are based on the algae inhibition growth and results are expressed as IC_x – median concentration that inhibited x % of algae culture growth (Kusk et al., 2018; OECD, 2011). However, other measures such as chlorophyll and cell viability

have been used to determine toxicity, because they are more sensitive than growth (Saul da Luz et al., 2016; Suresh Kumar et al., 2014).

Regardless the parameter analyzed, the statistical method used to estimate toxicity can be variable and, consequently, the effect concentration for x % or organisms (EC_x). In toxicity test is important to consider the origin of the variable (continuous or discrete), the endpoint (e.g. biomass, chlorophyll) and the statistical model used to estimate toxicity (OECD, 2006; Sebaugh, 2011). To increase the accuracy of the result (e.g. LC_x or EC_x), it is important to use the appropriate statistical model, which best fits the data of the test (best dose-response curve). The complete description of a statistical model involves specifying how the mean ($E(y)$) is described by a linear or nonlinear function $f(x, \beta)$ of doses or concentrations (x) (Equation 1) and the applicable assumptions (Piegorsch and Bailer, 2005; Ritz et al., 2015). Traditionally the logistic, log-logistic and probit functions have been widely used for this purpose.

Equation (1)

$$E(y) = f(x, \beta)$$

The Bayesian statistical approach is still uncommon in ecotoxicological data analysis because historically, as in ecology, the traditional statistical frequentist methods have prevailed over Bayesian methods (Fox, 2010; McCarthy, 2007). However, this is changing over the last two decades, since Bayesian statistic has been used very successfully in many statistical applications in ecology (e.g. Billoir et al., 2008; Fox, 2010; Grist et al., 2006). The main difference between the frequentist and Bayesian approach is in the concept of probability. While in the frequentist tradition probabilities are restricted to the description of random variables arising from replicated events, the Bayesian approach defines probability as a very broad measurement of uncertainty. Hence, incomplete knowledge about a fixed parameter is described by a probability distribution in Bayesian analysis. Prior (pre-data) uncertainty about some unknown model parameter is described by a prior probability distribution and combined with the data (using Bayes Theorem) to obtain new updated posterior knowledge described by the posterior probability distribution (McCarthy, 2007; Billoir et al., 2008; Kinas and Andrade, 2010). If prior knowledge about

the situation in analysis is vague or non-informative, the result obtained from the resulting posterior distribution tend to be close to results obtained from a frequentist analysis (Billoir et al., 2008). But, how can the uncertainties measurements help in the calculation of EC₅₀ in a doses-response experiment? The Bayesian approach will produce a posterior distribution for EC₅₀ that expresses current up-to-date knowledge. This posterior distribution allows, among other things, building credibility intervals (CrI); which constitute a range of possible values with an attached known probability (Feckler et al., 2018). For instance, a 95% credibility interval (CrI95%) defines a range of the 95% most plausible values of EC₅₀ (Kinas and Andrade, 2010).

Assuming that the toxicity is expressed in the form EC₅₀ (effect concentration at which 50% of the algae expect to have an effect), the Bayesian analysis will start with large prior uncertainty associated with the parameters involved in the EC₅₀ calculation, update to the posterior distribution for these parameters after observing the results from the experiment and finally, it will be obtained the posterior distribution and credibility interval for EC₅₀ will be calculated. This allows a range of plausible values that can be used for ecosystem management and regulatory frameworks.

In this study, toxicity of diclofenac to the microalga *Desmodesmus communis* was evaluated based on growth rate (biomass), chlorophyll-a (Chl-a) concentration and cellular viability. Different mathematical models were used to estimate values of EC₅₀ of diclofenac for the microalga, through different approaches such as frequentist models (based on Logistic and Weibull regressions) and Bayesian Model (Beta regression),

4.2. Methodology

2.1 Algae toxicity test

The toxicity tests were carried out according to OECD guideline 201 and ABNT 12648:2011. The alga used were *Desmodesmus communis* (E. Hegewald), obtained from Collection of Freshwater Microalgae Cultures of the Biological Sciences Institute of the Universidade Federal do Rio Grande (FURG). The strains were cultivated in WC medium under a temperature of 21°C, in white fluorescent light ($\sim 100 \mu\text{mol m}^{-2} \text{s}^{-1}$) and photoperiod 12L:12D, without shaking. Diclofenac sodium salt (DFC - C₁₄H₁₁Cl₂NO₂), with analytical

grade of 99% purity was added to algae culture media in interval from 10 to 160 mg L⁻¹ (with a factor x2). A control without the drug was kept over the test. The concentrations series was prepared by mixing the WC medium with a stock solution of the diclofenac, and then algae were inoculated. Algae concentration in each flask was 10⁴ cells mL⁻¹. They were obtained from a pre-culture at exponential growth. The final test vials volume was 50 mL, and tests were conducted in the same conditions as the culture. Exposure time was 72 h and endpoints analyzed were: growth rate, chlorophyll-a concentration and cellular viability. The pH was (adjusted to 7.0 ± 0.2), measured in the control group at the beginning and at the end of the test, and variation was lower than 1.5 (pH = 7.21 ± 0.05 at beginning and 7.17 ± 0.12 at the end), with a coefficient of variation (cv) lower than 20%. In addition, reference tests with NaCl were conducted and the mean result from five tests was EC₅₀ = 161.53 mM, ± 33.34, cv = 20.6 based on optical density (OD).

During the test, the optical density (OD) was analyzed (daily up to 72 h) by spectrophotometry at 750 nm. At the end (72 h) an aliquot was fixed and stained with Neutral Red (2 h) for cell viability (V) evaluation as described by da Luz et al. (2016). After incubation with Neutral Red, the algae were fixed with 4 % formaldehyde. Cells were counted in Newbauer chamber using an optic microscopy and counted. A total of 200 cells or 16 quadrants of the chamber were counted. Red cells were considered as viable and green cells as non-viable (supplementary material Table 2.4-A). Another aliquot (10 mL) was reserved for Chl-a assay. The Chl-a was extracted with methanol and estimated using a spectrophotometer at 665 nm. Results were expressed as µg of Chl-a L⁻¹ (Mackinney, G, 1941).

2.2 Statistical

2.1 Toxicity test

A one-way ANOVA variance analysis was used to compare the effects of diclofenac on algal growth and Chl-a content to the control (residuals analysis were performed to validate the assumptions: normality, homocedasticity and independence). Tukey post hoc test was used for the significant differences (p < 0.05) (software R-Studio

3.5.1 - R Core Team, 2018). The Kruskal-Wallis test and the post hoc Dunn were performed for the viability data (for non parametric data) with the same software.

2.2 Growth inhibition calculation

The inhibition vector (I) (Equation 3) (Bruce and Versteeg, 1992) was calculated based on the growth rate (Equation 2) according to OD estimation (the absorbance values were transformed into values in cells per mL through the growth curve for the *D. communis* [$y = 2E-07x - 0.0023$ $R^2 = 0.9988$]). For the vector $I < 0$, we considered 0, since negative values indicate non-inhibition of growth. Otherwise, if $I > 1$, we considered 1, meaning full inhibition caused by the drug (Nyholm et al., 1992). This condition $0 \leq I \leq 1$ was programmed in software R-Studio 3.5.1.

Equation (2)

$$r = (\ln(df) - \ln(di)) / (tf - ti)$$

where,

r = Growth rate

d(f,i) = final (f) and start (i) optical density,

t(f,i) = final (f) and start (i) time.

Equation (3)

$$I = (r_0 - r_j) / r_0,$$

where,

I = inhibition vector

r_0 = growth rate in the control

r_j = growth rate in the treatment or concentration j.

2.3 Classical analysis to estimate diclofenac toxicity

In the algae toxicity test, response or endpoint was a continuous variable (e.g., biomass, optical density, yield, chlorophyll, growth rate). Commonly the response is treated

as binary (binomial) (i.e. present or absent, dead or alive). In this case it should be considered the type and nature of the response previously choosing a model (Ritz et al., 2015). When a model is selected, it is important to have a clear understanding of the assumptions implying e.g. monotonic response. The main problem is to obtain a good or best fit dose-response curve for the parameter (analytic or numerical techniques). (Nyholm et al., 1992; Ritz and Streibig, 2008).

The purpose was to obtain a functional relationship between inhibition vector (described above) and the concentration vector (Nyholm et al., 1992). Nonlinear regression models relating y (response) to x (concentration), through a nonlinear function f (Equation 1) generally requires numerical methods to estimate toxicity (Christensen et al., 2009; Ritz and Streibig, 2008). In this process the first step is find reasonable initial values for the parameters to start the numerical iterations. Some ways of finding these initial values are describing below. The models used to fit the results of toxicity test with diclofenac were the Logistic and Weibull.

2.3.1 Logistic Model

This model is widely used and expresses a relationship through a sigmoid curve, symmetric in relation to EC_{50} . The logistic function Equation (4.a) is also known as Boltzman equation or sigmoid model. Important do not confuse with the log-logistic model (Ritz, 2010). It was used this Logistic Model with three parameters Equation (4.b) attending the parsimony principle.

Equation (4.a)

$$f(x; b, c, d, e) = c + (d-c) / (1 + \exp [b(x-e)])$$

The parameter c corresponds to the lowest asymptote (in the same units as the response) and equals zero for us because negative values for the response is equal to that there was no inhibition, therefore they are considered zero inhibition.

Equation (4.b)

$$f(x; b, d, e) = d / (1 + \exp [b(x-e)]) \text{ (Ritz, 2010)}$$

The parameter d is a metric regarding the control; many models define $d = 1$, as the maximum value for the control. So, the domain or response is restricted to the interval 0 to 1. In the present study it was not achieved 100% inhibition, for this reason, this parameter was kept. The parameter b is associated with the slope and corresponds to EC_{50} .

EC_{50} is the inflexion point in a sigmoid curve; by definition it is the value for x for which $f'' = 0$. The numeric method used to solve the equation is Levenberg-Maquardt (LM) that is a derivation of Newton's method; which has the advantage of be faster to converge (De França et al., 2009). For the implementation of this process we used the package `minipack.lm` (Elzhov et al., 2016) with the `nlsLM()` function that implements the algorithm LM in software R-Studio 3.5.1 (R Core Team, 2018). The code used to implement the algorithm is provided in the supplementary material.

2.3.2 Weibull Model

A sigmoid curve based on the exponential operator, is a nonsymmetrical function (Equation 5) that is less traditional than the logistic or the log-logistic in doses-response models. However, according to Christensen et al. (2009) (see also Christensen and Nyholm, 1984), this seems to be a good model to represent this kind of doses-response relationship. It was used Weibull Model of two parameters. The logarithm of parameter c represents the intercept associated with the slope at this point, and d is related with the slope. The function of the Weibull regression was programmed and was solved using the same numerical method LM of the package `minipack.lm` in the same statistical software R-Studio 3.5.1; thus obtaining the values of the parameters c and d (note that these parameters are different from the c and d of the logistic regression). The implemented algorithm is in the supplementary material.

Equation (5)

$$f(x; c, d) = 1 - \exp[-c(xd)] \text{ (Christensen et al., 2009)}$$

2.3.4 Starting values

This is an important step to fit the models. It was tested several forms to obtain starting values in the following order: 1. Graphically, when we identified visually the possible value for each parameter (3 parameters for the logistic equation, and 2 parameters for the Weibull equation). 2. Linearly, when we use the linear transformation of the logistic and Weibull equations (analytic solution). 3. Self-starter functions, provided in R by the available `SSlogis()` and `SSweibull()` to logistic and Weibull function, respectively. 4. Finally, we used the estimative obtained from an appropriate `glm` (generalized linear model) as starting values (Bruce and Versteeg, 1992; Ritz and Streibig, 2008)

2.3.5 Parameter Estimation

Nonlinear least squares minimization was the method used to estimate the parameters under the assumption of normally distributed residual values, Equation (6) (Ritz et al., 2015). As mentioned in previous sections, to get the parameter estimation we applied the function `nlsLM()` from package `minpack.lm` (Elzhov et al., 2016) that uses the Levenberg-Marquardt algorithm for nonlinear regression (it is less sensitive).

Equation (6)

$$\sum (R_i)^2 = \sum (y_i - f(x_i - \beta))^2$$

where,

R = residue

Y_i = response variable

$f(x_i - \beta)$ = mean function

2.3.6 Assumptions.

Normally distributed errors (I.e, residuals) were checked with the `qqnorm` plot or Shapiro's test. Variance homogeneity was checked by Levene's test. Independence of errors was checked by the typical autocorrelation plot. For the adjusted regression curves (dose-response), a hypothesis test of lack of adjustment was performed comparing the non-linear regression model used with the more general ANOVA model (Ritz and Streibig, 2008) (Supplemental material - Model diagnostic).

2.4 Statistical Bayesian analysis for DFC data toxicity.

2.4.1 Beta regression.

The beta distribution (Equation 7) is a flexible probability distribution (shapes depending on the values of two parameters) for modeling proportions, where the continuous random variable is delimited to the interval 0 to 1. In a convenient parameterization of the beta distribution (see Ferrari and Cribari-Neto, 2004), in terms of its mean, it became an option to modelling rates and proportions as a function of explanatory variables. Denoted as “beta regression” it has already wide applications (Cepeda-Cuervo, 2015; Ferrari and Cribari-Neto, 2004). It was chosen for present study because it seems to be a good model for the data generated with algae tests.

Equation (7)

$f(y; \mu, \phi) = \Gamma\phi / \{\Gamma(\mu\phi) \cdot \Gamma[(1-\mu)\phi]\} \cdot [y\mu\phi-1 \cdot (1-y)(1-\mu)\phi-1]$, $y \in (0,1)$ (Ferrari and Cribari-Neto, 2004)

The mean of the y response assumes a Beta distribution (μ, ϕ). The beta regression model is defined as follows:

Equation (8)

$$g1(\mu_i) = \eta1_i = f1(x_i^T; \beta) \quad 8.1$$

$$\text{Logit} = \log(\mu/(1-\mu)) = \eta1 \quad 8.2 \text{ Logit link function}$$

$$\eta1 = a + bxi \quad 8.3 \text{ Linear transformation}$$

(Ferrari and Cribari-Neto, 2004; Simas et al., 2010)

The logit function of the expected growth inhibition probability (μ) is assumed to be a linear function of diclofenac concentration (xi). In contrast to the methods described previously, in beta-regression the response variable is treated like a continuous variable, with a Beta distribution (Cepeda-Cuervo and Garrido, 2015). The parameter estimation is

performed within a Bayesian framework. The posterior distribution is obtained with stochastic simulation. For this purpose, we applied the Markov chain Monte Carlo (MCMC) methods with the use JAGS (Cepeda-Cuervo and Garrido, 2015; Kinas and Andrade, 2010) using the library rjags (Plummer et al., 2018) as an interface with software R. All posterior summaries and further statistical analyses were performed within software R-Studio 3.5.1.

To obtain initial values to start the Markoc chains, first we fitted the conventional `betareg()` function available in the package `betareg` in R (Zeileis et al., 2018). Subsequently, assuming independent vaguely informative (i.e. with very large variance) normal prior distribution for the regression parameters, 2.000 samples of the posterior distribution were generated, using R software with the package `rjags` (Plummer et al., 2018). The posterior parameter estimation was obtained from the sample of the posterior distribution taking a thinning of 5 (i.e. retaining only every fifth simulation in the Markov chain) after a burning of 2.000 observations (the initial warm-up fase of the chain) (se McCarthy, 2007; or Kinas and Andrade, 2010 for details on stochastic simulated posterior simulation and MCMC). The posterior parameter estimation given by the mean and credibility intervals extracted from the marginal posterior distributions are given in Table (1) (Cepeda-Cuervo, 2015). The full code is available in the supplementary material.

2.5 EC₅₀

In the frequentist Logistic and Weibull models, the EC₅₀ were estimated following the methodology of Christensen et al. (2009). In the Bayesian Model, the EC₅₀ was obtained by posterior distribution (see Table 1 and Figure 6 in section 3). Calculations are detailed in the supplementary material.

3. Results

3.1 Endpoints of toxicity

Figure 1 shows the growth rate (GR) of *D. communis* exposed to a range of diclofenac concentration (10 to 160 mg L⁻¹) for 72 h. A significant decrease of GR was observed on the two highest concentration tested (80 to 160 mg L⁻¹). Chl-a concentrations are presented in Figure 2. Despite non-significant, lower concentrations (10 to 40 mg L⁻¹ of

diclofenac) induced Chl-a synthesis, and only at 160 mg L⁻¹ of diclofenac, there was a reduction in Chl-a concentration.

Viability of microalgae *D. communis* was assessed by Neutral Red stain and estimated for 200 cells. In the absence of 200 cells, it was cells present in 16 quadrants of Neubauer Chamber. Control reached the highest viability (98%), followed by 10 mg L⁻¹ of diclofenac (92%). Concentration of 20 mg L⁻¹ of DFC affected cell viability (83% of viable cells), but did not reduced density of cells, in fact, at 20 mg L⁻¹ of DFC density of cells augmented in comparison to the control. Concentrations of 40, 80 and 160 mg L⁻¹ caused significant decrease in *D. communis* viability (68, 0 and 0 %, respectively) in comparison to control group. In addition, number of cells also significantly decreased at 80 and 160 mg L⁻¹ of diclofenac.

3.1 Statistical approaches

Table 1 summarizes results of diclofenac toxicity to *D. communis*, expressed as EC_x, estimated by Logistic, Weibull and Beta. It was calculated the EC₁₀, EC₅₀, and EC₉₀ of diclofenac based on the microalgae GR, which are represented in Table 1. In addition, the dose-responses curves and inhibition vector are presented in Figure 4. It is clear that logistic model is symmetric at EC₅₀, the Weibull model is a more linear and stricter curve, and the Beta regression displays a curve with a lower slope. In general, the dataset presented a good response decreasing in comparison to the control up to the higher concentration with a maximum effect of 83% (average inhibition of growth of the highest concentration). This is an important point because the EC values were based in the maximum effect. Moreover, in Figure 4, the 0.5 (as it is usually called) in the y axis does not represent 50% of effect; in fact, it corresponds to $0.83 \times 0.5 = 0.415$.

In the Bayesian Beta regression model we assumed that the observations were fit in beta distribution. Figure 5 shows all possible curves that could be adjusted to our experimental dataset, based on the posterior distribution. The marginal posterior distribution for the EC_x values (Figure 6) was built directly from the posterior distribution for the parameters of the beta model (see supplementary material).

4. Discussion

In this study, ecotoxicity of diclofenac to the microalgae *D. communis* was assessed by GR, Chl-a concentration and cell viability. GR was used to calculate the inhibition vector (EC_x) because it was the parameter with the better-fit dose-responses curves. On the other hand, Chl-a concentration did not present a monotonic pattern, while; viability was considered a measure of little accuracy because at high concentrations of DFC, there were no cells to be counted. This limits the use of analysis of cell viability to non-exposed cultures or exposed to sublethal treatments. Cell viability is a sensitive analysis indicated to determine viable and non-viable cells (Saul da Luz, et al., 2016; Zetsche and Meysman, 2012). However, in the present study, there were no cells for viable cell counting in the two highest concentrations of diclofenac. Thus, measurements of viability can be helpful to check the health status of a culture or for sublethal exposures, but care should be taken when using this endpoint to calculate the EC as the basis of expression of dose-response curves (Repetto et al., 2008).

Concerning Chl-a, many studies have been showed a decrease in chlorophyll efficiency and content after exposure to several kinds of contaminants, suggesting it as a sensitive parameter for toxicity estimation. Although non-significant, there was an increase in Chl-a content following exposure to 10 mg L⁻¹ of diclofenac. Perhaps, this increase could be a response of *D. communis* to compensate the reduction of photosynthetic efficiency in sublethal doses of the drug. This also may indicate that microalgae exposed to diclofenac is requiring higher amounts of Chl-a to cope with detoxification, which in general consumes more energy than a non-stressful situation. Couderchet and Vernet. (2003) found a similar trend in their study with *Scenedesmus obliquus* exposed to a pesticide. After 48 h of exposure they observed a slight increase in Chl-a content at concentration lower than EC_{50} . Liu et al. (2011) found diverse profiles of chlorophyll concentrations in *Selenastrum capricornatum* in response to exposure to different of antibiotics. For sulfamethoxazole, the authors observed an augment in chlorophyll concentration (non-significant) in the lowest concentration tested in the assay.

Regarding the models used to express EC_x , data were fitted in dose-response curves within the three models. However, the Weibull was the one that showed the lowest EC_{10} . In terms of risk assessment, this model may be the most protective approach for environment,

considering diclofenac as the toxicant. Christensen et al. (2009), suggest the Weibull as the best model to be use in the derivation of water quality criteria.

EC_x were based on the plateau that corresponds to the maximum effect (mean of 83 %), that is directly related to the estimation of toxicity. The conventional method keeps the plateau value as 1, and do not consider the importance of define a plateau value (depending on the control response) (Sebaugh, 2011). The Bayesian framework offers another form to estimate EC_x. It is expressed as marginal posterior probability distribution from which the estimates (mean of the distribution) and credibility intervals are readily available. It is an interesting analysis that should be more incorporated in ecotoxicological data (Fox, 2010). For the results of this work, there was a discrepancy in the EC₅₀ value delivered by the models. The credibility intervals obtained by the Bayesian analysis does not cover the EC₅₀ value delivered by logistic model and the value delivered by the Weibull model enter by few decimals (frequentist analysis). According to this, the logistic EC₅₀ is outside the range of 95% likely values for this parameter in the Bayesian context. In contrast, the opposite happens with the EC₁₀ value, for which the credibility intervals contain the values delivered by all the two frequentist models.

Comparing values of diclofenac toxicity, Cleuvers (2003; 2004) presented EC₅₀ of 72 mg L⁻¹ of diclofenac considering *Desmodesmus subspicatus* chlorophyll fluorescence after 72 h of exposure to the drug. The statistical software used was SIGMA PLOT 2000, and it was used a nonlinear curve fitting based on a sigmoid model (four-parameter logistic function). On the other hand, Ferrari et al. (2004) presented EC₅₀ of 163 mg L⁻¹ of diclofenac for *Pseudokirchneriella subcapitata* after 96 h of test by cellular density (counting the cell or absorbance at 550nm). The effect was estimated by regression using a log-logistic model known as Hill Model. Differences in EC_x values of same contaminant can be related to the sensitiveness of the species tested, to the endpoint that is being considered as well as to the static method used to calculate toxicity values, as shown in Table 1.

A common practice in ecotoxicology is the use of specialized softwares or models to estimate toxicity (e.g. TOXSTAT, DOSbox, ToxRat, Trimmed Speramn Karber, Probit). However, it is important to emphasize that these models are not always appropriate or precise, or that adjusting to the data. These softwares impose limitations to explore,

manipulate the datasets and are costly. Usually, they estimate EC_x in a black box and the result is provided without parameters and equations used to obtain the data (Christensen et al; 2009). In contrast, these work presented models available for free in the software R-Studio 3.5.1 to estimate toxicity, and compared different approaches (Bayesian and frequentist) to extend possibilities of dose-response relationship analyses. Nevertheless, the most important is to use a best-fit model to increase the reliability of toxicity data (Ritz, 2013).

5. Conclusion

The best parameter to express toxicity of diclofenac to *D. communis* is the GR. Chl-a did not show a monotonic profile and cell viability were sensitive but not applicable for toxicity test because of the decreasing in the number of the cells with the increasing drug concentration (there is no cell to estimate viability). The three models used in this study to estimate toxicity (frequentist and Bayesian) were adequate for the dataset, but the Weibull is suggested because of the lower value of EC_{10} . The Bayesian analysis provided a statistical framework more intuitive, producing results in form of probability distribution. Free software like R-Studio 3.5.1 is a tool that permits to develop the own model, and/or implement the different approaches, frequentist (e.g. minpack.lm) and Bayesian (e.g. rjags) to provide the more representative data of toxicity.

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Table 1. Parameters and EC values of the models fitted for the experimental data from DFC and *D. communis*. 95% confidence and credibility intervals for each value according to the approach frequentist and Bayesian. [confidence interval, 95%]; (credibility intervals, 95%). Each letter corresponds to different parameter for each model.

Statistic approach	Model	Parameters			EC (mg L ⁻¹)		
					EC ₁₀	EC ₅₀	EC ₉₀
Frequentist	Logistic	b = -0.072 [-0.10 to -0.041]	d = 0. 83 [0.78 - 0.88]	e = 82.36 [78.49 - 86.22]	52.10 [42.99 - 61.22]	82.36 [67.95 - 96.76]	112.61 [92.92 - 132.31]
	Weibull	c = 3.2e-05 [1.64e-05 - 8.22e-04]	d = 2.16 [1.83 - 2.48]		38.45 [29.76 - 47.14]	89.47 [69.25 - 109.69]	138.39 [107.12 - 169.67]
Bayesian	Beta	a = -3.87 (-4.57 to -3.11)	b = 0.03 (0.026 - 0.041)		47.22 (36.36 - 57.74)	101.70 (89.38 - 113.18)	134.43 (122.54 - 157.85)

Captions to Figures

Figure 1. Growth rate of *D. communis* exposed to different concentrations of diclofenac. Results are expressed as means $\pm$ standard deviation ($n = 5$). The asterisk (*) indicates significant differences between treatments with diclofenac and the control ($p < 0.05$).

Figure 2. Chlorophyll-a ($\mu\text{g L}^{-1}$) concentration in culture of *D. communis* exposed to diclofenac for 72 h. Results are expressed as means $\pm$ standard deviation ($n = 5$). The asterisk (*) indicates significant differences between treatments with diclofenac and the control ($p < 0.05$).

Figure 3. Cells viability (%) vs cells density (Cell mL^{-1}) of *D. communis* exposed to diclofenac for 72 h. Results are expressed as means $\pm$ standard deviation ($n = 5$). The asterisk (*) indicates significant differences between treatments with diclofenac and the control ($p < 0.05$).

Figure 4. Dose-responses relationship between growth rate of *D. communis* and diclofenac concentration using Logistic, Weibull and Beta regression models (solid, dashes line and dashes line fine).

Figure 5. Beta regression posterior curves generated with the 2000 iteration of Markov chain Monte Carlo - MCMC. The points represent the dataset (average inhibition vector), the emphasized curve represent the best curve fitted to experimental dataset.

Figure 6. Posterior marginal distributions of EC_{10} , EC_{50} and EC_{90} values (mg L^{-1} diclofenac) for with *D. communis*. Values on the x-axis represent the posterior mean and line represents the 95% credibility intervals.

Figure 1

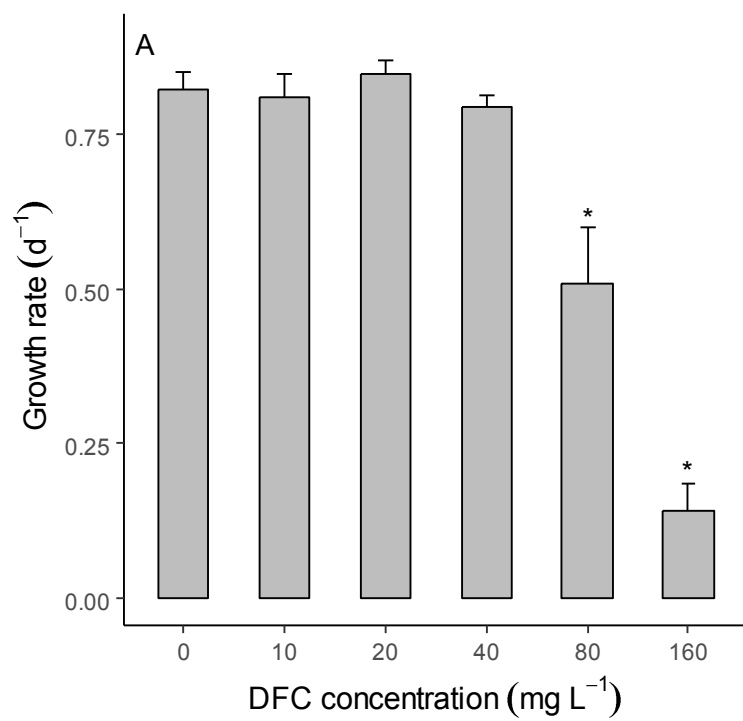


Figure 2

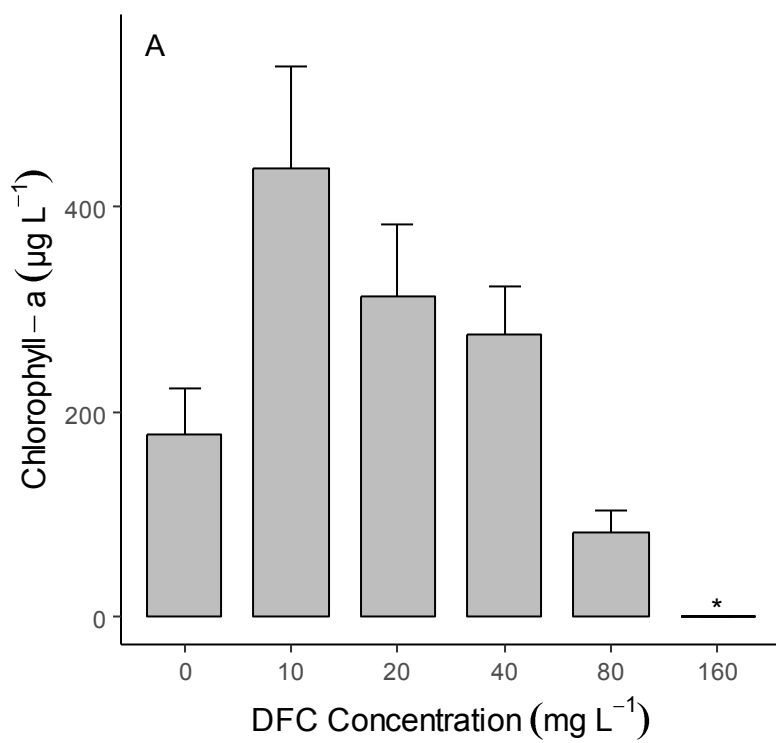


Figure 3

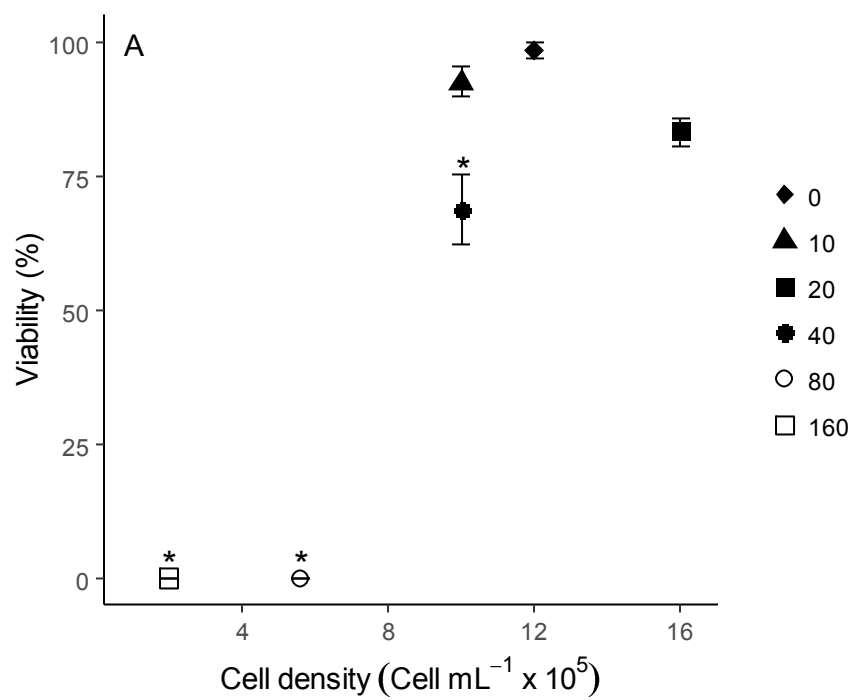


Figure 4

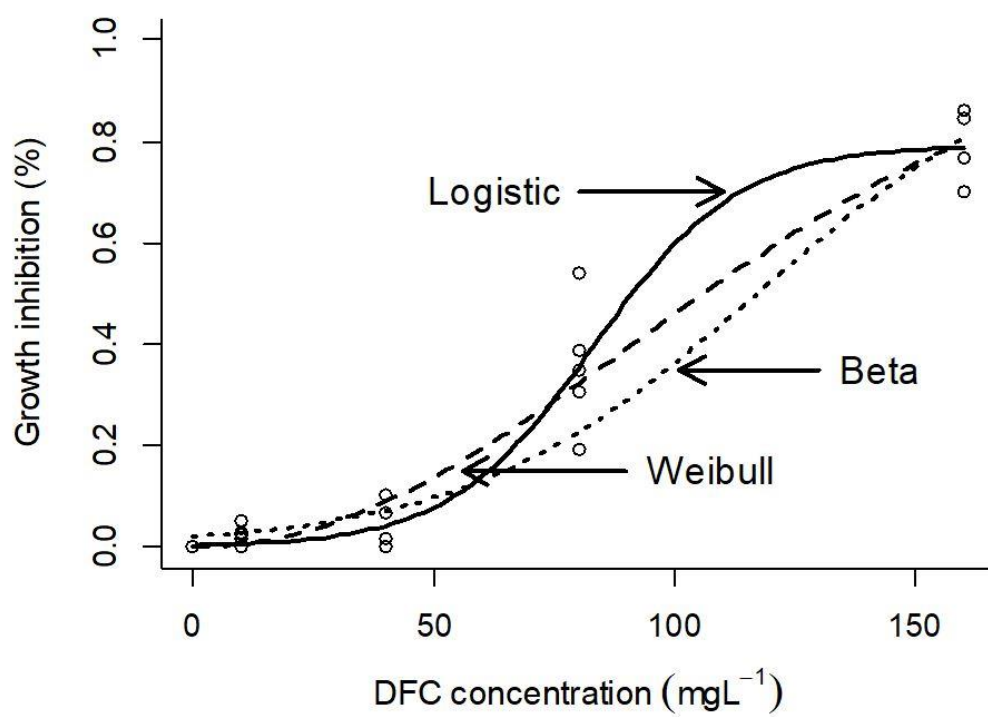


Figure 5

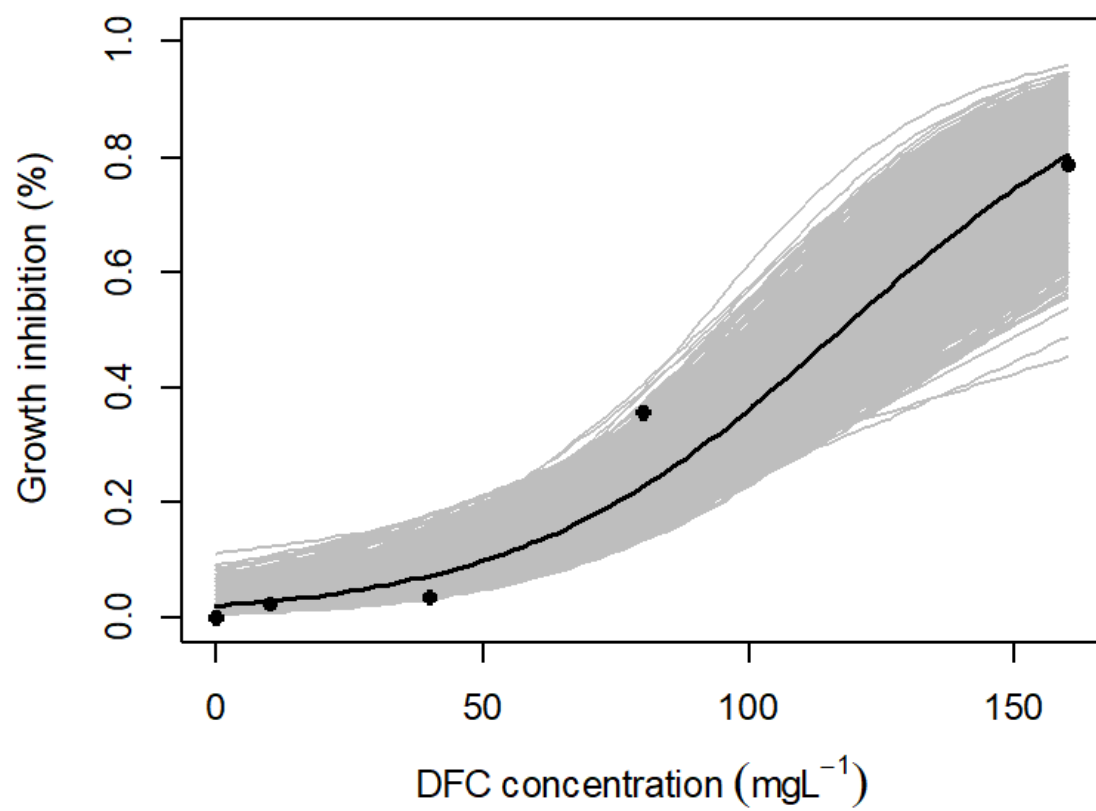
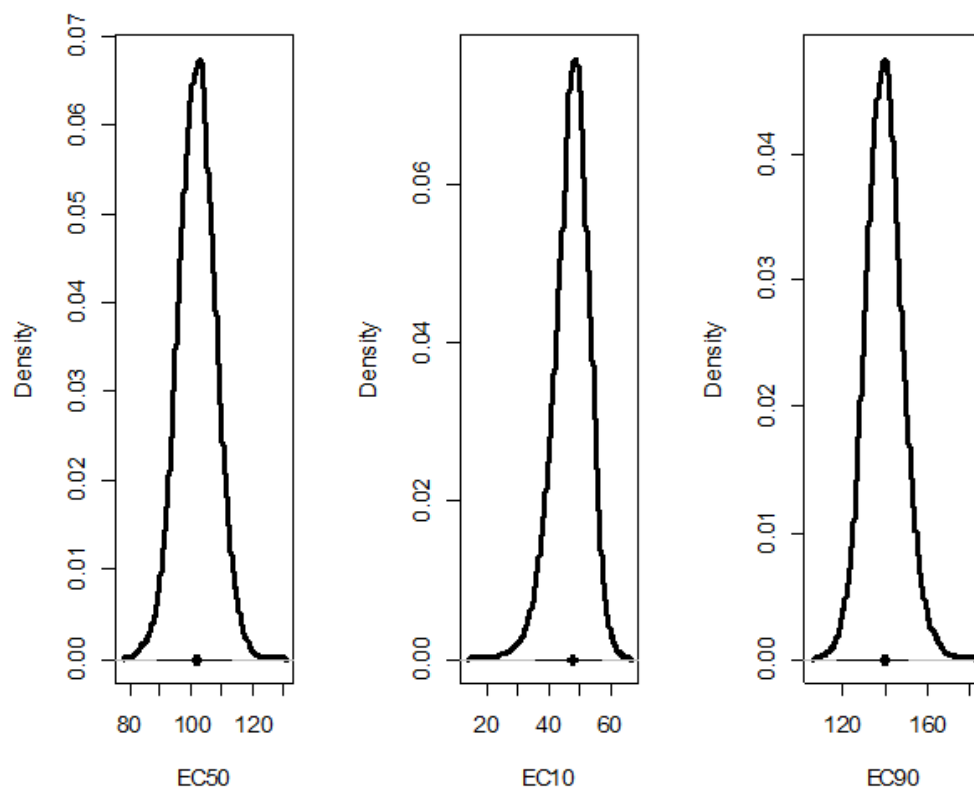


Figure 6



4.1 Supplemental material

1. Dataset

The table to show the inhibition vector and the concentration x. This vector was obtaining from growth rate.

Inhibition	x
0.00	0
0.00	0
0.00	0
0.00	0
0.00	0
0.00	10
0.00	10
0.05	10
0.00	10
0.08	10
0.00	20
0.01	20
0.00	20
0.00	20
0.00	20
0.01	40
0.04	40
0.01	40
0.07	40
0.03	40
0.52	80
0.43	80
0.34	80
0.39	80
0.22	80
0.82	160
0.90	160
0.75	160
0.86	160
0.82	160

2. Models: below are show the models used to fitted the dataset.

2.1 Logistic model

$$f(x; b, c, d, e) = c + (d - c) / (1 + \exp [b(x - e)]) \text{ (Ritz, 2010)}$$

We load the package library(minpack.lm) to we can use the nlsLM function to estimate the parameters of model. With five iterations we obtain the convergence, thanks to the good initial value and the Levenberg-Marquardt method.

```
Logis.model <- nlsLM(inhibition ~ (d/(1+exp(b * +(x - e)))),
                    data = Dr, start = list(b= -0.06, d = 0.83, e =
                    83.16), trace = TRUE)
It.    0, RSS = 0.0736174, Par. = -0.06      0.83      83
It.    1, RSS = 0.0705539, Par. = -0.0693952 0.831286 82.3797
It.    2, RSS = 0.0703919, Par. = -0.0722475 0.832185 82.375
It.    3, RSS = 0.0703903, Par. = -0.0726005 0.832175 82.3603
It.    4, RSS = 0.0703903, Par. = -0.0726214 0.83217 82.3592
It.    5, RSS = 0.0703903, Par. = -0.0726225 0.83217 82.3592
```

All parameters are significantly different of zero. Is a form to justify the use of three parameters in this model.

```
summary(Logis.model)

Formula: inhibition ~ (d/(1 + exp(b * +(x - e))))

Parameters:
  Estimate Std. Error t value Pr(>|t|)
b -0.07262    0.01516  -4.79 5.36e-05 ***
d  0.83217    0.02348  35.44 < 2e-16 ***
e 82.35919    1.88455  43.70 < 2e-16 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.05106 on 27 degrees of freedom

Number of iterations to convergence: 5
Achieved convergence tolerance: 1.49e-08

AIC(Logis.model)
[1] -88.51062
```

2.1.1 Model diagnostic

2.1.1-A Model fit

We start with the adjustment of the model verified graphically with the residuals and the mean function seems to

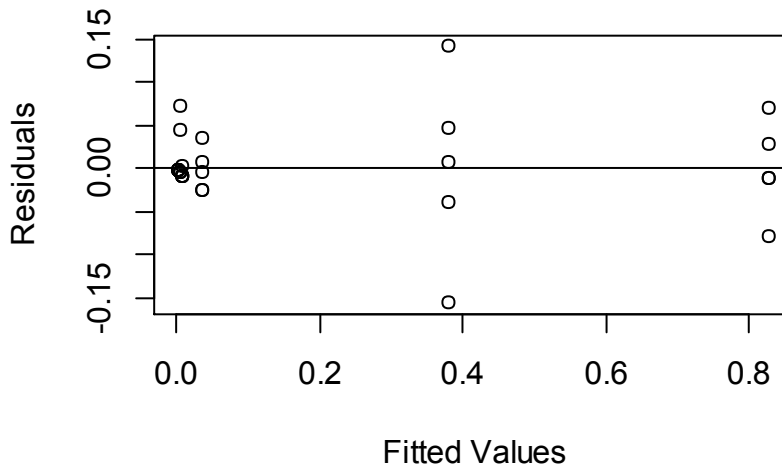


Figure 2.1.1-A. Model residuals versus fitted values from Logistic model

We apply the Lack-of-fit tests to supplement the graphic observation.

```
Linear.model <- lm(inhibition ~ as.factor(x), data = Dr)
anova(logis.model, Linear.model)
```

Analysis of Variance Table

Model 1: inhibition ~ (d/(1 + exp(b * +(x - e))))

Model 2: inhibition ~ as.factor(x)

	Res.Df	Res.Sum Sq	Df	Sum Sq	F value	Pr(>F)
1	27	0.070390				
2	24	0.067934	3	0.0024561	0.2892	0.8327

The null hypothesis (H_0 : lack of fit) is rejected because the p-value is > 0.05 . Thus we accepted the model fitted. We conclude that the logistic model with three parameters is appropriate to describing the doses response relationship.

2.1.1-B Variance homogeneity

First we look the absolute residuals with a plot the absolute residuals and fitted values. This figure below evidences an increasing in the center fitted value of the graphic.

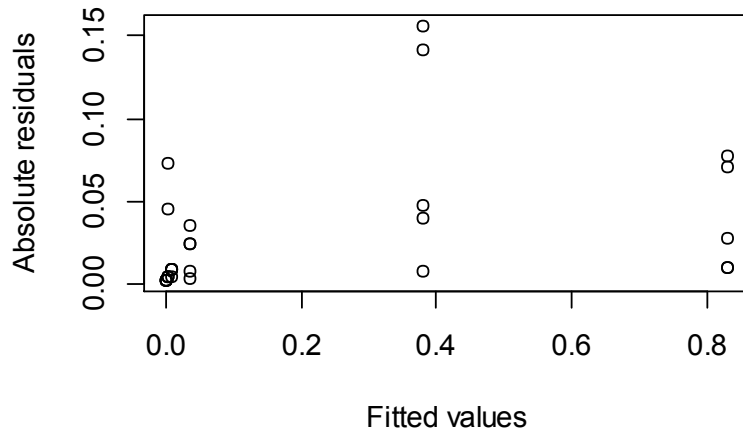


Figure 2.1.1-B. Absolute residuals and fitted values

We apply the Levene's test to supplement the graphic observation. The null hypothesis (H_0 : homoscedasticity) is accepted p-value is < 0.05 . Thus the homogeneity of the variance it is not true. But according to the figure above the violation to this assumption does not seem to be so serious.

```
Levene's Test for Homogeneity of Variance (center = median)
  Df F value Pr(>F)
group 5  3.1402 0.02547 *
  24
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

2.1.1-C Normal distribution

1
2
3

data set, the residuals
are that the normality

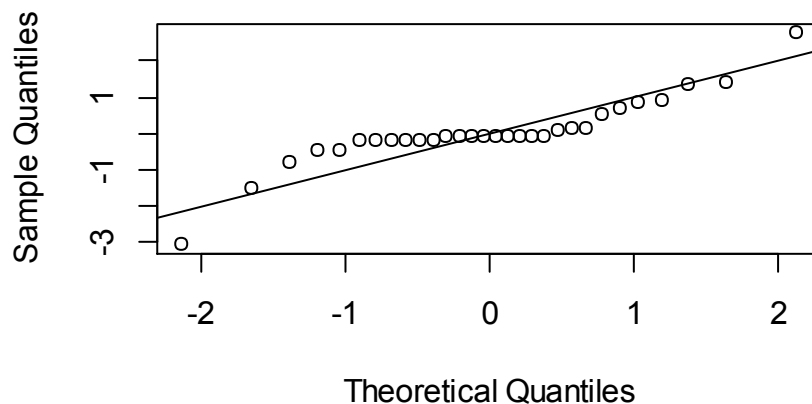


Figure 2.1.1-C. Normal QQ plot for the model fit Logistic (Logis.model)

We also verified normality by Shapiro hypothesis test but did not pass the test, which is attributed to the small number of data mainly.

2.1.1-D Independence: It is assumed from the beginning of the test that independence is assured, because each test bottle is prepared individually.

2.1.2. calculation of EC values (Logistic model)

The parameter and the confidence intervals

```
coef(logis.model)
      b          d          e
-0.07262249  0.83217009 82.35918589

confint2(logis.model)
      2.5 %      97.5 %
b -0.1037324 -0.0415126
d  0.7839949  0.8803453
e 78.4924112 86.2259606
```

We calculate the EC concentration following the method used by Christensen et al. (2009). Doing zero the parameter c of the logistic model and linearizing the equation we obtain

$$f(x; b, d, e) = d / (1 + \exp [b(x-e)]) \text{ (Ritz, 2010)}$$

We do $u = b(x-e)$ and the linear transformation is $u = \ln((d - (y*d)) / (y*d))$ y is the percentage of inhibition that we want to obtain b, d, e are the parameters obtained with the logistic model see box above

```

##ECx values and confidence intervals for the logistic function

# coefficients obtained from the regression
b <- coefficients(m7)[1]
-0.07262249
d <- coefficients(m7)[2]
0.8321701
e <- coefficients(m7)[3]
82.35919

# function of linear transformation of the logistic equation
Ulogis <- function(y) {
  u <- log((d-(y*d))/(y*d))
  return(u)}

# EC50 value
u50 <- Ulogis(0.5)
0
EC50 <- (u50+(b*e))/b
82.35919

# EC10 value
u10 <- Ulogis(0.1)
2.197225
EC10 <- (u10+(b*e))/b
52.10376

# EC90 value
u <- Ulogis(0.9)
-2.197225
EC90 <- (u+(b*e))/b
112.6146

```

2.1.3. Confidence intervals (Logistic model)

```
# Derivative representing the slope in 50% of the response
dfunçãoL <- deriv(~d/(1+exp(b*(z-e))), "z")

Solving the system of equations for b and replacing in the derivative
expressed as a function of b,
we obtain the slope expressed as a function of EC10 and EC50.
This way we then arrive in the form EC10 = EC50 - a/slope
u.50 <- b*(EC50-e)
u.10 <- b*(EC10-e)

# Thus a for the logistic function assumes the value of:
a <- 0.4339

# The slope corresponding to the 10% inhibition was calculated with
the
derivative evaluated at the value of u10
s10 <- -0.747*b

# Norma p.d.f. 1-alfa confidence coefficient of the
# interval alfa 0.05
tz <- qnorm(0.95)

# standard error associated with parameter b
errorb <- 0.01516

# Logit error function (DFC and D. communis data)
EcX <- function(x) {
  er <- sqrt(((x^2)/EC50^2)+ (((a/s10))^2)*(((tz)*errorb)/b)^2)
  return(er)
}

# error factor
ferr <- max(EcX(0:EC50)) - min(EcX(0:EC50))
0.1749067

# Confidence interval to EC10
EC10-ferr*EC10
42.99046
EC10+ferr*EC10
61.21705

# Confidence interval to EC50
EC50-ferr*EC50
67.95401
EC50+ferr*EC50
96.76436

# Confidence interval to EC90
EC90-ferr*EC90
92.91756
EC90+ferr*EC90
132.3117
```

2.2. Weibull model

$$f(x; c, d) = 1 - \exp[-c(xd)] \text{ (Christensen et al., 2009)}$$

We use the `nlsLM` function to estimate the parameters of model. With eight iterations we obtain the convergence, using adequate initial value and the Levenberg-Marquardt method.

```
fw2 <- function(x, c, d) {1-(exp(-(c)*(x^d)))}

weib.model <- nlsLM(inhibition ~ fw2(x, c, d), data =
Dr,
                start = list(c = 0.00005, d = 2), trace =
TRUE)

It.    0, RSS =    0.194921, Par. =      5e-05      2
It.    1, RSS =    0.096681, Par. =  4.64644e-05  2.08086
It.    2, RSS =    0.0960735, Par. =  4.32258e-05  2.0991
It.    3, RSS =    0.0957664, Par. =  3.64817e-05  2.13297
It.    4, RSS =    0.0955455, Par. =  3.25812e-05  2.15865
It.    5, RSS =    0.0955007, Par. =  3.30969e-05  2.15682
It.    6, RSS =    0.0955003, Par. =  3.28843e-05  2.15826
```

In the model summary is displayed the parameter values

```
summary(weib.model)

Formula: inhibition ~ fw2(x, c, d)

Parameters:
      Estimate Std. Error t value Pr(>|t|)
c 3.290e-05  2.409e-05   1.366   0.183
d 2.158e+00  1.555e-01  13.877 4.49e-14 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.0584 on 28 degrees of freedom

Number of iterations to convergence: 8
Achieved convergence tolerance: 1.49e-08

> AIC(weib.model)
[1] -81.35838
```

2.2.1 Model diagnostic

2.2.1-A Model fit

Residual and fitted value show that mean function seems to be an adequate model.

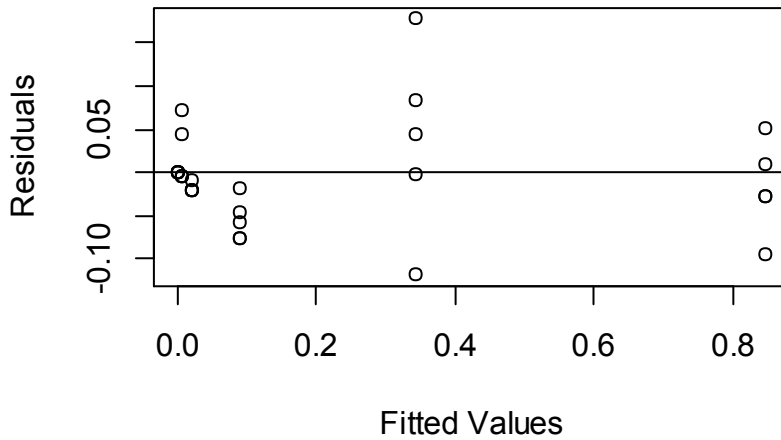


Figure 2.2.1-A. Model residuals versus fitted values from Weibull model

Now, we apply the Lack-of-fit tests to supplement the graphic observation. We have:

```
Linear.model <- lm(inhibition ~ as.factor(x), data = Dr)
anova(Weib.model, Linear.model)

Analysis of Variance Table

Model 1: inhibition ~ fw2(x, c, d)
Model 2: inhibition ~ as.factor(x)
  Res.Df Res.Sum Sq Df    Sum Sq F value    Pr(>F)
  1      28   0.095500
  2      24   0.067934  4  0.027566   2.4347 0.07494 .
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

The null hypothesis (H_0 : lack of fit) is rejected because the p-value is > 0.05 . Thus we accepted the fit of model. We conclude that the Weibull model with two parameters is appropriate to describing the doses response relationship.

2.2.1-B Variance homogeneity

First we look the absolute residuals with a plot the absolute residuals and fitted values. Similar to the logistic model the graphic below evidences an increasing of residuals absolute values in the center fitted value of the graphic.

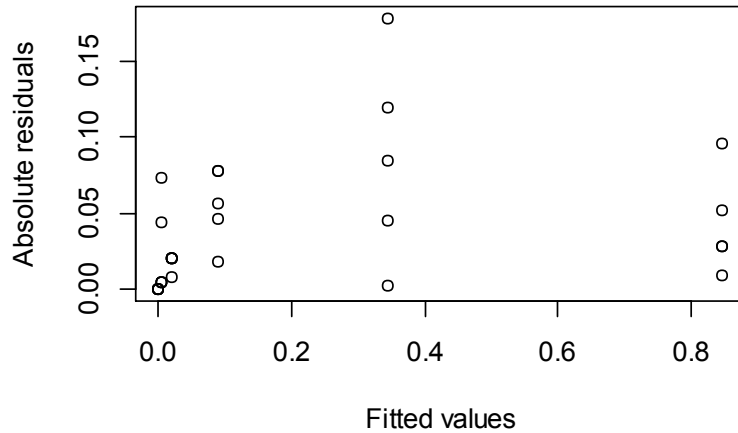


Figure 2.2.1-B. Absolute residuals and fitted values

2.2.1-C Normal distribution

Similar to logistic model the QQ graphic below the residuals seem be follow a normal nption is satisfied.

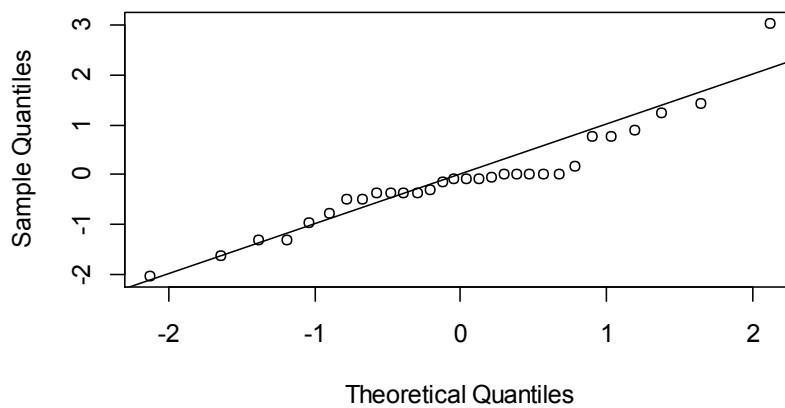


Figure 2.2.1-C. Normal QQ plot for the model fit Weibull (Weib.model)

2.2.1-D Independence: It is assumed from the beginning of the test that independence is assured, because each test bottle is prepared individually.

2.2.2. calculation of EC values (Weibull model)

```
coef(weib.model)
      c      d
3.290264e-05 2.158152e+00

confint(weib.model)
      2.5 %      97.5 %
c -1.644875e-05 8.225402e-05
d  1.839574e+00 2.476729e+00
```

-We calculate the EC concentration following the method used by Christensen et al. (2009)

$$f(x; c, d) = 1 - \exp(-cx^d)$$

We do $u = \ln c + d \ln x$ and the linear transformation is $u = \ln[-\ln(1 - y)]$

Example calculating the EC50: replacing the values of the parameters in the linear equation like this:

$$u = \ln[-\ln(1 - (0.5 \times 0.83))], \quad u = -0.623$$

$$-0.623 = \ln(0.0000329) + 2.16 * \ln EC50, \quad EC50 = 89.47$$

Calculating the EC10

$$u = \ln[-\ln(1 - (0.1 \times 0.79))], \quad u = -2.44$$

$$-2.44 = \ln(0.0000329) + 2.16 * \ln EC10, \quad EC10 = 38.45$$

Calculating the EC90

$$u = \ln[-\ln(1 - (0.9 \times 0.83))], \quad u = 0.32$$

$$0.32 = \ln(0.0000329) + 2.16 * \ln EC90, \quad EC90 = 138.39$$

```

##ECx values and confidence intervals for the weibull function

# coefficients obtained from the regression
c <- coef(weib.model)[1]
3.290264e-05
d <- coef(weib.model)[2]
2.158152

# function of linear transformation of the weibull equation
Uweib <- function(y) {
  u <- log(-log(1-(0.83*y)))
  return(u)}

# EC50 value
u50 <- Uweib(0.5)
-0.6233536
EC50 <- exp((u50-(log(c)))/d)
89.47331

# EC10 value
u10 <- Uweib(0.1)
-2.445904
EC10 <- exp((u10-(log(c)))/d)
38.4533

# EC90 value
u90 <- Uweib(0.9)
0.3179924
EC90 <- exp((u90-(log(c)))/d)
138.3964

```

2.2.3. Confidence intervals (Weibull model)

```
# Derivative representing the slope in 50% of the response
dfunçãow <- deriv(~1-(exp(-(c)*(z^d))), "z")

Solving the system of equations for d and replacing in the derivative
expressed as a function of d, we obtain the slope expressed as a
function of EC10 and EC50.

This way we then arrive in the form  $EC10 = EC50 - a/slope$ 
u.50 <- log(c) + d*log(EC50)
u.10 <- log(c) + d*log(EC10)

# Thus a for the logistic function assumes the value of:
a <- 1.3118

#The slope corresponding to the 10% inhibition was calculated with the
derivative evaluated at the value of u10
s10 <- 0.07989*d

# Norma p.d.f. 1-alfa confidence coefficient of the
# interval alfa 0.05
tz <- qnorm(0.95)

# standard error associated with parameter b
errorb <- 1.555e-01

# Weibull error function (DFC and D. communis data)
EcX <- function(x) {
  er <- sqrt(((x^2)/EC50^2)+ ((log(10)*(a/s10))^2)*(((tz)*errorb)/d)^2)
  return(er)
}
# error factor
ferr <- max(EcX(0:EC50)) - min(EcX(0:EC50))
0.2259774

# Confidence interval to EC10
EC10-ferr*EC10
29.76372
EC10+ferr*EC10
47.14288

# Confidence interval to EC50
EC50-ferr*EC50
69.25436
EC50+ferr*EC50
109.6923

# Confidence interval to EC90
EC90-ferr*EC90
107.1219
EC90+ferr*EC90
169.6709
```

2.3. Bayesian model - Beta regression

Here we assume that the growth inhibition (y) is continuous variable, that it come from the beta distribution $Y_i \sim \text{Beta}(p, q)$.

$$p = \mu \times \phi,$$
$$q = (1 - \mu) \times \phi$$

$$f(y; \mu, \phi) = \Gamma(\phi) / \{\Gamma(\mu \phi) \cdot \Gamma[(1 - \mu) \cdot \phi]\} \cdot [y^{\mu\phi-1} \cdot (1-y)^{(1-\mu)\phi-1}], \quad y \in (0, 1)$$

first, we defined the logit function like a link function

$$\text{logit}(\mu_i) = a + b x_i$$

Second, to find the initial values thus: As the beta function is not defined in zero, so we add plus 0.0001 to the inhibition vector.

```
inhibiplus <- inhibition+0.0001
Dr <- data.frame(x, inhibiplus)

library(betareg)
beta.initial <- betareg(inhibiplus ~ x, data = Dr)
summary(beta.initial)

Call:
betareg(formula = inhibiplus ~ x, data = Dr)

Standardized weighted residuals 2:
      Min       1Q   Median       3Q      Max
-1.2461 -0.5508 -0.0552  0.3248  1.9264

Coefficients (mean model with logit link):
              Estimate Std. Error z value Pr(>|z|)
(Intercept) -4.236368   0.339636  -12.47  <2e-16 ***
x             0.038069   0.003472   10.96  <2e-16 ***

Phi coefficients (precision model with identity link):
              Estimate Std. Error z value Pr(>|z|)
(phi)    11.384       3.534    3.221  0.00128 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Type of estimator: ML (maximum likelihood)
Log-likelihood: 94.45 on 3 Df
Pseudo R-squared: 0.7443
Number of iterations: 35 (BFGS) + 5 (Fisher scoring)
```

Now, we describe the code used to implement the Bayesian analysis. First we loading the data list:

```
D.dados<-list(n = length(Dr$inhibiplus),
              x = (Dr$x),
              y = Dr$inhibiplus)
```

Second, we writing the model in JAGS language

```
sink('BetaRalga.txt')
cat('model
{ #priors
  a ~ dnorm(0, .00001)
  b ~ dnorm(0, .00001)
  t0 ~ dnorm(0, 0.0010)
  phi <- exp(t0)

# verosimilhanca
for (i in 1:n){
y[i] ~ dbeta(p[i], q[i])
p[i] <- mu[i] * phi
q[i] <- (1-mu[i]) * phi
logit(mu[i]) <- a + b*x[i]
}
}')
sink()
#-----
```

Third, we defined the parameters for which we want posterior distribution, we defined the chains number, and we initialize the parameters with initial value obtained previously.

```
params <- c("a", "b", "phi")
n.cad <- 3

t0inicio <- c(0,0.5,-0.5) # não sei bem o que é?
set.seed(1234)
ainicio <- rnorm(3,-4.236368,0.372853)
binicio <- rnorm(3,0.03806935,0.003567 )

inicio <- list(list(a=ainicio[1], b=binicio[1], t0=t0inicio[1]),
               list(a=ainicio[2], b=binicio[2], t0=t0inicio[2]),
               list(a=ainicio[3], b=binicio[3], t0=t0inicio[3]))
```

Fourth, we load up the rjags library, we run the model, we burn-in the chains and we obtain of MCMC samples after to complete the burning.

```

library(rjags)
beta.alga <- jags.model("BetaAlga.txt",data = D.dados,
                       inicio,n.chains=n.cad,n.adapt=1000)
Compiling model graph
  Resolving undeclared variables
  Allocating nodes
Graph information:
  Observed stochastic nodes: 25
  Unobserved stochastic nodes: 3
  Total graph size: 89

Initializing model
|+++++| 100%

n.aq <- 2000
update(beta.alga, n.iter = n.aq)
|*****| 100%

comp.cadeia <- 10000; salto <- 5
beta.mcmc <- coda.samples(beta.alga,params,
n.iter=comp.cadeia,thin=salto)
|*****| 100%

```

Ready we can obtain summary of posterior distribution for the parameters.

```

summary(beta.mcmc)$stat[,1:2]
      Mean      SD
a  -4.21419233 0.319790767
b   0.03819083 0.003834296
phi 11.03553740 3.218493425

```

The credibility intervals are obtained loads up the library *HDInterval*, we used the `hdi()` function.

```

library(HDInterval)
betapost.samp <- as.matrix(beta.mcmc)
hdi(betapost.samp,0.95)
      a      b      phi
lower -4.842572 0.03059646  4.941318
upper -3.592427 0.04583490 17.172945
attr(,"credMass")
[1] 0.95

```

The posterior distribution to each parameter

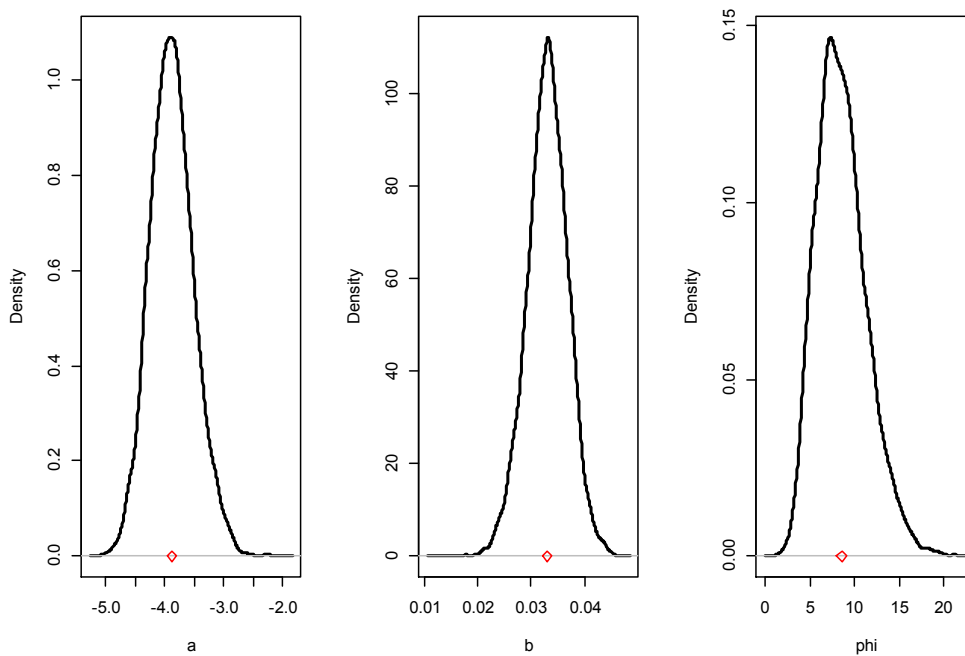


Figure 2.3-A. Posterior distribution of the parameters of the Beta model

2.3.1. calculation of EC values (Beta model)

With the mean of this parameter, we can calculate the EC50, EC10, and EC90 value thus:

$$\text{logit}(\mu/(1-\mu)) = a + bxi$$

$$\eta = \ln((0.5 \times 0.83) / (1 - (0.5 \times 0.83))) = -0.343$$

$$\text{EC50} = (-0.343 - a) / b = 101.70$$

Calculating the EC50

```
#EC50 calculation
eta50 <- log((0.5*0.83)/(1-(0.5*0.83)))
[1] -0.34

# eta = a + b*x[i]
EC50 <- (eta50-betapost.samp[,1])/betapost.samp[,2]
[1] -0.3433333

mean(EC50)
[1] 101.7026

hdi(EC50,0.95)
      lower      upper
 89.38085 113.18972
attr("credMass")
[1] 0.95
```

Calculating the EC10

```
#EC10 calculation
eta10 <- log((0.1*0.83)/(1-(0.1*0.83)))
[1] -2.402267

# eta = a + b*X[i]
EC10 <- (eta10-betapost.samp[,1])/betapost.samp[,2]

mean(EC10)
[1] 47.22703

hdi(EC10,0.95)
      lower      upper
35.15339  56.93435
attr(,"credMass")
[1] 0.95
```

Calculating the EC90

```
#EC90 calculation
eta90 <- log((0.9*0.83)/(1-(0.9*0.83)))
[1] 1.082676

# eta = a + b*X[i]
EC90 <- (eta90-betapost.samp[,1])/betapost.samp[,2]
> mean(EC90)
[1] 134.4321

> hdi(EC90,0.95)
      lower      upper
122.5442  157.8465
attr(,"credMass")
[1] 0.95
```

2.4. Raw data cell viability count

Table 2.4-A Cell viability data of the assay with DFC and *D. communis*

Concentration	Total number of quadrants	Cells viability	Number of counted cells	Viability (%)	Final Cells density (OD)
C2	11	197	200	99	3.E+05
C2	15	196	200	98	3.E+05
C2	13	194	200	97	3.E+05
C2	12	198	200	99	3.E+05
C2	11	197	200	99	3.E+05
CA	16	2	22	9	2.E+05
CA	16	0	32	0	2.E+05
CA	16	20	31	65	2.E+05
CA	16	5	28	18	2.E+05
CA	16	2	31	6	3.E+05
T1(10)	16	72	117	62	2.E+05
T1(10)	16	28	77	36	2.E+05
T1(10)	16	37	95	39	2.E+05
T1(10)	16	41	67	61	2.E+05
T1(10)	16	59	91	65	2.E+05
T2(20)	16	45	67	67	2.E+05
T2(20)	16	39	62	63	1.E+05
T2(20)	16	18	31	58	2.E+05
T2(20)	16	22	36	61	2.E+05
T2(20)	16	35	53	66	2.E+05
T3(40)	16	0	19	0	1.E+05
T3(40)	16	0	37	0	1.E+05
T3(40)	16	0	21	0	1.E+05
T3(40)	16	0	18	0	1.E+05
T3(40)	16	3	27	11	2.E+05
T4(80)	16	0	5	0	1.E+05
T4(80)	16	0	11	0	1.E+05
T4(80)	16	0	3	0	1.E+05
T4(80)	16	0	18	0	1.E+05
T4(80)	16	0	6	0	1.E+05
T5(160)	16	0	0	0	7.E+04
T5(160)	16	0	0	0	8.E+04
T5(160)	16	0	0	0	8.E+04
T5(160)	16	0	0	0	7.E+04
T5(160)	16	0	0	0	8.E+04

4.2 Guide for authors (ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY)

Your Paper Your Way

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article. To find out more, please visit the Preparation section below.

INTRODUCTION

Scope of the EES journal A multi-disciplinary journal that focuses on understanding the exposure and effects of environmental contamination on organisms including human health. The scope of the journal covers three main themes. The topics within these themes are indicated below (but are not limited to) the following:

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Aquatic and terrestrial ecotoxicology of organisms including microbes, invertebrates, vertebrate animals and plants. Also mesocosm or field studies informing on fate and effects. Mechanistic studies relating exposure, bioavailability and effects. Molecular to whole organism studies, including animal behaviour and population effects. Methods in ecotoxicology that address modes of action including omics, systems biology, quantitative measurements in living cells/tissues, biomarkers, histopathology, ecophysiology.

Environmental Chemistry

Soil, sediment and water chemistry that explains the fate, behaviour, sinks and effects of toxic substances such as metals, industrial and agrochemicals, nanomaterials, plastics and emerging contaminants. Studies that identify source apportionment, spatial or temporal distribution of toxic substances, chemical speciation, persistence and transformations in the environment. Mixtures effects and interaction with environmental factors such as pH, organic matter, temperature and salinity. Remediation of wastewater and sludge, biosorbants, biochars, green chemistry, chemical sensors and biosensor technology.

Environmental Safety

Epidemiological studies linking environmental contamination to human health effects. Molecular mechanism-based environmental toxicological studies using cell or animal models to evaluate hazards of air pollution, groundwater and drinking water contaminations, and environmental health and safety impacts from persistent toxic substances in the environment and food chain. Mechanistic elucidation of detrimental effects upon exposure to various contaminants at cellular and molecular levels and novel method development for ecotoxicological and environmental toxicological research. Evaluation of exposure, toxicity, and environmental risk using computational methods, such as big data and quantitative structure toxicity relationship modeling.

Types of paper

The journal publishes regular research articles. Regular research articles must not exceed 8,000 words. Word limit here is for text only. In principle the number of tables and figures should not collectively exceed seven. Any exceptions should require approval from editors. Review articles are welcome, but authors are requested to contact the editors for prior approval. Authors may provide supporting information (for manuscripts exceeding the recommended length), and this material will be made available online.

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5. Capítulo 2

Toxicity of Methylparaben to green microalgae species and derivation of a predicted no-effect concentration (PNEC) in freshwater ecosystems

(Submetido para a revista *Chemosphere*)

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Phone: +55 53 32935449

Abstract

Methylparaben (MeP) is one of the most used preservatives in the industry; however, the toxic effects on aquatic ecosystems are still poorly understood. Therefore, this study was conducted (1) to identify and compare the toxic effects of MeP on physiological parameters of different green microalgae species, using suitable mathematical models; and (2) to estimate a PNEC value for MeP in freshwater ecosystems, adopting either the deterministic or the probabilistic approaches. Toxicity tests were carried out with three green microalgae (*Pediastrum boryanum*, *Desmodesmus communis*, *Raphidocelis subcapitata*), in which different endpoints such as growth rate, chlorophyll-a, and cell viability were measured and compared through the effective concentration which caused a response in x% of test organisms (EC_x). EC_x were obtained by adjusting different non-linear regression models for each microalgae dataset. Chlorophyll-a endpoint resulted in the lowest EC₅₀ values, respectively 124.88, 81.18, 18.27 mg L⁻¹ for *D. communis*, *P. boryanum* and *R. subcapitata*, showing *R. subcapitata* as the most sensitive, and *D. communis* as the most tolerant species to MeP (P <0.05). PNEC was estimated from the present study and previous reports resulting in 5.7 and 65 µg L⁻¹, respectively for the deterministic (PNECd) and the probabilistic (PNECp) approach. The development of chronic assays using test organisms from different ecological groups is encouraged to provide robust PNECp. In this meantime, we recommend the use of the estimated PNECd to support MeP risk assessments and policy formulation.

Keywords: PPCPs, microalgae, sensitive species, statistical modeling reliability, species sensitivity distribution (SSD)

1. Introduction

Emerging contaminants are chemical compounds of emerging concern that have been present in the environment for a long time. Since the industrial revolution, the introduction of various products for use designed to improve the quality of life (e.g., agricultural, domestic, pharmaceuticals) has been growing and continuous. On the other hand, the environmental concern on the toxic effects of such compounds is an emerging science. Unveiling the biological effects of emerging contaminants is a challenge addressed by a growing number of studies in the area (see Daughton, 2016). The pharmaceutical and personal care products (PPCPs), such as drugs, cosmetics, household and animal care products, constitute a group of emerging compounds continuously delivered into aquatic ecosystems through different pathways (e.g. domestic wastewater, runoff, effluent of treated wastewater), reaching ecosystems as an unaltered product or in its degraded form (Althakafy et al., 2018; Kasprzyk-Hordern et al., 2009). These chemicals have been detected continuously at low concentrations in freshwater environments (Pal et al., 2010; Tarpani and Azapagic, 2018), and may affect the ecosystem health (Yap et al., 2018), so that they have become priority substances for agencies like the European Union (EU) and the United States Environmental Protection Agency (USEPA) (Ebele et al., 2017).

Among the emerging PPCPs are the chemicals alkyl esters of p-hydroxybenzoic acid, known as parabens, which have antimicrobial properties (Casoni and Sârbu, 2009). Their formulations, particularly methyl and propyl-paraben (Madsen et al., 2001), are employed in many domestic products as a preservative in food, cosmetics and pharmaceutical products (Yamamoto et al., 2011), effectively protecting against microbial, yeasts and molds actions (EMA, 2015). Parabens act by inhibiting the growth of

microorganisms strains through direct action on the cell membrane and interference in transport processes (Tade et al., 2018; Valkova et al., 2001).

The Methyl 4-hydroxybenzoate, also known as methylparaben (MeP), has an alkyl group formed by a methyl radical (Table 1). It is the most common paraben added to cosmetic products (Melo and Queiroz, 2010). Concerning biodegradation and half-life of MeP, data are controversial as they range from seconds to years. Wu et al. (2017) estimated the half-life of MeP under aerobic and anaerobic conditions from 15.8 min to 43.3 h. Terasaki et al. (2013) estimated MeP half-life of 3.45 years (pH = 8) using the HYDROWIN Program (2013) (US Environmental Protection Agency). In data repositories of the European Chemicals Agency (ECHA), total biodegradability of MeP was reported in 10 days, and it was considered readily biodegradable and with no potential to bioaccumulate (Carlsson et al., 2006; ECHA, 2019).

Studies have reported the presence of MeP in mineral and drinking waters, aquatic ecosystem (surface and groundwater) and wastewater. Carmona et al. (2014) reported an average MeP concentration in drinking and mineral waters of 0.012 and 0.04 $\mu\text{g L}^{-1}$; in the surface water of 0.119 $\mu\text{g L}^{-1}$; in influent and effluent wastewater of 0.334 and 0.011 $\mu\text{g L}^{-1}$; and in sediment river of 0.152 $\mu\text{g L}^{-1}$ (watershed Turin river in Valencia, Spain). Stuart et al. (2012) reported 5 $\mu\text{g L}^{-1}$ to the UK groundwater. Kasprzyk-Hordern et al. (2009) reported MeP concentration ranging from 0.3 to 150 $\mu\text{g L}^{-1}$ in waters of river Taff in the UK; and average concentrations from 2819 to 50 $\mu\text{g L}^{-1}$ in the influent and effluent of a UK wastewater treatment plant Cilfynydd, respectively.

The impact of the presence of MeP in aquatic ecosystems, including the effects on the aquatic life, is not fully elucidated. There are a few studies conducted to determine MeP

ecotoxicity to aquatic organisms. Some studies have reported the effective concentration which caused a response in x% of test organisms (EC_x). For the microalgae *Raphidocelis subcapitata*, EC₅₀ values from 35 to 91 mg L⁻¹ (growth inhibition test 72 h) have been reported (Di Poi et al., 2018; Madsen et al., 2001; Yamamoto et al., 2011). For the cladocerans *Daphnia magna*, it was reported EC₅₀ values from 5.6 a 60 mg L⁻¹ (acute immobilisation test 48h) (Bazin et al., 2010; Lee et al., 2017; Kamaya et al., 2005; Terasaki et al., 2009; Yamamoto et al., 2011). For fish species, LC₅₀ (lethal concentration for 50 % of exposed organisms) values ranging from 50 to 160 mg L⁻¹ (mortality and development test, 48, 96, 120 h) have been reported for fish *Oryzas latipes*, *Danio rerio*, *Oreochromis niloticus* and *Pimephales promelas* (Ateş et al., 2018; Dobbins et al., 2009; Silva et al., 2018). Moreover, Zhang et al. (2015) have suggested that some parabens may also lead to narcotic effects and endocrine disruption in fish *O. latipes* and *P. promelas*.

The effects of MeP in natural waters are still poorly understood, so that toxic effects of MeP and its metabolites may be underestimated. That calls for more studies aiming to investigate toxicity endpoints, as a first step towards an integrative analysis of MeP, allowing future improvements in good practices of using MeP as well as in regulatory tools for environmental protection, such as estimate of predicted no effect concentrations (PNEC) and environmental quality standards (EQS) (ECB, 2003; EC, 2011), providing stakeholders with toxicity data so that ecological risk assessment (ERA) can be performed and regulations adequately addressed.

The PNEC is established as a relevant value below which toxic effects are not expected to occur, thus ensuring the protection of aquatic environments against the toxic effects of the compound of interest. PNEC value is estimated over a toxicity dataset from

single species (deterministic PNEC) or species group (probabilistic PNEC), based on the premise that “ecosystem sensitivity depends on the most sensitive species” (ECB, 2003). Therefore, PNEC values are useful for establishment of environmental quality standards (EQS) by regulatory agencies, so that a growing number of studies have attempted at estimating PNEC for different chemical pollutants in the last years (e.g. Huang et al., 2018; Gredelj et al., 2018; Martins et al., 2018, Zhao et al., 2017). An additional constraint to the few existing ecotoxicity data for MeP is the reliability of the available data. Many studies rely on statistical softwares to calculate EC_x, but the best mathematical model that adjusts to the dataset is not often checked, which hedges the accuracy and reliability of the analysis (Sebaugh, 2011). In this sense, different methodologies have been developed to calculate the effect concentration based on different non-linear regression models (Christensen et al., 2009; Ritz et al., 2015).

Given the importance of increasing the knowledge on the ecotoxicity of MeP to freshwater species, and further estimate safe concentrations of MeP in freshwater ecosystems, the main goals of the present study were: (1) to identify and compare the toxic effects of MeP on physiological parameters of different green microalgae species, using suitable mathematical models; and (2) to estimate a general PNEC value for MeP in freshwater ecosystems, adopting either the deterministic or the probabilistic approaches.

2. Material and methods

This work was performed in two steps: the first part was focused on the exposure of microalgae to MeP, to which the toxic effects were compared using different endpoints through the EC₅₀ values. The mathematical model with the best fit of the concentration-

response curve to each set of algae was selected. In the second part, results from the first part, complemented by MeP ecotoxicity values reported in the literature for different ecological groups were gathered for the derivation of the predicted no effect concentration (PNEC), which was obtained using both the probabilistic and deterministic approaches.

2.1 MeP toxicity to microalgae

2.1.1. MeP preparation

Methylparaben, analytical grade, purity > 99% (Table 1) was used for preparing stock and work solutions. A stock solution of 480 g L⁻¹ was prepared by diluting MeP in acetone (purity > 99%) for 50 to 60 min in ultrasound. From the stock solutions, a work solution of 2.4 g L⁻¹ (acetone 0.5 %) was prepared by diluting the stock solution in MiliQ water. Work solution was then added to the experimental media as described in the section below (2.1.2). All solutions were prepared on the same day of the beginning of each toxicity test.

2.1.2. Algae culture and growth inhibition test

The ecotoxicity tests were carried out according to the OECD guideline 201 (OECD, 2011) and ABNT NBR 12648:2011. The algae species used were: *Pseudopediastrum boryanum* (Turpin) E.Hegewald, 2005; *Desmodesmus communis* (E.Hegewald) E.Hegewald, 2000; and *Raphidocelis subcapitata* (Korshikov) Nygaard, Komárek, J.Kristiansen & O.M.Skulberg, 1987. Both were obtained from the Collection of Freshwater Microalgae Cultures of the Biological Sciences Institute, Federal University of Rio Grande – FURG, Brazil. Culture medium was WC and Culture conditions were

temperature of $23\pm 2^{\circ}\text{C}$, white fluorescent light of 7000 Lux, photoperiod 12 h D:12 h L, without stirring.

The exposure media were prepared by mixing the growth medium (WC) with the work solution to the final MeP nominal concentrations from 30 to 480 mg L^{-1} (with a factor of 2), acetone 0.1%. A pre-culture of exponentially growing algal cells were then inoculated reaching from 10^5 to 10^6 cells mL^{-1} . From the pre-culture, an aliquot of the inoculated was added to all treatments reaching the final concentration of 10^4 cells mL^{-1} . Tests were conducted in the same abiotic conditions as the culture in 50 mL of experimental media, for 72 h. Endpoints analysed were growth rate, chlorophyll-a content, and cellular viability.

Validity conditions for the tests were achieved, as pH variation was lower than 1.5 during all tests, with coefficients of variation (cv) in the control groups below 20%. In addition, reference tests with NaCl were conducted and results showed similar NaCl toxicity for each algae species, with $\text{cv} = 27\%$, $\text{cv} = 20.6\%$ and $\text{cv} = 13.5\%$, respectively for *P. boryanum*, *D. communis* and *R. subcapitata*

2.1.2.1. Growth rate inhibition

After 24, 48 e 72 h of exposure, the optical density (OD) was analyzed by spectrophotometry at 750 nm, and the growth rate (Gr) was estimated based on the OD measurements, according to the Equation 1 (Table S1, Supplemental Material).

$$\text{Gr} = (\ln df - \ln ds) / (tf - ts) \quad \text{Eq. (1)}$$

Where,

Gr = Growth rate

df/ds = final and initial optical density,

tf/ts = final and initial time.

2.1.2.2. Chlorophyll-a

After 72h of exposure, the chlorophyll-a (Chl-a) was extracted from an aliquot of 10 mL of each test vial, which was centrifuged at 4000 rpm, the supernatant was removed and 3 mL of methanol was added to the pellet for extraction for 24 hours. Subsequently, Chl-a was measured by spectrophotometry at 665 nm. Chl-a concentration in $\mu\text{g L}^{-1}$ was calculated according to Equation 2 (Mackinney, 1941) (Table S2, Supplemental Material).

$$\text{Chl-a} = [(A_{665}) \times 12.63 \times \text{methanol volume} \times 1000] / \text{filtration volume} \quad \text{Eq. (2)}$$

Where,

A₆₆₅ = absorbance at 665 nm

12.63 = Chlorophyll extraction correction factor

x 1000 = conversion for L

2.1.2.3. Cell viability

The cellular viability (V) was estimated at the end of the experiment (i.e., after 72h) through the Neutral Red (NR) method (Saul da Luz et al., 2016). An aliquot of 1 mL was incubated for 2 h with NR, fixed with formaldehyde and counted. A total of 200 cells or two complete Neubauer counting chambers (equivalent to 16 quadrants) were counted under an optic microscope (x400). Stained cells with the cell membrane intact were scored as viable cells, while non-stained cells were considered membrane-disrupted cells (Saul da

Luz et al., 2016). Cell viability was estimated based on the viable cells (> 85%) in the control group (Tables S3.1 to S3.3, Supplemental Material).

2.1.2.4. Inhibition vector

The inhibition vector (I) was calculated using the results of the Gr, Chl-a concentration and V (Equation 3). When the vector I resulted in a negative value ($I < 0$), we attributed 0 to this value, indicating a lack of inhibition. When $I \geq 1$, the value 1 was attributed indicating 100% of inhibition (Nyholm et al., 1992).

$$I(\text{Gr}, \text{Chl}, \text{V}) = (\text{Gr}_0, \text{Chl}_0, \text{V}_0 - \text{Gr}_i, \text{Chl}_i, \text{V}_i) / \text{Gr}_0, \text{Chl}_0, \text{V}_0 \quad \text{Eq. (3)}$$

Where,

$I(\text{Gr}, \text{Chl}, \text{V})$ = inhibition growth rate (Gr), chlorophyll (Chl), viability (V)

$\text{Gr}_0, \text{Chl}_0, \text{V}_0$ = growth rate (r), chlorophyll (Chl), viability (V) in the control group

$\text{Gr}_i, \text{Chl}_i, \text{V}_i$ = growth rate (r), chlorophyll (Chl), viability (V) in the beginning of exposure

2.1.2.5 Data analysis

The appropriate model to each inhibition vector and fit of the concentration of exposure was tested using the drc specialized package (Ritz, 2013). Non-linear regression dose-response models were adjusted through the drm() function. In order to determine the appropriated fit, the regression curve was analyzed, and a test of the hypothesis of lack of fit was applied ($p > 0.05$ rejects the lack of fit) (Ritz and Streibig, 2008) (Supplemental Material S0).

One-way ANOVA was used to compare the effects of different treatments (including the solvent control) on algal growth and Chl-a content, with respect to the control group. Diminished responses in relation to control were considered as toxic effects. Residuals analyses were performed to validate the assumptions of normality (Shapiro-Wilk test), homoscedasticity (Levene test) and independence (Autocorrelation function (acf)). The Tukey's post hoc test was applied when statistical differences were found. Data were nonparametric for the cell viability tests, so the Kruskal Wallis test followed by the post hoc Dunn test was applied. Endpoints (Gr, Chl-a, V) and microalgae species (*P. boryanum*, *D. communis* *R. subcapitata*) were compared by one-way ANOVA. The software R was used for all analysis.

2.2. Derivation of the predicted no effect concentration (PNEC)

2.2.1. Ecotoxicity data survey

The ecotoxicity data of MeP were obtained through a systematic review from several databases available online (TOXNET, ECOTOX, ETOX, ECHA), from published papers available in search engines like Google Academic, Web of Science, Science Direct and PubMed, and from technical reports of environmental agencies. The main words used in the search were: "Methylparaben EC₅₀", "Methylparaben Aquatic", "Methylparaben Toxicity", "Methylparaben Concentration", "Parabens Risk", "Parabens Aquatic", and "Parabens Toxicity OR Ecotoxicity", "methyl 4-hydroxybenzoate", CAS number of the compound and freshwater toxicity. Only toxicity data meeting the following criteria were used: test performed under laboratory conditions; biological model exposed to freshwater; exposure to a single compound; endpoint(s) and exposure time clearly indicated in the text.

2.2.2. Ecotoxicity data evaluation

All ecotoxicological studies reported in scientific papers were evaluated for reliability using the SciRAP website (<http://www.scirap.org/>), which is a tool for evaluating reliability and relevance of toxicity data, being useful for the regulatory assessment of chemicals (ACES and IMM, 2016; Moermond et al., 2016). A total of 28 ecotoxicity test results were scored and all studies with a reliability > 57% were used for PNEC estimation. The dataset was organized in an excel library, resulting in 13 reliable studies, including the toxicity data obtained in the present study (Table 2).

2.2.3. Calculation of PNEC

The statistical normal distribution of the toxicity dataset for MeP was checked (Shapiro-Wilk test; $p > 0.05$) (Supplemental Material S1). Due to the limited dataset available, PNEC was calculated based on both the most sensitive species (deterministic approach) and derived from a species sensitivity distribution (SSD) curve (probabilistic approach).

2.2.3.1 Deterministic PNEC (PNECd)

For this approach, the lowest toxicity value of the dataset was divided by an assessment factor (AF1), following the European TGD on Risk Assessment (ECB, 2003; EC, 2011) (Equation 4).

$$\text{PNECd} = \text{Lowest toxicity data of MeP} / \text{AF1} \quad \text{Eq. (4),}$$

2.2.3.2 Probabilistic PNEC (PNEC_p)

This approach is a statistical extrapolation of the dataset, allowing the construction of an SSD curve. SSD was performed according to the TGD on Risk Assessment (ECB, 2003; EC, 2011). Within the ecotoxicity dataset, the organisms were sorted in ecological groups, as proposed by Martins et al. (2018). Ecological groups were: (1) microalgae (MIC), including cyanobacteria; (2) zooplankton (ZP), including all holoplankton animals and the meroplankton invertebrates in their planktonic phase; (3) benthic invertebrates (BI), considering juvenile/adult organisms that have already settled to their substrate; (4) fish (FISH); (5) amphibian (FROG); and (6) bacteria (BAC).

The ecotoxicity dataset was analysed and compared using the following probability distributions: (1) ranked log-normal (*ln*) toxicity; (2) ranked logistic toxicity; and (3) Weibull distribution. The PNEC was derived using an equation based on the confidence intervals (c.i.), as proposed by Aldenberg and Slob (1993) (Equation 5).

$$\text{PNEC}_p = 5\% \text{SSD}(50\% \text{c.i.}) / \text{AF}_2 \quad \text{Eq. (5)}$$

Where,

5%SSD = 5% percentile of the toxicity values in the dataset

AF₂ = assessment factor applied. The assessment factor (AF₂) was established as 20 due to the limited and low robust dataset (Martins et al., 2018).

Plotting positions on the SSD curve were calculated according to Equation 6 (EC, 2011), as follow.

$$(i-0.5)/n \quad \text{Eq. (6),}$$

Where:

i = the rank of the datum

n = total number of points in the dataset

The `fitdistrplus` package (Delignette-Muller and Dutang, 2018) was used to build the SSD graph. The function `fitdist()` was applied to fit the log-normal, logistic and Weibull distribution. The function `cdfcomp()` was used to observe and compare the empirical cumulative distribution against the different adjustments. Finally, the function `gofstat()` was used for obtaining a goodness-of-fit statistic to each distribution curve, this through the Anderson-Darling, Kolmogorov-Smirnov and Cramer-von Mises tests; this same function also delivers the value of Akaike information criterion (AIC) (Supplemental Material S2 - S4).

3. Results and Discussion

Figure 1 shows concentration-response curves of different nominal concentration of MeP for each microalgae (*P. boryanum*, *D. communis* and *R. subcapitata*) based on the growth rate (Gr), concentration of chlorophyll-a (Chl-a) and cellular viability (V). Data for *P. boryanum*, *D. communis*, and *R. subcapitata* were fitted respectively to the Weibull regression model of three parameters, the Logistic model of three parameters, and the asymptotic model of two parameters (see S0, Supplemental Material). Constructing concentration-response curves using the best fit model improves the accuracy and reliability of estimated EC_x values by reducing uncertainties (Baharith et al., 2006; Christensen et al., 2009; Ritz, 2010; Ritz et al., 2015), leading to the generation of more

reliable and robust tools that may be applied in general risk assessments (Christensen et al., 2009) such as the derivation of PNECs.

EC₅₀ values showed that MeP was significantly more toxic to *R. subcapitata* than to *D. communis* ($P = 0.0455$), while EC₁₀ values showed that *D. communis* was more tolerant to MeP toxicity when compared to both *P. boryanum* and *R. subcapitata* ($P=0.00136$) (Table 3). In fact, the species *R. subcapitata* has been recommended in several technical guidelines (such as OECD 201, ISO 8692, ABNT 12648) as a suitable biological model for testing the toxicity of chemicals, due to its high sensitivity to a wide range of contaminants (Fairchild et al., 1998; Yamagishi et al., 2017). In the present study, the growth inhibition EC₅₀ for *R. subcapitata* was 92.7 mg L⁻¹. This finding is similar to results reported by Yamamoto et al. (2011) (EC₅₀ 80 mg L⁻¹ of MeP) and Madsen et al. (2001) (EC₅₀ = 91 mg L⁻¹ of MeP).

For Chl-a, this study reported an EC₅₀ of 18.27 mg L⁻¹ of MeP. Di Poi et al. (2018) reported an EC₅₀ value of 35.25 mg L⁻¹ of MeP for the same species. Intra-specific differences in toxicity of compounds may be attributed to the intrinsic sensitivity of the strain used in the experiment (Eigemann et al., 2013). However, the methodology used for data analysis may also yield different results, calling for attention that selecting and reporting the best model that fits the dataset is important to produce accurate and reliable results. While Di Poi et al. (2018) estimated EC₅₀ based on the Hill equation, which is a modification of the logistic model, a different model (asymptotic regression model) was used in the present study. Moreover, Di Poi et al. (2018) calculated the growth rate based on the fluorescence and then calculated EC₅₀ for the endpoint Chl-a, while a direct analysis of Chl-a was performed in the present work.

Interspecific sensitivity to MeP may be related to the microalgae morphology and chemical composition, mainly of the cell wall; and their mode of life, i.e., if they live in colonies or as isolated cells (Debelius, 2009; Kasai and Hatakeyama, 1993). The present study was performed with three algae species belonging to the same family Chlorophyceae, but their characteristics of size, structure and way of life are different. *P. boryanum* and *D. communis* are colonial algae with a complex wall configuration that conserves the parental wall (Barsanti and Gualtieri, 2006; Bicudo and Menezes, 2006). *R. subcapitata* is smaller in size, and live as a solitary cell because there is a breakdown of the cellular wall when the new cells are released, preventing the formation of colonies (Yamgishi et al., 2017).

To the best of our knowledge, this is the first study reporting MeP toxicity for *P. boryanum* and *D. communis*. These are cosmopolitan colonial species, which inhabit a variety of freshwater ecosystems including highly eutrophic environments (Bicudo and Menezes, 2006; Weckström et al., 2010). Results on the toxicity of MeP to different algae species contribute for a better portrait of the fragility of freshwater ecosystems to MeP as a whole, where a diversity of species of algae and other biological groups occur together, so that it is important to identify the most sensitive species within representative ecological groups to better estimate safe concentrations of chemical compounds to be delivered into water bodies.

In regard of the different endpoints tested, there were no significant differences among the sensitivity of each microalgae species (Table 3), neither for EC₁₀ (P = 0.966) nor EC₅₀ (P = 0.676). The selection of the endpoint for estimating EC_x is important and may influence in the analysis. In the present study, MeP EC₅₀ to *R. subcapitata* for the endpoint Gr was found to be five times higher than for Chl-a, so that safe limits of the chemical

compound could be mistakenly derived, as an example of misusing. Therefore, because EC₅₀ values based on Chl-a were lower than Gr or V, Chl-a was selected to proceed PNEC estimate for microalgae, combined with other ecological groups as shown in Table 2.

Deterministic PNEC (PNECd) calculated from Equation 4 was 5.7 µg L⁻¹ of MeP with an assessment factor (AF) of 1000. Our result agrees with Ortiz de García et al. (2014), who reported an acute PNECd of 6.8 µg L⁻¹ of MeP, based on MeP toxicity to *V. fischeri*. Carlsson et al. (2006) reported acute PNECd to be two times higher (11.2 µg L⁻¹) than in the present study, based on MeP toxicity to *D. magna*, and Zhang et al. (2015) found PNECd to be three times higher than in the present study (15 µg L⁻¹), also based on the toxicity to *D. magna* and a further AF of 50.

Probabilistic PNEC (PNECp) was plotted as an SSD curve (Figure 2). PNECp was estimated based on the available ecotoxicity dataset (Table 2) through Equation 5, resulting in 65 µg L⁻¹ of MeP. Figure 2 showed that all tested models resulted in fitted distributions adequate for the dataset after passing the adjustment tests of Anderson-Darling, Kolmogorov-Smirnov and Cramer-von Mises ($P > 0.05$). Following the Akaike information criterion (AIC), the Weibull distribution was selected as the best fit (Supplemental Material S4).

It is worthy to note that enough information about MeP ecotoxicity that could be used to enhance the SSD curve and PNEC estimate is available, but the way to report methodology and findings can often jeopardise the reliability of the study. Out of approximately 30 reports on the MeP ecotoxicity to freshwater organisms that have been found, only 13 were used to create the SSD curve. The lack of details on the experimental design, information on compounds and test organisms, and statistical analysis resulted in

the exclusion of seven (7) studies from the dataset because they did not meet the arbitrary minimum score for reliability ($\text{SciRAP} \geq 57\%$). In addition, some reliable studies focused on the same species, but only the study in which the lowest toxicity value was found (if reliable) for each species was used for SSD and PNEC derivation.

In this sense, the PNEC_p derived in the present study is not flawless and care should be taken if the resulting value ($\text{PNEC}_p = 65 \mu\text{g L}^{-1}$) will be used for regulation or risk assessments purposes. Within the factors of uncertainty, it can be highlighted the little reliable dataset of ecotoxicity data for MeP (Table 2), the need to base SSD on chronic endpoints, and the need for more species and ecological groups in the ecotoxicity dataset (EC, 2011; ECB, 2003). Nevertheless, to our knowledge, this was the first attempt to estimate PNEC_p of MeP from the available ecotoxicity dataset and should be used as a starting point to direct future studies on environmental regulation of MeP. Therefore, the SSD curve presented in this study (Figure 2) constitutes a basis for further analysis of the sensitivity of freshwater species to MeP.

Despite MeP has been found at low concentrations in the aquatic environment ($0.04 - 2819 \mu\text{g L}^{-1}$) (Carmona et al., 2014; Kasprzyk-Hordern et al., 2009; Stuart et al., 2012), it may pose a potential risk due to its continuous release into natural waters and some efforts have already been made to assess the ecological risk of MeP to freshwater ecosystems. Ortiz de García et al. (2014) classified MeP as a low-risk substance (risk quotient (RQ) = 0.0067) for the Ebro River Basin (Spain). Similarly, Carlsson et al. (2006) found MeP to pose a low risk to some areas in Sweden. Yamamoto et al. (2011) assessed the ecological risk of seven parabens including the MeP to urban streams in Tokushima and Osaka (Japan) and also reported low risk ($\text{RQ} \ll 1$) of all parabens. Dobbins et al. (2009) also reported a

low risk of MeP to aquatic life based on hazard quotients ($HQ = 9 \times 10^{-5}$). Conversely, Molins-Delgado et al. (2016) calculated an $HQ < 0.5$ for MeP based on the chronic toxicity to marine and freshwater species, suggesting that harmful effects cannot be ruled out.

Risk depends on the combination of the bioavailability of the toxicant and the sensitivity of non-target species to the toxicant. In regard to MeP bioavailability, studies have shown that appropriate wastewater treatment may effectively reduce MeP concentration in receiving waters. Kasprzyk-Hordern et al. (2009) investigated MeP concentrations in two wastewater treatment plants (WWTPs) in England and found MeP levels from 661 to 30,688 $\mu\text{g L}^{-1}$ in influent and reduced MeP concentrations ranging from < 3 to 155 $\mu\text{g L}^{-1}$ in effluent after treatment, indicating that those WWTPs present high efficacy in MeP removal resulting in low concentration downstream the WWTPs. That raises a concern about the current sanitation status worldwide. A recent report from the United Nations (2018) reported that only 39 % of wastewater is adequately managed in the world, calling for attention that measures to improve sanitation are urgent. Sanitation for all is part of the sustainable development goals 6 (SDG 6) compiled in the Agenda 2030 committed to build a better world for people and our planet by the year 2030, on a global scale. The SDG 6.3 targets: “By 2030, improve water quality by reducing pollution, eliminating dumping and minimizing release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally” (UN, 2018).

Derivation of PNEC is very important as it provides information on the sensitivity of the biota to environmental pollutants. In special, PNEC_p is more attractive as it takes into account the sensitivity of the whole ecosystem instead of relying on the sensitivity of a

single species. In the present study, PNECd was lower than PNECp. This result is expected because there are many more uncertainties in deriving PNECd so that higher AF should be applied and may overestimate the actual toxicity of compounds (Martins et al., 2018). However, because the lack of robustness in deriving PNECp to MeP, we recommend the use of the deterministic PNEC value ($5.7 \mu\text{g L}^{-1}$) for risk assessment and policy formulation until minimum criteria for PNECp required by Regulatory Agencies are met.

4. Conclusion

Different ecotoxicity concentration-response curves fit better to different mathematical models so that selecting the appropriate model improves the accuracy and reliability of estimated ECx values by reducing uncertainties. EC₁₀ and EC₅₀ values showed that the microalgae *R. subcapitata* and *D. communis* were respectively the most sensitive and the most tolerant species to MeP, in this pioneering study evaluating the toxicity of MeP to *P. boryanum* and *D. communis*, whose outcomes contribute to enhancing ecotoxicity database for PNEC derivation. Despite PNECp is more attractive for policy formulations because it takes into account the sensitivity of many representative groups of the ecosystems, more information should be added to make PNECp robust and reliable, particularly the development and reporting of reliable tests with organisms from different ecological groups (e.g., macrophyte, benthic invertebrates, fungi), especially chronic studies. In this meantime, we recommend the use of the estimated PNECd in the present study, which represent the lowest MeP PNEC estimated to date ($5.7 \mu\text{g L}^{-1}$), to support MeP risk assessments and policy formulation.

Conflict of interest

None.

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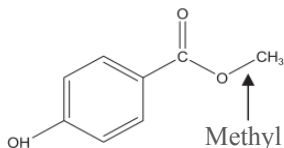
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Table 1. Physicochemical properties of Methylparaben

Chemical name	Methyl 4-hydroxybenzoate
^a CAS number	99-76-3
^b log K _{ow}	1.96
^b pK _a	8.5
^b Molecular weight	152.148 g/mol
^b Solubility in water	2.5 g L ⁻¹ at 25° C
^d Solubility in acetone	64 g.100 g ⁻¹ at 25° C
^c Half-life (short-chain parabens) (days)	1.8 – 3.7
^c Hydrolysis half-life at pH 8 (days)	1260
^c Bioaccumulation	not expected to bioaccumulate
Molecular formula	C ₈ H ₈ O ₃
Molecular structure	

^aECHA, 2019. ^bTOXNET, 2017. ^cNICNAS, 2017. ^dNCBI, 2019

Table 2. MeP toxicity dataset used in the SSD curve for estimating PNEC and their respective score after passing through SciRAP.

Ecological group	Species	Endpoint	EC ₅₀ (mg L ⁻¹)	SciRAP score (%)	References
ZP	<i>Daphnia magna</i>	Immobilization ^a	5.7	68.42	Lee et al. (2017)
BAC	<i>Vibrio fischeri</i>	Luminescence	5.9	61.11	Terasaki et al. (2009)
ZP	<i>Ceriodaphnia dubia</i>	Reproduction	7.3	66.67	Terasaki et al. (2013)
MIC	<i>Raphidocelis subcapitata</i>	Chlorophyll	18.27	89.47	This study
FISH	<i>Danio rerio</i> - embryo	Development	50	84.62	Ateş et al. (2018)
ZP	<i>Tetrahymena thermophila</i>	Growth inhibition	58	NS ^b	Bazin et al. (2010)
FISH	<i>Oryzias latipes</i>	Mortality	59.5	NS ^c	The Ministry of the Environment Japan
FISH	<i>Oreochromis niloticus</i>	Mortality	67.11	78.95	Silva et al. (2018)
IB	<i>Dugesia japonica</i>	Mortality	77	72.22	Li (2012)
MIC	<i>Pediastrum boryanum</i>	Chlorophyll	81.18	89.47	This study
MIC	<i>Desmodesmus communis</i>	Chlorophyll	124.88	89.47	This study
BAC	<i>Tetrahymena pyriformis</i>	Growth	125.34	57.89	Schultz et al. (1996)
FROG	<i>Xenopus laevis</i>	Mortality	178.14	61.54	San Segundo et al. (2013)

^aOutdoor immobilization (sunlight); NS = not scored but considered reliable for the source of information or methodology; ^bProtoxkit, ^cECHA

Table 3. Effect Concentrations (ECx) of MeP to three microalgae after exposure for 72-h growth-inhibition test. Values are presented as estimated ECx (mg L⁻¹) and the respective 95% confidence interval. Different letters indicate a significant difference between microalgae species ($p < 0.05$).

Species	Endpoint - concentration (mg L ⁻¹)					
	Growth rate		Chl-a		Viability	
	EC ₁₀	EC ₅₀	EC ₁₀	EC ₅₀	EC ₁₀	EC ₅₀
<i>P. boryanum</i>	62.87 ^b	134.96 ^{a,b}	46.06 ^b	81.18 ^{a,b}	27.33 ^b	55.3 ^{a,b}
	(58.18-	(121.11-	(40.60-	(74.09-	(24.47-	(49.83-
	67.57)	148.81)	51.51)	88.27)	30.18)	60.78)
<i>D. communis</i>	97.97 ^a	169.68 ^a	95.33 ^a	124.88 ^a	142.07 ^a	262.16 ^a
	(89.76-	(161.82-	(75.53-	(120.55-	(115.03-	(245.28-
	106.18)	177.53)	115.14)	129.22)	169.11)	279.03)
<i>R. subcapitata</i>	14.1 ^b	92.78 ^b	2.78 ^b	18.27 ^b	6.18 ^b	40.68 ^b
	(10.86-	(71.45-	(2.57-2.98)	(16.90-	(4.16-8.21)	(27.38-
	17.34)	114.10)		19.64)		53.99)

Captions to Figures

Figure 1. Inhibition curves calculated for Growth rate (A), Chlorophyll-a (B) and Cellular Viability (C) for *R. subcapitata* (—□—), *P. boryanum* (—●—), and *D. communis* (—▽—).

Figure 2. Species sensitivity distribution (SSD) of MeP based in different probability distributions. × = microalgae; ◇ = zooplankton; + = benthic invertebrates; ○ = fish; Δ = amphibian; □ = bacteria.

Figure 1

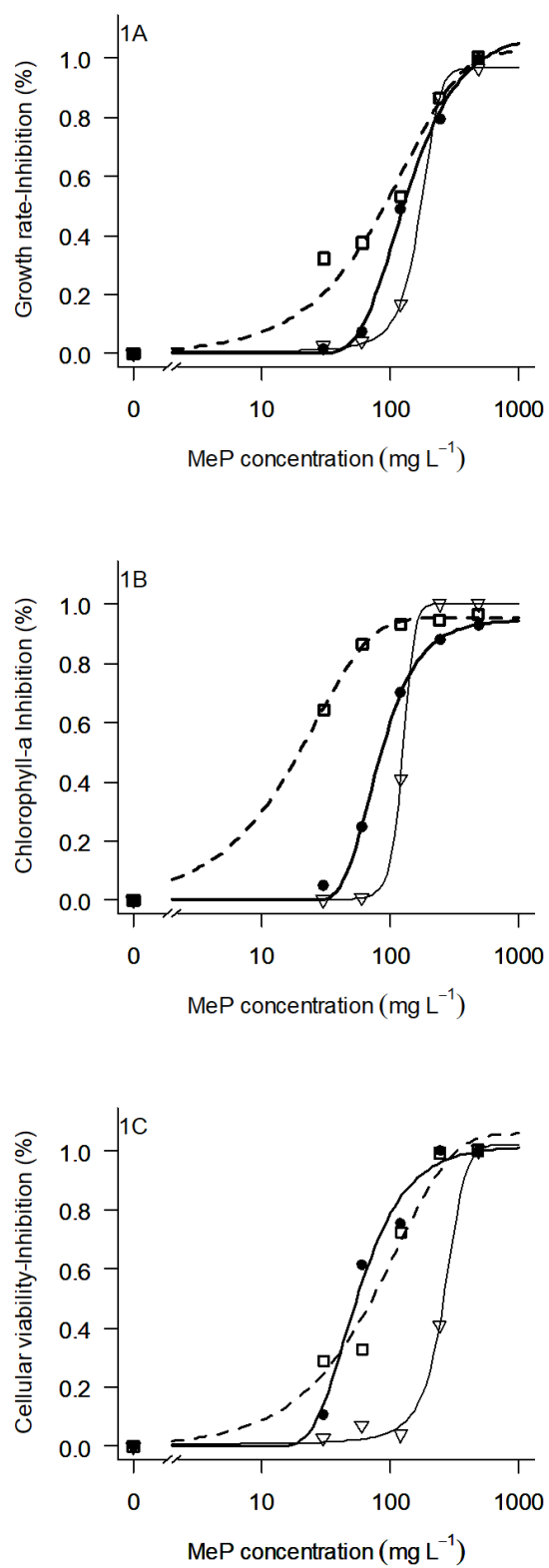
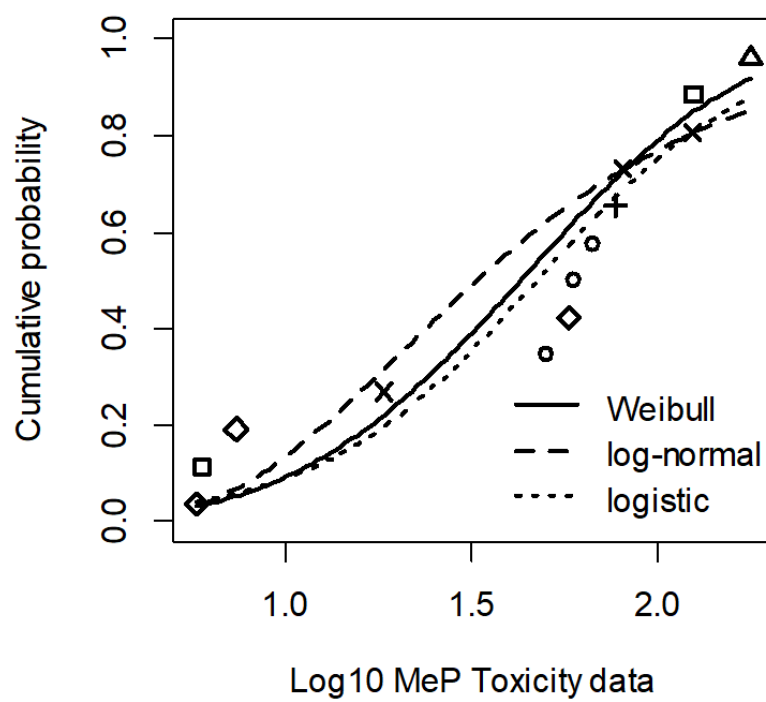


Figure 2



Supplemental Material

S0. Test of the Hypothesis of lack of fit

The analysis to select the best model was made using the `mselect()` function of the `drc` package, delivering the Akaike information criterion (IC) as a result; for the lack of fit test (against a one-way ANOVA model), the larger the p-value, the better. Log-Likelihood (LogLik) is another fit criterion, the larger, the better.

S0.1 Test of the Hypothesis of lack of fit to *P. boryanum*

	logLik	IC	Lack of fit
weibull(3)	57.54593	-107.09186	1.764977e-01
Log.Nomal(3)	52.60199	-97.20397	4.638974e-03
Log.Logistic(3)	51.25851	-94.51701	1.662657e-03
Log.Logistic(4)	51.73888	-93.47776	8.542889e-04
Logistic(3)	39.52092	-71.04185	1.721871e-07
Asymptotic.Reggression(3)	29.12708	-50.25416	4.513934e-11
Asymptotic.Reggression(2)	27.96946	-49.93892	5.482909e-11

S0.2 Test of the Hypothesis of lack of fit to *D. communis*

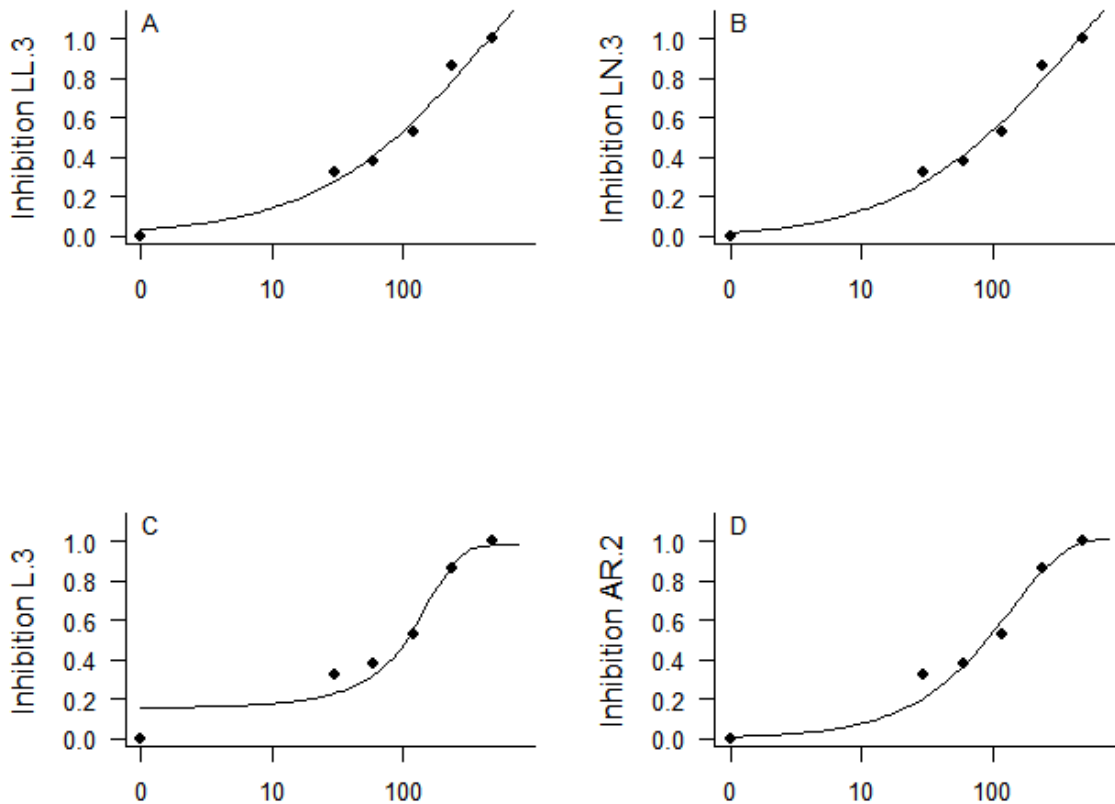
	logLik	IC	Lack of fit
Logistic(3)	60.44109	-112.88217	7.277948e-01
Log.Logistic(4)	59.42842	-108.85684	2.345912e-01
Log.Logistic(3)	56.80011	-105.60023	6.442893e-02
Log.Nomal(3)	56.17889	-104.35777	4.092935e-02
Weibull(3)	56.06265	-104.12530	3.757577e-02
Asymptotic.Reggression(3)	16.46820	-24.93639	1.094655e-15
Asymptotic.Reggression(2)	15.44194	-24.88389	1.522088e-15

S0.3 Plot of the fitted regression curve *R.subcapitata*

The following models were tested but data did not fit to them ($p < 0.05$):

	logLik	IC	Lack of fit
Log.Logistic(3)	35.69147	-63.38293	1.618890e-02
Log.Nomal(3)	35.34269	-62.68539	1.246409e-02
Weibull(3)	34.96055	-61.92109	9.348628e-03
Asymptotic.Reggression(3)	33.55847	-59.11695	3.226761e-03
Logistic(3)	27.09191	-46.18381	2.155551e-05
Asymptotic.Reggression(2)	32.44225	-58.88449	3.171495e-03

Therefore, the asymptotic regression model (AR.2 - D) was selected based on the estimated regression curve.



S1. Normality test to the set of data that are part of the SSD curve

```

Entering the acute toxicity data of MeP:
SSD <- read.table("SSD.txt", header = T)

Using the normal test Shapiro with the original data:
shapiro.test(SSD$EC50mg.L)

Shapiro-wilk normality test
data:  SSD$EC50mg.L
W = 0.91748, p-value = 0.2319

P > 0.05 it is accepted the null hypothesis the normal distribution

Applying the base ten logarithm (log10) for toxicity data MeP:
log10Tox <- log10(SSD$EC50mg.L)

```

S2. Building the SSD curve

To building the SSD curve we going to use the *fitdistrplus* package:

```

Loading the package
library(fitdistrplus)

Use the function fitdist to Fit of univariate distributions. weibull (fw),
log-normal(fln), logistics(fl)

fw <- fitdist(log10Tox, "weibull")
summary(fw)
Fitting of the distribution ' weibull ' by maximum likelihood
Parameters :
      estimate Std. Error
shape 3.979074  0.9639850
scale 1.788125  0.1299528
Loglikelihood: -9.04346   AIC:  22.08692   BIC:  23.21682

fln <- fitdist(log10Tox, "lnorm")
summary(fln)
Fitting of the distribution ' lnorm ' by maximum likelihood
Parameters :
      estimate Std. Error
meanlog 0.4148346 0.10479072
sdlog    0.3778283 0.07409589
Loglikelihood: -11.18595   AIC:  26.3719   BIC:  27.5018

fl <- fitdist(log10Tox, "logis")
summary(fl)
Fitting of the distribution ' logis ' by maximum likelihood
Parameters :
      estimate Std. Error
location 1.675379 0.14415101
scale    0.293654 0.06826612
Loglikelihood: -9.857558   AIC:  23.71512   BIC:  24.84502

```

S3. Plotting the empirical cumulative distribution vs distribution functions to build the curved SSD.

```

creating the plot
plot.legend <- c("weibull", "log-normal", "logistic")

cdfcomp(list(fw, fln, fl), legendtext = plot.legend, do.points = T,
fitcol = 1, vhorizontal = F, datapch = c(0:25)[as.numeric(SSD$EG)],
lwd = 2, xlab = Log10~MeP~Toxicity~data, ylab = Fraction~Affected,
main = "")

```

S4. Testing goodness-of-fit statistics for the three distributions adjusted to the curved SSD.

```

Use the function gofstat
gofstat(list(fw, fln, fl))

Goodness-of-fit statistics
                                1-mle-weibull 2-mle-lnorm 3-mle-logis
Kolmogorov-Smirnov statistic      0.2500492   0.3120742   0.2123812
Cramer-von Mises statistic        0.1449851   0.2210768   0.1122010
Anderson-Darling statistic        0.9220563   1.2460889   0.7794427

Goodness-of-fit criteria
                                1-mle-weibull 2-mle-lnorm 3-mle-logis
Akaike's Information Criterion    22.08692    26.3719    23.71512
Bayesian Information Criterion    23.21682    27.5018    24.84502

```

Table S1. Inhibition with growth rate (Gr) *P. boryanum*, *D. communis*, *R. subcapitata*.

X	Inhibition <i>P. boryanum</i>	x	Inhibition <i>D. communis</i>	x	Inhibition <i>R. subcapitata</i>
0	0.0	0	0.0	0	0.0
0	0.0	0	0.0	0	0.0
0	0.0	0	0.0	0	0.0
0	0.0	0	0.0	0	0.0
0	0.0	0	0.0	0	0.0
30	0.0	30	0.0	30	0.4
30	0.0	30	0.1	30	0.2
30	0.0	30	0.1	30	0.3
30	0.0	30	0.0	30	0.3
30	0.0	30	0.0	30	0.4
60	0.2	60	0.0	60	0.4
60	0.1	60	0.0	60	0.3
60	0.0	60	0.0	60	0.4
60	0.0	60	0.0	60	0.3
60	0.1	60	0.1	60	0.4
120	0.5	120	0.2	120	0.7
120	0.5	120	0.2	120	0.5
120	0.5	120	0.1	120	0.5
120	0.5	120	0.1	120	0.4
120	0.5	120	0.2	120	0.5
240	0.8	240	0.9	240	0.9
240	0.8	240	0.9	240	0.9
240	0.8	240	0.9	240	0.8
240	0.8	240	0.9	240	0.7
240	0.8	240	0.9	240	0.9
480	1.0	480	0.9	480	1.0
480	1.0	480	1.0	480	1.0
480	1.0	480	1.0	480	1.0
480	1.0	480	1.0	480	1.0
480	1.0	480	1.0	480	1.0

Table S2. Chlorophyll-a (Chl-a) *P. boryanum*, *D. communis*, *R. subcapitata*.

Treatment	Chlorophyll-a <i>P. boryanum</i>	Treatment	Chlorophyll-a <i>D. communis</i>	Treatment	Chlorophyll-a <i>R. subcapitata</i>
C1	669.39	C1	303.12	C1	1477.71
C1	694.65	C1	303.12	C1	1250.37
C1	732.54	C1	290.49	C1	1894.50
C1	530.46	C1	593.61	C1	1604.01
C1	732.54	C1	479.94	C1	1111.44
C2	694.65	C2	138.93	C2	1376.67
C2	719.91	C2	151.56	C2	555.72
C2	719.91	C2	164.19	C2	1477.71
C2	631.50	C2	328.38	C2	1326.15
C2	656.76	C2	227.34	C2	1124.07
T1(30)	555.72	T1(30)	479.94	T1(30)	404.16
T1(30)	669.39	T1(30)	391.53	T1(30)	568.35
T1(30)	694.65	T1(30)	442.05	T1(30)	530.46
T1(30)	783.06	T1(30)	505.20	T1(30)	631.50
T1(30)	618.87	T1(30)	606.24	T1(30)	492.57
T2(60)	416.79	T2(60)	517.83	T2(60)	189.45
T2(60)	492.57	T2(60)	378.90	T2(60)	252.60
T2(60)	555.72	T2(60)	467.31	T2(60)	202.08
T2(60)	517.83	T2(60)	606.24	T2(60)	176.82
T2(60)	543.09	T2(60)	454.68	T2(60)	189.45
T3(120)	151.56	T3(120)	277.86	T3(120)	88.41
T3(120)	189.45	T3(120)	214.71	T3(120)	101.04
T3(120)	189.45	T3(120)	202.08	T3(120)	138.93
T3(120)	214.71	T3(120)	252.60	T3(120)	75.78
T3(120)	252.60	T3(120)	214.71	T3(120)	101.04
T4(240)	63.15	T4(240)	0	T4(240)	113.67
T4(240)	88.41	T4(240)	0	T4(240)	101.04
T4(240)	101.04	T4(240)	0	T4(240)	12.63
T4(240)	88.41	T4(240)	0	T4(240)	101.04
T4(240)	63.15	T4(240)	0	T4(240)	88.41
T5(480)	37.89	T5(480)	0	T5(480)	50.52
T5(480)	37.89	T5(480)	0	T5(480)	37.89
T5(480)	50.52	T5(480)	0	T5(480)	88.41
T5(480)	50.52	T5(480)	0	T5(480)	50.52
T5(480)	63.15	T5(480)	0	T5(480)	37.89

C1 is the control and C2 is the solvent control (acetone); T1 to T5 are the tested concentrations

Table S3.1 Cell viability data of the assay with MeP and *P. boryanum*

Concentration	Total number of quadrants	Cells viability	Number of counted cells	Viability (%)	Final Cells density (by optical density)
C1	8	192	200	96.0	6.E+05
C1	8	192	200	96.0	6.E+05
C1	8	191	200	95.5	6.E+05
C1	8	193	200	96.5	5.E+05
C1	8	192	200	96.0	6.E+05
C2	8	182	200	91.0	6.E+05
C2	8	179	200	89.5	5.E+05
C2	8	174	200	87.0	6.E+05
C2	8	186	203	91.6	6.E+05
C2	8	178	200	89.0	5.E+05
T1(30)	8	172	200	86.0	5.E+05
T1(30)	8	176	200	88.0	6.E+05
T1(30)	8	175	200	87.5	5.E+05
T1(30)	8	164	200	82.0	6.E+05
T1(30)	8	167	200	83.5	6.E+05
T2(60)	8	69	200	34.5	4.E+05
T2(60)	8	81	200	40.5	5.E+05
T2(60)	8	74	200	37.0	5.E+05
T2(60)	8	74	200	37.0	6.E+05
T2(60)	8	72	200	36.0	5.E+05
T3(120)	16	35	141	24.8	3.E+05
T3(120)	16	55	197	27.9	3.E+05
T3(120)	16	32	165	19.4	3.E+05
T3(120)	16	39	176	22.2	3.E+05
T3(120)	16	40	168	23.8	3.E+05
T4(240)	16	0	34	0.0	2.E+05
T4(240)	16	0	48	0.0	2.E+05
T4(240)	16	0	29	0.0	2.E+05
T4(240)	16	0	31	0.0	2.E+05
T4(240)	16	0	38	0.0	2.E+05
T5(480)	16	0	0	0.0	1.E+05
T5(480)	16	0	0	0.0	1.E+05
T5(480)	16	0	0	0.0	1.E+05
T5(480)	16	0	0	0.0	1.E+05
T5(480)	16	0	0	0.0	1.E+05

C1 is the control and C2 is the solvent control (acetone); T1 to T5 are the tested concentrations

Table S3.2 Cell viability data of the assay with MeP and *D. communis*

Concentration	Total number of quadrants	Cells viability	Number of counted cells	Viability (%)	Final Cells density (by optical density)
C1	8	185	200	92.5	4.E+05
C1	8	184	200	92	3.E+05
C1	8	181	200	90.5	3.E+05
C1	8	187	200	93.5	4.E+05
C1	8	195	200	97.5	4.E+05
C2	8	159	200	79.5	4.E+05
C2	8	155	200	77.5	4.E+05
C2	8	180	200	90	3.E+05
C2	8	195	200	97.5	3.E+05
C2	8	181	200	90.5	3.E+05
T1(30)	8	178	200	89	4.E+05
T1(30)	8	195	200	97.5	3.E+05
T1(30)	8	182	200	91	3.E+05
T1(30)	8	174	200	87	4.E+05
T1(30)	8	185	200	92.5	3.E+05
T2(60)	8	167	200	83.5	3.E+05
T2(60)	8	182	200	91	3.E+05
T2(60)	8	173	200	86.5	3.E+05
T2(60)	8	173	200	86.5	3.E+05
T2(60)	8	169	200	84.5	3.E+05
T3(120)	8	186	200	93	3.E+05
T3(120)	8	156	200	78	3.E+05
T3(120)	8	184	200	92	3.E+05
T3(120)	8	182	200	91	3.E+05
T3(120)	8	187	200	93.5	3.E+05
T4(240)	16	48	72	66.6	1.E+05
T4(240)	16	38	70	54.3	1.E+05
T4(240)	16	36	69	52.2	1.E+05
T4(240)	16	38	67	56.7	1.E+05
T4(240)	16	31	69	44.9	1.E+05
T5(480)	16	0	32	0	1.E+05
T5(480)	16	0	36	0	1.E+05
T5(480)	16	0	23	0	1.E+05
T5(480)	16	0	23	0	1.E+05
T5(480)	16	0	24	0	1.E+05

C1 is the control and C2 is the solvent control (acetone); T1 to T5 are the tested concentrations

Table S3.3 Cell viability data of the assay with MeP and *R. subcapitata*

Concentration	Total number of quadrants	Cells viability	Number of counted cells	Viability (%)	Final Cells density (OD)
C1	8	169	200	84.5	1.E+05
C1	8	160	200	80.0	1.E+05
C1	8	181	200	90.5	1.E+05
C1	8	164	200	82.0	1.E+05
C1	8	172	200	86.0	1.E+05
C2	8	177	200	88.5	1.E+05
C2	8	182	200	91.0	1.E+05
C2	8	171	200	85.5	1.E+05
C2	8	190	200	95.0	1.E+05
C2	16	188	200	94.0	1.E+05
T1(30)	16	132	200	66.0	5.E+04
T1(30)	16	113	200	56.5	7.E+04
T1(30)	16	116	187	62.0	6.E+04
T1(30)	16	120	200	60.0	7.E+04
T1(30)	16	89	157	56.7	5.E+04
T2(60)	16	18	50	36.0	5.E+04
T2(60)	16	39	62	62.9	6.E+04
T2(60)	16	18	31	58.1	5.E+04
T2(60)	16	22	36	61.1	6.E+04
T2(60)	16	35	53	66.0	5.E+04
T3(120)	16	0	3	0.0	3.E+04
T3(120)	16	1	2	50.0	4.E+04
T3(120)	16	2	6	33.3	4.E+04
T3(120)	16	1	3	33.3	5.E+04
T3(120)	16	0	5	0.0	4.E+04
T4(240)	16	0	18	0.0	1.E+04
T4(240)	16	0	11	0.0	1.E+04
T4(240)	16	0	13	0.0	2.E+04
T4(240)	16	0	5	0.0	2.E+04
T4(240)	16	1	26	3.8	2.E+04
T5(480)	16	0	67	0.0	8.E+03
T5(480)	16	0	21	0.0	1.E+04
T5(480)	16	0	52	0.0	1.E+04
T5(480)	16	0	45	0.0	1.E+04
T5(480)	16	0	85	0.0	8.E+03

C1 is the control and C2 is the solvent control (acetone); T1 to T5 are the tested concentrations

5.2. Guide for authors (CHEMOSPHERE)

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6. CONSIDERAÇÕES FINAIS

O fármaco Diclofenaco (DCF) e o conservante Metilparabeno (MeP) classificados como PPCPs, foram estudados quanto a sua toxicidade. De forma clara, os valores de toxicidade do DCF apresentados no Capítulo 1 são bem inferiores ao de MeP, apresentados no Capítulo 2. Dentre os parâmetros analisados, a taxa de crescimento da cultura de *Desmodesmus communis* foi o parâmetro mais adequado para expressar a toxicidade do DCF, pela melhor curva dose-resposta; enquanto a clorofila foi o parâmetro mais sensível para expressar a toxicidade do MeP. A viabilidade das culturas não foi o melhor parâmetro para expressar a toxicidade, pois nas maiores concentrações de contaminantes não foi possível fazer a contagem de células, por sua ausência.

Alguns fatores podem alterar os valores de CEx, e, portanto, devem ser escolhidos de forma criteriosa. Valores de taxa crescimento, teor de clorofila-a e viabilidade celular geraram diferentes valores de CEx, e a escolha de qual deve ser utilizado, deve ser avaliada em cada caso. Além disso, o modelo estatístico usado para estimar a relação dose-resposta deve ser escolhido com critérios de ajuste padrão que melhore a confiabilidade das estimativas, para que possam ser usadas em ferramentas de análises do risco ambiental, como o PNEC, com segurança. Abordagens clássicas como a frequentista e modelos tradicionais como o logístico são referência para cálculo da CEx, embora seja recomendado usar novas abordagens estatísticas que auxiliem no entendimento e na medição da incerteza, tal como a Bayesiana, que tem emergido na ecologia nos últimos tempos.

Através do parâmetro de CEx calculado para cada microalga é possível fazer comparação entre a sensibilidade das espécies. Para o MeP, espécie a *Raphidocellis subcapitata* foi a mais sensível e a *D. communis* mais tolerante. Diferenças na sensibilidade podem estar associadas à características morfológicas, fisiológicas e genéticas dos organismos-teste.

Quanto ao PNEC, recomenda-se por hora o valor do PNEC determinístico (5.7 µg/L) para o MeP, uma vez o PNEC probabilístico não contou com uma base de dados robusta e suficiente para derivar um PNEC confiável.