THE ROLE OF MANGROVE ECOSYSTEMS IN THE DYNAMICS OF ORGANOCHLORINE PESTICIDES, WITH PARTICULAR FOCUS ON DICOFOL AND ITS MAIN METABOLITE

A Thesis

Presented to

The Academic Faculty

by

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ENDORSEMENT

I certify that this report is solely my work, and that it has never been previously submitted to any other higher education institutions for any academic award.

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I, the supervisor, believe that this Thesis is ready for assessment, and reaches the accepted standard for the degree of PhD in _____Science_____.

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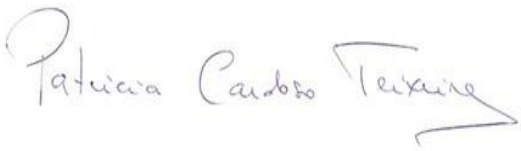

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Abstract

Mangroves are a unique group of plants, which offer a great variety of goods and services to the ecosystem and to the society. Regrettably, they have been globally threatened by urbanization and industrialization, among others, triggering overexploitation of the world's mangrove forests despite their ecological and economic importance. As a result, mangroves are often under pollution stress as sinks or receivers for numerous man-made pollutants such as pesticides, which are the main focus of this thesis. One of the most widely applied chemicals in the word are the organochlorine pesticides (OCPs) that even after their supposedly worldwide ban between 1950s-1990s, they can still be detected in the environment. Numerous studies have been done in phytoremediation of pollutants by mangroves, but little attention has been given to the role of mangroves in the remediation of OCPs. For this reason, part of this thesis will focus on the occurrence and distribution of OCPs in intertidal tropical and sub-tropical areas around the world with and without mangroves. As a **first goal (I)**, we evaluate —in a theoretical way— if the presence of mangroves affects or modifies the levels of OCPs in the surrounding environment. For this purpose, data from different matrices, such as water, sediment, benthic fauna and plants were included and discussed in this work. Moreover, and considering Macao's location, we also quantified OCPs from surface waters of this region from areas with and without mangroves and included in this task.

Besides this theoretical approach, this thesis also included some laboratoy and field work specifically focused on dicofol and 4,4'-dichlorobenzophenone (4,4'-DCBP, its main metabolite). Dicofol is an OCP strongly related to dichlorodiphenyltrichloroethane (DDT), which has been extensively used in China and more specifically, in the Pearl River Delta (PRD), a region under anthropogenic

pressure. However, due to dicofol's instability (i.e., sensitive to low pH, light exposure and high temperature), we expected to quantify 4,4′-DCBP (which is also common to DDT) as the main form present in the environment. As a **second goal (II),** we conducted a monitoring study in surface waters from Macao and Hong Kong, to evaluate the contamination status and water quality of these regions. Concentrations of 4,4'-DCBP, nutrients and physicochemical parameters were measured during transition and wet season, and at high and low tide. In addition, since the toxicity of this metabolite was totally unknown, we assessed it via two biological models: *Daphnia magna and Artemia salina*. Since 4,4'-DCBP was detected and quantified in both regions (2.8-30.0 ng/L), this thesis also includes experimental work focused on the assimilation and depuration pattern by a marine organism. For that purpose and as a **final goal (III)**, we selected the common edible bivalve *Meretrix* as a model to evaluate the dynamics of accumulation and depuration of the pesticide dicofol. The Vietnamese clams were exposed during 15 days under two different concentrations of dicofol, and decontaminated for the same period of time. Quantification of 4,4'-DCBP was done during both phases (uptake and depuration) and at different sampling times.

In summary, all these different works helped us to conclude that:

I.1) As expected, vegetated areas with mangroves presented lower concentrations of OCPs for all the matrices, and also better quality in terms of pesticide pollution for water and sediments. Results obtained from Macao's waters also revealed the same pattern, with mangroves areas having lower levels of contamination. Although the gathered data presented methodological variability (i.e. different quantification methods, extraction protocols, equipment used), the same pattern was observed among matrices, showing how robust and solid the results herein obtained are.

II.1). Hong Kong presented higher concentrations of 4,4'-DCBP than Macao, which may be due to the use of dicofol as a pesticide and the use of antifouling-paint for ships. Moreover, concentrations of 4,4'-DCBP during wet season were below limits of quantification, demonstrating a seasonal pattern and a dilution effect due to higher river discharges during this period.

II.2). Both regions showed possible eutrophication problems due to the high nutrient concentrations. These levels presented also a seasonal variability, with dissolved inorganic nitrogen and total dissolved solids higher during transition; and dissolved inorganic phosphorous, total suspended solids and chlorophyll a higher during wet season.

II.3). Toxicity of 4,4'-DCBP was lower than the parent compound dicofol, and the levels quantified indicated a low environmental risk. However, it is important to pay attention to this compound since interaction with other contaminants could enhance their toxicity, or processes such as biomagnification or bioaccumulation could make low concentrations a threat for the environment.

III.1). Different concentrations of dicofol presented different uptake and depuration kinetics. Animals exposed to higher concentrations (500 ng/L), had levels above limits of quantification (LOQ) after 24h exposure, unlike the ones exposed to lower concentrations (50 ng/L), which had levels <LOQ after the same period. The first ones also, presented lower uptake rates, and this could indicate that high dicofol concentrations in the system could affect the respiration rates of the organism. In addition, this work also showed that animals exposed to high concentrations of dicofol will need more than 15 days to depurate in order to reach safe levels for human consumption.

The compilation of the work done in this thesis allowed us to better understand the role of mangroves ecosystems on the accumulation of OCPs and to provide solid information that could create strategies for mangroves management and conservation. Moreover, and as a first attempt, we were able to quantify this pesticide metabolite in the PRD (one of the most seriously contaminated areas in China), to determine its toxicity and to define its kinetics in an important organism such as the edible bivalve *M. meretrix.*

We intend that this thesis will be helpful for the scientific community providing new insights regarding metabolite interactions (within and with other molecules) and toxicity (LC_{50} and theoretical risk assessment), which were unknown until now.

Keywords: mangroves, organochlorine pesticides, surface water, surface sediment, benthic fauna, dicofol, dichlorobenzophenone, *Meretrix meretrix*.

Resumo

Os mangais constituem um habitat particular capaz de oferecer uma grande variedade de bens e serviços ao ecossistema e à sociedade. Infelizmente, eles têm sido ameaçados por diversos fatores, tais como a urbanização, a industrialização, entre outros, desencadeando uma sobre-exploração dos mangais a nível mundial, apesar da sua importância ecológica e económica. Assim, os mangais estão frequentemente sob stress causado pela poluição e descargas de poluentes, tais como os pesticidas, que são o foco desta tese. Um dos compostos mais usados a nível mundial são os pesticidas organoclorados (OCPs), que mesmo após a sua proibição mundial entre 1950-1990, ainda continuam a ser detetados no ambiente. Numerosos estudos têm sido feitos acerca da fitoremediação de poluentes por mangais, mas tem sido dada pouca atenção ao papel dos mangais na remediação dos OCPs. Por esta razão, parte desta tese irá focar-se na ocorrência e distribuição de OCPs em áreas tropicais e sub-tropicais em locais com e sem mangal. Como **primeiro objetivo** (I), vamos avaliar, de uma forma teórica, se a presença de mangal afeta ou modifica os níveis de OCPs no ambiente envolvente. Para tal, dados de diferentes matrizes (ex. água, sedimento, fauna bentónica e plantas) foram considerados e discutidos neste trabalho. Para além disso, e considerando a localização de Macau, procedeu-se à quantificação de OCPs em águas superficiais dessa região em áreas com e sem mangal.

Para além desta abordagem mais teórica, esta tese engloba também trabalho laboratorial e de campo, especificamente focado no pesticida dicofol e no seu principal metabolito, a 4,4-diclorobenzofenona (4,4-DCBP). O dicofol é um pesticida organoclorado fortemente relacionado com o diclorodifeniltricloroetano (DDT), que foi muito usado na China e mais especificamente no delta do Rio das Pérolas (PRD),

uma região sob forte pressão antropogénica. Contudo, devido à instabilidade do dicofol (ex. sensibilidade a pH baixo, exposição luz e elevada temperatura), é esperado quantificar a 4,4-DCBP como a sua principal forma no ambiente.

Como **segundo objetivo** (II), foi conduzido um estudo de monitorização em águas superficiais de Macau e Hong-Kong, para avaliar o estado de contaminação e qualidade da água destas regiões. Concentrações de 4,4-DCBP, nutrientes e parâmetros físico-químicos foram medidos durante as estações húmida e de transição, em maré baixa e maré alta. Por outro lado, dado que se desconhecia a toxicidade deste elemento, foi feita uma avaliação da mesma usando dois modelos, a *Daphnia magna* e a *Artemia salina*. Dado que 4,4-DCBP foi detetado e quantificado em ambas as regiões (2.8-30.0 ng/L), esta tese também inclui trabalho experimental focado nos padrões de assimilação e depuração por um organismo marinho tipico. Para tal, e **como objetivo final** (III), foi selecionado uma espécie de bivalve comestível, a ameijoa vietnamita *Meretrix meretrix*, para avaliar a dinâmica de acumulação e depuração do pesticida dicofol. As ameijoas foram, assim, expostas durante 15 dias a duas concentrações diferentes de dicofol e após esse período foram colocadas em ambiente limpo, a depurar por mais 15 dias. A quantificação de 4,4-DCBP foi feita em ambas as fases (exposição e depuração) e em diferentes dias de amostragem.

Em resumo, estes trabalhos ajudaram-nos a concluir que:

I.1) tal como esperado, as áreas cobertas com mangal apresentaram menores concentrações de OCPs em todas as matrizes e também melhor qualidade de água e sedimentos. Os resultados obtidos das águas da região de Macau revelaram o mesmo padrão, com as áreas de mangal a apresentar menores níveis de contaminação. Apesar dos dados recolhidos apresentarem variabilidade metodológica (ex. diferentes métodos de quantificação, protocolos de extração, equipamento usado), o mesmo

padrão foi observado nas diferentes matrizes, demonstrando robustez dos resultados obtidos.

II.1) Hong-Kong apresentou concentrações mais elevadas de 4,4 DCBP do que Macau, que pode estar relacionado com o uso do dicofol como pesticida e do uso de tintas anti incrustantes nos barcos. Para além disso, as concentrações de 4,4-DCBP durante a estação húmida foram abaixo dos limites de quantificação, demonstrando um padrão sazonal e um efeito de diluição devido a maiores descargas durante este período.

II.2) Ambas as regiões apresentaram possíveis problemas de eutrofização devido a elevadas concentrações de nutrientes. Estes valores apresentaram também uma variabilidade sazonal, com níveis de azoto inorgânico dissolvido e sólidos dissolvidos totais mais elevados na estação de transição, enquanto o fósforo inorgânico dissolvido, sólidos suspensos totais e clorofila a foram mais elevados durante a estação húmida.

II.3) A toxicidade de 4,4-DCBP revelou ser menor do que a do composto parental dicofol e os níveis quantificados revelaram um baixo risco ambiental. Contudo, é importante prestar atenção a este contaminante, pois a sua interação com outros pode aumentar a sua toxicidade ou processos como a biomagnificação ou bioacumulação podem tornar baixas concentrações num risco para o ambiente.

III.1) Concentrações diferentes de dicofol apresentaram diferentes cinéticas de "uptake" e depuração. Animais expostos a concentrações mais elevadas (500 ng/L), apresentaram níveis acima dos limites de quantificação após 24h de exposição, ao contrário dos expostos a baixas concentrações (50 ng/L), que tiveram valores abaixo do limite de quantificação, para o mesmo período. Os primeiros, também, apresentaram taxas de "uptake" mais baixas, e isto pode indicar que concentrações de

dicofol elevadas no sistema poderiam afetar as taxas de respiração do organismo. Ainda, este trabalho revelou que organismos expostos a elevadas concentrações de dicofol necessitarão mais do que 15 dias para depurar, de forma a alcançar níveis de segurança para consumo humano.

A compilação destes trabalhos permitiu-nos compreender melhor o papel dos mangais na acumulação de OCPs e fornecer informação sólida que poderá ajudar na melhor gestão e conservação destes ecossistemas. Por outro lado, como primeira tentativa, foi possível quantificar este pesticida no delta do Rio das Pérolas e determinar a sua toxicidade e definir as cinéticas em organismos relevantes como o bivalve *Meretrix meretrix*.

Assim, esta tese será relevante para a comunidade científica fornecendo novas ideias sobre este pesticida e seu metabolito, tais como possíveis interações com outras moléculas, e informação sobre a sua toxicidade, que eram desconhecidos até ao momento.

Palavras-chave: Mangais, pesticidas organoclorados, água superficial, sedimento superficial, fauna bentónica, diclorobenzofenona, *Meretrix meretrix*.

Publications arising from this thesis

Journal articles

Published publications

L. Ivorra, P.G. Cardoso, S.C. Kiu, K. Tagulao, C. Cruzeiro, 2019. Environmental characterization of 4,4′-dichlorobenzophenone in surface waters from Macao and Hong Kong coastal areas (Pearl River Delta) and its toxicity on two biological models: *Artemia salina* and *Daphnia magna*. Ecotoxicology and Environmental Safety 171, 1–11. DOI: 10.1016/j.ecoenv.2018.12.054

L. Ivorra, C. Cruzeiro, S.C. Kiu, K. Tagulao, P. G. Cardoso, 2019. Uptake and depuration kinetics of dicofol metabolite 4,4'-dichlorobenzophenone, in the edible Asiatic clam *Meretrix meretrix*. Chemosphere 235, 662-669. DOI: 10.1016/j.chemosphere.2019.06.155

Submitted publications

L. Ivorra, P. G. Cardoso, S.C. Kiu, C. Cruzeiro, K. Tagulao, 2020. Can mangroves work as an effective phytormediation tool for pesticide contamination? A worldwide overview. Journal of Cleaner Production.

Conference abstracts

Poster presentations

L. Ivorra, B. L. Lo, S.C. Kiu, P.G. Cardoso and K. A. Tagulao· Heavy Metals in Macao's Coastal Environment: Can Mangroves Help?

16th IWA International conference on wetlands systems for water pollution control, 1-4 October 2018, Valencia

Oral presentations

L. Ivorra, K. Tagulao, S.C. Kiu, P.G. Cardoso and C. Cruzeiro. "Dicofol" levels in the mouth of the Pearl River Delta and its environmental implications.

22nd International conference on wetlands on environmental indicators. Organised by international society of environmental indicators. 1-5 August 2017, Helsinki.

L. Ivorra, C. Cruzeiro, P.G. Cardoso and K. Tagulao. Environmental risk assessment of 4,4'-dichlorobenzophenone (4,4'-DCBP) in the mouth of the Pearl River Delta (PRD). 10th The Asian Conference on Sustainability, Energy and the Environment. 8-10 June 2018, Kobe.

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Glossary of acronyms and abbreviations

ANOVA: Analysis of Variance

AF: Assessment Factor

ASW: Artificial Sea Water

Chl-a: Chlorophyll a

CCME: Canadian Councils of Ministers of the Environment

CA: Concentration Action

CI: Condition Index

BCF: Bioconcentration Factor

DCBP: Dichlorobenzophenone

DDD: Dichlorodiphenyldichloroethane

DDE: Dichlorodiphenyldichloroethylene

DIN: Dissolve Inorganic Nitrogen

DIP: Dissolve Inorganic Phosphorous

DDT: Dichlorodiphenyltrichloroethane

DO: Dissolved Oxygen

DT: Degradation Time

DW: Dry Weigth

EC: Effect Concentration

EF: Elimination Factor

ERL: Effect Range Low

ERM: Effect Range Median

FAO: Food Agriculture Organization

GC-MS: Gas Chromatography-Mass Spectrometry

HCB: Hexachlorobenzene

HCH: Hexachlorocychlohexane

HT: High Tide

IA: Independent Action

ISQG: International Sediment Quality Guideline

ISO: International Organization for Standardization

LC: Lethal Concentration

LT: Low Tide

LOD: Limit Of Detection

LOQ: Limit Of Quantification

Max: max

MD: median

ME: Matrix Effect

MEC: Measured Environmental Concentration

Min: min

MRL: Maximum Residue Level

N: Nitrogen

N_2: Nitrogen gas

OCP: Organochlorine Pesticides

OECD: Organisation for Economic Co-operation and Development

QC: Quality Control

QuEChERS: Quick Easy Cheap Rugged and Safe

P: Phosphorous

PAH: Polycyclic Aromatic Hydrocarbons

PCN: Polychlorinated Naphtalenes

PEL: Probable Effects Level

PNEC: Predicted No-Effect Concentrations

POP: Persistent Organic Pollutants

PPDB: Pesticide Properties Data Base

PRD: Pearl River Delta

RQ: Risk Quotient

SD: Standard Deviation

SPE: Solid Phase Extraction

STU: Sum of Toxic Units

T: Temperature

TDS: Total Dissolved Solids

TEL: Threshold Effect Level

TSS: Total Suspended Solids

WW: Wet Weight

Chapter 1. Introduction

1.1 Research background

Mangroves environments are unique habitats restricted to intertidal coastal zones and adjacent communities in the tropical and subtropical regions of the world (Tomlinson 1986; Nagelkerten et al. 2008). These ecosystems provide wide variety of ecological services (Rönnbäck, 1999) and have relevant importance for the surrounded flora and fauna, as well as for human communities. Some of the services provided by mangroves include flood protection (which is very important in areas susceptible of tsunamis such as South-East of Asia (Alongi 2008)), prevention of shoreline erosion, salinity buffering, carbon sequestration, important role on contaminants trapping leading to an improvement of water quality of adjacent ecosystem or habitat for a wide range of species (Manson et al. 2005; Lewis et al, 2011; Bayen et al. 2012). Besides their ecological importance, mangroves have an important socioeconomic role for humans due to their implications in some activities such as aquaculture, agriculture, forestry or source of building material (Bradley et al. 2008).

However because of their location, they have been globally threatened by urbanization and industrialization which have triggered widespread overexploitation of the world's mangrove forests despite their ecological and economic importance mentioned above (Bayen 2012; Lewis et al. 2011). It has been recognized that mangrove ecosystems are anthropogenically stressed (Lugo 1978; Pi et al. 2016), and within a wide range of stressors, pollutants such as pesticides are of great concern. Pesticides can be divided in different classes, including insecticides, herbicides and fungicides. These are (mixed) substances that are poisonous and efficient to target organisms but are not safe to non-target organism and environments. Since 1945, man-made organic pesticides have been a significant mark of human civilization, which greatly protects and facilitates agricultural productivity (Zhang et al. 2011b). In

the earlier period of organic synthesized pesticides, there were mainly three kinds of insecticides: carbamated, organophosphorus and organochlorined insecticides. The latter also known as OCPs, have been used for pest and insect control for more than half a century (Guan et al. 2009). These kinds of compounds are hardly degraded and hence, are capable of remaining in the environment for up to decades (Guo et al. 2008). Because of this, they are one of the most common pollutants present in the marine environment, bringing negative impacts on ecosystems and human health (Grung et al. 2015). OCPs were widely produced and used in China from 1950 to 1983. Due to their negative effects on organisms, many of OCPs, such as dichlorodiphenyltrichloroethanes (DDTs), hexanochlorocyclohexanes (HCHs) and chlordane, were prohibited from production and use since 2001 (Breivik et al., 2002), and have been gradually phased out due to their high bioaccumulation, toxicity, and persistence in the environment (Jones and de Voogt 1999; Nakata et al. 2002). However, these phased-out OCPs, are still commonly detected in air, water, soil and biota (Fu et al. 2003; Zhang et al. 2002; Huang et al. 2014; Qiu et al. 2018). As one of the most prosperous regions in China, the Pearl River Delta —one of the largest rivers— is considered an area with significant OCP pollution, compromising the regional air and water quality (Fu et al., 1997; Yang et al., 1997). In this region, and due to population growth and crop areas reduction, insecticides have been increasingly used to improve agricultural output. For instance, the annual pesticide application (37.2 kg/yr ha) in the PRD from 1980 to 1995 was four times higher than the average national level (Guo et al., 2006). As a result, OCPs have been frequently detected in water, soil, sediment, and biota sampled in the PRD (Guan et al., 2009; Guo et al., 2009; Li et al., 2006; Ma et al., 2008; Yu et al., 2011). Besides the accumulation of past uses, illegal uses or production of new pesticides related to

DDTs such as dicofol, can also contribute for OCPs persistence in the environment (Liu et al., 2009; Qiu et al., 2010). Dicofol is an organochlorine acaricide, that has been used in agriculture since the late 1950s (WHO/FAO, 1996). It is produced as two isomers (80% 4,4'-dicofol and 20% 2,4'-dicofol) and it is structurally similar to DDT, possessing similar concerns with respect to its release and presence in the environment (Eng et al. 2016).

Dicofol is well-known to readily degrade to 4,4´-DCBP (both within crops and at various steps of analytical procedures) and to be pH and light sensitive (EU Reference Laboratories for Residues of Pesticides, 2013). Therefore, 4,4'-DCBP will be the most expected form in the environment. For this reason, and considering the lack of information on dicofol, and its main metabolite (4,4'-DCBP), this compound will be further studied in this Thesis (Chapter 3 and 4).

With all the information mentioned above, it is therefore important not only to quantify the presence of OCPs in the environment but also to understand the potential of mangroves on the remediation of their surrounding habitats. In this thesis, the idea of using mangroves (due to their unique features such as location, high primary productivity, rich organic carbon, anoxic/reduced conditions, root physiology or rhizospheric microbial activity (Zheng et al. 2000)) for the phytorremediation of OCPs from the environment is assessed (Chapter 2). In addition, and considering the importance of dicofol as an active source of DDT pollution for the environment, characterization, risk assessment and toxicity of 4,4'-DCBP in the mouth of the PRD was done (Chapter 3). Finally, considering that estuarine environments can be susceptible to dicofol pollution, and that bivalves, as a filters-feeding animals, have been widely used to monitor pollutant compounds in aquatic ecosystems, uptake and

depuration kinetics of the Vietnamese clam, *Meretrix meretrix* after dicofol exposure was also studied (Chapter 4).

All of this provided a new insights about OCPs in areas with and without mangroves, and brought new and relevant information regarding dicofol and its metabolite, since information for these compounds is still scarce.

1.2 Main Goal and Research questions

The research presented in this thesis consists of a combination of field and laboratory work aimed to study the occurrence, distribution and toxicity of certain OCPs and related compounds in different biotic and abiotic compartments of mangroves ecosystems in the PRD and from other tropical and sub-tropical areas worldwide.

The main questions addressed in this thesis are listed below, together with the steps followed to answer them.

A. Could mangroves be considered as good natural remediators of OCPs from the environment?

A.1 Are OCPs concentrations different between areas with and without mangroves?

A.2 How is the accumulation pattern of OCPs in the associated benthic fauna?

A.3 How is the quality of the surrounding ecosystem in areas with or without mangroves?

The steps taken to answer these questions and sub-questions include:

(1) Extended literature review and data collection regarding OCPs levels reported in non-mangroves and mangroves areas worldwide.

(2) Analyses and data arrangement from the different compartments such as water, sediment, benthic fauna and mangrove plants.

(3) Quantification of OCPs in Macao surface waters from non-mangroves and mangroves areas as experimental work.

(4) Evaluation of the ecosystem status by comparison of the concentrations quantified with international guidelines and theoretical risk assessments of the abiotic compartments.

B. How are the levels of 4,4'-DCBP and the physicochemical characteristics of surface waters from the mouth of the PRD?

B.1 Are the quantified levels potentially risky for the environment?

B.2 How toxic can the metabolite 4,4'-DCBP be when compared to the parent compound dicofol?

The steps taken to answer these questions and sub-questions include:

(1) Water collection in different sampling points from Macao and Hong Kong regions.

(2) Validation of the extraction (via solid phase extraction), identification and quantification method of 4,4'-DCBP through Gas Chromatography coupled to Mass Spectrometry (GC-MS/MS) in surface water samples.

(3) Evaluation of chronic toxicity of 4,4'-DCBP in two biological models: the shrimp *Artemia salina* and the crustacean *Daphnia magna*.

C. Do edible bivalves have the ability to accumulate and depurate 4,4'- DCBP after dicofol exposure?

C.1 Will uptake and depuration kinetics of these organisms be different between both dicofol concentrations tested?

C.2 Will depurated clams reach acceptable levels for human consumption?

(1) Animals were bought from the market and kept in lab conditions. These conditions (mainly temperature and salinity) needed to be optimized in order to ensure the survival of these organisms at least during 2 months.

(2) Validation of the extraction (via QuEChERs), identification and quantification method of 4,4'-DCBP through GC-MS/MS in the bivalve *Meretrix meretrix*.

(3) Quantification of water samples from each aquarium in order to control the amount of 4,4'-DCBP present in the matrix.

1.3 Thesis outline

This Thesis addresses the questions mentioned above, which are summarized into the following five chapters:

Chapter 1 defines the research background, questions and objectives of the Thesis.

Chapter 2 presents the result of the extended literature review done together with data quantified in surface water samples from Macao; this chapter will study the pollution levels in the different matrixes and will discuss the possible ability of mangroves for OCPs phytoremediation from the environment.

Chapter 3 presents the results regarding concentration values of 4,4'-DCBP found in Macao and Hong Kong surface waters, together with a risk assessment associated to the values quantified; this chapter will also evaluate the toxicity levels of this main metabolite in *Artemia salina* and *Daphnia magna* considered as ideal biological models for determination of LC_{50} and EC_{50}.

Chapter 4 presents the results regarding uptake and elimination kinetics of 4,4'-DCBP in the bivalve *Meretrix meretrix* after being exposed to an environmental and a supra-environmental concentration of the parent compound (dicofol).

Chapter 5 summarizes the key findings of this research and discusses their implications towards answering the research questions. Further research opportunities identified on the basis of this research were also presented.

Chapter 2. Can mangroves work as an effective phytoremediation tool for pesticide contamination? A worldwide overview.

Submitted as:

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Abstract

Mangroves are a unique group of plants growing along tropical and sub-tropical coastlines, with the ability to remove several types of contaminants such as heavy metals and other persistent organic compounds in coastal waters. However, little attention has been given to the possible role of mangroves in the removal of organochlorinated pesticides (OCPs) from the environment. Used worldwide, these pesticides were banned in the late 80s, withal they can still be quantified in aquatic environments due to their high stability. Moreover, as persistent and lipophilic compounds, OCPs are known for their tendency to bioaccumulate and biomagnify through the food chain, affecting local ecosystems, and potentially human health. This work aimed to investigate the potential benefits of mangrove ecosystems as OCP phytoremediators. For this purpose, a total of seventy-three articles from non-mangrove and mangrove areas around the world were gathered, integrated and re-analysed as a whole. These data include information from four different matrices (water, sediment, benthic fauna and mangrove plants). A common trend of less pesticide contamination in mangrove areas was observed for all the selected matrices. As a complement, average concentrations were discussed considering International Directives, such as the European legislation 2013/39/EU for water policy and the Dutch List together with the International Sediment Quality Guideline, for sediments. Additionally, theoretical risk assessments were also included. Since information regarding OCPs in mangroves ecosystem is very scarce compared to non-mangrove areas, this review provides valuable insights regarding these environments, and the importance of preserving them as a relevant remediation unit.

Keywords

Surface waters, surface sediments, benthic fauna, intertidal environments, organochlorinated pesticides, toxicological assessment

2.1 Introduction

Mangroves are a unique group of plants thriving along with the tropical and sub-tropical coastal areas worldwide, providing a wide variety of ecosystem services such as flood protection, prevention of shoreline erosion, salinity buffering, habitat for a wide range of species, carbon sequestration and are reported to play a major role in the export of carbon and nutrients to the coastal zone and oceans (Bayen 2012; Lewis et al. 2011). Moreover, mangrove ecosystems are able to filter and remove contaminants to improve the water quality of adjacent ecosystems (Montgomery & Price 1979; Wong et al. 1997; Tam et al. 2005; Shete et al. 2009; Wang et al. 2017). Mangroves are also important to humans for a variety of reasons, including aquaculture, agriculture, and forestry as a source of fire-wood and building materials (Bradley et al. 2008). Unfortunately, they have been rapidly declining worldwide, with 50% of global mangroves areas destroyed (Rosen, 2000)) mainly due to land-use disturbance and proximity to areas exposed to high anthropogenic activity (Polidoro et al. 2010).

As a result, mangroves are often under pollution stress as sinks or receivers of several pollutants. However, the unique features of mangrove ecosystems—including physicochemical soil characteristics and complex root mechanisms that control contaminant uptake, accumulation, translocation, detoxification, and ion exclusion (Walsh et al. 1979; Sadiq & Zaidi 1994; MacFarlane et al. 2007)—make them a preferential tool for the uptake and preservation of a wide range of pollutants (Zheng et al. 2000). Among these pollutants, pesticides are of great concern because of their

broad use and persistence in the environment for up to decades (Guo et al. 2008), bringing negative impacts on ecosystems and human health (Laws, 2000; Grung et al. 2015; Aiyesanmi A.F. & Idowu G.A., 2012). In the last century, organochlorine pesticides (OCPs), have been extensively used worldwide (UNEP, 2003), raising environmental concerns due to their toxicity, persistence, bioavailability, endocrine-disrupting properties and long-range transportation (reviewed by El Shahawi et al., 2010; Bayen, 2012). Despite their worldwide ban between the 1970s-1990s (Wie et al. 2007), concentrations of these pollutants remain in the environment, being a threat to the ecosystem and human health (Páez-Osuna et al. 2002; Kishimba et al. 2009; Wang et al. 2013; Wang et al. 2017). Several studies have successfully evaluated phytoremediation of pollutants (i.e. nutrients, heavy metals, polycyclic aromatic hydrocarbons, polychlorinated biphenyls) by mangroves (Peters 1997; Páez-Osuna et al. 2002; MacFarlane et al. 2007, Qiu et al. 2018), but only a few studies have focused on the distribution and accumulation of OCPs in these ecosystems. Taking into consideration the ability of mangroves to accumulate pollutants, we hypothesized that mangrove plants may be effective on the removal of OCPs from the environment (H1). To verify this, the present work evaluated the tendency of concentrations for several OCPs in matrices like water, sediments and biota from tropical and sub-tropical areas around the world, with and without mangroves. For this, we compiled and analysed all the available data from published articles, over the last twenty-two years. Additionally, we have included data from Macao surface waters collected during 2017-2018 in non-mangrove (NM) and mangrove (M) areas. Overall, pesticides may be assimilated by plants, retained in the surrounding sediment, bioaccumulated by animals and/or metabolized by them and by a large diversity of microorganisms present in the nearby ecosystem (Hussain et al. 2009; Zhang et al.

2014). In accordance to H1, and due to favoured hydrological conditions (i.e. higher sedimentation, low water speed, and stagnant water) for uptake and metabolization of organic compounds, we expect to quantify lower amounts of OCPs in M waters (H1.1). Moreover, mangroves may trap these pollutants making them more available for further degradation processes held by the microbial community and plant remediation. Because of this, we also expect to quantify lower amounts of OCPs in sediments from M than NM areas (H1.2). As a consequence, mangrove environments will be less polluted reflecting this pattern at the benthic fauna from the surrounding environment, where a lower accumulation of persistent organic compounds (as OCPs) is also expected. Due to their hydrophobic character, these compounds will tend to accumulate in lipid tissues and increase their concentration over the trophic levels (biomagnification). Therefore, we expect that organisms from higher trophic levels will tend to accumulate more OCPs than those from lower ones (H2).

In summary, taking into consideration the hypotheses mentioned above, we expect higher OCPs concentrations in NM areas, increasing the potential toxicological risk in those environments (H3). In relevance to this, we included an evaluation of the ecosystem status through ecotoxicological risks assessments, considering the pesticide mixtures quantified in water and sediment samples. Given the significant concerns/issues about OCPs and the unique characteristics of mangroves and its ecosystem, could mangroves be considered as a possible green tool for OCPs remediation?

2.2 Methodology & data treatment

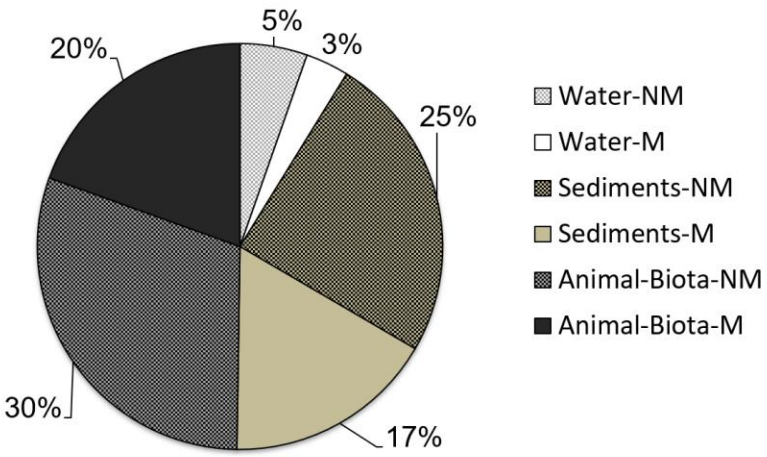
Data extraction: the present work reviews the current literature on OCPs in NM and M areas from tropical and sub-tropical regions around the world. PubMed (NCBI) and Google Scholar were the search engines used, applying the following keywords:

"OCPs+mangroves", "OCPs+biota coastal areas", "OCPs+water", "OCPs+sediments coastal areas", "OCPs+tropical regions" and "OCPs+sub-tropical regions". We included a total of seventy-three ISI papers, restricted to a period of twenty-two years (1998–2020) with 54 and 46% of the data focused on NM and M areas, respectively. Pesticides distribution and occurrence in various environmental compartments were included. We collected and expressed all the available data—median values (MD), minimum (min) of the median, maximum (max) of the median—as ng/L, ng/g DW (dry weight) and ng/g WW (wet weight) for water, sediments and biota compartment, respectively. Ranged values presented in the results section, were calculated considering the min and max of averaged quantified median values. To standardize the biota data, lipid or moisture content information was used to transform data and express it in wet weight. Figure 1, represents the distribution of data per studied matrix and the locations of each study.

Tables regarding data from water and sediment can be found in SM1, and were presented in two different ways: (1) considering the median values of individual compounds (Appendix A-Tables A1-A2), and (2) considering the median values of pesticides quantified by country (Appendix A-Tables A3-A4).

We assigned the classification of benthic animals based on feeding behaviour and habitat, resulting in six and two groups, respectively. Feeding behaviour was classified as carnivores, omnivores, herbivores, suspension feeders, surface deposit feeders and sub-surface deposit feeders, following Cardoso et al. (2008) classification. For the habitat, classification was done considering epifauna (animals living sediment-water interface) and infauna (animals living with the seafloor). Details for each organism were obtained from established databases, like BIOTIC (http://www.marlin.ac.uk/biotic/), and SeaLifeBase

(https://www.sealifebase.ca/search.php). Species with no information available were assigned based on descriptions of species behaviour and information on closely related species at the nearest taxonomic level (Appendix A-Table A5). Animals for which species name was not provided, were not included in the trait analyses; and when species belonged to two different categories, averaged median values were calculated, and 50% of the value was assigned to each corresponding category.



Water		Sediment		Fauna	
M	NM	M	NM	M	NM
China	Argentina	Brazil	Argentina	Bangladesh	Argentina
Hong Kong	Hong Kong	Cameroon	Brazil	Brazil	Brazil
India	India	China	China	China	China
Macao	Macao	Hong Kong	Hong Kong	Hong Kong	Hong Kong
Mexico	Mozambique	India	India	India	Nigeria
Mozambique	Nigeria	Mexico	Nigeria	Mexico	Singapore
Singapore	Singapore	Senegal	Puerto Rico	Nicaragua	Tanzania
	South Africa	Singapore	Singapore	Senegal	
		Taiwan	South Africa	Singapore	
		Tanzania	Taiwan	Taiwan	
		USA	Tanzania	Tanzania	
				USA	

Figure 1. Distribution (%) of data collected from non-mangrove (NM) and mangrove (M) worldwide areas, between 1998-2020.

Pesticide data organization: To make comparisons between NM and M areas and avoid misinterpretation, we decided to group the compounds by specific pesticide group. For that, a total of eight groups were created: (1) ∑DDT (2,4'/4,4'-DDT (dichlorodiphenyltrichloroethane) + 2,4'/4,4'-DDE

(dichlorodiphenyldichloroethylene) + 2,4'/4,4'-DDD (dichlorodiphenyldichloroethane), (2) ∑HCH (hexachlorocyclohexane (α/β/γ/δ isomers)), (3) ∑Endosulfan (α/β endosulfan + endosulfan sulfate), (4) ∑Heptachlor (Heptachlor + Heptachlor epoxide), (5) ∑Chlordane (α/γ chlordane), (6) ∑Aldrin (Aldrin, Dieldrin, Endrin, Endrin aldehyde), (7) Methoxychlor and (8) Hexachlorobenzene (HCB).

Data analyses: we carried out the statistical analyses and graphical representation using the software Prism 6. Differences between NM and M areas were assessed using the non-parametric Mann-Whitney test, to compare the distribution of two unmatched groups. Boxplot representation according to the pesticide group was used for water, sediments and fauna from NM and M areas, where lower and upper box boundaries are the 25^{th} and 75^{th} percentiles, respectively; median is represented by the line inside box, and lower and upper error lines represent 10^{th} and 90^{th} percentiles, respectively (Figure 3A/4A/5/6). Besides, frequency (%), accordingly to the groups of compounds (described above), was displayed as NM and M areas (Figure 3B/4B). Moreover, we assessed the correlation between OCPs concentration and lipid content using Spearman correlation.

Macao sampling: surface water samples were collected during ebb tide, in 2018-2019, at four different locations along Macao coastal waters; each location included NM and M areas; detailed information is available in Appendix B.

2.2.1 Legislation values

We compared averaged median concentrations from NM and M water (1) and sediments (2) samples, from tropical and sub-tropical coastal areas across the world to international guidelines. It is important to highlight that not all the quantified compounds are included in these directives:

(1) For this matrix, we considered the maximum allowable concentration for surface waters established by European Legislation (2013/39/EU) (EU, 2013).

(2) Since there is no specific and official EU Legislation for sediments, we used the threshold values presented in the "Dutch List" (VROM, 2000) and the "ISQG" (International Sediment Quality Guideline), from the Canadian Councils of Ministers of the Environment (CCME, 2002) in order to compare the environmental concentrations quantified. The Dutch List includes target and intervention values; meanwhile in the ISQG, contains two assessment values: (1) the lower value, referred to as the threshold effect level (TEL), represents the concentration below which adverse biological effects are expected to occur rarely, and (2) the upper value, referred as the probable effect level (PEL), defines the level above which adverse effects are expected to occur frequently.

2.2.2 Theoretical risk assessment

Risk assessment was done for water (1) and sediment (2) matrices:

(1) In order to predict the environmental hazard of the compounds detected in water, we used a two-tiered theoretical approach, as suggested by Backhaus and Faust (2012). These models, based on the two reference models—concentration addition (CA) and independent action (IA)—allow the calculation of expected combined effects purely based on concentration-effect information for the target components and their concentrations in the mixture (Silva & Cerejeira, 2014). The concentrations used for each compound were the max median values quantified in NM and M areas. The EC_{50} (mg/L) values from 4,4'-DCBP (4,4'-dichlorobenzophenone) was obtained from previous work (Ivorra et al. 2019) and the rest of the data were collected from Pesticides Property Database (PPDB, 2007).

Figure 2, shows all the steps calculations: the first-tier (based on CA), examines the most sensitive trophic level (algae, invertebrates or fish) and evaluates whether the concentration quantified in the environment might pose a potential environmental risk or not. This step only considers single compounds, and if Risk Quotient (RQ)>1 (i.e. high risk), step 2 will be examined. In the second one, we considered the potential risk of the mixture present in the environment. This step requires the calculation of the individual toxic units (TU) for each compound and trophic level (RQ_{TU}), and the Sum of Toxic Units ($STU=\Sigma RQ_{TU}$) for each trophic level. The highest RQ_{TU} is multiplied by an assessment factor (AF=100) to obtain the RQ_{STU}; if this value is higher than 1, the second tier (based on IA), is considered. The ratio $STU/max.RQ_{TU}$ can be used to predict it, giving us the max value from which CA may predict higher toxicity than IA (Junghans et al. 2006; Silva & Cerejeira 2014). In this step we can identify which compound(s) presents the highest toxicity among the others (mixture); these calculations are available in Appendix A-Table A7. When the average median values of a specific compound exceeded the established limit for water or sediment matrices, we identified the location/country presenting this supra environmental concentration.

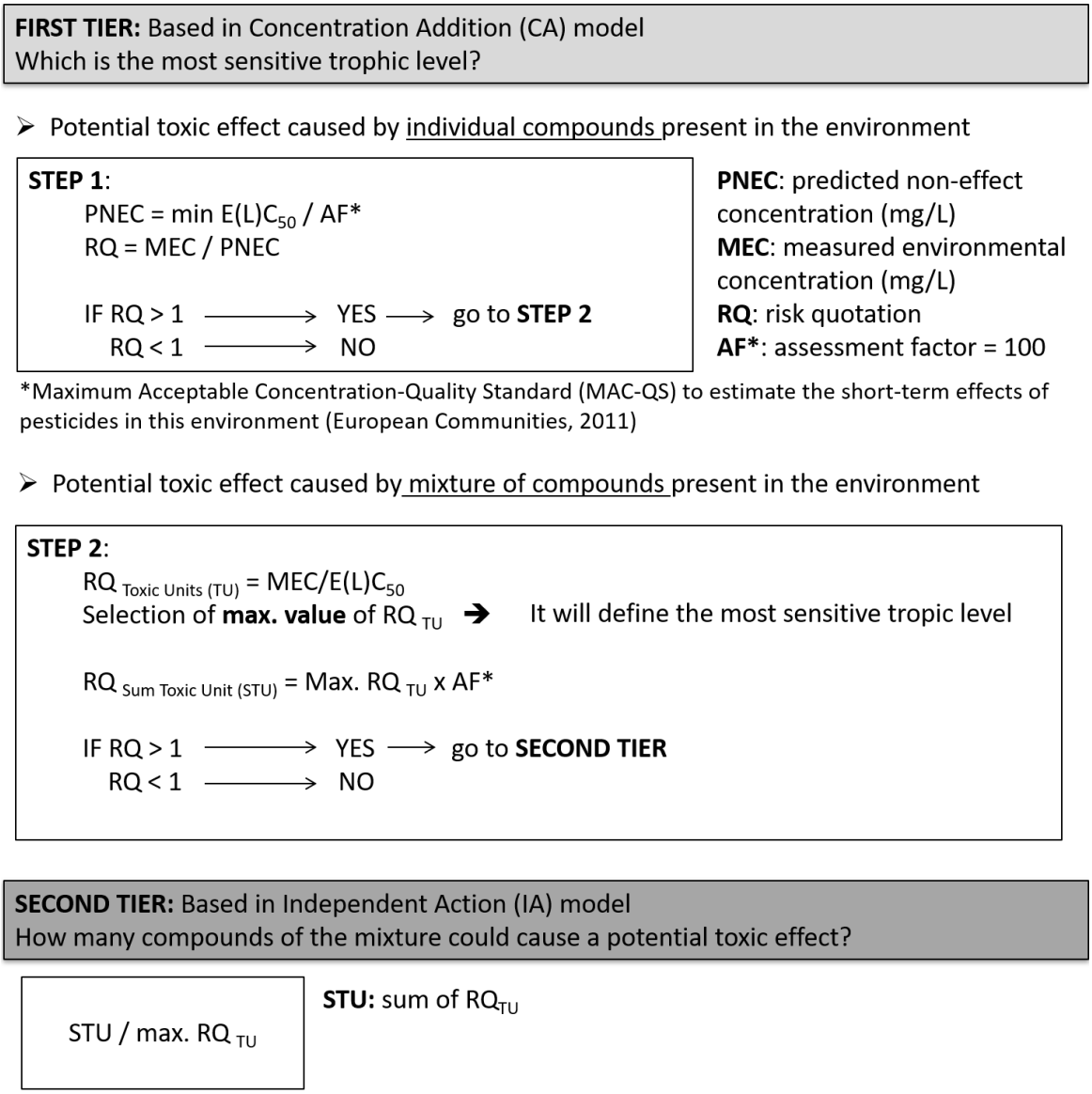


Figure 2. Illustration of the two-tiered approach to predict the potential environmental risk caused by a pesticide mixture in water.

(2) In environmental toxicology, effect range low (ERL) and effect range median (ERM) are measures of toxicity in sediments set by Long et al. (1995). To predict the environmental hazard of the compounds detected in the sediments, we compared the quantified levels against the ERL and ERM values. These measures are statistically derived levels of contamination and are used to assess the toxicity hazards from trace metals or organic contaminants to the benthic organism. The ERL indicates the concentration below which toxic effect is scarcely observed or predicted (<10%

frequency) and ERM indicates the concentration above which effects are generally or always observed (at least at 50% frequency).

2.3 OCPs occurrence and distribution

2.3.1 Water

We found a total of seventeen papers for this matrix, with 47 and 53% of the data corresponding to NM and M areas, respectively. Water samples data from Macao were also included (Appendix B). When data is arranged by pesticides group, NM areas presented 1.6-fold higher concentration of OCPs than M ones, although we did not find significant differences for this matrix (Figure 3A). The metabolite HCB (ca. 10.0 ng/L) and ∑Endosulfan (ca. 40.0 ng/L), presented the highest concentrations in NM and M areas, respectively (Figure 3B).

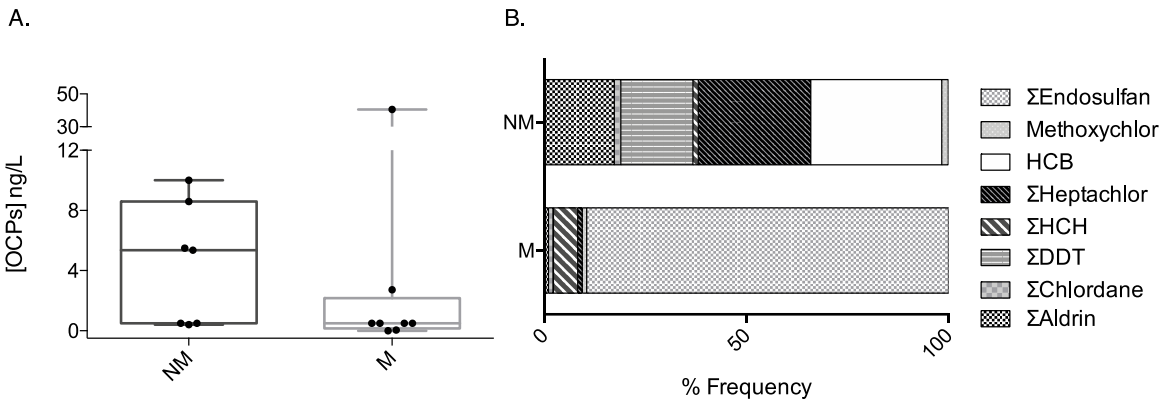


Figure 3. Distribution of pesticides in water samples displayed as NM and M areas (n=11). Graph A: average median values (ng/L) organized by pesticides group (n=11). Graph B: frequency (%) of each quantified pesticide group (n=11).

In the Appendix A, we provided information regarding concentration of each individual compound quantified for water (Table A1-a and A1-b); and the different concentrations quantified from tropical and sub-tropical coastal areas around the world (Table A3-a and a3-b). Overall, data collected between 2002 and 2017 show averaged median concentrations of pesticides ranging from <0.1 to 40.0 ng/L for NM

areas (Appendix A-Table A3-a), and <0.1 to 25.3 ng/L for M areas (Appendix-Table A3-b). Among the selected articles, the highest and lowest OCPs levels for NM areas were quantified in Nigeria and Hong Kong, respectively (Appendix A-Table A3-a). In contrast, M areas presented the highest concentration in Mexico and the lowest in Hong Kong (Appendix A-Table A3-b).

2.3.2 Sediments

We included a total of fifty-two papers for this matrix, with 54 and 46% of the data corresponding to the NM and M area, respectively. When data is arranged by pesticides group, we observed the same pattern as the one for the water matrix (Figure 4A), where NM areas presented 2.2-fold higher OCPs concentrations than M ones. However, for this matrix, we observed significant differences between both areas (U=6, $p < 0.005$, Figure 4A), and the compounds detected at highest concentration were ΣDDT with 1.4 ng/g DW and 0.8 ng/g DW in NM and M areas, respectively (Figure 4B).

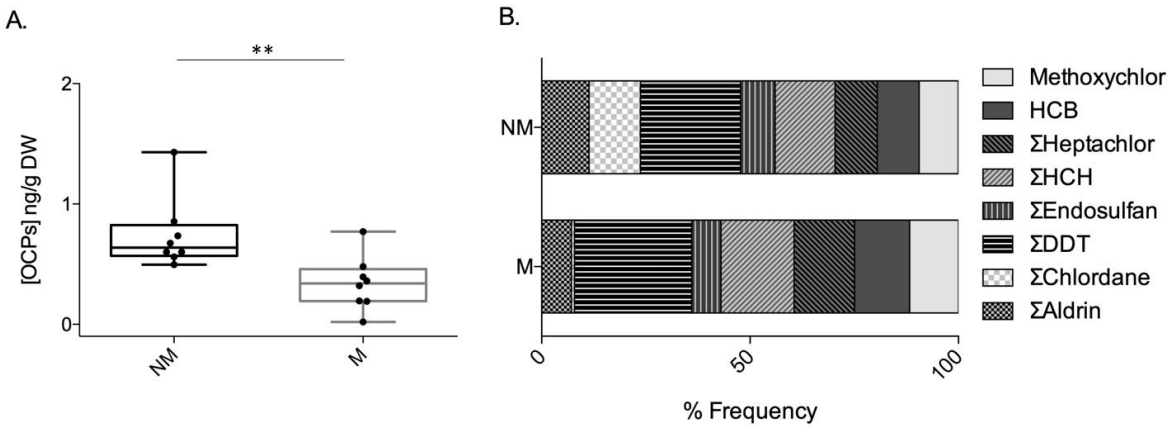


Figure 4. Distribution of pesticides in sediment samples displayed as NM and M areas (n=8). Graph A: average median values (ng/g DW) organized by pesticide groups. Graph B: frequency of each pesticides group quantified. The asterisk indicates significant differences between groups (U=6, *$p < 0.005$).

In Appendix A, we showed the information regarding concentration of each individual compound quantified for sediments (Table A2-a and A2-b); and the different levels quantified from tropical and sub-tropical coastal areas across the world (Table A4-a and A4-b). Overall, the data collected between 1998 and 2015 show average median concentrations of pesticides ranging from 0.3 to 8.9 ng/g DW in NM, and <0.1 to 30.0 ng/g DW for M areas (Appendix A-Tables A4). Among these concentrations, the highest and lowest OCPs levels for NM areas were quantified in Nigeria (1674 ng/g DW) and Argentina (<0.1 ng/g DW) (Appendix A-Table A4-a), respectively. In contrast, M areas presented the highest concentration in China (1906 ng/g DW) and the lowest in Mexico (<0.1 ng/g DW) (Appendix A-Table A4-b).

2.3.3 Fauna

We included a total of sixty studies for this matrix, with 42 and 58% of the data representing NM and M areas, respectively. Regarding the traits analyses, these were performed including and excluding the most representative species (i.e. mudskippers), whose presence could mask the behaviour of the remaining species. Results regarding feeding behaviour (Figure 5) indicated that NM areas presented always higher OCPs concentration than M ones; and that herbivores and carnivores, which tend to accumulate more OCPs than other traits, presented significant differences between NM and M areas ($U=2$, $p < 0.05$ and $U=4$, $p < 0.01$, respectively; Figure 5A). Considering all the species studied, herbivores had averaged median concentrations of 8.0 ng/g WW and 1.6 ng/g WW for NM and M areas, with ΣDDT (14.4 ng/g WW) and HCB (9.7 ng/g WW) as the compounds detected at the highest concentrations, respectively. Averaged median concentrations measured on carnivores were 5.1 ng/g WW and 0.6 ng/g WW in NM and M areas, respectively; for this group, Methoxychlor (20.4 ng/g WW) and ΣHeptachlor (1.1 ng/g WW) were detected at the

highest concentration in NM and M areas, respectively. When excluding the mudskippers from the analysis, the pattern changed in accordance and carnivores presented 3-fold higher average median OCPs concentrations than herbivores, as expected. In this scenario (without mudskippers), only information in M areas was available for group comparison. In this case, Details regarding concentrations quantified in biota samples are in Appendix A-Table A5.

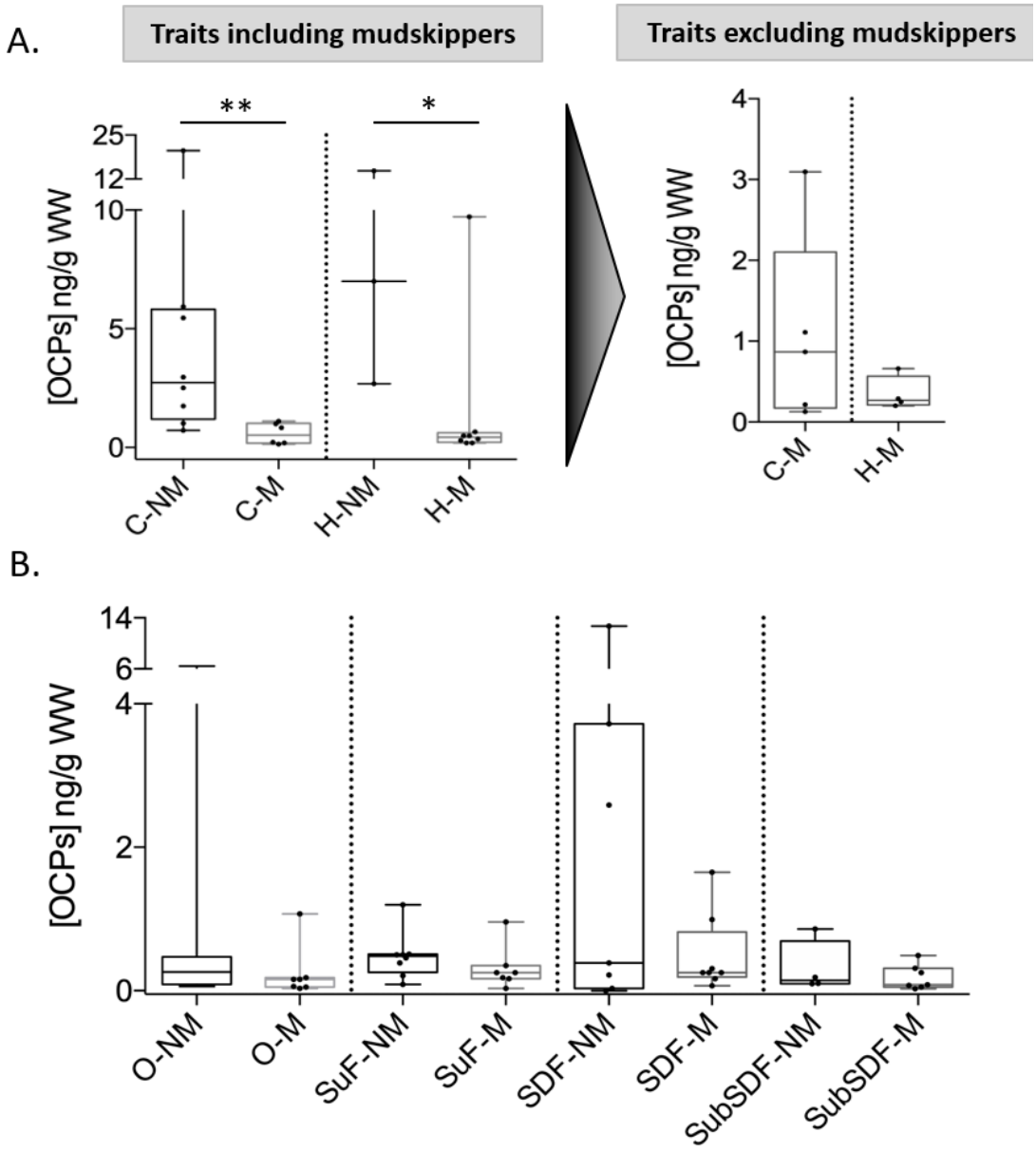


Figure 5. Distribution of the quantified group of pesticides (n=8) in benthic animals displayed by feeding trait and NM/M areas. Graph A: average median values (ng/g WW) for herbivores (H), carnivores (C) and omnivores (O), including (graph on the left) and excluding (graph on the right) the mudskippers data from both H and C

traits. Graph B: average median values (ng/g WW) for suspension feeders (SuF), surface deposit feeders (SDF) and SubSDF (sub-surface deposit feeders). Differences between groups are marked with an asterisk (*p < 0.05, **p < 0.01).

When data is arranged by habitat trait, epifauna presented higher OCPs concentration (2.3 ng/g WW) than in the infauna trait (0.5 ng/g WW). Epifauna (i.e. crustaceans, gastropods, mudskippers, mussels, oysters and shrimps) presented significant differences between NM and M areas (U=8, p < 0.05) with averaged median concentrations of 3.9 and 0.4 ng/g WW, respectively (Figure 6). In NM areas, Methoxychlor presented the highest concentration (22.9 ng/g WW) followed by ΣDDT (3.6 ng/g WW). ΣDDT were also the compounds quantified at the highest concentration in M areas (0.8 ng/g WW). On the other hand, infauna (i.e. clams and annelids) presented similar averaged concentrations in both areas (≈0.5 ng/g WW) where HCB was the compound quantified at the highest concentration for both areas (0.6 and 1.0 ng/g WW for NM and M, respectively). More details regarding concentration in this trait can be found in Appendix A-Table A5.

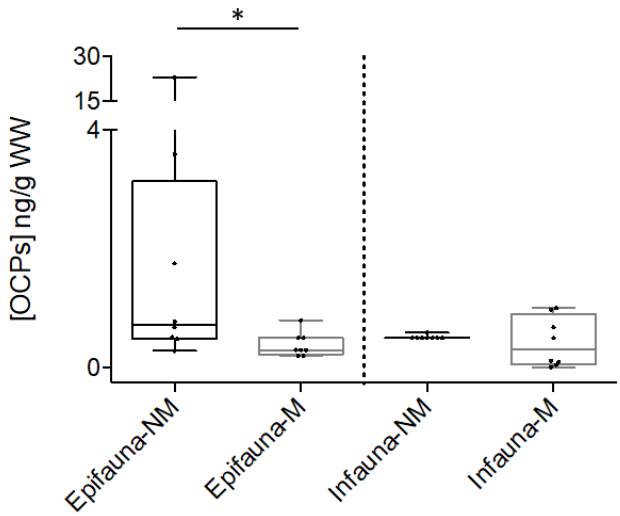


Figure 6. Distribution of the quantified group of pesticides (n=8) in benthic animals displayed by habitat trait and NM/M areas. Average median values (ng/g WW) organized by habitat trait; epifauna and infauna. Differences between groups are marked with an asterisk (*p < 0.05).

2.3.4 Flora

We found no substantial data regarding organochlorine pesticides in mangrove plants. Only two publications, from China and India, reported OCPs levels in mangrove plants (Shete et al. 2009; Qiu et al. 2019). Details regarding location, OCPs values, sampling year and species can be found in Appendix A-Table A6.

Shete et al. (2009) and Qiu et al. (2019), quantified OCPs in several plant compartments (from roots to leaves and fruits), showing a distribution of these compounds over the different mangrove tissues. Overall, the highest average amounts (3.1 ng/g DW) were quantified in roots, independently from the mangrove species and pesticide group (Figure 7A). ∑Chlordane presented the highest concentration in aerial parts, branches and fruits (3.0 ng/g DW and 5.5 ng/g DW, respectively). On the other hand, ∑DDT presented a more dispersed distribution, with highest values quantified in roots (8.6 ng/g DW) followed by leaves and fruits (≈1.9 ng/g DW) (Figure 7B).

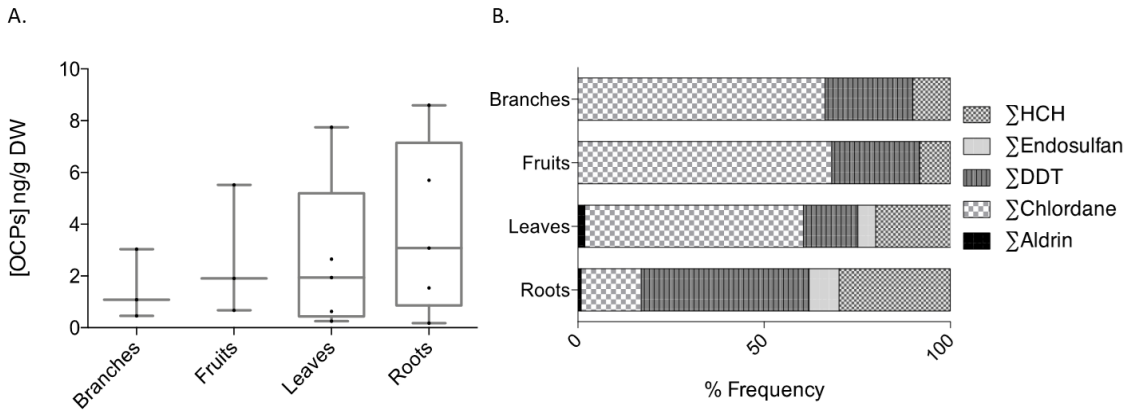


Figure 7. Distribution of pesticides groups (n=3 to 4) in mangrove samples. Graph A: average median values (ng/g DW) organized by mangrove tissues (n=3 to 4). Graph B: frequency of each pesticide group quantified in mangrove tissues.

2.4 Evaluation of water and sediments quality

2.4.1 Legislation limits

Higher pesticide concentrations were quantified in NM areas than in M ones, except for the metabolite Heptachlor epoxide and ΣEndosulfan, which was only present in M areas. Almost 43% of the compounds detected in NM areas were over the established limit versus the 38% detected in M ones (Table 1).

Table 1 shows several compounds presenting median concentrations above the levels established by the European Directive 2013/39/EU. For M areas, most of these high levels occurred in Asian countries (i.e. Singapore, Macao and India), with compounds such as ∑Endosulfan, Heptachlor and its metabolite with respectively 10-, 3- and 240-folds higher concentrations than the established European limits. For NM areas, ∑HCH, Heptachlor and its metabolite had a ubiquitous presence in Asian (i.e. India and Macao) and African countries (i.e. Nigeria and South Africa), with values of 1.6-, 63- and 86-folds, respectively above these thresholds.

Table 1. Average median concentrations of OCPs (ng/L) in surface waters from NM and M areas and the maximum allowed concentrations (ng/L) established by EU legislation framework for surface waters. *ΣEndosulfan (α/β); ΣHCH (α/β/γ/δ); ΣDDT (2,4'/4,4'-DDT, 2,4'/4,4'-DDE, 2,4'/4,4'-DDD). Bold numbers indicate concentration above the established limits.

Compounds (ng/L)	NM	M	EU limits (ng/L)
∑Endosulfan*	-	**41**	4
Endrin	5.4	0.5	10
∑HCH*	**32**	17	20
Heptachlor	**6.3**	**0.3**	<0.1
Heptachlor epoxide	**8.6**	**24**	<0.1
Hexachlorobenzene	10	<0.1	50
4,4'-DDT/DDE/DDD	5.1	<0.1	10
∑DDT*	16	1.5	25

With regards to the sediment matrix, all samples from both NM and M areas presented concentrations below the intervention values of the "Dutch List". However, 54% of the compounds quantified in NM areas were over the target values versus the 31% detected in M areas (Table 2). Aldrin, Endrin, ∑Endosulfan and Heptachlor epoxide presented concentrations above these intervention values, for NM and M areas; while dieldrin, ∑Chlordane and Heptachlor were above only for NM areas.

Regarding the Canadian guideline "ISQG", all compounds were below the PEL threshold values and 50% of the compounds quantified in NM (∑DDT and ∑DDE) and M (Heptachlor epoxide and ∑DDE) areas were above the TEL limits.

In this matrix, M areas also presented a lower percentage of compounds above the established limits by the Dutch List and ISQG guideline, indicating better matrix quality in M areas in terms of pesticide pollution.

Table 2. Average median OCPs levels (ng/g DW) in sediment samples from NM and M areas and the corresponding thresholds established by different guidelines; the Dutch List and the International Sediment Quality Guideline (ISQG); T.V.: Target Values, I.V.: Intervention Values, TEL: Threshold Effect Level; PEL: Predicted Effect Level; asterisk indicates concentrations above the target values established in the Dutch List; hashtag symbol (#) indicates concentrations above the TEL-ISQG. ∑DDT (DDD+DDE+DDT); DDD (2,4'/4,4'-DDD); DDE (2,4'/4,4'-DDE); DDT (2,4'/4,4'-DDT); ∑Aldrin (aldrin+dieldrin+endrin); ∑HCH (α/β/γ/δ); ∑Endosulfan (α/β); na:not available.

Compounds (ng/g DW)	**NM**	**M**	**Dutch List* (ng/g DW)**		**ISQG (ng/g DW)**	
			T.V.	**I.V.**	**TEL**	**PEL**
∑DDT	7.2	6.0	10.0	4000.0	na	na
2,4'/4,4'-DDD	2.3	2.4	na	na	3.5	8.5
2,4'/4,4'-DDE	2.8#	0.9	na	na	1.4	6.8
2,4'/4,4'-DDT	2.1#	2.7#	na	na	1.2	4.8
Aldrin	0.9*	0.3*	0.1	na	na	na
Dieldrin	0.6*	0.3	0.5	na	2.6	6.7
Endrin	0.5*	0.2*	<0.1	na	2.7	62.4
ΣDrins	4.0	0.9	5.0	4000.0	na	na
α-HCH	0.4	0.2	3.0	na	na	na
β-HCH	0.7	0.3	9.0	na	na	na
γ-HCH	0.6	0.5	0.1	na	0.9	1.4

ΣHCH	1.9	0.8	10.0	2000.0	na	na
Chlordane	0.9*	<0.1	<0.1	4000.0	na	na
∑Endosulfan	0.8*	0.5*	<0.1	4000.0	na	na
Heptachlor	0.7*	0.4	0.7	4000.0	na	na
Heptachlor epoxide	0.4*	1.3**#	<0.1	4000.0	0.6	2.7
Hexachlorobenzene	0.3	0.4	na	na	0.9	1.4

2.4.2 Risk assessment

Results regarding the water risk assessment for the first step show RQ>1, indicating that for both areas, the individual concentrations of the compounds quantified in the environment are a reason of concern. Therefore, the second step was performed and it is important to highlight that toxicity data regarding algae (LC/EC_{50}) was not available for all the targeted compounds (Appendix A-Tables A9). To evaluate the potential error of not including them, we calculated RQ_{STU} in two different ways: (1) considering only the common and available toxicity information for the three trophic levels, and (2) considering all the toxicity data available for each trophic level. In both scenarios, algae RQ_{STU} were always lower (less toxic), so we decided to consider the second option to avoid underestimations for the other trophic levels. Results indicated that, in both areas, $RQ_{STU}>1$, and that the most sensitive group for NM and M were fishes and invertebrates, respectively. Moreover, results from second-tier indicated that in NM, the toxicity was mainly due to endrin and γ-HCH; while in M, it was due to ΣDDD (2,4'/4,4'-DDD). All calculations are summarized in Appendix A-Tables A7.

Regarding the sediments, we used the ERL and ERM parameters as a way to evaluate the theoretical risk of OCPs quantified in the environment. NM and M areas did not have values above the ERM, suggesting that the concentration quantified in the sediments, would only trigger occasionally adverse effects in local biota. However, 100% of the compounds quantified in NM areas presented concentrations above the

ERL, while for M areas, only 67% were above this limit. This indicates that NM areas may present concentrations that could eventually compromise the status of these ecosystems and/or affect directly the benthic biota. Results regarding risk assessment for sediments are summarized in Appendix A-Table A8.

2.5 Discussion

2.5.1 OCPs distribution in abiotic compartments

Mangrove ecosystems are subjected to strong flushing and periodic flooding cycles influenced by tides (Jia et al. 2016). These hydrological conditions may help to explain the results obtained in this review. Wong et al. (2006), showed lower OCPs values in areas with strong flushing and tidal effect, as the case of mangrove ecosystems; these strong flushing currents, create an upwelling force between surface sediments and water, which may increase the amount of suspended particle in the water column. Since the target compounds have medium to high lipophilic nature (logP values 1.7-6.2), we assume that they will be preferably absorbed onto suspended particles and subsequently accumulate in the mangrove sediments, as was already discussed by Tam et al. (2001). Therefore, the more suspended particles present in the water column, the less persistent compounds such as OCPs will be quantified, as we postulated in our H1.1. Moreover, Kathiresan (2003), observed a significant difference in the concentration of suspended sediments between high and low tide water in mangroves zones. Sediment particles are carried in suspension into mangrove forests at high tide, and retained there due to the turbulence caused by mangrove structures, as suggested in previous works (Wolanski 1995, Furukawa et al. 1997). Also, during low tide, the speed of the flow will be slow, leading to the settlement of the suspended particles. As a general trend, compounds will be retained in sediments during low tide, allowing mangroves to uptake and/or microorganisms to

degrade the carried compounds from these active sinks (Susarla et al. 2002), explaining also why sediments from M tend to have lower amounts of persistent compounds. This idea is supported by several published works (Kruitwagen et al. 2008, Bayen et al. 2019, Carvalho et al. 2009, Bodin et al. 2011, Wu et al. 2015), where lower OCPs levels were measured in surface sediments from mangroves when compared to other tropical regions, supporting herein the tendency observed and postulated in H1.2. Understanding the OCPs distribution in coastal sediments is quite complex, but the trend obtained in this review, together with the results obtained by Shete et al. (2009) and Qiu et al. (2019), suggest that mangroves play an important role in retaining OCPs, like ΣDDT, ΣHCH and ΣChlordane, as we postulated in our H1.

2.5.2 OCPs distribution in fauna

Benthic fauna followed the same pattern as the abiotic data, with OCP concentrations 5.3-fold lower in M than NM areas, which corroborates with H1. We also observed variations between trophic groups, which may be attributed to the different trophic level, feeding characteristics, metabolization ability or lipid composition of the organisms (Hu et al. 2010; Akinsanya et al. 2015).

Analysing in detail the feeding trait, we could conclude that in the presence of the mudskippers, the pattern obtained (i.e. herbivores > carnivores) goes against H2. But, when excluding them from the analysis higher concentrations were obtained for the carnivores as initially expected (H2). So, our conclusions must be taken carefully. The latter pattern agrees with works from Dietz et al. (2000), Kidd et al. (2001) and Bayen et al. (2005), which reported trophic biomagnification for compounds such as ΣPentachlorobenzene, ΣDDT, ΣHCH and HCB toward higher trophic levels (i.e, from green algae to archer fish). Hydrophobic substances with log K_{OW} > 5 (i.e. ∑DDT,

DDE, Heptachlor, Aldrin, Chlordane, Methoxychlor and so on) have proven to be particularly susceptible to biomagnification in aquatic organisms except when metabolism occurs (Nfon et al. 2008). This data supports why carnivores belonging to a higher trophic level presented higher OCPs concentrations than the others (omnivores, suspension feeders, surface deposit feeders or sub-surface deposit feeders). Moreover, in marine biota, elimination or depuration rates for lipophilic substances may decrease with organism size. Therefore, higher trophic levels (with usually bigger size), will tend to have lower elimination/depuration rate and so to accumulate higher amounts of persistent compounds (Gray 2002).

Nevertheless, our data analysis showed that in the presence of mudskippers, the pattern was different and herbivores accumulated higher pesticide amounts than carnivores. So, we can hypothesise, that biomagnification probably is not the only explanation for the finding of higher concentrations at higher trophic levels. Authors like Gray (2002), reported that from eighty-six marine and freshwater reviewed articles, 47% showed absence of biomagnification effect. Other authors also corroborated with the absence of biomagnification. For example, Falandysz and Rappe (1996), studied the spatial distribution and bioaccumulation features of polychlorinated naphthalenes (PCNs, log K_{ow}=4-8) in the Southern Baltic and observed decreasing PCN concentrations with increasing trophic levels in a pelagic food chain. Similarly, Lundgren et al. (2002), have studied the biomagnification of PCNs in a benthic food chain (surface sediment - amphipod - isopod - fourhorned sculpin), also from the Baltic Sea, and did not observe biomagnification effect. These evidences indicate that besides dietary accumulation through food uptake, other exposure pathways, such as dermal adsorption (Namdari and Law, 1996) might be involved in the exposure of biota to POPs (Bayen et al. 2005). Dermal absorption

should be considered carefully in the case of mangrove ecosystem as organisms are alternately exposed to air/sediment/water due to tidal movements. In this case, high concentrations may not be only due to biomagnification, as the organism may take up contaminants through their body surface or respiratory organs by diffusion, which is the process of bioconcentration (Gray 2002). A good example of this, are the results obtained for mudskippers, considered as very sensitive organisms to the surrounding environment and frequently used as bioindicators in monitoring pollution, especially in tropical/sub-tropical coastal ecosystems (Ansari et al. 2014). The mudskipper *Boleophthalmus pectinirostris*, was the main species analysed as herbivore in NM areas and presented the highest concentration of OCPs (Lam and Lam, 2004; Nakata et al. 2005). Since these animals can breathe through their skin, accumulation of contaminants present in the surrounding environment may occur by both routes: bioaccumulation and bioconcentration. It is important to remember that NM sediments had significantly higher amounts of OCPs than M ones, and that contaminant exposure period (i.e. chronic inputs, long exposure, sporadic spills, and others) is also an important factor that will affect the bioavailability and accumulation rated of the contaminants (Roche et al. 2009).

Another possible explanation for the high levels observed in herbivores could be the absence of metabolization of these compounds by plants. For example, Nfon et al. (2008), showed low levels of DDT metabolites and chlordane metabolites in phytoplankton suggesting a limited capability of metabolism, whereas the presence of these metabolites in higher trophic levels indicated biotransformation reactions within these species.

Considering that hydrophobic compounds tend to be accumulated in lipid tissues, the organism lipid content may play an important role in OCPs accumulation. As

described by Zhou et al. (2007) or Sun et al. (2015), positive correlations were found between ΣDDT and lipid content in fish species, like baby croaker and mullet. Within the biota analysed, mudskippers (*Periophtalmodon schlosseri* and *Periophthalmus argentilineatus,* as carnivores and *Boleophthalmus boddarti* and *Boleophthalmus pectinirostris,* as herbivores) were the animals with higher lipid content, and also the ones with higher concentration of OCPs. A positive correlation (although not significant) was also observed (r = 0.83) for the data gathered in this review when averaged median concentrations of OCPs (NM and M areas together) were plotted against the lipid content of the benthic animals.

Regarding the habitat trait, we could observe the same tendency as for the previous one in terms of NM areas presenting higher amounts of OCPs (9.8-fold) than M ones, and this is in accordance with our previous hypothesis. However, considering that sediments provide a major reservoir of pollutants in marine habitats (Chen et al. 2007; Dachs and Méjanelle, 2010), we were expecting higher contamination levels in infaunal organisms than in epifaunal ones; which was not the case. Polychaetes were the main organism representing the infaunal group and due to the reduced mobility, they may have developed some tolerance mechanism in order to minimise the uptake of contaminants and to maintain their homeostasis when exposed to them (Meyer and Di Giulo, 2003; Geracitano et al. 2004). As an example, Pilo et al. (2016) concluded that the lower levels quantified in the polychaete *Nephtys hombergii* when compared to the bivalve *Cerastoderma edule,* was affected by the ability of the polychaetes to select particles base on their metal contamination, reducing the uptake of contaminants. Nevertheless, there is not much information regarding other compounds such as pesticides, and therefore the idea of the development of tolerance mechanisms for these compounds should be carefully studied. Moreover, considering

that the duration of the entire life cycle is relatively short (on the order of days or weeks) (Dean et al. 2008), exposure to contaminants will be short corroborating the results observed in this review.

2.5.3 OCPs distribution in flora

Several authors, such as Shete et al. (2009) and Qiu et al. (2019), highlighted the capability of mangrove plants to uptake POPs (such as OCPs). Root exudates—a wide range of compounds, such as amino acids, organic acids, carbohydrates and other secondary metabolites—may play an important role in the interception or assimilation of these pollutants (Jia et al. 2015). These small molecules are actively or passively secreted by the plant and tend to bind to the soil organic matter (SOM), modifying the mobility of the pollutant in the soil, lowering its hydrophobic character, and therefore, facilitating the uptake by the roots (Campanella & Paul, 2000; Ling et al. 2009; Liu et al. 2015). For example, Jia et al. (2016), proved that root exudates from mangroves promote the release of SOM and the desorption of organic pollutants from sediment, being then more available for plant uptake. In addition, Luo et al. (2006) showed that root exudates promote the desorption of 4,4'-DDT from soils, increasing its bioavailability to plants and soil organisms. However, we know that phytoremediation by wetlands plants is not an isolated process. It is also dependent on the synergy with rhizosphere microorganisms to remove and degrade toxic pollutants (Miglioranza et al. 2004; Calvelo-Pereira et al. 2006; Abhilash et al. 2011; Becerra-Castro et al. 2013; Miguel et al. 2013). Lu et al. (2011) showed that mangrove root exudates, besides modifying physicochemical conditions of the soil (i.e. increased humification) and of the pollutant (i.e. increased hydrosolubility), may increase the number of soil microorganisms in the rhizosphere, and this could enhance microbial biodegradation of organic compounds such as polycyclic aromatic hydrocarbons

(PAHs) or pesticides, and facilitate their uptake by the plant (White 2000; Miya and Firestone 2001; Jeremy et al. 2004; Phillips et al. 2012). Besides, mangrove roots are characterized by having a rich microbial diversity with a potential for hydrocarbon degradation (Tian et al. 2008). For example, Tam & Wong (2008), reported that the contaminated mangrove sediment had sufficient indigenous PAH-degrading microbes to intrinsically remediate mixed PAHs. Considering this previous evidence, we could assume that root exudates together with the microbial metabolic activity in the root zone will contribute to lower OCPs concentrations in the surrounding environment (Miglioranza et al. 2004). A similar conclusion was obtained by Calvelo-Pereira et al. (2006) and Abhilash et al. (2011), when the dissipation of HCHs levels was observed in the rhizosphere of crops (*Vicia sativa L.* and *Avena sativa L.*) and plant species (*Whitania somnifera*).

Additionally, the presence of lipids in roots and leaves could be one of the reasons for higher bioaccumulation of organic compounds in these tissues compared to others. The roots always carry the finest particles of sediment and this could explain the elevated OCPs concentrations in this tissue, especially for ΣDDT (Shete et al. 2009). Apart from the uptake of OCPs from water/sediment, atmospheric deposition and/or air-leaf exchange processes may be the response for the relatively higher OCPs levels in mangroves leaves (Qiu et al. 2019). Absorption through leaves is also an important pathway for OCPs entering the terrestrial compartment (Nizzetto et al., 2008; Salamova & Hites, 2013).

As observed in the other matrices, results in plants also corroborate the main hypothesis (H1). Nevertheless, this is just an overview of available data that shows a tendency for OCPs reduction in environments when mangroves are present. Further research will be needed to confirm these possible pieces of evidence.

2.5.4 International guidelines and hazard assessment

According to the concentrations quantified in water, M areas presented less targeted compounds with concentrations above the Directive 2013/39/EU, indicating an improvement of the water quality in these areas when compared to NM ones. The results also highlighted Asia and Africa as the continents with the worst scenarios in terms of pesticide water pollution, and this is in accordance to what was already described by Cruzeiro et al. (2018), which already mentioned higher concentration of insecticides in Asian and African countries; and by FAO (Food and Agriculture Organization), which concluded that the highest application rates of insecticides, attaining 6.5–60 kg/ha, occurred also in Asia (FAO, 2013). As a whole, there is still a need to improve in the regulation of OCPs, since they are still a threat to the environment and due to trans-boundary pollution, these high concentrations could affect other worldwide locations.

With regards to the theoretical risk assessment, fish and invertebrates (for NM and M, respectively) revealed to be the two taxonomic groups with the highest predicted sensitivity to the tested pesticide mixtures. This might be explained by the higher sensitivity that both groups might have to insecticides (except HCB) when compared to plants. When Silva and Cerejeria (2004), did the same analyses for several Portuguese water basins, they observed an opposite trend, since most of the quantified compounds were herbicides and due to their mode of action, higher toxicity in algae was obtained than in animals.

Although theoretical RQ from our data indicated that samples from both areas have a potential risk of concern, higher STU/max. RQ_{TU} was calculated in NM (1.94) in comparison to M (1.02), which means that waters from NM areas presented a higher

number of pollutants at concentrations capable to induce environmental risks; and that toxicity of all pesticide mixtures is dominated by a very small fraction of the present compounds. These results, together with the higher percentage of compounds above the European Legislation limits (Directive 2013/39/EU) quantified for NM areas, indicate better water quality (in terms of pesticide pollution) in M than NM areas, as was postulated in our H1 and H3.

Considering the guidelines available for sediments, countries from Asia, America and Africa presented high concentrations that are beyond acceptable limits for compounds that have been banned for several years (official ban 1983). However, they may persist in these environments and/or illegal recent applications might also be the main reason for these high amounts, as discussed by Zhang et al. (2012) and Olisah et al. (2020).

In summary, these results support also our hypotheses H1 and H3, since M areas presented better quality in terms of pesticides pollution than NM ones.

2.6 Final remarks

In the present work, we evaluated studies focused on the distribution and accumulation of OCPs in abiotic and biotic matrices from areas with and without mangroves, worldwide. Considering the gathered data, and the results obtained among the selected matrices, M areas tend to have lower OCPs amounts than NM ones, indicating that this ecosystem might be a potential tool to deal with persistent contamination. Due to the unique hydrological conditions of these areas, which favour the increase of suspended particles and their subsequent settlement into the sediments, mangroves, with their special structures, could have the ability to trap this kind of compounds, degrade and assimilate them with the possible help of the rhizospheric microorganisms. This idea was also reinforced by the results obtained for

water and sediment risk assessment, where better conditions in terms of pesticide pollution were found in mangrove areas.

Nevertheless, this study is mainly a theoretic approximation, and since concentrations of OCPs are still detectable in the environment (even after their official ban), further efforts should be undertaken to better understand the assimilation and degradation of OCPs by mangroves. Moreover, we also want to highlight that although mangroves provide a variety of good ecological services to the environment, they are under anthropogenic stress; and therefore, it is paramount to support resource management and restoration activities to protect this valuable ecosystem.

2.7 Acknowledgements

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Chapter 3. Environmental characterization of 4,4′-dichlorobenzophenone in surface waters from Macao and Hong Kong coastal areas (Pearl River Delta) and its toxicity on two biological models: *Artemia salina* and *Daphnia magna.*

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Abstract

The Pearl River Delta (PRD) is one of the areas with higher environmental concentration of organochlorine pesticides (OCPs), being DDT one of the most abundant. In this work, 4,4′-dichlorobenzophenone (4,4′-DCBP), a common metabolite of dicofol (DDT related) and DDT, was quantified in surface waters of Hong Kong and Macao, together with the analysis of physicochemical and nutrients parameters. Hong Kong presented higher 4,4′-DCBP mean levels (12.50 ng/L) than Macao (4.05 ng/L), which may be due to the use of dicofol as a pes- ticide and DDT as antifouling-paint for ships. The region presented a possible eutrophication state due to the high nutrients' concentration. For the first time, toxicity evaluation of this metabolite in *Artemia salina* and *Daphnia magna* was done, in order to compute valid EC50s and theoretically evaluate the risk in the PRD. The toxicity results (EC_{50} = 0.27 mg/L for *A. salina*; and EC_{50} = 0.17 mg/L and LC_{50} = 0.26 mg/L for *D. magna*), together with the 4,4′-DCBP levels quantified, indicated a low environmental risk.

Keywords:

Organochlorine pesticides, dicofol, DDT, metabolite, LC_{50}, EC_{50}

3.1 Introduction

Pesticides contamination in coastal areas has become an important worldwide problem since the 1950s, due to the agriculture system, runoff from treated plants and soil, atmospheric exchange or sewage discharge (Scholtz et al., 2002; Guan et al., 2009; Özkara et al., 2016). These chemicals persist in the environment, affecting surface water quality and having an ecotoxicological effect on aquatic flora, fauna and human health (Miyamoto et al., 1990; Ongley, 1996; Lozowicka et al., 2014; Skretteberg et al., 2015). Organochlorine pesticides (OCPs) are a group of persistent organic pollutants (POPs) used in agriculture worldwide, mainly from the 1950s to the 1980s, and are characterized for their stable chemical structure that allow them to accumulate, persist and biomagnify in the environment for decades (Dimond and Owen, 1996; Nakata et al., 2002; Carvalho, 2017). Because of their characteristics, OCPs are common pollutants in the marine environment (Luo et al., 2004) and have been a worldwide concern due to their reported toxic effects on humans and wildlife (Guan et al., 2009; Mrema et al., 2013). Especially in China, considered as the largest producer and consumer of pesticides in the world, 80% of the pesticides produced before 1983 (year of the official ban) were OCPs (Grung et al., 2015). The Pearl River Delta (PRD) in South China is considered one of the areas with high environmental concentrations of pesticides due to fast industrial and agricultural development in the region (Tieyu et al., 2005; Guo et al., 2009). During the last two decades, overfishing and pollution problems (Duan et al., 2009) also compromised the water and air quality in the area (Fu et al., 2003; Guan et al., 2009). One of the most common OCPs present in the PRD waters, is dichlorodiphenyl-trichloroethane (Grung et al., 2015), also known as DDT, a pesticide that has been widely used for pest control and mosquito abatement prior to the global ban in the 1970s and 1980s (Guo

et al., 2009). Previous studies suggested that DDTs concentration levels in the PRD have remained considerably high despite China's official ban in 1983 (Fu et al., 2003; Guo et al., 2009). Zhang et al. (2002), found no sign of declining concentrations of DDTs in sediment cores collected from the PRD during the summer of 1997, with mean values ranging from 2.5 to 22.7 ng/g DW. A more recent study, carried on between December 2009 and March 2010, showed average levels of 18.4 ng/g DW (Wei et al., 2015). In addition, sediment samples from Hong Kong and Macao, collected during 2007 showed alarming concentrations (i.e. 76–7 350 and 967–5 810 ng/g DW, respectively), which are 3.8-fold higher than the concentrations found in previous years in the PRD (Lin et al., 2009). The amount of DDTs detected in this environment may be associated to a historical contamination where excessive soil runoff enhanced by the large-scale land modifications and regional flooding might have contributed to the transport of OCPs from soil to the sedimentary system (Zhang et al., 2002; Guo et al., 2009). However, some authors have indicated the existence of currently fresh inputs or unknown sources of DDTs, which may also contribute to these environmental levels (Qiu et al., 2005; Wang et al., 2007), and can be targeted by different DDT isomers ratios. If the ratio between DDT and the sum of its metabolites (DDT/(DDE+DDD)) is higher than 1, and the ratio between DDT isomers (o,p′-DDT/p,p′-DDT) ranges between 1.3 and 9.3, often indicates a new source of DDT pollution, which can be explained by the use of technical dicofol, as mentioned by Fu et al. (2003) and Qui et al. (2005), respectively.

The annual average production of DDTs was about 6 000 t from 1988 to 2002, and nearly 80% of that was used to produce dicofol, a pesticide responsible for some of the new inputs of DDT in the environment (Qiu et al., 2005). According to Guo et al. (2008), the amount of dicofol used in China was almost 9 000 t between 1988 and

2002, and in 2003, an average of more than 14 t of dicofol was applied in the PRD region. Zheng et al. (2016), detected dicofol as the most frequent OCPs in water and sediment samples from Jiulang River (North East China). Since dicofol is produced from technical DDT — through a pathway including chlorination followed by hydrolysis to form the final product — its molecular structure is similar and it is associated with the same concerns as DDT and its metabolites (Fujii et al., 2011). It is estimated that 93% of 4,4′-DDT is converted to 4,4′-dicofol while only 37% of 2,4′-DDT is converted to 2,4′-dicofol (Qiu et al., 2005), with 4,4′-conformation as the main isomer present in the final product, and therefore the 4,4′-dichlorobenzophenone (4,4′-DCBP) as the main breakdown isomer present after the degradation of dicofol (Thiel et al., 2011). Owing to the instability and easy degradation of dicofol in water — when exposed to a higher pH, light or higher temperature — 4,4′-DCBP is the main expected form in surface waters (Fujii et al., 2011; Thiel et al., 2011; Yin et al., 2017). Moreover, it has also been reported that degradation of the main metabolites of 4,4′-DDT can contribute to the levels of 4,4′-DCBP in the environment (Purnomo et al., 2008; Ricking and Schwarzbauer, 2012).

To our knowledge, no previous studies have focused on the environmental characterization of 4,4′-DCBP in PRD; and its toxicity effects. This study may be considered as the first work in which these topics will be addressed, using two biological models, *Artemia salina* and *Daphnia magna*. The crustaceans *A. salina* (brine shrimp) and *D. magna* (water flea) are two invertebrate models that have been widely used for ecotoxicological studies in saline and freshwater environments, respectively (Cleuvers, 2003; Favilla et al., 2006). The life cycle of *A. salina* begins by hatching of dormant inactive cysts (0.2–0.3 mm), into free-swimming nauplii (0.45 mm; instar II/III), in a period of 24–36h and after being rehydrated in salty water. The

larvae are very adaptive to a wide range of salinities (5–250) and temperatures (6–35°C), having a short life cycle (3–5 weeks to reach adult life) or a high adaptability to adverse environmental conditions (Lu et al., 2012). The life cycle of *D. magna* begins by hatching of dormant inactive eggs (ephippia). The eggs develop in about 3 days into neonates, which can then be used immediately for the toxicity test. The measurement endpoints generally evaluated for this animal model are the 48 h-LC_{50} (for survival), and the 48h-EC_{50} (for immobility) (Jonczyk and Gilron, 2005). All these characteristics, make them appropriate models for short toxicological tests with low costs in routine and research practices (Cruzeiro et al., 2017; Lu et al., 2012).

Due to the limited knowledge in the potential environmental occurrence and toxicological effects of 4,4′-DCBP, the main goals of the present work were: a) to optimize and validate an analytical GC-MS/MS method to analyse 4,4′-DCBP in water samples; and for the first time b) conduct an environmental characterization of the 4,4′-DCBP levels in surface waters collected from the east and west mouth of the PRD, Hong Kong and Macao, respectively; and c) evaluate the toxicity of 4,4′-DCBP on two aquatic species, *A. salina* and *D. magna*, considered as ideal biological models for determination of LC_{50} and EC_{50}.

3.2 Materials and methods

3.2.1 Study area

The Pearl River Delta (PRD), embraced by Hong Kong S.A.R. and Macao S.A.R., is located in southern China (112°00'~115°25'E and 22°30'~23°45'N) (Duan et al., 2009). It has a land area of approximately 40,000 km^2 (Guo et al., 2008) and includes the third largest river (331.9 × 109 m3/yr) in China and the largest river system flowing into the South China Sea (SCS) (Zhao, 1990).

The PRD belongs to the subtropical climatic zone, characterized by high precipitations (1 600–2 200 mm, annually), mild temperature all year around (19.5–22.3°C) and humidity ranging from high to low, during summer and winter, respectively (Guo et al., 2009). Flood periods occur for at least three months in the summer, and 80% of the total flow befalls between April and September (Chen et al., 2004), where April is considered as a transition month.

Due to the population rise in this region, the amount of sewage discharge increased to around 14% from 2.61 billion m^3 to 2.97 billion m^3, only between 2005 and 2015 (Liu et al., 2018).

3.2.2 Water collection and quality measurements

Water samples were collected during the transition season (April) and middle of the flood season, also named as wet season (June) of 2017. A total of 10 sampling locations, distributed around Hong Kong (HK1-HK5) and Macao coastal areas (M1-M5), were sampled (Figure 8).

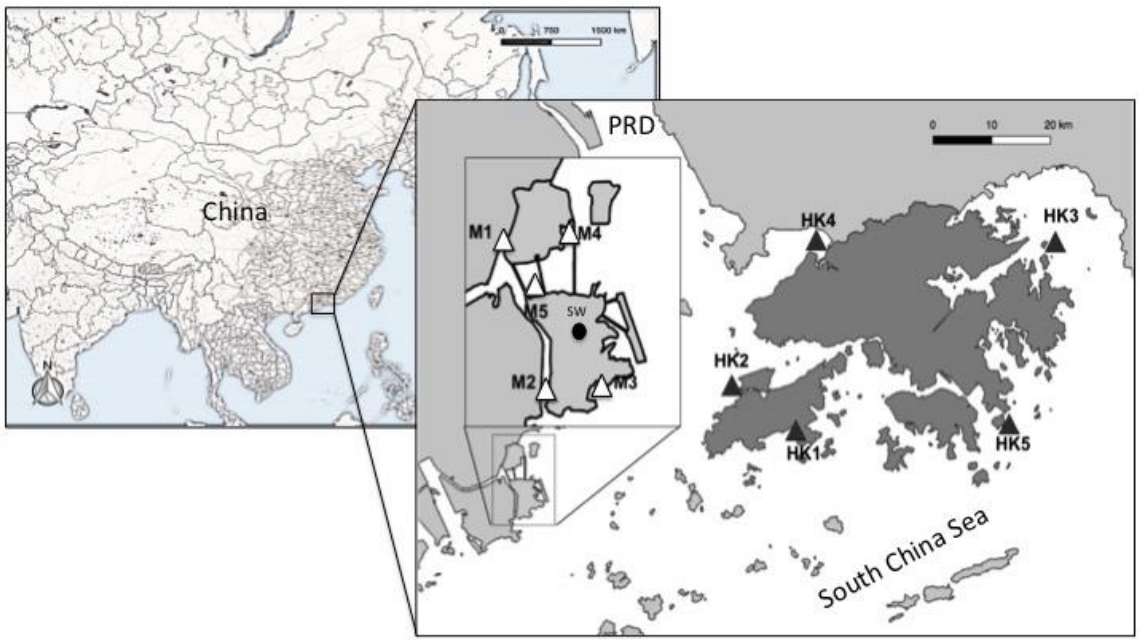

Figure 8. Map of the Pearl River Delta (PRD) region and the distribution of the sampling sites in Hong Kong (HK1 to HK5) and Macao (M1 to M5); SW indicates

the location were the spring water was collected. QGIS 2.18 Desktop, version 2.18.15.

At each site and sampling occasion, water samples (2 L) were collected during low tide (LT) and high tide (HT) into pre-rinsed amber glass bottles for quantification of 4,4′-DCBP, nutrients and psychochemical parameters. For 4,4′-DCBP quantification, all samples (0.5 L) were filtered (0.45 μm glass fibre filter; Sartorious, Germany) and acidified to pH 5 with acetic acid (CH_3COOH; Sigma-Aldrich, USA) prior to extraction for a higher sample stability. During transport and after filtering, water samples were kept at 4°C in the dark, for a maximum period of 24 h. Details about the chemicals and the reference standards are described in the Appendix C. Nutrients analysis (i.e. dissolved inorganic nitrogen (DIN, mg/L) and dissolved inorganic phosphorous (DIP, mg/L)), were measured in the laboratory, with a photometer device from Palintest (YSI 9500 photometer, UK). Physicochemical parameters, such as temperature (T, °C), dissolved oxygen (DO, %), total dissolved solids (TDS, g/L), pH, and salinity were measured in situ (using a portable meter (YSI pro plus, USA)), while Chlorophyll a (Chl-a, mg/m3) and total suspended solids (TSS, g/L) were quantified in the laboratory. Chl-a, was quantified by filtering 500 mL of water, through a Whatman GF/C glass fibre filter, following the protocol of Parsons et al. (1985); and TSS were quantified using 200 mL of the water samples following the protocol described by APHA (1995).

3.2.3 Water sample pre-concentration (SPE)

In the final and optimized protocol, cartridges were conditioned sequentially with 5 mL of methanol (MeOH), followed by 5 mL of ultrapure water, at a flow rate of 1–2 mL/min. Then, water samples (500 mL) spiked with the surrogate, were loaded into SPE cartridges at a constant flow rate of 5 mL/min and then allowed to dry.

Subsequently, the samples were eluted with 2.5 mL of ethyl acetate, followed by 2.5 mL of dichloromethane and 2.5 mL more of a 1:1 mix of dichloromethane and ethyl acetate (v/v), at a rate of 1 mL/min. The extracts were evaporated to dryness, under N_2 stream (99.995%) and then reconstituted into 200 µL of MeOH. The method optimization is fully described in Appendix C.

3.2.4 Instrumental methods, quality assurance and quality control procedures

Analyses were carried out using a gas chromatograph (Trace 1310 GC, Thermo Scientific), coupled with a triple quadrupole mass spectrometer detector (TSQ 8000 EVO, Thermo Scientific), an autosampler (Thermo ScientificTriPlusTM) and a Trace Pesticides column (TR-pesticides II, 30 m × 0.25 mm × 0.25 µm). Column oven temperatures were programmed for a 14 min period using several ramps: a) from 75°C with an initial equilibrium time of 3 min to b) 180°C at 30 °C/min until c) 280°C at 5 °C/min, where the temperature was maintained for 1 min. The injector port temperature was set to 250°C, and both ion source and MS transfer line were at 280°C. Helium (99.999% purity) was used as carrier gas and was maintained at a constant flow rate of 1.3 mL/ min. Sample injection (1 µL) was in the split-less mode (4 mm straight liner, 453A1925), using a 50 mm long needle.

The performance of the method was checked daily, using method blanks (solvent controls), quality controls (two-fold higher than the limit of quantification), fortified samples spiked with both surrogates, and using, weekly, new calibration curves. The limits of detection (LODs) and quantification (LOQs) were defined as LOD = 3.3 α/S and LOQ = 10 α/S; here, α is the standard deviation slope and S is the average slope of the calibration curves. Linearity, precision, accuracy, and recoveries were evaluated following the criteria established by SANCO/825/00 rev 8.1 (SANCO, 2010) (more details in Appendix C).

3.2.5 Biological assay

3.2.5.1 *Artemia salina* acute toxicity test

The hatching and the standard operational procedure for *Artemia*

toxicity screening test for estuarine and marine waters followed the Artoxkit M protocol from the company Microbiotest (Artoxkit M, Microbiotest), which is based on the ASTM standard Guide E1440-91 (ASTM American Society for Testing and Materials, 1987). Dry cysts (Ocean Nutrition, batch number: ONG01805) were incubated in artificial salty water (35 Sea Salt), previously aerated, at 25°C and 3000–4000 lx (light intensity). Thirty-six hours later, groups of 10 free-swimming nauplii (animals in instar II and III) were randomly transferred into 2 mL glass beakers and placed in a 24-multiwell plate to a final volume of 1 mL/well. This test was performed in four independent replicates, using one plate with three wells per treatment, and one plate for the standard toxicant reference ($K_2Cr_2O_7$). Animals were exposed during 24 h (maintained in the dark), at 25°C. The concentrations used for 4,4′-DCBP were 0, 0.019, 0.039, 0.078, 0.156, 0.312, 0.625, 1.25 and 2.5 mg/L (in consideration of its max. solubility in MeOH); and for the $K_2Cr_2O_7$ were 0, 10, 18, 32, 56 and 100 mg/L according to the standard operational procedure of the protocol. The saline control (just saltwater) and the solvent control (0.1% of MeOH) were included in all four plates in triplicates. The same procedure was repeated in three different days. Toxicity was analysed by counting the dead nauplii (no movement in 10 s of observation), using a binocular stereomicroscope (6.5 x of magnification).

In addition, sub-lethal effects in swimming behaviour (i.e. displacement (cm) and speed (cm/s)), were also analysed in order to determine the EC_{50} for this compound. For this purpose, the same range of concentrations (mg/L) were used. A total of 192 videos (four animals per treatment, per replica) of 50 s duration each, were recorded

and analysed using the UMAtracker software, version 0.1. For both cases, the plate results were valid if mortality of the control group was below 10%.

3.2.5.2 *Daphnia magna* acute toxicity test

The test was done using the DaphTox F magnaTM kit procedure (MicroBioTests, 2006; Kit number DM232; Batch number DM140217), which is based on the OECD guideline 202. Briefly, pre-rinsed ephippia were incubated in a standard freshwater solution (ISO 6341) at 21°C, for 72 h with a continuous light exposure of 6000 lx. Afterwards, the hatched animals were collected and fed, with spirulina, for a period of 2 h before the subsequent test exposure. This test was performed in four independent replicates, using one plate with three wells per treatment. Five daphnia neonates were placed per 10 mL well, and stored in the dark at 21°C, for 48 h. Eight different concentrations (ranging from 0.0195 to 2.5 mg/L, as in the *Artemia* assay), plus the control and solvent control (0.1% MeOH), were tested. After 48 h exposure, mortality of four different plates and same sub-lethal effects (following the same procedure as in *A. salina*) were analysed. For the analysis of the mortality, the number of dead neonates was recorded; and for the analysis of the swimming behaviour, a total of 160 videos (four animals per treatment, per replica), of 50 s duration each, were recorded and analysed using the same software mentioned above. The plate results were valid if mortality rate of the control group was below 10%. To validate the assay, an additional control was performed submitting the animals to different $K_2Cr_2O_7$ concentrations (0.32, 0.56, 1, 1.8, and 3.2 mg/L), as it was described in the protocol.

3.2.5 Data analysis

Method validation: results represented in Figure C1 and C2, and Table C2 (Appendix C) are expressed as mean ± standard deviation of the mean (SD). Statistical analyses were done with the soft- ware Prism 6 version 6.0c. After checking assumptions of

normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test), data sets were analysed by one-way analysis of variance (ANOVA) with post-hoc comparison via Tukey's test. Logarithmic transformations were applied when assumptions were not accomplished.

Environmental data: water samples were analysed for 4,4′-DCBP and only concentrations above LOQs were used for posterior analyses (data in Table C3). For graphical representation, data were organized according to sampling site (n = 10) and tide (n = 2; Figure 9) and expressed as final quantified environmental concentrations (ng/L). Normality and homoscedasticity were assessed and differences between sampling sites and tides were analysed using two-way ANOVA, with post hoc comparison via Tukey's test. For the physicochemical parameters and nutrients, Kruskal-Wallis and Mann-Whitney tests were applied, and heatmap was performed in order to observe how samples were grouped (through cluster analysis), using Euclidean distance and the R software (heatmap.2, version 3.5.0) (Figure 10). In addition, correlations were explored using Spearman correlation (Figure 11).

Toxicity tests: for determination of LC_{50} and EC_{50}, mortality and abnormal swimming behaviour data was verified for outliers using Rout (alpha = 0.5%). Data was treated as: % = (4,4′-DCBP/Solvent Control)*100 before logarithmical transformation and data normalization; then, a non-linear regression was applied (Figure 12c and d, 13c and d, 14b). Mortality rates, for *D. magna* and *A. salina*, were also analysed and presented as mortality (%) vs. toxic concentration (μg/L) (Figure 14a). Considering that the solvent control group is the appropriate control group for comparisons with treated groups (OECD, 2006) and no significant differences were observed between control and solvent control, the “solvent only method” (Green, 2014) was the one followed to do all the comparisons. One-way ANOVA followed by Dunnett's post-

hoc test, were performed to study differences between the different concentrations and the solvent control.

Risk assessment: a simplified theoretical model approach was used, to predict the environmental hazard of 4,4′-DCBP detected levels, as suggested by Backhaus and Faust (2012). The EC_{50} (mg/L) values used in this theoretical approach were the ones obtained in the acute-toxicity tests with *D. magna* and *A. salina*. The Predicted No-Effect Concentrations (PNECs) was calculated by the ratio between the EC_{50} (mg/L) levels and an assessment factor (AF) of 100, as: PNEC = EC_{50}/AF; the assessment factor was stabilised according to the Water Framework Directive (2000/60/EC, European Commission 2000), and considering that only the EC_{50} data from one trophic level was used in this approach. Then, the risk quotient (RQ) was calculated as the ratio between measured environmental concentration (MEC) and PNEC: RQ = MEC/PNEC. If the RQ was higher than 1, indicates high risk, if 0.1 < RQ < 1, medium risk and if RQ < 0.1, low risk.

3.3 Results

3.3.1 Method validation

Eight nominal calibration standards mixtures, with concentrations ranging from 3 to 400 ng/L and a fixed surrogate concentration of 50 ng/L, were spiked in the spring water matrix (salinity ca. 20). The calibration curves proved to have good fits with r^2 ranging from 0.986 to 0.999 and a final LOD and LOQ of 0.272 and 0.824 ng/L were obtained, respectively. Considering the three studied concentrations (2LOQ = 1.60 ng/L, 20LOQ = 16.48 ng/l and 100LOQ = 82.40 ng/L), all validation criteria presented successful ranges established by SANCO/825/00 rev 8.1 (SANCO, 2010). The final recovery rates ranged from 72.13% to 121.24%, while precision results (0.67–13.95%) were always below the maximum established, and accuracy ranged

from 72.74% to 116.00%, demonstrating high robustness during the extraction process (detailed data in Table C2).

3.3.2 Water quality

4,4′-DCBP levels measured in Macao and HK coastal areas presented a different spatial pattern during the transition season (Figure 9). Significant differences were found between Macao and HK sites (2-Way ANOVA, $F_{(9,20)} = 166.5$, $p < 0.05$). Namely, sites HK3, HK4 and HK5, presented significantly higher levels of contamination, with a range of 10.85–29.87 ng/L, than the remaining ones. Intermediate levels of 4,4′-DCBP (6.17–4.26 ng/L) were detected in both coastal areas, specifically in HK1 and HK2 (corresponding to Lantau island) and M3 and M5. The lowest levels of 4,4′-DCBP were detected in Macao, encompassing M1, M2 and M4, ranging from 3.77 to 2.98 ng/L.

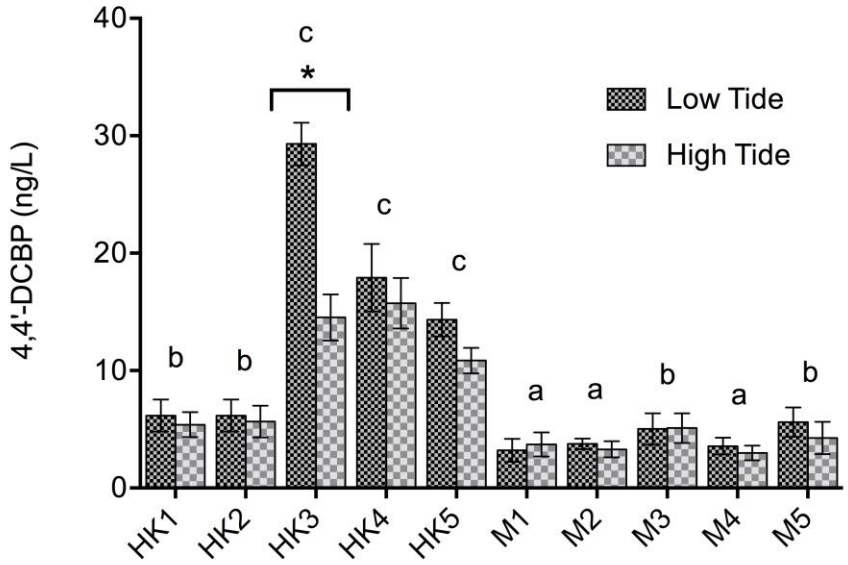

Figure 9. 4,4'-DCBP levels (ng/L) in Hong Kong (HK1-HK5) and Macau (M1-M5) during high and low tide. Different letters indicate significant differences between sites, while the upper asterisk indicates significant difference between low and high tide for the site HK3. Data are presented as means ± (SD).

Comparing tides, no significant differences ($p > 0.05$) were observed between high and low tide for all sampling sites, except for HK3 (2-Way ANOVA, $F_{(1,20)} = 28.11$,

$p < 0.05$). However, a significant interaction was found between tides and sites (2-Way ANOVA, $F_{(9,20)} = 4.73$, $p < 0.05$) indicating an influence of the tide in the levels of 4,4′-DCBP detected only in HK3.

Regarding the wet season campaign, 95% of the obtained values were below LOQ, except for M1-LT, presenting similar concentrations (≈ 2.80 ng/L) to the ones detected in the transition season but no significant differences were found between them (t-test, $p > 0.05$). Detailed data can be consulted in Table C3.

Nutrients and physicochemical data for the different sampling sites are summarised on Figure 10 and Tables C4-a and C4-b.

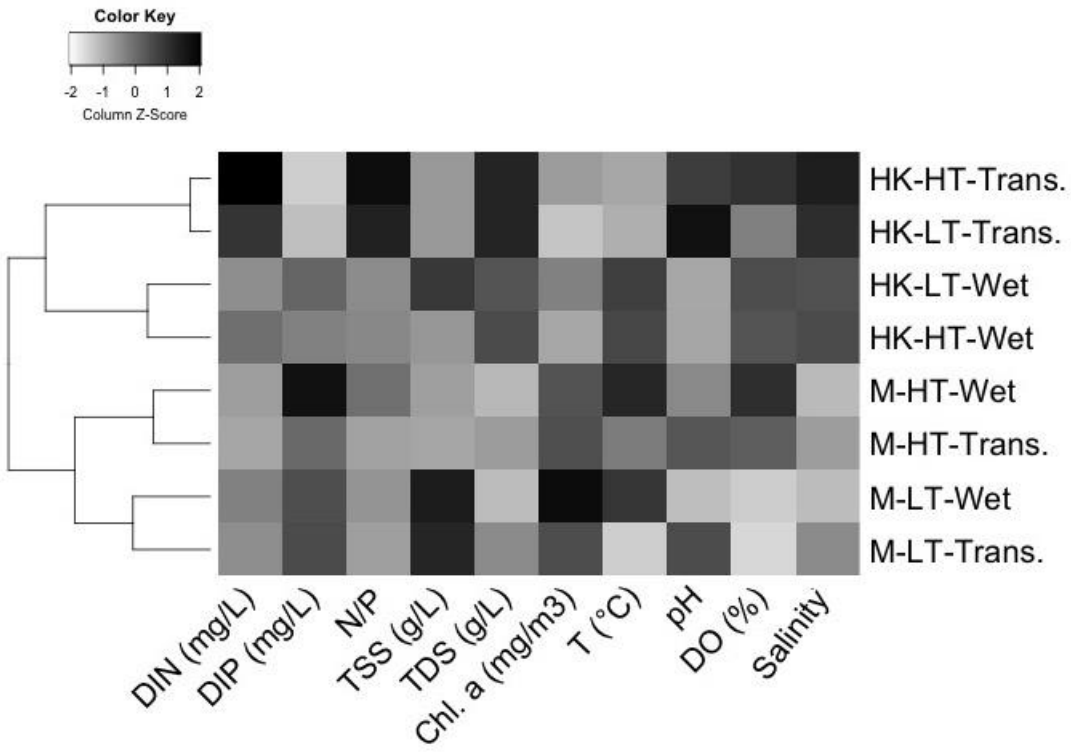

Figure 10. Heat map representing the physicochemical parameters and nutrients quantified in the surface waters of Macao and Hong Kong during wet and transition (Trans.) season. Scale is indicated by the color key; the higher the value the darker the color.

Higher DIN levels were recorded during the transition season, mainly for HK1-HT (16.43 mg/L), HK2-LT (14.73 mg/L), and HK2-HT (13.19 mg/L). Significant DIN

differences were found between transition (5.52 mg/L) and wet (2.21 mg/L) seasons (Mann-Whitney, $U = 21$, $p < 0.05$) but only for Hong Kong waters. Significant differences were also found between Macao (0.14 mg/L) and Hong Kong (0.09 mg/L) coastal areas for DIP levels (Mann-Whitney, $U = 126.5$, $p < 0.05$), where higher values were observed during wet season and especially in M4-HT (0.59 mg/L) and M1-LT (0.22 mg/L). For the N/P ratio, higher levels were found in Hong Kong waters during the transition season, registering the highest ratios for HK2-HT (1106.47), HK3-LT (662.94), and HK2-LT (429.73).

Concerning the physicochemical parameters, higher TSS levels were found in Macao in M5-LT, during wet season (0.76 g/L) and in M5-LT, during transition season (0.40 g/L). Only for Macao coastal waters, significant differences were observed between tides (0.26 g/L and 0.05 g/L for LT and HT, respectively; Mann-Whitney, $U = 17.50$, $p < 0.05$). Macao waters also presented higher Chl-a levels (Table C4-a), mainly during wet season for M4-LT and M1-LT (49.66 and 44.72 mg/m^3, respectively). In addition, during the transition season, significantly higher levels were observed in Macao (19.06 mg/m^3) than in Hong Kong (7.74 mg/m^3; Mann-Whitney, $U = 10$, $p < 0.05$).

Higher TDS levels were found in HK3 during transition and in HK4 during wet season (35.68 and 33.95 g/L, respectively). Significant differences were found between Hong Kong and Macao locations, for both seasons (Kruskal-Wallis, $H = 24.52$, $p < 0.05$). Concerning DO (%) levels, most of the sampling locations presented acceptable levels (78.52–151.3%) according to the 75/440/EEC Directive, which establishes a minimum of 70% DO for surface waters (European Economic Commission, 1975). However, locations like HK2 (except for HT transition season), HK4-LT, M1 (except for HT wet season), and M4-LT (during transition season)

presented levels below the optimum established. Average temperature levels in both coastal waters were 23.86°C and 29.46°C for transition and wet season, respectively. Salinity ranged from 3 to 35 for all the locations, except for M1 (< 3). As can be observed in the cluster analysis (Figure 10), physicochemical and nutrients distribution showed a different pattern between Hong Kong and Macao coastal waters. Within Hong Kong territory, differences between seasons were stronger than between tides, while in Macao differences between tides were stronger than between seasons.

3.3.3 Toxicity assays

3.3.3.1 Artemia salina

Acute toxicity tests using *A. salina* (24 h exposure) were performed in order to obtain the mortality rate (LC_{50}) and the sub-lethal effects (EC_{50}). An average LC_{50} of 48.46 mg/L was computed for the positive control ($K_2Cr_2O_7$), which is in accordance with the ARC-test range (28–64 mg/L; Vanhaecke andPersoone, 1984). Considering the effects of this metabolite in the swimming behaviour, a final average 24 h-EC_{50} value of 0.26 mg/L and 0.27 mg/L, were obtained for total displacement and speed, respectively (Figure 12). Significant differences were also found between SC and C5-C8 concentrations (0.315 and 2.5 mg/L; respectively) for total displacement (Figure 12-a) and speed (Figure 12-b) analyses (One-way ANOVA, $F_{(8)} = 9.878$, $p < 0.05$ and One-way-ANOVA, $F_{(8)} = 9.927$, $p < 0.05$, respectively).

3.3.3.2 Daphnia magna

Lethal and sub-lethal effects of 4,4′-DCBP were studied using an acute test in *D. magna* (48 h exposure). Considering the effects of this metabolite in the swimming behaviour, a final average 48 h-EC_{50} value of 0.17 mg/L, was obtained (Figure 13).

Significant differences were also found between C8 (2.5 mg/L) and SC for total displacement (Figure 13-a) and speed (Figure 13-b) analysis (One-way ANOVA, $F(8) = 5.165$, $p < 0.05$, and $F(8) = 5.356$, $p < 0.05$ respectively). Regarding the mortality rate, a good fit line response was achieved ($r^2 = 0.79$), reaching an average 48 h-LC50 of 0.26 mg/L (Figure 14).

3.4 Discussion

3.4.1 Method validation

The method validated in the present study, for the analysis of 4,4′-DCBP in surface waters, accomplished all the criteria (i.e., evaluation of linearity, accuracy, precision and recoveries) established by SANCO/ 825/00 rev.8.1 (SANCO, 2010), and the stability of the extracts showed ideal recovery rates, in the first 48 h, demonstrating the importance of analysing all the extracted samples within this period. Due to the lack of pesticide-free coastal water, a spring water source from Coloane (SW, Figure 1) was used as a matrix for the method validation with a previous addition of aquarium reef salt to simulate the average salinity conditions of the selected coastal areas (ca. 20); this matrix demonstrated to be valid for 4,4′-DCBP extraction and quantification method. With this optimized method, it was possible to quantify the metabolite at very low range of concentrations (0.8–50 ng/L) in different surface coastal waters with a wide range of salinities (0.19–36.07), confirming its robustness.

3.4.2 Water quality

The average 4,4′-DCBP values obtained for HK were similar to the ones reported in the mouth of the Yongdingxin River (16.75 ng/L), north of China (Wan et al., 2005), while the levels quantified in Macao (4.05 ng/L) were comparable with the ones found in the River Elbe (3.80 ng/L), Germany (Federal Environmental Agency,

2008). The strong and frequent rainy periods commonly observed during wet season, may have contributed to the vestigial concentrations found due to dilution effects. This temporal pattern was also observed by Yang et al. (2012) when several OCPs (including DDTs) were detected from Guangzhou (Pearl River Delta) from March to August 2005. Also, Zheng et al. (2016) observed a similar seasonal pattern for dicofol in Jiulong River (South China). No further information regarding 4,4′-DCBP has been reported in other countries, which once more shows the importance of this study.

The highest 4,4′-DCBP concentrations found in Hong Kong (mainly in HK3, HK4 and HK5), may be due to an extensive use of the main precursor pesticides, like dicofol, DDT, chloropropylate and chlorobenzilate, in the area. Although, chloropropylate and chlorobenzilate have also been reported as possible sources (Knowles and Ahmad, 1971; Yin et al., 2017), information regarding its use in China is scarce and only some residues of those pesticides have been described, in tea leaves from Indonesia (1968) and India (1969) (Bartsch et al., 1971). Therefore and considering the usage and detected levels of dicofol and DDT in China (Tieyu et al., 2005; Grung et al., 2015; Zheng et al., 2016), these pesticides can be considered as the main precursors of 4,4′-DCBP in this aquatic environment.

Since 1950s, DDT began to serve as efficient additive for antifouling, which are considered as a potential regional source of DDT in the PRD (Guo et al., 2008; Xin et al., 2011). The shipping and fishery industry ─ that use those paints to prevent the adhesion of sea organisms ─ could explain in part, the higher levels of 4,4′-DCBP detected in Hong Kong. Air samples analysed by Wang et al. (2007), showed higher levels of DDT in Hok Sui (corresponding to our HK3) and Tap Mui (corresponding to our HK5), when compared to other areas of Hong Kong. Other studies done in this region also reported higher DDTs levels in Hong Kong (0.80–5.60 ng/L) when

compared to Macao water samples (0.48–2.8 ng/L)(Luo et al., 2004; Wurl et al., 2006).

Dicofol, other potential source of DCBP since late 1950s, has been mainly used as a pesticide in southern China, including provinces such as Guangdong, Guangxi and Fujian (World Health Organization, 1996; Qiu et al., 2005). Due to its biomagnification potential in terrestrial environments (DT_{50} 30–60 days) and its susceptibility for hydrolysis with increasing pH (DT_{50} pH 5 = 47–85 days; DT_{50} pH 7 = 8–64 h; Reregistration Eligibility Decision (RED) Dicofol, 1998; OSPAR Commission, 2002), degradation of dicofol to 4,4′-DCBP can occur during its transport from land to coastal areas. While its usage is not allowed in Europe, and although with a decline from 27% (in 1999) to less than 8% after 2008 (Yang et al., 2008; Li et al., 2015), dicofol is still being used in China, especially to control mites, like *Tetranychus cinnabarinus*, *Tetranychus viennensi* and *Phyllocoptruta oleivora* on cotton, citrus and apple trees (United Nations Environmetal Programme, 2016). This could explain the higher concentrations of dicofol reported in surface waters (64.66 ng/L) from the Jiulong River (China; Zheng et al., 2016), when compared to rivers from Greece (< 0.1 ng/L) and United States (2.5 ng/L) (OSPAR Commission, 2002). As reported by the Stockholm Convention on Persistent Organic Pollutants, the current use of dicofol in Asia is below 1000 t/y (United Nations Environmetal Programme, 2016) and Hong Kong is one of the locations where dicofol can still be used, as it is indicated by the Agriculture, Fisheries and Conservation Department of Hong Kong Government (The Government of the Hong Kong Special Administrative Region, 2006). According to the European 2013/39/EU Directive, dicofol has a limit of 0.32 ng/L for surface waters (European Union, 2013). It is expected that 4,4′-DCBP, as the main and the most persistent metabolite of dicofol, will be present in

the environment. All samples above LOQ presented higher 4,4′-DCBP levels than the established limit for dicofol (precursor).

Average DIN concentrations of 1.58 mg/L and 3.89 mg/L were obtained, for Macao and Hong Kong, respectively, presenting higher DIN values than the ones reported by Zhang et al., (2014, 2017) (i.e. 0.42–1.36 mg/L and 0.07–0.14 mg/L) for PRD water samples collected during 2005–2007. The DIN levels detected were also higher than the values reported in the Marine Water Quality report for Hong Kong (Marine Water Quality in Hong Kong, 2016). However, similar values to the ones observed in our study were quantified by Chen et al. (2012) for water samples from Yuqiao reservoir (north of China; 1.21–5.22 mg/L), between 1989 and 2007. The DIN levels obtained for Hong Kong and Macao were considerably higher than the ones reported by the European Environmental Agency (2012) for Mediterranean and Baltic seas, and thus could become a potential threat for the trophic state of the PRD. A similar pattern, regarding the significant difference observed between seasons in Hong Kong samples (wet = 2.21 mg/L; transition = 5.52 mg/L), was also observed by Zhang et al. (2014) in surface waters from PRD. The decrease in DIN levels generally in spring and summer (corresponding to wet season) was probably attributed to the decreasing trend of Pearl River runoff downstream (Zhang et al., 2014).

DIP average levels found in Macao water samples, presented values above the limit established by the Environmental Protection Agency (U.S. Environmental Protection Agency, 1988; 0.1 mg/L for surface waters). In addition, during wet season and as a consequence of an increase in the precipitations rate, a worst scenario regarding DIP levels was observed. Overall, the average DIP levels obtained in this study for both sampling campaigns (0.14 mg/L for Macao and 0.09 mg/L for Hong Kong) were higher than the maximum levels (0.04mg/L) reported by Li et al. (2017) in PRD water

samples (in 2015–2016) and higher than the levels reported in the Hong Kong water quality report (Marine Water Quality in Hong Kong, 2016).

Almroth and Skogen (2010), classified the southeastern part of the North Sea (DIN = 0.19 mg/L; DIP = 0.02 mg/L), Kattegat (DIN = 0.08 mg/L; DIP = 0.05 mg/L), Gulf of Riga (DIN = 0.14 mg/L; DIP = 0.09 mg/L), and Golf of Finland (DIN = 0.05 mg/L; DIP = 0.05 mg/L) as problematic areas regarding eutrophication; Cardoso et al. (2010) reported levels of DIN < 1 mg/L and DIP < 0.12 mg/L in the Mondego River (Portugal), during eutrophication period. The levels obtained in our study for Macao (DIN = 1.6 mg/L; DIP = 0.14 mg/L), and Hong Kong (DIN = 3.87 mg/L; DIP = 0.09 mg/L), are similar or higher than those ones, suggesting some signs of eutrophication in these areas too. Regarding the N/P ratio, higher levels were found in Hong Kong waters during the transition period, mainly in HK2-HT (1106.5), HK3- LT (662.9), followed then by HK2-LT (428.7). The N/P ratio in a water body indicates which element will be the limiting factor, and consequently which one has to be controlled in order to reduce a possible increase in algae population (algal bloom) (Eutrophication and Health, 2002). The majority of our sampling locations (77.5%) presented a N/P ratio greater than the N-limitation boundary (16N:1P; Redfield, 1934), suggesting that DIP is the limiting factor for Macao and Hong Kong coastal areas. These results are in agreement with those from the Mondego estuary (Portugal) during the eutrophication period (Cardoso et al., 2010), which reinforces the idea about the eutrophic state of HK and Macao coastal waters.

Overall, the heatmap analysis (Figure 10), showed distinct patterns between Hong Kong and Macao surface waters. Higher levels of DIN and TDS were quantified in Hong Kong, while DIP and Chl-a showed higher values in Macao waters.

In addition, significant correlations ($p < 0.05$; Figure 11) were found between the quantified 4,4′-DCBP levels during the transition season and physicochemical parameters, like TDS, DIP and Chl-a (Figure 11). A positive correlation was observed between 4,4′-DCBP vs. TDS levels ($R = 0.704$, $p < 0.05$), which can be explained by the high hydrophobicity (octanol-water partition, log K_{ow} = 4.01; Han et al., 2011) of 4,4′-DCBP. Considering the theoretical log K_{ow} =4.44, calculated through ECOSAR version 1.11 (EPISuite Kowwin v1.68 Estimate), it is expected that a substantial fraction of this metabolite will be adsorbed to suspended sediment rather than the water column (Reregistration Eligibility Decision (RED) Dicofol, 1998). In our study, higher levels were detected in waters with higher TDS amounts, which may have an implication in the flora and fauna of the area.

On the other hand, significant negative correlations were observed for 4,4′-DCBP vs. DIP and 4,4′-DCBP vs. Chl-a ($p < 0.05$; $R = -0.514$ and $R = -0.657$, respectively). These results may be due the lower precipitation levels (≈ 137.9 mm) observed during the transition season (the only data used for these correlations), which can influence DIP and Chl-a levels, leading to negative correlations in both cases.

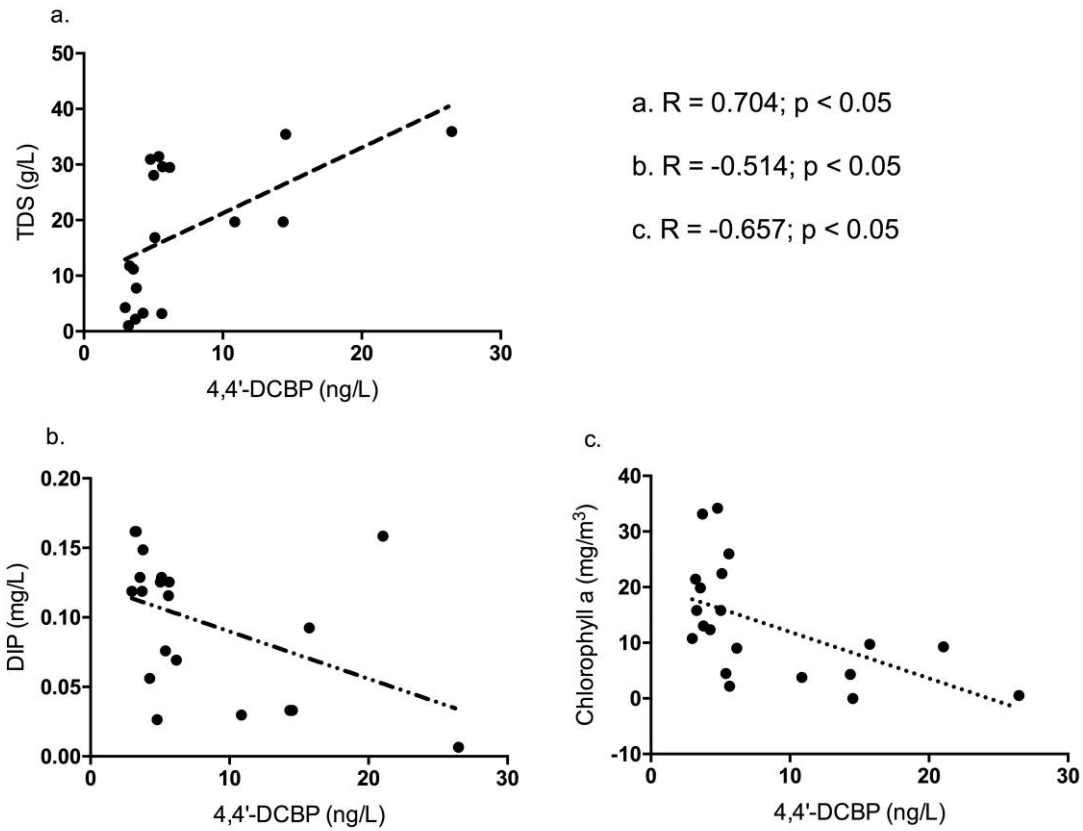

Figure 11. Spearman correlations of 4,4'-DCBP levels during transition season vs. (a) TSS, (b) DIP and (c) Chl-a.

However, for the first correlation (4,4′-DCBP vs. DIP), other external factors may had contributed to the DIP levels found, like different sources of phosphates (i.e. detergents, fertilizers, and organophosphate pesticides), since this metabolite lacks a phosphate group. Considering the second correlation, our results also showed that locations with higher 4,4′-DCBP levels presented lower Chl-a levels, indicating a possible negative effect on algal population or biomass in the area. In vitro studies, using the organochlorine endosulfan (at ranges of 0.001–0.05 mg/L), showed the negative effect of the pesticide on microalgae population growth, through the reduction of chlorophyll content (Ebenezer and Ki, 2014; Sinha et al., 2015). Further in vitro studies should be conducted with 4,4′-DCBP to provide better insights on its effect on other important aquatic organisms, like microalgae.

3.4.3 Toxicity assays

For both animal models, the reference test ($K_2Cr_2O_7$) results were in accordance with the ARC-test range (28–64 mg/L; Vanhaecke and Persoone, 1984) and with ISO 6341 range (0.6–2.1 mg/L, after 24 h; Persoone et al., 2009), defined for *A. salina* and *D. magna*, respectively, indicating a normal resistance to this compound, thus allowing to compare these data to other published assays.

For *A. salina*, average 24 h-EC_{50} of 0.27 mg/L was successfully obtained, considering results from both swimming behaviour parameters. However, it was not possible to compute a mortality dose response because of the maximum solubility limit of the compound (2.5 mg/L in MeOH).

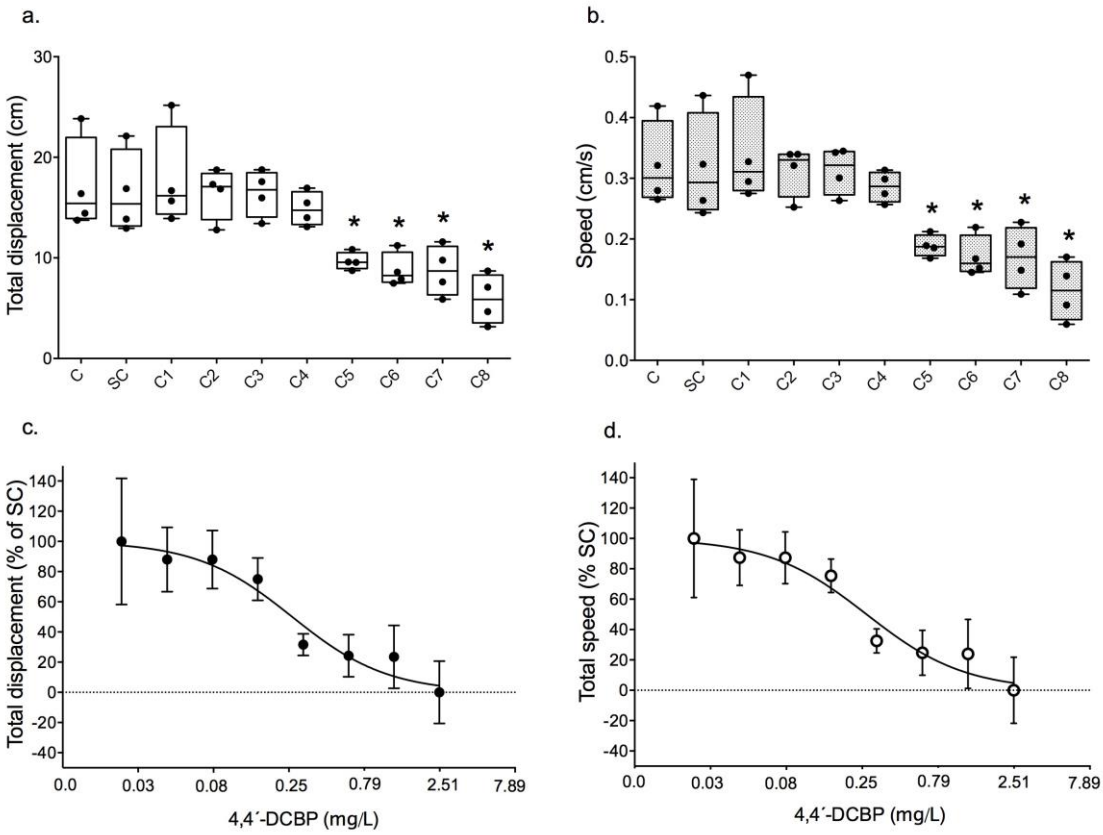

Figure 12. Total displacement and speed of *A. salina* (n=4 plates) after 24 h exposure to different 4,4'-DCBP concentrations: C=control, SC=solvent control, C1=0.019 mg/L, C2=0.039 mg/L, C3=0.078 mg/L, C4=0.156 mg/L, C5=0.315 mg/L, C6=0.625 mg/L, C7=1.25 mg/L and C8=2.5 mg/L. The figures a) and b) represent box and whisker plots of the total displacement (cm) and speed (cm/s), respectively. The horizontal line within the box indicates the median, boundaries of the box indicate the

25th- and 75th -percentile, and the whiskers indicate the highest and lowest values of the results. Upper asterisk indicates significant differences among the treatments and the SC. The figures c) and d) represent a dose-response experiment considering displacement (%) and d) speed (%), respectively. The values at each concentration were calculated as follows: %= (4,4'-DCBP/Solvent Control)*100, and vertical bars represent ± (SD).

For *D. magna*, we were able to compute both 48 h-LC_{50} and 48 h-EC_{50} values, where the average 48 h-LC_{50} value (0.26 mg/L; Figure 14) was higher than the average 48 h-EC_{50} value (0.17 mg/L). However, and as shown by the extensive databases on acute effects of chemicals on Daphnids and in our own results, LC_{50s} and EC_{50s}, do not differ markedly and this probably explains why the Commission of the European Communities in the section on acute toxicity testing for Daphnia, in Directive 92/69/EEC, specifies that "the Directive requirement for the LC_{50} for Daphnia is considered to be fulfilled by the determination of the EC_{50} as described in this method" (EEC, 1992).

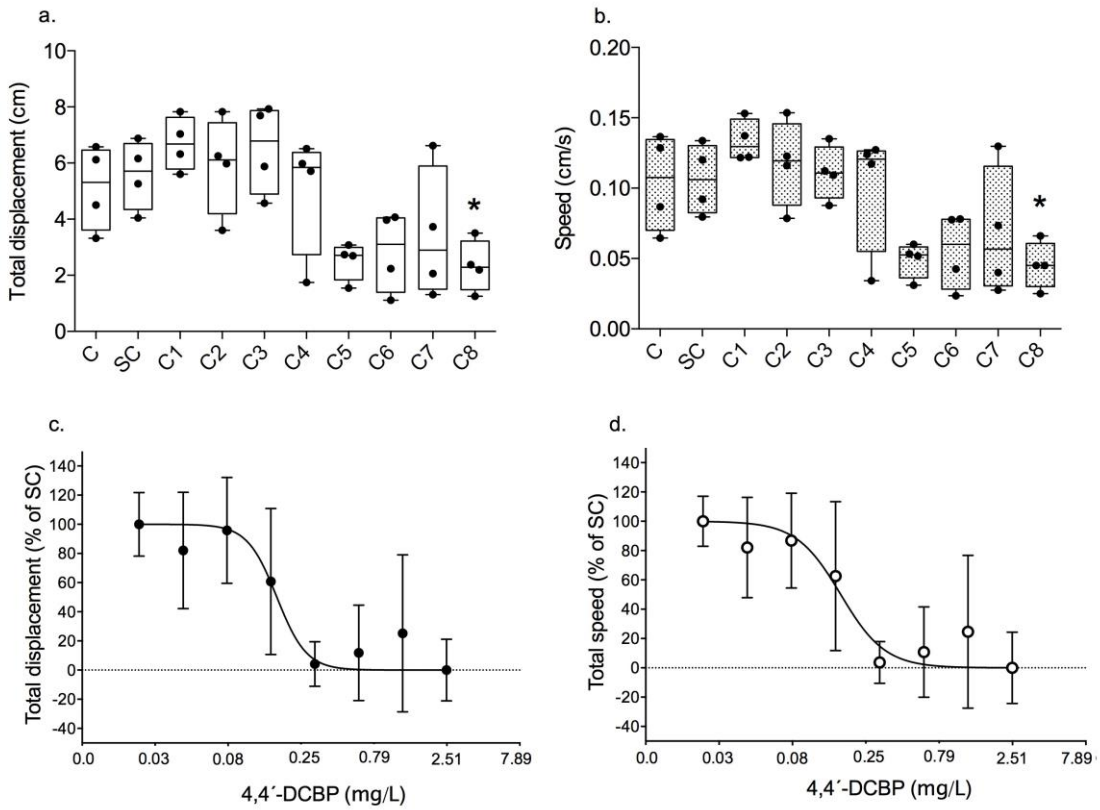

Figure 13. *D. magna* (n=4 plates) total displacement and speed after 48h exposure to different 4,4'-DCBP concentrations; C=control, SC=solvent control, C1=0.019 mg/L,

C2=0.039 mg/L, C3=0.078 mg/L, C4=0.156 mg/L, C5=0.315 mg/L, C6=0.625 mg/L, C7=1.25 mg/L and C8=2.5 mg/L. The figures a) and b) represent box and whisker plots of the total displacement (cm) and speed (cm/s), respectively. The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentile, and the whiskers indicate the highest and lowest values of the results. Upper asterisk indicates significant differences among the treatments and the SC. The figures c) and d) represent a dose-response experiment considering displacement (%) and d) speed (%), respectively. The values at each concentration were calculated as follows: %= (4,4'-DCBP/Solvent Control)*100, and vertical bars represent ± (SD).

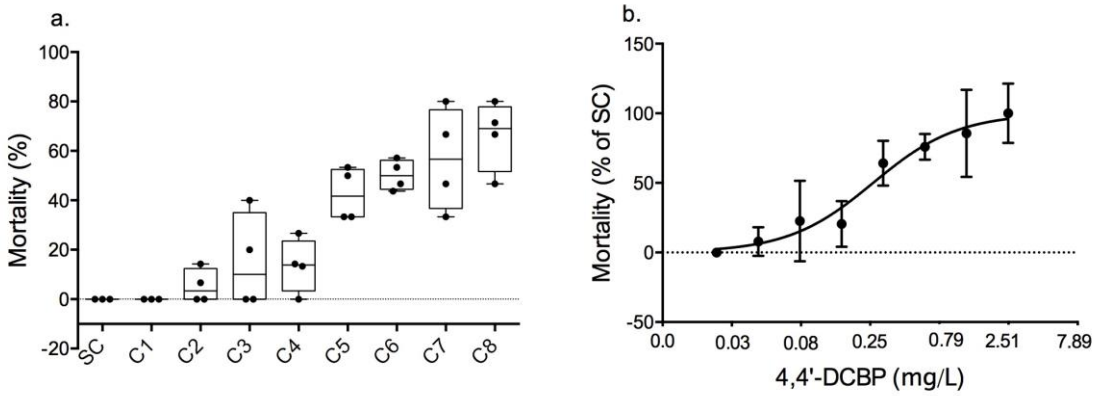

Figure 14. *D. magna* (n=4 plates) mortality rates (%) after 48h exposure to different 4,4'-DCBP concentrations; C=control, SC=solvent control, C1=0.019 mg/L, C2=0.039 mg/L, C3=0.078 mg/L, C4=0.156 mg/L, C5=0.315 mg/L, C6=0.625 mg/L, C7=1.25 mg/L and C8=2.5 mg/L. The figures a) represents box and whisker plots of the mortality rate (%). The horizontal line within the box indicates the median, boundaries of the box indicate the 25th- and 75th -percentile, and the whiskers indicate the highest and lowest values of the results. The figure b) represents a dose-response experiment considering the mortality. The values at each concentration were calculated as follows: % = 4,4'-DCBP/Solvent Control)*100, and vertical bars represent ± (SD).

D. magna 48 h-EC_{50} of DDT and dicofol, were fixed as 0.005 mg/L and 0.14 mg/L, respectively (FOOTPRINT PPDB), indicating a higher toxicity of the original forms in comparison with this metabolite. Considering all locations and both toxicity levels obtained for both animal models, a RQ < 0.1 was calculated, indicating that the quantified 4,4′-DCBP values in the surface waters of PRD mouth, represent a potential low environmental risk. However, there are still several reasons to consider these levels a concern to the environment. As it was mentioned before, 4,4′-DCBP has a high log K_{OW}, which may lead to bioaccumulation and biomagnification processes

contaminating all the edible fauna, which directly or indirectly can constitute a risk to human health. Moreover, concentrations of this metabolite were already registered in bird eggs, showing a capacity of direct transmission to off-spring (United Nations Environmetal Programme, 2016). In vitro studies also showed the antagonistic effect of 4,4′-DCBP towards the androgen receptor (with concentration range of 2.5 mg/L-25 g/L), leading to possible endocrine disrupting effects in wild life population (Thiel et al., 2011). All these facts alert us to potential risks that may affect the ecosystem and future generations.

3.5 Final remarks

With the successfully validated method, the metabolite 4,4′-DCBP was detected in surface waters from Macao and Hong Kong coastal areas, mainly during the transition period, demonstrating a seasonal pattern; also DIN and TDS levels were higher during this period in comparison with the wet season. During the wet season, and due to a higher river discharge, 4,4′-DCBP levels were below the LOQ, and an increase in DIP, TSS and Chl-a values was also observed. This demonstrates the importance of temporal samplings to characterize the current status of an ecosystem. Overall, levels of DIN and DIP were higher than in other water systems (as the Baltic sea), which reveals signs of lower water quality in this region.

Due to the lack of information regarding 4,4′-DCBP, toxicological tests (using two well-established models) were done in parallel with the monitoring campaign, obtaining EC_{50s} values for both species; this data is important to further apply theoretical approaches and evaluate the possible impact of the concentrations found in surface waters. In this case, a RQ < 0.1 was obtained indicating a low potential risk.

As final remarks, this work highlights the importance of studying metabolites and the need of analyse different matrices and trophic levels (since metabolites are very stable

in the organic matter content), assess the toxicological effect in additional biological models (like algae and cell line cultures), and evaluate theoretically the potential effects of this metabolite in the ecosystem and in human health risk through the ingestion of contaminated edible species (bioaccumulation and bio- magnification effect through trophic levels).

Acknowledgments

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Chapter 4. Uptake and depuration kinetics of dicofol metabolite 4,4'-dichlorobenzophenone, in the edible Asiatic clam *Meretrix meretrix*.

Published as:

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Abstract

Uptake and depuration kinetics of 4,4'-dichlorobenzophenone (main metabolite of dicofol) in the edible clam *Meretrix meretrix* were evaluated through a mesocosm experiment. *M. meretrix* was exposed to different dicofol concentrations (environmental concentration, D1−50 ng/L; supra-environmental concentration, D2−500 ng/L) for 15 days, followed by the same depuration period. To accomplish this goal, an analytical method was successfully optimized for 4,4'-DCBP using QuEChERS as extraction method with a range of concentrations 0.3-76.8 ng/g WW quantified by gas chromatography coupled to tandem mass spectrometry. Our results demonstrated different kinetics of accumulation depending on the two dicofol treatments. For D1, the uptake kinetic was best fitted using a plateau followed by one phase association kinetic model, while for D2 a one phase association kinetic model suited better.

Similar bioconcentration factors were obtained for both concentrations but only animals exposed to D2, showed 4,4'-DCBP levels above the limits of quantification after 24 h exposure. These animals also showed lower uptake rate (k_u) than organisms exposed to D1.

During the depuration period, only organisms exposed to D1 successfully depurated after 24 h. On the other hand, although animals exposed to D2 presented higher elimination factor, they did not reach the original levels after depuration. Moreover, values detected in these clams were higher than the Maximum Residue Level (10 ng/g) established by the European legislation. This indicates that longer periods of depuration time than the ones used in this study, may be needed in order to reach safe levels for human consumption.

This work also demonstrated that studies on metabolite kinetics during uptake/depuration experiments, could be a new alternative to understand the impact and metabolism of pesticides in the marine environment.

Keywords:

QuEChERS bioconcentration, Seafood, Bivalves, Organochlorine pesticides, GC-MS/MS

4.1 Introduction

Organochlorine pesticides (OCPs), are a classical example of persistent organic pollutants (POPs) of worldwide concern due to their persistence in the environment, bioaccumulation ability, and potential negative impacts on biota and human health (Guan et al., 2009; Guo et al., 2008). Among OCPs, dicofol is of special interest due to its high biomagnification potential, similarity with dichlorodiphenyltrichlorethane (DDT), and extensive use, predominantly in Southeast Asia (Guo et al., 2008; United Nations Environmental Programme, 2016). It is true that the global production of DDT and dicofol have shown a significant decline since the Stockholm Convention adoption, however these pesticides are still being used, i.e. DTT is used in response to the development of resistance in malaria vectors (mainly in Asia and Africa) (Berg et al., 2017) and dicofol as a pesticide (mainly in Asia). Moreover, these prohibited compounds could also be used in Europe in case of emergency situations that pose a danger to plant production and ecosystems (SANCO, 2013). Therefore, despite the pesticides ban from different countries, dicofol could be a global problem affecting not only China, but also other countries where this compound has been or is still being used in special situations. Dicofol is an organochlorine acaricide that has been used in agriculture since the late 1950s to protect mainly citrus and cotton cultivations from mites (Thiel et al., 2011; WHO/FAO, 1996). In a previous work, dicofol was quantified as the most frequent OCP in water and sediment samples collected in 2009 from Jiulang River (North East China) (Zheng et al., 2016). It is also identified as a potential “endocrine disrupting compound” due to its animal toxicity, cancerogenic and negative estrogenic effects (Liu et al., 2004; Reynolds et al., 2005; Thiel et al., 2011). The technical product is usually synthesized from DDT via chlorination and

subsequent hydrolysis and consists of approximately 80% and 20% of 4,4'- and 2,4'-dicofol isomers, respectively (Qiu et al., 2005).

Owing to the instability and easy degradation of dicofol in water −when exposed to higher pH (85 days, 64-99 h or 26 min of half- life at pH 5, pH 7 and pH 9, respectively), light (sensitive to sun light) and higher temperature (3.3 days of aqueous half-life photolysis at 20 °C and pH 7)− 4,4'-dichlorobenzophenone (DCBP) is the main metabolite and probably the most available form in surface waters (Fujii et al., 2011; Thiel et al., 2011; Yin et al., 2017; FOOTPRINT PPDB). In fact, 4,4'-DCBP, was quantified in surface waters (2.79-29.87 ng/L) from the mouth of the Pearl River Delta in a previous study (Ivorra et al., 2019a).

Some metabolites are often more persistent than their corresponding parent compounds and exhibit similar or even greater toxicity, e.g. the major biodegradation product of nonylphenol ethoxylates (nonylphenol) or endosulfan I/II (endosulfan sulfate) (Jahan et al., 2007; Stanley et al., 2009). In some cases, metabolites were quantified in aquatic environments in even higher levels than those of the parent compounds (Farre et al., 2008). Therefore, it is crucial to study the effect of metabolites in aquatic organisms.

Bivalves, as filter-feeding organisms, have been widely used to monitor pollutants in aquatic ecosystems due to their wide geographical distribution, sessile lifestyle, resistance to stress and high and rapid accumulation of toxic substances (Goldberg et al., 1978; Suarez et al., 2013; Walker and Livingstone, 1992), and also because of their economic interest and their implications in the food chain (Cardoso et al., 2013; Metian et al., 2008). For this study, we selected a common bivalve, *Meretrix meretrix* −known as Asiatic hard clam− which is widely consumed around the world and widespread in the Indo-West Pacific region (Poutiers, 1998).

Considering the chemical instability of dicofol, we assume that 4,4'-DCBP is possibly the most persistent form in the aquatic environment. Therefore, and regarding the lack of information about metabolites, the main goal of this work is to study the pattern of bioaccumulation and elimination kinetics of the metabolite 4,4'-DCBP in clams exposed to environmental and supra-environmental concentration (10x more) of dicofol. Thus, this work investigated if edible bivalves have the ability to accumulate and depurate 4,4'- DCBP, if the kinetics of these organisms will be different between both dicofol concentrations and if depurated clams will reach acceptable levels for human consumption.

4.2 Materials and methods

4.2.1 Sample description

Bivalves, originally collected in Guangzhou province, were acquired from a local market and transported immediately to the lab. During acclimation period (approximately 4 days), animals (ca. 700) were distributed in two containers (15 L each) and kept under oxic conditions ensured by air-bubbling the water. Temperature and salinity were gradually adjusted (1 °C/day and 2 ppt/day, respectively) until a final temperature of 27°C and salinity of 16-18 ppt. Animals were fed daily with 600 mL (1:10 dilution) of a commercial mixture of spirulina and kelp (Kent Marine Microvert) under a photoperiod regime of 12:12 light/dark cycle.

4.2.2 Experimental set up

The experimental set-up included a total of 60 sub-experiments (3 replicates*4 treatments*5 sampling times) corresponding to 60 different glass aquaria. The conditions for each treatment were: 1) control (C) only with seawater, 2) solvent

control (SC, methanol 0.1%), 3) dicofol at environmental concentration (D1, 50 ng/L), and 4) a supra-environmental dicofol concentration (D2, 500 ng/L).

The experiment ran for 30 days and was divided into the exposure phase (15 days exposed to dicofol) and the decontamination phase (15 days free of dicofol). During each phase, five sampling times were established: day 1, 2, 3, 7 and 15. Figure 15 shows the schematic representation of the experimental design.

After the initial acclimation period, 10 clams were distributed per aquarium, 24 h before the beginning of the experiment (to ensure the stability of the system). All aquaria were placed randomly into water baths (8 aquaria/water bath) with heater and aeration to assure a stable and homogeneous temperature. Each glass aquarium, containing 1 kg of pre-washed commercial sand (Xin Jing aquarium gravels) and 2.5 L of artificial seawater (ASW), was maintained at the same oxic conditions as described above.

Owing to the instability and easy degradation of dicofol mentioned above, the medium was renewed daily. For this purpose, a peristaltic water pump (BT100 M, Generic) was used to remove and replace the water, completely. Moreover, to ensure a homogenous concentration in the spiked aquaria, an aliquot of the water from the aquarium (250 mL approx.) was taken, spiked and mixed previously. 4,4'-DCBP quantification in water was performed regularly (right after spiking and 24 h later) to control the concentration levels throughout the experiment. The same food proportion was kept as in the acclimation period. All aquaria were individually covered with a glass to avoid cross-contaminations. Moreover, at the pre-defined sampling times, three organisms were removed and placed in constantly aerated clean seawater for 24 h depuration (to remove pseudo-fecal and fecal material from the digestive tract) (Coelho et al., 2006; Metian et al., 2008). After this period, clams were measured,

weighed (with and without shell), cut opened and the soft tissue frozen (-80°C) for later 4,4'-DCBP quantification. Condition index (CI) was also calculated according to CI−(fresh weight/shell weight) x 100, as complementary information about the health status of the organisms (Hyötyläinen et al., 2002). Survival rate (%) of the organisms was also controlled during the whole experiment.

Physical parameters were measured daily for temperature and weekly for pH and dissolved oxygen (DO). The water temperature in the aquaria was 26.92 ± 0.17°C, and pH and DO were 8.45 ± 0.14 and 106 ± 4.01%, respectively.

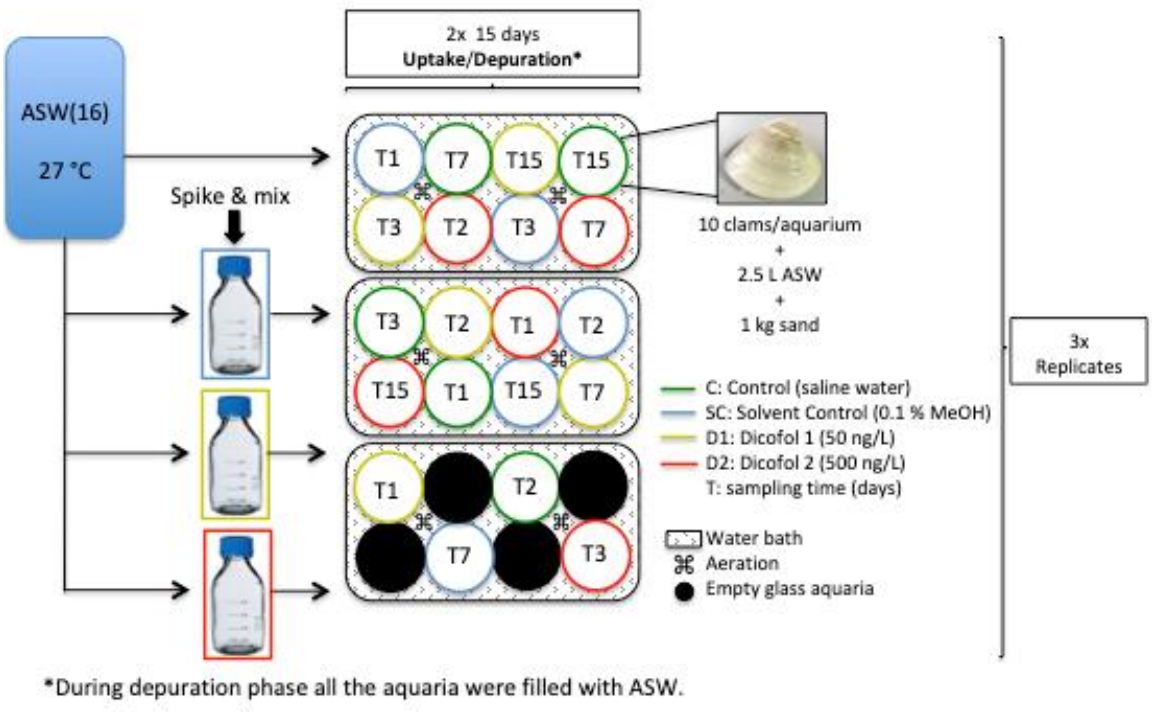

Figure 15. A schematic representation of the experiment set-up with 4 different treatments (C/SC/D1/D2) at 5 different sampling times (T1/T2/T3/T7/T15), randomly distributed in 8 water baths.

4.2.3 4,4'-DCBP quantification by GC-MS/MS

4.2.3.1 Reagents

LC/GC grade solvents such as, methanol (CH_3OH), acetonitrile (CH_3CN), ethyl acetate ($C_4H_8O_2$), and dichloromethane (CH_2Cl_2)

were purchased from Merck Limited Company (Germany). Ultra-pure water was obtained from a Milli-Q water system (resistance 1⁄4 5.1 mU/cm at 25°C).

Anhydrous magnesium sulfate ($MgSO_4$), sodium acetate ($C_2H_3NaO_2$), and Supelclean PSA SPE Bulk Packing (polymerically bonded, ethylenediamine-N-propyl phase that contains both primary and secondary amines), were acquired from Sigma-Aldrich (Steinheim, Germany); $MgSO_4$ was preheated (5 h at 500°C) to eliminate residual water and phthalates.

Dicofol-d_8 (used as surrogate and internal standard (IS)), dicofol and 4,4'-DCBP with purity > 98%, were purchased from Sigma-Aldrich (Steinheim, Germany). All compounds were individually prepared in CH_3OH with 0.1% acetic acid (CH_3COOH; Sigma-Aldrich, USA) to produce the final stock solution of 1000 mg/L and kept in the dark at -20°C. D-sorbitol and 3-ethoxy-1,2-propanediol (used as protectants) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solution of 182 mg/mL in 70% CH_3OH:H_20 and 800 000 mg/L in 100% CH_3OH were prepared for D-sorbitol and 3- ethoxy-1,2-propanediol, respectively. Protectants were used as 0.1:1 mg/mL (D-sorbitol:3-ethoxy-1,2-propanediol). Stock solutions of 3-ethoxy-1,2-propanediol were kept at 4°C, and D-sorbitol and the protectant mixture were stored in the same conditions as the surrogate and standard. For quantification purposes, an aliquot of each sample (195 mL) was taken and mixed with a protectants' solution (5 mL) at a final concentration of 0.0025:0.025 g/mL, respectively.

4.2.3.2 Bivalve and water samples preparation

Biological samples: 4,4'-DCBP extraction was performed using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) technique as was previously described by Cruzeiro et al. (2016). First, the frozen bivalve tissue was thawed, chopped, and then ground with a high-speed disintegrator model number FW80

(Faithful). A homogenate sample of 5 g was transferred into a 50 mL Teflon centrifuge tube (Nalgene Oak Ridge High-Speed, Thermo-Fisher, NY, USA), and spiked with 50 mL of the surrogate (0.5 mg/L) and/or calibration curve concentrations. The fortified sample was settled for 5 min and vortexed, then 5 mL of CH_3CN was added and vortexed again. The rest of the extraction was done by adding subsequently a combination of different salts followed by vortex and centrifugation (4°C, 4024 rcf, 5min) between steps; 1) 2 g $MgSO_4$ and 500 mg $C_2H_3NaO_2$; 2) collect upper layer (2.5 mL) and add 125 mg PSA and 375 mg $MgSO_4$; 3) collect the final extract (2 mL).

Water samples: 2 replicates of 500mL from each treatment group were collected in amber flasks, just after the dicofol addition (T0) and before the water renewal (T24). Samples were filtered (0.45 mm glass fibre filter; Sartorious, Germany) and acidified to pH 5 with CH_3COOH for higher sample stability.

The compound was extracted by solid phase extraction (SPE) using the OASIS HLB cartridges (200 mg, 6 cc; Waters, Ireland) following Ivorra et al. (2019a) protocol. Briefly, fortified water samples were loaded into pre-conditioned cartridges (5 mL CH_3OH followed by 5mL ultrapure water), allowed to dry, and eluted (2.5 mL $C_4H_8O_2$ followed by 2.5 mL of CH_2Cl_2 and 2.5 mL more of a 1:1 mix of CH_2Cl_2 and $C_4H_8O_2$ (v/v)). The extracts were evaporated to dryness, under N_2 stream (99.995%) and then reconstituted into 200 mL of CH_3OH.

4.2.3.3 Method validation and quality assurance

The validation procedure followed the European guidance document on pesticide residue analytical methods SANTE/11813/ 2017 rev 0 (SANTE, 2017). Linearity was evaluated using three independent calibration curves, each with seven nominal standard concentration of 4,4'-DCBP, (ranging from 0.06 to 3.84 mg/L) spiked (50

mL) into 5 g of homogenate organism matrix with the surrogate (0.5 mg/L). Curves were plotted using the ratio between the standard (4,4'-DCBP) and the IS area (dicofol-d_8). The limits of detection (LOD) and quantification (LOQ) were determined with the same curves, using the following formulas: LOD = 3.3 a/S and LOQ = 10 a/S, where a is the standard deviation of the response and S is the average slope of the calibration curves.

Recoveries, accuracy and precision were evaluated by analysing three independent replicates of each quality control samples (QCs) at two levels of concentration (low and medium) calculated as, QC_{low} = LOQ (4.01 mg/L) and QC_{medium} = 4LOQ (16.04 mg/L). Recoveries were determined by comparing the area ratio in spiked matrix with the area ratio of the same concentration in a matrix blank spiked after extraction. Precision was expressed as the relative standard deviation (%RSD) of the replicate measurements, and the accuracy was evaluated as the percentage of agreement between the methods results and the nominal amount of added compound.

As part of the validation, the matrix effect (ME) was also evaluated at both concentrations (LOQ), where matrix samples were spiked after extraction ($A_{standard}$ in matrix) and compared with those of injected standards ($A_{standards}$), as indicated in the following equation:

$$ME = \frac{\text{Astandard} - \text{Astandard in matrix}}{\text{Astandard}} x\ 100$$

The ions selection and the collision energies for quantification purposes were obtained from the auto selected reaction monitoring. Information from published methods, regarding the target ions were also taken into consideration (de Kok et al., 2005; EU Reference Laboratories for Residues of Pesticides, 2013; Pereira et al., 2014). The software Xcalibur (version 4.0.27.10, Thermo Scientific), together with

the NIST library, were used for ion products confirmation and quantification (Table D1, Appendix D).

For the water samples, the validation procedure followed the European guidance document on pesticide residue analytical methods SANCO/825/00 rev 8.1 (SANCO, 2010). In this matrix, the range of concentrations used were 3-400 ng/L, and three different QCs were included during validation (QC_{low} = 2LOQ (1.6 ng/L), QC_{medium} = 20LOQ (16.48 ng/L) and QC_{high} = 100LOQ (824 ng/L). More details can be found in Ivorra et al. (2019a).

4.2.3.4 Instrumental methods

Analyses were carried out using a gas chromatograph (Trace 1310 GC, Thermo Scientific), coupled with a triple quadrupole mass spectrometer detector (TSQ 8000 EVO, Thermo Scientific), an autosampler (Thermo ScientificTriPlusTM) and a Trace Pesticides column (TR-pesticides II, 30 m x 0.25 mm x 0.25 μmm 5 m Guard).

For the animal samples, column oven temperatures were programmed for a 35 min period using several ramps: a) from 80°C with an initial equilibrium time of 2 min to b) 180°C at 20 °C/min until c) 290°C at 5 °C/min, where the temperature was maintained for 7 min. The injector port temperature was set to 200°C, and both ion source and MS transfer line were at 290°C.

For the water samples, column oven temperatures were programmed for a 14 min period instead using several ramps: a) from 75 °C with an initial equilibrium time of 3 min to b) 180°C at 30 °C/ min until c) 280°C at 5 °C/min, where the temperature was maintained for 1 min. The injector port temperature was set to 250°C, and both ion source and MS transfer line were at 280°C.

In both analyses, helium (99.999% purity) was used as carrier gas and was maintained at a constant flow rate of 1.2 mL/min. Sample injection (2 and 1.5 mL for animal and

water samples, respectively) was in the split-less mode (4 mm straight liner, 453A1925), using a 50 mm long needle. New liners were used every 200 injections.

4.2.3 Data analyses

Uptake and depuration kinetics of the soft tissues were expressed in terms of change of 4,4'-DCBP concentration over time. The data obtained for 4,4'-DCBP uptake or depuration per unit of time was modelled by nonlinear regression analysis, using Graph- Pad Prism version 6.00, that uses the least-squares fitting method: the plateau followed by one phase association (eq. (1)) and the one-phase association kinetic model (eq. (2)) were applied for the uptake data for D1 and D2, respectively.

$C_t = IF(t<t_0, C_0, C_0+(C_{ss}-C_0)*(1-\exp(-K_u*(t-t_0))))$ (1)

$C_t = C_0+(C_{ss}-C_0)*(1-\exp(-k_u t))$ (2)

$C_t = (C_0-C_{ss})*\exp(-k_e t)+C_{ss}$ (3)

In addition, the one-phase exponential decay model (eq. (3)) was used to fit data from 4,4'-DCBP depuration for D2 concentration, where C_t and C_{ss} are the concentrations at time t (d) and at steady-state, respectively; k_u is the uptake rate constant (d^{-1}) and k_e is the depuration rate constant (d^{-1}); C_0 is the concentration at time 0 (He_douin et al., 2011).

In order to assess the experimental data goodness of the fit, the coefficient of determination (R^2) and the standard deviation of residues ($S_{y.x}$) were determined. A relatively high R^2 and low value of $S_{x/y}$ were used as criteria for good fit. For each case, the fitting was tested using the mean 4,4'-DCBP concentration at each studied time.

In addition, a biological half-life (the time it takes to reach half of the equilibrium value) was calculated ($T_{b1/2}$) from the corresponding uptake (k_u) and depuration (k_e) rate constants, according to the relation $T_{b1/2} = \ln 2/k_u$ and $T_{b1/2} = \ln 2/k_e$, respectively. Bioconcentration factors (BCFs) were generally calculated as the ratio of internal biota concentration (ng/kg) to the water exposure concentration (ng/L). The elimination of 4,4'-DCBP was expressed in percentage of lost 4,4'-DCBP concentration. Elimination factor was described by equation $EF = 100 - [(C_e/C_t) \times 100]$, where EF is the percentage of lost 4,4'-DCBP concentration, C_e is the 4,4'-DCBP concentration in the bivalve tissue after depuration period, C_t is the 4,4'-DCBP concentration in the tissue of transplanted bivalves after 15 days exposure.

To infer differences between treatments and sampling times, all data were initially checked for normality (Kolmorgorov-Smirnov test) and homogeneity of variances (Levene's test). In order to determine differences between treatments and sampling times a 2-way ANOVA was applied. Transformations of the data were needed to fit the assumptions for the analysis. The Tukey post-hoc test was applied, to assess differences in sampling times for each treatment; and Dunnett's test to assess differences between the solvent control (SC) and the treatments (D1 and D2). Finally, for each treatment, comparisons between uptake and depuration phase were done using Wilcoxon matched-pairs signed rank test. All statistical analyses were done using Graph Pad Prism version 6.00.

4.3 Results

4.3.1 Bivalves QuEChERS validation

LOD and LOQ were quantified with a final value of 1.33 and 4.02 mg/L, respectively. All validation criteria were successfully established, with final average percentages of

108.95%, 93.17% and 3.98%, for LOQ, and 108.75%, 90.15% and 4.11%, for 4LOQ (Figure D1, Appendix D), respectively for recovery, accuracy and precision.

Regarding the matrix effect results, an enhancement of the signal of 40.07% was observed for LOQ.

4.3.2 4,4'-DCBP uptake and depuration rates

4,4'-DCBP was not detected in control aquaria and control clams, thus indicating the absence of contamination. Survival rate of the organisms presented average values higher than 85% for all the treatments, except for D2, which showed an average value of 82.2% during uptake. Moreover, no differences were observed between control (C) and solvent control (SC) treatments, therefore SC was chosen for graphical representation and data comparison. Concentrations between LOD and LOQ were transformed as LOQ/2, as described by Beal (2001), and estimated as if all the values were real; values < LOD were not included in the analysis.

Generally, the bivalves exhibited an increase on 4,4'-DCBP concentration, in relation to solvent control, through the entire exposure period (Figure 16), however the kinetics of accumulation were different for the two dicofol treatments. For D1, the uptake kinetic was best fitted using a plateau followed by one phase association kinetic model, while for D2 a one phase association kinetic model suited better. In addition, for the lowest concentration (D1), the bivalves showed a faster accumulation than when exposed to the highest concentration (D2), since for the first case, after three days of exposure they reached 89% of the final concentration while for the second case, for the same period of time they just reached 52% of the final concentration.

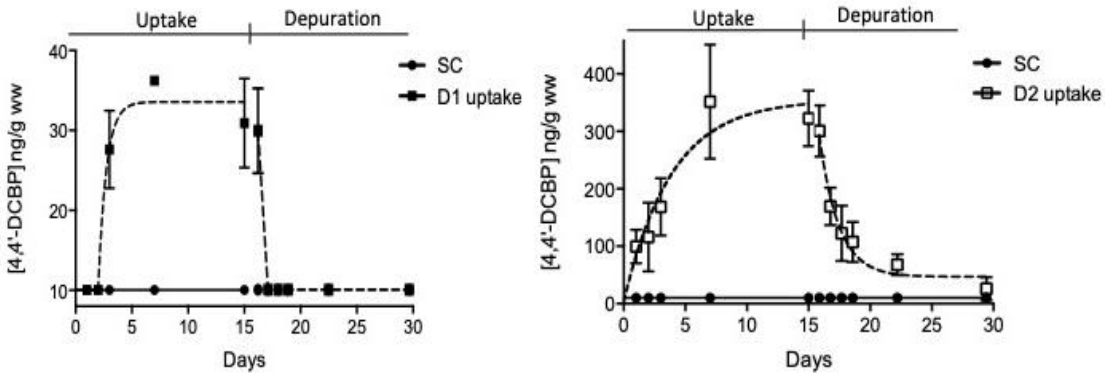

Figure 16. Kinetics of accumulation (days 0-15) and depuration (days 15-30) of 4,4'-DCBP in *M. meretrix* (ng/g ww) exposed to 50 ng/L (D1, left) and 500 ng/L (D2, right). Results are expressed by mean ± standard error (n 1⁄4 3 per sampling time).

This pattern was corroborated by the kinetic parameters that indicated an uptake rate constant (k_u) of 1.35 d^{-1} for D1 and 0.25 d^{-1} for D2 (Table 3). A stabilization of 4,4'-DCBP accumulation was attained after 5−7 days and 12−15 days for D1 and D2, respectively. After 15 days of exposure, final concentrations of 30.93 ng/g WW and 322.53 ng/g WW of 4,4'-DCBP were detected for D1 and D2, respectively. Finally, and considering the kinetic model, the biological half-life ($T_{b1/2}$) was also determined for D2 with a value of 2.79 d during uptake. For D1 it was not possible to estimate the biological half-life.

Table 3. Estimated uptake (upt.) and depuration (dep.) parameters of 4,4-DCBP in the bivalve *M. meretrix* exposed for 15 days to the pesticide and then kept for 15 days in clean water. C_0, concentration at time 0; C_{ss}, concentration at steady state; K_u: uptake rate constant (d^{-1}); K_e: depuration rate constant (d^{-1}); Tb½: biological half-life (d); SE: standard error; R^2: determination coefficient.

		C_0±SE (ng g^{-1})	**C_{ss}±SE (ng g^{-1})**	**K_u±SE (d^{-1})**	**K_e±SE (d^{-1})**	**$T_{b1/2}$**	**R^2**	**Sy.x**
Upt.	D1	10.05	33.57±1.34	1.38±0.4		-	0.98	1.9
	D2	10.05	356.4±41.4	0.25±0.1		2.8	0.94	36.7
Dep.	D1	-	-	-	-	-	-	-
	D2	315.2±21.7	49.60±16.9		0.57± 0.1	1.2	0.97	22.4

After 15 days of exposure, the log BCFs of 4,4'-DCBP were slightly higher in animals exposed to higher concentrations (3.86 for D2) than those exposed to lower concentration (3.79 for D1) (Table 4).

Table 4. Bioconcentration factors (log BCF15 d) and elimination factors (EF15 d) of *M. meretrix* tissues in the two contaminated treatments (D1 and D2) considering 15 d exposure. Results are expressed by mean ± standard error (n = 3 per sampling time).

	Uptake Log BCF	**Depuration EF (%)**
Treatment D1	3.79 ± 0.03	67.14 ± 2.56
Treatment D2	3.86 ± 0.04	92.63 ± 3.95

Significant differences between sampling times (2-way ANOVA, $F_{(5,36)} = 81.87$, $p < 0.0001$) and treatments (2-way ANOVA, $F_{(2,36)} = 724$, $p < 0.0001$) were observed. For D1, SC group was significantly different ($p < 0.0001$) from day 2 to day 15, while no significant differences ($p > 0.05$) were found for D1 between T3, T7 and T15. In the case of D2, significant differences ($p < 0.001$) from SC group were observed after 24 h exposure (T2) and initial accumulation (T1 - T3) showed significant differences from T7 and T15. Moreover, interaction between both factors (sampling time x treatment) was also significant ($F_{(10,36)} = 36.32$, $p < 0.0$).

On the other hand, the depuration kinetics for D2 treatment was best fitted using one-phase exponential decay model, with 43.7% decay after 24 h. D1 treatment did not match any kinetics model due to its rapid decay (67.5%) after 24 h transfer to a clean system. D2 treatment showed an elimination rate constant (k_e) of 0.57 d^{-1}. For D1 it was not possible to estimate this parameter. After 15 days of depuration, 27.16 ng/g WW of 4,4'-DCBP was detected in organisms from D2 treatment, in contrast to D1, which presented values < LOQ. The biological half-life ($T_{b1/2}$) for D2 depuration was

1.20 d, and an EF of 67.14% and 92.63% (Table 4) was calculated for D1 and D2, respectively.

In this case, D2 treatment also presented significant differences between sampling times (2-way ANOVA, $F_{(5,23)} = 11.99$, $p < 0.001$) and treatments (2-way ANOVA, $F_{(1,23)} = 540.75$, $p < 0.001$). D2 treatment was significantly different from SC during the whole depuration except for T15. At this sampling time no significant differences were observed with T0, which corresponds to the initial point of the experiment. Initial sampling times (T1 to T3) showed significant difference from T15; and T1 was also significantly different from T7. Moreover, interaction between both factors (sampling time x treatment) was also significant ($F_{(5,23)} = 11.99$, $p < 0.05$). Finally, results from Wilcoxon test showed that uptake and depuration kinetics for each treatment did not have a significant difference ($p > 0.05$).

Considering the CI, no significant differences ($p < 0.05$) were observed between SC and both dicofol treatments during the whole experiment (Figure D2).

4.4 Discussion

4.4.1 Biological method validation

The validated method accomplished all the criteria (i.e., evaluation of linearity, accuracy, precision and recoveries) established by SANTE/11813/2017 rev 0 (SANTE, 2017), demonstrating to be valid for 4,4'-DCBP extraction and quantification. This was only possible because all calibration curves were done in matrix, avoiding overestimation during the quantification due to higher ME. The target metabolite was quantified at very low range of concentrations (0.3-76.8 ng/g WW) indicating that this method is acceptable to detect the Maximum Residue Level (MRL) of pesticides in food (10 ng/g) established by the European Union (European

Commission, 2019), and therefore it can be used for future studies regarding food safety.

4.4.2 4,4'-DCBP uptake and depuration rates

Awareness of contamination and depuration processes in organisms, such as bivalves is an important issue to understand the possible biomagnification of contaminants through the food web, especially in the case of edible organisms such as the *M. meretrix*. This study focused on the kinetics of the metabolite 4,4'-DCBP rather than the parent compound, dicofol, providing new data in a topic where the information is still scarce. To our knowledge, there are no published data on 4,4'-DCBP kinetics with which it is possible to compare our results. Therefore, most of the comparison will be done using data from similar/related compounds (i.e. organic compounds, organochlorinated pesticides, DDT). Moreover, we also discuss the importance of using a very high concentration (10x D1) to mimic possible spills and understand the possible impact of extreme situations, which may occur in sporadic cases. For example, it has been reported that 119 spills incidents occurred from 1947 to 2011, which contained a total of 187 substances spilled. From these substances, the third largest group involved in marine accidental spills was pesticides, such as lindane or endosulfan (Cunha et al., 2015).

In this study, different kinetic patterns were observed between environmental (D1) and supra-environmental (D2) concentrations. For example, D1 treatment showed a baseline at initial time (plateau) (Figure 16), where accumulation of the compound was not remarkable. After this initial phase, both treatments could be explained by pseudo-first order association kinetics. The initial absence of plateau in D2 may be due to the higher quantified concentrations (4,4'-DCBP was > LOQ), which were significantly different from SC, after 24 h exposure. In both cases, during uptake, the

organisms assimilated a certain fraction of the compound until a steady state was reached. The steady state for D1 was reached faster than for D2, which was expected considering the k_u. Higher contaminant concentrations, like the ones spiked in D2 treatment (500 ng/L), may induce alterations in respiration rates and filtration capability (Bourdelin, F., 1996; Vijayavel, K., et al., 2007). The same behaviour in bivalves has been reported in other studies. For example, Cardoso et al. (2013) observed the same when *Cerastoderma edule* was exposed to different mercury concentrations while Gomez et al. (2012) when *Mytilus galloprovincialis* was exposed to different concentrations of tetrazepam.

Regarding the k_u, Richardson et al. (2005) estimated values of 9.66 x 10^3 and 3.82 x 10^4 in mussels exposed for 20 days to 100 ng/ L of α-HCH and dieldrin, respectively. These values are much higher than the ones obtained in this work (1.38 and 0.35 for D1 and D2, respectively). Several studies have reported that, in a sediment-water system in which the direct source of contaminant is the dissolved phase, the tendency for accumulation of organic contaminants can be correlated with n-octanol/water partition coefficients (K_{ow}) of the compounds (Geyer et at. 1982; Mackay, 1982; Pruell et al., 1986). In such systems, organic compounds are bioaccumulated through passive diffusion across the gills, rather than ingestion. Dicofol, as well as 4,4'-DCBP, tends to bind to particulate matter (WHO/FAO, 1996) rather than the water column, therefore lower amounts will be available in the dissolved phase for passive diffusion. This could explain the difference between 4,4'-DCBP k_u rates and other OCPs like α-HCH and dieldrin, that tend to accumulate in the dissolved phase (Richardson et al., 2005).

The bioconcentration of organic compounds is often associated with the molecule lipophility (high K_{ow}) and the molecule aqueous solubility (low S_w), which are

inversely related (Arnot and Gobas, 2006). For example, Katagi (2009) showed a strong positive correlation between log BCF and log K_{ow} in fish for pesticides developed in the most recent 10-years period. The more hydrophobic a pesticide is, the higher bioconcentration is observed with more distribution in the organs having higher lipid content (Katagi and Ose, 2014). In this study, the average log BCF values obtained for D1 and D2 treatment was 3.83, which is in the same range of values obtained by Richardson et al. (2005) for dieldrin (5.43), aldrin (3.92) and α-HCH (3.76). These values, and considering the low S_w and high K_{ow} (Table D2, Appendix D), indicate that the compound can concentrate more in the organism than in the surrounding water, and therefore biomagnification process may happen affecting the food web (Kanazawa, 1981).

Regarding the depuration period, this study revealed different kinetic patterns between treatments. We observed a fast recovery of the animals exposed to the lower concentration (D1) after 24 h (showing values < LOQ) while organisms exposed to higher concentration (D2) did not fully depurate over a period of 15 days. The same behaviour was observed by Richardson et al. (2005) in mussels depurated for 8 days, after being exposed to 100 ng/L of DDT, although k_e observed by this author was (0.015 d^{-1}) lower than in our study for 4,4'-DCBP (0.58 d^{-1}).

Our organisms also showed an elimination rate (k_e) 2.32x times higher than the uptake rate (k_u). Contrarily to these results, Uno et al. (1997) obtained higher k_u (338 d^{-1}) than k_e (0.054 d^{-1}) in clams exposed to 1700 ng/L of thiobencarb (30mg/L, solubility) during 14 and 15 days, respectively. Studies previously mentioned (Richardson et al., 2005; Gomez et al., 2012) also showed the same trend as Uno et al., (1997). Kinetic studies focused on parent compounds and metabolites behaviour, may follow different patterns. For example, during uptake phase the animal is

continuously exposed to the parent compound and it will start its accumulation in the organism. It is expected to get higher concentrations of the parent compound during this phase than the metabolite. However, during depuration, when no more parent compound is added, it will be expected to get higher metabolite concentrations due to metabolisation or degradation of parent compound in the system. This hypothesis could explain the differences obtained between our results and other previous studies, where the parent compound instead of the metabolite was measured during uptake and depuration. Our study showed the importance of understanding the behaviour of the metabolites, since they can still be very active, and may present different kinetics pattern.

In addition, the efficiency of eliminating contaminants seems to be higher when there is more concentration in the system. The EF between treatments (Table 4) after 15 days of depuration indicated that organisms exposed to D2 were able to eliminate more 4,4'-DCBP than those exposed to D1. Another elimination route could be depuration by passive diffusion into surrounding water. However due to the hydrophobic character of the compound this way is less expected. In an open system, and depending on the affinity of the compound for the feces, the contaminants may then desorb and reenter the water column. In this case, 4,4'-DCBP levels in the water during depuration (data not shown) were similar to the controls (SC + C) ones, which could indicate that 4,4'-DCBP besides being accumulated by the organisms, could be adsorbed to the substrate (i.e. sand), to some remaining fecal material or to the glass of the aquarium, rather than to the water column. More studies may be needed in order to fully understand the metabolism process of 4,4'-DCBP in marine invertebrates.

Furthermore, 4,4'-DCBP levels obtained after 15 days of depuration (26.19 ng/g WW) in animals previously exposed to D2, presented concentrations 2.6-fold higher than the MRLs established by the European legislation (10 ng/g) for any kind of food for Human consumption (European Commission, 2019).

In summary, considering all the information mentioned above, we can highlight that although depuration of 4,4'-DCBP is happening in a more effective way than uptake ($k_e > k_u$), longer depuration may be needed to fully eliminate higher concentrations to reach levels that are safe for human consumption.

4.5 Conclusion

There is still a lack of data on the toxicity and effects of pesticides' metabolites on bivalves, whether individually or in mixture with their parent compounds.

In the present work, we studied the kinetics of the metabolite 4,4'-DCBP, considered as the main degradation product of dicofol. Our results showed that uptake of the contaminant was less effective than elimination, which could be associated with high metabolism of the compound by the organism. Therefore, quantification of metabolites could be a new alternative and a better approach to understand pesticide metabolism in bivalves, and its impact on the marine environment.

Moreover, these results raise to a certain extent issues of concern, since for both dicofol exposures, the organisms reached limit values accepted by the EU (i.e. 10.05 ng/L for D1) or did not have the ability to return to safe values (in case of D2) for food consumption after 15 days of depuration.

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Chapter 5. Key findings and future research

5.1 Research questions answered

The research developed in this thesis required multi-disciplinary approaches to investigate hazardous compounds such as pesticides, and more specifically, organochlorine pesticides (OCPs). A combination of literature review, field and lab work, were done in order to answer the research questions initially postulated, which are directly addressed in this section.

A. Could mangroves be considered as good natural remediators of OCPs from the environment? (Chapter 2)

After the extensive literature review done, and all the secondary data collected from different tropical and sub-tropical regions around the world, and further analysis as a whole, indicated that not only the plant itself but also the other components of the mangroves ecosystems, are interesting remediation tools for OCPs compounds. Through theoretical approach, we cannot ensure that mangroves can degrade and eliminate these compounds from the environment. However, mangroves ecosystems seem to have a better condition in terms of level of contamination, indicating that interaction between biotic and abiotic matrices (i.e. water, sediments, benthic biota or microorganism) present in these environments are important key factors for OCPs remediation (Tam et al. 2001; Susarla et al. 2002; Wong et al. 2006).

A.1 Are OCPs concentrations different between areas with and without mangroves?

We were able to observe differences in OCPs concentrations in abiotic and biotic compartments. Water and sediments from non-mangrove areas always presented higher concentrations of OCPs (1.6- and 2.2-fold, respectively) than in mangroves areas. Statistically significant differences were observed in sediment matrix, and this could be (1) due to the hydrophobic nature of the contaminants (Han et al. 2011)

and/or (2) due to the ability of the sediments to act as an effective sink of pollutants (Susarla et al. 2002). Moreover, the same pattern was observed in the benthic fauna, with mangrove organisms having lower concentrations of OCPs; this led us to conclude that sediments-benthic fauna might be close interlinked.

A.2 How is the accumulation pattern of OCPs in the associated benthic fauna?

We decided to address this question dividing the benthic fauna in two different traits; according to (1) feeding behaviour and (2) habitat. For both cases we observed a different accumulation pattern between organisms, with non-mangrove areas presenting higher concentrations of OCPs than mangrove ones. For the first case, we saw that carnivores and herbivores were the main OCPs accumulating groups. These results were a bit surprising, since herbivores presented higher OCPs values than carnivores. However, further analysis, demonstrated that these high concentrations observed for the herbivores was related to mudskippers OCPs body burden. Nevertheless, as some authors mentioned (Falandysz and Rappe 1996; Gray 2002; Lundgren et al. 2002), the lack of biomagnification can occur, explaining why in some occasions higher concentrations are not quantified in organisms from higher trophic levels. Other factors, such as metabolism, lipid content or bioconcentration ability are important to consider when this kind of studies are carried out (Kidd et al. 2001; Gray 2002; Zhou et al. 2007; Nfon et al. 2008).

For the second trait, surprisingly, animals living in the water-sediment interface (epifauna) presented higher concentrations of OCPs than the ones living within the sediment (infauna). Benthic animals from higher trophic levels were included in the epifaunal group, meanwhile infauna was only represented by the polychaetes. The low levels quantified in polychaetes may be due to (1) their relatively short-life cycle

(on the order of days or weeks) (Dean et al. 2008), and (2) their adaptation to the environment due to the lack of mobility (Geracitano et al. 2004).

A.3 How is the quality of the surrounding ecosystem in areas with or without mangroves?

Quality assessment of the abiotic factors, such as surface water and sediments was done using international guidelines/directives and theoretical risk assessments. For surface water, we used legislation levels ("European Legislation") and risk assessment calculations as a theoretical approach; and for sediments (since there is no official legislation) we used two different guidelines ("Dutch List" and "ISQG") and risk assessment based in some established reference levels (ERL/ERM).

In all the cases and matrices, better quality in terms of OCPs contamination was observed in environments where mangroves were present. This corroborates with the previous statements, and demonstrates the interesting role that these environments could play in the removal of these compounds.

B. How are the levels of 4,4'-DCBP and the physicochemical characteristics of surface waters from the mouth of the PRD? (Chapter 3)

The average levels of 4,4'-DCBP quantified in surface waters from Macao and Hong Kong were higher (12-fold and 39-fold for Macao and Hong Kong, respectively) than the European limit established for its precursor (dicofol). Moreover, Hong Kong surface waters presented concentrations 3-fold higher than in Macao. Concentrations quantified in both areas followed a seasonal pattern, meaning that rainy periods may have contributed to the dilution effect observed in water samples collected during the wet season. The results obtained can be explained due to (1) use of antifouling-paints in shipping and fishery industry, (2) recent use of dicofol as a pesticide in Hong Kong

or (3) trans-boundary pollution mainly from China (Qiu et al. 2005; Guo et al. 2008; Xin et al. 2011).

Regarding the nutrients quantified in these waters, both areas had dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP) levels higher than European systems (i.e. Mediterranean and Baltic sea) and those limits established by EPA, respectively; indicating some signs of eutrophication. Nutrients were also affected by seasonal sampling, improving in case of DIN or declining in case of DIP, during heavy rainy periods. In sum, Hong Kong and Macao waters had distinct patterns, with higher levels of DIN and TDS for Hong Kong, and DIP and chlorophyll a for Macao.

B.1 Are the quantified levels potentially risky for the environment?

In order to evaluate the potential risk associated to the observed environmental concentrations, we used the same theoretical risk assessment mentioned above (Chapter 2). In this case, the RQ was below 1, and therefore a low potential risk was predicted. However, considering the characteristics of 4,4'-DCBP (i.e. its capability for biomagnification or ability for off-spring transmission), low concentrations could lead to a potential risk affecting the ecosystem and future generations.

B.2 How toxic can the metabolite 4,4'-DCBP be when compared to the parent compound dicofol?

Considering that 4,4'-DCBP will be the main expected form in the environment after dicofol's degradation, it is very important to address its toxicity to the surrounding environment. After assessing the toxicity in two different biological models (*D. magna* and *A. salina*), we can say that dicofol (as a parent compound) is 1.3-fold more toxic than its main metabolite (4,4'-DCBP). However, it may not be a threat to the environment since its degradation form presented lower toxicity. However, there

are other problems associated to the use of dicofol, such as the release of DDT as an intermediate product, or the possible toxicity effects caused by the mixture of contaminants present in the environment (Qiu et al. 2005; Silva and Cerejeira 2014). It would be highly recommended to keep a track of this compound and perform monitorization studies along time and locations.

C. Do edible bivalves have the ability to accumulate and depurate 4,4'- DCBP after dicofol exposure? (Chapter 4)

The edible bivalve *Meretrix meretrix,* was able to accumulate and depurate 4,4'-DCBP after dicofol exposure. Considering the bioconcentration factor (BCF) obtained and the hydrophobic characteristic of the compound, we could say that 4,4'-DCBP can concentrate more in the organism than in the surrounding water. Overall, depuration of the metabolite was more successful than accumulation, since elimination rate was 2.3-fold higher than the uptake one. It would be also important to include parent compounds and metabolites in kinetics studies, since they may follow different patterns. For example, during exposure the ratio between parent compound and metabolite will be higher due to continue exposure to dicofol. Meanwhile, during depuration the ratio will decrease, since there is no more addition of dicofol into the system and metabolisation or degradation of dicofol to 4,4'-DCBP may predominate.

C.1 Will uptake and depuration kinetics of these organisms be different between both dicofol concentrations tested?

Our results showed different kinetic pattern between both concentrations tested: environmental concentration (D1 = 50 ng/L) and supra-environmental concentration (D2 = 500 ng/L). For D1, the uptake kinetic was best fitted using a plateau followed by one phase association kinetic model, while for D2 a one phase association kinetic model suited better. During the uptake phase, D1 reached faster the steady state (5-7

days) than D2 (12-15 days), and presented also higher uptake rates, indicating that concentrations in D2 could modify respiration rates and filtration capability (Bourdelin, F., 1996; Vijayavel, K., et al., 2007). Depuration period also showed different kinetic patterns, with a higher elimination factor (EF) in organisms exposed to D2 than to D1. This indicates that the efficiency of eliminating contaminants seems to be higher when there is more concentration in the system.

C.2 Will depurated clams reach acceptable levels for human consumption?

After 15 days of depuration, we observed that only clams exposed to the lowest concentration (D1) were able to reach acceptable levels for human consumption, within 24 hours of depuration. On the other hand, organisms exposed to the highest concentration (D2), presented 2.6-fold higher levels than the Maximum Residue Level established by the European legislation, after the same time of depuration than D1 animals, demonstrating that these ones were not suitable for human consumption. These results indicate that in case of an accident (e.g. spill), longer periods of depuration will be needed for filter feeders present in affected areas.

This work also demonstrated that studies on the uptake/depuration kinetics of a certain contaminant could be interesting to understand the dynamics of that element on specific organisms and their possible implications for the marine environment and lastly, for human health.

5.2 Further research

To conclude this research work, we would like to highlight some of the questions that remain open, and could be very interesting to address in a near future.

1). Since information regarding bioremediation of OCPs by mangroves is not as extensive as for other pollutants and concentrations of these pesticides are detectable

in the environment, mesocosms experiments could help to understand better the specific role of mangroves, alone or together, with benthic fauna and microorganisms in the uptake, degradation, or elimination of OCPs. For example, further efforts should be taken to identify rhizosphere microorganisms capable of degrading complex molecules, such as OCPs, or to understand the uptake mechanism of these compounds by the mangroves' roots.

2). Although mangroves are well-known for the variety of good ecological services provided, they have been and continue being under anthropogenic stresses. Therefore, more financial resources should be provided to support and manage restoration and rehabilitation programs, which will help in the protection of this valuable ecosystem.

3). Within the benthic fauna, it would be interesting to focus on mudskipper species since it was the organism with highest OCPs concentrations. Besides, mudskippers are very sensitive to the surrounding environment and they are considered good ecological indicators since they can absorb and accumulate many different pollutants. As an additional study, we suggest controlled experiments (mesocosms) with and without mangrove plants in combination with mudskippers, since these benthic fishes have been suggested as potential bioindicators for the health of mangrove ecosystems.

4). Since our work was the first one quantifying 4,4'-DCBP in the mouth of the Pearl River Delta, we suggest a continuous environmental monitoring of 4,4'-DCBP in surface waters from Macao and Hong Kong to control the use of this compound. It would be also very interesting to include other OCPs and their metabolites. Understanding the behaviour of the metabolites is very important, since they can still be very active, and they could be more available in the environment than the parent compound itself.

5). In addition, and since some signs of eutrophication were observed, continued monitoring of physicochemical and nutrients parameters from these surface waters would be highly recommended.

6). This thesis proved that bivalves, like *M. meretrix*, are able to uptake and eliminate dicofol to a certain extent, but we do not know which modifications or damages could be caused at other levels. The implementation of other works focused at the genotoxicity or histopathological levels would give an extra information about specific effects of these compounds at metabolic or physiological levels.

7). Since the kinetics study of 4,4'-DCBP did not follow the same pattern as in other published works where the parent compounds were studied, we propose to do other OCPs studies with focus on metabolites uptake and depuration or even both (metabolite and parent compound).

Appendices

The appendices that accompany this thesis, contain:

Appendix A: Detailed tables regarding OCPs concentration in several matrices from Non-mangroves and mangrove areas around the world.

Appendix B: Validation method and physicochemical parameters regarding surface water samples from Macao collected in Non-mangrove and Mangrove areas.

Appendix C: Validation method of 4,4'-DCBP for surface water.

Appendix D: Optimization process for the clams' survival, validation method in *M.meretrix*, condition index values and OCPs properties.

Appendix A

Table A1-a. Pesticide concentrations [MD (median of the average), SD (standard deviation), Min (minimum of the average), Max (maximum of the average)] and n (frequency) in surface water samples from NM areas.

WATER: Non-Mangrove areas (ng/L)					
Compound	**MD**	**Min**	**Max**	**SD**	**n**
Endrin	5.36	0.50	80.00	25.21	8
α-chlordane	0.50	0.50	0.50	-	59
γ-chlordane	15.30	0.50	20.60	8.38	4
2,4'-DDD	3.00	0.02	5.50	2.63	4
2,4'-DDE	4.75	0.01	18.60	7.71	4
2,4'-DDT	6.00	0.01	20.00	7.30	5
4,4'-DCBP	43.92	4.42	691.77	7.65	1
4,4'-DDD	5.09	0.01	40.00	13.00	8
4,4'-DDE	0.12	0.06	10.00	4.67	3
4,4'-DDT	5.09	0.04	30.00	13.22	8
ΣHCH	31.80	3.60	60.00	28.20	2
γ-HCH	0.20	0.05	170.00	56.22	8
Heptachlor	6.25	0.05	190.00	61.11	8
Heptachlor epoxide	8.60	3.22	50.00	1.40	2
Hexachlorobenzene	10.00	0.09	58.57	23.06	5
Methoxychlor	0.50	0.01	45.00	16.34	6

Table A1-b. Pesticide concentrations [MD (median of the average), SD (standard deviation), Min (minimum of the average) Max (maximum of the average)] and n (frequency) in water samples from M areas.

WATER: Mangrove areas (ng/L)					
Compound	**MD**	**Min**	**Max**	**SD**	**n**
Endrin	0.50	0.02	2.90	1.26	3
α-chlordane	0.50	-	-	-	38
trans-nonachlor	0.00	-	-	-	1
γ-chlordane	0.01	-	-	-	1
2,4-DDD	0.05	0.04	0.05	0.01	2
2,4-DDE	0.02	0.01	0.03	0.01	2
2,4-DDT	0.04	0.03	0.04	0.01	2
4,4'-DCBP	16.94	4.17	72.13	2.85	1
4,4'-DDD	0.80	0.03	2000.0	865.79	4
4,4'-DDE	0.59	0.02	1.32	0.59	4
4,4'-DDT	0.07	0.04	9.42	0.01	3
Endosulfans	40.53	30.00	51.06	10.53	2
ΣHCH	16.73	11.25	770.00	328.68	4
γ-HCH	0.45	0.02	4.99	1.94	6
Heptachlor	0.25	0.00	0.50	0.25	2
Heptachlor epoxide	23.88	1.65	59.98	-	1
Hexachlorobenzeno	0.00	0.00	0.27	0.13	3
Methoxychlor	0.50	-	-	-	1

Table A2-a. Pesticide concentrations [MD (median of the average), SD (standard deviation), Min (minimum of the average), Max (maximum of the average)] and n (frequency) in sediment samples from NM areas.

SEDIMENT: Non-Mangrove areas (ng/g DW)					
Compound	**MD**	**Min**	**Max**	**SD**	**n**
α-chlordane	0.43	0.01	4.42	1.34	11
β-chlordane	0.53	0.46	3.34	1.36	5
α,β-chlordane	0.95			1.90	6
γ-chlordane	1.01	0.03	3.35	1.29	7
α-endosulfan	0.34	0.06	67.77	15.05	19
β-endosulfan	0.49	0.02	2.88	0.76	17
Endosulfan sulfate	1.01	0.06	7.15	2.21	12
Endosulfans	0.30	0.11	7.22	2.67	9
Aldrin	0.92	0.13	6.47	1.87	15
Endrin	0.63	0.01	4.13	1.23	14
Dieldrin	0.63	0.05	31.81	6.93	20
Aldrin, Endrin, Dieldrin	1.09	0.48	1.25	0.33	4
Endrin aldehyde	1.81	1.54	2.08	0.27	2
α-HCH	0.41	0.05	6.19	1.73	30
β-HCH	0.68	0.05	5.24	1.41	28
γ-HCH	0.61	0.03	1673.57	290.96	33
ΣHCH	2.34	0.06	573.84	109.82	26
Heptachlor	0.78	0.02	33.50	9.36	19
Heptachlor epoxide	0.50	0.02	1122.74	298.80	13
Hexachlorobenzene	0.29	0.13	9.79	3.78	5
Methoxychlor	0.57	0.08	11.99	3.08	13
2,4’-DDD	1.62	0.01	10.87	3.20	9
2,4’-DDE	1.15	0.02	4.81	1.57	8
2,4’-DDT	1.42	0.10	9.65	2.56	13
4,4’-DDD	0.81	0.01	14.80	3.37	30
4,4’-DDE	1.65	0.10	21.16	5.54	31
4,4’-DDT	0.69	0.01	26.43	5.29	34
4,4’-DDTs	2.13	0.33	9.12	2.94	16
2,4’/4,4’-DDTs	10.38	1.10	872.21	236.48	12

Table A2-b. Pesticide concentrations [MD (median of the average), SD (standard deviation), Min (minimum of the average), Max (maximum of the average)] and n (frequency) in sediments samples from M areas.

SEDIMENT: Mangrove areas (ng/g DW)					
Compound	**MD**	**Min**	**Max**	**SD**	**n**
α-chlordane	0.01	0.00	1.05	0.40	6
β-chlordane	0.01	0.01	0.02	0.00	2
γ-chlordane	1.11	0.00	4.52	1.75	4
Chlordanes	0.02	0.01	0.52	0.19	6
α-endosulfan	0.20	0.00	814.00	255.63	10
β-endosulfan	0.01	0.00	1.69	0.62	7
α,β-endosulfan	11.89	0.18	23.60	11.71	2
Endosulfans	0.20	0.01	0.20	0.09	3
Aldrin	0.25	0.00	12.20	2.80	18
Endrin	0.15	0.01	2.21	0.62	14
Dieldrin	0.32	0.01	12.20	4.27	8
Endrin aldehyde	0.15	0.10	0.33	0.09	7
α-HCH	0.23	0.00	5.72	1.41	15
β-HCH	0.28	0.00	5.35	1.29	16
γ-HCH	0.51	0.00	9.20	2.21	19
ΣHCH	1.15	0.06	134.00	32.14	16
Heptachlor	0.26	0.02	5.72	1.47	14
Heptachlor epoxide	1.30	0.00	4.26	1.81	4
Hexachlorobenzene	0.36	0.00	70.50	20.89	10
Methoxychlor	0.32	0.11	11.50	3.51	9
2,4’-DDD	0.55	0.01	1.23	0.33	14
2,4’-DDE	0.26	0.04	3.51	0.90	15
2,4’-DDT	0.71	0.00	2.19	0.57	15
4,4’-DDD	1.84	0.06	6.13	1.61	16
4,4’-DDE	0.64	0.08	247.00	54.93	19
4,4’-DDT	2.12	0.02	1906.00	448.03	17
4,4’-DDTs	3.01	0.17	92.60	24.88	12
4,4’/ 2,4’-DDTs	7.23	0.86	26.81	6.76	11

Table A3-a. Overview of the concentrations (ng/L) quantified in water samples from NM areas in different tropical and sub-tropical regions around the world.

WATER: Non-Mangrove Areas							
Country	**MD**	**Min**	**Max**	**SD**	**N**	**S.Year**	**Reference**
Argentina	3.6	1.9	5.0	1.3	3	2016	Miglionanza et al. 2013
Hong Kong	0.1	<0.1	0.5	0.2	38	2003	Wong et al. 2006
India	10.0	7.0	90.0	24.9	23	2003	Singh et al. 2006
Macao	2.8	0.5	87.0	27.1	10	2017	Our Data (SM2)
Mozambique	0.2	<0.1	0.3	0.1	3	2015	Sturve et al. 2016
Nigeria	40.0	20.0	190.0	56.8	11	2014	Akinsaya et al. 2015
Singapore	0.2	<0.1	17.9	1.6	21	na	Basheer et al. 2003
South Africa	15.9	5.5	58.6	12.7	28	2002	Fatoki et al. 2004

Table A3-b. Overview of the concentrations (ng/L) quantified in water samples from M areas in different tropical and sub-tropical regions around the world.

WATER: Mangrove Areas							
Country	**MD**	**Min**	**Max**	**SD**	**N**	**S.Year**	**Reference**
China	3.7	1.1	18.9	5.9	8	2015	Yang et al. 2017
Hong Kong	<0.1	0.0	0.5	0.2	18	2003	Wong et al. 2006
Macao	2.8	0.9	24.5	8.7	13	2017	Our Data (Appendix B)
Mexico	<0.1	<0.1	2000.0	384.6	26	2000-2002	Carvalho et al. 2009, Romero et al. 2004
Mozambique	0.2	0.1	0.5	0.1	4	2015	Sturve et al. 2016
Singapore	25.3	0.0	770.0	208.4	12	2004-2012	Bayen et al. 2019, Bayen et al. 2005

Table A4-a. Pesticide concentrations (ng/g DW) in sediment samples from NM areas in different tropical and sub-tropical regions around the world.

SEDIMENTS: Non-Mangrove Areas							
Country	**MD**	**Min**	**Max**	**SD**	**N**	**S.Year**	**Reference**
Argentina	0.3	<0.1	7.2	1.5	79	2002-2005-2006	Miglionanza et al. 2004, Miglionanza et al. 2013
Brazil	0.7	<0.1	67.8	14.4	38	2008-2010-2011	Combi et al. 2013, Galvao et al. 2014, Oliveira et al. 2016
China	1	<0.1	872.2	78.7	174	1998-2000-2003-2007-2009-2011-2012	Fu et al. 2001, Nakata et al. 2005, Zhang et al. 2009, Zhang et al. 2011a,Yu et al. 2013, Li et al. 2015, Kaiser et al. 2016, Adeleye et al.2016
Hong Kong	1.5	<0.1	15.1	2.9	48	1999-2003	Zheng et al. 2000, Wong et al. 2006
India	0.9	<0.1	9.7	2	39	2003-2005-2015	Zanardi-Lambardo et al. 2019, Guzzella et al. 2005, Binelli et al. 2008, Singh et al. 2006
Nigeria	5	0.6	1673.6	512.9	12	2014	Akinsaya et al. 2015
Puerto Rico	5.1	0.1	10.1	5	2	2009	Whitall et al. 2014
Singapore	2.3	0.5	21.9	3.8	40	2003	Wurl et al. 2005
South Africa	1.1	0.8	18	8	3	2012	Vogt et al. 2018
Taiwan	0.5	<0.1	4.9	1	32	2000-2001	Doong et al. 2008, Hung et al. 2007
Tanzania	8.9	0.7	24	7.5	7	2000	Mwevura et al. 2003

Table A4-b. Pesticide concentrations (ng/g DW) in sediment samples from M areas in different tropical and sub-tropical regions around the world.

SEDIMENTS: Mangrove Areas							
Country	**MD**	**Min**	**Max**	**SD**	**N**	**S.Year**	**Reference**
Brazil	0.1	<0.1	26.8	3.6	67	2003-2008-2012	Souza et al. 2008, Rizzi et al. 2017,Galvao et al. 2014
Cameron	30	-	-	-	1	2009	Fusi et al. 2016
China	0.5	0.1	1906	162.2	139	2000-2011-2015-2010-2014-2013	Wu et al. 2015, Yang et al. 2017, Kaiser et al. 2016, Qiu et al. 2019, Zhang et al. 2019
Hong Kong	1.7	<0.1	17.7	3.5	38	1999-2002	Zheng et al. 2000, Wong et al. 2006
India	0.2	<0.1	136.2	38.3	15	1999-2003-2005-2015	Shete et al. 2009, Bhattacharya et al. 2003, Zanardi-Lambardo et al. 2019, Guzzella et al. 2005, Binelli et al. 2008
Mexico	<0.1	<0.1	814	158.9	27	2000-2002	Romero et al. 2004,Carvalho et al. 2009
Senegal	0.4	0.1	5.9	2.2	5	2005	Bodin et al. 2011
Singapore	0.4	<0.1	8.7	2.5	12	2004-2012	Bayen et al. 2005, Bayen et al. 2019
Taiwan	0.2			-	1	2017	Das et al. 2020
Tanzania	1.1	0.2	3	0.9	6	2003	Kruitwagen et al. 2008
USA	17.9	9.2	70.5	22.8	6	2010	Lewis et al. 2015

Table A5. Classification of the benthic fauna included in the analysis according to feeding habit and habitat, and OCPs concentration quantified in each specie from NM and M areas, expressed as MD (median of the average, ng/g WW).

Feeding habit	Specie	Family	Habitat	NM (MD)	M (MD)
Filter feeder	*Cyclina orientalis*	Veneridae	Infauna	0.5	na
	Anomalocardia brasiliana	Veneridae	Infauna	na	0.3
	Polymesoda expansa	Cyrenidae	Infauna	na	1.3
	Balanus spp.	Balanidae	Epifauna	na	0.5
	Mytella guayensis	Mytilidae	Epifauna	na	33.3
	Perna perna	Mytilidae	Epifauna	0.6	0.3
	Arca senilis	Arcidae	Epifauna	na	0.5
	Perna viridis	Mytilidae	Epifauna	2.1	1.3
	Crassostrea rhizophorae	Ostreidae	Epifauna	na	83.2
	Crassostrea spp.	Ostreidae	Epifauna	na	1.8
	Isognomon ephippium	Isognomonidae	Epifauna	na	2
	Austinogebia edulis	Upogebiidae	Epifauna	na	0.3
Surface Deposit Feeder*	*Callinectes sapidus*	Portunidae	Epifauna	na	1
	Cyrtograpsus altimanus	Varunidae	Epifauna	<0.1	na
	Exopalaemon styliferus	Palaemonidae	Epifauna	0.4	na
	Melicertus kerathurus	Penaeidae	Epifauna	13.4	na
	Metapenaeus ensis	Penaeidae	Epifauna	0.3	0.5
	Neohelice granulata	Varunidae	Epifauna	<0.1	na
	Penaeus monodon	Penaeidae	Epifauna	na	<0.1
	Uca arcuata	Ocypodidae	Epifauna	1	3.7
Sub-surface Deposit Feeder	*Diopatra neapolitana* *	Onuphidae	Infauna	na	0.1
	*Neanthes glandicincta***	*Nereididae*	Infauna	0.5	0.2
Carnivores	*Callinectes amnicola*	Portunidae	Epifauna	4	1.6
	Callinectes danae	Portunidae	Epifauna	1.8	na
	Conus spp.	Conidae	Epifauna	na	0.2
	Hepatus pudibundus	Aethridae	Epifauna	3.5	na
	Hexaplex duplex	Muricidae	Epifauna	na	0.9
	Melongena corona	Melongenidae	Epifauna	na	3.7
	Myomenippe hardwicki	Menippidae	Epifauna	na	2
	Periophtalmodon schlosseri	Gobiidae	Epifauna	na	0.2
	Periophthalmus argentilineatus	Gobiidae	Epifauna	na	1.1
	Pleuroploca trapezium	Fasciolariidae	Epifauna	1.8	na
	Pugilina morio	Melongenidae	Epifauna	na	0.2
	Scylla serrata	Portunidae	Epifauna	5.6	24.3
	Thai gradata	Muricidae	Epifauna	na	0.7

Omnivores	*Varuna litterata*	Varunidae	Epifauna	1.6	na
Herbivores	*Alpheus microrhynchus*	Alpheidae	Epifauna	na	0.1
	Boleophthalmus boddarti	Gobiidae	Epifauna	na	0.7
	Boleophthalmus pectinirostris	Gobiidae	Epifauna	9.3	62.3
	Nerita lineata	Neritidae	Epifauna	na	0.3
	Peneaus spp.	Penaeidae	Epifauna	na	0.3
	Telescopium telescopium	Potamididae	Epifauna	na	0.3

* also consider as Omnivores; ** also consider as Carnivores

Data extracted from: Aguirre-rubi et al. 2017, Akinsaya et al. 2015, Bayen et al. 2004, Bayen et al. 2005, Bodin et al. 2011, Borrell et al. 2019, Carvalho et al. 2009, Das et al. 2019, Commendatore et al. 2018, Galvao et al. 2012, Galvao et al. 2014, Kruitwagen et al. 2008, Lam et al. 2004, Lewis et al. 2015, Liebezeit et al. 2011, Magalhães et al. 2016, Mwevura et al. 2003, Nakata et al. 2005, Wong et al. 2006, Zhang et al. 2019.

Table A6. Pesticide concentrations [MD (median of the average), Min (minimum of the average), Max (maximum of the average) and SD (standard deviation)] in mangroves samples. The number of compounds quantified (N), sampling year and species were also added. *Mix of mangroves, means average values considering the following species: *Sonneratia hainanensis, Sonneratia caseolaris Bruguira sexangula, Brugueira gymnorrhiza, Rhizophora stylosa, Rhizophora apiculata, Kandelia candel, Lumnitzera racemosa, Aegiceras corniculatum.* (na: not available).

Species	MD	Min	Max	SD	N	S.Year	Country	Reference
*Mix of mangroves**	2.7	0.5	7.8	3.9	15	2014	China	Qiu et al. 2019
Avicennia marina	1.4	0.2	15.4	5.3	8	na	India	Shete et al. 2009

Table A7-a1. Risk assessment in water samples from NM areas. Tier I, step 1.

Compound	EC_{50} Algae mg/L (72h)	EC_{50} Invert. mg/L (48h)	LC_{50} Fish mg/L (96h)	MEC(max.) ng/L	MEC(max.) mg/L	PNEC	RQ MEC/PNEC
Fenobucarb	-	0.10	1.70	109.63	1.10E-04	1.00E-03	0.110
Pirimicarb	140.0	0.02	100.00	1.04	1.04E-06	1.70E-04	0.006
Pyrimethanil	1.20	2.90	10.56	9.11	9.11E-06	1.20E-02	0.001
Endrin	-	0.004	0.00	80.00	8.00E-05	7.30E-06	**10.959**
α-chlordane	-	0.59	0.09	0.50	5.00E-07	9.00E-04	0.001
γ-chlordane	-	0.59	0.09	20.60	2.06E-05	9.00E-04	0.023
o,p'-DDD	-	0.01	0.07	45.50	4.55E-05	9.00E-05	0.506
p,p'-DDD							
o,p'-DDE	-	0.001	0.03	28.60	2.86E-05	1.00E-05	**2.860**
p,p'-DDE							
o,p'-DDT	-	2.50	0.01	50.00	5.00E-05	5.00E-05	**1.000**
p,p'-DDT							
p,p'-DCBP	-	0.17	-	691.77	6.92E-04	1.70E-03	0.407
γ-HCH	0.03	54.00	0.00	170.00	1.70E-04	2.90E-05	**5.862**
Heptachlor	-	0.04	0.01	190.00	1.90E-04	7.00E-05	**2.714**
Heptachlor epoxide	200.0	0.24	0.02	50.00	5.00E-05	2.00E-04	0.250
Hexachlorobenzene	0.50	0.01	0.03	58.57	5.86E-05	4.80E-05	**1.220**
Methoxychlor	0.60	0.001	0.05	45.00	4.50E-05	7.80E-06	**5.769**

Table A7-a2. Risk assessment in water samples from NM areas. Tier I, step 2 and Tier II.

Compound	RQ TU algae (MEC/EC_{50})	RQ TU invert. (MEC/EC_{50})	RQ TU fish (MEC/EC_{50})		
Fenobucarb	-	1.10E-03	6.45E-05		
Pirimicarb	7.43E-09	6.12E-05	1.04E-08		
Pyrimethanil	7.59E-06	3.14E-06	8.63E-07		
Endrin	-	1.90E-02	**1.10E-01**		
α-chlordane	-	8.47E-07	5.56E-06		
γ-chlordane	-	3.49E-05	2.29E-04		
2,4'-DDD	-	5.06E-03	6.50E-04		
4,4'-DDD	-	-	-		
2,4'-DDE	-	2.86E-02	8.94E-04		
4,4'-DDE	-	-	-		
2,4'-DDT	-	2.00E-05	1.00E-02		
4,4'-DDT	-	-	-		
4,4'-DCBP	-	4.07E-03	-		
γ-HCH	6.30E-03	3.15E-06	5.86E-02		
Heptachlor	-	4.52E-03	2.71E-02		
Heptachlor epoxide	2.50E-07	2.08E-04	2.50E-03		
Hexachlorobenzene	1.17E-04	1.22E-02	1.95E-03		
Methoxychlor	7.50E-05	5.77E-02	8.65E-04		
STU (∑)	6.50E-03	1.33E-01	**2.13E-01**	Tier II	STU/max RQ TU
RQ-STU=max RQ-TU * AF			**21.251**	Base on IA	**1.939**

Table A7-b1. Risk assessment in water samples from M areas. Tier I, step 1.

Compound	EC_{50} Algae mg/L (72h)	EC_{50} Invert. mg/L (48h)	LC_{50} Fish mg/L (96h)	MEC(max.) ng/L	MEC(max.) mg/L	PNEC	RQ MEC/PNEC
Fenobucarb	-	0.10	1.70	231.13	2.31E-04	1.00E-03	2.31E-01
Pirimicarb	140.00	0.02	100.00	2.92	2.92E-06	1.70E-04	1.72E-02
Pyrimethanil	1.20	2.90	10.56	2.04	2.04E-06	1.20E-02	1.70E-04
Endrin	-	0.00	0.00	2.90	2.90E-06	7.30E-06	3.97E-01
α-chlordane	-	0.59	0.09	0.50	5.00E-07	9.00E-04	5.56E-04
γ-chlordane	-	0.59	0.09	0.01	9.55E-09	9.00E-04	1.06E-05
2,4'-DDD	-	0.01	0.07	2000.05	2.00E-03	9.00E-05	**2.22E+01**
4,4'-DDD							
2,4'-DDE	-	0.00	0.03	1.34	1.34E-06	1.00E-05	1.34E-01
4,4'-DDE							
2,4'-DDT	-	2.50	0.01	9.42	9.42E-06	5.00E-05	1.88E-01
4,4'-DDT							
4,4'-DCBP	-	0.17	-	72.13	7.21E-05	1.70E-03	4.24E-02
γ-HCH	0.03	54.00	0.00	4.99	4.99E-06	2.90E-05	1.72E-01
Heptachlor	-	0.04	0.01	0.50	5.00E-07	7.00E-05	7.14E-03
Heptachlor epoxide	200.00	0.24	0.02	59.98	6.00E-05	2.00E-04	3.00E-01
Hexachlorobenzene	0.50	0.00	0.03	0.27	2.69E-07	4.80E-05	5.60E-03
Methoxychlor	0.60	0.00	0.05	-	-	7.80E-06	-

Table A7-b2. Risk assessment in water samples from M areas. Tier I, step 2 and Tier II.

Compound	RQ TU algae (MEC/EC_{50})	RQ TU invert. (MEC/EC_{50})	RQ TU fish (MEC/EC_{50})		
Fenobucarb	-	2.31E-03	1.36E-04		
Pirimicarb	2.09E-08	1.72E-04	2.92E-08		
Pyrimethanil	1.70E-06	7.03E-07	1.93E-07		
Endrin	-	6.90E-04	3.97E-03		
α-chlordane	-	8.47E-07	5.56E-06		
γ-chlordane	-	1.62E-08	1.06E-07		
2,4'-DDD	-	**2.22E-01**	2.86E-02		
4,4'-DDD	-	-	-		
2,4'-DDE	-	1.34E-03	4.19E-05		
4,4'-DDE	-	-	-		
2,4'-DDT	-	3.77E-06	1.88E-03		
4,4'-DDT	-	-	-		
4,4'-DCBP	-	4.24E-04	-		
γ-HCH	1.85E-04	9.24E-08	1.72E-03		
Heptachlor	-	1.19E-05	7.14E-05		
Heptachlor epoxide	-	-	-		
Hexachlorobenzene	-	-	-		
Methoxychlor	-	-	-		
STU	1.86E-04	**2.27E-01**	3.64E-02	Tier II	STU/maxRQ TU
RQ-STU=max RQ-TU * AF		**22.701**		Base on IA	**1.022**

Table A8. Concentration median (ng/g DW) values found in NM and M areas for the compounds included in the sediment risk assessment. ERL: Effect Range Low; ERM: Effect Range Median. Bold numbers indicate values above ERL. ∑DDT (DDD+DDE+DDT); DDD (2,4'/4,4'-DDD); DDE (2,4'/4,4'-DDE); DDT (2,4'/4,4'-DDT). na: not available.

Compound	NM	M	ERL	ERM
∑DDT	**7.2**	**6**	1.6	46.1
DDD	**2.3**	**2.4**	2	20
DDE	**2.8**	0.9	2.2	27
DDT	**2.1**	**2.7**	1	7
Endrin	**0.5**	**0.2**	<0.1	45
Heptachlor	**0.7**	0.4	0.5	6

Appendix B

B1. Quantification of pesticides from surface water samples (Macao, China).

B1.1 Studied area

Macao is a subtropical coastal city (21°50′-23°25′N/112°33′- 114°10′E) (Figure S1) located at the western shore of the Pearl River Estuary (PRE), one of the largest estuaries in the world, covering an area of ~2100 km^2. The PRD is subject to a typical Asian monsoon climate, i.e., hot and humid in summer with strong southeastern monsoon breezes from the South China Sea and strong precipitations; frequent but light rainfalls in spring and autumn (also known as transition period); and relatively cool and dry winter influenced by northeastern monsoon winds from northern China. The average ambient temperature ranges from 19°C to 28°C all year round, and an annual volume of rainfall ranges from 1300 to 2280 mm (Guangzhou Planning Association and Guangzhou Territory Planning Association, 1994).

Despite its small size (land area of 30 km^2, approximately) and highly urbanized environment, Macao is home to a healthy stand of mangrove forest along approximately 4 km of the Taipa-Coloane coastline (Tagulao, K.A., 2018). According to the recent survey conducted in this area, mangrove plants are limited to five different species: *Avicennia marina, Sonneratia apetala, Kandelia obovata, Aegiceras corniculatum* and *Acanthus ilicifolius.* The majority of these mangroves grow within the 40-hectare Ecological Zone II in Cotai managed by the Environmental Protection Bureau of Macao. Some patches can also be found in a small protected area in the eastern side of Coloane as well as Macao Peninsula (Tagulao, K.A., 2018) (Figure B1).

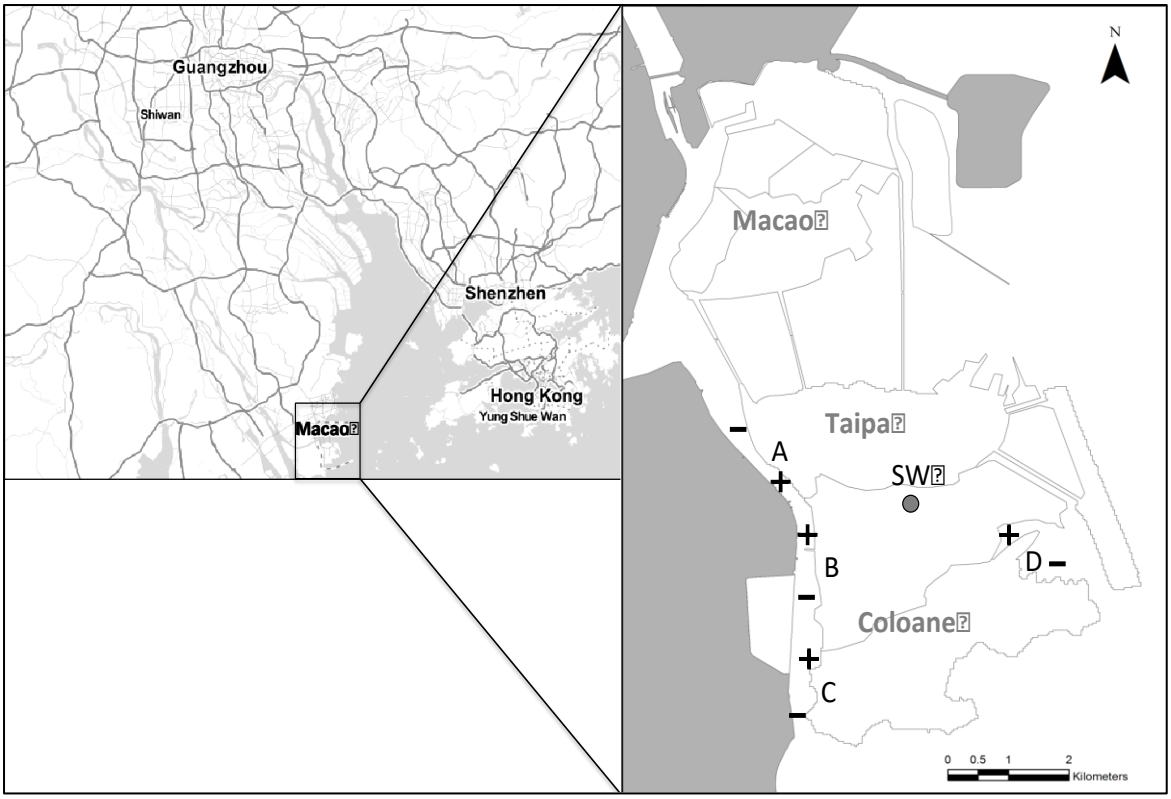


Figure B1. Distribution of the sampling sites (A to D) along Macao coastal area. Mangrove (M) and non-mangrove (NM) areas are represented by + and -, respectively. SW indicates the location of the spring water source used for validation purposes (QGIS 2.18 Desktop, version 2.18.15).

B1.2 Water collection and pre-concentration

Sampling was based on the geographical location and spatial distribution of the mangrove species. Water samples were collected from four different sampling sites along the coast of Taipa and Coloane (A to D). In each sampling location, M (+) and NM(-) areas were defined with a minimum of 600 m distance between them. Water samples were collected between April (2018) and February (2019), encompassing all the different seasons: transition I (April-May), wet (June-September), transition II (October-November) and dry (January-February). A total of sixty-four water samples (4 sampling sites x 4 seasons x 2 replicates per site) were analysed in the study.

At each sampling location, water samples (2 L) were collected with pre-rinsed amber glass bottles for pesticides, nutrients and physicochemical parameters quantification.

Detailed descriptions of sample pretreatment and analysis can be found in our previous publications (Ivorra et al. 2019a, Ivorra et al. 2019b). For pesticides quantification, samples (500 mL) were filtered (1,12 μm glass fibre filter; Sartorious, Germany) and acidified to pH 5 with acetic acid (CH_3COOH; Sigma-Aldrich, USA) for high sample stability prior to extraction. During transport and after filtering, water samples were kept at 4°C in the dark, for a maximum period of 24 h.

Pesticides were extracted by solid-phase extraction (SPE) using the OASIS HLB cartridges (200 mg, 6 cc; Waters, Ireland). Briefly, fortified water samples were loaded into pre-conditioned cartridges (5 mL CH_3OH followed by 5 mL ultrapure water), allowed to dry, and eluted (2.5 mL $C_4H_8O_2$ followed by 2.5 mL of CH_2Cl_2 and 2.5 mL more of a 1:1 mix of CH_2Cl_2 and $C_4H_8O_2$ (v/v)). The extracts were evaporated to dryness, under N_2 stream (99.995%) and then reconstituted into 200 μL of CH_3OH.

Nutrients analysis (i.e. dissolved inorganic nitrogen (DIN, mg/L) and dissolved inorganic phosphorous (DIP, mg/L)), were measured in the laboratory, with a photometer device from Palintest (YSI 9500 photometer, UK). Physicochemical parameters, such as temperature (T, °C), dissolved oxygen (DO, %), total dissolved solids (TDS, g/L), pH, and salinity were measured in situ (using a portable meter, YSI pro plus, USA), while chlorophyll a (Chl-a, mg/m^3) and total suspended solids (TSS, g/L) were quantified in the laboratory. Chl-a, was quantified by filtering 500 mL of water, through a Whatman GF/C glass fibre filter, following the protocol of Parsons et al. (1985); and TSS were quantified using 200 mL of the water samples following the protocol described by APHA (1995).

B1.3 Chemicals and reagents

All water samples were analyzed considering the nineteen selected compounds: eleven organochlorine pesticides (OCPs) [fenobucarb, pyrimethanil, pirimicarb,

aldrin, heptachlor, endosulfan (α - β isomer), dieldrin, endrin, methoxychlor, 4,4'-DDT and HCH (α - β - γ isomer)], five OCPs metabolites [endosulfan sulfate, heptachlor epoxide, 4,4'-dichlorodiphenyldichloroethylene (4,4'-DDE), 4,4'-dichlorodiphenyldichloroethane (4,4'-DDD), and 4,4'-dichlorobenzophenone (4,4'-DCBP)], two carbamates [fenobucarb and pririmicarb], and one anilinopyrimidine [pyrimethanyl]. Pirimicarb-d_6 and dicofol-d_8 were used as internal standards (IS) at a final concentration of 5 µg/L in the matrix; each IS, was assigned to the target molecule according to their molecular structure similarity and/or retention time (pirimicarb-d_6: 8.00-13.49 min; dicofol-d_8: 13.50-23.00 min).

All standard solutions (98-99%) were acquired from Sigma-Aldrich (Seelze, Germany). Each compound was prepared in CH_3OH with 0.1% acetic acid (CH_3COOH; Sigma-Aldrich, USA) to produce the final stock solution of 1000 µg/L and kept in dark at -20°C to avoid possible decay.

D-sorbitol and 3-ethoxy-1,2-propanediol (used as protectants) were also purchased from Sigma-Aldrich (Steinheim, Germany). Stock solution of 182 mg/mL in 70% CH_3OH:H_20 and 800 000 mg/L in 100% CH_3OH were prepared for D-sorbitol and 3-ethoxy-1,2-propanediol, respectively. Protectants were used as 0.1:1 mg/mL (D-sorbitol:3-ethoxy-1,2-propanediol). Stock solutions of 3-ethoxy-1,2-propanediol were kept at 4°C, and D-sorbitol and the protectant mixture were stored in the same conditions as the IS and standard solutions. For quantification purposes, an aliquot of each sample (57 µL) was taken and mixed with the protectant mix solution (3 µL) at a final concentration of 0.005:0.05 mg/L, respectively.

The analytical grade solvents, methanol, ethyl acetate, and dichloromethane were purchased from Merck Limited Company (Germany). Ultrapure water was obtained from a Milli-Q water system (resistance = 5.1 μΩ/cm at 25°C).

B1.4 Instrument analysis, quality assurance and quality control procedures

Analyses were carried out using a gas chromatograph (Trace 1310 GC, Thermo Scientific), coupled with a triple quadrupole mass spectrometer detector (TSQ 8000 EVO, Thermo Scientific), an autosampler (Thermo ScientificTriPlusTM) and a Trace Pesticides column (TR-pesticides II, 30 m x0.25 mmx0.25 mmx5 m Guard). Column oven temperatures were programmed for a 35 min period using several ramps: a) from 80°C with an initial equilibrium time of 2 min to b) 180°C at 20 °C/min until c) 290°C at 5 °C /min, where the temperature was maintained for 7 min. The injector port temperature was set to 200°C, and both ion source and MS transfer line were at 290°C.

Helium (99.999%) was used as the carrier gas and was maintained at a constant flow rate of 1.2 mL/min. Sample injection (1.5 μL) was in the split-less mode (4mm straight liner, 453A1925), using a 50 mm long needle. New liners were used every 200 injections.

The ions selection and the collision energies for quantification purposes were obtained from the auto-selected reaction monitoring. Information from published methods, regarding the target ions, were also taken into consideration (Lehotay et al., 2005; EU Reference Laboratories for Residues of Pesticides, 2013; Pereira et al., 2014). The software Xcalibur (version 4.0.27.10, Thermo Scientific), together with the NIST library, were used for ion products confirmation and quantification (Table B1).

Table B1. Information about the MS/MS method used for environmental quantification of the target compounds.

Intervals	Pesticides	Molecular mass (g/mol)	RT (min)	GC-MS/MS		CE
				Precursor	Product	
8.00	Fenobucarb	207.3	9.46	121.1	77.1	20.0
	α-HCH	290.8	10.59	181.0	145.0	15.0
	β-HCH	290.8	11.12	218.9	183.0	10.0
	γ-HCH	290.8	11.35	183.0	147.0	10.0
	Pyrimethanil	199.1	11.68	199.2	198.2	10.0
11.90	**Pirimicarb-D6**	**244.3**	**12.09**	**166.1**	**96.1**	**10.0**
	Pirimicarb	238.4	12.13	238.2	166.2	10.0
	Heptachlor	373.3	13.26	100.0	65.1	10.0
14.00	Aldrin	364.9	14.29	262.8	192.9	30.0
	Dicofol-D8	**378.5**	**14.48**	**143.0**	**115.0**	**15.0**
	4,4'-DCBP	252.1	14.56	141.0	113.0	15.0
	Heptachlor epoxide	389.3	15.55	182.9	155.0	15.0
16.20	α-endosulfan	406.9	16.56	240.9	206.0	10.0
	4,4'-DDE	318.0	17.26	246.0	176.1	25.0
	Dieldrin	380.9	17.43	262.9	193.0	30.0
17.80	Endrin	380.9	18.10	262.9	193.0	30.0
	β-endosulfan	406.9	18.44	195.0	159.0	10.0
19.40	Endosulfan sulfate	422.9	19.72	271.8	236.9	10.0
	4,4'-DDD + 4,4'-DDT	320.0	19.90	235.0	165.1	30.0
	Methoxychlor	345.7	21.82	227.1	169.1	25.0

The validation procedure followed the European guidance document on pesticide residue analytical methods SANTE/11813/ 2017 rev 0 (SANTE, 2017). Linearity was evaluated using three independent calibration curves, each with six nominal standard concentration of the compounds included in the analysis (ranging from 0.75 to 24 ng/L), and spiking (200 µL) into the 500 mL of filtered and acidified water matrix with the IS (at a final concentration of 4 ng/L). Curves were plotted using the ratio between the standard and the IS area (pirimicarb-d_6 or dicofol-d_8). The limits of detection (LOD) and quantification (LOQ) were determined with the same curves, using the following formulas: LOD = 3.3 α/S and LOQ = 10 α / S, where α is the standard deviation of the response and S is the average slope of the calibration curves.

Recoveries, accuracy and precision were evaluated by analyzing three independent replicates of each quality control samples (QCs) at two levels of concentration (low and medium) calculated as, QC_{low} = LOQ and QC_{medium} = 10LOQ.

Recoveries were determined by comparing the area ratio in the spiked matrix with the area ratio of the same concentration in a matrix blank spiked after extraction. Precision was expressed as the relative standard deviation (%RSD) of the replicate measurements, and the accuracy was evaluated as the percentage of agreement between the methods results and the nominal amount of added compound.

As part of the validation, the matrix effect (ME) was also evaluated at the lowest concentration (LOQ), where matrix samples were spiked after extraction ($A_{standard\ in\ matrix}$) and compared with those of injected standards ($A_{standards}$), as indicated in the following equation: $ME = -((A_{standard} - A_{standard\ in\ matrix})/A_{standard}) * 100$.

For quantification purpose, and considering environmental levels in Asia (Cruzeiro et al. 2018), different range of concentrations was used for the calibration curve (CC), IS and QC. The CCs included 7 points and a range of 2.4-153.6 ng/L with an IS of 20 ng/L, and 40LOQ as a QC.

B2. Method validation

The calibration curves proved to have good fits with r^2 ranging from 0.956 to 0.989. The final recovery rates ranged from 66.53% to 112.96%, while precision results (3.17–14.63%) were always below the maximum established (30 %), and accuracy ranged from 79.00% to 114.88%, demonstrating high robustness during the extraction process (detailed data in Table B2). Therefore, all validation criteria presented accepted ranges established by SANTE/11813/ 2017 rev 0 (SANTE, 2017).

Table B2. Results of recoveries, accuracy and precision (RSD) of the two quality controls (QCs) used for the validation (LOQ and 10LOQ). The results are expressed by mean.

Pesticide	QCs (µg/L)	Recovery (%)	SD	RSD (%)	SD	Accuracy (%)	SD	LODs (µg/L)	LOQs (µg/L)
Fenobucarb	0.25	97.50	36.26	7.21	4.70	102.04	21.66	0.08	0.25
	2.47	87.76	9.11	5.00	2.09	98.17	16.63		
α- HCH	3.64	83.80	12.45	7.43	7.71	110.54	21.72	1.20	3.64
	36.42	75.05	18.49	3.17	2.41	114.88	23.04		
β-HCH	0.30	91.80	10.99	11.44	7.00	99.06	14.35	0.10	0.30
	3.00	108.29	5.80	5.14	2.92	96.83	8.34		
γ-HCH	0.60	95.54	9.07	9.68	7.42	111.38	17.44	0.20	0.60
	5.97	99.58	16.83	3.88	2.44	98.78	14.21		
Pyrimethanil	0.28	83.92	9.55	11.45	7.32	119.33	7.71	0.09	0.28
	2.84	110.83	6.37	3.77	2.71	97.79	17.45		
Pirimicarb	0.15	79.18	17.88	11.67	6.90	88.93	17.95	0.05	0.15
	1.47	93.73	5.79	4.54	4.68	97.08	5.36		
Heptahchlor	0.34	82.42	16.54	9.14	7.40	93.13	23.50	0.11	0.34
	3.45	71.49	12.27	8.29	6.76	97.03	13.38		
Aldrin	0.48	66.53	5.57	10.11	5.22	79.00	29.72	0.16	0.48
	4.76	108.71	14.97	3.88	3.48	89.03	32.69		
4,4'-DCBP	0.59	90.49	24.09	4.43	3.76	94.89	15.17	0.19	0.59
	5.88	67.69	3.92	8.07	6.40	91.49	17.86		
Heptachlor epoxide	1.11	89.13	27.70	7.95	7.60	90.66	22.07	0.37	1.11
	11.11	112.96	10.72	5.05	4.70	99.77	10.10		
α-endosulfan	1.15	65.18	2.41	9.11	4.00	92.60	17.04	0.38	1.15
	11.50	69.58	8.12	6.10	4.91	82.50	24.43		
4,4'-DDE	0.80	76.77	12.24	4.70	1.41	113.80	33.95	0.26	0.80
	8.00	90.63	5.67	4.82	6.13	104.28	24.56		
Dieldrin	0.83	83.08	17.35	13.53	8.42	87.32	20.58	0.27	0.83
	8.26	98.91	17.08	7.37	6.54	102.80	35.81		
Endrin	0.33	71.76	7.56	10.34	8.81	84.03	9.42	0.11	0.33
	3.33	89.07	10.43	9.19	7.48	107.03	20.52		
β-endosulfan	0.48	84.55	14.76	11.58	7.15	101.93	41.37	0.16	0.48
	4.76	98.46	14.96	9.57	6.59	103.40	26.09		
4,4'-DDT + 4,4'-DDD	0.74	81.01	19.96	14.63	4.40	89.99	8.63	0.25	0.74
	7.43	87.05	14.78	5.47	3.91	81.57	11.94		
Endosulfan sulfate	4.15	99.42	12.22	9.64	6.78	86.65	16.36	1.37	4.15
	41.54	90.13	19.23	6.47	7.50	88.30	21.89		
Methoxychlor	2.89	92.04	10.35	9.46	5.66	103.00	10.10	0.95	2.89
	28.92	75.78	14.20	4.54	3.53	86.93	26.36		

In addition, the stability of the pesticides in water samples was evaluated by comparing the initial results of the LOQs with those obtained after a period of 24 h, 48 h, 72 h, and 7 d, 10 d, 14 d (h=hours and d=days) kept at −20°C. Results showed, that all the compounds were stable during the whole period except for methoxychlor and endosulfan sulfate (did not show stability after the first 24 h); aldrin and DDT (only stable during the first 72 h); and β-endosulfan and endosulfan sulfate (stable during the first 7 d). Finally, the matrix effect with signal enhancement and diminution was observed. Therefore, matrix-matched calibration curves were used during the whole study. Information regarding data stability and matrix effect can be found in Tables B3 and B4, respectively.

Table B3. Stability of the compounds extracted at LOQ concentration from spring waters after 24, 48, 72 h and 7, 10 and 14 d. Values in bold represent accuracies out of the acceptable range for SANTE guidance (SANTE, 2017). The results are expressed by mean (± SD).

Compound	Accuracy (%)					
	24 h	48 h	72 h	7 d	10 d	14 d
Fenobucarb	107.58±1.09	105.22±2.90	115.13±1.15	111.31±14.00	123.21±14.48	120.1±3.11
α-HCH	108.22±2.66	112±1.58	115.85±0.56	96.13±1.60	100.28±9.86	113.66±6.68
β-HCH	106.32±1.57	104.89±2.47	113.28±6.17	106.26±2.35	107.58±8.58	113.43±13.35
γ-HCH	98.09±1.58	98.77±3.48	105.78±3.62	100.23±4.35	93.5±3.45	95.75±3.34
Pyrimethanil	111.58±3.99	108.6±6.11	114.36±1.32	88.36±3.18	112.69±14.21	100.94±7.35
Pirimicarb	108.03±1.01	108.05±8.14	117±1.11	111.09±10.02	99.99±8.34	113.38±2.21
Heptachlor	110.01±2.76	106.29±17.19	110.74±27.05	75.03±6.84	85.17±0.44	71.61±1.38
Aldrin	118.25±5.05	107.82±16.70	107.65±15.80	**58.63±2.64**	**52.58±1.84**	**159.66±100.02**
4,4'-DCBP	110.22±1.63	120.61±3.40	106.73±30.75	61.00±1.71	101.35±17.86	63.05±7.99
Heptachlor epoxide	112.82±3.51	114.09±17.90	88.14±4.15	71.76±14.87	70.13±6.30	61.3±10.35
α-endosulfan	111.57±3.06	111.35±17.62	100.21±11.45	63.74±9.28	61.35±7.63	106.10±15.62
4,4'-DDE	113.09±2.80	112.84±17.11	108.51±13.55	69.67±1.82	89.15±6.92	71.76±2.90
Dieldrin	111.71±2.49	112.02±19.25	115.77±11.19	76.42±15.40	69.08±0.00	115.39±4.39
Endrin	99.72±1.76	95.03±12.92	81.80±10.02	114.63±18.79	75.66±6.18	-
β-endosulfan	95.01±3.11	92.89±10.95	93.64±9.76	61.65±3.97	**39.08±0.00**	-
Endosulfan sulfate	**27.84±1.17**	**31.39±3.44**	**17.14±6.42**	102.06±6.84	**50.65±7.33**	**40.02±11.24**
4,4'-DDT	84.64±2.62	79.51±13.13	62.5±28.32	**145.68±5.71**	**61.55±13.81**	**58.00±22.49**
Methoxychlor	**40.11±3.71**	**42.2±7.21**	**16.54±7.99**	**150.15±7.63**	**51.57±0.00**	**51.47±21.59**

Table B4. Evaluation of ME at LOQ concentrations for all selected compounds. The results are expressed as a percentage (%).

Compound	LOQ		Min	Max
	Mean	SD		
Fenobucarb	-85.62	3.78	-89.96	-79.93
α-HCH	-23.19	5.73	-28.3	-12.59
β-HCH	-48.74	29.52	-94.54	-20.04
γ-HCH	11.81	8.54	0.78	26.55
Pyrimethanil	6.16	10.16	-11.72	17.49
Pirimicarb	33.99	15.38	13.45	60.11
Heptachlor	150.22	81.99	30.38	270.74
Aldrin	194.27	56.21	120.46	286.89
4,4'-DCBP	-70.76	17.40	-95.64	-45.62
Heptachlor epoxide	95.58	32.48	50.01	146.75
α-endosulfan	259.46	99.05	117.3	384.1
4,4'-DDE	99.15	47.87	42.37	158.14
Dieldrin	-13.25	59.11	-83.83	63.35
Endrin	308.61	140.13	85.09	454.1
β-endosulfan	-21.93	52.27	-82.45	19.9
Endosulfan sulfate	26.82	92.67	-92.22	151.86
4,4'-DDD + 4,4'-DDT	47.26	143.73	-89.77	331.66
Methoxychlor	-12.53	62.24	-91.39	34.84

B3. Occurrence of OCPs

Since some pesticides such as α-HCH, heptachlor, 4,4'-DDT, 4,4'-DDD and methoxychlor were not detected, a total amount of 14 compounds were quantified in water samples from Macao, Considering all sampling campaigns, pesticides levels ranged 0.20 – 700 ng/L, presenting a global average concentration of 9.52 ng/L (Table B5). Compounds like 4,4'-DCBP, followed by fenobucarb, heptachlor epoxide, α-endosulfan and endosulfan sulfate presented the highest range of concentrations (4.17-691.77 ng/L, 5.93-231.13 ng/L, 1.65-59.98 ng/L, 1.41-40.61 ng/L and 2.03-23.78 ng/L, respectively).

Table B5. Summary table of the environmental levels (ng/L) quantified for each compound from surface waters of Macao's coastal environment.

Pesticide (ng/L)	Min	Max	Average	MD	SD	Frequency of samples > LOQ (%)
4,4'-DDE	0.82	1.78	1.32	1.27	0.17	100
Pirimicarb	0.21	2.92	0.71	0.39	0.77	83.33
Dieldrin	2.11	4.52	2.34	2.16	0.50	100
Endrin	2.73	4.75	2.90	2.74	0.50	100
γ-HCH	0.31	4.78	2.23	2.54	0.78	96.43
β-HCH	0.31	5.00	1.27	0.64	1.47	43.75
β-endosulfan	0.90	6.05	1.45	1.14	0.99	100
Pyrimethanil	0.29	9.11	1.82	1.34	1.66	100
Aldrin	1.33	15.33	2.92	1.99	2.75	100
Endosulfan sulfate	2.03	23.78	7.04	5.85	4.15	90.91
α-endosulfan	1.41	40.61	4.27	2.73	7.43	100
Heptachlor epoxide	1.65	59.98	9.28	5.68	10.75	100
Fenobucarb	5.93	231.13	41.65	30.57	38.89	100
4,4'-DCBP	4.17	691.77	54.04	25.14	103.08	100

Pesticide levels measured in Macao surface waters from NM areas presented significantly higher values (1.85x) than M areas. (Mann-Whitney, $U = 93.00$, $p < 0.05$)(Figure B2).

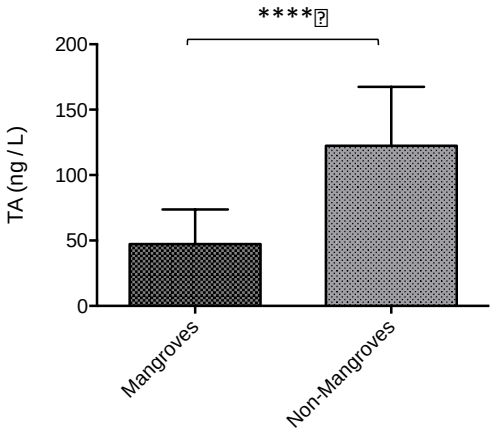


Figure B2. Total amount (TA, ng/L) of pesticides detected in M and NM areas (n = 32). Significant differences are indicated with an asterisk (****, $p < 0.0001$). Data are expressed as a sum of the average of all pesticides median values ± interquartile range.

Although no significant differences were found between seasons (Kruskal-Wallis, $p > 0.05$), transition periods showed a higher total amount of pesticides (TA) (Figure B3). Samples collected during transition II had the highest amount (TA = 103.77 ng/L), followed by transition I (TA = 86.55 ng/L). In both seasons, significant differences were found between M and NM areas (Mann-Whitney, $U = 4.0$, $p < 0.05$; Mann-Whitney, $U = 9.0$, $p < 0.05$; for transition I and II respectively). On the other hand, dry season (TA = 81.48 ng/L), was the only period that did not show a significant difference between M and NM areas. Finally, the lowest values (TA = 76.79 ng/L) were detected during the wet season. In this case, significant differences were also obtained between M and NM areas (Mann-Whitney, $U = 0.00$, $p < 0.05$).

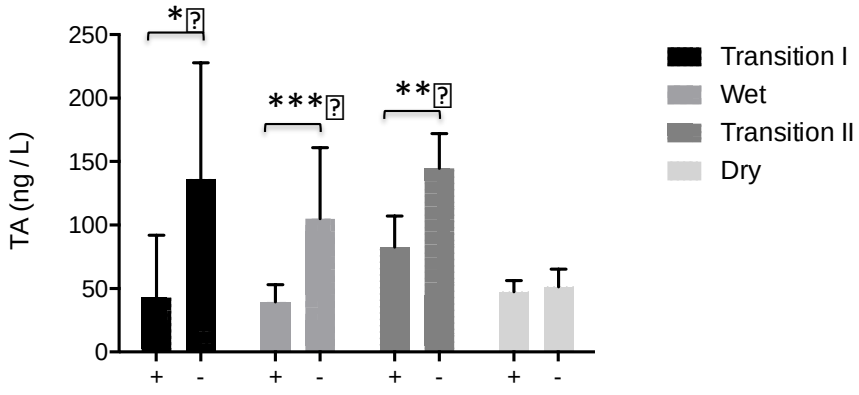


Figure B3. Seasonal distribution of the total amount of pesticides (TA, ng/L) quantified in M (+) and NM (-) areas. Significant differences are indicated with asterisk (*, $p < 0.05$; **, $p < 0.008$, ***, $p < 0.0007$). Data is expressed as sum of the average of the median values ± interquartile range, n = 8.

B4. Physicochemical analysis and nutrients

Detailed data regarding nutrients and physicochemical values are summarized in Tables S6. DIN and DIP presented higher values in M than NM areas, except for DIN values during transition II, although these differences were not significant. M areas presented a range of values of 0.79-2.49 ng/L and 0.06-0.10 ng/L for DIN and DIP, respectively. Lowest DIN values were quantified during wet season and the highest during transition II. A different pattern was observed for DIP, where the lowest values were detected during dry season and the highest during wet season. Regarding NM areas, a range value of 1.50-3.43 ng/L and 0.08-0.15 ng/L were quantified for DIN and DIP, respectively. In this case, the lowest DIN and DIP values were detected during wet and dry season, respectively; and the highest concentration of these nutrients were detected during transition I. With regards to chlorophyll-*a*, concentrations ranged from 20.98-34.86 mg/m^3 in M areas with a max. value during transition I, and a min. value during transition II while NM areas ranged from 1.10-18.34 mg/m^3 with a max. value during transition II and a min value during dry

season. Regarding TSS, a range between 0.04-1.33 mg/L and 0.03-0.10 mg/L was detected for M and NM areas, respectively. In both cases, max. values were detected during transition I and min. values during dry season. Finally, TDS values were higher than TSS, with ranges from 1.96-10.41 mg/L for M areas and 1.82-10.52 mg/L for NM. M areas presented the highest amount during transition I, and transition II for NM areas. The lowest values were detected during wet season for both areas.

The temperature fluctuated from 21˚C (dry season) to 33˚C (wet season), with an annual average temperature of 26.91˚C. Concerning DO (%) levels, most of the water samples presented acceptable levels (70.00–107.20%) according to the 75/440/EEC Directive, which establishes a minimum of 70% DO for surface waters (European Economic Commission, 1975). However, water collected during transition I-II (in M areas) and wet season (in M and NM areas) presented levels below the optimum established. Salinity ranged from 1.69 to 9.61 with the lowest values found during wet season, and pH was constant during the whole sampling period, with an average value of 7.77 (Table B6).

Table B6. Physicochemical parameters analysed in each sampling location during transition (I and II), wet and dry season.

		DIN (mg/L)	DIP (mg/L)	N/P	TSS (g/L)	TDS (g/L)	Chl. a (mg/m^3)	T (°C)	pH	DO (%)	Salinity (ppm)
Transition I	M	2.6±0.7	0.4±0.5	35.9±31.5	1.6±0.7	9.9±1.9	31.8±16.9	30.0±3.3	7.5±0.2	64.2±8.7	8.9±2.1
	NM	4.2±2.7	0.4±0.6	19.3±8.5	0.1±0.04	8.9±3.9	9.0±6.4	30.8±2.3	7.8±0.1	78.8±10.7	7.6±3.3
Wet	M	0.9±0.4	0.1±0.02	14.2±9.8	0.5±0.4	2.5±1.6	20.9±8.1	31.4±2.8	7.6±0.2	67.6±28.1	2.1±1.4
	NM	1.6±0.6	0.2±0.2	11.0±6.9	0.1±0.06	2.0±0.4	13.9± 5.5	33.2±2.6	7.5±0.3	69.7±19.7	1.7± 0.4
Transition II	M	2.8±0.9	0.1±0.03	40.4±6.3	0.8±0.4	6.4 ± 2.8	34.5±10.7	22.9±0.6	7.9±0.08	45.6±4.5	5.9±2.8
	NM	2.9±1.6	0.2±0.2	17.4±4.4	0.1±0.1	10.5±2.6	20.4±11.0	23.3±0.5	7.8±0.1	77.8±18.9	9.6±2.6
Dry	M	2.4±1.6	0.1±0.03	29.3±9.1	0.2± 0.2	4.7±3.1	29.7±19.3	22.1±0.2	8.2±0.3	87.7±7.9	4.2±2.2
	NM	2.4±1.3	0.1±0.1	21.3±11.5	0.03±0.01	8.5±4.5	1.4± 1.4	21.8±0.2	7.8±0.5	107.2±24.6	8.0±4.5

Appendix C

C1. Chemicals and reference standards

The analytical grade solvents methanol, ethyl acetate, and dichloromethane were purchased from Merck Limited Company (Germany). Ultrapure water was obtained from a Milli-Q water system (resistance = 5.1 μΩ/cm at 25°C).

Dicofol-d_8 (used as surrogate and internal standard) and 4,4'-dichlorobenzophenone (4,4'-DCBP) were purchased from Sigma-Aldrich (Steinheim, Germany). Both compounds were individually prepared in methanol with 0.1% acetic acid to produce the final stock solution of 1 000 000 ng/L and kept in the dark at -20°C. D-sorbitol and 3-ethoxy-1,2-propanediol (used as protectants) were purchased from Sigma-Aldrich (Steinheim, Germany). Stock solution of 182 mg/mL in 70% methanol:H_20 and 800 000 mg/L in 100% methanol were prepared for D-sorbitol, and 3-ethoxy-1,2-propanediol, respectively. Different concentrations of the mixture in a proportion of 1:10 (D-sorbitol:3-ethoxy-1,2-propanediol) were used in order to select the one presenting better response. Stock solutions of 3-ethoxy-1,2-propanediol were kept at 4ºC, and D-sorbitol and the protectant mixture were stored in the same conditions as the surrogate and standard. For the quantification purpose, an aliquot of each sample (57 μL) was taken, mixed with a protectants solution (3 μL) at a final concentration of 0.005:0.05 mg/L, respectively.

C2. Water sample pre-concentration (SPE)

The selected pesticide was extracted by solid-phase extraction (SPE) using the OASIS HLB cartridges (200 mg, 6cc; Waters, Ireland) following three different published methods (M1 to M3): Pepich et al., (2005), Cruzeiro et al., (2015) (Figure C1) and the standard Oasis HLB methodology. After analysing the 4,4'-DCBP recovery rates with these methods, the best protocol was adapted and improved.

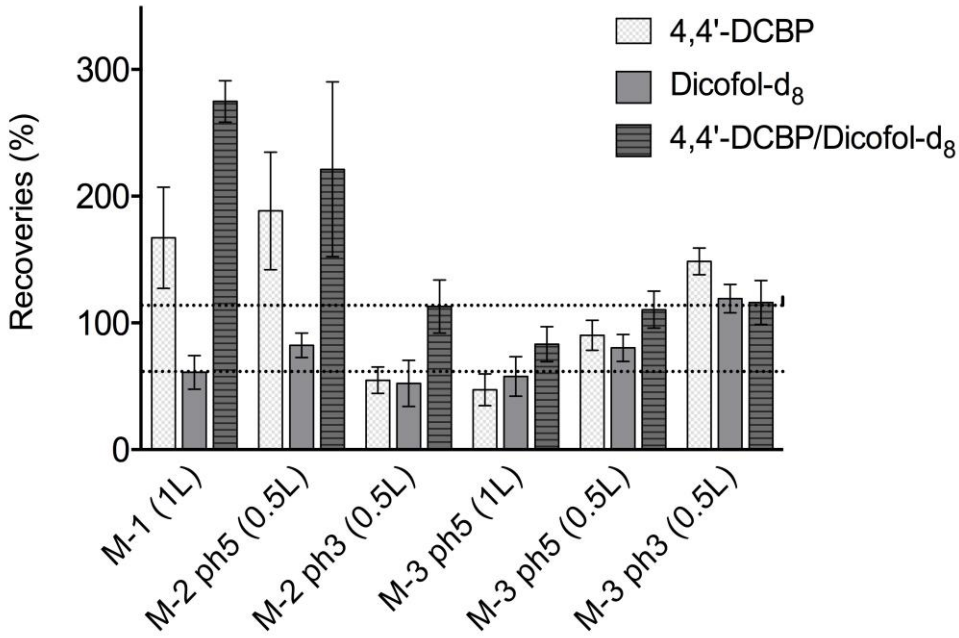


Figure C1. Recoveries (%) obtained using different extraction methods adapted from Oasis SPE standard protocol (M-1); Cruzeiro et al. 2015 (M-2) and Pepich et al. 2005 (M-3). The dash lines indicate the accepted range of recoveries according to the SANCO/825/00 criteria.

Considering the optimum recovery ranges (70-120%) defined by SANCO/825/00 rev 8.1 (European Commission Directorate General Health and Consumer Protection, 2010), M-3 (Pepich et al., 2005) presented the best response (80.3-110.5%) when adapted to pH 5, therefore it was chosen for the next validation steps. The original pH was changed due to the known instability of these compounds in alkaline waters.

C3. Ion selection and optimization

The ions selection and the collision energies for quantification purposes were obtained from the auto selected reaction monitoring (auto SRM). Information from published methods, regarding the target ions were also taken into consideration (A. de Kok et al., 2005; EU Reference Laboratories for Residues of Pesticides, 2013; Pereira et al., 2014). The software Xcalibur (version 4.0.27.10, Thermo Scientific), together

with the NIST library, were used for ion products confirmation; final data in Table C1.

Table C1. Information about the ions from 4,4'-DCBP and dicofol-d_8. For the characterization purpose the ions 141 and 143 were used to quantify 4,4'-DCBP and dicofol-d_8, respectively.

MW (g/mol)	Mass of parent ion	Mass of product ion	Collision Energy
4,4'-DCBP	**141**	113	15
251.11	215	152	20
	250	215	5
Dicofol-d_8	**143**	115	15
375.96	145	117	15
	258.1	143,1	10

C4. Validation studies and matrix effect

The validation procedure followed the European guidance document on pesticide residue analytical methods SANCO/825/00 rev 8.1 (European Commission Directorate General Health and Consumer Protection, 2010). This process includes the evaluation of linearity, accuracy, precision, recoveries, limit of detection (LOD), and limit of quantification (LOQ). Linearity was evaluated using three independent calibration curves, each with seven nominal standard concentration (ranging from 3–400 ng/L) spiked (200 µL) into spring water matrix (previously filtered and acidified) with the surrogate (50 ng/L) and at 20 ppm salinity. Curves were plotted using the ratio between the standard (4,4'-DCBP) and the IS area (dicofol-d_8). In order to avoid interferences derived from the matrix (spring water), the fortified samples were subtracted from a non-fortified sample (blank). The LOD and LOQ were determined with the same curves, using the following formulas: LOD = 3.3 α/S and LOQ = 10 α/S, where α is the standard deviation of the response and S is the average slope of the calibration curves. Taking into consideration the LOD and LOQ values, the range of

concentrations of the calibration curve were adjusted (ranging from 0.8-50 ng/L) for the environmental sample characterization.

Recoveries, accuracy and precision were evaluated by analysing three independent replicates of each quality control samples (QCs) at three levels of concentration (low, medium and high) calculated according to the SANCO/825/00 rev 8.1 (European Commission Directorate General Health and Consumer Protection, 2010), *i.e.* QC_{low} = 2LOQ (1.6 ng/L), QC_{medium}= 20LOQ (16.48 ng/l) and QC_{high}= 100LOQ (82.4 ng/L). Recoveries were determined by comparing the area ratio in spiked spring water with the area ratio of the same concentration in a matrix blank spiked after extraction. Precision was expressed as the relative standard deviation (%RSD) of the replicate measurements, and the accuracy of the method was evaluated as the percentage of agreement between the methods results and the nominal amount of added compound (data in Table C2).

Table C2. Results of recoveries, accuracy and precision (RSD) of the three quality controls (QCs) used for the validation (2LOQ, 20LOQ and 100LOQ). The number of replicates is indicated by R1, R2 and R3. Values are expressed by mean (± SD).

QC (µg/L)	Recovery (%)	SD	Accuracy (%)	SD	RSD (%)	
4.12	107.92	10.94	110.22	9.20	10.14	R1
	121.24	10.92	110.07	9.12	9.00	
	104.08	3.02	98.26	2.83	2.90	
	104.14	13.63	90.69	11.28	13.09	R2
	72.13	1.99	72.74	15.45	2.76	
	83.40	1.45	99.35	16.15	1.74	
	107.03	1.55	91.44	1.94	1.45	R3
	107.70	6.80	90.15	8.50	6.31	
	99.93	2.04	82.17	1.67	2.04	
41.2	119.88	9.31	116.00	5.41	7.76	R1
	115.77	2.03	105.50	1.69	1.75	
	97.39	2.09	92.00	1.96	2.15	
	119.60	3.28	102.65	5.21	2.74	R2

	93.94	3.77	95.10	20.65	4.02	
	86.53	0.58	90.98	4.21	0.67	
	119.59	11.17	107.45	13.94	9.34	
	119.38	2.86	100.36	9.23	2.40	R3
	112.44	8.26	92.38	6.74	7.35	
	98.85	11.82	99.55	8.34	11.95	
	117.39	9.02	106.85	7.54	7.69	R1
	106.76	12.36	100.76	11.57	11.58	
	119.95	8.23	99.95	4.81	6.86	
206	80.29	3.63	77.01	10.47	4.52	R2
	100.32	14.00	108.14	14.97	13.95	
	118.64	6.71	101.69	8.37	5.65	
	105.06	4.01	83.67	3.20	3.82	R3
	107.25	11.80	88.15	9.63	11.00	

New liners were used every 200 injections. During all processes, solvent (methanol) with protectants and matrix blanks (spring water) were systematically analysed to prevent potential contamination, and triplicate samples were used in every day injection.

In gas chromatography, matrix-effect is described as one of the main sources of errors in multiresidue analytical methods, and it is attributed to the presence of active sites in the injector, which causes the differences in the observed response for the given analyte in solvent compared to response in sample matrix (signal suppression or enhancement; Peček et al., 2013). One common way to solve the matrix effect problem is the use of analyte protectants (Peček et al., 2013; Sánchez-Brunete et al., 2005). In this study, a mix of the protectants D-sorbitol and 3-ethoxy-1,2-propanediol at three different concentrations were evaluated in order to counteract the enhancement of the chromatographic response. The lowest and the medium concentrations of protectants gave the best results (data not shown), so the mix

containing 0.005:0.05 mg/l of D-sorbitol:3-ethoxy-1,2-propanediol respectively, as a final concentration in the extract, was chosen for further analysis.

The matrix effect (ME) was evaluated at the lowest concentration (2LOQ), where six water matrix samples were spiked after extraction ($A_{standard\ in\ matrix}$) and compared with those of injected standards ($A_{standards}$), as indicated in the following equation:

$$ME = -\frac{Astandard - Astandard\ in\ matrix}{Astandard}\ x\ 100$$

If the ME results equal to zero, no matrix effect is presented, whereas ME above or below zero represents a signal enhancement/suppression, respectively. In this case, an average value of -53.73% was obtained indicating a suppression effect. To compensate this matrix effect and avoid underestimation, matrix matched calibration curves were used.

Finally, the stability was analysed immediately after preparation and later at 24, 48, 72 and 96h after samples were being kept at -20°C, where the best results were obtained only after 24 and 48 h of extraction (Figure C2).

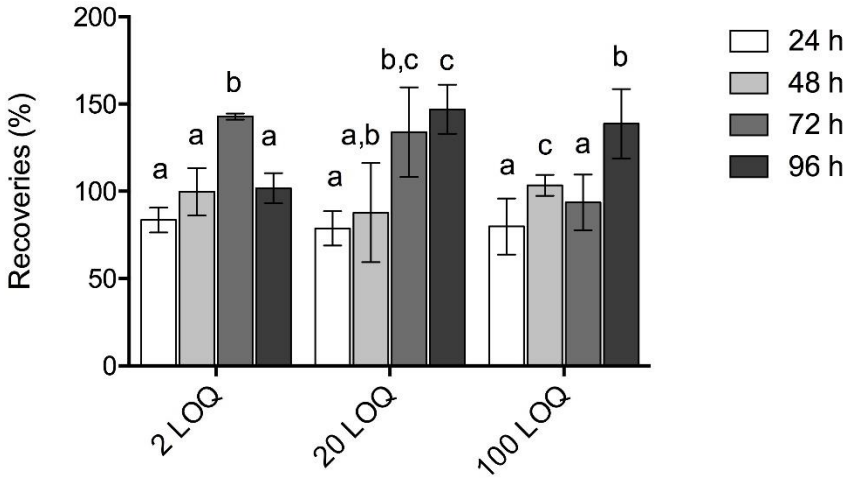


Figure C2. Stability of 4,4'-DCBP extracted at three different concentrations (2LOQ, 20LOQ and 100LOQ) from spring waters after 24, 48, 72 and 96 h; the results are expressed by mean (± SD).

C5. Monitoring results

Table C3. Levels of 4,4'-DCBP (ng/L) measured in the PRD during two different seasons (Transition and Wet) at high and low tide (HT and LT, respectively). Values are expressed by mean (± SD).

Location	Transition season				Wet season			
	HT		LT		HT		LT	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
M1	3.71	1.03	3.21	0.97			2.79	0.6
M2	3.29	0.68	3.77	0.45				
M3	5.1	1.26	5.03	1.35	< LOQ		< LOQ	
M4	2.98	0.63	3.56	0.71				
M5	4.26	1.38	5.62	1.24				
HK1	5.66	1.35	6.17	1.37				
HK2	4.79	1.18	5.39	1.06				
HK3	14.53	1.97	29.87	2.04	< LOQ		< LOQ	
HK4	15.75	2.15	17.91	2.87				
HK5	10.85	1.09	14.34	1.42				

Table C4-a. Physicochemical parameters analysed in each sampling locations during transition (trans) and wet season (wet).

		DIN (mg/l)		DIP (mg/l)		N/P		TSS (g/l)		Chl-a (mg/m^3)	
		Trans	Wet	Trans	Wet	Trans	Wet	Trans	Wet	Trans	Wet
HK1	HT	16.43	0.75	0.13	0.09	290.12	17.95	0.01	0.02	2.17	21.23
	LT	2.39	0.77	0.07	0.11	76.42	15.55	0.03	0.29	9.02	31.72
HK2	HT	13.19	0.96	0.03	0.1	1106.47	20.78	0.02	0.02	34.18	2.29
	LT	14.73	1.03	0.08	0.09	429.73	23.72	0.02	0.02	4.47	7.34
HK3	HT	0.95	0.69	0.03	0.02	63.54	93.27	0.04	0.05	-	1.6
	LT	1.98	1.57	0.01	0.02	662.94	175.58	0.03	0.06	0.52	4.69
HK4	HT	1.07	0.94	0.09	0.18	25.71	11.44	0.11	0.09	9.71	13.29
	LT	1.47	0.99	0.16	0.23	20.48	9.58	0.09	0.48	9.26	10.29
HK5	HT	1.13	9.85	0.03	0.12	84.1	178.68	0.04	0.04	3.76	5.39
	LT	1.9	4.52	0.03	0.15	127.76	68.85	0.05	0.2	4.32	10.51
M1	HT	1.41	1.19	0.12	0.01	26.34	397.56	0.01	0.03	33.16	27.3
	LT	3.71	3.72	0.16	0.22	50.85	37.77	0.02	0.01	21.44	44.73
M2	HT	1	0.97	0.12	0.09	18.71	23.17	0.03	0.02	15.83	14.15
	LT	1.44	2.22	0.13	0.17	24.76	29.79	0.29	0.11	13.01	24.55
M3	HT	1.08	2.01	0.16	0.16	14.72	28.1	0.03	0.05	22.45	15.14
	LT	1.39	1.32	0.15	0.18	20.85	16.45	0.26	0.19	15.83	17.99
M4	HT	1.12	1.08	0.06	0.59	44.36	4.03	0.01	0.03	10.78	10.53
	LT	1.13	1.27	0.12	0.05	21.68	53.34	0.28	0.27	19.84	49.67
M5	HT	0.96	1.47	0.13	0.05	16.55	65.94	0.03	0.04	12.34	25.19
	LT	1.18	2.01	0.13	0.05	20.91	90.05	0.39	0.76	25.97	10.21

Table C4-b. Physicochemical parameters analysed in each sampling locations during transition (trans) and wet season (wet).

		T (°C)		pH		DO (%)		Salinity		TDS (g/l)	
		Trans	**Wet**	**Trans**	**Wet**	**Trans**	**Wet**	**Trans**	**Wet**	**Trans**	**Wet**
HK1	HT	22.4	28.1	8.06	7.64	86.25	104.8	29.53	15.16	29.6	17.15
	LT	22.8	30.3	8.47	7.39	93.75	84.3	29.42	7.34	29.49	8.09
HK2	HT	22.5	27.9	8.56	7.27	95.3	68.02	31.3	14.64	30.94	15.78
	LT	22.6	26.7	8.4	7.18	66.9	68.23	31.6	15.85	31.44	16.94
HK3	HT	25.2	29.3	7.76	7.01	101.2	93.83	36.07	30.22	35.43	30.19
	LT	25.8	30.6	7.82	7.19	104.2	96.94	34.32	31.32	35.94	30.31
HK4	HT	24.9	30.7	7.55	7.5	72.78	75.5	20.74	7.92	-	8.98
	LT	22.3	29.9	7.57	7.5	44.79	68.8	14.66	7.73	-	8.77
HK5	HT	24.4	26.8	7.09	7.99	146.2	120.41	27.16	33.37	19.67	34.3
	LT	24	28	7.64	8.13	108.4	151.3	18.77	33.1	19.67	33.6
M1	HT	24.8	30.2	7.76	7.5	62.49	102.03	1.77	0.19	2.19	0.26
	LT	23	28.1	7.4	7.19	20.66	24.16	0.8	0.36	1.03	0.48
M2	HT	26.5	31.4	7.8	7.6	94.06	108.43	10.7	4.7	11.79	5.59
	LT	21.4	28.6	7.48	7.28	86.39	74.66	6.86	1.25	7.79	1.6
M3	HT	27.3	32.5	7.77	7.3	112.12	98.62	15.77	8.98	16.84	10.1
	LT	22.6	29.5	7.9	7.15	78.54	87.42	27.29	10.5	28.08	11.65
M4	HT	26	29.9	7.37	7.9	105.19	103.44	3.57	0.4	4.26	0.69
	LT	22.7	29	8.4	7.95	64.43	75	10.15	0.42	11.19	0.47
M5	HT	24.8	28.9	7.89	7.5	77.75	94.2	2.71	0.18	3.28	0.24
	LT	21.2	32.6	7.58	7.49	87.75	86.12	2.64	0.24	3.19	0.33

Appendix D

D1. Clams' survival optimization

First of all, it was important to decide the target organism for this study. For this purpose, two different species (*Philipinarum* and *Meretrix)* were kept in the lab, but only *Meretrix* were able to survive more than 8 weeks in lab conditions.

Then, three different salinities were tested (17, 22 and 26 ppt). The salinity range was based on the natural conditions registered in the Pearl River Delta, where the clams come from (Poutiers, 1998). Since fluctuations in the salinity due to tide it will not happen, it was important to test which permanent salinity is more appropriate for the organisms. The results showed that lower mortality it was observed at 17 ppt. Clams were able to survive at these conditions during 2 months.

Finally, the temperature was set at 27°C, according to the average temperature in Macao between September and October (time period when the experiment was done).

D2. Method validation

For characterization purpose, the same ions as indicated in Figure C1 were used to quantify 4,4'-DCBP and dicofol-d_8.

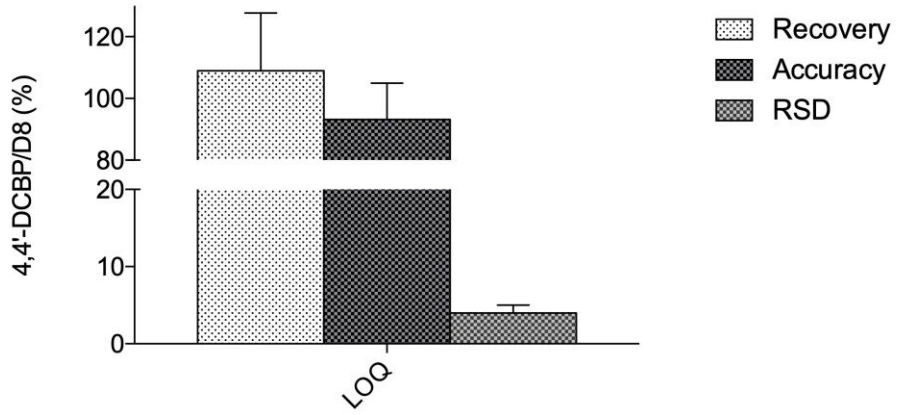
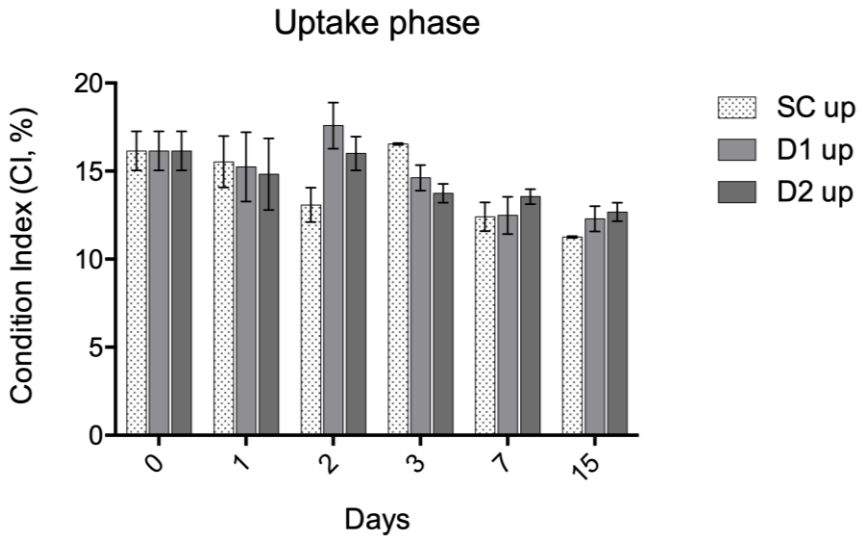


Figure D1. Recovery (%), accuracy (%) and RSD (%) obtained during the method validation. Results are expressed by 4,4'-DCBP/dicofol-d_8 mean ± standard deviation.

D3. Condition index

In the graphs below, information regarding the condition index (%) is presented. No significant differences were observed between SC and dicofol treatment during the whole experiment.



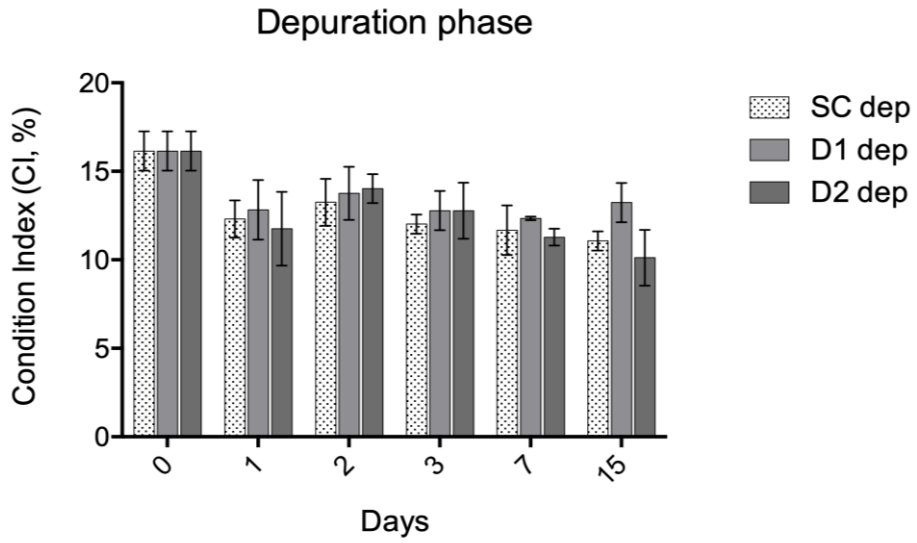
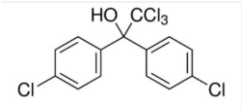
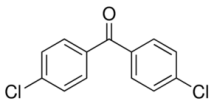
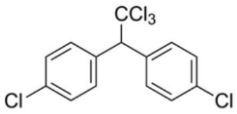
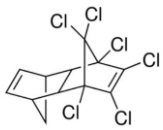
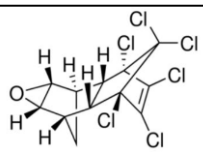
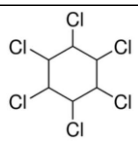
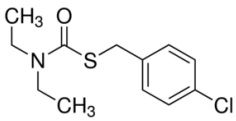


Figure D2. Condition index (%) of the bivalve *M. meretrix* during uptake phase (upper graph) and depuration phase (bottom graph). Results are expressed by mean ± standard deviation, (n = 3 per sampling time).

D4. Pesticides comparison table

Compound Name	CAS Name	Structure	Sw (mg/L)	log Kow	log BCF
Dicofol	115-32-2		0.80	4.30	4.00
4,4'-DCBP	90-98-2		0.84[a]	4.62	3.83[b]
DDT	50-29-3		0.06	6.91	3.5
Aldrin	309-00-2		0.027	6.50	3.52 3.92[c]
Dieldrin	60-57-1		0.14	3.70	4.54 5.43[c]
α-HCH	319-84-6		2.00	3.82	3.76[c]
Thionbencarb	28249-77-6		16.10	4.23	na

Sources: PPDB, Pesticides Properties Data Base; a) Theoretical value obtained with Ecosar version 1.11; b) BCF value obtained in our study; c) Experimental values obtained by Richardson et al. 2005.

Figure D3. Structure and properties of some pesticides (mainly OCPs) used for data discussion.

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