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FATE AND EFFECTS OF CHLOROPHENOLS TO PHYTOPLANKTON SPECIES FROM PORTUGUESE FRESHWATERS

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Pesadelos cor-de-rosa, azuis, violeta,
mios guturais, rugidos de mansinho,
corro e corro mais e nunca chego à meta,
sou perseguido apesar de estar sozinho,
acomodado neste terrível pesadelo!
Estou livre, preso na lã deste novelo
que desnovelo ao ler novelas de novo,
estou quentinho e apertadinho no ovo
até perceber que sou da geração rasca
e então começo a bicar a minha casca
e não me deixam porque assim entra a luz!
Para muita gente é esse o caminho da cruz,
essa libertação, esse passeio, o calvário,
ter mais um dia a perder no calendário
quando após 366 tudo está na mesma!
Nem um centímetro ao lado, nem uma lesma
no seu vagar conseguia andar tão devagar!
Arrasto e deixo que o meu rasto se vá apagar
pois trilho o sarilho deste feio passeio
sem encontrar ou sequer tentar um meio
de lá voltar e aprender com o passado!
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do que um discurso sempre repetido, gasto,
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This is a scientific thesis
dedicated to passion,
a wonderful feeling
common to both
lovers and
scientists
…

RESUMO

O pentaclorofenol (PCP), o mais tóxico dos clorofenóis (CPs), é um pesticida de largo espectro, considerado um poluente prioritário devido à sua persistência no ambiente e elevada toxicidade em todos os tipos de organismos, mesmo em concentrações baixas. O conhecimento dos efeitos dos CPs em cianobactérias, e nomeadamente do PCP e da sua remoção por espécies fitoplanctónicas, é escasso. Neste trabalho, estudou-se os efeitos do PCP em duas espécies fitoplanctónicas comuns e ubíquas: a cianobactéria *Microcystis aeruginosa* e a microalga *Chlorella vulgaris*.

Inicialmente, foi desenvolvido um método de microextracção para fase sólida, no espaço emerso, acoplado a cromatografia gasosa com detecção por captura electrónica para determinação dos CPs. O método permitiu a determinação simultânea de sete CPs, com graus de cloração diferentes, e foi aplicado com sucesso pela primeira vez em amostras de água ambientais. Foram estudados vários processos para a conservação de amostras de CPs em diferentes matrizes aquosas de modo a seleccionar os melhores procedimentos para o respectivo armazenamento a longo prazo antes de serem submetidas a análise.

Posteriormente, o método optimizado foi usado em estudos de ecotoxicidade para a determinação de PCP em meio de cultura Fraquil, dopado em níveis ambientais, na presença das espécies fitoplanctónicas isoladamente e, posteriormente, em culturas mistas. A toxicidade do PCP nas espécies foi avaliada através do estudo da variação do respectivo crescimento, durante dez dias, usando a densidade óptica e o conteúdo em clorofila *a* para testes em espécies isoladas e contagens celulares ao microscópio para experiências com culturas mistas.

A toxicidade do PCP segue o modelo hormético e foi bastante elevada para a cianobactéria para concentrações do composto superiores a dezenas de $\mu g\ L^{-1}$. Contrariamente, a microalga foi insensível ao PCP excepto para níveis de concentração muito elevados. As espécies estudadas exibiram perfis de toxicidade e aptidões de remoção ou estabilização do PCP assaz distintas. A cianobactéria *M. aeruginosa* foi capaz de remover parte do PCP do meio de cultura enquanto a microalga *C. vulgaris* estabilizou este composto. Foram observadas interacções

interespecíficas complexas em culturas com a mistura das espécies. A exposição ao PCP poderá conduzir a alterações da estrutura da comunidade fitoplanctónica dulciaquícola. O eventual aparecimento de florescências de cianobactérias tóxicas pode ser facilitado por influência do PCP.

Palavras-chave: Pentaclorofenol · Ecotoxicidade · *Microcystis aeruginosa* · *Chlorella vulgaris* · Água doce

ABSTRACT

Pentachlorophenol (PCP), the most toxic of the chlorophenols (CPs), is a broad-spectrum pesticide, considered a priority pollutant due to its persistence in the environment and high toxicity to all kind of organisms, even at low concentrations. The knowledge of the effects of CPs on cyanobacteria, and namely PCP and its removal by phytoplankton species, is scarce. In this work, the effects of PCP on two very common and ubiquitous freshwater phytoplankton species were studied: the cyanobacterium *Microcystis aeruginosa* and the microalga *Chlorella vulgaris*.

First, a headspace solid phase microextraction coupled to gas chromatography with electron capture detection method was developed for the determination of CPs. The method allowed the simultaneous determination of seven different CPs, with different degree of chlorination, and it was successfully applied for the first time in environmental water samples. Several processes for CPs samples' preservation in different water matrices were studied in order to select the best procedures for the respective long-term storage before analysis.

Second, the optimized method was used in ecotoxicological studies for the determination of PCP in Fraquil culture medium, spiked at environmental levels, in the presence of the phytoplankton species separately and, posteriorly, in mixed cultures. The PCP toxicity to the species was evaluated by studying the growth variation, during ten days, using optical density and chlorophyll *a* content for single species tests and microscopy cell counts for mixed culture experiments.

The toxicity of PCP to the cyanobacterium follows the hormetic model and was very high at PCP concentrations higher than tens $\mu g\ L^{-1}$. Conversely, the microalga was rather insensitive to PCP except in very high levels. The studied species had very different toxicity profiles and abilities to remove or stabilize PCP. The cyanobacterium *M. aeruginosa* was able to remove part of the PCP from the culture medium while the microalga *C. vulgaris* stabilized this compound. Complex interspecific interactions were noticed in a mixed culture of the species. PCP exposure might lead to changes in the freshwater phytoplankton community structure. The possible appearance of toxic cyanobacterial blooms may be eased by the influence of PCP.

Keywords: Pentachlorophenol · Ecotoxicity · *Microcystis aeruginosa* · *Chlorella vulgaris* · Freshwater

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ABBREVIATIONS AND SYMBOLS

2-CP	2-Chlorophenol
3-CP	3-Chlorophenol
4-CP	4-Chlorophenol
2-C-5-MP	2-Chloro-5-methylphenol
4-C-2-MP	4-Chloro-2-methylphenol
4-C-3-MP	4-Chloro-3-methylphenol
4-C-3,5-DMP	4-Chloro-3,5-dimethylphenol
2,3-DCP	2,3-Dichlorophenol
2,4-DCP	2,4-Dichlorophenol
2,5-DCP	2,5-Dichlorophenol
2,6-DCP	2,6-Dichlorophenol
3,4-DCP	3,4-Dichlorophenol
3,5-DCP	3,5-Dichlorophenol
2,3,4-TCP	2,3,4-Trichlorophenol
2,3,5-TCP	2,3,5-Trichlorophenol
2,3,6-TCP	2,3,6-Trichlorophenol
2,4,5-TCP	2,4,5-Trichlorophenol
2,4,6-TCP	2,4,6-Trichlorophenol
3,4,5-TCP	3,4,5-Trichlorophenol
2,3,4,5-TeCP	2,3,4,5-Tetrachlorophenol
2,3,4,6-TeCP	2,3,4,6-Tetrachlorophenol
2,3,5,6-TeCP	2,3,5,6-Tetrachlorophenol
3,5-DM-4-CP	3,5-Dimethyl-4-chlorophenol
AA	Acetic anhydride
Acetyl	Acetylation
AES	Atomic emission spectrometry
ASE	Accelerated solvent extraction
AUC	Area under growth curve
C_0	Initial concentration
CAR/PDMS	Carboxen/polydimethylsiloxane
CC	Cell count
CC_C	*Chlorella vulgaris* cell count
CC_M	*Microcystis aeruginosa* cell count
CF	Continuous flow
chl_a	Chlorophyll *a* content
CPs	Chlorophenols
CRM	Certified reference materials
CT	Cryogenic trapping
CW-TPR	Carbowax-templated resin
DAD	Diode array detection
DLLME	Dispersive liquid-liquid microextraction
DVB/CAR/PDMS	Divinylbenzene/carboxen/polydimethylsiloxane
DMSO	Dimethyl sulfoxide
EC	Electrochemical detection
EC_{20}	Effective concentration to 20% of the organisms
EC_{50}	Median effective concentration
ECD	Electron capture detection
EEC	European Economic Community
EU	European Union
FCC	Ferrocenecarboxylic acid chloride
FID	Flame ionization detection

FLD	Fluorescence detection
FLLabel	Fluorescence labelling
GC	Gas chromatography
GCB	Graphitized carbon black
GR	Average growth rates
GR_C	*C. vulgaris* average growth rate
GR_M	*M. aeruginosa* average growth rate
HLB	Hydrophilic lipophilic balance
HS	Headspace
IC	Ion chromatography
IC_{25}	Inhibitory concentration to 25% of the organisms
IC_{50}	Half maximal inhibitory concentration
K_{oc}	Adsorption coefficient
K_{ow}	Octanol-water partition coefficient
LC	Liquid chromatography
LC_{50}	Median lethal dose
LD	Liquid desorption
LLE	Liquid-liquid extraction
LLLME	Liquid-liquid-liquid microextraction
LOD	Limits of detection
LOEC	Lowest observed effect concentration
LOQ	Limits of quantification
LPME	Liquid phase microextraction
LVI	Large volume injection
MAC	Maximum admissible concentration
MASE	Membrane-assisted solvent extraction
MIC	Minimum inhibitory concentration
MRL	Maximum residue limit
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MSPE	Magnetic solid phase extraction
MW	Microwave
Na-PCP	Sodium pentachlorophenate
NOEC	No observed effect concentration
OD_n	Optical density at $_n$ nm
OECD	Organization for Economic Co-operation and Development
P_0	Vapour pressure
P-A	Purge-assisted
PA	Polyacrilate
PCP	Pentachlorophenol
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimehtylsiloxane/divinylbenzene
PEG	Polyethylene glycol
PFBenzyl	Pentafluorobenzylation
PFBenzoyl	Pentafluorobenzoylation
pKa	Acidity constant
POLE	Polyoxyethylene-10-lauryl ether
POPs	Persistent organic pollutants
PT	Purge and trap
PTFE	Polytetrafluoroethylene
PTV	Programmed temperature vaporization
QuEChERS	Quick, easy, cheap, effective, rugged and safe
rpm	Rotations per minute
RSD	Relative standard deviation

RT	Retention time
RTC	Resource Technology Corporation
S-DVB	Styrene-divinylbenzene
SBSE	Stir bar sorptive extraction
SD	Steam distillation
SDE	Steam distillation extraction
Silyl	Silylation
SM	Stir membrane
SPE	Solid phase extraction
SPME	Solid phase microextraction
TD	Thermal desorption
TEA	Triethylamine
US EPA	United States Environmental Protection Agency
USA	United States of America
UV	Ultraviolet detection
VPDB	Poly(vinylpyrrolididone-divinylbenzene)
WHO	World Health Organization

PART I

INTRODUCTION

Background

A never-ending challenge

Organization of the thesis

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A never-ending challenge

I would say that water is the main challenge for mankind in the beginning of the new millennium, namely its accessibility and quality. Of all the water in the planet, only a very small part, freshwater, is available for human consumption. Freshwater is so important that life is not possible without it. Despite the scarcity and preciousness of this global consumption good, freshwater is constantly being polluted by anthropogenic sources in the name of the so-called “development”, with negative consequences to the environment, including our own species.

We, humans, are not alone in the connection to freshwater. Many species rely on freshwater to survive as well. Some of them, like freshwater cyanobacteria, are much older and far more important than us in the well being of the environment, since we depend much more on them than the opposite. Cyanobacteria were here first and helped “creating” the conditions to our own evolution. Together with other phytoplanktonic species, particularly microalgae, they are the basis of the aquatic ecosystem as primary producers due to their photosynthetic ability. It is vital for the following so-called “higher” species in this complex web of biotic relationships that they can succeed in their “function” of organic matter producers. Even the word “higher”, as found in the previous sentence, can be deceptive. It should be understood only as a description comparing the levels that the different species occupy in the food web and not as a higher position in the evolutionary development. Fortunately, the traditional anthropocentric vision of the environment is changing in the last decades and awareness is being raised to the problems concerning our planet.

In a time of economical crisis, austerity and political misleading, even when the scientific investigation and the environmental protection suffer the most from funds shortage, it is very important to continue focusing on the environmental issues. I believe that our society is now ready to consider difficult questions. What will decrease more the standards of life, less money or less health? How much money are we ready to pay for a clean planet? Future generations are going to reap the consequences from a past century of wild industrialization and an indecisive present. Such harmful consequences, already being felt, will drive us to a future where “the children will be held responsible for the crimes of their parents”.

The responsibility must be shared by a starving mankind. The lion’s share of the environmental pollution problem comes from agriculture: nowadays, we can’t imagine our industrial society without pesticides. Too much people to feed and limited arable fields, whether by economical or environmental reasons; productivity must be high and pesticides

are very helpful in this purpose. Pesticides were crucial in the development of modern agriculture. The control of pests, plagues and diseases markedly improved the efficiency of food production [1]. Nevertheless, undesirable side effects occur frequently, such as damage to non-target organisms or general environmental contamination, caused specially by persistent pesticides [2]. Industrial societies will have public health problems to solve and economical losses related to the necessary decontamination due to pesticides misuse, not to mention the ecological issues like biodiversity decrease and damage to non-target organisms. This might lead, and eventually actually will, to a societal response such as banning of some toxic compounds, always with associated costs. The decision will, most probably, be a political one, or, in other words, it will be a matter of interpretation of the reality; it is difficult to reach a consensus about these subjects. Must a toxic compound be banned? Must its use be restricted? Which are the acceptable levels in the environment? Works like the present one will help in the always difficult decision making process. However, new pesticides, as well as new formulations of the existing ones, and new uses for them, are emerging. The challenge is in a constant update: new studies are always required.

Pesticides, as well as other anthropogenic pollutants, were commonly regarded in an anthropocentric view, as if their effects were exclusive to human health. Fortunately, in the last decades, the broader view of the environmental implications of these pollutants has been arising. Not only direct effects on humans are important but also the effects to lower levels of the trophic chain can be implicated in the well being of the entire planet, mankind included. Thus, any environmental change can have, at least, indirect effects on the human beings. By understanding the role of these pollutants in “simple” species, we can have an idea of the concrete effects to the phytoplanktonic communities, their role for the removal and environmental transformation of contaminants, the possible mechanisms of detoxification and the extrapolation of the data to other species.

The definition of this environmental problem is difficult. “Misuse” of pesticides is commonly the scapegoat of this complex issue but may not be the best term to define the problem. The first pesticides are contemporary with the first agricultural based societies, being all natural products. The use of pesticides has been growing since and in the early 20th century synthetic pesticides were introduced in the agriculture. For long, people had no idea of the environmental implications of pesticides; simply, they didn’t even think about it. Legislation for the regulation of pesticides also dates from the beginning of the last century but it was very scarce, bashfully applied and couldn’t prevent the widespread overuse of synthetic pesticides and its consequences. This consciousness arose only in the 1960s, being remarkable the 1962 book “Silent Spring” by Rachel Carson, marking the beginning of

the global environmental movement [3]. However, she could not see the accomplishment of her dreams. The society, namely the industry and the governments, was not yet ready to accept the existence of environmental problems and violently attacked everyone who dared to be different and to raise the voice against the environmental hostility. In spite of other environmental activists have followed the example of Rachel Carson, concrete actions for environmental protection took place only a decade after “Silent spring” was published, with the noteworthy ban of the well-known pesticide DDT for agricultural use, in the USA, in 1972 [3].

Other factors are certainly involved in the awakening of our society. The American scientist Neil deGrasse Tyson even suggests that the environmental protection acts were only possible after the publication of the picture of the Earth seen from the moon, in December 1968. It is very probable that this picture (Fig. 1) raised the public consciousness to another level. The planet Earth could no longer be considered just a “battlefield” with borders and national rivalries but, on the contrary, a global place where everyone has to share the clouds, the oceans and the continents.

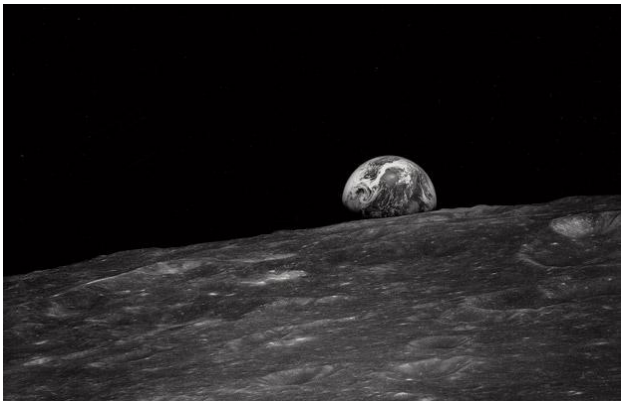

Fig. 1. The Earth seen from the Moon [7].

While environmental legislation is much more advanced and efficiently applied nowadays, it is still far from being present in all the countries in the World. Even when legislation is present, it is not always implemented, due to economical reasons or general lack of knowledge. The adoption of less-polluting farming practices can only be made with the full cooperation of the agriculture community and largely depends on demonstrating both the environmental and economic benefits of such practices to land managers [4]. Global legislation is urgent but, again, it is difficult to reach a consensus due to the estimated economic losses subsequent to the pesticides restrictions and unwillingness of the governments to spend money in broad education programmes for proper pesticide application. The European citizens have demonstrated a growing interest in understanding how their tax money is used in efforts to guarantee the safety and quality of the consumer

goods and the environment [4]. The crisis in Europe started to have some economic consequences on the food and environmental safety in the recent years, affecting tourism and food industry. Anyway, can the environmental costs be estimated?! This is a huge issue that requires diplomacy, foresight and patience to be solved.

It is not by chance that pesticides adverse effects were, and still are, much neglected. The study of pesticides, and their effects in living species, is very difficult due to the complexity of biotic and abiotic processes involved [5]. Furthermore, historically, the ecotoxicity studies were based mainly on effects in mammals and in target species, largely forgetting the coexistence of non-target species that could be also affected. The huge number of possible interactions between all these entities in the environment turns prediction of the effects a complex exercise of uncertainty [6]. It is not easy to accept a truth based in such uncertainties. It is sure that environmental risk is present and we suffer negative consequences derived from pesticides overuse but the mechanisms are so intricate that we must rely on prevention first of all. In nature, a multiplicity of processes tends to remove toxic compounds from water [2]. In evaluating the effects of pesticides on non-target species, like phytoplankton, a comparison is intended for understanding the mechanisms in the real environment. Ecotoxicity tests using single species, including phytoplankton, allow easy control of the conditions and interpretation of direct effects. Adding additional ecological layers, like the simultaneous presence of two phytoplankton species, as in this study, will increase the resemblance of natural conditions at the cost of time consuming labour and higher price. In that case, the results may be more difficult to interpret but they are definitely closer to the real conditions [6].

Having in mind the now common motto “Think globally, act locally!”, and the normal limitations of environmental studies, this work intends to be a small contribution to help solving the problem of environmental pollution. By studying the interactions between chlorophenols (CPs), widespread organic pollutants, and two ubiquitous phytoplankton species from temperate freshwaters, also common in Portugal, I hope that this data can be useful, in the near future, not only to Portugal but to all similar freshwater environments, and that it can be helpful in the control of the undesirable cyanobacteria blooms and destructive pesticides’ pollution.

Apart from these practical purposes, I believe this work has the advantage of treating fundamental problems and proposing new investigation methods both in Analytical Chemistry and Ecotoxicology. The interdisciplinary character of this research is a direct consequence of the complexity of the environmental issues. A combination of methods

belonging to previously separated scientific areas is a necessary condition to solve these problems.

Structure of the thesis

This thesis is composed of four parts and eleven chapters. Each chapter ends with a list of the references enclosed in it. The first part, “Part I – Introduction”, comprises the first three chapters of the thesis. This section is a broad introduction concerning the two main subjects of this project: the determination of CPs in environmental samples and the ecotoxicity of CPs in the freshwater environment. Chapter 1, entitled “Phytoplankton in the freshwater environment”, will briefly discuss the role of phytoplankton in the freshwater environment and the species selected for the ecotoxicity tests. In Chapter 2, named “Fate and toxicity of chlorophenols in the freshwater environment”, the presence of CPs in the freshwater environment and its implications are considered, leading to the target compound chosen to the studies. Throughout Chapter 3, called “Chromatographic determination of chlorophenols in environmental samples”, the existing analytical chromatographic methodologies for determination of CPs are reviewed, mainly regarding water analysis. All the objectives of this work are summarized in Chapter 4, “Objectives”.

The second part, “Part II – Experimental conditions”, comprehends a description of the practical laboratory work carried out to accomplish the aims of this thesis. It consists of Chapter 5 – “Materials and methods”, which describes all the methodology, materials, reagents and techniques used throughout this study.

The third part, named “Part III – Results and discussion”, contains a collection of the obtained results and their discussion, pointing out some conclusions. It is arranged in four chapters, the first two including the analytical chemistry results and the second two the ecotoxicology results. Chapter 6, “A headspace SPME-GC-ECD method suitable for determination of chlorophenols in water samples”, describes a method that was developed for the determination of CPs in water samples. Chapter 7, entitled “Stability of chlorophenols in water: sample storage procedures”, is a study of the stability of CPs in different water samples and the best procedures for their long-term storage. In Chapter 8, “Behaviour of the cyanobacterium *Microcystis aeruginosa* when exposed to pentachlorophenol and comparison with that of the microalga *Chlorella vulgaris*”, ecotoxicity studies appear, namely the fate and effects of the chosen toxicant on cyanobacteria, the main novelty of this thesis, and also comparison with the results obtained for microalgae. Chapter 9, named “Toxicity of pentachlorophenol to a mixture of freshwater phytoplankton: *Microcystis aeruginosa* and

Chlorella vulgaris", expands the knowledge obtained in the previous chapter to more realistic conditions, particularly a mixture of the same phytoplankton species, in the presence of the same toxic compound.

The last part of this essay, termed "Part IV – Conclusions", is divided in two chapters: "Chapter 10 – General Conclusions" and "Chapter 11 – Future research". The first chapter comprises the general outcomes drawn from this study, pointing to their environmental meaning, and the latter reflects on the perspectives of future work that arise from the present thesis and on some questions still waiting for an answer.

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Chapter 1

Phytoplankton in the freshwater environment

1.1. Eutrophication and phytoplankton blooms

Eutrophication is the process of nutrient enrichment, particularly P and N, in their diverse forms, that stimulates phytoplankton blooms, primary productivity and massive growth of macrophytes [1]. Eutrophication has been recognised as a significant problem for more than four decades and it's far from being solved.

The eutrophication can happen naturally by leaf decay, lixiviation of nutrients from upstream water bodies and natural forest fires. However, anthropogenic pressure, like industry, urbanization and its effluents [2], agricultural activities, particularly the use of pesticides and fertilizers, and inadequate management of watersheds, like the increase of irrigation and deforestation, all of these considered major causes of eutrophication, may accelerate the process [1, 3]. Warmth, over 20 °C, sunlight exposure and quiescent water also promote blooms [4]. The intensification of farming and growth of world population, with consequent increase of waste disposal, actually contributed to the large impact of eutrophication in the last decades [1].

Currently, it is known that not only the excess of nutrients is responsible for phytoplankton blooming. Pesticides, despite being generally toxic to phytoplankton, may promote their growth and, consequently, blooming [5]. Thus, the potential role of CPs, extensively used as pesticides, in promoting phytoplankton blooms, or inhibiting it by its toxic effects to phytoplankton species, must be investigated.

Generally, eutrophication studies are focused on surface freshwater ecosystems. Considering the relationship between surface area and water volume, they are much more influenced by any external or internal sources of contaminations than open ocean areas [3]. The occurrence of phytoplankton blooms in eutrophic rivers is the most common effect of eutrophication and can cause severe damages to ecosystems and human health, as well as economic losses. Several groups of phytoplankton can produce blooms but the most severe negative effects are caused by dinoflagellates, diatoms, and cyanobacteria, being the latter the most common in the eutrophication of freshwater systems [3].

Blooming of high cell densities and biomass, and consequent collapse, may lead to hypoxia, or anoxia, and therefore mass mortality of sensitive species, like fish, and major changes in the ecosystem [1]. Biodiversity losses can occur at all trophic levels due to habitat deterioration, with increased turbidity and decreased oxygen concentration [2]. The variation in pH, with high values (over 9) during daytime and the warmer months and low

levels at night, may also stress the environment and cause changes in the biogeochemistry that can increase the negative effects stressed before. The odour and taste of water can be negatively affected as well [2, 3].

When Portugal joined the European Economic Community (EEC) in 1986, its main agricultural indices contrasted significantly with those of the other member states. Agriculture made up 12% of gross domestic product, and provided employment to roughly 20% of the active population. In Portugal, the adoption of new technologies, namely improved seeds, higher inputs of fertilizers and pesticides, and mechanization, were introduced later than in other European countries. Sparingly used fertilizers and pesticides partially avoided the negative impact in terms of soil and underground water contamination that took place in many European countries [6]. Currently, the situation is expected to have changed after the progressive convergence with the current European Union (EU) standards. This probably means higher levels of eutrophication and, consequently, more phytoplankton blooms despite knowing that the development of freshwater blooms is highly irregular and far from being completely understood [7, 8]. Nevertheless, data about eutrophication in Portugal in the last decades is scarce and discrete but research is increasing lately. Most of the reports show increasing levels of eutrophication [9-12], with one exception [13], in different water bodies throughout the country.

1.2. Freshwater phytoplankton

Phytoplankton lives in fluctuating environments where many factors, such as sinking, light availability and nutrient uptake, influence the distribution of phytoplankton in time and space [14]. The composition of phytoplankton community is a critical component in environmental monitoring, ecosystem restoration and management [15]. The current work treats representatives of two of the most important groups of phytoplankton: cyanobacteria and microalgae. Their role in the freshwater environment may be affected by pollutants and it is important to know how such interactions occur, namely in the possible event of blooming [16]. Diatoms represent another major group of phytoplankton that, despite being ubiquitous, is specially important in oceans where they are estimated to contribute up to 45% of the total oceanic primary production [17, 18]. The current work will not focus on this group.

1.2.1. Cyanobacteria

As can be stated by the stromatolites found in Australia, cyanobacteria have evolved as the most primitive oxygenic phototrophic organisms, appearing about 3200 million years ago [19], almost as old as known life itself, and so are responsible for the atmospheric oxygen we rely on for living [3]. They are a remarkably diverse group of bacteria, although highly specialized, adapted to a variety of ecological habitats such as terrestrial, glaciers, aerial, marine, brackish and freshwater environments [20].

The cyanobacteria usually are a main component of the phytoplankton in surface freshwater ecosystems. Wrongly, cyanobacteria are sometimes considered microalgae; the historic, and informal, term “blue-green algae” enhances the confusion but actually cyanobacteria constitute a separate phylum in the domain Bacteria [21]. They are gram-negative prokaryotes that contain chlorophyll *a*, like the eukaryotic algae and plants. Some species are also able to fix N_2 via specialized cells, heterocysts. A number of species can also regulate their buoyancy, by specialized intracellular gas vesicles, and move vertically within the water column. Blooms can have a multiplicity of aspects, from blue-green to black, in colour, and from colonies to mats and scums, in shape [4].

Nowadays, cyanobacteria take part in no less than one fifth of the global photosynthetic primary production of biomass, accompanied by the cycling of oxygen [19]. Giving this fact states their current importance, they are also very well studied because of their longevity as taxonomic group, their capability of forming blooms, some of them toxic, and also because of the interesting production of natural products with many applications, most notably pharmaceuticals [3]. Having high chemical stability and water solubility, these natural products are already, and will continue to be, an important subject of scientific research [20].

As already stated, the most common phytoplankton organisms associated with the eutrophication of freshwater systems are cyanobacteria and they form blooms leading to very high cell densities that cause major changes in the ecosystem, related with the loss of water quality, harm to human health and to economy. The direct consequences of these blooms are the decrease of transparency and oxygen levels, the production of off-flavours and toxins [3]. There are multiple toxin-producing cyanobacteria genera and cyanotoxins. Harmful cyanobacteria are known to produce various types of cyanotoxins, such as hepatotoxins, neurotoxins, cytotoxins, irritants or gastrointestinal toxins [4]. Cyanotoxins may have lethal effects on many aquatic or terrestrial organisms, including humans [22]. In spite of the diversity of toxins found so far, some cyanobacteria species and some strains within

reported toxic species are not able to produce these toxins [3]. Toxic blooms can also have substantial economic impacts, namely on tourism (which is a particularly relevant activity in Portugal), human health, restriction of fish and water consumption and the considerable costs of mitigation of blooms and restoration of lakes and rivers [3, 4].

The occurrence of toxic blooms of cyanobacteria has been increasing worldwide in recent decades, and will continue to increase with warming climates and continued nutrient loading from human activities, with blooms appearing in previously unaffected areas [4]. In Portugal, most of the reported toxic occurrences are due to the cyanotoxins microcystins. Cyanobacteria can be found in many different types of aquatic systems, tending to bloom in less disturbed and slow flowing rivers such as Minho, Douro, Tejo or Guadiana and in natural lakes such as those of the central Portugal region between Aveiro and Figueira da Foz. Reservoirs used as drinking water sources, like Torrão, Bravura and Aguieira, as well as wastewater treatment plants, also have blooms [3].

1.2.1.1. *Microcystis aeruginosa*

Microcystis aeruginosa Kützing, 1846, is a ubiquitous cyanobacterium often linked with the unwelcome production of toxic blooms. It is the most common toxin-producing cyanobacterium, producing hepatotoxic microcystins, the main type of cyanobacterial toxin, responsible for liver disease and liver and colon cancer [23]. Its morphophysiological characteristics promoted their great success in the phytoplankton community. It requires little energetic input to sustain cellular balance, being capable of withstanding low nutrient levels, has high affinity to N and P and contains gas vacuoles that permit buoyancy [22]. The cells are about 2 to 3 μm in size and colonies can grow bigger than 200 μm in size [24]. The *M. aeruginosa* doubling time was calculated as 3.25 days, in one experiment [25], and 1.24 to 1.39 days in *in situ* experiments when N was not limiting growth [26].

Off all cyanobacteria genera, *Microcystis* is the most important genus responsible for water blooms and production of microcystins. Not all *Microcystis* strains produce toxins, though. Morphologically identical strains can vary in toxicogenicity in a same bloom or regulate the level of toxicity under varying laboratory conditions. The reason for such natural variations in toxicity is unknown, as well as the conditions inducing the toxin release are not clear [22]. However, it is known that toxicity is determined by microcystin production genes and a wide range of environmental factors can regulate their expression. Some strains can spontaneously lose their toxicity under laboratory conditions [27].

Microcystis aeruginosa is a common cyanobacterium in Portugal and presents a significant water quality problem. It blooms, namely during the summer, in water bodies used for drinking and recreation, with consequent toxin production, sometimes at high concentrations [28]. Different *M. aeruginosa* strains isolated in Portugal have been reported as toxic, with production of microcystins [2].

Limited information is available on the toxic effects of *M. aeruginosa* caused by pollutants and endocrine disruptors [27, 29]. Data is particularly scarce about toxicity of CPs to this cyanobacterium. This fact, together with its ubiquity and decisive role in the freshwater environment, justify why this is the chosen cyanobacterium species for this study.

1.2.2. Microalgae

Algae are a miscellaneous and large group of autotrophic organisms, chiefly, ranging from unicellular (microalgae) to multicellular forms, some of them with several metres in length. Green algae, the large group of algae from which plants are thought to have emerged, are known to be comparatively sensitive to many chemicals; they have been considered indicators of the bioactivity of industrial wastes and they vary in their response to a variety of toxicants [30].

Microalgae are simple microscopic photosynthetic unicellular eukaryotes, notably responsible for about half of all of the atmospheric carbon fixation and typically inhabiting aquatic environments, soil surfaces and other locations exposed to light [31]. Microalgae, together with cyanobacteria, form the basis for most of the food webs in aquatic ecosystems as major representatives of the phytoplankton [32]. As primary producers in the aquatic environment, their essential role in nutrient cycling and oxygen production is critical to many ecosystems [30], so microalgae are vital for water quality monitoring programs as well [32]. They provide food and oxygen by photosynthesis to the next levels of the food chain, like zooplankton, therefore they directly affect the structure and functioning of the entire ecosystem [29].

Nowadays, besides ecotoxicity studies, microalgae are mainly grown for the production of several compounds of economical interest, like β-carotene, food and biofuel [33-35]. In general, they have a high ratio between surface area and volume and so have a high potential reactivity with pesticides, namely sorption, which has been specially recognized with respect to the persistent and lipid partitioning organochlorine contaminants, where CPs are included. Microalgae were considered to be useful for studying pesticides'

accumulation since they are known to metabolize some pesticides. Evidence suggests that different types of microalgae possess, or acquire, the capability of fission of the aromatic and heterocyclic substrates so it is possible that microalgae play an important role in the degradation of aromatic compounds and pesticides [31].

Organic pollutants greatly influence the growth of microalgae [29]. It is important to study the effects of organic pollutants on algal growth for controlling algal bloom, monitoring and eliminating organic pollution, and assessing ecological risk of organic pollution. Microalgae can be used in risk assessment due to its sensitivity to toxicants, easy acquisition, small size and fast reproduction. There are many studies about effects of nutrients, such as N and P, and heavy metals on the growth of microalgae but in recent years attentions have focused on the effects of organic pollutants [29].

While degradation and toxicity of CPs have been well documented using bacteria (excluding cyanobacteria, obviously) and fungi, only few toxicity studies have been conducted with microalgae. The knowledge on the toxicity of CPs toward algal growth is of great importance due to its role in natural water bodies as a major primary producer and its possible existence in the microbial communities of wastewater treatment plants [36].

1.2.2.1. *Chlorella vulgaris*

Chlorella vulgaris Beyering, 1890, is a ubiquitous unicellular green algae belonging to the phylum Chlorophyta. This microalga is spherical in shape, about 2 to 10 μm in diameter, and without flagella. Microalgae contain the green photosynthetic pigments chlorophyll *a* and *b* in its chloroplast. They multiply through photosynthesis and requires only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce [37]. This low nutritional requirement may be one of the reasons for the success of the genus. *Chlorella* doubling time was found to be between 1.5 and 1.8 days [38, 39]. Like some other microalgae, *C. vulgaris* can have more than one type of metabolism, for instance heterotrophic besides photoautothrophic, and undergo metabolic shifts in response to changes in environmental conditions [34].

Interestingly, the microalga *C. vulgaris* has immunological effects such as antibacterial and antiviral action and protection to oxidative stress. Ingesting *Chlorella* spp. can lower blood sugar levels and increase haemoglobin concentrations among other beneficial effects. Feeding microalgae to elderly people or animals has been demonstrated to protect against age-dependent diseases, particularly cardiac hypertension or

hiperlipidemia. Moreover, it has been proved to have immune-modulating and anticancer properties [37]. Such positive effects do not seem to be very well known but research has been carried out recently, for instance the study of the addition of microalgae, such as those of the genera *Chlorella* and *Spirulina*, to fermented dairy products like yogurt in order to raise the viability of probiotics [37]. The good control of cultivation and low-cost harvesting, owing to the associated high cell densities, is very advantageous in the production of goods of interest like biofuel [34].

1.3. Population dynamics

1.3.1. Seasonal variations

Season is an important factor influencing the phytoplankton community composition. Several studies reported phytoplankton seasonal succession in lakes. For example, diatoms dominate under conditions of low temperatures and high levels of nutrients and cyanobacterial dominance coincides with the highest temperatures and lowest nutrient concentrations [2]. Phytoplankton assemblage was highly correlated with conductivity, temperature and nutrient concentrations, namely P. This may be due to the known competitive advantage and/or allelopathy of the bloom-forming cyanobacteria towards microalgae [2], as will be further discussed in this chapter.

The study of the effects of pesticides to phytoplankton regarding seasonal variation is an interesting subject. This was performed for pentachlorophenol (PCP), for example. It was reported that, in mesocosms experiments, the phytoplankton community responded differently to PCP throughout the year. The seasonal plankton community response could be ranked in the following order of decreasing sensitivity to PCP: autumn ≥ spring / winter ≥ summer [40]. Different biological factors may have contributed to such variations: different species assemblage, physiological states or physicochemical conditions. The community varied little regarding the dominant species of each group of phytoplankton, which were present during the whole year. Other factors, abiotic, that seasonally changed were temperature and dissolved oxygen. All these factors may have influenced PCP toxicity [40].

1.3.2. Shifts in the community composition

Phytoplankton lives in fluctuating environments where many factors such as grazing pressure, sinking, light availability, nutrient uptake and turnover influence the distribution of

phytoplankton in time and space. Determining the change of phytoplankton populations in nature may provide insights into the mechanisms by which phytoplankton populations maintain themselves [14]. Cyanobacteria, along with the environmental parameters, may work as an important modulator in the composition of phytoplankton community. This may be very significant when discussing the phytoplankton seasonal dynamics in a water body that suffers from potentially toxic cyanobacterial blooms [2]. This can be explained by the competitive advantages of cyanobacteria over other phytoplankton groups, like microalgae: the abilities to vertically migrate by regulating the buoyancy, fix N_2, store P, allelopathy [2], survival in low irradiance and low growth constant [41]. *M. aeruginosa*, in particular, has a great influence in the phytoplankton community structure. Its abundance is negatively correlated with that of the microalgae and diatoms [2].

As they are primarily photoautotrophs, cyanobacteria depend closely on irradiance. In general, cyanobacteria seem to have similar light requirements when compared with other phytoplankton groups, although the level at which photosynthesis becomes light-limited is lower for cyanobacteria than for microalgae. This is due to the accessory photopigments phycocyanin and phycoerythrin [41]. Buoyancy regulation is an additional advantage for the utilization of optimum light intensity [41]. On the other hand, it is known that, at the surface of water bodies, cyanobacteria are frequently exposed to high or excessive light intensities which can lead to surface scum undergoing photo-oxidation and subsequent death. However, at least for *Microcystis*, the photo-oxidation can be avoided by the production of protective carotenoid pigments. The growth responses of cyanobacteria to a wide range of light intensities are important for their survival and competitiveness [41].

The cyanobacteria optimum temperatures, around or even more than 25 °C, are higher than those for diatoms and microalgae and might explain why cyanobacterial blooms usually occur during the warmer months [41]. The temperature affects the cyanobacteria growth by changing the rates of enzymatic reactions, the molecular configuration of cellular constituents and other physiological phenomena. However, whilst a general relationship of accelerated cyanobacterial growth with elevated temperature is often observed, temperature alone is unlikely to be the most important environmental variable [41].

It was found that the number of the phytoplanktonic taxa was near the maximal at the time of a cyanobacterial bloom, with development of diatoms and particularly microalgae species that were not particularly prevalent over the remainder of the year. Phytoplankton community dynamics during a cyanobacterial bloom may give important information about the noxious potential of the bloom due to allelopathy and/or competitive advantage of cyanobacteria over microalgae [2].

The presence of cyanotoxins must be assessed along with allelopathic tests using the bloom-forming cyanobacteria in order to gain a better understanding of the phytoplankton community dynamics [2]. If a direct relation between phytoplankton assemblage dynamics, environmental parameters and cyanotoxin production could be established, it could be a useful mechanism to help understand, predict and prevent the development of cyanobacterial blooms (through control of nutrient inputs, for example) [2]. In what concerns microalgae allelochemicals, many of those already described affect higher levels of the trophic chain but little is known for those affecting co-occurring phytoplankton. Nevertheless, photosynthesis inhibition and decrease in growth rate under the influence of allelochemicals have been shown [42].

Moreover, cyanobacterial dominance may also change the cladoceran community since the toxicity of cyanobacteria such as *M. aeruginosa* towards cladocerans is well known and blooms can be toxic [2]. Cladocerans include *Daphnia magna*, a zooplankton representative, known to graze on phytoplankton species like *C. vulgaris* [43], composing the next trophic level. Mesocosms studies including different trophic levels in a same experiment must be carried out [30].

When in the presence of pollutants, if microalgae and cyanobacteria have strongly differential sensitivity, and specially if cyanobacterial sensitivity is strongly lower than green algae sensitivity, the contamination may result in a shift of the community structure from dominance by microalgae to dominance by cyanobacteria and may sustain cyanobacterial blooms at particular times. The contaminations can lead to more risks to the ecosystem [30]. The interactions of pollutants with phytoplankton species will be further discussed in the next sections.

1.3.3. Blooming

In aquatic ecosystems, as most research has shown, the formation of phytoplankton blooms is attributed to the overabundance of phytoplankton growth and the gradual shift of the phytoplankton community structure, usually from dominance by green algae to dominance by cyanobacteria or a gradual shift within the cyanobacterial population from dominance by one species to dominance by another species. The reasons for these shifts are not only due to physical factors, like light and temperature, but also nutritional factors, like N and P in water, and biological factors, like food chain and food web. In general, low N to P ratio levels enhance bloom occurrence in cyanobacteria [2].

At the time of a cyanobacteria bloom, several changes in the aquatic ecosystem occur. The availability of phosphate seems to be required for the formation of blooms, its concentration in water increases significantly and it can be the dominant anion for a long time [2, 44]. However, *M. aeruginosa* bloom formation is known to be related with the depletion of phosphate in the environment [2]. On the other hand, *M. aeruginosa* blooms occurred after the dissolved oxygen depletion and the sudden increase of the ammonium levels. The *M. aeruginosa* density remained relatively high even with phosphate depletion in the water. This is most likely due to its ability to store P and occurs in the presence of depleted environmental P levels although it requires environmental sources of N [2]. Moreover, in the phytoplankton community, there was a severe reduction in the chlorophyll *a* concentration at the time of the cyanobacterial blooms, despite the chlorophyll *a* concentration being highly variable [2, 9]. Conductivity and temperature, followed by the total suspended solids and nutrient concentrations, have shown to be the parameters with high correlation with the phytoplankton assemblages. Nevertheless, the occurrence of cyanobacterial blooms (mainly of *M. aeruginosa*) proved to be also an important parameter correlated with the phytoplankton assemblage during that time. This may be due to the cyanobacterial competitive advantage over microalgae but also to the toxicity of the bloom-forming cyanobacteria [2].

Environmental factors like light, temperature and nutrients greatly affect the growth of *M. aeruginosa* in laboratory cultures and do not exist individually in nature. The interactive effects of these factors can provide a better understanding of the growth of the cyanobacterium, by applying statistically based experimental design, as proposed by some authors [45]. The growth of the cyanobacteria under plentiful availability of nutrients becomes a function of light and temperature [41].

The cyanobacteria blooms pose potential health risks for humans and other organisms due to the release of different toxins to the environment [30], hence the need to control and to diminish cyanobacterial proliferation. Natural control of cyanobacterial blooms seems to exist. For instance, certain macrophytes produce and release metabolites, namely polyphenols and fatty acids as allelochemicals to inhibit cyanobacterial growth, which is consistent with experimental evidence and suggests the appeal of macrophytic vegetation for control of cyanobacterial blooms [46]. In what concerns artificial control of blooms, several studies have been conducted intending the inhibition or even removal of toxic blooms by a variety of methods, in order to reduce the amount of P or to decrease the abundance of nuisance phytoplankton species directly in the water bodies (in-lake measures) [23, 47]. These methods include chemicals, the so-called algicides, *sensu lato*.

Actually, instead of algicides, the word should be “cyanocides”, meaning a substance used to kill or inhibit the growth of cyanobacteria, but such word does not seem to exist; however, it will be used throughout herein. Nevertheless, most of the cyanocides, *sensu stricto*, are unusable due to the consequent secondary environmental pollution, inapplicability in the complex natural systems and high costs associated [23, 47]. Other methods for removal of cyanobacteria include grazing, biomanipulation, allelopathy, usage of barriers, aeration and destratification [48]. Cyanocides include metals (like Al and Fe), photosensitizers (like hydrogen peroxide), pesticides and chemicals derived from natural compounds. Although still pricey, the latter seems to be a good alternative to other cyanocides, since non-persistent and ecotoxicologically acceptable compounds are desirable to manage cyanobacterial blooms [48].

An important factor that can alter the blooming process is the presence of pollutants, as already referred. The wrong traditional view of both cyanobacteria and microalgae as representatives of algae might have negatively influenced the current knowledge on this matter. Thus, research comparing the differential sensitivity of cyanobacteria and microalgae is of important scientific significance [30].

All things considered, more ecological data are needed using different cyanobacteria species [41] and other surface freshwater species regarding allelopathic effects [2] to better understand the occurrence of cyanobacteria blooms.

1.4. Phytoplankton interaction with pesticides

The investigation of the surface freshwater environment where cyanobacteria and microalgae are known to co-occur and interact with different chemicals can provide valuable data about how pollutants alter the growth and development of organisms, the response of such organisms to different pollutants and the adverse impacts of these pollutants in the environment [16]. Phytoplankton species, as primary producers in the aquatic food chain, are good indicators of ecotoxicological risk from exposure to chemicals, namely pesticides. Data obtained from phytoplankton is specially useful as specific responses to toxicants may be expressed at lower contaminant doses. Prediction of injurious consequences to the environment is always desirable [49].

Microalgae are known to be comparatively sensitive to many chemicals. They have been considered indicators of the bioactivity of industrial wastes and they vary in their response to a variety of toxicants. Their ecological position at the base of most aquatic food

webs, along with cyanobacteria and other groups of phytoplankton, and their essential roles in nutrient cycling and oxygen production are critical to many ecosystems. Much information on the toxicological aspects of pesticides on microalgae has been obtained but little is known about the toxicological aspects of pesticides on cyanobacteria [30, 50].

Several factors affect phytoplankton ecotoxicity tests, including the medium composition, pH, CO_2 supply, light, temperature, test endpoint, exposure time, phytoplankton biomass concentration, interspecific variability and the pesticides' concentrations and sorption. If such factors are uncontrolled, differences in growth rate may cause different results with different species, although the test systems are claimed to be identical. Considerable genuine variability certainly exists among species with regard to their sensitivities to chemicals [49].

Phytoplankton plays a central role in the biogeochemical cycles of pesticides in the aquatic environment. Various possible pathways exist for the introduction of pesticides into food webs, such as interaction with the sediment and direct absorption during fish respiration. Nevertheless, since phytoplankton is at the base of the food chain, its uptake is thought to be a key process in the transfer of pollutants from water to the next trophic levels. Also, phytoplankton uptake of pollutants influences the transport, occurrence and distribution of pesticides in the aquatic environment. For instance, the vertical distribution of some pesticides was found to be correlated with the vertical profile of phytoplankton biomass [51].

1.4.1. Phytoplankton response to pesticides

1.4.1.1. From an ecosystem point of view

Ecological systems are hierarchically structured, from the non-living subcellular structures to the highly complex biosphere. Biosphere is composed of ecosystems, ecosystems are composed of communities and communities are composed of populations that are composed of organisms of a same species. Therefore, looking at a single population does not reveal much about the entire freshwater ecosystem, for example. A population is composed of different individuals with different tolerance to stress factors. This is due to the natural genetic and physiological diversity of a population [52]. Populations are involved in various and complex interactions between each other and with the surrounding environment. A dynamic pattern of sequential declines and recoveries of the populations naturally occurs. However, the situation becomes more complex and difficult to predict when new variables are added, like the presence of pollutants.

Exposure to a toxicant affects not only the individual organisms but the entire population as well as its interactions with other populations and, in fact, it affects all the other levels of complexity. Toxicants, such as pesticides, may affect different species to significantly different degrees, even when the species are phylogenetically or environmentally closely related [49]. The most tolerant individuals of a population will thrive under stress conditions [52].

Generally, substances of environmental concern, namely pesticides, are repeatedly discharged in the ecosystem or are persistent in it or even both. Chronic exposure to a toxic compound may represent a selective pressure to which a population responds by adapting. Microcosms' experiments have found examples of increased tolerance at the community level due to changes in the genetic structure after chronic exposure to a toxicant, leading to an increase in the species' diversity. This is in agreement with the evolutionary theory, which predicts that exposure to a stress factor, such as a toxicant, should affect the diversity of a biological system [52].

1.4.1.2. From a population point of view

Exposure to toxicants can, and probably will, affect the growth rates of a population. An initial exposure causes a fast decline of most of the individuals of a population. This will lead to a lower population density, or biomass, and a decrease in the tolerance to the toxicant. Under prolonged exposure, tolerance to the toxicant will increase. Low concentrations of the toxicant will affect a non pre-exposed population but not a pre-exposed one. Furthermore, the selective pressure generated by the exposure should be stronger for a biological system that has never been exposed to it than for a pre-exposed one. The latter shall have a better tolerance in case of further exposures. This tolerance increase shall conduct to a higher population growth rate under a given exposure, a broader tolerance range, a higher EC_{50} (median effective concentration) and a smaller variability in the growth rates [52].

It was observed, for a non-axenic laboratory culture of *M. aeruginosa*, that stress reduces the genetic and physiological variability of a population by selecting against the individuals least able to adapt, for a time presumably extending over a few generations. This may reduce the potential of the population to resist further stresses [52]. However, this can be reversed after prolonged and repeated exposures, as referred [52].

Nowadays, biological populations are being exposed to a great number of many new substances and this shall be a matter of concern. Such level of stress will, probably, accelerate the rates of adaptation and changes in the ecosystem. The dynamics of adaptation involve so many different processes that it is hardly possible to understand and evaluate them without the aid of computer modelling [52].

1.4.1.3. Effects of pesticides on phytoplankton

Generally, the responses of phytoplankton to pesticides are little or nonexistent at low doses, ranging up to growth inhibition at high concentrations [49]. At least, this is the common assumption of toxic effect of pesticides on phytoplankton. Nevertheless, some tendency for phytoplankton growth stimulation by low doses of pesticides has been noticed. For instance, PCP on the microalga *Chlorella ellipsoidae* increased the algal biomass, peaking after 48 hours of incubation [49], and low doses of nonylphenol enhanced the growth of both toxic and nontoxic strains of the cyanobacterium *M. aeruginosa* [29]. This suggests a hormesis effect of pesticides on phytoplankton species. Hormesis is a biphasic dose-response mechanism characterized by a modest growth stimulation at a low dose of pollutant and a growth inhibition at a high dose, resulting in an inverted U-shaped dose response [29]. This seems to be a very common and important mechanism that is still not very well understood [53].

The effects of pesticides on phytoplankton seem to be mainly related with photosynthesis, phosphorylation and protein synthesis [5]. Probably, a combination of several factors and distinct mechanisms occurring simultaneously, most of which related with energy transduction, may be the main responsible for the pesticides toxicity [54, 55]. Certain herbicides inhibit the photosynthetic electron flow and this is likely to be the most important way how chemicals impact phytoplankton. For example, ureide herbicides could inhibit the Hill reaction of isolated chloroplasts, an effect that could be correlated with chemical's lipophilicity. This can lead to changes in the chlorophyll content [49]. Several other related parameters can be studied. For instance, there has been relatively little research on the effect of pesticides on carbohydrate metabolism in phytoplankton. The total carbohydrate accumulation is a parameter related to the photosynthetic activity of phytoplankton [49, 56].

1.4.2. Different sensitivity to pesticides among phytoplankton species

There is a considerable diversity in phytoplankton sensitivity to pesticides. This sensitivity depends on the species, the pesticides and the experimental conditions [49]. There are few reports concerning the differential responses of various cyanobacteria and microalgae to pollutants. Nevertheless, differential sensitivity of cyanobacteria and microalgae was found for several pollutants like the organophosphate insecticides fenitrothion [57] and methyl parathion [49], carbamate insecticides (carbosulfan, propoxur, carbofuran, carbaryl and metolcarb) [30], organochlorine pesticides (lindane and PCP) [49], herbicides metribuzin [58] and isoproturon [49] and endocrine disruptors (4-octylphenol, 4-nonylphenol and β-estradiol) [59].

The difference in sensitivity between cyanobacteria and microalgae is highly expected. The organization of prokaryotes, like cyanobacteria, is very different from that of the eukaryotes, like microalgae. The adaptations to a same habitat, the surface freshwater environment, may relieve such phylogenetic differences but not extinguish them. In cyanobacterial cells, the photosynthesis and oxidative phosphorylation occur in the cytoplasm [59], while in eukaryotic cells such phenomena occur in the chloroplasts and mitochondria, respectively. These organelles are enclosed by a double membrane [60]. Therefore, pesticides must pass through an additional barrier, the double membrane, to arrive to its site of action in eukaryotic cells. Moreover, prokaryotes may have less elaborated enzymatic antioxidant pathways [59]. Also, cyanobacterial cells have a bigger surface to volume ratio compared to microalgae, which is known to increase the transfer rate of hydrophobic toxic substances [51]. For example, a study on twelve different phytoplankton species exposed to the organophosphorus insecticide fenitrothion suggested that tolerance to this compound may be inversely correlated with the cell surface area to volume ratio, cell lipid content, and the inherent bioconcentration potential of the cell [57].

Considering all these data, it seems that eukaryotic cells, due to their higher degree of organization, may be less sensitive to pesticides, in general. For instance, prokaryotes, like *M. aeruginosa*, are, in fact, generally more sensitive to antibacterial agents than microalgae and, because of this, prokaryotes must be always included as aquatic toxicity test species [61]. Nevertheless, this fact can be balanced with some particularities of cyanobacteria. The photosynthetic apparatus of some cyanobacterial strains showed to be extremely adaptable under exposure to different pollutants, allowing the maintenance of their photosynthetic performance, as for example in *Anabaena* sp. [62] and *M. aeruginosa* [63, 64]. To perform photosynthesis, cyanobacteria contain protein complexes called phycobilisomes, anchored to the stromal surface of thylakoid membranes [62]. These light-

harvesting complexes deliver the light energy, mostly absorbed by phycobiliproteins, to the photosystem II reaction centres through chlorophyll *a* and carotenoids [62, 65]. It has been shown that these photosynthetic pigments can have different sensitivity to toxicants [62-64]. Microalgae, during their evolution, have lost the cyanobacterial phycobilisome light-harvesting system [60]. Therefore, this can be a disadvantage to microalgae in the event of exposure to pesticides affecting photosynthesis.

Studies on a molecular level are still lacking and are necessary to identify and quantify the contribution of each possible mode of action of the pesticides toxicity on phytoplankton [55]. This will clarify the reasons for the different sensitivity to pollutants observed among phytoplankton species. The ability to detect early molecular responses to chemicals is crucial to the understanding of the biological impact of pollutants [66].

1.4.3. Microcosms and mesocosms experiments

Biotic and abiotic factors prevailing in terrestrial and aquatic ecosystems affect both phytoplankton and pesticides prior to and during their interactions. However, direct comparison between pesticide concentrations in natural waters and those added to the medium must be interpreted with caution because, in nature, several processes occur and tend to change or remove the chemicals from the water. Pure culture studies separate the responses of one species in defined conditions from the interaction of many organisms, which is an advantage [49]. Therefore, single species toxicity tests have historically been the sources of biological data for hazard evaluation. A common practice is the use of manifold single species toxicity tests, for each toxic compound in study and each species, individually. Some reports of comparative sensitivity of pesticides toward various microalgae species have been published [30]. However, though both sensitive and reproducible, single species tests lack environmental realism because species seldom occur in isolation but rather as part of complex communities and the crucial interspecific interactions are not considered [67]. Nevertheless, it remains to be further studied whether there exists a correlation between the effect on the laboratory scale single species growth tests and the actual effect of multiple mixed species growth in the field [30].

Multiple species toxicity tests such as microcosms and mesocosms tests enable observation of the indirect effects of chemicals caused by interactions among species. However, conducting mesocosms tests to assess the impact of chemicals on ecosystems has its disadvantages. Mesocosms tests involve skilled and time consuming labour, are expensive and the obtained data is not easy to interpret [30].

Although numerous studies have evaluated the toxicity of a single chemical to phytoplankton, few studies have estimated toxicity of two or more chemicals [49]. Nevertheless, as mixtures of phytoplankton occur naturally in the environment, mixtures of toxicants are present in the freshwater ecosystems as well. Therefore, complexity can always be increased in ecotoxicity studies.

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Chapter 2

Fate and toxicity of chlorophenols in the freshwater environment

2.1. Chlorophenols

Chlorophenols are among the most toxic compounds widespread in the environment [1]. The different CPs have been extensively used for more than 60 years and have been detected in soil, sediments, natural waters, food and human urine [1]. They are ubiquitous contaminants, due to their persistence and abundance, and represent the largest group of phenols [2]. The long half-lives and the widespread occurrence of CPs are particularly relevant in water where they are propelled by its relatively fair solubility in water and consequent high mobility in the aqueous environment [3]. Their dechlorination rates depend on the substrate and their half-lives range from some hours to a few months [4].

2.1.1. CPs in humans

The action of CPs in humans include mutagenic, carcinogenic and also estrogenic effects [5]. PCP has been classified as a weak mutagen, despite of its ability to form DNA adducts, and it is a potential carcinogen [6]. Therefore, the effects of CPs in humans are well-known and such data are useful in toxicity studies with other species, for comparison. At μg L^{-1} levels, PCP could interfere with the transport of thyroid hormones in the human plasma [7]. There is also evidence that CPs are precursors of extremely toxic dioxins and furans either upon incineration [8] or after metabolism in humans [9]. It is interesting to notice the natural *in vivo* conversion of PCP to a chlorinated dioxin, in cow faeces, and to chlorinated dioxins and dibenzofurans in humans [10].

To enter the human body, CPs have different routes. For example, PCP can be absorbed by ingestion, inhalation and by the skin [6]. CPs are retained in the blood and only to a lesser extent in the adipose tissue [7]. Numerous toxic effects caused on human erythrocytes were observed, like lipid peroxidation and decreased membrane acetylcholinesterase activity. They changed ATPase activity and membrane fluidity and also damaged membrane proteins. These compounds also oxidized haemoglobin [2]. CPs also modulate the activity of ion channels in the nervous system; TCPs may block ion channels in a mg L^{-1} concentration level [2]. Moreover, toxicity studies on reproductive effects revealed that 3,4-dichlorophenol (3,4-DCP) disrupted the sperm acrosome [11].

In breast milk, CPs were measured with maximum values in the order of tens to a hundred μg kg^{-1}. The most frequently detected, and with highest levels, were 2,4-dichlorophenol (2,4-DCP), 2,4,5-trichlorophenol (2,4,5-TCP) and PCP [12]. The CPs in

human urine appear as a result of occupational or environmental (food, water, air) exposure to CPs or as metabolites of other chlorine-containing compounds [13, 14]. The excretion of PCP in the urine of the general population is related to its use, specially in the past, as a wood and leather preservation agent [15, 16]. The concentrations of free (non-conjugated) CPs in urine samples are usually less than 1 μg L^{-1}, or less than 0.1 μg L^{-1} for the case of PCP [14]. After de-conjugation of CPs in the urine samples, the maximum concentrations, depending on the compound and the level of exposition, were from less than 1 μg L^{-1} to a few mg L^{-1} [13, 15-20].

Concerning workers exposed to CPs, major health effects seem to be related with dioxins and furans generated as low-level by-products of CPs, which are found in higher levels in employees assigned to, particularly, synthesis operations [21, 22]. Dioxins serum concentrations can be correlated with total years of employment in the plant, the type of work, age, body fat and eating local game [21]. Exposure to dioxins may be associated with a small increase in overall cancer risk and in the risk for specific cancers [22-24], chloracne [21], circulatory diseases, specially ischemic heart disease, and possibly diabetes [25]. An admissible daily dose of individual CPs that may be taken by a man without inducing carcinogenic changes is 5 μg kg^{-1} of body weight for 2- chlorophenol (2-CP) and 3 μg kg^{-1} of body weight for 2,4-DCP, 2,4,6-trichlorophenol (2,4,6-TCP) and PCP [2]. Risks tended to be higher for 10 to 19 years after the first exposure [25].

2.1.2. Sources and utilization of CPs

Halogenated compounds found in the environment have two different origins: biogenic and anthropogenic. The natural sources of CPs were neglected for a long time, as they were considered to be isolation artifacts or abnormalities of nature [10]. The anthropogenic sources of halogenated compounds are dominated by fluorinated and chlorinated compounds, where CPs are included [26].

Chlorophenols are naturally formed by chlorination of mono and polyaromatic compounds present in soil and water. Synthesis of CPs proceeds with the participation of chloroperoxidases contained in plants and microorganisms in the presence of hydrogen peroxide and inorganic chloride [3]. CPs are produced by biochemical and geochemical processes, like *de novo* synthesis by fungi [3, 27], for instance 2,4-DCP produced by a *Penicillium* sp. soil fungus [10], plants [2] and bacteria [3]. Other natural sources include 2,6-dichlorophenol (2,6-DCP) as a sex pheromone in at least a dozen tick species, including *Dermacentor variabilis* [10, 28] where 2,4-DCP and 2,4,6-TCP were detected after ingestion

of blood [29]. 2,5-dichlorophenol (2,5-DCP) has been isolated from the common grasshopper and it is an ant repellent [10]. Aquatic and terrestrial plants are known to contain various phenols [10].

The anthropogenic sources of CPs to the environment are related to their widespread use as pesticides and impregnation agents. As pesticides, they can be found in insecticides, herbicides, fungicides [30] and molluscicides [7]. CPs may also be formed after degradation of pesticides [2]. As impregnation agents, they are used in leather, textiles [2] and, mainly, wood [31].

As raw materials for chemical industry, CPs are used in the production of several biocides, like the herbicide 2,4-dichlorophenoxyacetic acid [11], the insecticide lindane, the fungicide hexachlorobenzene [32] and the bactericide triclosan [33]. Moreover, CPs are used or formed in chemical industries [34], like in the manufacture of plastic, dyes and in the petrochemical [35] and metallurgic industries [2]. PCP is an additive to drilling fluid and certain food packaging [6]. The incineration plants are a source of CPs to the air [11].

In addition, CPs can be formed by the combustion of organic material, in chlorobleaching of pulp in paper mills, and even as by-products during the chlorination of drinking water [36, 37].

Pentachlorophenol was introduced in the 1930s as a preservative for timber and lumber [38, 39]. It is considered a priority pollutant since it is harmful to organisms even at very low concentrations and has a long half-life in the environment [40]. In spite of being a broad-spectrum pesticide (bactericide, fungicide, herbicide, insecticide and molluscicide), its major use is, still, the preservation of wood products. Due to its numerous applications and low price, PCP has a worldwide distribution. The reason for its common use as preservative is its relative resistance to biological degradation, which creates a pollution problem [41]. The misuse, accidental spillage and improper disposal [42], as well as leaching out of treated wood and run off from agricultural fields, lead to extensive soil and surface and ground water contamination [38].

2.1.3. Physicochemical properties of CPs

All the CPs are composed of a benzene ring, bonded to a hydroxyl group, and a different number of chlorine atoms, raising their toxicity, stability against decomposition and the capability for bioaccumulation from the simple mono-CPs, with one chlorine atom, to the most substituted PCP, with five [4, 43]. All CPs are solids (melting points from about 33 to

191 °C). Their octanol-water partition coefficients (K_{ow}) and the degrees of dissociation (indicated as descending acidity constant (pKa) values) increase and the water solubilities, or hydrophilicity, decrease with the increasing number of chlorine atoms [44]. CPs are weakly acidic, so in the aquatic environment they occur in both dissociated and undissociated forms [45]. The physical properties of CPs vary greatly, depending on the number of chlorine atoms and their position relative to OH group, as can be seen in Table 2.1.

As a weak acid (pKa 4.7-4.9, Table 2.1), PCP exists in both deprotonated (ionic, negatively charged) and protonated (non-ionised, neutral) forms. The degree of dissociation is pH-dependent, with its deprotonated form dominating at higher pH [41, 46] and exhibiting lower toxicity [47]. In natural waters, PCP ionic form dominates. Both sodium pentachlorophenate (Na-PCP) and PCP dissociate to approximately the same extent. The ionic form is between 5000 and 25000 times more water-soluble than PCP [46]. PCP is a hydrophobic molecule that has high partition coefficients with organic phases. As solubility, the apparent octanol-water partition coefficient of PCP varies strongly with pH [41], with a log K_{ow} between 3.81 and 5.86 [48], making it the most lipophilic CP. Therefore, PCP easily bioaccumulates and trophic biomagnification is particularly acute within the aquatic food web [46]. The adsorption coefficient (K_{oc}), a measure of how strongly a molecule adheres to a surface, is a function of the sample pH and the ionic strength [41, 46] and its estimation for PCP is highly variable [44]. PCP tends to adsorb to the sediment and can be released back to the water through desorption [46].

2.1.4. Concentrations of CPs in the aquatic environment

The concentrations of CPs found in the aquatic environment are presented in Table 2.2. In open ocean waters, CPs concentrations are about 5 to 10 ng L^{-1} or even less [2]. Relatively high concentrations were measured in coastal seawater, over 1500 ng L^{-1} for both 2,4-DCP and PCP [49], but values below 1 ng L^{-1} were also registered for some CPs [50]. Much more variable levels have been observed in natural freshwaters, from low ng L^{-1} [20, 46, 51-59] to tens mg L^{-1} range [51, 60]. Irrespectively of the huge variation, the medium concentrations are usually very low. For instance, Gao et al. [51] have found medium levels of 5, 2, and 50 ng L^{-1} for 2,4-DCP, 2,4,6-TCP and PCP, respectively, in China´s Rivers.

Table 2.1. Vapour pressures (P_0 at 25 ºC), octanol-water partition coefficients (log K_{ow}) and acidity constants (pKa) of different chlorophenols commonly found in environmental and biological samples.

Chlorophenol	P_0 (mm Hg)	log K_{ow}	pKa
2-CP	1.0-2.4 [a]	2.03-2.29 [a]	8.3-8.6 [c]
3-CP	0.25-0.32 [a]	2.17-2.63 [a]	8.8-9.1 [c]
4-CP	0.21 [a]	2.17-2.88 [a]	9.1-9.4 [c]
4-C-2-MP			9.5-10.5 [d]
4-C-3-MP	0.05 [a]	2.18-3.10 [a]	9.4-9.7 [d]
2-C-5-MP			7.8-9.7 [d]
4-C-3,5-DMP			9.4-10.6 [d]
2,3-DCP		3.15-3.19 [c]	6.4-7.8 [c]
2,4-DCP	0.09-0.16 [a]	2.87-3.61 [a]	7.5-8.1 [c]
2,5-DCP		3.20-3.24 [c]	6.4-7.5 [c]
2,6-DCP	0.08-0.10 [a]	2.34-3.36 [a]	6.7-7.8 [c]
3,4-DCP		3.05-3.68 [a]	7.4-8.7 [c]
3,5-DCP		2.57-3.56 [c]	6.9-8.3 [c]
2,3,4-TCP	0.008-0.026 [a]	3.51-4.07 [a]	6.5-7.7 [c]
2,3,5-TCP	0.022 [b]	3.84-4.56 [c]	6.8-7.4 [c]
2,3,6-TCP	0.0025 [b]	3.88 [c]	6.0-7.1 [c]
2,4,5-TCP	0.02-0.057 [a]	3.52-4.19 [a]	7.0-7.7 [c]
2,4,6-TCP	0.006-0.032 [a]	2.67-4.03 [a]	6.0-7.4 [c]
3,4,5-TCP	0.0025 [b]	4.01-4.39 [c]	7.7-7.8 [c]
2,3,4,5-TeCP	0.00034 [b]	4.21-5.03 [a]	6.2-7.0 [c]
2,3,4,6-TeCP	0.004-0.006 [a]	4.10-4.45 [a]	5.3-6.6 [c]
2,3,5,6-TeCP	0.00067 [b]	3.88-4.90 [a]	5.2-5.5 [c]
PCP	0.00005-0.0017 [a]	3.81-5.86 [a]	4.7-4.9 [c]

[a] [61]; [b] [62]; [c] [63]; [d] [64]

Table 2.2. Concentrations of chlorophenols in the aquatic environment.

Water sample type	Chlorophenol	Concentration (ng L^{-1})	Ref.
River water (UK)	2,4-DCP	<200-2000	[65]
River water (Poland)	PCP	<10-300	[55]
	2,3,4,5-TeCP	<10-32	
	2,4,6-TCP	<10-227	
	2,4-DCP	<10-53	
	2-CP	<10-317	
River water (Portugal)	2,4,6-TCP	20-2300	[66]
River water (China)	2,4-DCP	<5-330	[53]
	4-CP	<27-2300	
	3-CP	<31-120	
Tigris river water (Iraq) [a]	PCP	<12-4596	[54]
	2,3,4,6-TeCP	<9-1550	
	2,4-DCP	<8-981	
	2-CP	<7-1086	
Kupa River (Croatia)	PCP	<4-95	[56]
	2,3,4,6-TeCP	<4-42	
	2,4,5-TCP	<2-23	
	2,4,6-TCP	3-15	
	2,4-DCP	<2-18	
	4-CP	<2-50	
River water (India) [b]	PCP	8790	[60]
	2,4,6-TCP	24460	
	2,4-DCP	7210	
	2,3-DCP	560	
	4-CP	7280	
Tama River (Japan)	2,4,6-TCP	6.4-6.7	[20]
	2,4-DCP	9.9-12.3	
Surface freshwater (China)	PCP	<140-560	[67]
	2,4,6-TCP	<110-720	
	2,4-DCP	<130-490	

Nakdong River (Korea)	2,4,6-TCP	<0.19-21.6	[52]
	2,6-DCP	<0.14-3.14	
	4-CP	<0.15-91.1	
Surface freshwater (Germany)	PCP	<8-230	[57]
	4-C-3-MP	<15-140	
Surface freshwater (Italy)	PCP	<7-505	[58]
	2,4,6-TCP	<3-109	
	2,4-DCP	<1-68	
	2-CP	<4-52	
	4-C-3-MP	<2-33	
Surface freshwater (Croatia) [c]	PCP	5-238	[59]
	2,3,4,6-TeCP	2-93	
	2,4,6-TCP	17-62	
Surface freshwater (Belgium) [c]	PCP	500-1500	[46]
Surface freshwater (Germany) [c]	PCP	20-410	
Surface freshwater (the Netherlands) [c]	PCP	20-210	
Surface freshwater (France)	PCP	0.5-3300	
Surface water (Portugal) [b]	PCP	1000	[68]
	2,4,6-TCP	2300	
Surface water (China)	PCP	<1.1-594; median 50	[51]
	2,4,6-TCP	<1.4-28650; median 2	
	2,4-DCP	<1.1-19960; median 5	
Rhone delta and ditch water (France)	4-C-2-MP	<15-1120	[69]
Coastal Seawater (North Sea) [d]	PCP	19-49	[70]
Coastal seawater (the Netherlands) [c]	PCP	10-50	[46]
Coastal seawater (Baltic)	PCP	<200-1560	[71]
	2,4-DCP	<50-6000	
	2-CP	<50-700	
	4-C-3-MP	<50-800	
Coastal seawater (Sweden)	PCP	1-132	[50]
	2,3,4,6-TeCP	<1-100	
	2,4,6-TCP	<1-370	

	2,6-DCP	5-40	
	2,4-DCP	<2-400	
Coastal seawater (Singapore)	PCP	<26-1650	[49]
	2,4-DCP	<22-1550	
Groundwater (Croatia) [b]	PCP	151	[59]
	2,3,4,6-TeCP	46	
	2,4,6-TCP	24	
Groundwater (Spain)	4-CP	<10-200	[72]
Rain water (France)	4-C-2-MP	<15-120	[69]
Rain water (Croatia) [b]	PCP	19	[59]
	2,3,4,6-TeCP	63	
	2,4,6-TCP	69	
Snow (Croatia) [b]	PCP	131	[59]
	2,3,4,6-TeCP	527	
	2,4,6-TCP	210	
Drinking water (Poland) [b]	2,3,4,5-TeCP	221	[37]
	2,4,6-TCP	75	
	2,4,5-TCP	486	
	4-CP	10	
Drinking water (Croatia)	PCP	<4-474	[56]
	2,3,4,6-TeCP	<4-10	
	2,4,5-TCP	<2-22	
	2,4,6-TCP	<2-9	
	2,4-DCP	<2-17	
	4-CP	<2-6	
Drinking water (Iraq) [a]	2,4-DCP	94	[54]
	2-CP	23-33	
Effluent wastewater (pulp mill) [a]	PCP	3.6	[50]
	2,3,4,6-TeCP	5.8-7.5	
	2,4,6-TCP	22-40	
	2,4-DCP	8.3-24	
Raw wastewater	2,4,6-TCP	180-1000	[73]

	2,4-DCP	600-2200	
Effluent urban wastewater	PCP	<30-40	[74]
	2,4,5-TCP	<30-40	
	2,4,6-TCP	<30-100	
	2,4-DCP	<30-40	
	4-CP	<30-80	
	2-CP	<30-200	
Influent urban wastewater [a]	2,4,6-TCP	<0.04-0.57	[75]
	2,5-DCP	0.31-41.8	
	2,4-DCP	<0.02 -30.7	
	2,6-DCP	<0.04 -28.4	
	4-CP	13.2 [b]	
	2-CP	10.6-101	
	3,5-DM-4-CP	404 [b]	
Effluent urban wastewater [a]	2,4,6-TCP	<0.04-0.25	[75]
	2,5-DCP	1.9 [b]	
	2-CP	<0.03-0.14	
Urban influent wastewater	2,4-DCP	<4-2222	[73]
Urban wastewater effluents [a]	2-CP	<3-14.9	[76]
Wastewater effluent	2,6-DCP	<17-42	[53]
	2,5-DCP	<9-220	
	2,4-DCP	540-12500	
	4-CP	<27-780	
	2-CP	<48-130	

[a] μg L^{-1}
[b] maximum values
[c] range of maximum values
[d] range of mean values

Depending on the type of wastewater, the concentrations of CPs with different degree of chlorination can be very variable, from low ng L^{-1} [53, 73] to mg L^{-1} range [53, 73]. Most often detected CPs (or those often found at the highest concentrations) include 2-CP; 4-chlorophenol (4-CP); 4-chloro-3-methylphenol (4-C-3-MP); 2,4-DCP; 2,6-DCP; 2,4,6-TCP; 2,3,4,6-tetrachlorophenol (2,3,4,6-TeCP) and PCP. The sewage treatment plants can be

considered as sources of these compounds in aquatic environments, specially if the treatment processes cannot remove them effectively. Levels of CPs in landfill leaches are usually about 100 ng L^{-1} or less; higher levels (µg L^{-1} range) were sometimes reported, specially for PCP [14, 77, 78].

Chlorophenols were also detected in drinking water, at concentrations from low to hundreds ng L^{-1}, in Croatia [56] and Poland [37], therefore above the legal limits (section 2.3), and at tens µg L^{-1}, in Iraq [54].

Several CPs were found in Portuguese freshwaters: 2-CP [66, 68]; 3-chlorophenol (3-CP) [68]; 2,4-DCP [79]; 2,6-DCP [80]; 2,4,6-TCP [66, 68, 80]; 2,3,4,6-TeCP [80] and PCP [66, 68, 79], at concentrations from low ng L^{-1} level to low µg L^{-1} level.

2.1.5. Toxicity of CPs to aquatic organisms

The CPs are chemicals with very high toxicity to a wide range of organisms, interfering with oxidative phosphorylation, inhibiting ATP synthesis [2] and accelerating the utilization of lipid reserves [81]. CPs are believed to be fetotoxic, embryotoxic and teratogenic [32]. The overall toxic effect of CPs is caused by a combination of several distinct mechanisms, most of which interfere with energy transduction, mainly in mitochondria, chloroplasts and bacterial cytoplasmic membranes, where they may act as uncouplers, inhibitors or merely as narcotic agents [82, 83]. The mechanism of toxicity of CPs depends on the number of chlorine atoms: mono-CPs seem to act as non-specific polar narcotics [83, 84] while for other CPs the mechanism of toxicity is related with the uncoupling of the oxidative phosphorylation, which increases with the number of Cl atoms [84], and inhibition of the electron transport [83]. The position of the chlorine atom on the aromatic ring is also important in the toxicity of CPs. The *ortho*-substituted chlorine results in lower toxicity compared with *meta*- and *para*-substituted congeners [85]. Moreover, CPs may act simultaneously as an uncoupler and an inhibitor [83].

The CPs physicochemical properties facilitate the toxicity to aquatic life, namely their relatively high lipophilicity, and their accumulation in the aquatic environment, specially in bottom sediments and on suspended matter [36, 63]. The CPs toxicity also depends on the degree of chlorination, isomeric position of the chlorine atoms [36], the presence of other compounds and the pH of ambient water [63]. Their toxic effects increase with the number of chlorine atoms in the molecule [86]. Although exceptions occur, as the toxicity of CPs with different degree of chlorination can be similar, PCP usually is the most toxic one [1, 47].

The toxicity of different CPs to different aquatic organisms is presented in Table 2.3. Toxicity depends on the species, the toxic compound, the endpoints assessed, the techniques used and the chosen exposure time. Therefore, the results obtained from ecotoxicity studies are difficult to compare. Toxicants, such as pesticides, are known to affect individual, though related, species to significantly different degrees [87].

Toxicity of PCP to aquatic organisms is emphasized in Table 2.3 as its EC_{50} values are in bold. Most studies about CPs toxicity were, specifically, with PCP. This compound interferes with the oxidative phosphorylation process and inhibits ATP synthesis [88] as well as the electron flow process in photosynthesis [89]. These may be the main effects on phytoplankton. As for other organisms, PCP seems to be the most toxic of the CPs, which is expected given its physicochemical properties.

Pentachlorophenol, as a widespread contaminant, represents a risk of acute or chronic toxicity to aquatic organisms. In order to predict potential adverse effects of PCP, the knowledge of the level and the mechanisms of toxicity to aquatic organisms is important [116]. It is known that, for instance, it affects the energy metabolism of fish by partially uncoupling phosphorylation and increasing oxygen consumption [46]. PCP is also believed to have endocrine disrupting properties [116]. Effects of this endocrine disruptor to non-target organisms like phytoplankton, lacking an endocrine system, are considerable as the energy flux at the photosystem II level is severely affected [117].

2.1.5.1. Toxicity of CPs to cyanobacteria

The toxicity of CPs is hardly known to cyanobacteria. There are some works on the toxicity of CPs to microalgae but almost none with cyanobacteria. Microalgae have some important similarities with cyanobacteria that justify a comparison similar to the one discussed in Chapter 8. These phytoplanktonic groups are not as phylogenetically close as most studies assume. This confusion may be related with the classical term “blue-green algae” used for cyanobacteria. Microalgae are eukaryotic and cyanobacteria are prokaryotic but they do share the same freshwater environment.

Table 2.3. Toxicity of chlorophenols to aquatic organisms.

Organism	Endpoint	CPs	EC_{50} (mg L^{-1})	Ref.
Cyanobacteria				
Microcystis aeruginosa	Growth (10 days)	PCP	**0.117**	[90]
Anabaena inaequalis (soil)	Growth (96 hours)	PCP	**0.13-1.13**	[87]
Microalgae				
Chlorella vulgaris	Growth (96 hours)	PCP	**10.3**	[91]
Chlorella vulgaris	Growth (7 days)	PCP	**1.66**	[1]
		TeCPs	1.29-1.31	
		TCPs	1.53-3.20	
		DCPs	1.48-2.10	
Chlorella vulgaris	Growth (19 days)	PCP	**10.03**	[92]
Chlorella vulgaris	Growth (10 days)	PCP	**12.6**	[90]
Chlorella vulgaris	Growth (11 days)	PCP	**12-13 (IC_{50})**	[38]
Chlorella emersonii	Growth (19 days)	PCP	**5-6 (IC_{50})**	[92]
Chlorella emersonii	Growth (11 days)	PCP	**3-4 (IC_{50})**	[38]
Chlorella VT-1	Growth (19 days)	PCP	**17.93**	[92]
Chlorella VT-1	Growth (11 days)	PCP	**26-27 (IC_{50})**	[38]
Chlorella kessleri (soil)	Growth (96 hours)	PCP	**34.3-512.0**	[87]
Tetraselmis marina	Growth (10 days)	4-CP	25.5-34.8	[93]
Scenedesmus obliquus	Dissolved O_2 (8 days)	2,4-DCP	41	[94]
		MCPs	89-117	
Scenedesmus obliquus	Growth (96 hours)	PCP	**6.2**	[95]
		2,4,6-TCP	30.5	
		2,4-DCP	39.1	
Scenedesmus obliquus	Growth (72 hours) pH 6.5-9	PCP	**0.26-24.06**	[96]
Pseudokirchneriella subcapitata	Growth (72 hours)	2,4,6-TCP	2	[97]
Pseudokirchneriella subcapitata	Growth (48 hours, closed system)	PCP	**0.007**	[98]
		2,3,4,6-TeCP	0.012	
		DCPs	1.23-2.43	

		MCPs	8.63-8.81	
Pseudokirchneriella subcapitata	Dissolved O_2 /growth (48 hours, closed system)	PCP	**0.004/0.013**	[99]
		2,3,4,6-TeCP	0.072/0.061	
		2,4,6-TCP	0.801/−	
		DCPs	3.03-4.20/2.53-3.82	
		MCPs	20.6-20.9/13.0-14.8	
Chlamydomonas reinhardtii	Growth (10 days)	PCP	**360**	[100]
Selenastrum capricornutum	Growth (96 hours)	PCP	**0.052 (IC_{25})**	[101]
Freshwater phytoplankton	Growth (7 days)	PCP	**0.263-0.264-0.290**	[102]
Fish				
Tilapia zilli (Perciformes)	Mortality (48 hours)	PCP	**0.645**	[1]
		TeCPs	1.41-2.46	
		TCPs	1.29-3.73	
		DCPs	0.85-2.30	
		MCPs	4.49-6.55	
Tilapia mossambica (Perciformes)	Mortality (96 hours)	2,4,6-TCP	5.66	[103] [104]
		2,4-DCP	8.35	
Ctenopharyngodon idellus (Cypriniformes - carp)	Mortality (96 hours)	2,4,6-TCP	3.54	[103] [104]
		2,4-DCP	5.25	
Carassius auratus (Cypriniformes - goldfish)	Mortality (96 hours)	2,4,6-TCP	4.31	[103] [104]
		2,4-DCP	7.94	
Carassius auratus (Cypriniformes - goldfish)	Mortality (5 hours)	PCP	**0.3**	[105]
		2,3,4,6-TeCP	0.8	
		TCPs	0.92-4.0	
		DCPs	2.5-35	
		MCPs	30-93	
Carassius auratus (Cypriniformes - goldfish) (pH 6/pH 8)	Mortality (5 hours)	PCP	**0.2-0.3/0.85**	[106]
		2,3,4,6-TeCP	0.2-0.3/1-1.5	
		TCPs	0.7-1.5/1.5-7	
		DCPs	2-30/3->80	
		MCPs	50-100/50-150	
Oryzias javanicus	Mortality	4-CP	3.0	[107]

(Beloniformes)	(96 hours)			
Oryzias latipes (Beloniformes)	Mortality (96 hours)	PCP	**0.125 (NOEC)**	[34]
Cladocera				
Daphnia magna	Immobilization (24 hours)	2,4,6-TCP	4.9	[97]
Daphnia magna	Immobilization (24 hours)	PCP	**0.76**	[108]
		2,3,5,6-TeCP	2.27	
		TCPs	2.08-7.38	
		DCPs	2.09-9.38	
		MCPs	8.07-17.95	
Daphnia magna	Mortality (48 hours)	2,4,6-TCP	1.73	[103] [104]
		2,4-DCP	2.12	
Daphnia magna	Growth (48 hours) pH 7.13-8.81	PCP	**0.06-3.77**	[96]
Daphnia magna	Immobilization (48 hours)	2,4-DCP 2,4,6-TCP PCP (mix)	**0.46-1.21**	[47]
Other animals				
Bufo bufo gargarizans (amphibian)	Mortality (96 hours)	2,4,6-TCP	8.63	[103] [104]
		2,4-DCP	9.46	
Rana nigromaculata (amphibian)	Mortality (96 hours)	2,4,6-TCP	7.46	[103] [104]
		2,4-DCP	9.85	
Limnodrilus hoffmeisteri (annelid)	Mortality (96 hours)	2,4,6-TCP	7.52	[103] [104]
		2,4-DCP	9.89	
Chaetogammarus marinus (crustacean)	Mortality (96 hours)	PCP	**0.26**	[32]
	Swimming efficiency (7.5 days)	PCP	**0.04**	
	Embryogenesis (20 days)	PCP	**0.02**	
Penaeus aztecus (postlarval brown shrimp)	Avoidance (96 hours)	PCP	**0.317 (LC_{50})**	[109]
Lumbriculus variegatus (oligochaeta)	Growth and reproduction (28 days)	PCP	**0.057-26.3**	[88]

Chironomus riparius (larvae, Diptera)	Mortality (48 hours)	PCP	**0.898-1.192 (LC_{50})**	[36]
Hydra viridissima (cnidarian)	Growth (4 days); Growth (6 days)	4-CP	45; 22.3 (LOEC)	[110]
Hydra vulgaris (cnidarian)	Growth (4 days); Growth (6 days)	4-CP	32; 1.1 (LOEC)	[110]
Aquatic plants				
Lemna polyrhiza (Araceae)	Growth (8 days)	PCP	**8.1**	[111]
Lemna minor (Araceae)	Growth (7 days)	2,4,6-TCP	0.01	[112]
Lemna gibba (Araceae)	Growth (7 days)	2,4,6-TCP	0.02	
Landoltia punctata (Araceae)	Growth (7 days)	2,4,6-TCP	0.22	
Lemna gibba (Araceae)	Growth (7 days)	PCP	**0.53**	[89]
		2,4,5,6-TeCP	0.28	
		2,4,5-TCP	0.41	
		2,4-DCP	1.5	
		4-CP	23.5	
Protozoa				
Tetrahymena pyriformis (protozoan)	Growth (40 hours)	PCP	**2.26**	[113]
		TeCPs	0.44-1.53	
		TCPs	1.56-7.07	
		DCPs	4.56-30.3	
		MCPs	9.7-41.1	
Bacteria				
Vibrio fischeri	Luminescence (15 minutes)	2,4,6-TCP	14.4	[97]
Pseudomonas fluorescens (lux marked)	Luminescence (20 minutes)	TCPs	1.9-18.2	[114]
		DCPs	2.9-78	
		MCPs	12.2-122	
Burkholdeira RASC c2 (lux marked)	Luminescence (20 minutes)	TCPs	0.87-14.8	[114]
		DCPs	4.1-11.4	
		MCPs	21.9-28.3	
River bacteria (mix)	Growth (24 hours)	2,4-DCP	8.2	[115]
		MCPs	8.3-20.9	

Among all CPs, only PCP has been studied with cyanobacteria, to date. There are only two previous works about the toxicity of PCP to cyanobacteria, carried out at relatively high concentrations of PCP. The first, back in 2000, was conducted at the phytoplankton community level, without taxonomic identification details [102], and the second was with a soil species, *Anabaena inaequalis* [87]. Additionally, the toxicity of Na-PCP was also checked for aquatic cyanobacteria, in a plate incubation test during 9 days, by measuring only OD [118]. That article, however, focused on the development of toxicity tests for veterinary antimicrobial products, and used Na-PCP as a model substance. Apart from data on the EC_{50}, non-observed effect concentration (NOEC) and minimum inhibitory concentration (MIC), no other results about the effects of PCP were demonstrated. Another study dealing with PCP and *M. aeruginosa* focused on the biotic removal of the compound by dried biomass of the cyanobacterium [40]. Therefore, the current work can be considered the first systematic ecotoxicity study of the effects of PCP on an aquatic cyanobacterium.

2.1.5.2. Toxicity of CPs to microalgae

The knowledge on the toxicity of CPs toward algal growth is of great importance due to the role of microalgae in natural water bodies as a major primary producer and their possible existence in the microbial communities of wastewater treatment plants. While degradation and toxicity of CPs have been well documented using bacteria and fungi, only few toxicity studies have been conducted with microalgae [119]. Studies on the physiological–biochemical changes in algae induced by PCP are also rare [116]. The effects of PCP on biosynthesis of chlorophyll were studied with the microalgae *Scenedesmus obliquus*, *Spirulina subsalsa* and *Chlorella pyrenodoisa* [116]. However, these studies were conducted under different experimental conditions, using different algal species or even different endpoints, which make it difficult to compare the results [116]. This is a common problem on ecotoxicity studies with phytoplankton species.

Some studies show that a low dose of PCP seems to activate chlorophyll *a* synthesis in the microalga genus *Chlorella*, while high concentrations of PCP inhibit it, proportionately more than the concomitant reduction in total cell density. Hence, PCP effects on microalgae seem to be related with the photosynthetic process, which in turn changes their growth rate [87, 120]. Increases of algal cell count at 5 mg L^{-1} of PCP [87], and carbohydrate content, in a mixture of phytoplankton composed mainly of microalgae, at PCP levels up to 0.669 mg L^{-1} [102], were reported. Increasing PCP levels, however, showed to decrease algal growth, photosynthetic pigment and soluble protein levels [87, 116]. The algal protein content and

synthesis were always shown to be inhibited by any level of PCP [102]. PCP toxicity to microalgae seems to be affected by the medium used for growth [121] and all the factors present in Table 2.3, like the different techniques, endpoints and exposure times used in the studies.

Concerning environmental pollution, *C. vulgaris*, the chosen microalga to this study, is one of the dominant algal species in freshwater and has potential to either degrade or adsorb a variety of organic pollutants present in the wastewaters originating from industries [119]. It is able of bioconcentrating, for instance, natural and synthetic estrogens [122, 123]. Particularizing, the presence of CPs may induce algal growth, like 4-CP and 2,4-DCP (both at concentrations up to 20 mg L^{-1}) [119]. For this microalga, it was observed that toxicity of CPs increased from mono-CPs to di-CPs, and was similar among the more chlorinated ones, including PCP [87].

The microalga *C. vulgaris* could not biodegrade 4-CP and 2,4-DCP over a 30 days' incubation period [119]. A species of the same genus, *Chlorella* VT-1, was found to completely remove 2,4-DCP within 13 days [124]. Moreover, in the presence of increasing initial 4-CP concentration, the chlorophyll *a* content (chl_a) of *C. vulgaris* cells linearly decreased [119]. It was previously shown that the chl_a of *C. vulgaris* is highest under autotrophic growth condition and it decreases with the addition of organic compounds and elimination of light [119].

Other microalgae species were studied in the presence of CPs. Then, a few examples will be presented. *Chlorella* VT-1 growth was inhibited by 10 to 30 mg L^{-1} of 2,4-DCP and was able to remove part of this compound from the medium [124]. The same microalga species, a PCP-tolerant strain, was exposed to several other CPs and showed some tolerance to all of them except 2,4,5-TCP, which was toxic at all concentrations studied [121]. In earlier studies, the same microalgae strain, which was then isolated from PCP treated water, showed great resistance to PCP, as expected, when compared with *C. emersonii* and *C. vulgaris* [38, 92]. *Chlorella* VT-1 was even able to mineralize PCP [38]. The effects of 2,4-DCP and 3,4-DCP on the microalga *Pseudokirchneriella subcapitata* were studied. The first compound was more toxic. Toxicity varied within the short exposure times used, though [11]. A study on the effects of 4-CP on the marine microalga *Tetraselmis marina* showed that the compound is toxic to the microalga and the species is able to metabolize 4-CP, which involves conjugation of 4-CP with glucose [93, 125]. The same species was found to metabolize 2,4-DCP, again by glucosidation. The compound was found to be more toxic to *Tetraselmis marina* than 4-CP [126]. From this few examples it becomes clear that a lot of interspecific variability seems to exist among microalgae species

and to different CPs. Therefore, each species must be studied with each compound, regarding both toxicity and removal.

Calculated EC_{50} values for different microalgae species can be very disparate (Table 2.3). The species *Pseudokirchneriella subcapitata* seems to be very sensitive to PCP, with EC_{50} values between 0.004 [99] and 2 mg L^{-1} [97] but it must be stated that the exposure times were short, as low as 2 days for the first and 3 days for the latter work (studies with short exposure times, up to 96 hours, can be considered acute toxicity tests while over than 4 days can be considered chronic toxicity exposure [127]); early PCP toxicity seems to be always higher. Contrarily, some species are remarkably tolerant to PCP, like the soil species *Chlorella kessleri* (EC_{50} 34.3-512.0 mg L^{-1}) [87], *Selenastrum capricornutum* (IC_{25} 51.5 mg L^{-1}) [101], the marine species *Tetraselmis marina* (EC_{50} 25.5-34.8 mg L^{-1}) [93, 125] and *Chlamydomonas reinhardtii* (EC_{50} 360 mg L^{-1}) [100], being the first two exposed during 4 days and the other two during 10 days. This huge variation covers five orders of magnitude but, as discussed, results are difficult to compare due to different exposure times, different endpoints assessed, different culture media used, natural interspecies variability and using data from different sources with subsequent issues related with inter-laboratory variance [82].

2.1.5.3. Toxicity of CPs to other aquatic organisms

Concerning other groups of aquatic organisms, the toxicity of CPs to fish, measured as EC_{50} values, is more uniform. PCP seems to be the most toxic CP, as expected, with EC_{50} values ranging from 0.2-0.3 to 0.654 mg L^{-1} [1]. For instance, mono-CPs EC_{50} values vary from 4.5-6.6 [1] to 5-10 mg L^{-1} [106]. Again, it must be stressed that this is a very rough comparison since different species, endpoints and exposure times are being compared.

The knowledge of the toxicity of CPs to the order Cladocera, namely the crustacean *Daphnia magna*, is important given this is a model species used in numerous studies, specially those regarding different trophic levels, as this crustacean, a good representative of zooplankton, is a grazer of phytoplankton [81]. The calculated PCP EC_{50} values were from 0.06-3.77 [47] to 0.46-1.21 [96] and to 0.7 mg L^{-1} [108]. Again PCP is the more toxic of the CPs. The less toxic mono-CPs EC_{50} values range from 8 to 18 mg L^{-1} [108]. In this case, the same species is compared but still the endpoints and exposure times are different.

When the toxicity of CPs is meant to other aquatic animals, like amphibians, annelids, other crustaceans and cnidarians, among others, not many CPs were studied and

the EC_{50} values assessed are usually from low to tens mg L^{-1} except for PCP, where such values decrease to less than 1 mg L^{-1} [32, 36, 88, 103, 104, 110]. However, it is necessary to have in mind that very different species are considered in these studies.

Interestingly, toxicity of CPs to some aquatic plants seems to be high. As seen in Table 2.3, different species from the same family Araceae were studied with growth as endpoint during an exposure period of 7 or 8 days. The PCP EC_{50} values were determined for *Lemna gibba* and *Lemna polyrhiza* as 0.33 and 8.1 mg L^{-1}, respectively [89, 111]. All EC_{50} values, always for plants of the same *Lemna* genus, assessed for other CPs were, usually, lower than 1 mg L^{-1} [89, 111, 112].

In studies using bacteria, with luminescence as endpoint, for 15 to 20 minutes, assessed EC_{50} values for TCPs vary from 0.87-14.8 [114] to 14.4 mg L^{-1} [97]. The toxicity of different CPs to the protozoan *Tetrahymena pyriformis* was studied and the EC_{50} values were 2.26 mg L^{-1} for PCP and from 9.7 to 41.1 mg L^{-1} for mono-CPs, studying growth as endpoint during 40 hours [113].

Apart from the difficult comparison, some other problems arise. For instance, it was proposed that the toxicity of ionizable compounds such as CPs to aquatic organisms should be evaluated as their accumulated amounts *in vivo* instead of the concentration in the media because toxicity depends largely on the pH of the media [106], which is very logical but may be difficult to put in practice. Moreover, the behaviour of pollutants in the environment is complex, since it simultaneously reflects the interplay between biotic and abiotic processes. The complicated physicochemical properties of a compound associated with the complex patterns of mixing and transport in the aquatic environments hamper the prediction of its fate [70]. Mixtures of compounds can be more realistic, if are found in the environment, as well as mixtures of species. Pure cultures, however, separate the response of one species in defined conditions from interaction with other organisms and chemical compounds found in the environment [87]. The obtained data in the current work and in the studies presented in Table 2.3 is necessary but of limited environmental application as the laboratory conditions are still far from the field conditions. Progressively closer conditions to the environment may be an advantage in ecotoxicity studies and, therefore, mesocosms experiments are required, as will be discussed in chapters 8 and 9.

Pentachlorophenol was the chosen toxic compound to the ecotoxicity studies due to its high toxicity to all organisms and its importance in the environment, leading to the classification of priority pollutant. Some generalized lack of knowledge is still verified for such relevant compound. This thesis will focus on this CP.

2.1.6. Environmental fate of CPs

Natural depuration of CPs is effective. However, as they degrade slowly in the environment, due to their chlorinated nature, contamination can last for decades [38, 121]. The biodegradation of CPs by bacteria and fungi is well studied while data is relatively scarce for aquatic plants, microalgae and, specially, cyanobacteria. Some microalgae, like *C. vulgaris,* can metabolize phenol and PCP [124]. In plants, the detoxification of CPs is usually by conjugation with glutathione, glucoside and glucuronide and transport to the vacuole or medium [124]. These toxicants become more soluble and easier to degrade by microorganisms. Such strategy is also used by fungi to degrade CPs [119]. Plants and actinomyces can facilitate biodegradation of CPs by making them more soluble and easier to degrade [121]. The potential of some cyanobacteria and microalgae in the removal of CPs by biodegradation and biosorption was recognised [128, 129]. Abiotic mechanisms of CPs removal include photodegradation, oxidation and evaporation [63].

Despite the long half-life and resistance to degradation, PCP can be transformed by biotic and abiotic factors. When thinking in diminishing the environmental pollution, the most effective method is the complete breakdown of an organic molecule to water, carbon dioxide and various salts [38]. This is naturally assured by bioremediation, a biological process that consists in the degradation of a chemical compound into innocuous simpler products [38]. The application of bioremediation has been industrially successfully exploited in the last decades and has been extensively studied for several biological species and hazardous compounds. This is the case of microorganisms capable of utilizing PCP as a carbon and energy source, namely microalgae. However, for maximum biodegradation yield, the selected species must have, simultaneously, high growth rate, high production of biomass, high degradation rate for PCP and high tolerance to it [38].

Microalgae, like *C. vulgaris* and *Chlorella* VT-1, a microalga pre-exposed to PCP, were capable of mineralizing PCP in the presence of light [38]. PCP mineralization studies have been carried out with bacteria and fungi also; microalgae have the advantage of being easily grown, needing only light and carbon dioxide [38]. Microorganisms can degrade CPs by different routes. In the case of PCP, it can be mineralized by para-hydroxylation to the corresponding chlorohydroquinone that has the chlorines removed by further hydrolytic and reductive steps, which resulting trihydroxybenzene is subject of ring cleavage and complete mineralization to carbon dioxide. Other routes, involving the methylation of the hydroxyl group followed by conjugation to glucoside, glucuronide and glutathione, are not able to completely degrade CPs but can reduce the toxicity of PCP or sequester it [121]. PCP is presumed to be very resistant to microbial degradation due to its highly chlorinated organic

nature, hence its broad biocidal effect. However, the ability to degrade PCP has been demonstrated among bacterial and fungal populations in both pure and mixed cultures. The detoxification of PCP by plants is also possible and, perhaps, is occurring by mechanisms that are similar to those of microalgae, since both groups share fundamental metabolic similarities [38].

Bioaccumulation of PCP in the aquatic food chain starts with sorption, by living or non-living phytoplankton, which plays an important role in eutrophic lakes in the biogeochemical cycles of PCP [40, 130]; it is likely that the same principle is valid to other eutrophicated water courses like rivers. The sorption of PCP by cyanobacteria is not very well known although it is widely studied on soil, sediment, clay, mineral and black carbon. Biosorption of PCP on biomasses of fungi, bacteria, chitin, seaweed, pine bark, and mixed microbial consortia of aerobic and anaerobic sludge has been investigated in order to remove PCP from wastewater [40]. The sorption of PCP by the cyanobacteria genus *Microcystis* in water was recently studied. Dried biomass of cyanobacteria derived from a natural bloom may be used as an efficient biosorbent for the removal of PCP [40]. Biosorption of PCP by cyanobacteria can also be influenced by metal ions, nutrients and pH. It significantly decreases with pH until pH 9. Ionic strength is, so, important for the sorption of PCP [40]. As the pH increases, sometimes over 9, almost all PCP is in its ionized form and sorption is expected to decrease due to electrostatic repulsion as deprotonation of the polar functional groups of cyanobacteria also increase with pH [40]. This could be a natural defence to PCP and other weak organic acids. Sorption of ionized PCP can have place by interaction of PCP with metals, forming an ion pair of pentachlorophenolate-metal or interaction between the hydrophobic parts of the PCP anion and the surface of the cyanobacterium [40].

A study on the biosorption of PCP onto two types of inactive biomass, bacteria and fungi, concluded that biosorption process involves uptake by both the cell walls and other cellular components of the microorganisms [131]. Moreover, biosorption of PCP was found to be nonlinear and correlated with hydrophobicity [131]. Another work, with anaerobic granular sludge, showed that lipid cellular content would influence the adsorption capacity, as well as hydrophobicity, and that PCP was more strongly sorbed than the other CPs with lower degree of chlorination. They attributed these differences in biosorption capacity for anaerobic and aerobic biomass to the varying lipid composition and content of different biomasses. Temperature also influence adsorption, assuming it is exothermic [131].

It was shown that PCP is rapidly accumulated in organisms and in sediments from the water column. Contaminated organisms in a clean environment exhibited the ability to

cleanse themselves of most of the PCP within a few days [132]. The aquatic environment is particularly sensitive to PCP. Although its solubility in water is considerable, and may even be enhanced in estuarine water, accumulation in the sediment is known to occur [132]. PCP can be released back to the water through desorption [46].

The abiotic possible losses of PCP from the environment comprise photolysis, oxidation and evaporation [63, 132]. The immediate oxidation process utilizing the atmospheric oxygen dissolved in the water does not play an important role in the environmental degradation of CPs and those with the greater number of chlorine atoms, such as PCP, are usually immune to oxidation processes in ambient temperature [63]. The evaporation of CPs may occur from shallow surface waters, when the ambient temperature is above 20 °C, as it was observed in the natural environment during rapid mixing of waters. Nevertheless, the evaporative processes play an insignificant role in the removal of CPs from the aquatic environment [63].

Photolysis must be the most significant abiotic degradation process of PCP [63, 132]. Some authors proved that all CPs can undergo photolysis leading to dechlorination by cleavage of the C-Cl bond. Direct photolysis of PCP in distilled water, assessed using different experimental conditions, gives numerous degradation products, including other CPs, dioxins and furans. These PCP by-products are known to be extremely toxic and their influence in toxicity cannot be discarded [45].

It is assumed that, in laboratory conditions, PCP is easily degraded and removed from samples but environmental conditions like its persistence, sorption to soils and dissolution in organic matter and water decreases degradation rates. Additionally, the degradation of CPs in environmental conditions and the organic sorption mechanisms are still a matter of debate and no consensus was reached [39, 131], so research must go on.

2.2. Legislation on CPs

The risks posed by CPs led to restrictions in their use in the EU [46] and in the USA, since the 1990s, and the production of some CPs was banned in most countries [6]. Several CPs are included in most lists of priority substances in the field of water policy [48, 58, 121, 133, 134]. In the EU, legislation was created and the levels set for CPs depend on the purpose of the water distribution. The EU set a maximum admissible concentration (MAC) of total phenols in drinking water to be 0.5 μg L^{-1} and 0.1 μg L^{-1} for individual compounds in

environmental waters (inland and other surface waters) [48, 66, 74, 80, 135]. The minimum quality objectives of wastewater impose 20 μg L^{-1} for 2,4-DCP [136].

2.2.1. Legislation on PCP

In spite of being widely used, and having many different applications, PCP has been banned in many countries and its use has been severely restricted in others, leading to a worldwide reduction of the consumption since the 1980s [6, 116]. PCP production was banned in the EU in 2000 [6] and any risks posed by it to the freshwater environment in Europe is likely to be attributed to sediments contaminated with PCP from historic usage [46]. In the USA, PCP production was banned in 1992 [6] and industrial sources can contribute to environmental contamination persisting for many years after the closure of the facilities [137].

Pentachlorophenol is not in the group of persistent organic pollutants (POPs) regulated by the Stockholm Treaty but it is regarded as a POP according to OECD (Organization for Economic Co-operation and Development) guidelines [34]. It is included in most priority pollutant lists and has also been classified by the World Health Organization (WHO) as a possible carcinogenic agent to humans [133]. The United States Environmental Protection Agency (US EPA) has classified PCP as a probable human carcinogen based on evidence from animal toxicity studies and human clinical data [138].

The US EPA set a maximum legal limit for PCP in drinking water of 1 μg L^{-1} [136]. For marine and freshwaters, the Council of State and Territorial Epidemiologists (CSTE) of the USA, in 1994, set a water quality objective of 1 μg L^{-1} for PCP [46].

In 1991, the EU adopted some directives that placed a number of restrictions on the use of PCP and Na-PCP in EU Member States [46]. The EU set a MAC of 1 μg L^{-1} for PCP in environmental waters [48]. While maximum discharge levels of 1 mg L^{-1} are permitted for PCP–Na industries, the minimum quality objectives of wastewater are of 2 μg L^{-1} for PCP [136]. Austria, Belgium, Finland, Germany, the Netherlands and Sweden introduced national legislation effectively banning the use of PCP. The production of PCP has ceased in the EU and all current uses rely on imports [46]. The German Regulation on the Prohibition of Chemicals prescribed a tolerable PCP content below 5 mg kg^{-1} in materials and products [134].

In spite of all the restrictions, PCP continues to be an important pollutant since it is stable and persistent. It is clear that legislation is necessary and after its application, nevertheless, the problem of PCP pollution will last for several years.

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Chapter 3

Chromatographic determination of chlorophenols in environmental samples

3.4. Quality control

3.5. Conclusions

3.6. References

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3.1. Introduction

Various methods for analysis of CPs in environmental samples have been proposed, mainly based on chromatographic separation. In most cases, a previous preconcentration/cleaning step is necessary. However, even using preconcentration, some of the methods presented relatively high limits of detection (LODs) and, therefore, can be used only for very contaminated samples. Methods for CPs determination (with low LODs) that can be applied to real environmental samples (waters, sediments, soils, biological tissues) and food are reviewed in this chapter.

For practical purposes, in this chapter the LODs of the different methods will be compared to the concentration range for CPs found in real samples. The concentrations of CPs found in the aquatic environment were already focused in Chapter 2 and presented in Table 2.2. The concentrations of polychlorinated CPs (biocides) in wood and cork samples are variable, from less than ng g^{-1} to tens ng g^{-1} [1, 2] and even µg g^{-1} or more [3, 4]. The presence of CPs in food results from environmental contamination or migration from food storage containers treated with biocides. PCP has been found in fruits in concentrations from ng g^{-1} to several hundreds ng g^{-1} fresh weight [3]. The levels of CPs in the total diet (Slovak Republic) were on average in the order of ng g^{-1} and several tens of ng g^{-1} fresh weight, the highest values being observed for 2,4-DCP and 2,6-DCP [5]. In clam tissues [6] and in honey [7], CPs have been found in the low ng g^{-1} range. CPs have also been found in wines, mainly after bleaching of wooden vessels or treatment with biocides of vessels and cork stoppers. The presence of CPs and of their methylated metabolites chloroanisoles are the reason of a bad flavour of wine [8]. Levels of CPs in the order of ng mL^{-1} were found in wine with such sensory problems [9]. In milk samples, CPs have been found at levels up to several µg L^{-1} [5].

Due to their lipophilic properties, the CPs tend to sorb onto solid material and to accumulate into soils, sediments, sludge and ash samples. Depending on the type of soils and sediments and the pollution sources, the concentration of CPs can range from bellow ng g^{-1} [10] to tens and hundreds ng g^{-1} or even more than µg g^{-1} [11-14]. In sludge samples, 2,4-DCP and 2,4,6-TCP were found in the levels 55-350 ng g^{-1} and 7.5-38 ng g^{-1}, respectively [10]. In ash samples, levels of CPs in the order of tens ng g^{-1} were observed [15].

Some CPs (2-CP, 4-C-3-MP, 2,4-DCP, 2,4,6-TCP and PCP) have been included in the list of priority pollutants established by the US EPA [13]. The EU legislation has set a MAC of total phenols in drinking water to be 0.5 µg L^{-1} and 0.1 µg L^{-1} for individual compounds [16] and 1 µg L^{-1} for PCP in inland and other surface waters [17].

The current trends of CPs determination in environmental and biological samples using chromatography are summarized in this chapter. Special emphasis is given to sampling, storage conditions and the application of preconcentration techniques for the determination of CPs using chromatographic methods. Solid phase extraction (SPE), solid phase microextraction (SPME), stir bar sorptive extraction (SBSE), liquid phase microextraction (LPME), dispersive liquid-liquid microextraction (DLLME), liquid-liquid-liquid microextraction (LLLME) and purge and trap (PT) methods are considered. Methods for microwave (MW) and ultrasonic extraction of CPs from solid matrices are also focused. The methods are compared with respect to the matrices, analytes, LODs and sample size.

3.2. Sampling and storage

3.2.1. Liquid samples

Water samples are usually collected in amber bottles and stored at 4 ºC until analysis [18-24] but storage at 10 ºC in darkness has also been reported [25]. CPs have different stability when stored in acidified river water samples at 4 ºC and some of them, like 2-CP and 4-CP, suffered 15% losses in 28 days [26]. It is advisable to analyze the samples within 24 hours [22] to 48 hours after collection [27, 28]. It is worth noticing that, in biologically active samples, CPs can be rapidly degraded [29]. To prevent losses and to save storage space, it is advantageous to preconcentrate CPs on SPE cartridges, like Isolute EVN+ (a polystyrene-divinylbenzene polymer) and freeze them before elution and analysis [30]. Other possibility can be static sampling using LLLME in which the CPs were extracted into an acceptor phase situated in the lumen of a hollow fibre [31]. Samples can be collected directly in vials, containing NaCl, and acidified and stored at 4 ºC until analysis involving SPME [32]. The authors reported that the standard solutions in reagent water (ultrapure) were stable in these conditions up to 25 days. For CPs analysis in tap water, sodium thiosulphate pentahydrate, at concentration between 80 mg L^{-1} [32] and 1000 mg L^{-1} [33], has been added just after sampling to prevent the oxidation of the analytes by the residual chlorine. Sodium sulfite (1000 mg L^{-1}) [34] and ascorbic acid (175 mg L^{-1}) [35] have been also used with the same purpose. Wine samples, when not analyzed immediately, can be stored at 4 ºC in order to prevent losses from the most volatile analytes [9].

Clear water samples may be analyzed without previous filtration [27, 36, 37]. However, even for relatively clean samples, filtration can be required to prevent blocking of SPE cartridges [22]. Additionally, if suspended particles are left unfiltered, it is possible that the partitioning of CPs between the particulate and dissolved phases would change during

storage. Filters with different pore size in the range 0.22 μm [18, 21, 38, 39] - 1.5 μm [19] have been used. It seems there is no consensus about the most suitable pore size of the filters to be used in sample filtration in order to make easier the comparison of field data.

Different filtration materials have been used so far: nylon [21, 23, 34, 40-42], glass fibre filters [16, 19, 20, 43-48], cellulose [31, 38, 39, 49], cellulose acetate [18] and nitrocellulose [28]. A proper choice of the filtration material is important. For example, filtration of non acidified water samples using nylon filters led to marked losses of CPs, specially the polychlorinated ones, while glass fibre filters could be used without adsorbing or destroying the analytes [44]. However, nylon has been successfully used by other authors to filter samples after acidification [40], or even without acidification [17, 21]. Since no consensus over the proper filtration material is achieved, centrifugation of the samples may be an alternative [50].

3.2.2. Sediment, soil, ash and sludge samples

For the determination of CPs in solid samples there is no consensus regarding the most suitable way of drying and sieving the samples. Literature reports drying of sediment and soil samples by lyophilisation [10, 11] or at room temperature [13, 14, 51-53], 105 ºC [54] and 120 ºC [55]. Sediment, soil or sludge samples have been sieved to particle size below 2 mm [12, 13, 51, 52], 841 μm [55], 300 μm [10, 14, 53, 54, 56] and 120 μm [11]. In most of the cases, the samples have been stored until analysis at 4 ºC [11-14, 51, 52, 54], rarely at room temperature [55]. For ash samples, sieving to obtain particle size below 60 μm and storage at 4 ºC until analysis have been used [15].

In case of spiking, most researchers agree it is very important to keep the sample spiked with the analytes for a certain period before analysis to allow the equilibrium to be attained. The ageing of the solid samples was carried out at 4 ºC for different periods: one day [11], three days [12], three weeks [57], one month [14, 15, 54], six months [56] as well as at room temperature for one week [52]. Low recoveries were observed for sediment samples after six months of ageing [56]. Another study [53] demonstrated that the recovery of CPs decreased during the first two weeks storage of spiked soil at 4 ºC and after that it does not decrease further for ageing times up to two months. The recovery of 2-CP even increased with time after the first two weeks of storage. The authors raise the question of microbial and chemical dechlorination of polychlorinated CPs during storage at 4 ºC. To answer this question, experiments with isotope-enriched spikes could be necessary. Ageing of spiked soil was studied by Alonso et al. [11] for storage times between 12 hours and 48

hours and no significant difference of the recovery was observed. The authors concluded that ageing for 12 hours will be enough to attain equilibrium. The discrepancies among works may result from the fact that the ageing process depends not only on the ageing time but also on the matrix. Therefore, a previous optimization of the procedure for each particular kind of samples is recommended.

3.2.3. Biological tissues and food

The biological tissues and food samples generally require low storage temperatures. For analysis of CPs in cork, the samples were ground and stored at -20 ºC [2, 9] or 4 ºC [1]. Clam tissues samples were freeze dried, homogenized in a grinder and stored at -20 ºC until analysis [6]. Food samples (total diet) were homogenized and deep-frozen [5]. The homogenization of biological samples like algae, for example, can be difficult and freezing it with liquid nitrogen and subsequent grinding is recommended [58].

After storing in the dark for 8 weeks, the average extraction recovery of CPs from cork and wood samples decreased from 104% to 58% [59] and, therefore, similar to the other solid samples, the ageing is recommended to attain equilibrium since it will be more representative of the natural condition of the samples. For the determination of CPs in soft tissues, no ageing of the spiked samples was carried out [5, 6, 58].

3.3. Analytical methods

For the determination of CPs, the methods that have been used, as well as the degree of chlorine substitution in each compound and the respective LODs, are summarized in Table 3.1 for the case of liquid samples, like water and wine, in Table 3.2 for non-biological solid samples and in Table 3.3 for biological solid samples. When the procedures have also been used for other analytes this is stated in notes below the Tables 3.1 to 3.3. From the concentrations of CPs found in real samples (see section 3.1), target LODs for CPs can be established. For waters, methods with LODs below 10 ng L^{-1} can be considered useful for practical application in environmental analysis. For wine samples, the LODs should be less than about 100 ng L^{-1}; 1 ng g^{-1} being the limit set for food samples and 10 ng g^{-1} for solid non-biological samples and wood.

Table 3.1. Methods for analysis of chlorophenols in liquid samples.

Analytical technique	Sample type (volume)	Number of Cl atoms in CPs	LOD (ng L^{-1})	Reference
SPME-GC-MS	landfill leaches (12 mL)	1-5	1-40	[60]
Acetyl-HS-SPME-GC-MS	wastewater (12 mL)	1, 2 [a] 3-5	1-36 (LOQ) 2-21 (LOQ)	[61]
PFBenzoyl-SPME-GC-ECD	groundwater (3 mL)	1-3, 5	5-800	[62]
MW-HS-SPME-GC-ECD	landfill leaches (20 mL)	2-5	100-2000	[63]
SPME-GC-MS	landfill leaches (2 mL)	1-5 [a]	5-1000	[64]
SPME-GC-FID	river water (8 mL)	1-3, 5	5-276	[49]
HS-SPME-GC-FID	well, tap water (15 mL)	1-3, 5 [a]	700-58000	[65]
SPME-silylation-GC-MS	river, wastewater (20 mL)	2, 3 [a]	2-4 (LOQ)	[20]
P-A/HS-SPME-GC-MS	landfill leaches (0.1 mL)	2-5	10-40	[66]
SPME-GC-MS	reservoir, tap, groundwater (20 mL)	1-5 [a]	53-4100	[32]
SPME-GC-MS/MS	tap water, wine, lemon juice (5 mL)	3, 4 [a]	0.25-0.30	[67]
HS-SPME-GC-MS	lake water (10mL)	1, 2 [a]	40-60	[68]
Acetyl-HS-SPME-GC-MS/MS	river, reservoir, wastewater (10 mL)	2, 3 [a]	13-21	[43]
SPME-GC-ECD	deionised water (4 mL)	3, 5 [a]	500	[69]
Acetyl-HS-SPME-GC-MS	wastewater, landfill leaches (5 mL)	1-5 [a]	11-27	[46]
Acetyl-HS-SPME-GC-ECD	river, estuarine, wastewater (10 mL)	1-5	0.1-120	[44]
SPME-LC-EC	drinking water (20 mL)	1-5	5-9	[4]
SPME-LC-EC	river and wastewater (4 mL)	1-3, 5 [a]	13-60	[41]
SPME-LC-DAD	sea, ground, wastewater (4 mL)	1-3, 5 [a]	1000-6000	[18]
Acetyl-SBSE-TD-GC-MS	lake, groundwater (10 mL)	1-3, 5 [a]	100-400	[70]
Acetyl-SBSE-TD/CT-GC-MS	river, tap water (10 mL)	2-5	1-2	[71]
SBSE-LD-silylation-LVI-GC-MS	well, tap, river, wastewater (15 mL)	1-3, 5 [a]	6-65	[28]

SBSE-LC-DAD	tap, sea, wastewater (50 mL)	1, 2 [a]	720-1370	[72]
SBSE-TD/CT-GC-MS/MS	tap water, wine, lemon juice (5 mL)	3, 4 [a]	0.06-0.27	[67]
Acetyl-LPME-GC-MS	river water (1 mL)	1-3 [a]	10-21	[73]
Acetyl-LPME-GC-MS	river, mineral, tap water (10 mL)	1-5 [a]	5-210	[74]
LPME-silylation-GC-MS	reservoir, sea, tap water (5 mL)	2, 5 [a]	15	[75]
LPME-silylation-GC-MS	river water (3 mL)	1-3, 5 [a]	4-61	[42]
SM/LPME-silylation-GC-MS	river, tap water (30 mL)	1-3	15-23	[24]
LPME-tosylation-GC-MS	river, lake, wastewater (5 mL)	1-3 [a]	200-280	[76]
MASE-LVI-GC-MS	groundwater (15 mL)	1-3, 5 [a]	9-595	[25]
MW-HS-LPME-GC-ECD	landfill leaches (10 mL)	2-5	40-700	[77]
HS-LPME-LC-UV	lake, tap water (10 mL)	1, 2	6000-23000	[78]
SPE-methylation-HS-LPME-LC-DAD	river, tap, groundwater (40 mL)	1-3, 5 [a]	40-80	[35]
LPME-LC-DAD	lake, ground, wastewater (20 mL)	1-3, 5	100-300	[23]
LLLME-LC-UV	tap, river, ground, wastewater (15 mL)	1-3	500-1000	[79]
LLLME-LC-UV	ground, lake, river, wastewater (15 mL)	1-3	300-400	[31]
LLLME-LC-UV	seawater (10 mL)	1-3, 5 [a]	20-100	[37]
SM/LLLME-LC-UV	river, well, tap water (20 mL)	1-3 [a]	198-453	[80]
LLLME-LC-DAD	reservoir, tap water (14 mL)	1-3, 5	49-81	[81]
CF/LLLME-LC-DAD	river, tap water (100 mL)	1-3, 5	20-90	[33]
DLLME-acetyl-GC-ECD	well, tap, river water (5 mL)	1-5	10-2000	[27]
SPE-DLLME-acetyl-GC-ECD	well, tap, river water (100 mL)	1-5	0.5-100	[82]
SPE-acetyl-GC-ECD	river water (75 mL)	1-3, 5	3-110	[83]
SPE-acetyl-GC-ECD	river water (250 mL)	1-3, 5 [a]	1-40	[84]
SPE-acetyl-GC-MS	river water (250 mL)	1-3 [a]	15-75	[85]
SPE-acetyl-GC-MS/MS	wastewater (250 mL)	1-3, 5 [a]	10-30	[17]
SPE-silylation-GC-MS	wastewater (2 L)	1-5 [a]	4-44	[45]

SPE-FCC-GC-AES	lake, tap water (10 mL)	1-3	1.6-3.7	[16]
SPE-LC-MS/MS	river, lake, drinking water (2 L)	1-3, 5	1-7	[36]
SPE-LC-MS	groundwater (500 mL)	1-3, 5 [a]	10-25	[58]
SPE-LC-MS	river, wastewater (100 mL)	1, 2	5-48 (LOQ)	[40]
SPE-LC-UV	river, tap, wastewater (10 mL)	1-3, 5 [a]	570-1080	[22]
SPE-LC-UV	river water (200 mL)	1-2 [a]	3300-3700	[86]
SPE-LC-UV	river, tap water (200 mL)	1-3, 5	80-800	[38]
SPE-LC-UV	river, tap water (100 mL)	1-3 [a]	10-90	[34]
SPE-LC-UV	river, groundwater (500 mL)	1-5	20-100	[50]
SPE-LC-UV	wastewater (150 mL)	1 [a]	200-480 (LOQ)	[48]
micro SPE-LC-UV	river, tap, wastewater (60 mL)	1-3, 5 [a]	560-4500	[87]
MSPE-LC-UV	river, wastewater (150 mL)	1	170-220	[88]
MSPE-LC-DAD	river, spring, tap water (200 mL)	1, 2, 5	200-350	[39]
MSPE-LC-MS	river, tap, ground, wastewater (700 mL)	1-3, 5	110-150	[21]
Acetyl-PT-GC-AES	tap water (5 mL)	1-3	23-150	[89]
Acetyl-HS-PTV-GC-MS	tap, river, seawater (5 mL)	1-3	5-8	[90]
Capillary LC-EC	wastewater (2 µL)	1 [a]	1000	[91]
FLLabel-LC-FLD	tap, reservoir, wastewater (500 mL)	1-3 [a]	100-900	[92]
SPE-acetyl-GC-ECD	cork macerate (100 mL)	3-5	<10	[93]
SPE-acetyl-GC-ECD	wine (1 L)	3-5 [a]	8-20	[94]
SPE-acetyl-LVI-GC-MS/MS	wine (1 L)	3-5 [a]	0.2-0.5	[94]
Acetyl-HS-SPME-GC-ECD	cork macerate (5 mL)	3-5	0.8-1.5	[93]
Acetyl-HS-SPME-GC-ECD	wine (8 mL)	3-5	3-20	[8]
Acetyl-DLLME-GC-MS	wine (5 mL)	1-5 [a]	4-40	[9]
HS-SPME-GC-ECD	milk (0.5 mL)	3-5 [a]	560-1010	[95]

[a] Other compounds also analyzed

Table 3.2. Methods for determination of chlorophenols in sediment, soil, sludge and ash samples.

Analytical technique	Extraction procedure	Number of Cl atoms in CPs	LOD (ng g^{-1})	Reference
SPE-LC-UV	MW, *CH_3OH/H_2O (4/1)+2%TEA*; soil 10 g	1-3, 5 [a]	30-80	[11]
SPE-LC-MS	MW, *CH_3OH/H_2O (4/1)+2%TEA*; soil 10 g	1-3, 5 [a]	0.007-0.4	[11]
SPE-silylation-GC-MS/MS	MW, *acetone/CH_3OH (1/1)*; sludge 0.5 g	2, 3 [a]	0.8 (LOQ)	[10]
SPE-silylation-GC-MS/MS	MW, *acetone/CH_3OH (1/1)*; sediment 1 g	2, 3 [a]	0.4 (LOQ)	[10]
SPE-GC-FID	MW, *10^{-5} M NaOH*; sediment 5g	1, 2, 5 [a]	1.3-10.6	[14]
LC-UV	MW, *2% POLE*; sediment 2g	1-3, 5 [a]	2.4-25	[56]
GC-MS	MW-acetyl, *hexane/acetone (1/1)*; ash 1g	1-5	2-5 (LOQ)	[15]
GC-ECD	MW-SD-acetyl-SPE; soil 3g	1-5	13-194	[52]
SPE-LC-MS	Ultrasound, *CH_3OH/H_2O (4/1) +5%TEA*; sediment 30 g	1-3, 5 [a]	1	[58]
Acetyl-SBSE-TD-GC-MS	Ultrasound, *CH_3OH*; soil 1g	1-3, 5 [a]	0.2-0.9	[12]
Acetyl-SBSE-TD-GC-MS	Ultrasound, *CH_3OH/CH_2Cl_2 (9/1)*; sediment 0.5 g	2, 3, 5	1.8-3.8	[96]
SDE-GC-FID	Ultrasound, *0.1 M NaOH*; soil 15 g	1, 3 [a]	100	[97]
Acetyl-PT-GC-AES	Ultrasound probe, *5% K_2CO_3*; soil 7g	1-3	0.08-0.54	[89]
SPME-GC-MS	ASE, *H_2O + 5% CH_3CN*; soil 10g	1-5	1.1-6.7	[13]
Acetyl-GC-MS/MS	QuEChERS, *CH_3CN/H_2O (2/1) +1% acetic acid*; soil 10g	1, 3, 5 [a]	0.5-3	[51]
Acetyl-HS-SPME-GC-MS; soil 1 g	-	1-5 [a]	0.07-0.92	[54]
MW-HS-SPME-GC-ECD; soil 1g	-	2-5	0.1-2	[55]

[a] Other compounds also analyzed

Table 3.3. Methods for determination of chlorophenols in solid biological samples.

Analytical technique	Extraction	Number of Cl atoms in CPs	LOD [b] (ng g^{-1})	Reference
Acetyl-LLE-GC-ECD	*Pentane*; cork	3-5 [a]	0.5-1.7	[2]
GC-MS	Acetyl *(hexane)*; wood	1-5	<20	[3]
GC-MS	Acetyl *(hexane)*; fruit	1-5	<2	[3]
Acetyl-LLE-GC-ECD	MW, *CH_3OH*; cork	3-5 [a]	0.1-0.5	[1]
SPME-GC-MS	MW, *0.5% POLE*; wood	1-5	2-120	[59]
Acetyl-DLLME-GC-MS	Ultrasound probe, *pentane*; cork	1-5 [a]	0.02-0.11	[9]
Acetyl-LLE-GC-ECD	Ultrasound, *hexane/acetone (1/1)*; worms	2-5	20-130	[98]
SPE-LC-MS	Ultrasound, *CH_3OH/H_2O (4/1)+5%TEA*; algae	1-3, 5 [a]	2.5-5	[58]
SPE-IC-MS	Ultrasound, *CH_3OH/H_2O (4/1)+5% TEA*; clam	1-3, 5	0.05-0.5 [dw] (LOQ)	[6]
SBSE-TD-GC-MS	Ultrasound, *ethanol/H_2O (3/1)*; cork	3-5 [a]	0.7-380	[99]
PFBenzyl-SPE-GC-ECD	Alkaline digestion-SDE; food	2-5	0.5-1 (LOQ)	[5]
SPME-LC-EC; wood	-	5	45	[4]
Acetyl-HS-SPME-GC-AES; honey	-	1-5	0.1-2.4	[7]

[a] Other compound also analyzed.
[b] LOD expressed to fresh weight, except otherwise stated.
[dw] dry weight

3.3.1. Water samples

In certain waters with difficult matrices, like wastewaters, liquid chromatography (LC) has been used without preconcentration of the analytes. For example, capillary LC with electrochemical detection (EC) was used to determine 2-CP by direct injection of the samples and provided a LOD of 1 μg L^{-1} [91]. Different CPs were directly determined in tap and wastewater by LC with fluorescence detection (FLD) after fluorescence labelling (FLLabel) with coumarin-6-sulphonyl chloride [92], the LODs being in the range 100 to 900 ng L^{-1}. The direct analysis is simple and rapid, which is an important advantage over the time-consuming preconcentration techniques. Nevertheless, direct injection methods are not widely applied owing to relatively high LODs. However, their sensitivity could be improved, for example, by proper selection of FLLabel reagent [100, 101].

3.3.1.1. SPE

SPE is an exhaustive extraction method that, compared to the previously widely used liquid-liquid extraction (LLE), minimizes the use of organic solvents. However, it requires at least 100 mL of water sample in order to attain sufficiently low LODs (Table 3.1). The necessary sample volume is determined by both the breakthrough volume of the cartridge and the need to reduce the analysis time. Different sorbent materials have been used for SPE of CPs from water samples.

Styrene-divinylbenzene (S-DVB) based resins for preconcentration of free CPs and graphitized carbon black (GCB) for preconcentration of acetylated CPs [102-104] have been used in combination to gas chromatography (GC) with different detectors. S-DVB based resins and GCB were applied for preconcentration of free CPs and the analysis of the extracts was carried out by LC with ultraviolet detection (UV) [19, 26, 30]. A recent study by Elci et al. [16] used S-DVB cartridges to preconcentrate CPs from water samples and to analyze by GC with atomic emission spectrometry (AES) detection. However, instead of using chlorine emission lines, the authors derivatized the CPs by ferrocenecarboxyl acid chloride and used the much more sensitive and selective iron emission leading to LODs from 1.6 to 3.7 ng L^{-1}.

In recent years, conductive polymeric sorbents have been used for SPE of free CPs. Polyaniline [83], poly-N-methylaniline [84] and polypyrrole [85] resins have been used to preconcentrate non-derivatized CPs. Acetylation was carried out after SPE and

the extracts were analysed using GC with electron capture detection (ECD) or mass spectrometry (MS) detection and the obtained LODs were in the ng L^{-1} and tens ng L^{-1} range.

Polyaniline has enhanced performance only for the analysis of polychlorinated CPs, while poly-N-methylaniline and polypyrrole showed quantitative recoveries for all CPs. An important advantage of the polypyrrole sorbent is the very low consumption of desorption solvent which made possible its application on-line with LC-UV without derivatization, having LODs between 10 and 90 ng L^{-1} [34].

The hydrophobic divinylbenzene and the hydrophilic N-vinylpyrrolidone have been used in hydrophilic lipophilic balance (HLB) cartridges. Free CPs from acidified samples [40, 58], or at neutral pH [36], were preconcentrated and determined by LC-MS [40, 58] or LC with tandem mass spectrometry (MS/MS) [36]. Very high breakthrough volumes (2 L) were found, depending on the quantity of HLB sorbent in the cartridge, and CPs with different degree of chlorination were determined with relatively high recoveries. LODs in the order of several ng L^{-1} were obtained. A recent study demonstrated that coupling of HLB and C18 cartridges could be used for the simultaneous extraction of analytes with very different polarities [45]. After silyl derivatization of the extracts and analysis by GC-MS, the LODs were in the range 4 to 44 ng L^{-1}.

New materials for SPE have been used in recent years for CPs determination with quantitative recoveries, such as multi-walled carbon nanotubes [38] or co-polymers, molecularly imprinted with 2,4,6-TCP as a template molecule to increase the selectivity of SPE [22]. The extracts were analysed by LC-UV and LODs from a hundred to a thousand ng L^{-1} were obtained.

Silica particles covered with dialkylated cationic surfactant were excellent sorbents for CPs with different degree of chlorination, leading to LODs between 20 and 100 ng L^{-1} when LC-UV was used to analyze the extracts [50]. The sorption of CPs was based on both electrostatic and hydrophobic interactions. In contrast, when an anionic surfactant was used (alumina covered with sodium dodecyl sulfate), only hydrophobic interactions took place, leading to lower recovery of more polar mono- and di-CPs [105].

Nanosized sorbents have high extraction capacity. Unfortunately, it is difficult to pack the small particles into cartridges. Magnetic solid phase extraction (MSPE) has been used recently. Nanoparticles with magnetic properties (Fe_3O_4) were covered with

cationic surfactants or ionic liquids [21, 39] or combined with clay particles with high sorption capacity [88] and suspended in the water sample for SPE of CPs. After the extraction, the magnetic particles were isolated using a strong magnet. The analysis of the extracts was carried out by LC-MS or LC-UV with LODs of about a few hundreds ng L^{-1}. Quantitative recoveries were obtained. The suspension SPE method was fast because the loading of sample to the SPE cartridge was avoided.

3.3.1.2. PT

In PT techniques, the analytes are preconcentrated and separated as completely as possible from the sample matrix using purging agent (inert gas or water vapour) after which they are sorbed into a suitable solid or liquid trap. It is required that the analytes are volatile at the purging conditions used.

For instance, CPs were acetylated to reduce their polarity and increase the volatility and were subsequently purged by a flow of helium into a Tenax GC trap [89]. Care should be taken when choosing the right trap material since a recent study demonstrated that Tenax TA can cause decomposition of acetyl-CP species [90]. The analytes were thermally desorbed by heating the trap and purged into GC-AES for analysis. The method required only 30 minutes per sample. The LODs were between 23 and 150 ng L^{-1} but PCP was not volatile enough to be purged, even after the acetylation. The steam distillation extraction (SDE) method [97] used the volatility with water vapour of the non-derivatized CPs. Here, the purging agent was water vapour and the trapping was carried out by simultaneous LLE of the CPs from the condensate, with diethyl ether. The hydrophobicity of chlorine-substituted phenols strongly enhanced the extraction efficiency and quantitative recoveries were obtained. The organic extracts were analyzed by GC with flame ionization detection (FID) and LODs of 10 µg L^{-1} were obtained. The method has potential to be applied to real samples when using more sensitive and selective detectors. The steam distillation required about 90 minutes per sample but can be accelerated using MW heating.

3.3.1.3. Microextraction methods

The microextractions are non-exhaustive methods that strongly minimize or even completely eliminate the use of organic solvents. The extraction is not complete but the extracted quantity is proportional to the concentration of CPs in the sample.

They generally require 1 to 20 mL of sample, which is an important advantage having in mind storage space and limited sample availability, specially in model studies.

3.3.1.3.1. Headspace evaporation

This method, although similar to the PT techniques, will be considered together with the other microextraction techniques because it is also a non-exhaustive method. Pavon et al. [90] reported that acetyl-CPs were purged from the headspace and cryogenically trapped into empty or packed liners using programmed temperature vaporization (PTV). The analytes were analyzed using fast GC-MS after flash-heating of the liner to desorb the analytes. Since Tenax TA sorbent destroyed the acetyl-CPs, an empty liner was used. The LODs are between 5 and 8 ng L^{-1}.

3.3.1.3.2. SPME

Due to the polar nature of the CPs, a polar polyacrilate (PA) fibre was used for sampling of the non-derivatized analytes. The fibre was immersed in the sample at pH about 2, in the presence of NaCl or Na_2SO_4, during 40 to 60 minutes. Then, the CPs were desorbed from the fibre in the hot GC injector. The detection was carried out using MS [32, 60, 64, 106] or ECD [69]. However, the direct sampling of CPs using PA fibre may depend on the sample matrix, like the presence of surfactants and humic acids, but a simple increase of the extraction time could be sufficient to eliminate the matrix effect [106].

To decrease the matrix interferences and to increase the fibre life-time, sampling from the headspace (HS) was tested but it did not result for TeCPs and PCP when conventional heating of the samples was used [63, 64]. When MW energy was used to heat the sample, the SPME procedure was completed in about 5 minutes and it was possible to determine CPs from the headspace, including 2,3,4,6-TeCP and PCP [63]. Similar results were obtained by purging the sample with nitrogen and fast headspace extraction (30 minutes) of CPs (including 2,3,4,6-TeCP and PCP) [66].

Laboratory-made fibres have been also tested. Carbon monolith fibre permitted short extraction time and displayed high capacity to phenolic compounds [68]. Polyaniline fibres could be prepared by highly-reproducible electropolymerization process. Such fibres were used for HS sampling of phenols with different degree of

chlorination, even PCP, but they had low thermal stability [65]. It was found that calixarene and carbon aerogel fibres had higher thermal stability and presented no carry-over problems, usually encountered when PA fibres were used, specially for PCP [49, 106, 107].

Without derivatization, the LODs, depending on the analyte, have been, in most cases, between several ng L^{-1} and several µg L^{-1} (Table 3.1) [32, 49, 60, 63-66, 68, 69]. Such values are not sufficiently low to determine CPs in most environmental water samples. When the analytes were converted to less polar and more volatile analytes, chromatographic separations were improved and the LODs were usually enhanced [20, 43, 44, 46, 61].

A method to carry out the derivatization of the analytes was on-fibre silylation after direct SPME of the CPs with polar (PA) or bi-polar polydimethylsiloxane/divinylbenzene (PDMS/DVB) fibres [20]. The fibre with the sorbed CPs was exposed to N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide for 10 minutes and after thermal desorption the derivatives were separated and detected using GC-MS. Limits of quantification (LOQs) of 2 to 4 ng L^{-1} were obtained. Another tested approach was the *in situ* derivatization, using direct SPME at pH about 11, by pentafluorobenzoyl chloride previously sorbed on PDMS/DVB fibre [62]. The separation and detection were performed by GC-ECD. The LODs, depending on the analyte, were from 5 to 800 ng L^{-1}. Also in this case, an additional step of 10 minutes was required for the sorption of the derivatizing agent to the fibre, before SPME.

Acetylation has been another type of *in situ* derivatization, carried out by adding acetic anhydride to the sample at alkaline pH, maintained by alkaline metal carbonates [44], bicarbonates [46, 61] or hydrogen phosphates [43]. Since the derivatization occurred in the water sample, the number of analytical steps was minimized. The use of hydrogen phosphates had the advantage of preventing bubble formation (carbon dioxide) and eventual overpressure during the extraction. Owing to the high volatility and low polarity of the acetylated derivatives, it is convenient to sample them from the headspace using bi-polar PDMS/DVB [44], carboxen/polydimethylsiloxane (CAR/PDMS) [61], divinylbenzene/carboxen/polydimehtylsiloxane (DVB/CAR/PDMS) [43] or non-polar polydimehtylsiloxane (PDMS) fibres [46, 61]. Non-polar fibres are more appropriate to determine polychlorinated CPs owing to their low polarity [61]. CAR/PDMS fibre suffers from strong carry-over [44, 46]. To simultaneously determine CPs with different degree of chlorination in water samples after acetylation, the best fibre seems to be PDMS/DVB [44]. The detection has been carried out by MS [61],

MS/MS [43] and ECD [44]. The LODs obtained, depending on the analyte, were from less than ng L^{-1} to a hundred ng L^{-1}.

SPME with carbowax-templated resin (CW-TPR) fibre was coupled with LC after on-line desorption using the mobile phase [18] or off-line desorption in a small volume (40-60 µL) of organic solvent mixture [4] or a micellar solution [18]. Using diode array detection (DAD), the LODs obtained were from 1 to 6 µg L^{-1} [18]. With amperometric electrochemical detection, the LODs were from 5 to 9 ng L^{-1} but marked carry-over was observed with CW-TPR fibre [4]. Static desorption of the CPs from PA fibre in the injector of the LC-EC solved the carry-over problems and permitted to attain LODs from 13 to 60 ng L^{-1} [41].

3.3.1.3.3. SBSE

A drawback of SPME is the small volume of enrichment phase attached to the fibre (0.5 µL). In SBSE, a magnetic bar is covered with much larger quantity of acceptor phase which strongly enhances the extraction efficiency [28]. As with SPME, carry-over problems can be an important limitation caused by repeated analysis with the same stirring bar.

The acetylated derivatives of CPs, due to their low polarity, can be more efficiently sorbed into a stir bar covered with a PDMS phase. In a work from Montero et al. [70], after the extraction, the bars were heated in a thermal desorption unit and the desorbed derivatives analyzed by GC-MS, providing LODs between 100 and 400 ng L^{-1} or even two orders of magnitude less if the analytes were cryofocused after the thermal desorption [71]. The SBSE with PDMS coated bar has also been used to extract non-derivatized CPs. After the extraction, the CPs were desorbed into ethylacetate and the extracts were silylated and analysed by GC-MS using large volume injection [28]. The LODs were between 6 and 65 ng L^{-1}. Lower LODs (0.06 to 0.27 ng L^{-1}) were obtained using thermal desorption with cryofocusing and GC-MS/MS detection [67]. However, the method of liquid desorption is less expensive because avoids the use of thermodesorption device.

Until recently, only non-polar PDMS coatings for SBSE were available, thus limiting the selectivity of the method [28]. However, the new SBSE coatings, like poly(vinylpyrrolididone-divinylbenzene) monolithic material (VPDB) [72] or polyurethane

foams [108] have much higher affinity to non-derivatized CPs than PDMS coating do, which is a promising future development of the CPs determination.

3.3.1.3.4. LPME

Compared to SPME and SBSE, the LPME has the advantage of not suffering carry-over effects. It requires very small amounts of organic solvents. One approach to reduce the solvent consumption during the traditional LLE can be the use of membrane-assisted solvent extraction (MASE) [25]. A small volume (less than 1 mL) of organic solvent was put into a membrane bag made of dense polypropylene and the membrane was placed into the sample. After the extraction, the analysis was carried out by GC-MS equipped with large volume injector and the LODs obtained were between 9 and 595 ng L^{-1}. A recent study demonstrated that integrated stirring into the extraction unit could provide much better performance compared to the case of separated extraction and stirring units [24].

A larger reduction of solvent consumption was obtained using single drop microextraction and hollow fibre LPME. A single drop of 50% solution of acetonitrile in water was exposed to the headspace above the sample and heated with the help of ultrasound energy [78]. Due to the limited drop volume of about 5 µL, and to the difficulties to cool the solvent drop, the efficiency of this extraction procedure was relatively small. For this reason, the sampling from the headspace was carried out in 10 µL of 50% acetonitrile, placed in the bottom of a PCR tube inserted into the vial cap with the bottom upwards. The bottom of the PCR tube was placed out of the vial and was cooled in an ice bath, which, together with the larger volume of the acceptor solvent, increased greatly the efficiency of the process [78]. The use of ultrasound enabled a decrease of the extraction time to only 25 minutes. However, the analysis was carried out by LC-UV and relatively high LODs, in the range 6 to 23 µg L^{-1}, were obtained.

Other possibility, in order to increase the volume of the organic phase, is the application of polytetraflourethylene (PTFE) sleeve over the needle of the syringe [23, 35]. Vesicle-based coacervate drops with large volume (30 µL) were used as solvents, compatible with LC analysis [23]. These multifunctional coacervate drops interact with CPs by hydrophobic, π-cation and hydrogen bond interactions and have the ability to solubilise analytes with a wide range of polarity. LODs from 100 to 300 ng L^{-1} were reported [23].

Sampling from the headspace of non-derivatized CPs using disposable hollow fibre LPME could be achieved using MW energy [77]. In these conditions, very short extraction times (10 minutes) were required, even for polychlorinated CPs. However, an efficient cooling system was necessary to prevent solvent evaporation from the hollow fibre and to increase the extraction efficiency. The analysis of the extract was carried out by GC-ECD with LODs in the range 40 to 700 ng L^{-1}.

The CPs have been acetylated *in situ* and the derivatives extracted in a microdrop of butylacetate on the tip of a syringe needle [34] or in a floating drop of 1-undecanol which, after the fast extraction, solidified upon cooling [74]. Another type of derivatization, specially suitable for solvent microextraction techniques owing to the small volume of extractant required, is the silylation in the hot injector after the injection of the extract [75] or silylation inside the syringe before the injection [42]. Using GC-MS for analysis of drops, LODs in the order of tens ng L^{-1} were obtained for both acetylation and silylation derivatization [42, 74, 75]. The derivatization could be carried out inside the drop simultaneously with the extraction. For instance, the CPs were transported from the sample, containing an ion-pair agent, to the drop of the organic solvent containing tosyl chloride to derivatize the CPs [76]. After GC-MS analysis, the LODs were from 200 to 280 ng L^{-1}.

Sampling from the headspace with a single drop (10 µL) was applied after methylation of the CPs with dimethyl sulphate in alkaline media [35]. The methylation was required to both increase the volatility of the analytes for the headspace sampling and improve separation in the subsequent LC-UV analysis. Thus, despite derivatization has been mainly used for GC analysis, it can also improve LC performance. When combined with SPE, this method permits to attain relatively low LODs, between 40 and 80 ng L^{-1}.

Acetylated derivatives of the CPs have been extracted from water samples by means of a water soluble disperser solvent, containing small quantity of water insoluble and dense extraction solvent. After addition of the disperser solvent to the sample, a finely dispersed emulsion (microdrops of the extraction solvent) was formed leading to practically immediate extraction, called DLLME [27]. After centrifugation, the lower layer was analyzed by GC-ECD and the LODs varied from 10 ng L^{-1} to 2 µg L^{-1}. The highest LODs values were obtained for mono-CPs due to the lower sensitivity of ECD to these compounds. MS detection may improve the sensitivity of the method for mono-CPs. Additional improvement of the method was attained by combining it with SPE [82]. The elution solvent during the SPE phase should be chosen carefully because it

should also play the role of disperser solvent during DLLME. With GC-ECD, the LODs were in the range 0.5 to 100 ng L^{-1}. Advantage of DLLME is its extreme rapidity, decreasing strongly the time and cost of the analysis.

Additional improvement in selectivity can be obtained using LLLME of non-derivatized CPs. For this purpose, the CPs were extracted from the acidified sample, in their molecular forms, into an organic solvent (1,2,4-trichlorobenzene [81] or polar ionic liquid [37, 79]) impregnating the hollow fibre membrane from where the CPs diffuse into 10 to 15 µL of the alkaline acceptor phase. There, the CPs were transformed into their respective anions which cannot return back into the membrane and are concentrated into the acceptor phase. The analysis was carried out by LC-UV and the LODs were 500 to 1000 ng L^{-1} [79] or several tens ng L^{-1} [37, 81]. In the work of Lin and Huang [81], alkaline mobile phase was used since the CPs anions provided better ultraviolet spectra than did uncharged CPs. Disk-shaped supported liquid membranes, impregnated with dichloromethane, were used for LLLME from the sample to alkaline acceptor phase using continuous flow (CF) operation, offering an high enrichment factor and very good stability of the liquid membrane [33].

3.3.2. Other liquid samples

Some of the methods used for water sample analysis could be applied for CPs determination in other liquid samples (Table 3.1). For instance, SPE was used for determination of CPs in wine samples. A large volume (1 L) of red wine was successfully preconcentrated on HLB cartridges and, after acetylation, the extracts were analyzed using GC-MS/MS obtaining LODs in the range 0.2 to 0.5 ng L^{-1} [94].

The CPs from wine and cork macerate samples were acetylated and analyzed by HS-SPME-GC-ECD with PDMS fibre [8, 93] with quantitative recoveries for most of the CPs studied (compared to standards in hydroalcoholic solution). However, it is possible that PCP determination in wine depends on the matrix, since one of the studies reported very strong matrix effect from white wine, while it was possible to determine it in cork macerate [93]. The other study reported successful PCP determination in red wine using the same method [8].

A similar HS-SPME-GC-ECD method, but with PA fibre and without derivatization, was used to determine the CPs in human milk samples, obtaining LODs

from 560 to 1010 ng L^{-1} [95]. The samples were acidified with perchloric acid in order to de-conjugate the CPs.

3.3.3. Solid samples

3.3.3.1. Extraction of CPs from a solid matrix

The shaking of the sample with an organic solvent is cheap and effective in some cases but most often is time consuming or presents low recoveries. For instance, shaking for 30 minutes with hexane with simultaneous acetylation of CPs was applied to aqueous slurry of fruits and wood samples [3]. The recoveries were quantitative for fruits but only between 42% and 58% for the wood samples. Cork samples were extracted with hexane by shaking for 90 minutes [2], with quantitative recoveries, except for PCP (57-76%).

An alternative shaking method, which was classified by the authors [51] as a quick, easy, cheap, effective, rugged and safe (QuEChERS) procedure was reported. Extraction was carried out with a mixture acetonitrile/water (2/1) acidified with acetic acid (0.66%) for one hour. After salting out, the acetonitrile layer was removed. Quantitative recoveries were obtained, possibly because the method does not require evaporation step. However, further validation studies are necessary, since the authors did not apply their procedure to certified reference materials (CRM) and did not mention if ageing of the spiked samples was used in the validation protocol.

Accelerated solvent extraction (ASE) from soil samples with water containing 5% acetonitrile as organic modifier permitted to minimize the use of organic solvents and took only 30 minutes [13]. The recoveries were in the range 42 to 82%, the lowest ones being found for polychlorinated CPs. Most of the researchers opted to use ultrasound or microwave-assisted extraction to enhance the efficiency of the extraction or to speed-up the process, as will be detailed below.

3.3.3.1.1. Ultrasonic extraction

Organic solvents were widely used in ultrasonic extraction of CPs. Sediment and soil samples were ultrasonically extracted using methanol/dichloromethane (9/1) for 15 minutes [96], methanol for 30 minutes [12] and methanol/water (4/1) containing 5% triethylamine (TEA) for 20 minutes [58]. In the last case, TEA prevented the losses

of CPs in the subsequent evaporation of methanol. Recoveries higher than 80% were obtained with the exception of PCP (about 70%) which is, generally, the most problematic species of CPs to extract from the solid matrix due to its high hydrophobicity.

Soil samples were extracted with 0.1 M NaOH for 60 minutes in ultrasonic bath [97] or with 5% potassium carbonate using ultrasound probe for 30 seconds [89]. Both alkaline extraction methods provided recoveries higher than 75-80% but TeCPs and PCP were not analyzed.

The ultrasonic extraction of CPs from biological tissues generally displays less recovery problems than in the case of sediments or soil samples. Extraction from cork samples with ethanol/water (3/1), involving ultrasonication and shaking for about one day [99], or with pentane, using ultrasound probe for 3 minutes [9], provided quantitative recoveries, including for PCP. For algae samples, extraction for 20 minutes using methanol/water (4/1) containing 5% TEA [58] led to recoveries of CPs in the range 70-90%, the lowest value being observed for PCP.

Chlorophenols from soft biological tissues (worms) were ultrasonically extracted for 10 minutes with hexane/acetone (1/1) after acidification with sulphuric acid [98]. For clam tissues, 20 minutes of extraction with methanol/water (4/1) containing 5% TEA was used [6]. Recoveries higher than 80%, including for PCP, were obtained with both methods.

3.3.3.1.2. MW extraction

A MW extraction process requires optimization of the following parameters: composition and volume of the solvent, pressure or temperature, MW power and time of extraction and also, possibly, derivatization reagents quantities.

Most of the methods for MW extraction use organic solvents. The extraction time for CPs using this process was between 16 minutes [109] and 90 minutes [1] and the organic solvent volume was between 15 mL [109] and 50 mL [11]. Either acid or base additives to the solvent were found to be useful sometimes to increase the recovery. MW extraction was carried out both in closed [1, 10, 15, 109] and open vessel systems [11].

Methanol was used for extraction of CPs from cork samples with about 90% recoveries for polychlorinated CPs [1]. Acetone/hexane (1/1) was used for soils [109] or with simultaneous acetylation, in the presence of TEA as a base, for the case of ash samples [15]. Recoveries between 72 and 94% were obtained for ash samples; the lowest values were found for 2,6-DCP. Acetone/methanol (1/1) containing 1% formic acid was used for sludge and sediments [10] and the recoveries were independent on the matrix, between 85% and 94% on average, even for sludge samples containing very high levels of organic matter. Methanol/water (4/1) in the presence of 2% TEA was used for soil samples with recoveries higher than 77%, including for PCP [11].

Alkaline and micellar extractions have the advantages of completely eliminate the use of organic solvents and strongly decrease the extraction times. A solution of NaOH (pH=9) was used in closed vessels to extract CPs from sediments in 6 minutes [14]. A possible drawback of alkaline extraction could be the interference of humic material, co-extracted with CPs at high pH [13].

Micellar solution of polyoxyethylene-10-lauryl ether (POLE) was used in closed vessels to extract CPs from soil [53] or sediment samples [56] or in open vessels for wood samples [59]. It was possible to work without addition of acids or bases to the extraction medium. The method for CPs extraction showed higher recoveries than the Soxhlet extraction with organic solvent and the extraction time was only 2 to 3 minutes. Recoveries were quantitative and independent of the matrix, both for soil and sediments [53, 56].

The extraction under MW field is a very rapid method that may solve the problem of poor recoveries of polychlorinated CPs. However, it is necessary to check if catalytic reactions on the solid matrix induced by MW radiation occur [11], which could lead to poorer recoveries of some analytes. CPs were found stable in MW field during alkaline extraction of sediments [14] but studies for other procedures and other matrices are yet to come.

3.3.3.2. Extracts' clean-up

In the case of extraction with organic solvents, after evaporation, the extracts have been purified using SPE with Isolute EVN [11] or, more recently, with Oasis HLB cartridges [6, 58], followed of analysis using LC, without any further treatment. However, most often GC was used even if it required more complicated treatment, like

derivatization. When the matrices of the samples are very complex, the cleaning may require several steps. Removal of basic and neutral interferences by back-extracting them from an alkaline solution was used to clean-up extracts from sludge and sediment samples [10]. After that, SPE was used with Oasis HLB cartridges and the extracts were finally silylated before analysis.

If water miscible solvents were used, the extracts could be diluted with water and the CPs preconcentrated using SBSE with *in situ* acetylation [12, 96] or without any derivatization [99]. The organic extracts, containing the CPs to be analyzed could be evaporated [1, 9] or purified by extraction into alkaline solution [2, 109] and the CPs could be acetylated in aqueous medium. Then, the derivatives were re-extracted with organic solvent using LLE [1, 2, 98] or DLLME [9]. It seems to be possible to acetylate the CPs directly into the organic solvent used for the extraction of the solid sample, in the presence of pyridine as base [51].

Extracts in organic solvent of acetyl-CPs, obtained after simultaneous extraction and acetylation from fruits and wood [3] and ash samples [15], were analyzed using GC-MS without further treatment, apart from possible evaporation of the organic solvent.

The extraction with organic solvent usually required its evaporation, which, in conjunction with high toxicity and negative environmental impact, could lead to possible losses of the analytes. In contrast, the extracts of solid samples with alkaline and neutral aqueous media could be compatible with LC [53, 56], SPME [13, 59], PT [89] or SDE [97] without further purification of the extract. However, for some complex sample matrices, specially when alkaline water was used for extraction, extracts should be cleaned-up [14].

3.3.3.3. Extraction-preconcentration integrated procedures

3.3.3.3.1. SDE

The SDE of solid samples can be integrated with liquid extraction or SPE, although it has to be carried out *off-line* to the main analytical device. Such procedure was applied to total diet food samples, which were de-conjugated in alkaline conditions and, after acidification, distilled with water vapour with simultaneous extraction with toluene for 1 hour [5]. After that, the CPs were derivatized with pentafluorobenzyl bromide for 3 hours, the solution cleaned-up with Florisil and analysed by GC-ECD.

The method was very sensitive, in spite of being time consuming, with LOQs between 0.5 and 1 ng g^{-1} (Table 3.3) and quantitative recoveries were obtained. Microwaves can be used to speed-up the SDE. In another case [52], a soil sample was mixed with water, the CPs were acetylated and the acetyl-CPs were distilled with water vapour using MW energy. The distillate passed through *on-line* SPE cartridge. Both C18 and ENVI-18 were used and provided quantitative recoveries, mainly as a result of the lower polarity of the derivatized analytes. The whole distillation process took about 16 minutes. The analysis was carried out with GC-ECD and the LODs were between 13 and 194 ng g^{-1} (Table 3.2).

3.3.3.3.2. SPME

To analyze acidified slurries of solid samples, SPME can be used, which eliminates a previous extraction step. It can be easily automated to be carried out *on-line* with the main analytical device. SPME requires less amount of sample than usual methods to extract CPs from solids and it is much faster. Direct SPME with PA fibre was applied to determine 3-CP in slurry of contaminated soil, using GC-FID for analysis [110]. A CW-TPR fibre and analysis by LC-EC were used for PCP in wood slurry [4]. As adsorption of CPs to the fibre is a relatively slow process, non-equilibrium SPME has also been used, applying a suitable internal standard, given that it would reach equilibrium for the same time as the analytes [110]. However, as relatively high LODs were obtained (for instance, 45 ng g^{-1} for PCP in wood [4]), direct SPME could be used to analyze only relatively contaminated samples.

The transfer of CPs with different degree of chlorination from the acidified slurry to the HS could eliminate the matrix effects encountered with complex soil matrices [55]. The extraction from the headspace using PA fibre was accelerated by using microwaves and accomplished in less than 10 minutes, even for the least volatile PCP, after which the analysis was carried out with GC-ECD. Another approach for fast HS-SPME was to combine it with *in situ* acetylation. This method was applied for soil slurry analysis [54] and honey samples [7]. The acetyl-CPs from the soil slurry were preconcentrated using the PDMS fibre at 100 ºC and analyzed by GC-MS. At this temperature, the extraction time profile had a very peculiar shape, the analytical response being maximal at 20 minutes and strongly decreasing afterwards. The authors proved that this behaviour was a consequence of the soil matrix. The acetyl-CPs from the honey sample were adsorbed to PDMS/DVB fibre and analyzed by GC-

AES. The equilibrium was reached in 30 minutes at 90 ºC. The LODs for HS-SPME methods for solid samples were in the order of ng g^{-1}, or less, and can be used even for the analysis of non-contaminated samples.

3.4. Quality control

The Federal Institute for Materials Research and Testing [111] and the Institute for Reference Materials and Measurements [112] sell industrial soils certified for PCP and, in some cases, also with 3,4-DCP and 2,4,5-TCP, and wood samples certified for PCP. Specially good source of environmental reference materials is the Resource Technology Corporation (RTC) [113] which sells a rich variety of materials, namely lake sediment, sewage sludge, soils with different matrices and levels of contamination. CRM from RTC contain one or more of the following CPs: 2-CP; 4-C-3-MP; 2,4-DCP; 2,6-DCP; 2,4,5-TCP; 2,4,6-TCP and PCP. Unfortunately, for water and food samples, there are no CRM, and in such cases the accuracy has been estimated by spiking. To our knowledge, the ageing of the CPs spiked into solid food samples was not evaluated but at least one day ageing is advisable, based on the properties of CPs to undergo slow equilibration with solid matrices. Due to the very high price, even if the CRM are available, only a limited number of studies have included analysis of CRM into method development [52-54, 89, 96]. Participation into inter-laboratory exercises has been also reported [4].

3.5. Conclusions

A lot of recent methods aimed at the simultaneous determination of CPs with different degree of chlorination. However, the sampling storage, despite of being an important step of analytical process, has been largely underestimated in the literature. Application of organic solvent-free (or minimal solvent) techniques has been used in the last decade.

The target LODs for CPs in water samples (in the low ng L^{-1} range or lower) can be attained only if a preconcentration step (microextraction or SPE) with derivatization is included, and the analysis is carried out using GC with a sensitive detector, like ECD or MS. Apart from lower cost and easier maintenance, ECD has no advantage over MS detector because it is less sensitive for mono-CPs. The MS detector has also the advantage of making possible the use of stable isotope-labelled surrogate standards.

This enables to work with very difficult matrices and to check out for eventual CPs transformation during sample storage and analysis. The use of LC does not require derivatization but preconcentration and use of sensitive detectors (MS or EC) are necessary to analyze CPs in real water samples. More widespread detectors, like UV, can still be used successfully if high preconcentration is achieved with SPE or LLLME techniques. In non-biological and biological solids and wine samples, almost all cited methods permit attaining LODs suitable for real sample applications. The main trend in solid sample analysis has been the development of fast methods for CPs withdrawal from the solid matrices, like MW extraction, that are able to disrupt the strong interaction between the matrix and the lipophilic CPs, specially PCP. An alternative of extraction methods, integrating into one step extraction and analysis of solid samples, is a very attractive future trend.

3.6. References

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Chapter 4

Objectives

4.1. Aim

The aim of this study was to investigate the fate and effects of CPs to freshwater phytoplankton species commonly present in Portugal. Considering all the CPs and using all the phytoplankton species found in Portugal? This would be a far too broad goal in the context of a four years' project like the present one. An attainable aim would imply the choice of CPs and cyanobacteria and microalgae species. One of the CPs and at least one phytoplankton species, specifically a cyanobacterium, had to be selected. This selection was crucial to the work and the selected phytoplankton species, *M. aeruginosa* and *C. vulgaris* (Chapter 1), and the selected compound, PCP (Chapter 2), are considered in all the following objectives. The interactions between PCP and phytoplankton species, namely cyanobacteria, are hardly known and deserved particular attention. For comparison, the interactions between PCP and *C. vulgaris*, somehow better well known, were also studied, which later allowed more complex interactions to be investigated by mixing the two species and exposing them to the toxic compound. Having in mind this specific aim, the study of PCP, *M. aeruginosa*, *C. vulgaris* and their interactions, five objectives result and are further detailed.

4.2. Objectives

4.2.1. Development of an analytical method

In order to accomplish the aim of this work, an analytical method for the determination of CPs had to be previously developed. The characteristics of that method should include very low LODs and using a small volume of sample. These requirements arise from the necessity to measure environmental concentrations of PCP during ecotoxicity tests with a limited amount of sample. Based on the information from Chapter 3 (Table 3.1), such method could be SPME coupled to GC with an element-specific detector.

Therefore, the first objective of this thesis was to develop a method for the determination of PCP in the culture media. However, in order to expand the application of this research, the development of a new method, never used before for water sample analysis, was decided. The studied analytes were not limited to PCP but included some more CPs with different degree of chlorination, having different properties (Table 2.1), selected among those commonly found in the environment

(Chapter 6). In this way, the method will offer the freedom of choice to use other CPs in future studies, or even mixtures of CPs. This method can be applied to different kinds of water samples and will not be limited to laboratory scale ecotoxicity studies.

The second objective was to study the stability of environmental levels of CPs in different types of water samples for a variety of storage conditions (pH, temperature and derivatization). The integrated idea of an analytical process contains not only the measurement procedure but all the steps required to obtain information about the samples, including sampling and storage. It was expected to find out the best procedures for long-term sample storage. Furthermore, since the analytical method to be developed is expected to work with small sample volumes, the long-term storage procedures will be cost-effective. The conclusions drawn from this experiment (Chapter 7) can help in the design, and even in the validation, of future ecotoxicity studies with environmental samples.

4.2.2. Ecotoxicity studies

After the necessary analytical development, and in order to verify the suitability of the chosen phytoplankton species for the ecotoxicity studies, a series of preliminary experiments had to be carried out (detailed in the section 5.2.3 (Chapter 5)). Following this unavoidable step, the third objective was to study the fate and effects of PCP on *M. aeruginosa*, which is a main novelty of this work (Chapter 8). Environmental levels of PCP had to be studied, regarding application of the data in real cases. It was necessary an additional ecotoxicity study of PCP on the microalga *C. vulgaris*, similar to that of the cyanobacterium, in order to allow further expansion of the objectives of this thesis.

The fourth objective was to understand if *M. aeruginosa* is able to remove PCP from the cultures. This is also hardly known not only to *M. aeruginosa* but also to *C. vulgaris*, so both species were studied with this purpose (Chapter 8). The removal abilities were studied not only in pure cultures but also for the mixture of the two phytoplankton species.

The fifth objective, as a logical sequence from the previous two objectives, was the comparison of the toxicity profiles and removal abilities of *M. aeruginosa* and *C. vulgaris*, when exposed to environmental levels of PCP, in mixed cultures with both species (Chapter 9). The results obtained in the accomplishment of the previous two

objectives had to be verified in the mixture of the species. Previous to this experiment, the interspecific interactions in mixture, without PCP, required investigation. The mutual influence is species-dependent and difficult to predict. The population dynamics in phytoplankton mixtures is complex, even in the absence of external toxic substances (Chapter 1). Since the interactions of *M. aeruginosa* and *C. vulgaris* have barely been studied, this is an additional novelty of this work.

PART II

EXPERIMENTAL

Chapter 5

Materials and methods

5.1. Analytical method development

5.1.1. Reagents and solutions

Solvents and reagents were analytical grade except otherwise stated. Solutions of 2-CP, 4-CP, 2,6-DCP, 2,4-DCP (Pestanal®, Riedel-de Haen, Seelze, Germany), 2,4,6-TCP (Pestanal®, Fluka, Steinheim, Germany), 2,3,4,5-tetrachlorophenol (2,3,4,5-TeCP) (Supelco, Bellefonte, USA) and PCP (98%, Aldrich, Steinheim, Germany), with concentrations about 2 mg mL^{-1}, were prepared in absolute ethanol (Panreac, Barcelona, Spain) and stored in a freezer in the dark. Working standard mixed solutions, with concentrations about 4 μg mL^{-1}, were obtained daily by dilution with ethanol. Acetic anhydride (AA) (Merck, Darmstadt, Germany) and anhydrous sodium carbonate (Riedel-de Haen, Seelze, Germany) were used for *in situ* acetylation of the analytes. Sodium chloride was purchased from Merck (Darmstadt, Germany). Deionized water with conductivity < 0.1 μS cm^{-1} was used throughout.

For decontamination, all the glassware for the analytical measurements was rinsed with water, soaked overnight in 20% (v/v) nitric acid (Pronalab), rinsed with water again and dried.

Nylon filters (0.45 μm pore size, Whatman®) and glass fibre filters (1.2 μm, Whatman®) were used for filtering the samples. The glass fibre filters were cleaned with methanol (Chromasolv, Aldrich). In order to test the suitability of the method for the determination of the ecotoxicity effects of CPs to algae, some of the experiments were carried out in f2 algal culture medium (pH 7) [1].

5.1.2. HS-SPME Sampling

Headspace solid phase microextraction was performed by an automatic sampler CTC Analytics, CombiPal model, using successively, for comparison purposes, 100 μm thickness PDMS, 65 μm PDMS/DVB, 75 μm CAR/PDMS and 60 μm polyethylene glycol (PEG) fibres from Supelco. Each new fibre was conditioned for one hour before the first utilization, according to the manufacturer's specifications, and for 30 minutes daily at the same conditions.

In optimized conditions, for HS-SPME carried out in a 20 mL vial, to 9.5 mL of water sample (eventually spiked with the studied analytes), 1 g of NaCl, 0.5 mL of daily prepared solution of Na_2CO_3 in water (20% w/v) and 100 μL of AA were added. Then,

the vial was closed with Teflon-lined septa (Supelco). The obtained solution was pre-equilibrated at 70 ºC for 10 minutes, after which a fibre was introduced into the headspace for 60 minutes at the same temperature, the solution being then mechanically shaken at 500 rotations per minute (rpm) during pre-equilibration and at 250 rpm during SPME.

For testing the applicability of the optimized method to real samples, estuarine and river water, both from Cávado River (north of Portugal), and treated wastewater from a slaughterhouse were used.

5.1.3. Analysis

Analysis by GC-ECD were performed using a Varian CP-3800 gas chromatograph (prepared for simultaneous ECD and FID) with a split/splitless CP-1177 injector port, a SPME liner (0.75 µm) and a microseal septum system (Merlin, Half Moon Bay, CA) and a CP-Sil 8CB Low Bleed/MS column from Varian (60 m x 0.25 mm i.d., 0.25 µm film thickness). The column oven was initially set at 40 ºC for 5 minutes, then it was heated, at a rate of 20 ºC $minute^{-1}$, to 235 ºC, hold 8 minutes after which it was heated to 300 ºC at a rate of 30 ºC $minute^{-1}$. In optimized conditions, the temperature of the injector was set at 260 ºC. Desorption during fibre injections was performed in splitless mode for 6 minutes, decreasing the possibility for carry-over effects. The time of desorption was selected based on literature data [2, 3] and was set constant throughout the experiments. The carrier gas was helium (99.9995%) with a constant flow rate through the column of 1.5 mL $minute^{-1}$, mixed with make-up gas nitrogen (99.999%) with volume mixing ratio of 1:5. The gases were supplied from Air Liquid (France). The ECD oven temperature was set to 320 ºC. The Varian computer software Star Chromatography Work Station 6.30 controlled the equipment.

GC-MS was used to detect possible GC-ECD method interferences and to confirm the obtained results with the optimized method. GC-MS run was similar to the GC-ECD one, with the exception of the helium flow rate (1.0 mL $minute^{-1}$). MS detection was carried out using a Saturn 2000 mass spectrometer from Varian using conditions described before [2, 4].

5.1.4. Method optimization

Several parameters, namely type of fibre, derivatization conditions, time and temperature of extraction and salt addition were varied in order to find suitable conditions for the determination of CPs in water samples. Standards of CPs in deionized water were used for that purpose and a thoughtful study of the blanks was also carried out in order to avoid possible contaminations. The experiments were carried out randomly and in duplicates, except otherwise stated. One hour extraction time was used throughout, except for the experiments to determine the influence of the extraction time. The method was optimized using the following CPs concentrations, set to have similar peak heights for all the compounds (ng L^{-1}): 2-CP (6000), 4-CP (7000), 2,6-DCP (100), 2,4-DCP (100), 2,4,6-TCP (20), 2,3,4,5-TeCP (60) and PCP (24), except otherwise stated. The optimum conditions were selected when the peak areas were maxima.

5.1.5. Stability Study

To study the stability of CPs in relevant environmental conditions, two different matrices, river water from Cávado River and treated wastewater from a slaughterhouse were filtered (glass fibre) and used. All water samples were put in vials containing NaCl and were spiked with a mixture of CPs with the following levels (ng L^{-1}): 2-CP (500), 4-CP (1000), 2,6-DCP (50), 2,4-DCP (50), 2,4,6-TCP (10) and PCP (12). For each matrix, samples were prepared according to the required composition (presence or absence of Na_2CO_3 (C) and AA (A)): in the first condition, samples were stored with absence of both (C-, A-), in the second condition only Na_2CO_3 was added (C+, A-) and in the third condition both Na_2CO_3 and acetic anhydride were added (C+, A+). Half of all samples were stored in the refrigerator at 4 °C and the other half in the freezer at -18 °C. The summary of the storage conditions is presented in Table 5.1. The experiments were carried out in duplicate with the exception of the controls (day 0), which were performed eight and six times for river and wastewater, respectively. The samples were stored up to 39 days. At different intervals, the concentrations of CPs in the samples were determined. Na_2CO_3 and/or AA were added just before measurements (in case they were not added before). The frozen samples were completely defrosted before the addition of the chemicals. The analysis of the samples was carried out using the optimized HS-SPME procedure described (see section 5.1.2. and Chapter 6) using GC-MS (see section 5.1.3).

Table 5.1. Summary of the storage conditions. Presence (+) or absence (-) of sodium carbonate (C) and of acetic anhydride (A), as well as the storage temperature (T), are included.

Sodium carbonate (C)	Acetic anhydride (A)	T (°C)
−	−	4
−	−	-18
+	−	4
+	−	-18
+	+	4
+	+	-18

5.2. Ecotoxicity studies

5.2.1. Materials and decontamination

All non-disposable material for the ecotoxicity experiments was cleaned with a solution of detergent (Teepol N, Tensoquímica, Portugal), washed with tap water and rinsed several times with deionized water (conductivity < 0.1 μS cm^{-1}) before being acid cleaned (HCl 1 M) for 24 hours, again rinsed with deionized water and air dried in a sterile atmosphere. Microwave sterilisation at 700 W was performed during 10 minutes for the plastic material (polycarbonate bottles, Nalgene) and for 5 minutes for the pipettes' tips. Glass Erlenmeyers, plugged with cotton stoppers (cotton wrapped in cheesecloth), were dry heat sterilized in an oven at 170 °C, for 1 hour. The cotton stoppers were immediately covered with two layers of aluminium foil, before cooling. Unless otherwise stated, all experiments were carried out in sterile Fraquil culture medium [Morel, 1975]. Culture media were sterilized by filtration with a 0.1 μm polycarbonate filter (Millipore). Solutions of PCP, for spiking of the culture media, were prepared in dimethyl sulfoxide (DMSO) (Riedel-de Haën, Seelze, Germany) and stored in a freezer in the dark. Working standard solutions were obtained daily by dilution with ethanol (Panreac, Barcelona, Spain). All sample manipulations were carried out aseptically in a Class 100 laminar flow hood, in a clean room with Class 100 filtered air.

5.2.2. Cultures of *M. aeruginosa* and *C. vulgaris*

One unicyanobacterial culture of *Microcystis aeruginosa*, strain LEGE 05195, previously isolated from the Torrão reservoir in the Tâmega River (northern Portugal), and the microalgal strain *Chlorella vulgaris* LEGE Z-001, from Algoteca, University of Coimbra – ACOI 879, were used in the exposure experiments. The selected isolates were kept in Z8 medium [5] at LEGE culture collection and, thus, prior to the experiments they were acclimated and cultured in Fraquil medium [6]. The cultures in all the experiments were incubated in a controlled-environment cabinet at 22 ± 2 °C with a light intensity of 18 μE m^{-2} s^{-1} (16 hours light/8 hours darkness) provided by a cool white fluorescent lamp.

5.2.3. Preliminary experiments

5.2.3.1. Natural chlorination

The possibility of natural chlorination in the culture media (which contain chlorides) by the action of light was investigated in both abiotic (f2 medium) and biotic conditions (f2 and *M. aeruginosa*). Natural levels of phenol (4 μg L^{-1}) [7] or hydrogen peroxide (2.5×10^{-6} mol L^{-1}) [8] or both were also added. CPs were not produced *in situ* during the ecotoxicity tests or its production was in such low levels that could not affect the obtained results.

5.2.3.2. Selection of the carrier solvent

The effects of 15 μL of ethanol and 15 μL of DMSO (one at a time), in 150 mL of f2 medium, on the growth of *M. aeruginosa* were studied. It was noticed that ethanol led to the formation of some fungi in the cultures, macroscopically visible; the same did not happen with DMSO. The growth of *M. aeruginosa* did not depend on the presence or absence of DMSO. So, in all the experiments, DMSO was used as carrier solvent.

5.2.3.3. Selection of the culture medium

A study of the phytoplankton growth performance in different culture media was carried out. The different culture media tested were f2, Fraquil and Z8. The growth of *M. aeruginosa* was performed in duplicates and was assessed by optical density at 750

nm (OD_{750}). A study of the recoveries of CPs after spiking of the respective culture media was also conducted. The cells failed to grow with f2 culture medium in some experiments. Fraquil culture medium was then selected because its nutritional composition is closer from environmental conditions than Z8 medium. All the tested media assured quantitative recoveries of CPs.

5.2.4. Growth inhibition tests

Previous to the experiments, the correlation between the OD_{750} and the cell counts (CC), performed under an optical microscope (Nikon, Japan) with a haemocytometer (Marienfeld, Germany), was assessed with six flasks inoculated with *M. aeruginosa* or *C. vulgaris*. The measurements were carried out every two days during 10 days (n=36).

5.2.4.1. Single species cultures

For the single species toxicity studies (Chapter 8), the cells in exponential growth phase were incubated for 10 days in the presence of PCP at seven different nominal concentration levels: 0.01, 0.1, 1, 10, 100, 500 and 1000 μg L^{-1} for the cyanobacterium, and 0.01, 0.1, 1, 100, 500, 1000 and 5000 μg L^{-1} for the microalga, with three replicates for each concentration. The initial volume of Fraquil culture medium with cells transferred to the test cultures was previously determined in order to obtain an OD_{750} of about 0.020, corresponding to 4.510×10^5 and 2.444×10^5 cells mL^{-1} of *M. aeruginosa* and *C. vulgaris*, respectively.

To determine the population growth, OD_{750} was measured each 2 days; chl_a was measured in the last day of the experiment as described by Jeffrey & Humphrey [9]. The pH of the different cultures was also determined in the last day. Two controls were performed in duplicate for each concentration level: a chemical control (Fraquil medium with PCP) and, for each species, a biological control (Fraquil medium with cells). The purity of the cultures during the tests was confirmed by microscopy examination.

5.2.4.2. Mixed cultures

For the mixed cultures toxicity studies (Chapter 9), a mixture of *M. aeruginosa* and *C. vulgaris*, in exponential growth phase, was incubated for 10 days in the presence of PCP at six different nominal concentration levels: 0.1, 1, 10, 100, 1000 and 10000 μg L^{-1} with three replicates for each concentration. The initial volume of Fraquil culture medium with cells transferred to the test cultures was similar to the described in the previous section.

To determine the population growth, the cells of *M. aeruginosa* and *C. vulgaris* were counted each 2 days. The CC were performed by transferring 10 μL of sample, after stirring of the culture, to a haemocytometer (Marienfeld, Germany) and observing under an optical microscope (Nikon, Japan) with a magnification of 400 times. The OD_{750} was measured simultaneously as an additional quality control. A chemical control (Fraquil medium with PCP) was performed in duplicate for each concentration level. Four biological controls (Fraquil medium with both species), two cyanobacteria controls (Fraquil with *M. aeruginosa*) and two microalgae controls (Fraquil with *C. vulgaris*) were incubated simultaneously with the test flasks for each two nominal concentrations. The pH of all cultures was determined in the last day of the incubations.

5.2.5. PCP measurements

All samples were collected from each culture flask, in the first and last days of the experiment, and filtered using 1.2 μm glass fibre filters (Whatman®), previously cleaned with methanol (Chromasolv, Aldrich). PCP concentrations in the culture media were determined by a previously developed SPME method, combined with GC-ECD (Chapter 6). The recovery of PCP in Fraquil medium was previously calculated, in duplicate, as 92 ± 1% and, therefore, PCP concentrations were not corrected for recovery.

5.2.6. Data treatment

5.2.6.1. Single species cultures

The calculation of the effective concentrations EC_{20} (effective concentration to 20% of the organisms) and EC_{50} (Chapter 8) was adapted from the Organisation of

Economic Co-Operation and Development test guideline 201 [10], using the initial concentrations, C_0, of PCP in the culture media (calculated as the average of the duplicates of the chemical controls). The test endpoint was the inhibition of growth expressed as average growth rate during the exposure period. The growth variation was calculated by comparing the chl_a and area under growth curve (AUC) (OD_{750} *vs* time) of the treatments with the values for the respective biological controls. The AUC was calculated as described [10, 11]. Each individual treatment flask was compared with two respective biological controls.

A Student's t-test was performed to compare the cyanobacterium and microalga growth yield in the presence and in the absence of PCP (biological control). A two-way analysis of variance (ANOVA) was performed to compare the interactions between the different endpoints assessed and the different PCP concentrations on the toxicity to each species. PCP percentage of removal was calculated as the difference between the average PCP concentrations in the last and first days of the experiment divided by the average initial PCP concentration, for each concentration studied. This calculation was performed in abiotic (the chemical control) and biotic (PCP and phytoplankton species) conditions.

5.2.6.2. Mixed cultures

From the curves of CC *vs* incubation time, the AUCs were calculated. The effect of one species over the other in the absence of PCP was evaluated using AUCs for mixed and pure cultures. The effect of PCP was studied by means of AUCs for mixed cultures in the absence and in the presence of PCP. In all cases, the comparison of the average AUCs was performed using the Student's t-test.

The average growth rates (GR) for all experiments were calculated using the $CC_{(t)}$, for each period of incubation time, *t* (days 6, 8 and 10), for *M. aeruginosa* and *C. vulgaris* and the corresponding counts at the beginning of the experiment ($CC_{(0)}$) divided by *t*: $GR_{(t)}=(CC_{(t)}-CC_{(0)})/t$.

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PART III

RESULTS AND DISCUSSION

Chapter 6

A headspace SPME-GC-ECD method for determination of chlorophenols in water samples

Part of this chapter has been published as a paper:

Morais, P. de, Stoichev, T., Basto, M. C. P., Carvalho, P. N., Vasconcelos, M. T. (2010) A headspace SPME-GC-ECD method suitable for determination of chlorophenols in water samples. *Analytical and Bioanalytical Chemistry*, 399, 2531-2538.

Abstract: A HS-SPME-GC-ECD method was optimized for the determination of seven CPs with different levels of chlorination. This is the first time that HS-SPME-GC-ECD with acetylation of the analytes is used for the simultaneous determination of CPs in water samples. The influence of fibre type, derivatization conditions, salt addition, temperature and time of extraction and temperature of desorption was checked. Possible sources of contamination and analyte losses were considered. The best results were obtained with the PDMS/DVB fibre, derivatization by acetylation using 100 µL of AA and 0.1 g of anhydrous sodium carbonate per 10 mL of sample, salt addition of 100 g L^{-1} sodium chloride, extraction at 70 °C for 60 minutes and desorption in the GC injector at 260 °C for 6 minutes. The LODs for mono-CPs were 12 and 122 ng L^{-1} for 2-chlorophenol and 4-chlorophenol, respectively. For polychlorinated CPs, the LODs were lower than 6 ng L^{-1}, values similar to the existing methods that use SPME with derivatization for CPs determination in water samples. The method is suitable for the determination of CPs in most environmental aqueous samples. Repeatability and reproducibility were less than 16.8% and 11.7%, respectively. The optimized method was successfully applied for the analysis of waters with complex matrices such as river and estuarine water samples.

6.1. Introduction

As discussed in Chapter 3, in the recent years several methods were used to pre-concentrate CPs from water samples: SPE [1, 2], LPME [3, 4], DLLME [5], SPE-DLLME [6] and SBSE [7]. Separation is achieved using LC [2] or GC after derivatization of the target analytes by silylation [4, 7] or acetylation [1, 5].

Solid phase microextraction is a simple, easily automated technique that does not require organic solvents. SPME can be used coupled to GC, generally followed by MS detection, after acetylation of the analytes [8, 9], silylation [10], or even without derivatization [11, 12]. Some research groups opted for the use of ECD, instead of MS, due to its selective, highly sensitive response to halogen containing compounds and lower price but it has been rarely used for water sample analysis. In such cases, either the derivatization was not performed [13, 14] or achieved by benzoylation [15]. With derivatization, lower LODs are attained.

Headspace SPME, coupled to GC with ECD, was used as a sensitive and selective tool for the determination of acetylated derivatives of CPs with different degree of chlorination: 2-CP, 4-CP, 2,6-DCP, 2,4-DCP, 2,4,6-TCP, 2,3,4,5-TeCP and PCP. The analytes were selected among those most frequently found in the environment. The HS extraction mode is usually preferred to the direct immersion because it minimizes the matrix interferences and prolongs the fibre coating life-time. A similar method (HS-SPME-GC-ECD and acetylation), optimized for a different fibre, has already been used to analyse wine samples for the presence of only highly chlorinated CPs [16, 17] but to our knowledge this method has not been used for the determination of CPs in water samples. It was now successfully applied for the simultaneous determination of CPs with different degree of chlorination in analysis of

waters with complex matrices such as river, estuarine and wastewater samples. The influence of fibre type, derivatization conditions, salt addition, temperature and time of extraction and temperature of desorption was checked. Emphasis was given to distinguish possible sources of sample contamination or analyte losses during water sample preparation.

6.2. Results and discussion

6.2.1. Fibre choice

The extraction capability of four different types of fibres was compared (Fig. 6.1). The error bars on the figure represent minimum/maximum range for different fibres of the same lot because between-fibre variability within the same lot is important to consider in routine analysis. The CPs were derivatized with 100 µL of AA in the presence of 0,1 g of Na_2CO_3 in 10 mL of deionized water. The temperature of extraction was set at 80 ºC and the temperatures of desorption were 280, 250, 250 and 230 ºC for CAR/PDMS, PDMS/DVB, PDMS and PEG fibres, respectively. For mono-CPs the best results in terms of analytical signal intensity were obtained using the CAR/PDMS fibre but both CAR/PDMS and PDMS/DVB bipolar fibres can be used successfully for the determination of mono-CPs by HS-SPME. The CAR/PDMS fibres are better to extract compounds with smaller molecular size [18] and get progressively worse with increasing chlorination degree of the analytes. The non-polar PDMS, the bipolar PDMS/DVB and the polar PEG fibres improve the performances for polychlorinated phenols but the best results were obtained with PDMS/DVB fibre. A previous study reported that CAR/PDMS is best suited for the determination of low chlorinated phenols while PDMS fibre was used for high chlorinated phenols [8]. The present study demonstrated the suitability of the PDMS/DVB fibre for simultaneous determination of phenols with different degree of chlorination. Additionally, the PDMS/DVB showed generally smaller between-fibre variation compared to the CAR/PDMS fibre.

Polydimehtylsiloxane/divinylbenzene and CAR/PDMS fibres were also compared for the possible carryover effects (in duplicates). The PDMS/DVB fibre presented no carryover effect, except for 2,6-DCP (about 1.6%). The CAR/PDMS showed higher carryover for 2-CP, 2,6-DCP and 2,4,6-TCP (about 10, 11 and 4%, respectively). This carryover effect in the CAR/PDMS fibre has already been reported and the authors even discharged the use of the fibre [9].

Due to all the facts presented, PDMS/DVB fibre was selected to proceed with further method optimization. It should be noted that these fibres have reasonably long life-time of about 150 injections.

6.2.2. Derivatization conditions

The analytical signal for HS-SPME-GC-ECD determination of CPs was studied using factorial experimental design with two factors, namely the levels of carbonate (C) and acetic anhydride (A). The derivatization occurs when the acetylation rate is faster than the hydrolysis rate of the AA, which is determined by the pH of the reaction mixture [17]. The three different levels of the factors were: high, central and low, noted as "+", "0" and "–", respectively. The "+", "0", and "–" levels of Na_2CO_3 per 10 mL of deionized water sample were 0.12, 0.10 and 0.08 g, respectively; for AA, respective levels were 150, 100 and 50 µL. The temperatures of extraction and desorption were 80 °C and 250 °C, respectively, and no NaCl was added. The exit functions were the peak areas for the analytes normalised to the corresponding peak areas of the central point experiment.

For the highest level of AA tested (A), the relative peak areas were statistically significantly higher (Table 6.1) for all the analytes ($p<0.05$) (Table 6.2). The highest level of sodium carbonate (C) had negative effect on the analytical signal for the experiments with the lowest level of AA (Table 6.1) and the effect was statistically significant for all the analytes ($p<0.05$), except for 2,4-DCP and 2,3,4,5-TeCP (Table 6.2). The best results were obtained for (+, +) and (0, 0) experiments. For further experiments, the quantities of Na_2CO_3 and AA needed for the derivatization were fixed at their central levels (0.1 g of Na_2CO_3 and 100 µL of AA).

6.2.3. Blanks

It was found that NaCl from some suppliers might lead to the formation of interfering peaks in the sample chromatogram resulting in unacceptably high detection limits. Some samples of NaCl *pro analysi* contained substances that co-eluted with 2,4-DCP and PCP, whereas NaCl *pro analysi* used was of better quality for this purpose, even though it still contained traces of these compounds. In Na_2CO_3, trace levels of compounds that co-elute with PCP were also found. Heating NaCl for 3 hours at 180 ºC decreased the interference for 2,4-DCP by 46%. Similar treatment applied to

Na_2CO_3 decreased its interference for PCP by 73%. Using HS-SPME-GC-MS it was possible to confirm that these interferences were not caused by CPs. Complete elimination of the interference was not possible.

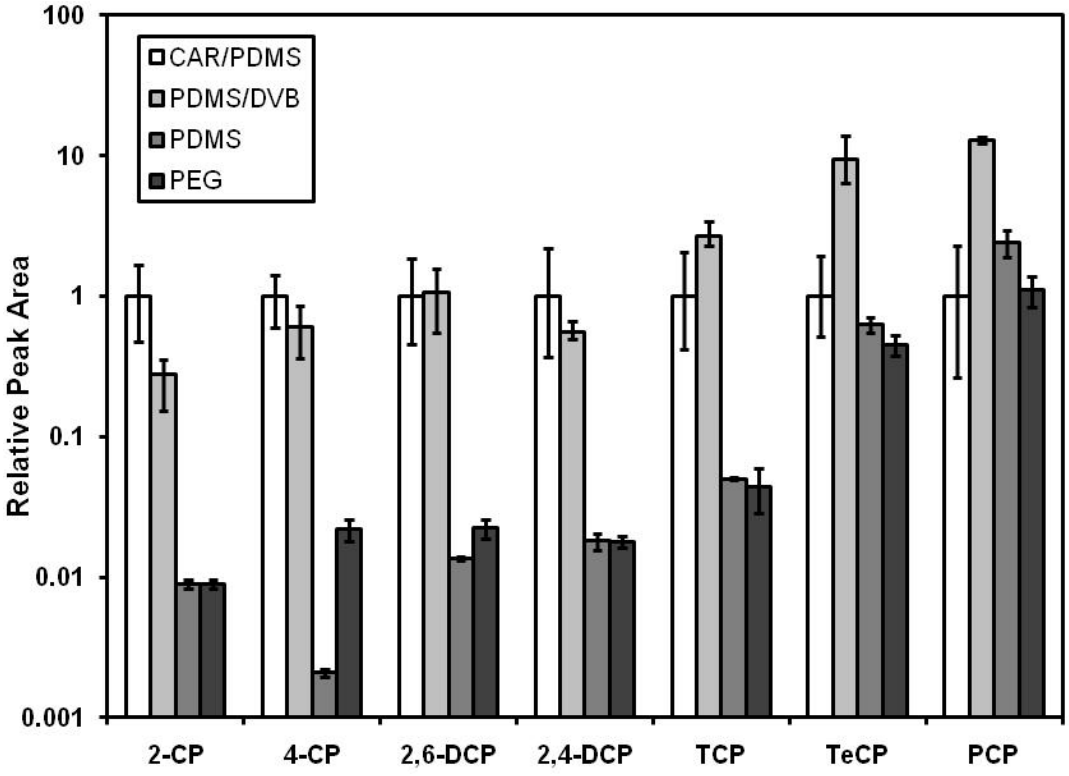

Fig. 6.1. HS-SPME-GC-ECD relative peak areas (normalised to that obtained using CAR/PDMS fibre) for chlorophenols (logarithmic scale). Error bars are maximum-minimum deviations of two (PDMS and PEG fibres) or three replicates (CAR/PDMS and PDMS/DVB fibres) using different fibres of the same lot.

Additionally, NaCl was also responsible for an unknown peak in the blank matrix that has very close retention time (RT) to 2,4,6-TCP (RT 15.08 minutes). However, it is possible to analyse the 2,4,6-TCP, regardless of this peak.

6.2.4. Salting out effect

The peak area of the CPs, after respective blank correction for each set of experiments, is shown on Fig. 6.2 in the absence and in the presence of NaCl (100, 200 or 300 g L^{-1}). The temperatures of extraction and desorption were 80 °C and 250 °C, respectively. The analysis of the CPs was improved by the addition of NaCl to the sample, specially for the case of mono-CPs. This is in accordance with the fact that the

effect of salt addition usually increases with the polarity of the compound [19]. The extraction yield of the higher chlorinated CPs was not found to increase at high NaCl concentrations. Similar results were found for the case of wine samples [16], while other authors [8, 9] stated maximum effect of NaCl in near saturation conditions. The opposite effect of NaCl is common in SPME and is due to the complex interactions of salt ions, both with water molecules and with the analytes [20]. Despite the best results for several of the studied analytes being obtained for 200 g L^{-1} NaCl, it was decided for any further experiments to use 100 g L^{-1} concentration of NaCl due to blank contamination. At 100 g L^{-1} NaCl, the sorption of CPs to the fibre (relative to absence of salt) is increased by a factor between 1.2 and 2.0.

Table 6.1. Relative peak areas for chlorophenols after 2^2 factorial experiment. The levels of acetic anhydride and sodium carbonate are A and C, respectively.

(A, C)[a]	2-CP	4-CP	2,6-DCP	2,4-DCP	2,4,6-TCP	2,3,4,5-TeCP	PCP
(−, −)[b]	0.63±0.16	0.51±0.09	0.71±0.09	0.33±0.0	0.59±0.07	0.19±0.03	0.28±0.06
(+, −)[b]	1.04±0.09	0.98±0.03	0.79±0.04	1.12±0.25	0.90±0.02	1.35±0.21	1.05±0.07
(−, +)[b]	0.09±0.04	0.11±0.03	0.03±0.01	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
(+, +)[b]	1.02±0.07	0.92±0.09	0.94±0.09	1.27±0.05	1.03±0.03	1.00±0.01	0.99±0.03
(0, 0)[c]	1.00±0.25	1.00±0.07	1.00±0.06	1.00±0.18	1.00±0.06	1.00±0.19	1.00±0.12

[a] "+", "0" and "−" levels of A and C are 150, 100 and 50 µL and 0.12, 0.10 and 0.08 g, respectively.

[b] Error expressed as minimum-maximum interval near the average (n=2).

[c] Error expressed as standard deviation (n=4).

Table 6.2. $F_{1,4}$ values for the main effects and their interaction. The underlined values show statistical significance at 95% confidence level. The notations A and C refer to the levels of acetic anhydride and sodium carbonate, respectively.

	2-CP	4-CP	2,6-DCP	2,4-DCP	2,4,6-TCP	2,3,4,5-TeCP	PCP
A	46.8	87.0	54.7	65.0	246.2	96.7	358.4
C	8.3	11.7	15.4	0.5	29.8	6.0	13.5
A+C	6.8	6.2	39.1	3.5	71.9	0.5	5.9

6.2.5. Influence of the desorption temperature

The influence of the desorption temperature on the signal intensity was checked at injector temperatures of 230, 250, 260 and 270 ºC (Fig. 6.3). The analytical signal was maximal at 260 ºC for all analytes with the exception of 2,4-DCP, which continued to increase with injector temperature. A single factor analysis of variance (ANOVA) demonstrated that the effect of desorption temperature was statistically significant for 2-CP ($p<0.05$), 4-CP ($p<0.01$) and 2,4-DCP ($p<0.05$). For any further experiments, the temperature of desorption selected was 260 ºC.

6.2.6. Influence of the extraction temperature

The dependence of the analytical signals on the extraction temperature was studied for temperatures between 50 and 90 ºC (Fig. 6.4). The choice of the temperature range was based on equilibrium considerations; lower temperature favours the sorption to the fibre but higher temperature is necessary for the transfer of the analytes toward the HS [21]. For mono-CPs, the optimal temperature is between 60 and 70 ºC while for the polychlorinated phenols it is between 70 and 80 ºC. This fact might reflect the lower volatility of the polychlorinated analytes, necessitating higher temperature to bring them up into the HS. In all cases, at 90 ºC extraction temperature, the analytical signal decreased due to desorption losses of the analytes. For the following experiments, the temperature chosen for HS-SPME was 70 ºC.

6.2.7. Influence of the extraction time

The influence of the extraction time on the peak heights is shown on Fig. 6.5. Data were fitted using the equation for non-equilibrium HS-SPME [22]. For most of the analytes, the equilibrium is reached after 60 minutes. Therefore, the optimum time for the HS-SPME was chosen to be 60 minutes. The automated sampler used provided precise control of the experimental conditions (e.g. time) and can be used for quantitative analysis even in non-equilibrium situations.

Globally, for CPs with different degree of chlorination, HS-SPME of the acetylated derivatives for 60 minutes at 70 ºC, after 100 g L^{-1} sodium chloride addition to the sample, showed to be suitable using PDMS/DVB fibre. After the extraction, the analytes are desorbed in the injector at 260 ºC for 6 minutes.

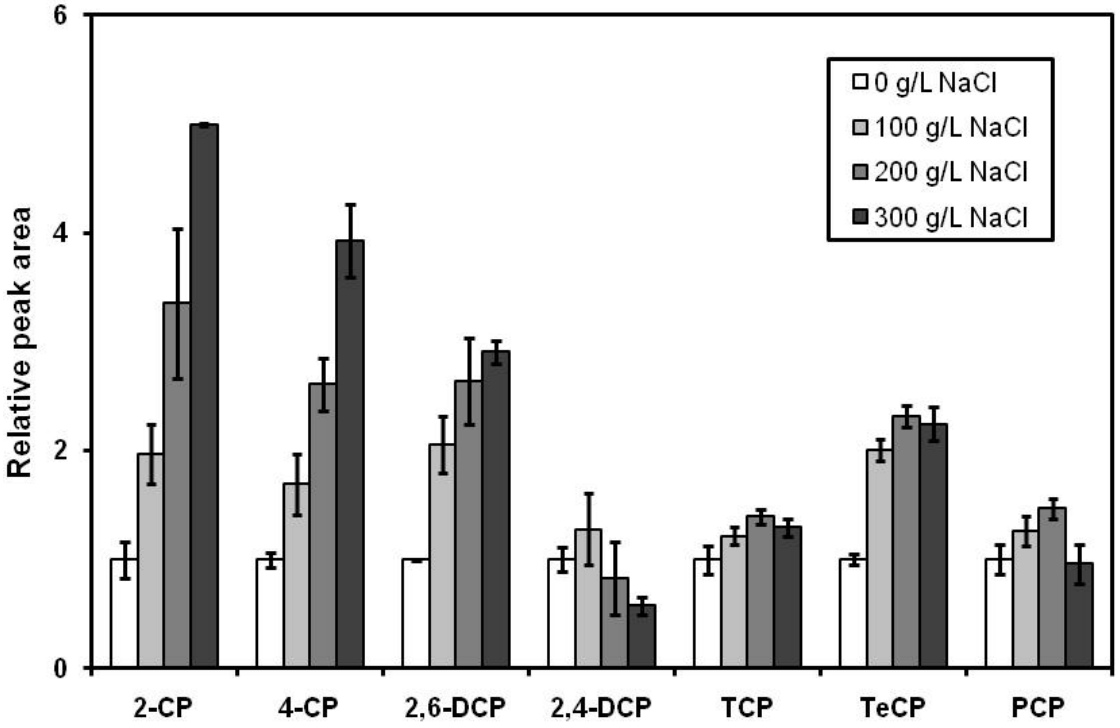

Fig. 6.2. Salting out effect for the determination of chlorophenols by HS-SPME-GC-ECD using PDMS/DVB fibre. The peak areas are corrected with the corresponding blanks for different NaCl levels and normalised to the corresponding peak areas in the absence of NaCl. Error bars are mean deviations of two replicates.

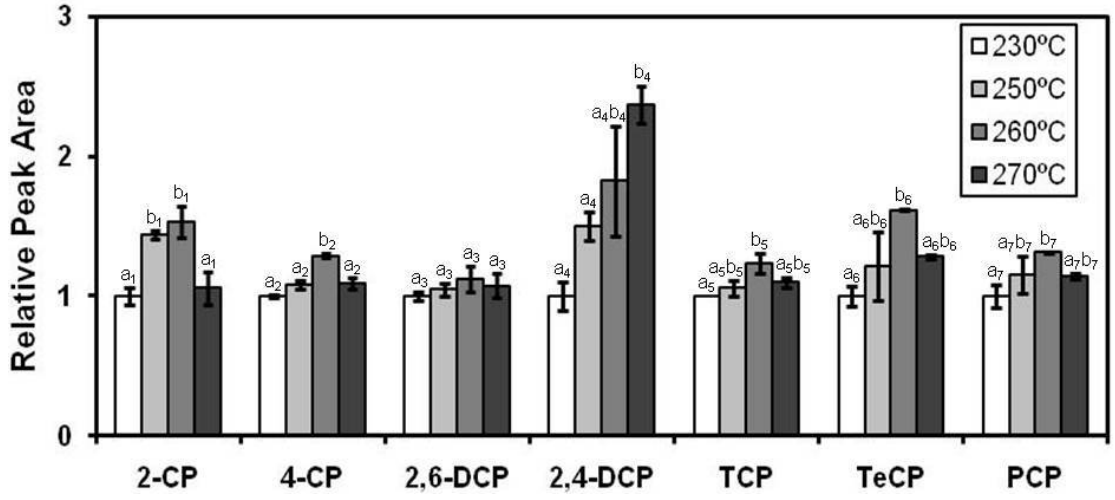

Fig. 6.3. Influence of the desorption temperature on the relative peak areas (normalised to the peak area at desorption temperature 230 ºC) for the determination of chlorophenols. Error bars are mean deviations of two replicates. Results significantly different ($p<0.05$, least significant difference) among the desorption temperatures are marked with different letters (index numbers for each CP).

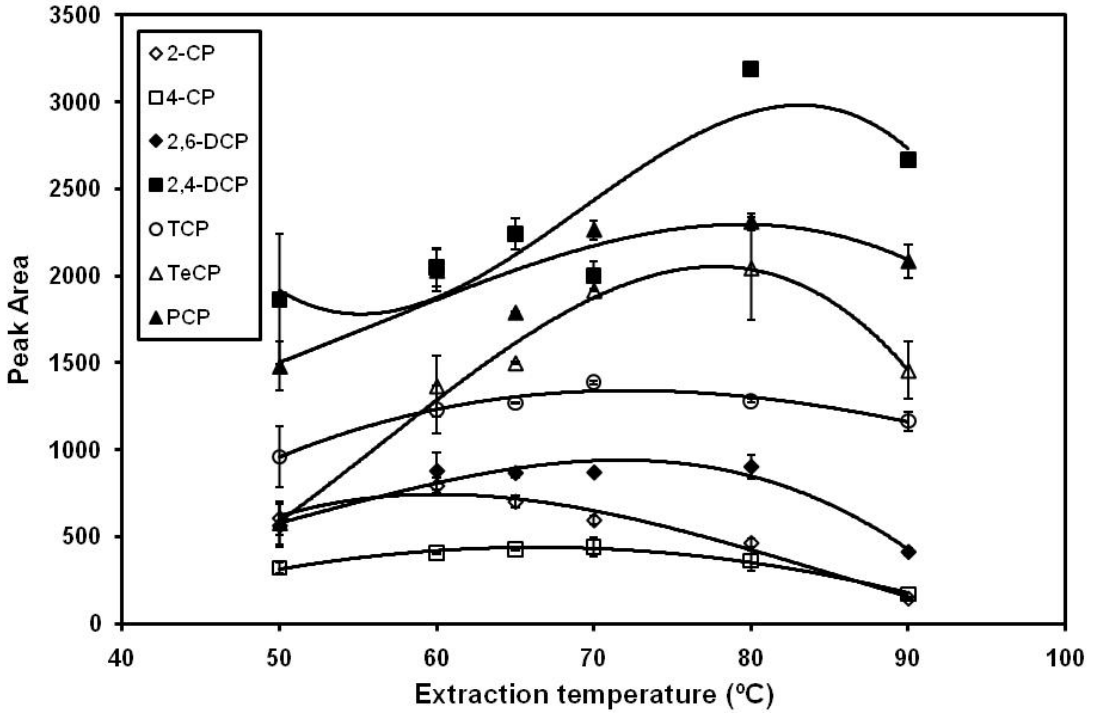

Fig. 6.4. Influence of the extraction temperature on the peak area for the determination of chlorophenols. Error bars are mean deviations of two replicates.

6.2.8. Problems during sample filtration

Possible contamination during sample filtration using nylon or glass fibre filters was also tested. It was found that nylon filter released into the solution an unknown substance that interferes with 2,4-DCP analysis. The glass fibre filters also introduced interferences, up to saturation of the analytical signal. However, washing of the glass fibre filters with methanol (ultrasonic bath) was found effective to completely remove this problem.

In addition, a comparison of CPs recovery in filtered and non-filtered samples was performed in spiked f2 media samples. Quantitative recovery was observed for all CPs in non-filtered f2 media samples (overall 103 ± 5%) and in those filtered through glass fibre filter (overall 98 ± 4%). When nylon filters were used, the recovery of the CPs markedly decreased, losses from 30 to even 100% being observed specially for polychlorinated phenols.

Sample filtration before analysis should be considered with care since it can both introduce interfering substances in the samples and sorb/decompose the analytes. Therefore, clean glass fibre filters were used further for all the samples analysed.

6.2.9. Analytical figures of merit

Limits of detection, repeatability, reproducibility and the linear ranges for the studied CPs in deionized water are presented in Table 6.3. The LODs were determined based on three times the standard deviation of ten blanks. The blanks were obtained following the developed analytical procedure, including filtration through previously cleaned glass fibre filters. The LODs for the mono-CPs are relatively high (12 and 122 ng L^{-1} for 2-CP and 4-CP, respectively) due to lower sensitivity of the method for these compounds. The LODs are lower for polychlorinated phenols, between 0.1 and 5.6 ng L^{-1}. The LODs are comparable, or sometimes better, to the results obtained for other methods for water analysis using SPME-GC-MS after acetylation [8, 9] or SPME-GC-ECD after benzoylation [15]. The obtained LODs are better than those found for aqueous samples analyses using SPME-GC-ECD without derivatization [13, 14]. The European legislation for drinking water has set the maximum residue limit (MRL) for total phenols to 500 ng L^{-1} and 100 ng L^{-1} for individual CPs [23]. The obtained LODs are smaller than the MRL, with the exception of 4-CP which LOD is in the same order of magnitude. Therefore, the present method could be used for CPs monitoring in drinking water. From a more general point of view, this method is sensitive enough to determine CPs in most environmental water samples, except for mono-CPs in the open ocean.

The repeatability is expressed as the average of the values of the relative standard deviation (RSD, %) obtained daily for 5 days experiment (8 measurements each day). Values ranged from 8.9% to 16.8%, the higher values being obtained for polychlorinated phenols due to the lower concentration levels tested (Table 6.3). The reproducibility, expressed as RSD from the average daily signals during five days measurements of eight standard solutions, ranged from 7.7% to 11.7%. The method gives very similar results from day to day.

The ranges of concentrations (Table 6.3) where the linear responses were observed were one (2,4-DCP), two (4-CP; 2,6-DCP and PCP) and three (2-CP; 2,4,6-TCP; 2,3,4,5-TeCP) orders of magnitude. For polychlorinated phenols, concentrations were in the ng L^{-1} range, while for mono-CPs were in the μg L^{-1} range. This will imply high dilutions when very contaminated samples with polychlorinated phenols are to be analysed.

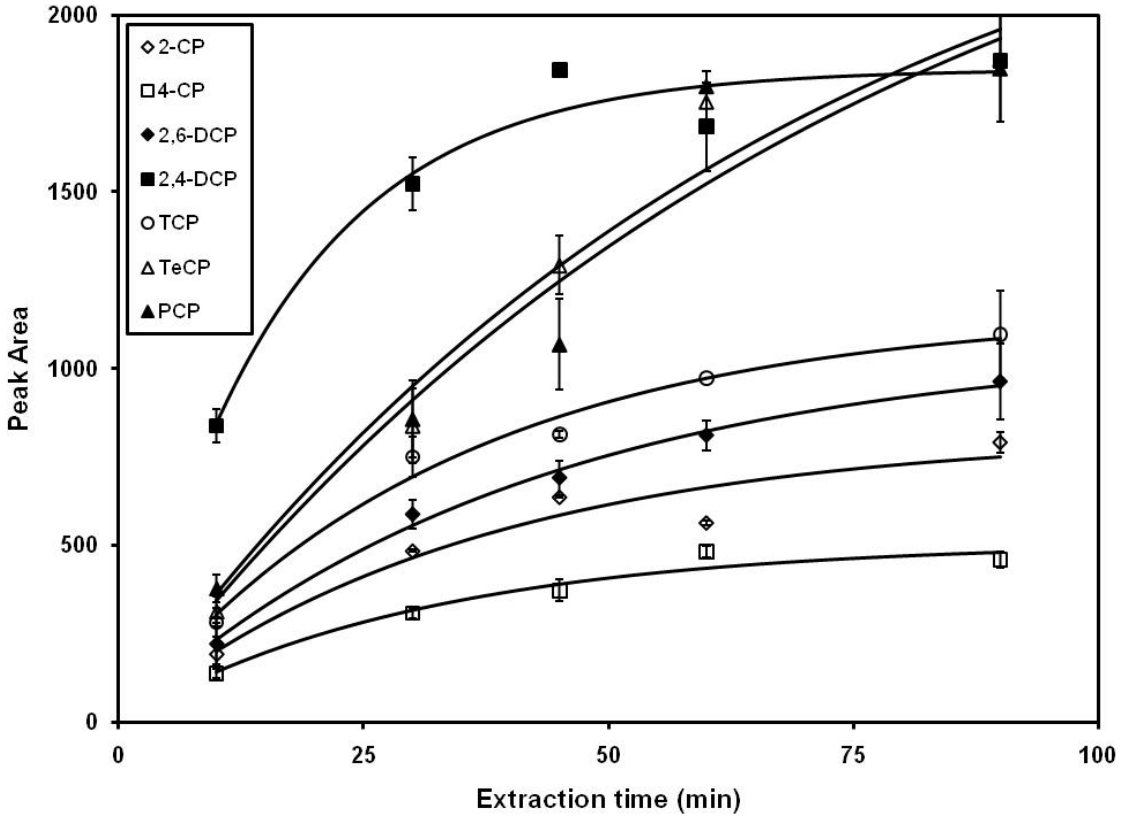

Fig. 6.5. Influence of the extraction time on the peak area for the determination of chlorophenols. Error bars are mean deviations of two replicates.

The recoveries of the tested CPs were studied in f2 algal culture medium, as well as in river, estuarine and treated wastewater samples, analysed in duplicates (Table 6.3). The spiking concentrations of CPs, in ng L^{-1}, were: 2-CP (5600); 4-CP (6200); 2,6-DCP (60); 2,4-DCP (60); 2,4,6-TCP (12); 2,3,4,5-TeCP (36) and PCP (14). Quantitative recoveries (Table 6.3) were obtained for most of the CPs, with exception of 2,4-DCP for river and estuarine water samples. This problem resulted of the presence of the last compound in the water sample in relatively high concentration (see below), which, after the spiking, resulted in a concentration out of the linear range. However, after appropriate dilution of the samples, and spiking with 2,4-DCP at lower levels (40 ng L^{-1}), the recovery of 2,4-DCP (in triplicate) was 104 ± 19% and 88 ± 8% for river and estuarine water, respectively. For the treated wastewater sample, relatively low recoveries of 2,3,4,5-TeCP were observed. Probably, components of this sample matrix interfere with the determination of 2,3,4,5-TeCP.

6.2.10. Application to real samples

Figure 6.6 illustrates the chromatograms obtained by the HS-SPME-GC-ECD method: for a blank solution (Fig. 6.6a), a standard mixture of CPs prepared in deionized water (Fig. 6.6b), river water (Fig. 6.6c) and spiked river water (Fig. 6.6d).

Application of the method to river and estuarine water samples resulted in the quantification of 2,4-DCP (from 47.4 to 59.6 ng L^{-1}) and PCP (from 5.9 to 8.9 ng L^{-1}). The presence of these compounds in the analysed samples was confirmed by GC-MS.

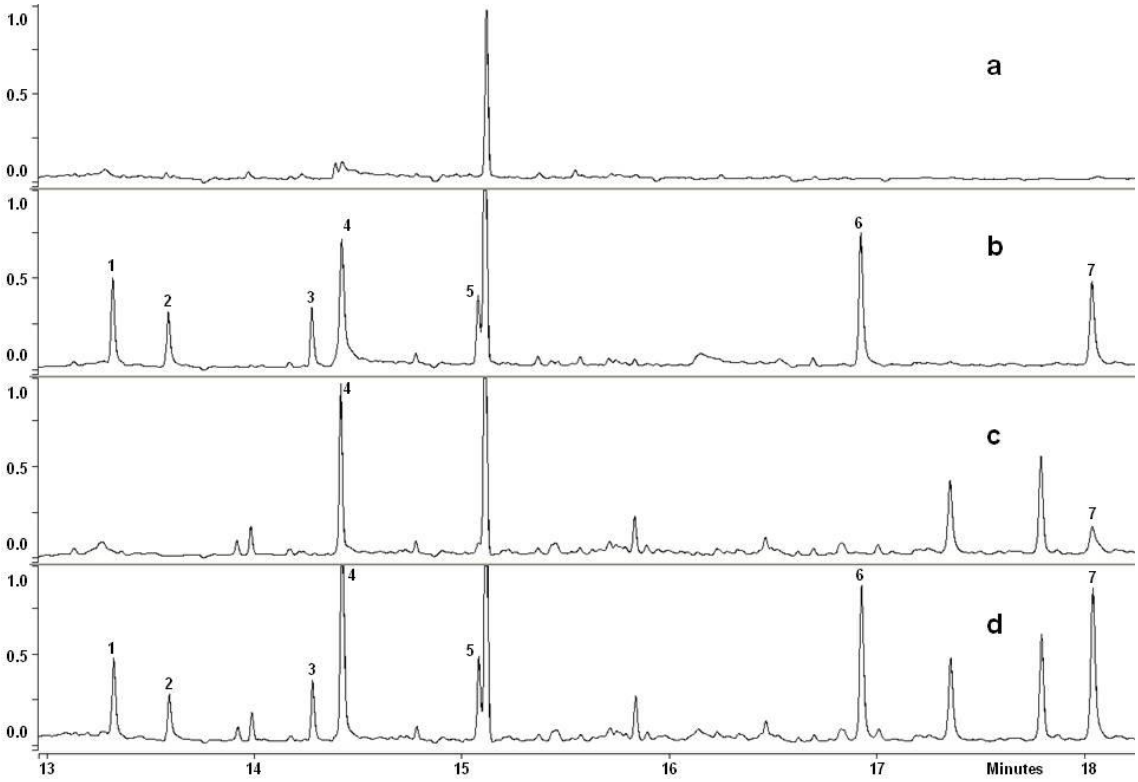

Fig. 6.6. Chromatograms for chlorophenols (1: 2-CP; 2: 4-CP; 3: 2,6-DCP; 4: 2,4-DCP; 5: 2,4,6-TCP; 6: 2,3,4,5-TeCP and 7: PCP): **(a)** blank in deionized water; **(b)** standard in deionized water; **(c)** river water and **(d)** river water spiked with CPs at the same levels as in the standard.

6.3. Conclusions

The present headspace SPME-GC-ECD method (using PDMS/DVB fibre) can be used as a routine method suitable for the simultaneous determination of acetylated derivatives of CPs having different degree of chlorination, in waters. However, whenever working with new type of samples, identification of the analytes and screening for possible interferences should be performed by a more selective technique, like GC-MS.

Table 6.3. Characteristics of the optimized method for determination of chlorophenols in water by HS-SPME-GC-ECD.

	2-CP	4-CP	2,6-DCP	2,4-DCP	2,4,6-TCP	2,3,4,5-TeCP	PCP
LOD (ng L^{-1})	**12**	**122**	**1.3**	**5.6**	**0.1**	**0.1**	**0.5**
Repeatability [a, b]	**10.9** (6.9-16.3)	**8.9** (6.0-11.4)	**15.8** (7.3-25.4)	**10.9** (4.4-18.5)	**14.9** (10.9-20.6)	**16.8** (8.4-27.5)	**15.8** (10.9-24.4)
Reproducibility [b, c]	**8.8**	**7.7**	**7.6**	**11.7**	**7.7**	**8.1**	**8.0**
Linear range (ng L^{-1})	**40-11000**	**400-6200**	**4-100**	**15-60**	**0.4-40**	**0.4-60**	**1.4-24**
R^2 [d]	0.986, n=6	0.994, n=5	0.987, n=7	0.997, n=5	0.988, n=8	0.999, n=6	0.999, n=6
RECOVERY (%) [b, e]							
f2 algal medium	**92±3**	**102±4**	**96±6**	**101±5**	**99±4**	**95±11**	**98±7**
River water	**95±3**	**101±10**	**101±2**	**33±6**[f]	**87±2**	**107±6**	**109±9**
Estuarine water	**106±4**	**111±6**	**108±3**	**22±1**[f]	**85±5**	**109±2**	**129±5**
Treated wastewater	**109±8**	**96±18**	**114±5**	**72±17**	**109±13**	**65±5**	**94±1**

[a] Average relative standard deviation (RSD) values for 5 days with 8 measurements each day; range is given in brackets

[b] Concentrations of chlorophenols (ng L^{-1}): 2-CP (5600); 4-CP (6200); 2,6-DCP (60); 2,4-DCP (60); 2,4,6-TCP (12); 2,3,4,5-TeCP (36); PCP (14)

[c] RSD based on the average (n=8) daily peak area values during 5 days

[d] Square of the correlation coefficient (R^2) and number of points per linear curve (n) are given

[e] Mean values and error expressed as minimum-maximum interval (n=2)

[f] Low recoveries for 2,4-DCP were due to concentration out of the linear range after the spiking. Recoveries of 104±19% and 88±8% for river and estuarine water, respectively, were obtained after a suitable dilution of the samples.

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Chapter 7

Stability of chlorophenols in water: sample storage procedures

Part of this chapter has been accepted as a paper:

Morais, P. de, Stoichev, T., Basto, M. C. P., Carvalho, P. N., Vasconcelos, M. T. (2013) Stability of chlorophenols and their acetylated derivatives in water: sample storage procedure. *Journal of AOAC International* (accepted).

Abstract: The determination of CPs in water samples is a subject of increasing interest. Reduction of the sample storage space and the stability of CPs when present at very low levels are still problems that deserve research. The stability of CPs (2-CP, 4-CP, 2,6-DCP, 2,4-DCP, 2,4,6-TCP and PCP) at ng L^{-1} levels at different temperatures and in the presence or in the absence of sodium carbonate and acetic anhydride was studied for up to 39 days. Stable and reproducible CPs concentrations for about a month of storage in both river and wastewater were achieved in two storage conditions as follows: at -18 °C and addition of 10% sodium chloride; and at 4 °C and addition of both 10% sodium chloride and 10 mg mL^{-1} sodium carbonate. These sample treatments are good alternatives to the immobilization of CPs on SPE cartridges, in terms of both analytes' stability and saving of storage space.

7.1. Introduction

Sample storage is an important part of the chemical analysis of CPs in waters but it is often neglected [1]. Stability of CPs up to five months in frozen samples and up to four months in chemically preserved samples at 4 °C has been reported [2]. However, Puig and Barceló [3] demonstrated that CPs have different stability when stored in acidified river water samples at 4 °C and some of them suffered 10% losses in one month. In addition, most of the stability studies were carried out using relatively high concentrations of CPs. The most commonly used SPE methods require a large sample volume and consequently a lot of storage space.

There are several possibilities to reduce the sample storage space but all of them require CPs stability studies. First, silyl derivatives of CPs can be rendered stable for long-term storage if the excess of derivatizing agent is removed [4]. However, the whole procedure involved time-consuming LLE of the water samples and evaporation of the extract before silylation. Second, it could be advantageous to preconcentrate CPs on SPE cartridges [5-7]. Very good stability for 1 to 3 months of the CPs was reported, sometimes even when cartridges were stored at room temperature. The third possibility is to use microextraction methods, which require small sample volume (1-20 mL) [1]. The stability has been studied in acidified standard solutions (20 mL) of CPs in pure reagent water stored at 4 °C until analysis by direct SPME without derivatization [8]. The authors reported that the CPs were stable for up to 25 days. However, in biologically active samples, CPs can be more rapidly degraded [9]. Stability could be dependent on water matrix, storage temperature and the properties of the analytes [6]. Therefore, possible losses during sample storage are problems that deserve research.

Preconcentration of the CPs by HS-SPME using acetylation of the analytes [10] would allow saving a lot of storage space in case the samples are collected into SPME vials. However, the HS-SPME procedure requires the presence of Na_2CO_3 and AA, in order to carry out the derivatization reaction, as well as NaCl for salting out the

analytes. In order to minimize the storage space using this procedure, a variety of storage conditions must be tested. In this work, the stability of CPs with different degree of chlorination in water samples with various matrix compositions was studied at environmentally relevant concentrations. The influence of storage temperature and the presence or absence of Na_2CO_3 and AA on the stability of CPs was investigated for a period of up to 39 days in order to find out the best procedure for sample storage for that analytical method.

7.2. Results and discussion

Natural occurring CPs levels were found in the samples. In the river water, 4-CP (169 ng L^{-1}) and, in the wastewater, PCP (20 ng L^{-1}) were quantified. The concentrations of CPs after the spiking, measured at different storage times, are presented in Fig. 7.1, for river water, and in Fig. 7.2, for wastewater.

7.2.1. Influence of the storage time

Storage time has no influence for at least one month on CPs concentrations at (C-, A-) conditions, irrespectively of the storage temperature and sample matrix. These results are in agreement with what has been found by other studies carried out at higher spiking levels and different storage conditions. Stability of CPs has been studied in spiked acidified samples (10 μg L^{-1}) and CPs were stable for at least one month with very slight decrease (10%) for the case of mono-CPs [3]. The CPs, spiked at levels of several μg L^{-1} to pure reagent water at pH 4 in the presence of 10% NaCl, were proven to be stable for 25 days but most of the concentrations significantly decreased after that [8]. The results from the current study demonstrate that it is not necessary to acidify the samples for long-term storage.

For all other conditions, the decrease of CPs concentrations and the statistical significance of the storage time (one-way ANOVA) are shown in Table 7.1. The phenolate salts at (C+, A-) conditions are stable, with the exception of PCP after more than three weeks of storage. Very slight decrease of PCP concentration (13%) was noticed at 4 °C in (C+, A-) conditions for the case of river water, while all the analytes were stable in wastewater in the same conditions. There was no influence of the storage temperature on the stability of the phenolate salts.

The stability of acetylated derivatives of the CPs was studied in (C+, A+) conditions. In most cases, at 4 °C, the acetylated derivatives, specially for polychlorinated CPs, were much less stable compared to non-derivatized CPs. The concentrations of acetyl-2,4,6-TCP and acetyl-PCP started to decrease only after 2-6 days of storage in both river and wastewater. The acetyl-CPs in the frozen samples were much more stable.

7.2.2. Influence of the sample matrix

The behaviour of the CPs during storage in the different matrices was studied using two-way ANOVA. For all analytes, the sample matrix has no influence on the stability, with the exception of PCP for some of the storage conditions. The interaction between sample matrix and the storage time was statistically significant ($p<0.05$) for PCP at 4 °C (C-, A-), -18 °C (C-, A-), -18 °C (C+, A-) and 4 °C (C+, A+) treatments (Table 7.1), pointing to the different behaviour of PCP in the tested conditions, depending on the matrix (Figs. 7.1 and 7.2).

7.2.3. Dispersion of the CPs concentrations

For some of the storage conditions, like (C+, A+) at -18°C, the CPs concentrations were largely scattered around the initial concentration without clear trend with time (Figs. 7.1 and 7.2). The RSDs of the CPs concentrations, measured at different storage times and different conditions, are shown in Table 7.2. The RSDs should be as small as possible to achieve lower dispersion of the measured concentrations throughout time. For both river and wastewater, relatively reproducible results were achieved in only two storage conditions: -18 °C (C-, A-) and 4 °C (C+, A-). Additionally, in these conditions, all CPs were stable for about a month and, therefore, they were considered suitable options for water sample storage.

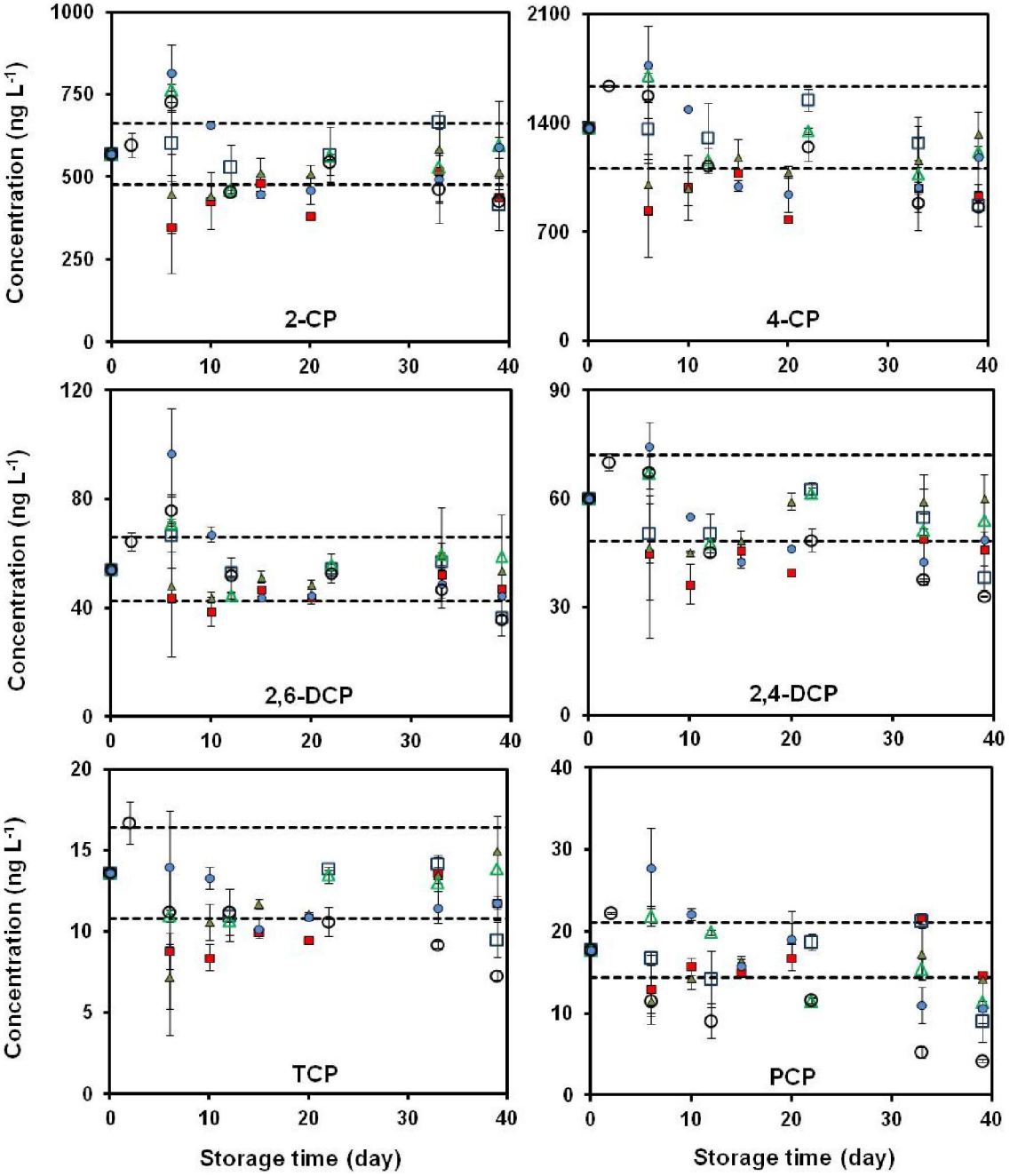

Fig. 7.1. Influence of the storage time on the chlorophenols concentrations in spiked river water samples at different storage temperature. The dotted line represents the relative standard deviation of the initial concentrations (n=8). Presence / absence of Na_2CO_3 (C) and acetic anhydride (A) during storage are denoted by + / -, respectively.

4 °C: ○ (C+, A+) △ (C+, A-) □ (C-, A-) -18 °C: ● (C+, A+) ▲ (C+, A-) ■ (C-, A-)

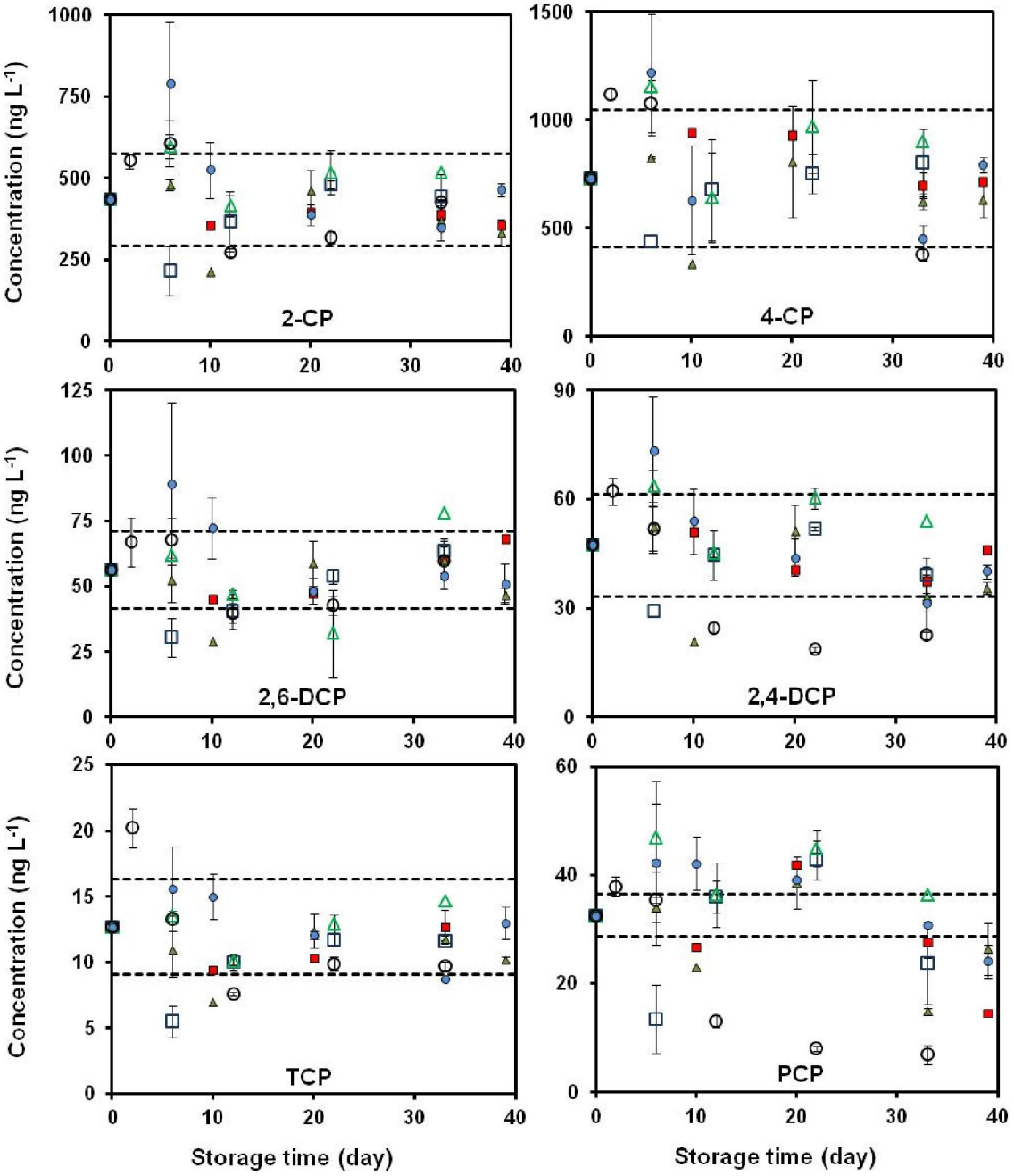

Fig. 7.2. Influence of the storage time on the chlorophenols concentrations in spiked wastewater samples at different storage temperature. The dotted line represents the relative standard deviation of the initial concentrations (n=6). Presence / absence of Na_2CO_3 (C) and acetic anhydride (A) during storage are denoted by + / -, respectively.

4 °C: ○ (C+, A+) △ (C+, A-) □ (C-, A-) -18 °C: ● (C+, A+) ▲ (C+, A-) ■ (C-, A-)

Table 7.1. Influence of the storage time on the stability of chlorophenols. Only analytes with statistically significant ($p<0.05$) effect of time are represented.

Conditions	Analytes / Concentration decrease after 33 days (%) [a]
River water:	
-18 °C; (C+, A-)	—
-18 °C; (C+, A+)	PCP / 38% (0.03)
4 °C; (C+, A-)	PCP / 13% (0.04)
4 °C; (C+, A+)	4-CP / 35% (0.02); 2,6-DCP / 13% (0.05); 2,4-DCP / 38% (0.01); 2,4,6-TCP / 32% (0.03); PCP / 71% (0.005)
Wastewater:	
-18 °C; (C+, A-)	PCP / 54% (0.05)
-18 °C; (C+, A+)	—
4 °C; (C+, A-)	—
4 °C; (C+, A+)	2,4-DCP / 52% (0.02); 2,4,6-TCP / 24% (0.02); PCP / 79% (0.0001)

[a] *p* values from ANOVA single factor (storage time) are given in brackets

Table 7.2. Average relative standard deviations (RSD, %) of the six chlorophenols (CPs) concentrations, measured at different storage times for different conditions (the second value in each entry represents the interval in which the RSD varies for individual CPs).

Sample matrix	Storage conditions				
	-18 °C			4 °C	
	(C-, A-)	(C+, A-)	(C+, A+)	(C-, A-)	(C+, A-)
River water	16.9 ± 3.1 [ab]	14.1 ± 4.3 [a]	24.9 ± 8.4 [cd]	18.9 ± 3.9 [bc]	16.7 ± 5.5 [ab]
Wastewater	17.1 ± 9.1 [abc]	26.1 ± 4.4 [d]	27.5 ± 7.0 [d]	26.9 ± 6.4 [d]	18.7 ± 6.9 [abc]

Statistically higher / lower values ($p<0.05$) are marked with different letters

7.2.4. Comparison with other storage methods

The use of SPE cartridges for immobilization of CPs improves their stability [8-10]. However, the LODs of the technique (LC with DAD), used to separate and detect

the CPs, were reported to be in the order of several tens to thousands ng L^{-1}. In such works, the levels of CPs present in the water samples used were from 5 to 50 μg L^{-1}, which are higher than those usually found in real environmental samples [8, 11-15].

Therefore, the proposed storage procedures, in terms of CPs stability and space saving, seem to be good alternatives to the immobilization of CPs on SPE cartridges. They may be used for HS-SPME-GC determination of CPs in water samples by MS or electron capture detection with LODs in the low ng L^{-1} range [1]. The acetylation greatly improved the LODs compared with direct SPME [8] and gave possibility for a variety of storage conditions to be studied for real water samples spiked at ng L^{-1} levels.

7.3. Conclusions

The acetylated derivatives, specially for polychlorinated CPs, are much less stable compared to non-derivatized CPs. For both river and wastewater samples, the two following storage conditions provided stable and reproducible CPs concentrations for about a month: freezing the sample in the presence of 10% NaCl (before analysis suitable amounts of both Na_2CO_3 and AA should be added); storage at 4 °C in the presence of both 10% NaCl and 10 mg mL^{-1} Na_2CO_3 (before CPs determination only AA should be added). These procedures are simple and no additional preservatives, like sulphuric acid, are necessary for long-term storage.

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Chapter 8

Behaviour of the cyanobacterium *Microcystis aeruginosa* when exposed to pentachlorophenol and comparison with that of the microalga *Chlorella vulgaris*

Part of this chapter has been submitted to publication as a paper:

Morais, P. de, Stoichev, T., Basto, M. C. P., Ramos, V., Vasconcelos, V. M., Vasconcelos, M. T. (2013) Behaviour of the cyanobacterium *Microcystis aeruginosa* when exposed to pentachlorophenol and comparison with that of the microalga *Chlorella vulgaris*. (submitted)

Abstract: Pentachlorophenol is a priority pollutant due to its persistence and high toxicity even at low concentrations. Its effects on a strain of the toxic and bloom forming cyanobacterium *M. aeruginosa* were investigated at laboratory scale. This is the first systematic ecotoxicity study of the effects of PCP on an aquatic cyanobacterium. The eukaryotic green microalga *C. vulgaris* was studied in the same conditions as the prokaryotic strain, in order to compare the PCP toxicity and removal ability between these two ubiquitous and ecologically important species. The cells were exposed to environmental levels of PCP during 10 days, in Fraquil culture medium, at nominal concentrations from 0.01 to 1000 µg L^{-1}, to the cyanobacterium, and 0.01 to 5000 µg L^{-1}, to the microalga. Growth was assessed by AUC (optical density *vs* time) and chl_a. The toxicity profiles of the two species were very different. The calculated effective concentrations EC_{20} and EC_{50} were much lower to *M. aeruginosa* and its growth inhibition expressed by chl_a was concentration-dependent while by AUC was not. The cells might continue to divide even with lower levels of chl_a. The number of *C. vulgaris* cells decreased with the PCP concentration without major impact on the chl_a. The effect of PCP on *M. aeruginosa* is hormetic: every concentration studied was toxic except 1 µg L^{-1}, promoting its growth. The legal limit of PCP set by the EU for surface waters (1 µg L^{-1}) should be reconsidered since a toxic cyanobacteria bloom might occur. The study of the removal of PCP from the culture medium by the two species is an additional novelty of this work. *M. aeruginosa* could remove part of the PCP from the medium, at concentrations where toxic effects were observed, while *C. vulgaris* stabilized it.

8.1. Introduction

The aquatic environment is particularly sensitive to PCP [1]. The phytoplankton composition in freshwater ecosystems is varied and oftentimes includes cyanobacteria and green microalgae as major components [2-4]. In the environment, the behaviour of pollutants and their interaction with phytoplankton is complex due to the interplay between abiotic and biotic processes [5], which hampers the extrapolation of laboratory data. On the other hand, experiments based on single species cultures, despite its drawback in terms of ecological relevance, separate the response of one species to changes in a particular variable from interactions with other organisms and/or chemical compounds found in the environment [3]. The identification to the organism level is important since the toxicity profile can be very different for closely related species [3]. This is particularly relevant for cyanobacteria, since only some species, or even some strains within a same species, may produce toxins [6].

The overall toxic effect of CPs is caused by a combination of several distinct mechanisms, most of which interfere with energy transduction, mainly in mitochondria, chloroplasts and bacterial cytoplasmic membranes [7, 8]. PCP interferes with the oxidative phosphorylation process and inhibits ATP synthesis [9] as well as the electron flow process in photosynthesis [10]. Therefore, chl_a can be an indicator of PCP toxicity [3].

The potential of some cyanobacteria and microalgae in the removal of CPs by biodegradation and biosorption was recognised [11, 12]. The uptake of pesticides by

phytoplankton involves adsorption on the cell surface followed by absorption within the cell, which can lead to the entry of toxic chemicals into the food chain [2]. Abiotic mechanisms of PCP removal include photodegradation, oxidation and evaporation [13].

As discussed in Chapter 2, there are two works about the toxicity of PCP to cyanobacteria, carried out at relatively high concentrations of PCP: one at the phytoplankton community level, without taxonomic identification details [2], and one for a soil species [3]. The toxicity of Na-PCP was also checked for aquatic cyanobacteria, in a plate incubation test during 9 days, by measuring only OD [14]. That article, however, focused on the development of toxicity tests for veterinary antimicrobial products, and used Na-PCP as a model substance. Apart from data on the EC_{50}, NOEC and MIC, no other results about the effects of PCP were demonstrated. Conversely, several studies treat toxicity of PCP to *C. vulgaris* [15-22]. The aforementioned studies on the toxicity of PCP to *C. vulgaris* and cyanobacteria, further discussed, used nominal concentrations of PCP and do not measure its initial concentrations and possible removal during the tests.

The objective of this work was to research the fate and effects of PCP at environmental levels on two common freshwater phytoplankton species, one prokaryotic and the other eukaryotic. The results reported for *M. aeruginosa* are among the first for the toxicity of CPs on cyanobacteria. For the first time, the toxicity of PCP on a single aquatic cyanobacterial species was evaluated using data for both OD and chl_a measurements. The results were compared with those for the chlorophyte *C. vulgaris*, obtained in the same conditions, as well as with data from the literature. An additional novelty of this work was the study of the ability of the two species in the removal of PCP from the culture medium.

8.2. Results

8.2.1. PCP removal by *M. aeruginosa*

For the nominal PCP concentrations 0.01, 0.1, 1, 10, 100, 500 and 1000 μg L^{-1}, the initial concentrations, C_0, were equal to 0.07, 0.11, 1.02, 11.1, 129, 248 and 1131 μg L^{-1}, respectively (Fig. 8.1A). The average initial and final concentrations of PCP in the blanks (biological controls) were 0.007 ± 0.004 μg L^{-1} (n=7) and 0.010 ± 0.009 μg L^{-1} (n=7), respectively (values ± standard deviations).

The removal of PCP, in abiotic conditions (n=2), for the initial concentrations 0.07, 0.11, 248 and 1131µg L^{-1} was 63.5 ± 12.3%, 29.1 ± 14.3%, 38.0 ± 5.0% and 34.8 ± 14.5%, respectively (Fig. 8.1A). In biotic conditions (n=3), at the same four concentration levels of PCP, the removal tended to be higher: 80.1 ± 7.0% ($p<0.1$), 38.4 ± 6.7%, 46.5 ± 7.8% and 70.4 ± 11.6% ($p<0.05$), respectively (Fig. 8.1A) (values ± standard deviations). For the other tested concentrations, no removal of PCP was observed.

8.2.2. PCP removal by *C. vulgaris*

For the nominal PCP concentrations 0.01, 0.1, 1, 100, 500, 1000 and 5000 µg L^{-1}, the initial concentrations, C_0, were equal to 0.02, 0.10, 0.99, 59.0, 165, 380 and 5539 µg L^{-1}, respectively (Fig. 8.1B). The average initial and final concentrations of PCP in the blanks (biological controls) were 0.005 ± 0.002 µg L^{-1} (n=7) and 0.009 ± 0.008 µg L^{-1} (n=7), respectively (values ± standard deviations).

The removal of PCP, in abiotic conditions (n=2), for the initial concentrations 0.02, 0.10, 0.99, 59.0 and 5539 µg L^{-1} was 29.8 ± 7.1%, 70.7 ± 5.4%, 77.5 ± 2.0%, 14.7 ± 8.6% and 67.0 ± 5.5%, respectively (Fig. 8.1B). In biotic conditions (n=3), at the same five concentration levels of PCP, the removal tended to be lower: 25.8 ± 16.4%, 38.4 ± 14.4% ($p<0.05$), 34.3 ± 3.9% ($p<0.001$), 11.4 ± 10.8% and 65.1 ± 3.2%, respectively (Fig. 8.1B) (values ± standard deviations). For other tested concentrations, no PCP removal was observed.

8.2.3. Growth variation of *M. aeruginosa* at different PCP concentrations

For the initial PCP concentrations 248 and 1131 µg L^{-1}, the average growth rates determined by chl_a (Fig. 8.2A) were significantly smaller than for the respective biological controls (one sided *t*-test, $p<0.01$). A slight enhancement of growth was observed for the initial PCP concentration 1.02 µg L^{-1} but the effect was not statistically significant. Above this concentration, growth inhibition was always observed and it was concentration-dependent, leading to the calculation of the EC_{20} and EC_{50} values 0.015 and 0.117 mg L^{-1}, respectively.

Growth assessed by AUC (Fig. 8.2B) led to much more variable results, in general, and not concentration-dependent; nevertheless, these results corroborate the

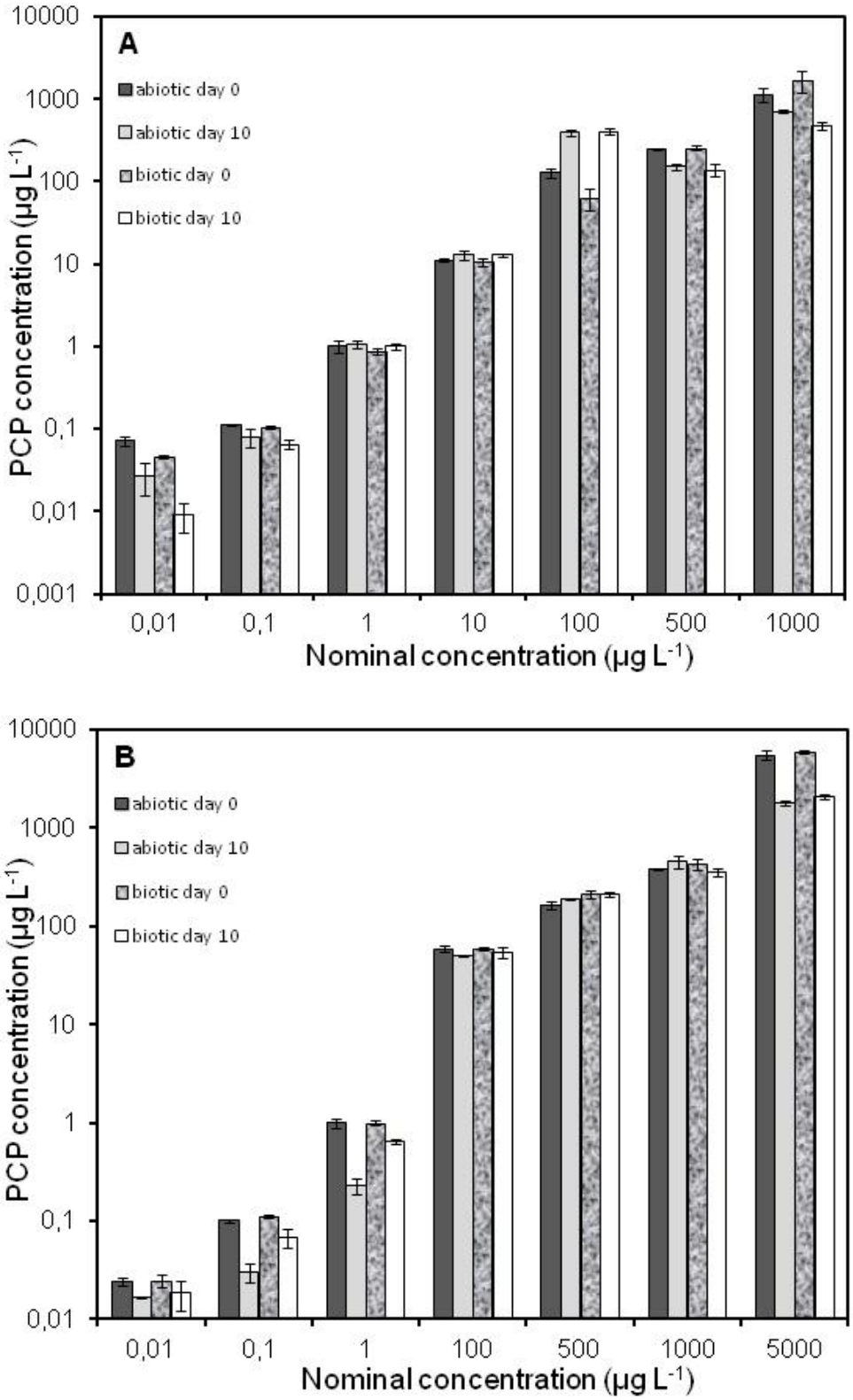

Fig. 8.1. Pentachlorophenol (PCP) concentration, in logarithmic scale, measured by HS-SPME-GC-ECD in the first and last days of a 10 days experiment, as a function of PCP nominal concentration in cultures of *Microcystis aeruginosa* (A) and *Chlorella vulgaris* (B) in Fraquil medium spiked with different concentration levels of PCP ("abiotic" refers to chemical control flasks with Fraquil medium and PCP; "biotic" refers to cultures of *M. aeruginosa* or *C. vulgaris* in the same conditions of the controls). Error bars are mean deviations of two replicates, for abiotic conditions, and standard deviations of three replicates, for biotic conditions.

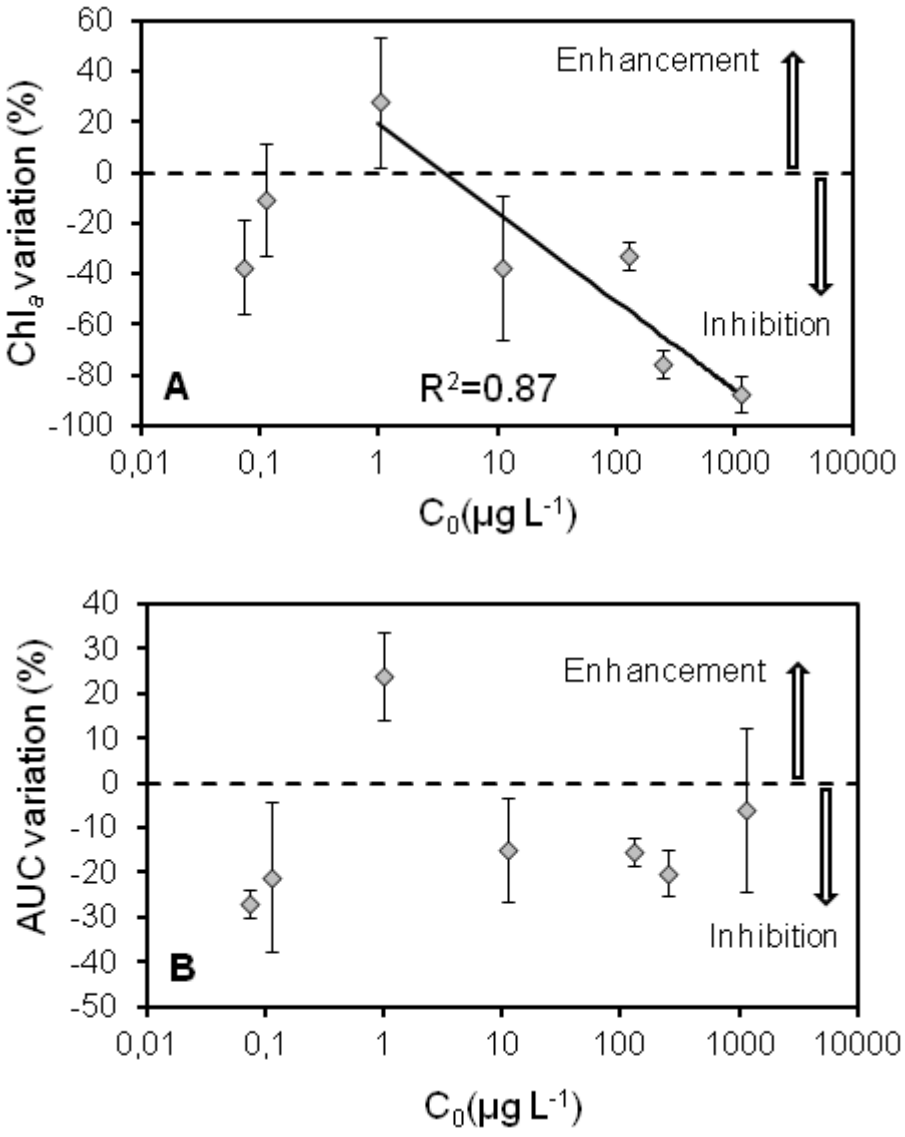

Fig. 8.2. Percentage of variation of chlorophyll *a* content (A) and area under curve (B), with *M. aeruginosa,* as a function of the initial concentration of pentachlorophenol (PCP), C_0, after 10 days of experiment in Fraquil medium cultures. The values for the growth variations and the error bars were obtained as averages of three replicates and their standard deviations, respectively.

promotion of growth of *M. aeruginosa* for 1.02 µg L^{-1} of PCP. For confirmation of this result, the experiment at that concentration of PCP was repeated with four replicates and compared with three new biological controls. The AUC was statistically higher at 1.02 µg L^{-1} of PCP, compared with the respective controls ($p<0.02$, 7 replicates, 5 biological controls). Bellow this concentration, there was inhibition of growth, significantly higher for the initial PCP concentration 0.11 µg L^{-1} ($p<0.02$), compared with the controls. Above 1.02 µg L^{-1} of PCP, the AUC was statistically smaller for the initial PCP concentrations 129 µg L^{-1} ($p<0.1$) and 248 µg L^{-1} ($p<0.05$), compared with the respective biological controls. The growth variations, assessed by chl*a* and AUC,

determined as a function of PCP concentration (Fig. 8.2) were different and the significant interaction between the endpoints and the PCP concentration (two-way ANOVA, $p<0.001$) demonstrated this difference is clearly seen at high PCP levels.

The difference of pH between the chemical control (average pH 7.07 ± 0.15, n=5) and the biological control (average pH 8.49 ± 0.6, n=5) was less than 1.5 pH units. In the chemical controls, the pH did not vary with the different PCP concentrations. In Fraquil medium with PCP and the cyanobacterium, the pH values were between these two (average pH 7.7 ± 0.48, n=5), tending to decrease at the two highest concentrations of PCP (data not shown). For the two lowest PCP levels and the respective controls, the pH was not measured.

8.2.4. Growth variation of *C. vulgaris* at different PCP concentrations

Growth assessed by chl_a (Fig. 8.3A) led to variable results and was not concentration-dependent. The average growth rates, expressed on the basis of chl_a, were significantly smaller than the respective biological controls for the initial PCP concentrations 0.02 µg L^{-1} ($p<0.1$) and 380 µg L^{-1} ($p<0.05$). Nevertheless, PCP inhibited the growth of *C. vulgaris* in all concentration levels above 0.99 µg L^{-1}.

The AUCs were significantly smaller for the initial concentrations of PCP 165 µg L^{-1} ($p<0.05$) and 380 µg L^{-1} ($p<0.1$), compared with the respective biological controls (Fig. 8.3B). Growth inhibition was concentration-dependent, leading to the calculation of the EC_{20} value 0.369 mg L^{-1}. The EC_{50} value could not be calculated because it is higher than the highest PCP concentration studied (5539 µg L^{-1}). The growth variations, assessed by chl*a* and AUC, determined as a function of PCP concentration (Fig. 8.3) were different and the significant interaction between the endpoints and the PCP concentration (two-way ANOVA, $p<0.1$) demonstrated this difference is seen at high PCP levels.

The pH in the biological control of the *C. vulgaris* cultures was about 1 pH unit higher (average pH 9.49 ± 0.38, n=7), comparing with the cyanobacterium cultures. In the presence of PCP and *C. vulgaris*, the pH values were always similar to those found for the biological control, irrespectively of the PCP concentration (average pH 9.54 ± 0.32, n=7). The pH for the chemical control (average pH 7.18 ± 0.13, n=7) was, in average, over 2.3 pH units lower in comparison to the biological control.

8.3. Discussion

To our knowledge, this is one of the first studies about the toxic effects of PCP to cyanobacteria and no other CPs were studied to date. The first attempt, back in 2000 [2], emphasized the impact of PCP on the growth and community structure of freshwater phytoplankton, including cyanobacteria and chlorophytes, in the Nile River, but the taxonomic resolution employed was restricted to the phylum level. Thus, an in-depth evaluation of which were the genera or species more or less susceptible to PCP cannot be retrieved from this study. Furthermore, the lowest concentration level tested in the above study was 244 μg L^{-1}, difficult to be found in the environment. In another study, the effects of PCP on a soil cyanobacterium were studied in low biomass densities and the lowest concentration level of PCP was 10 μg L^{-1} [3]. At last, the toxic concentrations of Na-PCP have been measured for aquatic cyanobacteria [14]. Nevertheless, the focus of that study was not the ecotoxicity of PCP; rather, the idea was to develop toxicity tests for veterinary antimicrobial agents using cyanobacteria.

The novelty of the current work is, therefore, focused in the toxicity and removal of PCP by freshwater cyanobacteria, with *M. aeruginosa* as example due to its global distribution and environmental and ecological relevance [23, 24]. Nonetheless, the interactions of PCP with this species are compared with that found for a widely studied common freshwater microalga, *C. vulgaris*, at the same conditions. Ideally, the biological data for interspecific comparisons should be obtained by the same researcher in a single laboratory using the same protocols [8], which was applied herein. Furthermore, the PCP concentrations surveyed in this study cover the entire range found in the freshwater environment.

8.3.1. PCP toxicity to *M. aeruginosa*

Pentachlorophenol was toxic to *M. aeruginosa* at all concentrations studied, except at 1.02 μg L^{-1}. The behaviour of *M. aeruginosa* exposed to increasing concentrations of PCP can be considered hormetic. Hormesis is a dose response phenomenon characterized by a low dose stimulation and high dose inhibition, resulting in an inverted U-shape dose response curve (see Fig. 8.2). At the PCP concentration 1.02 μg L^{-1}, the cyanobacterium grew faster than in the biological controls, both when it was measured by chl_a and AUC, leading to average growth promotions of 27.9% and 23.9%, respectively. This is in agreement with the statement that the maximum response from hormesis is typically modest [25]. The growth

stimulation could be a first sign of perturbation of the biological processes by the toxicants [26]. The toxicant might foster cell division and the consequent increase in biomass could be an attempt to provide a dilution effect of PCP as its concentration will be divided for a higher number of cells [18]. Despite the controversy over the hormetic model of dose response, some authors claim that this is an important and, possibly, very common mechanism that is still not very well understood [25]. Several compounds are known to promote the growth of *M. aeruginosa*, like nonylphenol [6], minocycline degradation products [27] and polycyclic aromatic hydrocarbons [28].

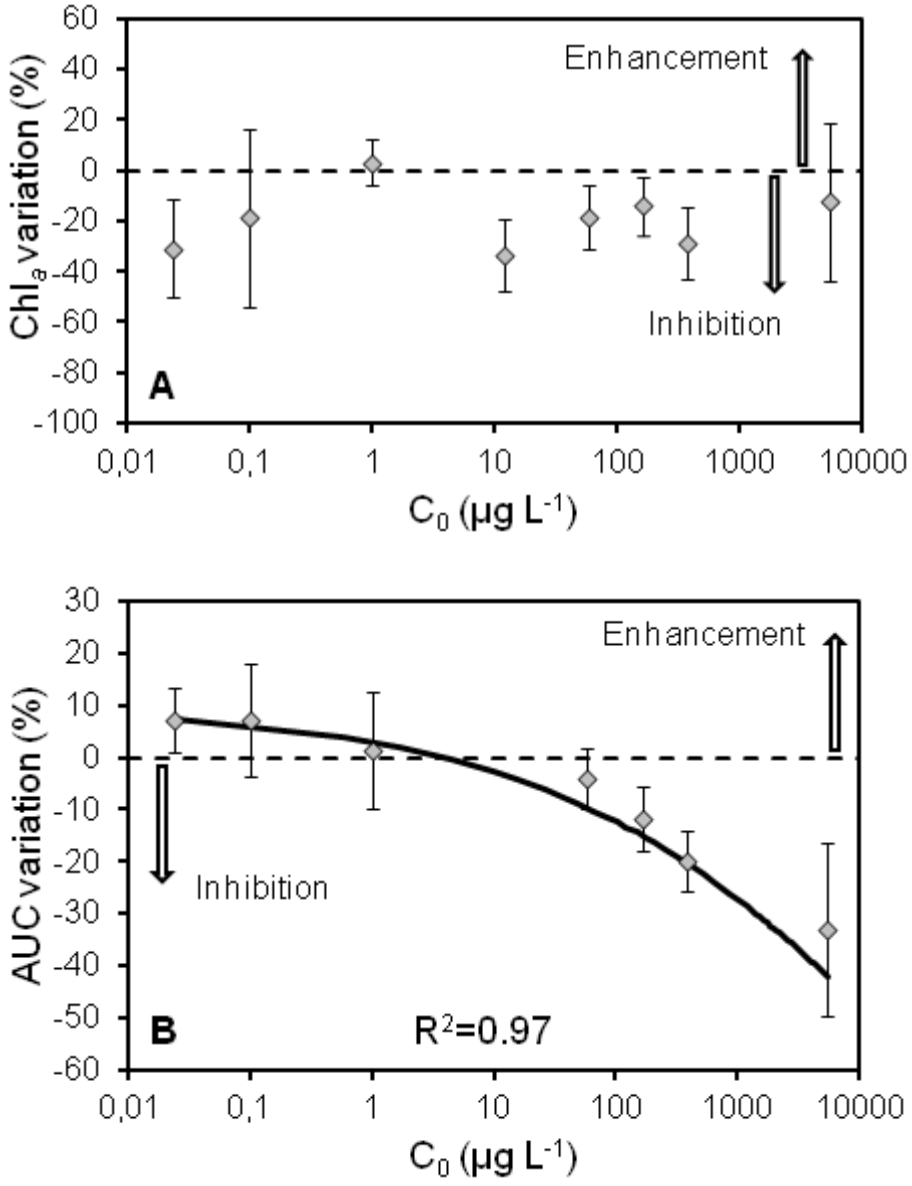

Fig. 8.3. Percentage of variation of chlorophyll *a* content (A) and area under curve (B), with *C. vulgaris,* as a function of the initial concentration of pentachlorophenol (PCP), C_0, after 10 days of experiment in Fraquil medium cultures. The values for the growth variations and the error bars were obtained as averages of three replicates and their standard deviations, respectively. The data were fitted to the Weibull distribution.

Pentachlorophenol concentrations lower than 1.02 μg L^{-1} could not trigger such growth stimulation response mechanism by the cyanobacterium, being slightly toxic rather, an effect already observed in a hormetic mechanism [25]. Probably, very low PCP concentrations, even in the pg L^{-1} level, could also exhibit toxicity to phytoplankton specimens since they are enough to initiate endocrine disrupting effects on wildlife and humans [29]. Other endocrine disruptors can affect the photosynthetic electron flow in microalgae and cyanobacteria, namely *M. aeruginosa* [30]. For PCP concentrations higher than 1.02 μg L^{-1}, the growth overreaction possibly is not enough to counteract the toxic effects.

The results obtained by chl_a were concentration-dependent of PCP (Fig. 8.2A), and therefore chosen for the calculation of the EC_{20} and EC_{50}, while OD_{750} results were not (Fig. 8.2B). This may indicate that the cells continued to divide even with their chl_a diminished. It is known that PCP may change the chl_a without altering the number of phytoplankton cells [2]. Moreover, to perform photosynthesis, cyanobacteria contain protein complexes called phycobilisomes, anchored to the stromal surface of thylakoid membranes [31]. These light-harvesting complexes deliver the light energy, mostly absorbed by phycobiliproteins, to the photosystem II reaction centres through chlorophyll *a* and carotenoids [31, 32]. It has been shown that these photosynthetic pigments can have different sensitivity to toxicants [31, 33, 34]. Therefore, photosynthesis in cyanobacteria might continue even with low levels of chl_a if other pigments, possibly less sensitive to PCP, assured its function. In fact, the photosynthetic apparatus of some cyanobacterial strains showed to be extremely adaptable under exposure to different pollutants, allowing the maintenance of their photosynthetic performance, as in *Anabaena* sp. [31] and *M. aeruginosa* [33, 34].

In a previous study, several aquatic cyanobacterial isolates have been exposed to PCP during 6 days [14]. The EC_{50} values were determined using AUC data (OD_{655} *vs* time) and were 1.2 mg L^{-1} for *M. aeruginosa* NIES-44 and between 0.17 and 9.9 mg L^{-1} for the other tested species. The lower value observed was for the other *Microcystis* strain used in the study, *M. wesenbergii* NIES-107. The obtained EC_{50} value for *M. aeruginosa* in the current study (0.117 mg L^{-1}) is even lower but it must be taken into consideration the different parameter and experimental conditions used to calculate it. However, several *Microcystis* morphospecies described, and still valid, under the Botanical Nomenclature Code have been seen to belong to one same species according to the Rules of the Bacteriological Code, demanding a unification of these taxa [35]. In fact, the abovementioned strain *M. wesenbergii* NIES-107 has been shown to be phylogenetically more related to *M. aeruginosa* strains than to other *M.*

wesenbergii isolates [36, 37]. Thus, despite the methodological differences of the two studies, the inter-comparison between the EC_{50} values determined for the three strains is not unreasonable, showing that *Microcystis* sp. (at its broad sense) may have a relatively large range of EC_{50} values for PCP.

Data from the literature have demonstrated that, at PCP concentrations of 10, 100 and 250 µg L^{-1}, the growth of the soil cyanobacterium *Anabaena inaequalis*, measured as hormogonia (i.e. short, motile reproductive filaments) density, was promoted at the exposure times 48 and 72 hours [3]. Chlorophyll *a* biosynthesis generally decreased with the increase of exposure time (up to 96 hours) and PCP concentration. Nevertheless, it was higher than the controls for the three referred concentrations at the same exposure times. At 96 hours of PCP exposure, the EC_{50} was 0.13 mg L^{-1}; this value is similar to the one obtained in the current study. Similarly, El-Dib et al. [2] stated that, at 244 µg L^{-1}, PCP enhanced total phytoplankton growth, cyanobacteria included, and chlorophyll *a* biosynthesis, while for higher concentrations PCP inhibited phytoplankton growth and reduced the cell counts. The authors noticed that cyanobacteria count generally decreased in the presence of PCP but also that a relative increase of the total cyanobacteria counts occurred at lower PCP concentrations after 5 days of exposure. From the cell counts [2], the EC_{50} of PCP to cyanobacteria (mixture) would have been between 0.2 and 0.4 mg L^{-1}, again in the same order of magnitude with the results from the present study.

8.3.2. PCP toxicity to *C. vulgaris* and comparison to *M. aeruginosa*

The toxicity profile of PCP to *C. vulgaris* seems to be different from that of the cyanobacterium. Growth enhancement could not be proven at any concentration level (Fig. 8.3). *C. vulgaris* is nearly insensitive to environmental concentrations of PCP (0.02, 0.10 and 0.99 µg L^{-1}) and toxic effects appear at levels above 0.99 µg L^{-1}. In *C. vulgaris*, the chl_a was not concentration-dependent of PCP while AUC results were (Fig. 8.3). This may indicate that the number of cells progressively decreased with increasing PCP concentrations without affecting so considerably the chl_a as with the cyanobacterium.

In the present study, it was observed that the highest inhibition of growth occurred on day 6. After that, the microalga tended to recover but inhibition was still observed, even at day 10. When the AUC and the growth variation were calculated for the first 6 days of exposure, the obtained EC_{50}, 7.6 mg L^{-1}, was lower (data not shown).

In another study, the addition of 10 mg L^{-1} of PCP reduced the initial growth rate of the PCP tolerant microalga *Chlorella* sp. VT-1 and increased the length of the lag phase [22]. This lag period possibly allowed a tolerant population of cells to adapt to PCP. This phenomenon may be related to some form of detoxification required before the cells can resume growth [22]. The obtained results for *C. vulgaris* do not demonstrate the importance of the length of the lag phase in possible detoxification. The lag phases were variable, roughly lasting from 2 to 4 days, irrespectively of the presence of PCP or its concentration, but similar between each treatment and the respective control (data not shown).

Although the EC_{50} value for *C. vulgaris* could not be calculated, due to the range of PCP concentrations studied, a theoretical value, 12.6 mg L^{-1}, could be estimated from the curve in Fig. 8.3A and used for the comparison with the values found in the literature. The EC_{50} of PCP to *C. vulgaris* have been calculated to be 10.3 mg L^{-1} (4 days of exposure) [16], 1.66 mg L^{-1} (IC_{50}, seven days of exposure) [21] and 6.66 mg L^{-1} (3 days) [20]. The strain *Chlorella* sp. VT-1 showed to be more tolerant to PCP (EC_{50} 17.93 mg L^{-1}) than *C. vulgaris* (EC_{50} 10.03 mg L^{-1}) [17]. Another study reported a lower EC_{50} for *Chlorella* sp. VT-1 (6.5 mg L^{-1}) [22]. Although the EC_{50} values can be very disparate and difficult to compare due to different exposure times, endpoints assessed, culture media and natural intraspecific variability, all cited values are in the same order of magnitude.

Comparing with other microalgae, *C. vulgaris* has an intermediate sensitivity to PCP. An example of a more sensitive species is *Pseudokirchneriella subcapitata*, with EC_{50} values of 0.004 to 0.013 mg L^{-1}, for 2 days of exposure using a closed system [38], and 0.16 mg L^{-1}, for 6 days of exposure using a well plate system [14]. Contrarily, some species are more tolerant to PCP, like the soil species *Chlorella kessleri*, with a EC_{50} of 34.3 mg L^{-1} for 4 days of exposure [3].

It is generally assumed that the responses of organisms to toxic substances are small or non-existent at low doses and gradually leading to growth inhibition at higher levels [3]. The results for *C. vulgaris*, reported here, support this assumption (Fig. 8.3B). It is interesting to notice that hormesis was not observed in this case, a different behaviour from that of *M. aeruginosa*.

Several studies indicate that pesticides might interfere with photosynthesis, phosphorylation and protein synthesis [39]. The toxic effects of PCP on the studied species were probably due to a combination of several factors and distinct mechanisms

occurring simultaneously, most of which related with energy transduction [7, 8]. It is likely that both photosynthetic activity and oxidative phosphorylation were affected by PCP in the studied phytoplankton species. It is known that PCP impaired the oxidative phosphorylation in *C. vulgaris* [8] and the photosynthesis in other microalgae [40, 41].

The much higher toxicity of PCP to *M. aeruginosa* compared to *C. vulgaris* might be related to numerous factors. First, cyanobacterial cells have bigger surface / volume ratio compared to microalgae, which is known to increase the transfer rate of hydrophobic toxic substances [42]. Second, in cyanobacterial cells the photosynthesis and oxidative phosphorylation occur in the cytoplasm [30], while in eukaryotic cells PCP must pass through an additional double membrane (chloroplasts and mitochondria, respectively) to arrive to its site of action. Third, prokaryotes may have less elaborated enzymatic antioxidant pathways [30]. However, studies on a molecular level are still lacking and are necessary to identify and quantify the contribution of each possible mode of action [7] since the ability to detect early molecular responses to chemicals is central to the understanding of biological impact of pollutants [43].

8.3.3. Removal of PCP by *M. aeruginosa*

The removal of PCP from the culture media, when observed, seemed to be faster in the presence of the cyanobacterium (Fig. 8.1A). As discussed, PCP was more toxic to the cyanobacterium at the lowest and highest concentrations studied (Fig. 8.2). It is worth noticing that, at the same levels, removal of PCP by the cyanobacterium was also observed. Possibly, PCP is more internalized by the cells, and therefore more toxic, at low and high concentrations. On the other hand, at intermediate PCP levels, where removal was not noticed, the cells might be able to avoid the entrance of PCP. Opposite behaviour has been observed for octylphenol where there is biotic removal by *M. aeruginosa* at low non-toxic levels and no biotic removal at toxic concentrations [44].

The biotic removal of PCP from the medium could be explained by biosorption, as already studied for dried biomass of *M. aeruginosa* [45]. At the pH of the incubation tests, all PCP is practically as deprotonated form (pentachlorophenolate anion). Biosorption could occur by interaction of the cellular membrane either with an ion pair formed between this anion and metals from the Fraquil medium or with the hydrophobic parts of the PCP anion [45].

For the highest PCP concentration tested, the removal in biotic conditions was already the double than that of abiotic conditions, the highest difference noticed between the two conditions for every PCP concentration studied (Fig. 8.1A). It is known that biosorption can be carried out by dead cells, as already noticed for removal of CPs by bacterial biomass [46]. The high toxicity might lead to an increased cell death and possibly larger sorption of PCP due to its lipophilicity.

8.3.4. Removal of PCP by *C. vulgaris*

Oppositely to *M. aeruginosa*, it seemed that *C. vulgaris* not only was unable to remove PCP but had a stabilizing effect on it, slowing down its removal from the medium. Possibly, the microalga is able to avoid the uptake of PCP. So, it is likely that the losses of PCP were due to abiotic factors, like photodegradation [47], oxidation and evaporation [48], rather than biosorption or biodegradation. The experiments in the current study did not permit to assess the relative importance of each abiotic factor but, according with the literature, photodegradation could have been the most prevalent mechanisms of abiotic removal of PCP [47].

To our knowledge, the removal of PCP by *C. vulgaris* has not been studied yet. Only the PCP tolerant *Chlorella* sp. VT-1 cultures were found to mineralize about 13% of isotopically-labelled PCP, by measuring the release of CO_2, ability that was not observed for *C. vulgaris* [18]. Since the PCP concentration was not checked during the tests, its removal has not been evaluated in other works.

It is difficult to explain the stabilizing effect of *C. vulgaris* on PCP found in the current work. One study of the interactions of octylphenol with *M. aeruginosa* demonstrated that exudates were able to stabilize it [44]. Even considering the enormous difference between the two microorganisms, it can be suggested that exudates from *C. vulgaris* could stabilize PCP during the incubation tests.

8.3.5. Environmental fate of phytoplankton species after a PCP contamination

In the freshwater environments, PCP concentrations are usually in the ng L^{-1} level and, as a consequence, PCP could be slightly toxic to *M. aeruginosa* and rather neutral to *C. vulgaris*. In contaminated surface waters, the concentrations of PCP can

reach μg L^{-1} or even mg L^{-1} levels. At these conditions, it can be expected that PCP will be much more toxic to *M. aeruginosa* than to *C. vulgaris*.

A PCP concentration of about 1 μg L^{-1} could promote the growth of *M. aeruginosa*; as a consequence, at this concentration, it may trigger the formation of a possibly toxic cyanobacteria bloom. The legal limits of PCP in drinking and surface waters are equal to this value. Additional micro- and mesocosms experiments should be carried out to check if PCP is able to promote the cyanobacterial growth at this level. In the event of growth promotion, the legal limit should be reconsidered, at least in water courses prone to form *Microcystis* spp. blooms. The same type of experiments can also be used to evaluate the competitive interactions between *C. vulgaris* and *M. aeruginosa* at different PCP concentrations.

8.4. Conclusions

Pentachlorophenol was toxic to *M. aeruginosa* in every concentration tested except for 1 μg L^{-1}. At this concentration, there was enhancement of growth, which suggests that possible toxic cyanobacteria blooms might occur in freshwater waterbodies, under favourable environmental conditions. PCP was not toxic to *C. vulgaris* unless at high levels of contamination (mg L^{-1} level). The mechanism of toxicity of the two species seemed to be different. Another marked difference between the two species is that, at environmental levels (ng L^{-1}), *M. aeruginosa* could remove PCP from the culture medium while *C. vulgaris* stabilized it.

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Chapter 9

Toxicity of pentachlorophenol to a mixture of freshwater phytoplankton

Abstract: Pentachlorophenol is a priority pollutant due to its persistence and high toxicity. For the first time, PCP effects were investigated at laboratory scale on co-cultures of two ubiquitous freshwater phytoplankton species: the cyanobacterium *M. aeruginosa* and the microalga *C. vulgaris*. The cells were exposed to environmental levels of PCP during 10 days, in Fraquil culture medium, at nominal concentrations from 0.1 to 10000 μg L^{-1}. Growth was assessed by AUC (CC *vs* time). The phytoplankton community structure can be changed as a consequence of a PCP contamination. Low μg L^{-1} levels of PCP are advantageous to *M. aeruginosa*. This is the first report on the promoting effect of PCP on the growth of aquatic cyanobacteria, using mixtures with microalgae. As a result of the direct toxic effects of high PCP concentrations on *M. aeruginosa*, *C. vulgaris* cell count increased given that in non-spiked co-cultures *M. aeruginosa* inhibited the *C. vulgaris* growth. At 16.7 mg L^{-1}, PCP already had direct toxic effects also on the microalga. The culture medium pH tended to decrease with increasing PCP concentrations, which was mostly related with the cyanobacterium growth inhibition caused by PCP. The PCP concentration was stable in the co-cultures, which differed from what have been observed in monocultures of the same two species. Short-term laboratory assays with two phytoplankton species give important information on the species interactions, namely possible direct and indirect effects of a toxicant, and must be considered in ecotoxicity studies regarding environmental extrapolations.

9.1. Introduction

Previously, the fate and effects of PCP at environmental levels on unialgal cultures of the toxic and bloom forming cyanobacterium *Microcystis aeruginosa* and the green microalga *Chlorella vulgaris* were studied [1]. The results demonstrated different PCP toxicity profiles and removal abilities of the studied species. It is necessary to confirm if the behaviour of *M. aeruginosa* and *C. vulgaris* when exposed to PCP in mixed cultures (i.e. co-cultures) is similar to that of the single species tested in monoculture experiments.

Laboratory monoculture bioassays with phytoplankton species [2-11] are useful to facilitate the achievement of results in well-controlled conditions, with high sensitivity and reproducibility. However, they lack environmental realism because microalgal species do not occur in isolation but as part of complex communities. Consequently, the plausible interspecific biological interactions (e.g. competition and mortality) are not considered in single species toxicity tests [12]. Therefore, not surprisingly, ecotoxicity studies using co-cultures of phytoplankton species are becoming common [12-20].

Before investigating the effects of PCP in co-culture bioassays, it is necessary to study the somehow unpredictable interspecific interactions in non-spiked cultures. For example, when *M. aeruginosa* and microalgae (other than *C. vulgaris*) were mixed, different responses have been observed, with the cyanobacterium being inhibited [17], stimulated [12] or having even more complex responses [20]. In a study with non-spiked mixed cultures containing *C. vulgaris* and other phytoplankton species, *C. vulgaris* inhibited the microalga *Ankistrodesmus braunii* [14]. Concerning the selected species for the current study, in a recent work the microalga *C. vulgaris* was exposed

to cyanobacterial cell extracts (from *M. aeruginosa* and also *Aphanizomenon ovalisporum*), leading to variable growth responses by the microalga depending on the cell extract used and the cyanotoxins present [21]. Nevertheless, a true mixture of cultures of the two selected species was never studied before.

Generally, PCP inhibits or is rather neutral to mixtures of phytoplankton species [13-16], despite the response being dependent on its concentration and the species. However, exceptions occur, like the microalga *Cryptomonas* spp. which growth was stimulated by PCP in a seasonal plankton mesocosms experiment [16], so each case must be studied separately. In the abovementioned studies, cyanobacteria were absent [14, 16] or present as a group of phytoplankton without identification to the species level [13, 15].

Therefore, the objective of this work was to understand the fate and effects of environmental levels of PCP on a mixture of two common phytoplankton species: *M. aeruginosa* and *C. vulgaris*. For the first time, the interactions between the two species and the effects of PCP on their population dynamics within co-cultures were investigated.

9.2. Results

9.2.1. Population growth at different PCP concentrations

The population growth during the period of incubation (single mono- and co-cultures, in the presence and in the absence of PCP) is shown in Figs. 9.1 (*M. aeruginosa*) and 9.2 (*C. vulgaris*). For *M. aeruginosa*, the AUCs for CC_M in the co-cultures without PCP were statistically identical ($p>0.7$) to the corresponding areas for the pure controls (Fig. 9.1). On the contrary, as can be seen in Fig. 9.2, *M. aeruginosa* had a negative effect on the population growth of *C. vulgaris*. This effect was noticed from the beginning of the experiment. The AUCs for CC_C in the co-cultures were significantly lower ($p<0.001$) than the AUC for the respective single monoculture controls (Fig. 9.2).

For *C. vulgaris*, the AUCs for CC_C (Fig. 9.2) in the co-cultures containing PCP, compared to the AUCs for the biological controls, were significantly higher for either 376 µg L^{-1} ($p<0.05$) or 16.65×10^3 µg L^{-1} ($p<0.02$) of PCP. Therefore, no toxic effect of PCP to *C. vulgaris* (based on cell counts) was observed at any of the tested concentrations. On the contrary, for *M. aeruginosa*, clear toxic effect was observed for

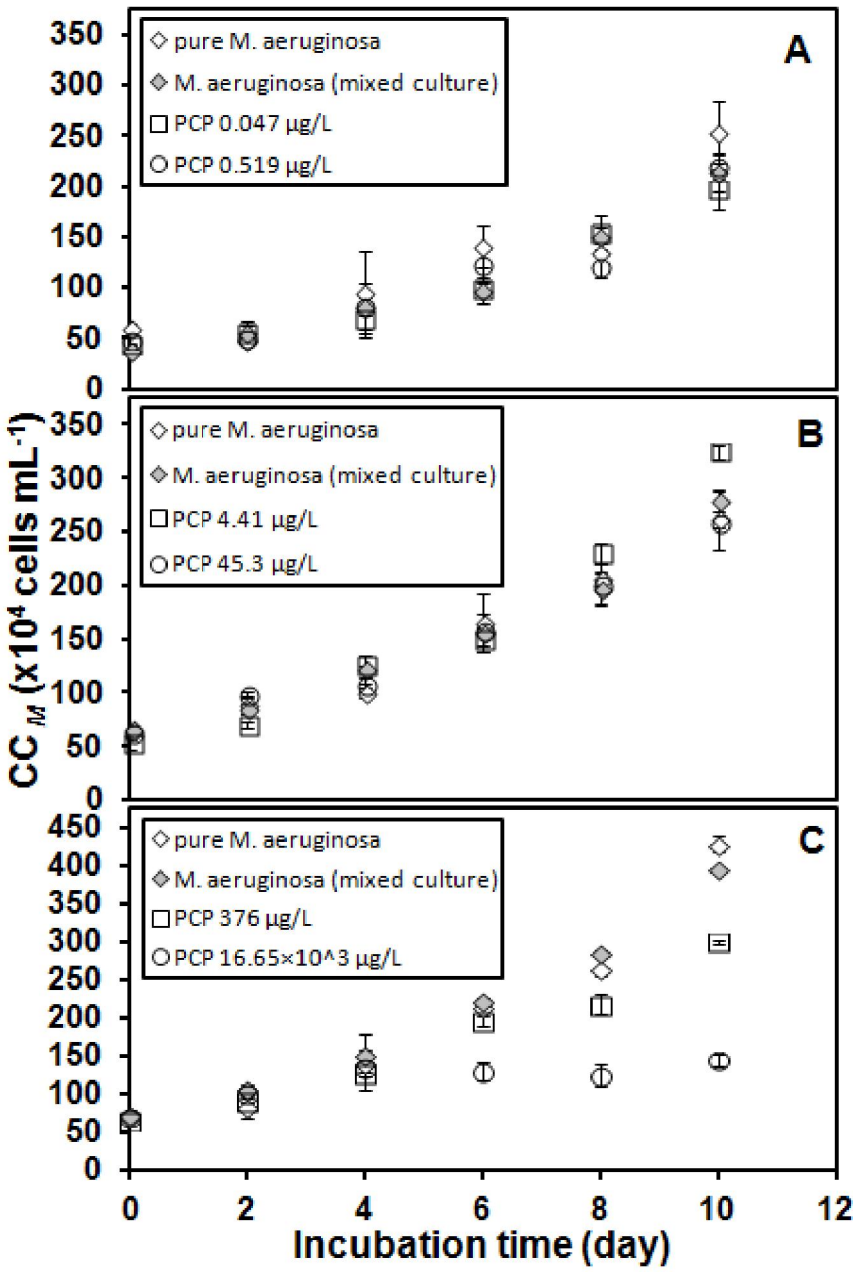

Figure 9.1. Cell count of *M. aeruginosa* (CC_M) in pure or in co-cultures with *C. vulgaris*, as a function of time, in Fraquil medium, during a ten days experiment. Test co-cultures were spiked with different initial pentachlorophenol concentrations: A - 0.047 and 0.519 µg L^{-1}, B - 4.41 and 45.3 µg L^{-1} and C - 376 and 16,65×10^3 µg L^{-1}. The error bars are mean deviations of two replicates for pure *M. aeruginosa* cultures, standard deviations of four replicates for *M. aeruginosa* in non-spiked co-cultures and standard deviations of three replicates for *M. aeruginosa* in spiked co-cultures.

the PCP concentrations 376 µg L^{-1} and 16.65×10^3 µg L^{-1}, especially after day 6 of the experiment (Fig. 9.1C). In the spiked co-cultures (Fig. 9.1C), the AUCs for CC_M were significantly lower than those observed for the biological controls, for 376 µg L^{-1} ($p<0.05$) and 16.65×10^3 µg L^{-1} ($p<0.001$) of PCP. The AUC for CC_M in the co-cultures containing 4.41 µg L^{-1} of PCP was significantly higher ($p<0.02$) when compared with

that of the biological controls (Fig. 9.1B). The measured PCP concentrations are summarised in Table 9.1.

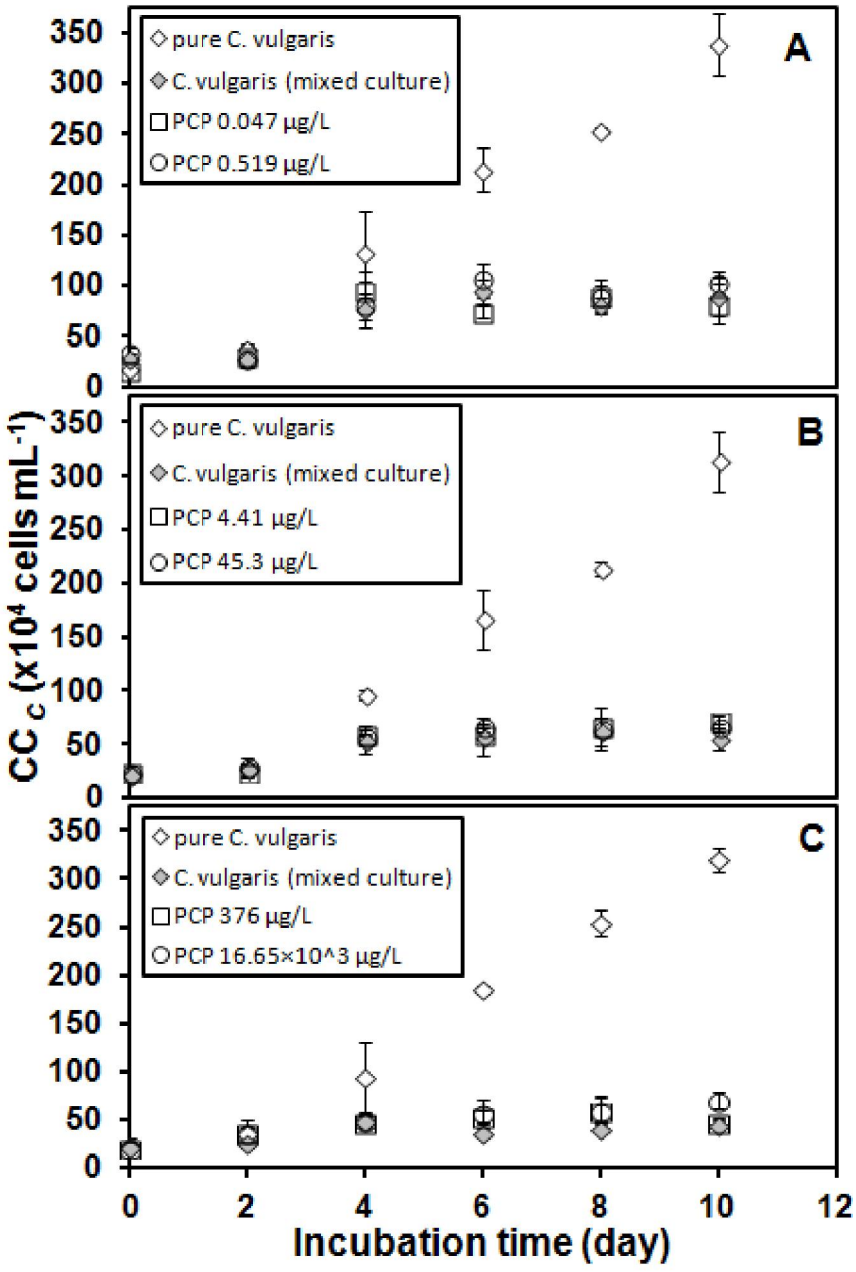

Figure 9.2. Cell count of *C. vulgaris* (CC_C) in pure or in co-cultures with *M. aeruginosa*, as a function of time, in Fraquil medium, during a ten days experiment. Test co-cultures were spiked with different initial pentachlorophenol concentrations: A - 0.047 and 0.519 µg L^{-1}, B - 4.41 and 45.3 µg L^{-1} and C - 376 and 16,65×10^3 µg L^{-1}. The error bars are mean deviations of two replicates for pure *C. vulgaris* cultures, standard deviations of four replicates for *C. vulgaris* in non-spiked co-cultures and standard deviations of three replicates for *C. vulgaris* in spiked co-cultures.

Table 9.1. Concentrations of PCP (μg L^{-1}) in Fraquil medium for abiotic and biotic (co-cultures of *M. aeruginosa* and *C. vulgaris*) conditions in the first and last days of the incubations.

	Abiotic tests [a]		Biotic tests	
$C_{NOMINAL}$	Day 0	Day 10	Day 0	Day 10
0	–	–	0.006 ± 0.002 [b]	0.008 ± 0.004 [b]
0.1	0.047 ± 0.001	0.047 ± 0.006	0.037 ± 0.001 [c]	0.044 ± 0.005 [c]
1	0.519 ± 0.056	0.328 ± 0.067	0.400 ± 0.034 [c]	0.406 ± 0.043 [c]
10	4.41 ± 0.79	4.07 ± 0.30	3.81 ± 0.60 [c]	3.93 ± 0.27 [c]
100	45.3 ± 3.16	46.8 ± 7.00	51.4 ± 7.04 [c]	45.2 ± 6.66 [c]
1000	376 ± 36	398 ± 1	441.5 ± 9 [c]	518 ± 154 [c]
10000	16.65×10^3 ± 672	12.81×10^3 ± 2114	25.04×10^3 ± 4994 [a]	22.50×10^3 ± 3427 [a]

[a] Median values (n=2) with one-/two-sided confidence interval;

[b] Average values (n=6) with standard deviations;

[c] Average values (n=3) with standard deviations.

9.2.2. Average growth rates

The growth rates for *C. vulgaris* controls (days 6, 8, 10) were, in average, (28.4 ± 4.1) $\times10^4$ cells mL^{-1} day^{-1} (n=18). For *M. aeruginosa* controls, the growth rates (days 6, 8, 10) were, in average, (20.0 ± 8.6) $\times10^4$ cells mL^{-1} day^{-1} (n=18), much more variable. The averages of the mean growth rates (days 6, 8 and 10) for *C. vulgaris* (GR_C) *vs* those for *M. aeruginosa* (GR_M) are plotted in Fig. 9.3. In the absence of PCP, a non-linear dependence of population growth rates for both species was observed. The GR_C was much higher at GR_M below 20×10^4 cells mL^{-1} day^{-1} and tended to approach the GR_C in pure culture.

The average GR_M and GR_C in the co-cultures containing different concentrations of PCP are also plotted in Fig. 9.3. Most of the points are within the trend described for biological controls, except for 16.65×10^3 μg L^{-1} of PCP, where both GR_M and GR_C were below the main trend for the biological controls.

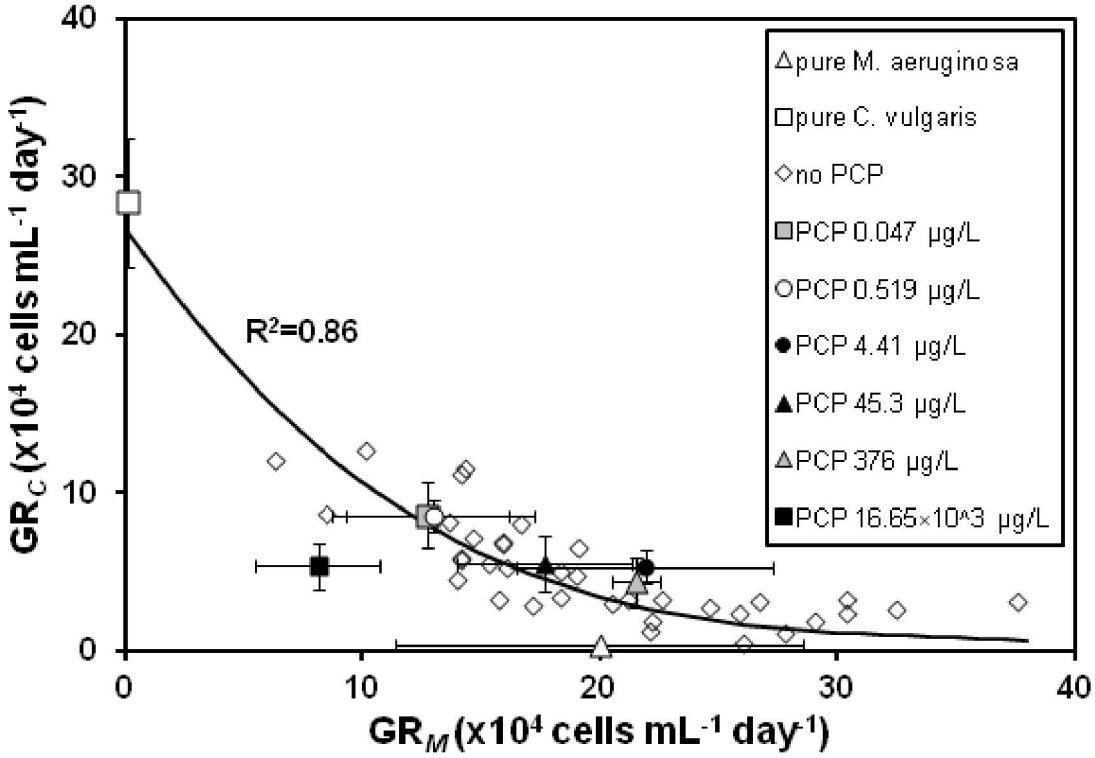

Figure 9.3. Average of the average growth rates of *C. vulgaris* (GR_C) as a function of the average of the average growth rates of *M. aeruginosa* (GR_M) (days 6, 8 and 10) in co-cultures of *M. aeruginosa* and *C. vulgaris* and in pure cultures of each species in Fraquil medium (ten days experiment). Test co-cultures were spiked with different pentachlorophenol (PCP) concentrations. The error bars represent the standard deviations for the average growth rates of three replicates (n=9) for co-cultures with PCP and of six replicates (n=18) for the pure cultures.

9.2.3. Variation of pH of the cultures at different PCP concentrations

The pH values observed in the cultures at the end of the experiment as a function of PCP initial concentration, C_0, are shown in Figure 9.4. The pH for the respective biological controls is also given for comparison. In the chemical controls, the pH for the first four tested levels of PCP was 7.73 ± 0.10 (n=4) and it was not different from the pH of non-spiked culture medium (7.67 ± 0.14, n=3). The pH values were slightly lower, 7.20 and 6.97, for the two highest PCP concentrations (376 and 16.65×10^3 µg L^{-1}, respectively).

For the biological controls, the pH at the end of the experiment was 9.18 ± 0.13 (n=6). It increased about 1.5 pH units, compared with the pH in the beginning of the experiment (7.73). The pH in the tests' cultures at the end of the incubation, compared to the respective biological controls, were significantly higher for the PCP initial concentration 4.41 µg L^{-1} ($p<0.05$) and significantly lower for the PCP initial concentrations of 376 ($p<0.1$) and 16.65×10^3 µg L^{-1} ($p<0.0001$).

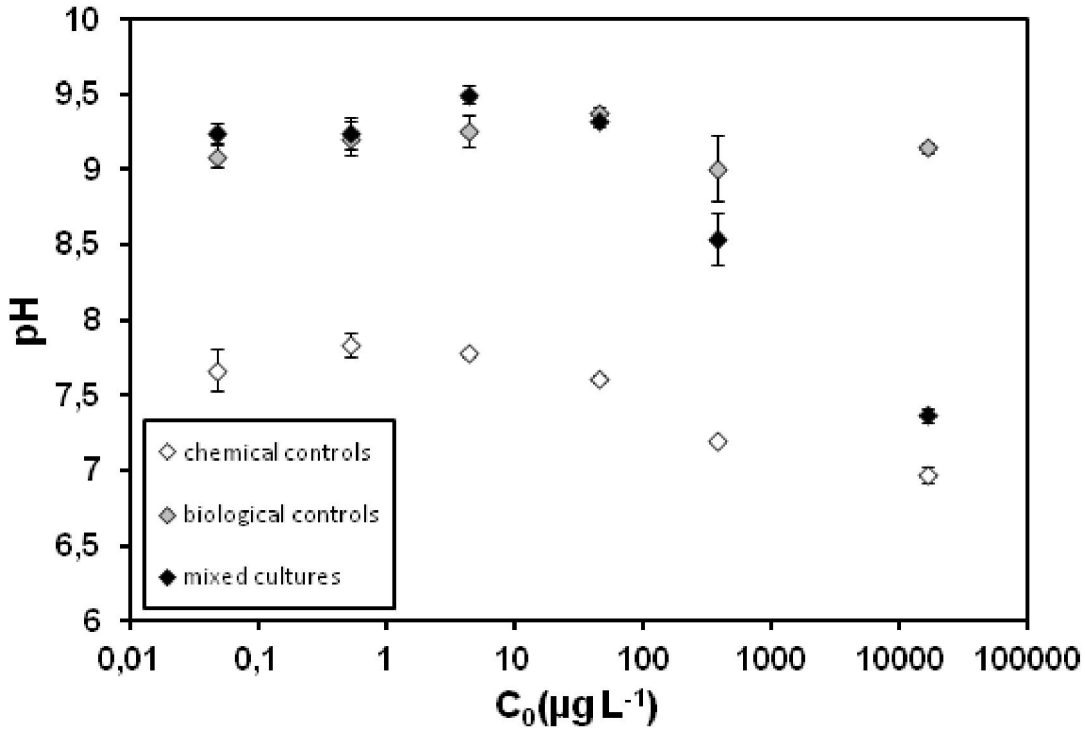

Figure 9.4. Average pH (measured in the last day of a ten days experiment) of co-cultures of *M. aeruginosa* and *C. vulgaris*, in Fraquil medium, spiked with different initial pentachlorophenol concentrations, C_0 (logarithmic scale). Respective chemical and biological controls are given for comparison. The error bars are mean deviations for two replicates, for the controls, and standard deviations for three replicates, for the spiked co-cultures.

9.3. Discussion

9.3.1. Interactions between *M. aeruginosa* and *C. vulgaris*

From the results presented in Figs. 9.1 and 9.2, it can be inferred that, in non-spiked co-cultures, *M. aeruginosa* had a negative effect on the growth of *C. vulgaris* while it was not observed any influence of *C. vulgaris* on the growth of *M. aeruginosa*. Whereas for *M. aeruginosa* the optimum pH is in the range 7.7 to 9.4 [22], *C. vulgaris* prefers slightly acidic conditions (pH 6.0 to 6.5) [23]. This may be one reason for the general success of the cyanobacterium over the microalgae in the biological controls, where the pH at the end of the experiment was about 9.2 (Fig. 9.4). Allelopathy is another possible explanation of these results [20] but the present work was not designed to unravel such interactions. The inhibition of *C. vulgaris* growth by *M. aeruginosa* in co-cultures was surprising since it is known that *M. aeruginosa* cell extracts, containing cyanotoxins, stimulated the growth of the same strain of *C. vulgaris* [21]. Consequently, it is unlikely that these compounds were responsible for this inhibitory effect. Nevertheless, other compounds are known to be released to the

medium by cyanobacteria [21] and can have different effects on the growth of the co-occurring species. For instance, another study states that allelopathic effects of both *M. aeruginosa* and the microalga *Desmodesmus armatus* may have been involved in the observed reciprocal inhibitory effects in co-cultures [20]. Given such diverse findings and interspecific variations, future research on allelopathy is needed.

9.3.2. Toxicity of PCP in co-cultures

The presence of 4.41 µg L^{-1} of PCP in a mixture of *M. aeruginosa* and *C. vulgaris*, led to a significant growth promotion of the cyanobacterium (Fig. 9.1B). To our knowledge, this is the first report on a promoting effect of PCP on the growth of aquatic cyanobacteria in a culture medium also containing microalgae. For the microalga, although an increase of the CC_C was not observed at 4.41 µg L^{-1} of PCP (Fig. 9.2B), the GR_C at this concentration was slightly higher than expected from the trend observed in biological controls (Fig. 9.3). It can be suggested that the higher growth rate of the microalga might be a mechanism to counteract the growth promotion of *M. aeruginosa* by PCP.

In a previous work, using a unicyanobacterial culture of the same strain of *M. aeruginosa*, it has been observed that a PCP concentration of 1.02 µg L^{-1} had a promoting effect on the growth of the cyanobacterium while such effect was not noticed for *C. vulgaris* [1]. The effect observed for *M. aeruginosa* is critical because the growth stimulation occurred at environmental levels of PCP, at low µg L^{-1} range, close to the MAC value of 1 µg L^{-1} for surface waters. Therefore, PCP at environmental concentrations shows the capacity to cause phytoplankton population shifts. Of even more concern is the fact that it might trigger the emergence and appearance of likely toxic *M. aeruginosa* blooms. The present study corroborates these findings using an environmentally more realistic mixed species approach. In water bodies prone to such blooms, the MAC value of PCP in surface waters should be reconsidered, as emphasized before [1].

Several inorganic and organic substances, in low doses, are known to promote the growth of *M. aeruginosa* in single species tests, like arsenate [24], lanthanum salts [19], nonylphenol [25], PCP [1], minocycline degradation products [26], polycyclic aromatic hydrocarbons [27] and the herbicides atrazine [28], uniconazole [29], glyphosate [30] and diclofop [31]. The growth promotion of the cyanobacterium from most of these compounds can be considered a hormetic effect, except for minocycline

degradation products [26] and uniconazole [29]. One possible explanation is that the growth promotion could provide a dilution effect as the toxicant concentration will be divided for a higher number of cells [1, 5]. However, the PCP concentration remained constant throughout the biotic experiments (Table 9.1). The growth stimulation, in this case, could be a first sign of perturbation of the biological processes by the toxicants, leading to an extra allocation of resources [32].

The two highest PCP concentrations studied, however, clearly decreased the CC_M (Fig. 9.1C). The estimated effective concentrations EC_{20} and EC_{50} were 0.252 and 5.775 mg L^{-1}, respectively. They were at least an order of magnitude higher than the values found for single species test of the same strain of *M. aeruginosa* in a previous study [1]. This difference in the response of the cyanobacterium to PCP may be explained by the different methods used to estimate the toxicity, given that, in the abovementioned work, EC_{50} and EC_{20} values were determined using chlorophyll *a* content measurements, which happened to be more sensitive to PCP toxicity than cell population growth, in the case of *M. aeruginosa* [1]. Other possibility is that such variation may be a consequence of the presence of the microalga in the co-cultures, occurring a positive reaction to its exudates, the so-called allelopathy, despite the fact that various allelopathic effects described for the same or similar species are contradictory [20, 21].

The decrease of the CC_M at the two highest PCP concentrations (Fig. 9.1C) corresponds to a slight increase of the CC_C (Fig. 9.2C). At 376 µg L^{-1} of PCP, the growth rates GR_M and GR_C were within the main trend described by the biological controls (Fig. 9.3). Therefore, at this PCP concentration, there is probably an indirect effect of PCP on *C. vulgaris* as a result of its direct toxicity to *M. aeruginosa*. However, for 16.65×10^3 µg L^{-1} of PCP, both GR_M and GR_C were below the main trend for the biological controls (Fig. 9.3). It would be expected that decreasing the GR_M would lead to a much higher GR_C (12.95×10^4 cells mL^{-1} day^{-1}) than observed (5.34×10^4 cells mL^{-1} day^{-1}). This decrease of GR_C, of about 59%, is in accordance with the estimated EC_{50} value (12,6 mg L^{-1}) for single species test with the same strain of *C. vulgaris*, using cell population growth measured by AUC (OD_{750} vs time) [6]. The increase of CC_C by PCP in this case (Fig. 9.2) is also an indirect effect from its toxicity to *M. aeruginosa*. However, direct toxic effect of PCP on *C. vulgaris*, was also observed (Fig. 9.3), even if it is not visible from Fig. 9.2C.

Concentrations of PCP above the estimated EC_{20} value for *M. aeruginosa* (0.252 mg L^{-1}) are expected to shift the phytoplankton community to the dominance of

C. vulgaris over *M. aeruginosa*. Although somehow unlikely, such PCP concentrations can be found in contaminated freshwater environments. A shift in the phytoplankton community structure, observed in co-cultures of *Scenedesmus obliquus* and *M. aeruginosa* [17], from dominance by microalgae to dominance by cyanobacteria, has been interpreted as being a consequence of the exposure to the photosynthesis-inhibiting herbicide metribuzin.

9.3.3. Stability of PCP in culture media

The PCP concentrations, presented in Table 9.1, remained stable throughout the experiment, except in two cases, in abiotic conditions, for which there was a significant decrease of PCP: 0.519 µg L^{-1} ($p<0.1$) and 16.65×10^{3} µg L^{-1} ($p<0.05$). Therefore, the current report has found a remarkable stability of PCP, especially in biotic conditions. In a previous work carried out in single species cultures [1], it was found that PCP could be biotically removed by single species cultures of *M. aeruginosa* and stabilized by *C. vulgaris* ones. Since the cyanobacterium outcompeted the microalga in the co-cultures (Figs. 9.1 and 9.2), it was expected that biotic removal of PCP would be also observed in the present study. However, biotic removal of PCP was not noticed but rather stabilization. Two possibilities might arise from the results to justify this unexpected behaviour: or *C. vulgaris* was still able to stabilize the PCP in the cultures despite the minute population growth, possibly releasing some exudates [1, 33], or the removal capability of the cyanobacterium might be lost in co-cultures. The PCP removal may be a mechanism of defense to PCP and possibly requires a considerable amount of energy [1]. This probable energy cost could have been a disadvantage in the competition with the microalga.

9.3.4. Role of pH in the PCP toxicity to the co-cultures

The total concentration of PCP in the media during the tests did not change with the incubation time but the pH changed (Fig. 9.4), leading to a shift between protonated and deprotonated chemical forms of PCP. The increase of pH (Fig. 9.4) corresponded to a higher CC_M (Fig. 9.1B) whereas the decrease of pH corresponded to a lower CC_M (Fig. 9.1C). This is consistent with the higher toxic effects of PCP on the cyanobacterium observed at the two highest concentration levels and the observed growth stimulation that occurred at a lower PCP level (4.41 µg L^{-1}). The increment of

the pH of the medium caused by the phytoplankton species is expected to decrease the toxic effects of PCP in the co-cultures, namely to *M. aeruginosa*, a tendency already noticed in the single species tests [1]. This pH increment was negligible at toxic levels of PCP.

9.4. Conclusions

In the absence of PCP, *M. aeruginosa* could outcompete *C. vulgaris*. Different factors might be involved in the interspecific competition, such as pH or allelopathy. Environmental PCP concentrations, in the low μg L^{-1} level, were able to stimulate the growth of *M. aeruginosa*, suggesting that this compound has the capacity to promote the appearance of blooms of this toxic cyanobacterium. Relatively high concentrations of PCP, above 0.25 mg L^{-1}, resulted in direct toxic effect to *M. aeruginosa*. The quite PCP insensitive *C. vulgaris* will indirectly benefit from this toxicity on the cyanobacterium and a shift on the species dominance may happen. Direct toxicity on the microalga was observed only at very high concentrations (16.7 mg L^{-1}), unlikely to occur in the environment. In the co-cultures, PCP could not be biotically removed but was stabilized instead. Previous findings with single species tests corroborate the PCP toxic effects described herein but could not corroborate the observed stabilization of PCP in the co-cultures experiments. The obtained results demonstrate that short-term laboratory assays with two phytoplankton species give important information on the species interactions, namely the possible direct and indirect effects of a toxicant. Freshwater phytoplankton species interactions are common and possibly crucial in naturally occurring communities and, thus, must be considered in ecotoxicity studies if regarding environmental extrapolations.

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PART IV

CONCLUSIONS

Chapter 10

General conclusions

10.1. Development of the analytical method

10.2. Ecotoxicity studies

10.3. Other achievements

The five objectives of this thesis (Chapter 4) were accomplished. The developed work (Chapters 6, 7, 8 and 9) allows drawing five general conclusions. They are described in two distinct, yet interrelated, parts of this chapter: the analytical method development and the ecotoxicity studies.

10.1. Development of the analytical method

For the first time, a SPME-GC-ECD method for the determination of CPs in water samples (after derivatization of the CPs by acetylation) was developed and it proved to be effective for water sample analysis. Due to the low sample volume required, it could be used in environmental model studies. The simultaneous determination of seven environmentally relevant CPs was possible, in spite of their very diverse properties. This method is accurate and sensitive enough to determine CPs in a large variety of water samples, like culture media from phytoplankton species, river, estuarine and wastewaters. Most of the detected interferences were overcome by carefully selecting the best conditions. However, when a new type of water samples is analysed, it is advisable to confirm the identity of the detected analytes by a more selective technique, like GC-MS.

The CPs measurement technique allowed studying the stability of the analytes for a variety of conditions. The acetylated derivatives were much less stable than the parent CPs. The stability was less dependent on the type of CPs than on other factors, like storage temperature. As a result of this study, it was possible to develop procedures that allowed long-term (about one month) conservation of all studied CPs in different types of water samples. No additional preservatives were needed, which simplified the method and avoided possible contaminations. Due to the low sample volume required, less storage space is necessary. Therefore, the storage procedure described here is a good alternative to the immobilization of CPs on some SPE cartridges. Additionally, its efficiency was proven at very low levels of CPs in the water while the storage method using SPE was checked for very high concentrations of CPs and its practical use to real environmental samples is not yet confirmed.

It can be stated, based on all the previous conclusions, that an integrated cost-effective analytical approach was developed, including water samples' storage and determination of CPs. It is possible to use it for routine water monitoring purposes and

for environmental chemistry research on CPs. Within the aim of this thesis, it was proven that the method worked for phytoplankton culture media and allowed accomplishing the ecotoxicity part of the present work.

10.2. Ecotoxicity studies

This work was the first systematic ecotoxicity study of the effects of PCP in aquatic cyanobacteria. The response of *M. aeruginosa* to PCP was hormetic. At about 1 μg L^{-1} of PCP, there was growth stimulation of the studied cyanobacterium strain, which suggests that possible toxic cyanobacteria blooms might occur in freshwater environments with this level of PCP contamination. Curiously, this is the same value of MAC of PCP in surface waters (1 μg L^{-1}) and such value should be reconsidered. At low concentrations, lower than 1 μg L^{-1}, PCP was slightly toxic to *M. aeruginosa*. At PCP concentrations higher than 1 μg L^{-1}, toxicity occurred and much more pronouncedly than at the low concentrations. The chl_a was a more sensitive endpoint, when compared with optical density, and concentration-dependent of PCP for its high concentrations. The toxicity profile of PCP to *C. vulgaris* was different from that to the cyanobacterium. The growth enhancement could not be proved at any concentration level studied. This microalga was rather insensitive to PCP at concentrations under 1 μg L^{-1} and mild toxic effects appear above this level. The chl_a was not PCP concentration-dependent while optical density was. Comparing with other phytoplankton species, namely other microalgae, PCP toxicity is intermediate for *C. vulgaris*.

Concerning another novelty of this work, the PCP removal by phytoplankton species, interesting conclusions could be drawn. A small part of PCP was removed from the culture medium by *M. aeruginosa*, particularly at concentrations where toxic effects were observed. At these concentrations, the removal in biotic conditions was higher than that in abiotic conditions by a factor of 1.2 to 2.0. Conversely, *C. vulgaris* stabilized PCP in the culture medium. When in mixed cultures with the two species, PCP was stabilized. The PCP removal capability of the cyanobacterium was lost in this case, unexpectedly.

The population dynamics in the mixture of *M. aeruginosa* and *C. vulgaris* was first studied in the absence of PCP. The cyanobacterium inhibited the growth of the microalga while the contrary was not observed. This result is a novelty and, given the small and contradictory amount of data available on this subject of the interspecific

interactions, additional research is needed. From a methodological point of view, it is important to state that, in order to study the toxic effect of a compound in a mixture of species, the biological control should be also a mixed culture and not single species.

Pentachlorophenol was able to change the species' distribution when the two species in mixed cultures were exposed to it. As expected from the single species experiment, PCP had a direct toxic effect on *M. aeruginosa*, noticed at concentrations higher than tens μg L^{-1}. At this high PCP levels, a positive indirect effect of PCP on *C. vulgaris* was noticed, due to the PCP toxicity to the cyanobacterium. However, at the highest concentration tested, it was possible to differentiate also a direct toxic effect of PCP on *C. vulgaris*. At PCP concentrations in the low μg L^{-1} range, there was a growth promotion of *M. aeruginosa*, which is in accordance with single species experiments. Therefore, the results obtained for the mixed species cultures experiments corroborate the possible role of PCP in the appearance of *M. aeruginosa* blooms and the need to reconsider the legal limits of PCP in the surface waters, particularly in water bodies prone to the appearance of cyanobacteria blooms. From the PCP toxicity and removal results obtained, it can be concluded that species interactions might play a decisive role in the environment and the ecotoxicity research shall consider it when regarding the environmental application of the obtained data because single species tests are not enough to derive valid conclusions. The research in the ecotoxicity field must advance to the more complex but realistic mesocosms experiments.

10.3. Other achievements

In addition to the conclusions drawn from the main objectives, secondary achievements could arise. Some results are interesting and, despite not having been further studied, they are useful. For example, the result that CPs were not produced in significant quantity by natural chlorination was important for conducting all ecotoxicity studies described in this thesis. The selection of the proper carrier solvent and the culture media was also very important.

Chapter 11

Future research

From the introduction of the thesis (Part I), it is clear that several questions, in the environmental pollution field, are still waiting for an answer. Some of them were answered in this work and are listed in the objectives (Chapter 4). Some other questions are waiting in line to be solved by research. Furthermore, from the results found and discussed in this thesis, new problems have arisen. One characteristic of the scientific research is the never-ending process of correcting errors and answering new questions after hypothesizing them. The resulting advances open new problems as a challenge for the future investigation. Following this thesis, some perspectives of future work emerge and are here discussed.

11.1. Analytical development

Given the main objectives of this work, most of the perspectives of future research will be related with the ecotoxicity studies. However, concerning possible analytical improvements, it is advisable to develop a similar method for the determination of CPs in other matrices, like environmental or clinical ones, because of its high sensitivity and low sample volume required as already proven for water samples. For example, in the case of sediment samples or biological tissues and fluids, a SPME-GC-ECD method is still waiting to be developed. Despite this perspective for future research is not related with the aim of this thesis, it directly follows the analytical development carried out. The future method could be used to study the adsorption of CPs on solid particles and its accumulation in sediments, for instance.

Furthermore, a similar method to determine CPs in urine samples could be used as a clinical practice in the labour medicine field. It is proven that CPs are biomarkers of exposure to some chlorine-containing organic substances while for others it is just a hypothesis that was never checked [1].

Finally, and establishing a connection to the ecotoxicity studies, the development of a method to determine CPs in phytoplankton biomass will result in a better understanding of the possible toxicants' removal processes by phytoplanktonic species, like those reported in this thesis.

11.2. Ecotoxicity studies

There is an important question that emerged from this thesis: how PCP was removed by single species cultures of *M. aeruginosa* and stabilized by cultures of *C. vulgaris*? To answer this question, a supplementary strategy during the ecotoxicity tests will be required: to examine the role of the exudates [2, 3] for the removal or stabilization of PCP, as well as the relative significance of physical sorption or chemical transformation for the biotic removal. With these additional efforts, another question will immediately arise and may even justify a deeper reflection: what implications will these results have in the possible prediction of the role the phytoplankton on the fate of PCP in the freshwater environment? The PCP removal by *M. aeruginosa* is a very interesting result that deserves further research.

The research can be expanded indefinitely with the use of other phytoplankton species, and other mixtures of species, and other CPs, and mixtures of CPs. Notwithstanding the huge number of possible combinations for similar ecotoxicity studies, the analytical method for the determination of CPs in water is already developed (Chapter 6), namely for other six environmentally-relevant CPs, other than PCP. Similarly, the phytoplankton species to be chosen must have relevance in the environment and the selected mixture of species must include species that actually co-occur in the environment. Likewise, the toxic effect of a mixture of compounds is more suited to real environmental cases, where substances with different toxicity coexist. Interesting results will be expected because, for instance, the CPs might have different mechanisms of toxicity to the studied species.

The interactions between phytoplanktonic species are diverse and research is needed, as discussed in Chapter 9. Some results for the same species are even contradictory. The single species tests with toxic compounds are justified but such conditions are unlikely in the environment and are not enough to derive conclusions applicable to the environment. The study of the exudates, again, released by competing species, deserves special attention by researchers. How these exudates can influence the environmental communities? Will they lead to changes in the communities' structure? And how will a toxicant exert its toxicity when such interspecific interactions occur? Probably, each case must be studied separately, as conclusions are difficult to draw, how it is suggested in Chapter 9.

The environmental significance of the ecotoxicity studies, and its complexity, can be increased even further by studying other species, apart from phytoplankton,

situated in higher levels of the food chain, for instance grazers like the cladoceran *Daphnia magna*. The presence of a sediment phase can be also important. Such mesocosms studies can be based on the results of this thesis but methods for determination of CPs in the sediment samples and in biological tissues will be also necessary, as already proposed in this chapter. Apart from observing direct and indirect effects on the species, it will be interesting to know the role of different biotic and abiotic compartments in the removal of the toxic substances. With the higher complexity of a mesocosms, other questions arise: how PCP is transferred through the food chain? Will it bioaccumulate? Will PCP suffer biodegradation? Will the growth promoting effect of PCP on *M. aeruginosa* (Chapters 8 and 9) be observed also in these more environmentally realistic cases? Will PCP actually lead to a *M. aeruginosa* bloom? In this event, how environmental legislation should be changed? And what other questions will follow? There is always space for investigation if the scientists are ready to embrace complexity.

It is difficult to predict how many more questions will be answered, and how many new problems will be disclosed for further investigation, if someone decides to step upon this work for further expansion towards the understanding of the interaction between CPs and the freshwater environment. The future work will, undoubtedly, be more in line with the global view of our planet, in the sense described in the background of this thesis. I will be very happy to see that my work will be used to contribute in attaining the dream of a clean planet.

11.3. References

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