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Phytoremediation of phenolic pollutants in natural and constructed wetlands

Inês Paes de Faria Monteiro Montenegro

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Inês Paes de Faria Monteiro Montenegro

Tese de Doutoramento apresentada à
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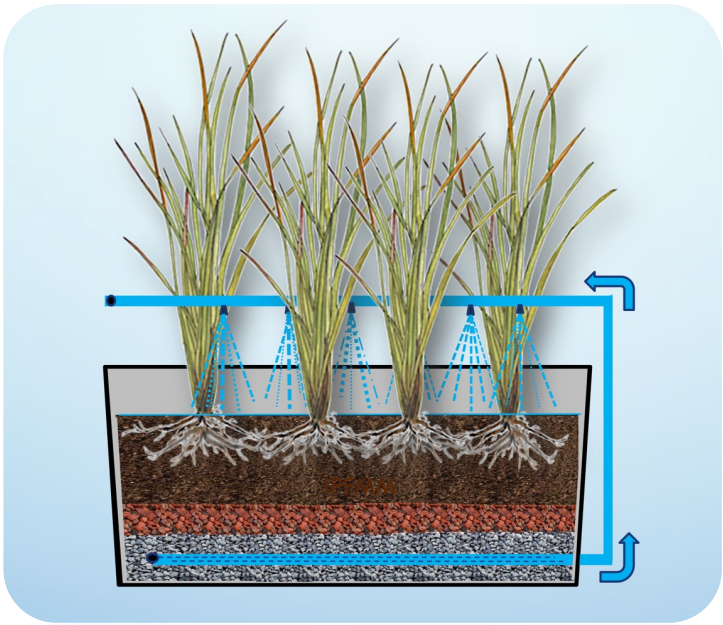

Phytoremediation of phenolic pollutants in natural and constructed wetlands

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2022

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Think about it:

“Your degree is just a piece of paper, your education is seen in your behavior”

Unknown

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Resumo

As zonas húmidas podem atuar como filtros naturais na purificação da água, sendo as plantas destas zonas, os solos e as comunidades microbianas, os principais intervenientes nestes processos naturais. No caso dos sapais estuarinos, vários processos de remoção de contaminantes têm sido observados (por exemplo, compostos fenólicos; degradação pela comunidade microbiana da rizosfera de plantas de sapal), no entanto, estas zonas húmidas naturais são caracterizadas por uma extrema variabilidade nos componentes funcionais, dificultando a previsão de respostas à purificação da água.

Ao contrário das zonas húmidas naturais, em que a purificação da água é promovida pela regulamentação ambiental, as zonas húmidas construídas (ZHC) são arquitetadas para promoverem o eficiente tratamento de águas contaminadas através de monitorização e controlo humano, atenuando os efeitos de variabilidade e potenciando processos biológicos, físicos e químicos.

Assim, as ZHC podem ser uma opção adequada para remover contaminantes de diferentes águas, resultante de complexas interações de fenómenos naturais envolvendo diversos processos físicos, químicos e biológicos, que constituem a fitorremediação. Abordagens de fitorremediação, envolvendo plantas e respetivas comunidades microbianas da rizosfera, têm sido exploradas como uma alternativa para despoluir e recuperar ambientes contaminados, nomeadamente águas e solos. No entanto, a contaminação de água / solo pode influenciar os processos de fitorremediação, pois os poluentes podem interagir com as plantas e a respetiva rizosfera.

Este estudo teve como objetivos:

a) investigar a presença de dois grupos diferentes de compostos fenólicos, nomeadamente alquilfenóis (nonilfenol - NP e octilfenol - OP) e clorofenóis (pentaclorofenol - PCP), no desempenho das ZHC para a remoção da matéria orgânica e de nutrientes (iões amônia, nitrito, nitrato e fosfato);

b) investigar a capacidade dos ecossistemas baseados em ZHC para a remoção, de dois grupos diferentes de compostos fenólicos, de água de uma ribeira urbana contaminada. Para tal, foi avaliada a resposta da comunidade microbiana presente no

substrato das ZHC, otimizando o conhecimento sobre os processos que ocorrem e promovendo o bom funcionamento desses sistemas verdes.

Phragmites australis, sedimento em contacto com raízes de plantas e areia foram recolhidos no estuário do Rio Lima e utilizados na montagem dos sistemas baseados em ZHC. Seis sistemas, cada um com cerca de 40 plantas e três camadas (cascalho, rocha lávica e sedimento / areia) foram montados em escala laboratorial simulando uma configuração de fluxo vertical (FV; Vertical Flow Constructed Wetland - VFCW). Os sistemas operaram com um circuito de recirculação automática de água (caudal ca. 10 mL min^{-1}), com um período de recirculação (12 h) seguido de um período de não recirculação (12 h).

A água, com contaminação de origem antropogénica, da ribeira urbana, dopada com diferentes compostos fenólicos, uma mistura de NP e OP (na concentração de 100 µg L^{-1} cada) ou PCP (na concentração de 100 µg L^{-1}), foi tratada em sistemas ZHC, em escala laboratorial, em condições controladas, por oito semanas, durante ciclos de uma semana.

A presença dos compostos fenólicos não interferiu com o desempenho das ZHC, na remoção da matéria orgânica, nem com o processo de nitrificação. A presença de PCP promoveu a remoção do ião nitrito e a remoção do anião fosfato foi promovida pela presença do NP e OP.

As taxas de remoção de NP e OP foram > 99 %, enquanto, que as de PCP variaram entre 87 % e 98 %. A elevada taxa de remoção de NP e OP poderá estar associada ao facto, do sedimento já ter estado exposto a um dos contaminantes, concretamente o OP, ou seja, poderemos estar perante um *efeito de memória* da comunidade microbiana, promovendo os processos de degradação. Adicionalmente, o processo de degradação do NP poderá ter sido influenciado por esta situação. Relativamente ao PCP, devido à baixa concentração detetada no sedimento, a remoção poderá estar associada principalmente a processos de biodegradação.

Em geral, os índices de *riqueza*, *diversidade* e *dominância* da comunidade microbiana presente no substrato da rizosfera da *P. australis*, não foram afetados pela presença do NP e OP. Este resultado e também a elevada percentagem de remoção de NP e OP, poderão estar associados ao facto de que a comunidade microbiana foi previamente exposta ao OP. Na presença do PCP verificou-se que a comunidade microbiana perdeu *diversidade* aumentando o número de espécies *dominantes*.

Observou-se também, que na presença dos dois grupos de compostos fenólicos, vários géneros *especialistas* foram enriquecidos após serem expostos à água da ribeira urbana dopada. Os três géneros *especialistas* mais abundantes foram *Methylotenera* e *Methylophilus* (família Methylophilaceae) e *Hyphomicrobium* (família Hyphomicrobiaceae) nos sistemas ZHC (CW) tratados com água da ribeira urbana dopada com uma mistura de NP e OP. Nos sistemas ZHC com água da ribeira urbana dopada com PCP, os três géneros mais abundantes foram *Denitromonas* (família Rhodocyclaceae), *Xenococcus_PCC_7305* (família Xenococcaceae) e *Rhodocyclaceae*_uncultured (família Rhodocyclaceae).

Estimular (adição de mais nutrientes) ou aumentar (o chamado bioaumento) a presença destas bactérias em sistemas ZHC, pode ser uma opção adequada para aumentar a eficiência dos mesmos, na eliminação de compostos fenólicos de águas contaminadas, nomeadamente com o composto PCP que não foi totalmente removido. De facto, para o PCP é necessária mais investigação, que também deve incluir processos de adsorção ao substrato dos sistemas ZHC.

O presente estudo contribui para aumentar o conhecimento sobre o desempenho dos sistemas ZHC, e promover o uso destas, como uma solução baseada na natureza para o tratamento de águas contaminadas com compostos fenólicos de diferentes famílias, uma questão relevante considerando o impacto negativo destes contaminantes no meio ambiente. Os sistemas ZHC, podem contribuir assim, para eliminar ou reduzir esse impacto, protegendo os ecossistemas.

Abstract

Wetlands can act as natural filters in water purification, being wetland plants, soils and microbial communities the main actors in these natural processes. In the case of estuarine salt marshes, several processes of contaminants removal have been observed (e.g. phenolic compounds; degradation by rhizosphere microbial community *of* salt marsh plants), however, natural wetlands are characterized by extreme variability in functional components, making difficult to predict responses to water purification.

Unlike natural wetlands, where water purification is promoted by environmental regulation, Constructed Wetlands (CW) are architected to promote efficient treatment of contaminated waters by monitoring and human control, mitigating the effects of variability and potential biological, physical and chemical processes.

In this way, CW can be a suitable option to remove contaminants from different waters / wastewaters, relying on several physical, chemical and biological processes, including phytoremediation. Phytoremediation approaches involving plants and respective microbial communities of the rhizosphere have been explored as an alternative to clean and recover impacted environment, namely contaminated waters and soils. However, contamination in water / soil might influence phytoremediation processes because the pollutants can interact with plants and the respective rhizosphere.

This study aimed to:

a) to investigate the presence of two different groups of phenolic compounds, namely alkylphenols (nonylphenol - NP and octylphenol - OP) and chlorophenols (pentachlorophenol - PCP), in the performance of CW for the removal of organic matter and nutrients (ammonia, nitrite, nitrate and phosphate ions);

b) investigate the capacity of ecosystems based on CW to remove two different groups of phenolic compounds from a contaminated urban stream water. To this end, the response of the microbial community present in the substrate of CW was evaluated, optimizing the knowledge about the processes that occur and promoting the proper functioning of these green systems.

Phragmites australis, sediment in contact with plant roots and sand were collected in the *Lima River* estuary and used in assembling CW systems. Six CW microcosms, each with ca. 40 plants and three layers (gravel, lava rock and sediment / sand) were assembled at laboratory scale simulating a vertical flow configuration (VFCW). Systems operated with an automatically recirculating water system (flow rate ca. 10 mL min^{-1}) with a recirculation period of 12 h followed by a 12 h non - recirculation period.

Water from an anthropogenic impacted urban stream doped with different phenolic compounds, either a mixture of NP and OP (at a 100 µg L^{-1} concentration each) or PCP (at a 100 µg L^{-1} concentration), was treated in CW at lab scale, in controlled conditions, for eight weeks, during one - week cycles.

The presence of phenolic compounds did not interfere with the performance of the CW in the removal of organic matter or with the nitrification process. The presence of PCP has promoted the denitrification process and the presence of the NP and OP promoted phosphate anion removal.

The removal rates of NP and OP were > 99 %, whereas PCP removal ranged between 87 % and 98 %. The high rate of removal of NP and OP could be linked to the fact that the sediment has already been exposed to one of the contaminants, in particular OP, i.e., we may be facing a *memory effect* of the microbial community that improved the degradation process. In addition, the degradation process of NP could have been influenced by this situation. For the PCP due to the low concentration detected in the sediment, removal may be mainly associated with biodegradation processes.

In general, the indices of *richness, diversity* and *dominance* of the microbial community present in *P. australis* 's rhizosphere substrate have not been affected by the presence of NP and OP. This result and the high removal percentage of NP and OP might be related to the fact that the microbial community has been exposed to OP, and we may be faced with a *memory effect*. In the presence of the PCP it was found that the microbial community lost *diversity* by increasing the number of *dominant* species.

It was also observed that in the presence of the two groups of phenolic compounds, several specialist genera were enriched after being exposed to doped urban stream water. The three more abundant specialist genera were *Methylotenera* and *Methylophilus* (Methylophilaceae family) and *Hyphomicrobium* (Hyphomicrobiaceae family) in CW systems treating water doped with a mixture of NP and OP. In CW systems treating water doped with

PCP, the three more abundant genera were *Denitromonas* (*Rhodocyclaceae* family), *Xenococcus*_PCC_7305 (Xenococcaceae family) and *Rhodocyclaceae*_uncultured (Rhodocyclaceae family).

Stimulating (adding more nutrients) or increasing (the so called bioaugmentation) the presence of these bacteria in CW systems could be a suitable option to increase CW efficiency for eliminating phenolic compounds from contaminated waters / wastewaters, namely PCP which was not totally removed. In fact, for PCP further research is needed which should also include adsorption processes to CW substrate.

The present study contributes to increase the knowledge regarding CW performance and to promote the use of this nature - based solution for the treatment of waters contaminated with different families of phenolic contaminants, a relevant issue taken in consideration the negative impact of these contaminants to the environment. CW can contribute to eliminate or reduce that impact, protecting ecosystems.

Keywords: Natural Wetlands, Constructed Wetlands, Phenolic Compounds, Nonylphenol, Octylphenol, Pentachlorophenol, *Phragmites australis,* Rhyzosphere Microbial community

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Abbreviations

Abund. - Abundance

ALMWOAs - Aliphatic Low Molecular Weight Organic Acids

ANAMOX - Anaerobic ammonium oxidation

ANOVA - Parametric one - way analysis of variance

ANR - Assimilative Nitrate Reduction

AP - Alkylphenols

APCI - Atmospheric Pressure Chemical Ionization

APEC - Alkylphenoxy Carboxylic Acid

AP_2EC - ((4 - Alkyphenoxy) Ethoxy) Acetic Acid

AP_1EC - (4 - Alkylphenoxy) Acetic Acid

ApnEOs - Alkylphenol Polyethoxylates

AP_2EO - 4 - Alkylphenol Diethoxylate

AP_1EO - 4 - Alkylphenol Monoethoxylate

ASE - Accelerated Solvent Extraction

BaμE - Bar adsorption microextraction

Benzoyl CoA - Benzoly Coenzyme A

BOD_5 - Biological Oxygen Demand

BPA - Bisphenol - A

Ca. - Close value

CAS - No. - Chemical Abstracts Service - Number

CLAM - Multinominal Species Classification Method

COD - Chemical Oxygen Demand

CW - Constructed Wetland

C_{18} - Reversed Phase C18 SPE Cartridge

DAD - Diode Array Detection

DBNSA - Dapeng Bay National Scenic Area

Dehydrogenase Activity - DHA

DNA - Deoxyribonucleic Acid

DNF - Denitrification

DNRA - Dissimilatory Nitrate Reduction to Ammonium

DO - Dissolved Oxygen

E.C. - European Community

EDC - Endocrine Disrupting Compounds

EEC - European Economic Community

e.g. - "*exempli gratia"* a Latin phrase that means "for example"

EI - Electronic Ionization

(ENV+) - Polystyrene copolymer phase

E.Q.S. - Environmental Quality Standards

Erα - Estrogen Receptors α

Erβ - Estrogen Receptors β

E.U. - European Union

FCW - Floating Constructed Wetland

FLD - Fluorescence detector

FWSCW - Free Water Surface Flow Constructed Wetland

GC - Gas Chromatography

GC - MS - Gas Chromatography - Mass Spectrometer

HCW - Hybrid Constructed Wetland

HF - Horizontal Flow

HFCW - Horizontal Surface Flow Constructed Wetland

HF - Horizontal Flow

HPLC - High Performance Liquid Chromatography

HQ - Hydroquinone

HRT - Hydraulic Residence Time

HSSF - Horizontal Subsurface Flow

HSSF - Horizontal Sub - Surface Flow

HS - SPME - Headspace - Solid Phase Microextraction

i.e. - Abbreviation for "*id est"* a Latin phrase that means "that is"

IPCS - International Program on Chemical Safety

$K_{p(sw)}$ - Sediment - water partitioning

LOD - Limit of Detection

Log K_{ow} - Log (octanol / water Partition coefficient)

Log K_{oc} - Log (organic carbon Partition coefficient)

LOQ - Limit of Quantification

MAE - Microwave Assisted Extraction

MDS - Multdimensional Scaling

MEA - Millennium Ecosystem Assessment committee

MS - Mass Spectrometry

MSD - Mass Selective Detector

MW - Molecular Weight

N.A. - No Relative

N.D. - Not Detected

NMDS - Non - metric Multdimensional Scaling

NP - Nonylphenol

NP_OP - Both nonylphenol and octylphenol are present

NPEO - Nonylphenol Ethoxylate

NP_1EO - Nonylphenol Monoethoxylates

NP_2EO - Nonylphenol Diethoxylates

OM - Organic Matter

OP - Octylphenol

OPEO - Octylphenol Ethoxylate

OTU - Operational Taxonomic Unit

PAH - Polycyclic Aromatic Hydrocarbon

PAO - Phosphorus Accumulating Organisms

PCA - Pentachloroanisole

PCB - Polychlorinated Biphenyl

PCP - Pentachlorophenol

PCR - Polymerase Chain Reaction

PcpB - PCP - 4 - monooxygenase

PcpC - Tetrachloro - p - hydroquinone - reductive dehalogenase

PcpA - 2,6 - dichlorohydroquinone - 1,2 - dioxygenase

PE - Person Equivalent

PEP - Phosphoenolpyruvate carboxylase

PFASs - Per- and polyfluoroalkyl

PFTBA - Perfluorotributylamine

pH - Potential of Hydrogen

PHBV - Poly - 3 - hydro - xybutyrate - co - 3 - hydroxyvalerate

pK_a - Acid dissociation constant

PLA - Polyacetic acid

PLE - Pressurized Liquid Extraction

PTBS - Propylene Tetramer Benzene Sulphonate

P. australis - *Phragmites australis*

POP - Persistent Organic Pollutant

RHDα - Ring - Hydroxylating - Dioxygenase

RNA - Ribonucleic Acid

RSD - Relative Standard Deviation

RuBisCO - Ribulose - 1,5 - bisphosphate carboxylase oxygenase

SILVAngs - SILVA next - generation sequencing

SFCW - Surface Flow Constructed Wetlands

SIS - Selected - Ion - Storage

SIM - Selected Ion Monitoring

S_o - Octanol solubility

sp. - Species

spp. - Several species

SPE - Solid Phase Extraction

SPME - Solid Phase Microextraction

SS - Suspended Solid

SSFCW - Subsurface Flow Constructed Wetlands

SSF - Subsurface Flow

S_w - Aqueous solubility

TOC - Total Organic Carbon

TSS - Total Suspended Solids

U.S. - United States

USEPA - United States Environmental Protection Agency

UV / VIS - Ultraviolet Visible

VF - Vertical Flow

VFCW - Vertical Flow Constructed Wetland

WHO - World Health Organization

WPF - Willow Planted Filter

WWTP - Wastewater Treatment Plants

ZHC - Zonas Húmidas Construídas

ZVI - Zero Valent Iron

4-CP - 4 - Chlorophenol

16s rRNA - 16s ribosomal RNA

1. Introduction

1.1. Phenolic compounds

Chemical compounds, like phenols compounds, are essential part of daily life of modern societies and in many economic activities, they provide a wide range of benefits, e.g. increased productivity and improvements in the control of disease [29].

Phenols compounds of anthropogenic origin exist in the environment due to the activity of the chemical, petrochemical, textile, polymers, pulp and paper mill, and pharmaceutical industries [30–32].These compounds enter into the aquatic ecosystems as a consequence of the drainage of municipal or industrial effluents, and also by direct application of pesticides in particular phenoxyherbicides like 2.4 - dichlorophenoxyacetic acid or 4 - chloro - 2 - methylphenolyacetic acid and also phenolic biocides like pentachlorophenol (PCP), dinoseb or diarylether pesticides [30].

Phenolic compounds are a very heterogeneous group of compounds with varying chemical and physical properties, being classified based on different kinds of substituents present on the aromatic ring of the phenolic moiety [33]. Specifically, this group includes alkyl-, chloro-, hydroxyl-, and nitro- phenolic compounds [33]. The presence of these substituents has effects in the compound physicochemical characteristics and toxicity as well as in its environmental behaviour. The possibility to form hydrogen bonds suggests that phenols compounds should have good water solubility, however this is not the rule [34]. The unsubstituted phenol is relatively well soluble in water (83 g L^{-1}), whereas substituted derivatives are not so soluble in water, solubility not exceeding 30 g L^{-1} for most of them [34].

Toxicity of phenolic compounds

The toxicity of phenolic compounds is associated with two main processes - unspecified toxicity related with hydrophobicity of the individual compounds and formation of free radicals [35].

The hydrophobicity affects the solubility and consequently the interaction of these compounds with specified cell and tissue structures [35]. For instance, the presence of alkyl and halogen groups enhance the hydrophobic character of the aromatic ring resulting in decreasing water solubility. Additionally, for phenolic compounds possessing functional groups with strong polar characteristics, difference in capacity to dissolve are more pronounced, even for isomers of the same compounds, there can be significant differences

[34]. For instance, in case of chlorophenol the hydrophobicity increases with the number of chlorine atoms that enhances toxicity of the individual compound [36]. According to Michalowicz and Duda [30] the degree of toxicity is also associated with the localization of the substituted group in the phenol ring. The proximity of the substituted group with the -OH group leads to formation of intramolecular hydrogen bonds and the -OH group becomes less active in the solvation process [34]. Due to this fact, the solubility of ortho is lower than meta or para isomers and consequently the toxicity rate significantly increases [34]. For instance, a chlorine atom substituted in ortho position in phenol ring decreases its toxicity in cell and tissue structures and meta substitution increases toxic action of the compound [35].

The formation of free radicals is another toxic effect for cell and tissue. The cell activates the detoxification process when the phenolic compounds penetrated into the cell, but sometimes this process leads to increase toxicity of individual compounds by the formation of electrophilic metabolites that may bind and damage DNA or enzymes [30].

Phenolic compound and endocrine disrupting effect

One of the main problems of phenolic compounds is their endocrine disrupting effect.

The most widely accepted definition of endocrine disruptor is the one by the International Program on Chemical Safety (IPCS) of the World Health Organization (WHO): “Endocrine disruptors have been defined as exogenous substances that alter function(s) of the endocrine system and consequently cause adverse (health) effects in an intact organism or its progeny (or (sub)populations)” [37].

How these exogenous compounds disrupt hormonal regulation? The endocrine system utilizes hormones secreted by the specific hormonal glands directly into the bloodstream and the secreted hormones are in part bound to serum proteins and transported to the target organ or cell [38]. The response of the cell to the active hormone is dependent for the most part on the presence of specific hormone receptors [38]. The affinity and specificity of the respective receptor to the external signal (hormone) determines whether the particular hormone is bound by the receptor [39]. The receptor with its bound ligand, i.e., the hormone, transmits the signal resulting in a specific biological response. In the case of steroid hormones the main cellular response is an activation of the gene transcription (genomic effects), although “non-genomic”, rapidly occurring effects have also been discussed [39].

Studies have shown that phenolic compounds, such as alkylphenols (NP and OP) [1] and chlorophenols (PCP) [40] have endocrine disruption activity in living organisms, with the most emphasized being the binding to the estrogen receptors α (ERα) and estrogen receptors β (ERβ) and acting competitively towards natural hormones (e.g. 17β – estradiol). The affinity

of these phenolic compounds towards the receptors are much weaker (weak estrogen activity) than the affinity naturally induced from natural hormones of a living organism [40, 41]. These endocrine disrupters also can bind to ERα or ERβ and subsequently alter the normal expression of estrogen-responsive genes [42–44].

The estrogen receptors ERα and ERβ distribution in human body and abundance are represented in Fig. 1.1. These estrogen receptors have an important role in reproductive system of living organisms. For instance, studies done with knock-out mice for estrogen receptors (α and β) indicate hypotrophy of the uterus, infertility and rudimentary mammary gland development, etc. [1].

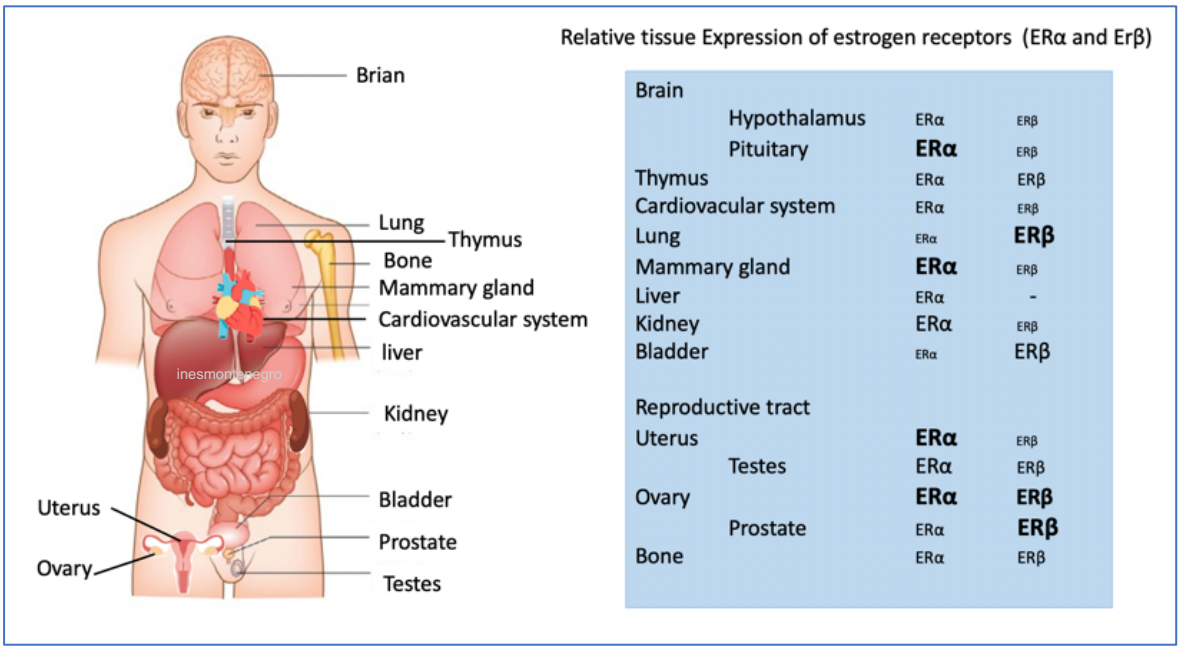


Fig. 1.1 - Summary of relative expression of ERα and ERβ in each human tissue (adapted from Mueller et al. [1]).

The endocrine disrupting effect induced by phenolic compounds such as alkylphenols (NP and OP) and chlorophenols (PCP) and their derivatives in humans and animals has been substantially investigated and motivated regulatory actions from governments.

So, along with the need to prevent inputs of these compounds into the environment, there is the need for methodologies to recover affected ecosystems.

1.1.1. Alkylphenols

Alkylphenols (AP) include many compounds, ranging from cresols to dodecylphenol [4]. AP consist of a phenol ring, which is mono or poly-substituted by alkyl chains with different length [4]. The dominant group of alkylphenols is the para– or 4 - isomers, this means that the hydroxylgroup is situated on the phenol ring opposite to the alkyl chain. AP and their

polyethoxylated derivatives are used directly as intermediates or as additives (emulsifiers, detergents and flotation and dispersing agents) for a wide range of industrial products and processes [45–47]. For instance, nonylphenol (NP) and octylphenol (OP), detected in aquatic environments, are the degradation products of alkylphenol ethoxylates (APnEOs, n is the number of ethoxylate units), specifically NPEOs (nonylphenol ethoxylates) and OPEOs (octylphenol ethoxylates). These APnEOs, due to the fact that are highly cost - effective surfactants with exceptional performance, are one of the most widely used classes of nonionic surfactants in industrial, commercial and household applications such as detergents, emulsifiers, dispersing agents, antistatic agents, demulsifiers, and solubilisers [48–55].

For many decades, these APnEOs, have been widely employed in formulation of a large variety of compounds and have been applied in many industrial sectors: textile industry, leather industry, plastics industry, agrochemical industry, pharmaceuticals and cosmetics industry, as well as in pulp and paper industry [56–58]. However, few industrial groups are responsible for the biggest part of the flows to wastewater of APnEOs and degradation products reported in wastewater treatment plants effluents (WWTPs) and also in fresh water [41, 59–62]. The textiles and leathers groups [62–65] and pulp and paper mill effluents [66, 67] are the major sources of alkylphenols and APEO in wastewater [62]. Personal care products, paint and lacquers are also importance, even if their contributions are smaller [62].

In the case of textile and leathers industries, APnEOs (mainly NPEOs and OPEOs) are applied in pretreatment operations or in additives as detergents or wetting agents in wool scouring, hydrogen peroxide bleaching and dyeing processes [68–72]. APnEOs were detected in products that are used in consumer goods that are bought and used every day, like clothes, bags and shoes made of leather or textile [68]. In the case of the paper industry, NPEO surfactant is commonly found in pulp and paper mills wastewater effluents and in river near paper mill industry [73–76]. In the paper industry, NPEO surfactants are used for de-linking in the production of recycled paper [74]. The de-linking solution is typically an aqueous blend of NPEO and linear alcohol ethoxylates mixed in a ratio of up to 5 % surfactant by weight of the paper to be de-inked [74].

Another source of APnEOs is municipal wastewater of large urban areas because the majority of household detergents and cleaners contain these nonionic surfactants in its formulation [62]. For instance, Cheng and Ding [77] detected NPnEOs in 41 % to 90 % household detergents at concentrations from 0.2 to 21 %. Månsson et al. [62] highlights washes of textile in households' washing machines and the release of APnEOs used in manufacturing process as sources. Probably a majority of APnEOs are released during the first washes since APnEOs are non-ionic surfactants that are readily water-soluble and not all

textiles are washed frequently (i.e. curtains, blankets, carpets) but are often washed sometimes over their lifetime [62].

Until 1960 the principal surfactant used in formulation of detergents was propylene tetramer benzene sulphonate (PTBS), however, due to the branched alkyl chain of PT benzene this compound become resistant to bacterial degradation and appear in sewage treatment discharges and foaming arose on rivers [78]. The prohibition of this non-biodegradable surfactant forced the switch to more biodegradable straight chain alkyl surfactants, such as APnEOs [78].

During the secondary wastewater treatment [79] the APEOs are biodegraded by a stepwise shortening of the ethoxylate chains, creating a complex mix of compounds including shorter chain ethoxylates, alkylphenoxy carboxylic acids (APECs), and alkylphenols (APs), such as OP and NP (Fig. 1.2) [2, 80].

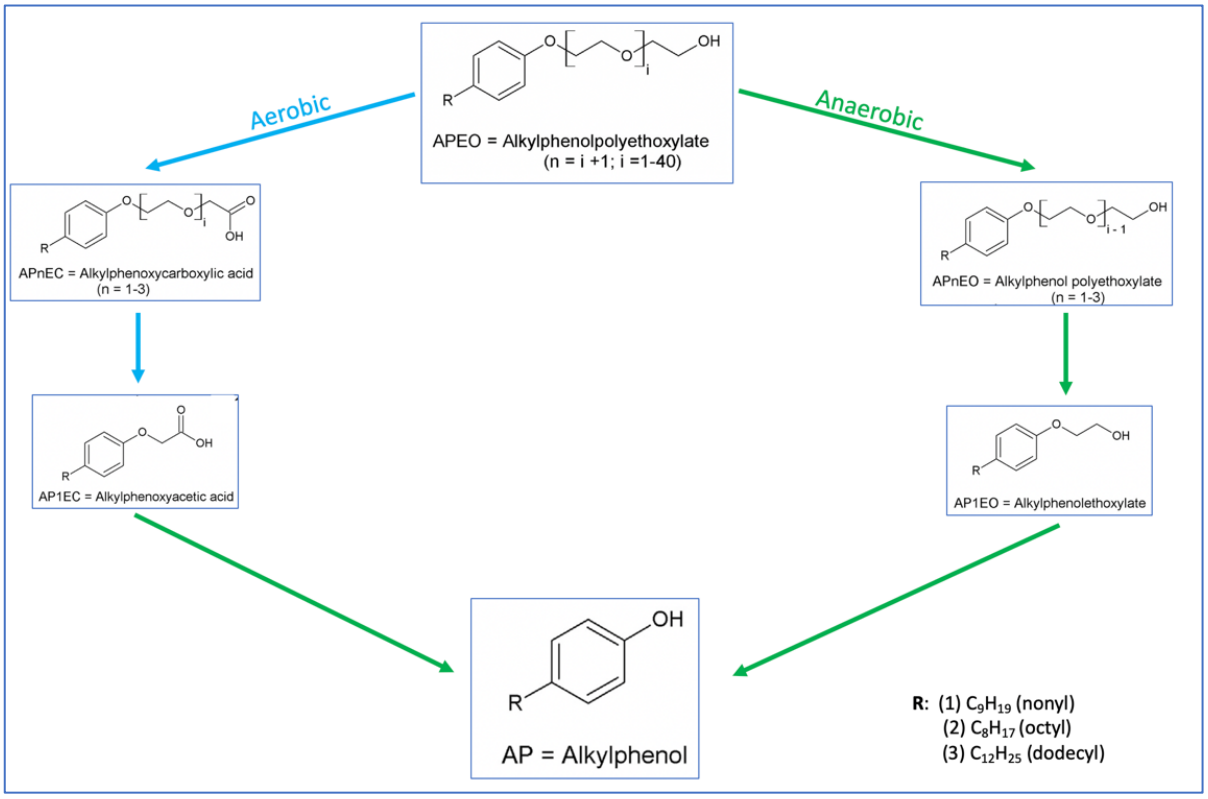


Fig. 1.2 - Breakdown processes of long-chain alkylphenol polyethoxylates (APEOs) under aerobic and anaerobic conditions, and the formation of their metabolites. The group of metabolites comprises: 4-alkylphenol diethoxylate (AP_2EO), 4-alkylphenol monoethoxylate (AP_1EO), ((4-alkyphenoxy) ethoxy) acetic acid (AP_2EC), (4-alkylphenoxy) acetic acid (AP_1EC), and 4-alkylphenols. Source: adapted from Acir et al. [2].

The metabolites of APEOs, such as NP and OP, are less biodegradable than their precursors, less water soluble, more lipophilic, resistant to further degradation, more toxic, and exhibit estrogenic properties [81]. OP and NP occur mainly in the environment as incomplete microbial transformation products of nonyl and octylphenol polytoxylates [82]. Data

from environmental monitoring indicate the presence of NP and OP in water and sediments of aquatic environments in many countries (Table 1.1). Generally, NP and OP occurs in the aquatic environment with concentration varying widely in surface water from tens of ng L^{-1} [83, 84] to dozens of mg L^{-1} [85]. Usually, NP has been found at higher concentration than OP, because octylphenol polyethoxylates are less common than other alkylphenol polyethoxylates such as nonylphenol polyethoxylates in industrial and domestic use [86].

Table 1.1 - Levels of nonylphenol (NP) and octylphenol (OP) in water and sediments collected in different countries.

Sampling area	Compounds	Concentration		References
		Water (µg L^{-1})	Sediment (µg g^{-1} dw)	
***Ria de Aveiro* – Portugal**	4-t-OP 4-NP	0.001 0.0015 - 0.035	a)	Jonkers et al. [87]
***Lima River* Estuary- Portugal**	4-NP 4-n-NP 4-n-OP 4-t-OP	0.006 - 0.65 0.009 - 0.028 0.006 - 0.047 0.008 - 0.105	a)	Rocha et al. [88]
***Leça River* - Portugal**	4-NP 4-n-OP 4-t-OP	n.d. – 0.0013 n.d. – 0.0054 0.0052 – 0.0083	a)	Rocha et al. [89]
***Ave River* – Portugal**	4-NP 4-n-NP 4-n-OP 4-t-OP	0.059 – 0.155 0.0008 – 0.0168 0.0006 – 0.0044 0.0034 – 0.0254	a)	Rocha et al. [90]
***Douro River* Estuary - Portugal**	4-NP 4-n-NP 4-n-OP 4-t-OP	0.0882 – 0.1701 0.0033 – 0.1157 < 0.0116 0.0051 – 0.0306	a)	Rocha et al. [91]
***River Sado* - Portugal**	4-NP 4-n-NP 4-n-OP 4-t-OP	0.1292 – 0.2399 0.0026 – 0.0273 0.0028 – 0.0260 0.0114 – 0.0220	a)	Rocha et al. [92]
***Ria Formosa* - Portugal**	4-NP 4-n-NP 4-n-OP 4-t-OP	0.0122 – 0.5470 0.0034 – 0.0146 0.0005 – 0.0085 0.0043 – 0.0410	a)	Rocha et al. [93]
Seine Estuary – France	4-NP	0.015 - 0.386	a)	Cailleaud et al. [84]
Elbe estuary – Germany	4-NP	< 0.03	a)	Bester et al. [94]
Rhine estuary – Holland	NP	0.031 - 0.150	0.002 - 0.092	Jonkers et al. [95]
Scheldt estuary – Holland	NP	0.035 - 0.901	< 0.0004 - 1.08	Jonkers et al. [95]

(continued)

Table 1.1 (continued)

Ria de Arousa Estuary - Spain	NP 4-n-NP 4-OP 4-t-OP	0.037 - 0.240 < 0.005 - 0.013 < 0.006 - 0.009 < 0.008 - 0.072	a)	Salgueiro - González et al. [96]
Ria de Vigo – Spain	NP 4-n-NP 4-OP 4-t-OP	< 0.030 - 0.337 < 0.005 - 0.016 < 0.006 < 0.008 - 0.010	a)	
Ria de Pontovedra - Spain	NP 4-n-NP 4-OP 4-t-OP	< 0.030 - 0.054 < 0.005 < 0.006 < 0.008	a)	
Ria de A Coruña - Spain	NP 4-n-NP 4-OP 4-t-OP	< 0.030 < 0.005 < 0.006 < 0.008	a)	
Bay of Biscay Santader - Spain	NP 4-n-NP 4-OP 4-t-OP	< 0.030 < 0.005 < 0.006 < 0.008	a)	
Tees estuary (Redcar Jetty) – UK	4-NP 4-OP	5.2 13.0	a)	Blackburn and Waldock [97]
Mersey estuary (Eastham Lock) - UK	4-NP	0.32	a)	Blackburn and Waldock [97]
Tyne estuary - UK	NP OP	a)	0.03 - 0.08 0.002 - 0.02	Lye et al. [98]
Savannah River estuary – USA	NP OP	a)	< 0.0003 - 0.0290 < 0.0003 - 0.0780	Senthil Kumar et al. [99]
Savannah River estuary – USA	NP OP	a)	< 0.0003 - 0.0290 < 0.0003 - 0.0780	Senthil Kumar et al. [99]
Lingding Bay – China	NP OP	0.030 - < 0.02 < 0.02	0.571 - 0.060 0.018 - 0.001	Cheng et al. [100]
Pearl River Estuary – China	4-NP 4-t-OP	0.230 - 3.35 0.0012 - 0.0040	0.0076 - 0.0210 < 0.00017	Diao et al. [101]
Daliao River estuary – China	NP	0.084 - 0.777	0.0015 - 0.4560	Li et al. [102]

a) not sampled

1.1.1.1. Alkylphenols legislation and restrictions

Since the 1980s, the alkylphenols NP and OP have been of environmental concern because of their toxicity for aquatic organisms due to their estrogenic properties and widespread contamination [103].

OP and NP were extensively used until recently in the European Union (E.U.) and in the United States (U.S.) as intermediates in the production of phenolic resins and alkyl phenol ethoxylates (non-ionic detergents) [104, 105]. Biodegradation of alkyl phenol ethoxylates was proven to be an important source of NP and OP environmental contamination [106].

Due to the endocrine disrupting effect on the growth and development of humans and animals, the E.U. started to take action against APnEOs and their degradation products, in particular NP and OP [107]. The NP and OP have been listed as priority hazardous substances in the field of water policy by the European Community Water Framework Directive 2000 / 60 / EC [108] and the final European Union decision No.2455 / 2001 / EC [109]. Marketing and use restriction of NP / NPEOs have been put into place under Directive 2003 / 53 / EC (Restrictions Directive) [107]. This “Restrictions Directive” prohibits the use of NP and NPEOs as a substance or constituent of preparations in concentrations equal or higher than 0.1% by mass for domestic cleaning, cosmetic products, co - formulants in pesticides and biocides, manufacturing of pulp and paper, textiles and leather processing and industrial and institutional cleaning except in controlled closed systems with no release into wastewater or systems with special treatment where the process water is pre-treated prior to biological wastewater treatment or recycled or incinerated [110]. According to Mao et al. [111] due to implementation of the European Directive 2003 / 53 / EC, NP concentration in European countries are now much lower than in Asia.

In 2008, the concentration of NP and also OP in surface waters has been regulated in the Directive 2008 / 105 / EC [112]. The Directive 2008 / 105 / EC [112] sets the Environmental Quality Standards (EQS) for these two alkylphenolic compounds. EQS have been proposed for priority substances to provide a benchmark for achieving the good surface water chemical status that EU member states are required to respect [106]. The EQS indicates the annual average concentration: for NP in seawater samples (or other surface waters) is 0.3 μg L^{-1}, whereas the maximum allowable concentration is 2 μg L^{-1} [113]. In the case of OP the maximum allowable concentration is not applicable since the annual average concentration (0.01 μg L^{-1}) is significantly lower than the values derived on the basis on acute toxicity [113].

In Europe due to the imposed use restriction for the Directive 2003 / 53 / EC, the main alternative for NPEOs includes linear and branched alcohol ethoxylates and glucose - based carbohydrates such as alkylpolyglucoside [114]. According to Campbell [115] these

alternatives are considered to be environmentally more safer as they degrade more rapidly [116].

While in European countries there are restrictions, the widespread use of NPEOs continues in many others countries without any type of legal control [117]. At present only a small number of countries (e.g. Asian countries) continue to use APEOs unrestricted [106], nevertheless, OP and NP are still widespread and detected world widely in diverse environmental matrices [106, 118–120].

1.1.1.2. Physicochemical properties and behavior in the environment

An alkylphenol is a phenol derivative wherein one of the ring hydrogens has been replaced by alkyl group(s) as it happens with NP and OP [121].

In aquatic ecosystems, NP and OP are subjected to factors that influence their concentration and dissipation, such as: volatilization, sorption, photodegradation and biodegradtion. The extension of these factors are associated with the physicochemical properties. Tables 1.2, 1.3 and 1.4 list some relevant physicochemical properties of NP (linear and branched alkyl chain) and OP, respectively.

The volatilization rate of these alkylphenols from soils is not significant, according to their vapour pressure and the Henry ' s law constant, 6.0×10^{-1} Pa m^3 mol^{-1} at 25 ºC for NP and 4.6×10^{-1} Pa m^3 mol^{-1} at 25 ºC for OP, being semi-volatile organic compounds [122]. In the case of NP, it was observed that only 0.22 % of 1 g NP per kg of soil was removed over a period of 40 days by volatilization [123].

The photodegradation is an important process in the reduction of NP and OP concentration in aquatic environment and this degradation increases when the dissolved oxigen concentration increases [3, 124]. In the photodegradation process the dissolved oxygen and hydroxyl radicals produced by other species attack the alkylphenol molecules, as observed by Li et al. [3] with NP. The hydroxyl radicals that promote the photodegradation of alkylphenols may come from NO_3^- photolysis [125].

Fig. 1.3 represents the photodegradation pathway of NP in natural seawater based on the study of Li et al. [3]. The phototransformation of alkylphenols is significantly dependent on the oxygen concentration. According to Mazellier [124] the rate of phototransformation decreased with decreasing oxygen concentration. The oxygen dissolved can capture the electron and energy from the excited states of the organic pollutant molecules and then accelerate the degradation rate [126]. 4-nonylcatecol [3] and 4-octylcatechol [124] are some of the intermediated products of NP and OP photodegradation process, respectively.

Table 1.2 - Physicochemical properties of nonylphenol with linear alkyl chain.

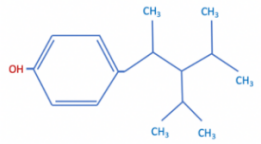
CAS Number : 104-40-05 4-Nonylphenol (linear alkyl chain) $C_{15}H_{24}O$ Molecular weight 220.36 g / mol	Physicochemical properties	
	Physical state at 20°C and 101.3 KPa*	Pale yellow viscous liquid
	Boiling point**	293 - 297 °C
	Melting point**	42 °C
	Density**	0.937 g / cm^3 at 20 °C
	Vapor pressure*	0.109 Pa at 25 °C
	Octanol / water partition (Log K_{ow})*	5.76
	Solubility in water *	7mg / L at 25 °C
	Dissociation constant (pK_a)*	Ca. 10 (Value could be higher than this)
	Organic carbon / water particion (Log K_{oc})*	4.58 (MCI method)[a]

*European Chemical Agency, Substance Registration Dossier for p-nonylphenol, 2016. https://echa.europa.eu/registration-dossier/-/registered-dossier/17497 (accessed Marsh 22, 2017)

**European Chemicals Agency, SVHC Support Document for 4-Nonylphenol, branched and linear, 2012. https://echa.europa.eu/documents/10162/d3c27f36-2704-597a-0b18-8e03d1a8e451 (accessed Marsh 22, 2017)

[a] MCI method - Molecular Connectivity Indices method

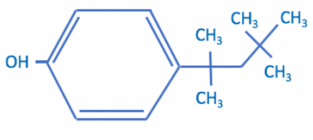
Table 1.3 - Physicochemical properties of nonylphenol with branched alkyl chain.

CAS Number : 84852-15-3 4-Nonylphenol (branched alkyl chain)  (One example of branched NP among several possible types) $C_{15}H_{24}O$ Molecular weight 220.36 g / mol	Physicochemical properties	
	Physical state at 20 °C and 101.3 KPa*	Yellow liquid with phenolic odour
	Boiling point *	302 °C at 101.3 kPa
	Melting point*	< -7 °C at 101.3 kPa
	Density*	0.95 g / cm^3
	Vapor pressure*	0.3 Pa at 25°C
	Log K_{ow}*	5.4 at 23 °C, pH = 5.7
	Solubility in water *	Ca. 5.7 mg / L at 25°C, 6 < pH < 7
	Dissociation constant (pK_a)*	Ca. 10 (Value could be higher than this)
	Organic carbon / water particion (Log K_{oc})*	4.41 (MCI method)[a]

**European Chemicals Agency, SVHC Support Document for 4-Nonylphenol, branched and linear, 2012. https://echa.europa.eu/documents/10162/d3c27f36-2704-597a-0b18-8e03d1a8e451 (accessed Marsh 22, 2017)

[a] MCI method - Molecular Connectivity Indices method

Table 1.4 - Physicochemical properties of octylphenol.

CAS Number : 140-66-9 4-t-Octylphenol  $C_{14}H_{22}O$ Molecular weight 206 g / mol	Physicochemical properties	
	Physical state at 20°C and 101.3 kPa*	Solid
	Boiling point *	289 °C at 101.3 kPa
	Melting point*	85 °C at 101.3 kPa
	Vapor density	-
	Density*	0.37 g / cm^3 at 22 °C
	Vapor pressure*	2 Pa at 38 °C
	Log K_{ow}*	4.8 at 22 °C and pH 6.6
	Solubility in water *	7 mg / L at 20 °C and pH 6 - 7* 5.1 mg / L at 25 °C **
	Dissociation constant (pK_a)*	10.25

*https://echa.europa.eu/pt/brief-profile/-/briefprofile/100.004.934 (accessed october 2019)
** https://pubchem.ncbi.nlm.nih.gov/compound/4-Tert-Octylphenol#section=Melting-Point (accessed october 2020)

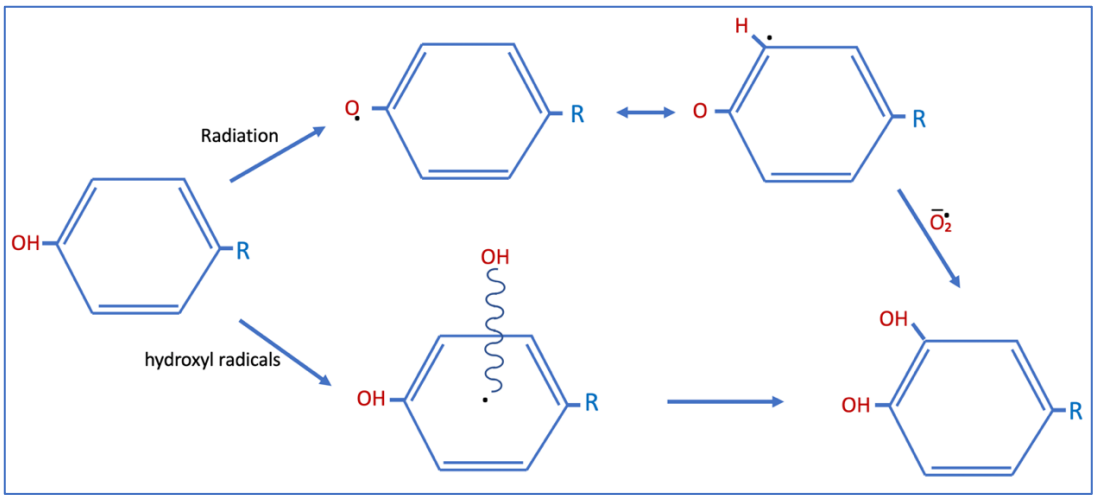


Fig. 1.3 - Photodegradation mecanism of nonylphenol. Source: adapted from Li et al. [3].

NP ($C_{15}H_{24}O$; 5.7-7 mg L^{-1} at 25°C) is signigficantly less solube in water, than OP ($C_{14}H_{22}O$; 7 mg L^{-1} at 20 °C) due to the nonyl chain with one carbon more [4, 127, 128]. The water solubility of NP and OP and octanol - water partition coefficient (Log K_{ow}), suggest that NP has a strong affinity to sorb to suspended solids in the water column or in the organic component of soils and sediments than OP [49, 129–131].

Several factors affect organic chemical adsorption on soil and sediments, such as molecular properties of adsorbed compounds, properties of adsorbents, and liquid-phase characteristics (composition and water content of adsorption medium) [132]. The molecular properties may be classified into three categories related to the electronic structure (nature of constituent atoms and functional groups), partition coefficients and the size and shape molecules [132]. The adsorption process in the case of phenolic compounds is closely related to the electron donor power and position of ring substituents [133]. According with Boyd [133] the adsorption increases with the electron donor power whatever the ring position ortho, meta or para, due to the increased ability of phenol OH to act as proton acceptors and thus to form hydrogen bonds [132]. The adsorption is lower if the substituent is in the ortho position due to steric hindrance of hydrogen bond formation [132].

The ionization of organic molecules is directly dependent on their electronic structure. In the case of strong bases, it always occurs in cationic form in soils and sediments. In the case of weak bases and weak acids, such as NP and OP, their ionization depends on ionic composition of the liquid phase, especially its pH as a basic characteristic of these compounds is their pk_a values [132]. In the case of NP and OP, they are weak acids ($pK_a \approx 10$) which can be present in aqueous solutions both as undissociated and deprotonated species [134]. In the natural pH of the aquatic environment NP and OP are present mainly as undissociated

species, and hence as hydrophobic species [3, 135] and less reactive than deprotonated species [134].

In this sense pH variation might have an effect on NP and OP adsorptive process [136, 137], because the pH variation interfere with the chemistry of both the adsorbate and the adsorbent by shifting the equilibrium dissociation process of solutes and surface functional groups of the sorbent towards ionized or unionized species [34]. Stronger sorption is often observed at pH values near the pK_a since a high degree of protonation results in increased interactions with the soil matrix [138]. In the case of weak acids, like NP and OP, these are more adsorbed from neutral or acidic solution when in undissociated species [34, 139, 140]. For instance, at lower pH values, oxonium ions are present in solution, preventing dissociation, and in these pH conditions formation of hydrogen bonds and π-π interations between phenolic adsorbates and sorbent surface is privileged and adsorption capacity is the highest [34]. At higher pH, alkaline conditons, the adsorption of alkylphenols decreases, because the molecules turn into phenolate ions and since the surface of the adsorbent is negatively charged in the presence of electrostatic repulsive forces prevent adsorption [141, 142].

In addition to all the above mencionated abiotic factors, it will be important to make reference also to the size of the adsorbate molecules, because porous structure of the adsorbent can act as a molecular sieve in sorption process [34]. The rate of mass transfer is controlled by diffusion of adsorbates in poros structure of the adsorbent [34]. Availability of the pores strictly depends on size of the molecule, in other words, smallest molecules penetrate almost all pores, while for large molecules, not all pores are accessible [34]. An increase in adsorption resulting from the rise of adsorption energy is supposed if the pore size is about twice the size of kinetic diameter of adsorbate molecule [143].

In the a contaminated environment there is usually a mixture of compounds with similar size molecules and a competition between them for the active sites in adsorbent takes place. Compounds possessing higher affinity to the adsorbent interact stronger with its surface and displace molecules interacting through weaker forces [144, 145]. Hence, it follows a selectivity of adsorbent towards the specific compounds. In the case of the presence of molecules with significantly differences in size, the larger molecules can block entrances of some pores effectively preventing adsorption of the smaller species [146].

Some works suggested that adsorption of phenolic compounds is mainly controlled by porous structure and other works say that it is not like that, and why there is no correlation between sorption and total surface area [147]. Johnson et al. [86] studied the potential of OP sorption in three different structure, clay (2 μm), silt (2 - 63 μm) and sand (63 - 900 μm). These authors observed that OP adsorption was particularly large in silt pores, when surface particle size predominated was between 2 - 63 μm. The results obtained by Terzyk et al. [147] can

give a possible explanation why it was not the structure with the largest area that adsorbed more compound. These authors suggested that competition between water adsorbing in pores and phenolic compound is mainly responsible for the results in adsorption process, in other words, in case of diluted phenol solutions, the competition between phenol and water molecules leads to substantial reduction in adsorption of phenolic compounds on oxidized surface. For that reason, Laszlo et al. [148] pointed out that not only molecules of phenol compounds but also water are present on the surface, therefore, the competition between them should be taken into account in the calculation of the cross-sectional area of adsorbed phenolic compounds, further, some pores can be physically inaccessible for adsorbed phenol-solvent aggregates.

This interaction with soil matrix is also conditioned by linear or branched isomers configuration. According to Düring [149] the branching isomers of NP probably hinders diffusion within aggregates and the organic matter may limit specific interactions with soil matrix. This could explain the lower partition coefficient (Log K_{oc}) when compared to those of NP linear. Additionally, the diffusion within aggregates and organic matter promotes non-complete reversibility of the compounds bond [149]. Höllrigl - Rosta [150] observed that NP linear binds strongly to humic acid in the dissolved organic matter through non-specific lipophilic interaction that are regulated by diffusion and are not completely reversible.

Then these three factors, volatilization, photodegradation and particularly sorption will condition NP and OP behavior in the environmental matrices. Another important factor is biodegradation.

1.1.1.3. Alkylphenols biodegradation

Biodegradation processes can reduce the toxicity and environmental persistence of long-chain alkylphenol, such as NP and OP and when the degradation of these compounds does not occur readily in nature they are often detected in alkylphenol – contaminated soils [151]. In the case of OP the estimated half-life is about 40 days in water and more than 150 days in sediment [152]. In the case of NP, the persistence in the environment is associeted with the length and branching of the alkyl chains [153].

Several bacterial strains isolated from different environments have been reported to be capable of degrading NP and OP. For instance, *Stenotrophomonas* sp. and *Pseudomonas mandelii* isolated from polluted soils [154], *Pseudomonas* sp. strain JC1 isolated from NP acclimated river sediments [155], *Rhizobium* strain WZ1 and *Sphingobium* strain WZ2 [156] and *Sphingobium amiense* sp. nov. [157] isolated from river sediments, *Sphingomonas* sp. TTNP3 [158] and *Sphingomonas xenophaga Bayram* [159] isolated from activated sludge in

wastewater treatment plants and from a laboratory - scale activated sludge reactor fed with NP, respectively, *Bacillus cereus* [160] and *Bacillus sphaericus* [161] isolated from sewage sludge, Ƴ- *Proteobacteria* and *Bacillus* isolated from granular sludge [162], *Acinetobacter* sp. [163] and *Shingomomas* sp. [164] isolated from activated sludge. *Pseudomonas nitroreducens* strain TX1 isolated from rice field drainage [165] and *Stenotrophomonas* sp. strain IT-1, *Sphingobium* spp. strains IT - 4 and IT - 5 isolated from *P. australis* rhizosphere [166]. All environmental matrices had been previously exposed to alkylphenols.

Besides, considerable work has been conducted to evaluate alkylphenol degradation in pure cultures of *Sphingomomnas xenophaga Bayram* [159] as well as in microbial consortia formed by *Sphingomonas*, *Pseudomonas*, *Alicycliphilus*, and *Acidovorax* genera [8].

The degradation mechanisms of alkylphenols are sparsely documented but have been hypothesized. Recent investigation of NP and OP bacterial metabolism showed that the alkyl moieties of the alkylphenol growth substrate were transformed to alkyl alcohols with retention of the structures of the original alkyl side chains [167]. According to Gabriel et al. [159], the aromatic ring of alkylphenols needs to be cleaved from the aliphatic substituent (alkyl side chains) in order to be degraded and utilized as a growth substrate, because only the aryl part of the compounds is utilized as a source of carbon and energy. In the most commonly accepted hypothesis the start of degradation is via ring fission (ring oxidation) similar to the metabolism of other aromatic compounds [164, 168], leaving intermediates of alkyl chains with different lengths [158]. This initial degradation via ring fission was observed by de Vries et al. [169] and Tanghe et al. [158] in branched alkylphenols and by Jeong et al. [170] in linear alkylphenols. The fission of the aromatic ring can occur in the following metabolic pathways: a) *ipso* - hydroxylation of the fourth carbon atom (C_4) of the aromatic ring, followed by the migration of the alkyl chain to an adjacent C atom of the ring [4, 5]; b) monohydroxylation at the C atom adjacent to the hydroxyl to produce alkylcatechol [6]; c) dihydroxylation of the phenol ring in the initial catechol step [7]; d) oxidation of the aromatic ring, followed by degradation of the intermediated [8] (Fig. 1.4).

Relative to microbial degradation of OP, several approaches confirmed that biodegradation is one of the natural pathways that lead to its elimination from the environment and according to Rajendran et al. [9] it can occur through different metabolic pathways as observed in Fig. 1.5. The degradation can occur by (a) aromatic ring hydroxylation followed by ortho or meta - cleavage of the aromatic ring in bacteria and yeasts [6, 166, 171, 172]; (b) oxidative radical polymerization in ligninolytic fungi [173]; (c) ipso - hydroxylation followed by aromatic ring hydroxylation; and by (d) alkyl - chain hydroxylation at the terminal carbon position followed by terminal or sub - terminal oxidation of the hydroxyl group in yeasts and

non - ligninolytic fungi [82, 172]. The *ipso* - substitution is the most referenced pathway of degradation of alkylphenols in the literature. Ipso substitutions are grouped into two types

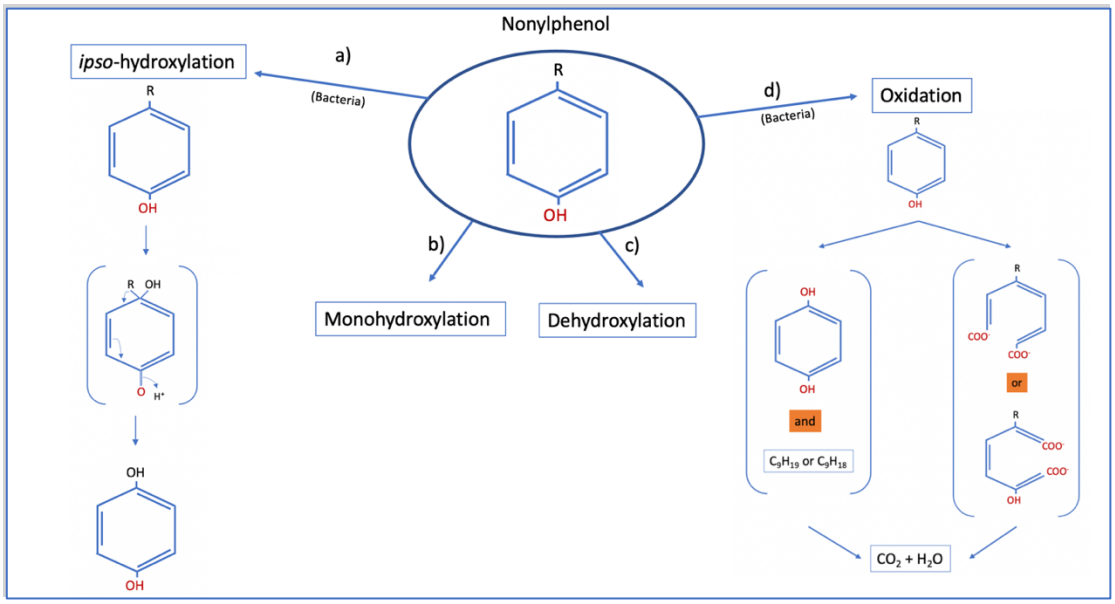


Fig. 1.4 - The metabolic pathways of nonylphenol fission of aromatic ring by microorganisms proposed by **a)** Corvini et al. [4] and Kohler et al. [5]; **b)**Tuan et al. [6]; **c)** Conneely et al. [7]; and **d)** Bai et al. [8]).

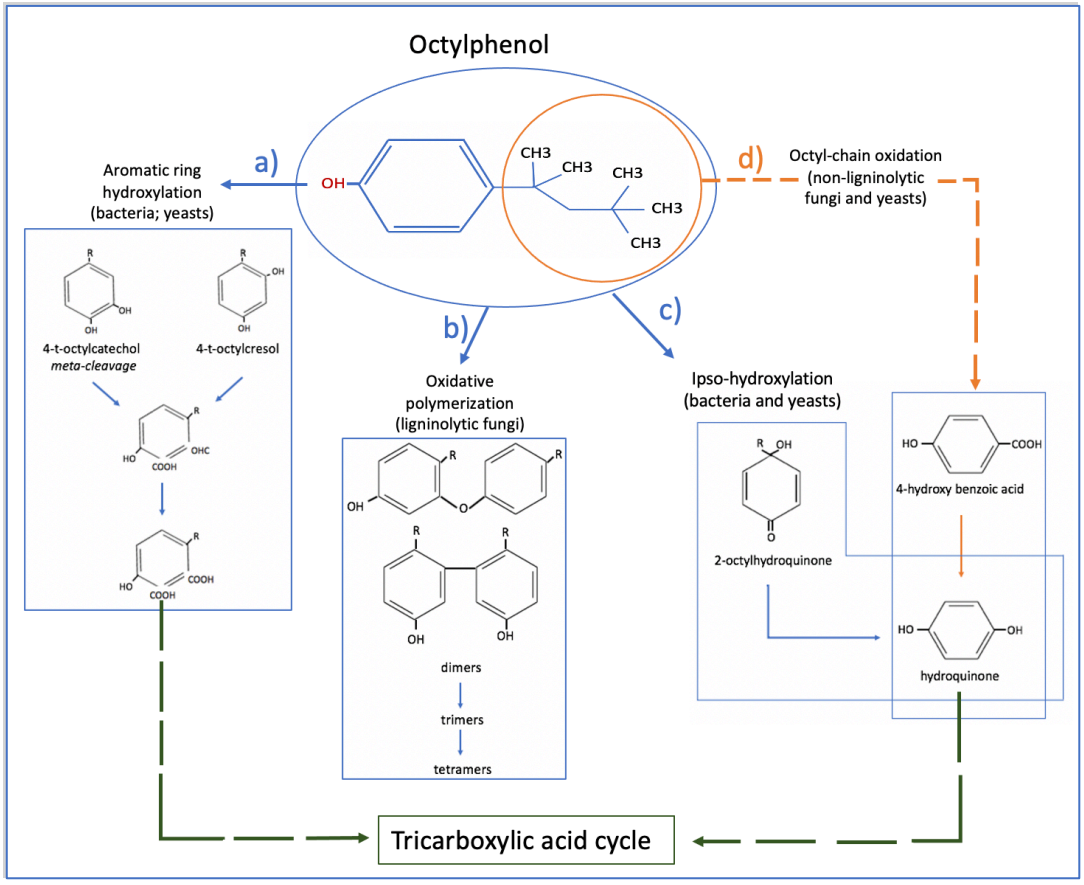


Fig. 1.5 - Different pathways of octylphenol degradation by aerobic bacteria and fungi (adapted from Rajendan et al. [9]).

according to the nature of the substituent eliminated from the quinol intermediate [174]. In the case of OP degradation via type I *ipso* - substitutions would result in the release of an anionic group and the formation of p - benzoquinone, whereas type II *ipso* - substitution of OP [171] and NP [175] would result in the formation of hydroquinone (HQ).

The metabolic pathways leading to alkylphenols degradation are important because they can allow the full elimination of the compounds from impacted ecosystem, ensuring that the by - products do not show toxicity. Then, the microbial degradation should be further explored for the remediation of water and soil/sediment contaminated with alkylphenols.

1.1.2. Chlorophenols

Chlorophenols are synthetic phenolic compounds, being the most widespread largest group of phenols and represent a major environmental concern, due to their toxicity, poor biodegradability and recalcitrant properties and endocrine disruption activity in living organisms as previously mentioned. They derivate from phenol and are composed by a benzene ring, a -OH group and atom(s) of chlorine [30, 176]. The atoms of chlorine are bond between first carbon (C_1) and fifth carbon (C_5) of phenol, which is an aromatic compound derived from benzene by adding a hydroxyl group to a carbon, replacing hydrogen [177]. Chlorophenols are a group of 19 compounds formed by chlorination and hydrolysis of chlorobenzenes [176]. Together with the 19 isomers, chloroderivatives of methyl and ethyl - phenol are also considered as chlorophenols [178]. Chlorophenols may also be present as salts, for example, sodium and potassium salts [179].

Within the chlorophenols group, PCP is the most toxic due to the degree of chlorination [180] and is one of eight chlorophenols that has industrial interest (2 - dichlorophenol, 4 - dichlorophenol, 2,4 - dichlorophenol, 2,4,5 - trichlorophenol, 2,4,6 - trichlorophenol, 2,3,4,5 - tetrachlorophenol, 2,3,4,6 - tetrachlorophenol and PCP) [176].

PCP, which is an artificial semivolatile, organochlorine and hydrophobic compound, was first described in 1841. It is considered a highly chlorinated organic compound and the most hazardous halogenated organic compound and is a kind of ionisable hydrophobic organic compound [181–183]. PCP is produced with the aim of preserving timber and lumber but also for a wide variety of agriculture and industry application, being still widely used mainly as a wood preservative and as a pre-harvest biocide [184, 185]. In fact, PCP and its sodium salt have been widely used on bacteria, mould, algae and fungi [186]. Due to the extensive use in agricultural and industrial application, different levels of PCP have been detected in the environment including in aquatic environment (Table 1.5). As so, it has been detected in a large number of matrixes [10], such as air, water, soil, sediments and biologic tissues [187].

PCP has been used as a biocide more specifically fungicide, herbicide, algicide, insecticide and molluscide [10] due to its capacity in promoting the permeability of cell membrane to protons, which results in dissipation of transmembrane pH gradients and electrical potentials [10]. For instance, uncoupling of oxidative phosphorylation result from the PCP protonophoric properties. PCP may also adsorb preferentially to disordered regions in the biological membrane leading to alterations in membrane function [10].

PCP has been categorized as a kind of persistent organic pollutant (POP) owing to the resistance to environmental degradation, persistence and bioaccumulation, with significant impact on human health and ecobiota [29]. According to the literature, PCP can induce the development of various toxicological effects in mammals such as carcinogenicity, teratogenicity, mutagenicity, immunotoxicity, neurotoxicity and various endocrine-modulating effect [188].

Table 1.5 - Levels of PCP in water (μg L^{-1}) and sediments (μg g^{-1} dw) collected in different countries.

Sampling area	Compound	Concentration		References
		Water (μg L^{-1})	Sediment (μg g^{-1} dw)	
Estuaries in Portugal: Minho (march 2003) Douro (may 2002) Aveiro (march 2003) Mondego (april 2003) Tagus (may 2002) Sado (november 2001) Formosa (june 2003) Guadiana (june 2001)	PCP	 0.0005 - 0.0007 0.0012 - 0.0017 - 0.0010 - 0.0127 - - 0.0005 - 0.0010 -	 0.051 - 0.079 0.0001 - 0.1138 - - - - - -	Almeida et al. [189]
River - Portugal (1999): Guadiana Nisa	PCP	 0.13 0.31	-	Azevedo [190]
Freshwater bodies in Belgium, Germany and Netherlands	PCP	0.01 - 0.17*	-	Muir and Eduljee [191]
Estuaries in Germany and Netherlands in the 1980s and 1990s	PCP	0.006 - 0.1*	-	Muir and Eduljee [191]
4 Southern estuaries in Netherlands in the 1990s	PCP	-	0.024 - 0.027	Muir and Eduljee [191]
Freshwater marine and estuarine waters in UK in the 1990s)	PCP	0.00002 – 0.00020 0.001 - 0.007*	-	Muir and Eduljee [191]
Estuaries in North Sea	PCP	-	0.027 - 0.20	EuroChlor [192]

(continued)

Table 1.5 (continued)

Pearl River Delta- China	PCP	-	0.0014 - 0.0340	Hong et al. [193]
Yinma River Basin - China	PCP (and others phenolic compounds)	nd – 0.0055	nd - 0.006*	Zhou et al. [194]
Jiaozhou Bay - China	PCP	-	0.0005 - 0.0037	Pan et al. [195]
Chinese rivers - China	PCP (and others chlorophenols)	< 0.0011 - 0.5940	-	Gao et al. [196]
Aojiang River - China	PCP (and others chlorophenols)	-	nd - 0.0714	Chen et al. [197]
Yangtze River - China	PCP	-	0.0006 - 0.0128	Tang et al. [198]
Pearl River Delta - China	PCP	-	0.0079	Hong et al. [193]

n.d. - not detected; * average concentration;

1.1.2.1. Chlorophenols legislation and restrictions

The presence of PCP in air, water and soil throughout different environments is a result of its use over many decades. Due to PCP persistence in soil and water environments, both the European and US Environment Protection Agencies have classified PCP as a 'priority pollutant' [199]. In 1991 the EU, adopted the Ninth Amendment of the Marketing and Use Directive 76 / 769 / EEC through Council Directive 91 / 173 / EEC [191]. This directive contains a number of restrictions on the use of PCP, its salts and esters, in member states of EU [191]. In 1996 restricted discharges to the environment were imposed by EU legislation in Directive 86 / 280 / ECC. This directive defines as quality goal for inland surface, estuary, internal coastal and territorial waters a PCP concentration lower than 2 μg L^{-1} [200]. Some EU countries (Austria, Belgium, Finland, Germany, Netherlands and Sweden) introduced national legislation effectively banning the use of PCP [191].

Recently, the production of PCP has ceased in the EU, and all current uses under Directive 91 / 173 / EEC rely from countries outside the EU like the US [191]. Production in the US has decreased from 45 million lbs (≈ 20.4 million kg) in 1983 to approximately 11 million lbs (≈ 5 million kg) and emissions within the UK fell from 538 tonnes to 437 tonnes between 1990 and 2002 [201]. So, despite its ban or significant reduction, this compound is still in use and consequently can end up in different environmental compartments. As so, methodologies are needed to recover impacted environments.

1.1.2.2. Physicochemical properties and behavior in the environment

The major differences between behavior profiles of organic chemicals in the environment is associated to the physicochemical properties of compounds. The physicochemical properties of PCP and the structure representation is shown in Table 1.6.

PCP (C_6Cl_5OH) is a weak acid, and the acidic characteristics are due to the presence of the chlorine atoms. For instance, the pk_a (i.e. negative logarithm of acid dissociation constants) for phenol is 9.3 while the same constant for PCP is 4.70 [202]. The electron withdrawing and effects of various substituent groups in PCP makes it more acidic than phenol (C_6H_5OH) [183].

Carswell and Nason [183] showed that 14 mg of PCP could be added per kg of neutralized distilled water prior precipitation. In this sense, 14 mg L^{-1} is the most widely reported value in literature for solubility of PCP, as commonly defined in unbuffered water with initial pH of 7 [184]. Nevertheless, as observed by Carswell and Nason [183] experiment, addition of PCP to neutral unbuffered water will decrease the pH so that 14 mg L^{-1} will be the solubility at pH somewhat lower than 7.

As PCP is a weak acid (pk_a value 4.71), partial dissociation occurs naturally in aquatic systems on earth's surface [203]. When PCP is added to pure water, part of it dissociates to form $C_6Cl_5O^-$ (or PCP^- or pentachlorophenate anion) and protonizes water (H_3O^+), bringing down the solution pH. The dissociate in aqueous phase involves an equilibrium between two chemical forms as shown by equation (1): the slightly soluble undissociated PCP (C_6Cl_5OH or PCP^o) and the very soluble pentachlorophenate anion [184]. While the aromaticity imparts hydrophobic or nonpolar characteristic to undissociated PCP, the ionic charge of pentachlorophenate enhances its hydrophilicity through ion-dipole interaction with water molecules [202]. The ratio of the equation (1) components changes, therefore, with pH [184].

$$C_6Cl_5OH + H_2O \Leftrightarrow C_6Cl_5O^- + H_3O^+ \qquad (1)$$

Due the nonpolar characteristic and presence of -OH group that promotes the ionization, PCP is commonly referred to as a hydrophobic ionizable organic compound (HIOCs) [202, 203].

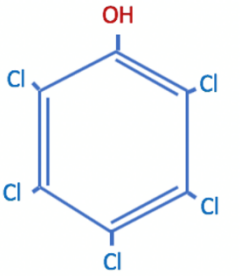
The partition coefficient K_{ow} value of PCP provide a direct estimate of hydrophobicity, which in this case is 10^5. This value shows that solubility of compounds in organic solvents are usually orders of magnitude higher than their aqueous solubility [204, 205]. For ionizable

compounds, such as PCP, the K_{ow} value is pH dependent because the speciation of the PCP is pH dependent [206]. In this sense, the total solubility of the PCP increases as the pH of the water increases and K_{ow} should decrease correspondingly [184, 206]. According with Nowosielski and Fein [206] for PCP the K_{ow} exhibits pH dependence in the range $pK_a - 1 < pH < pK_a + 3$, but at lower and higher pH values the behaviour of PCP is independent of pH.

Table 1.6 - Physicochemical properties of pentachlorophenol.

CAS number : 87-86-5

Pentachlorophenol



C_6HCl_5O

Molecular weight 266.3 g / mol

Physicochemical properties	
Physical state at 20 °C and 101.3 kPa	-
Boiling point *	309.5 °C
Melting point**	190 - 191 °C
Vapor density*	1.10 E-04 mm Hg
Density**	1.978 g / cm^3 at 22 °C
Vapor pressure**	< 0.1 Pa at room temperature
Log K_{ow}**	5.18
Solubility in water **	14 mg / L at 20 °C
Dissociation constant (pK_a)*	4.7 at 25 °C

*https://chem.nlm.nih.gov/chemidplus/rn/87-86-5 (accessed October 2019)
**http://gestis-en.itrust.de/nxt/gateway.dll/gestis_en/014000.xml?f=templates$fn=default.htm$3.0 (accessed October 2019)

As mentioned before, as observed for other chlorophenols the primary processes governing the fate and behavior of PCP in the different environmental matrices are sorption, volatilization, photolysis and biodegradation [11], processes that will, as indicated, depend on the physico - chemical characteristic mentioned above.

Based on the relatively low Henry's law constant (2.45×10^{-8} atm m^3 / mol at 22 ºC) it is unlikely that PCP will volatize from water [207]. So, the volatilization process is not taken into account in the remediation process of wastewater contaminated with PCP.

Photolysis is an important transformation processes that can contribute for PCP removal from aquatic environment [208]. However, it is dependent on the average depth of a stream or river or effluent, decreasing as depth increases due to the lower amount of light. For instance, chlorinated phenols, tetrachlorodihydroxyl benzenes and non-aromatic fragments such as dichloromaleic acid photodegradation products were detected by Crosby and Wong [209] after irradiating an aqueous solution containing 100 mg L^{-1} of PCP with sunlight or UV light.

Sorption will depend on the form, undissociated (PCP) or dissociated for (PCP^-). Adsorption of PCP^- on soil / sediment is different from undissociated PCP, because it does

not form micelles, exhibit strong amphiphilic characteristic or ionic exchange with soil/sediment [203]. The undissociated PCP can interact as a hydrophobic non - ionized organic compound in the environment [210]. The sorption of undissociated PCP results from *London van der Waals - London* dispersion forces reinforced by the hydrophobic interaction [210]. This combined effect is often referred to as hydrophobic sorption [211]. The thermodynamic driving force for the hydrophobic sorption is the increase in entropy which occurs upon dehydration of the non - ionizable organic molecules [212]. In aqueous phase, the non-ionizable PCP is surrounded by water molecules [212]. As the non - ionizable PCP is transferred from the aqueous phase to the non - aqueous solid phase, the structural of water molecules around non - ionizable organic molecules breaks down and the entropy of the system increases [212]. Therefore, PCP is more adsorbed to the soil / sediment than PCP^-.

1.1.2.3. Chlorophenols biodegradation

Among the mentioned processes governing the fate and behavior of PCP, the biologically mediated degradation is the main transfer pathway of PCP in soil, in which PCP acts as an electron acceptor and is then transformed into less toxic compounds [213, 214]. The microbial community structure changes to adapt to and engage in the process of PCP degradation under PCP-stress conditions [215–217].

The degradation of PCP has been observed in aerobic and anaerobic conditions [218] and diverse habitats indicate biological transformation potential, however, is manifested only under specific environmental conditions. These conditions include temperature, availability of electron acceptors and donors, nutrients, and toxic metals [218]. For instance, in an aquatic system, such as wetland, these properties are often present as gradients resulting in a continuum of microbial activities [218].

The biodegradation of PCP process can be completed through three ways: oxygenolysis, hydroxylation or reductive dechlorination [10, 219]. Degradation process of PCP result in lesser chlorinated products, and according to Häggblom [220] it can function as carbon and energy sources for those aerobic and anaerobic microorganisms involved in degradation processes. The enzymes and pathways of PCP degradation is shown in Fig. 1.6.

As PCP is a highly toxic recalcitrant compound with toxic properties [221, 222], it can be degraded only by a limited number of bacteria [223] that likely have the enzymes required for its catabolism [11]. Microbial cometabolism is considered to be an effective method of PCP degradation, in which PCP is used as a second substrate and may serve as a carbon source for the growth of microorganisms [11].

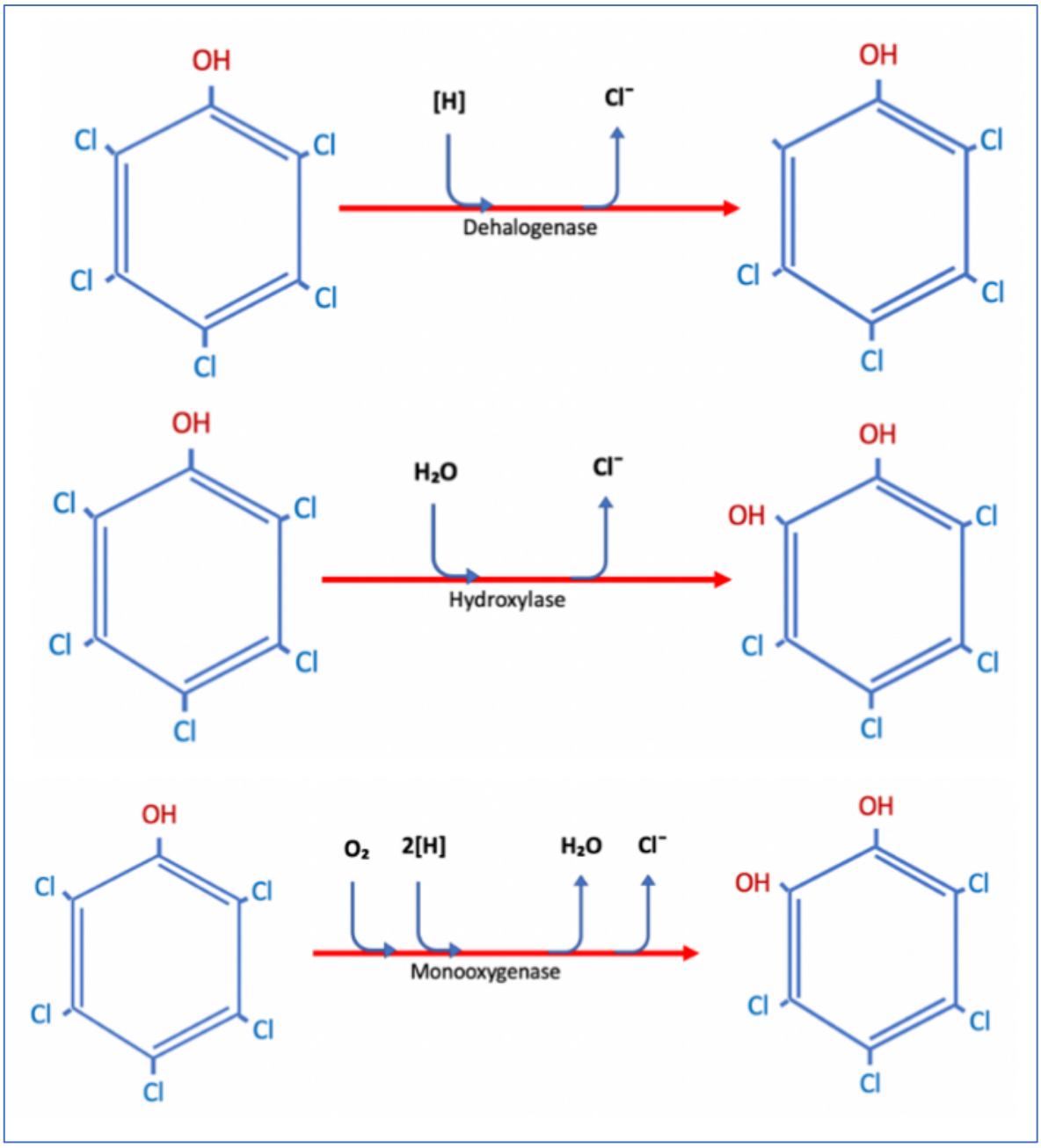


Fig. 1.6 - The three pathways and enzymes (dehalogenase, hydroxylase and monooxygenase) that are involved in PCP biodegradation (adapted from McAllister et al. [10]).

PCP can be degraded by soil bacteria in aerobic and anaerobic environment [224]. For complete degradation of chlorinated aromatic compounds, such as PCP, two steps are necessary, removal of the chloride atom and cleavage of the phenolic ring [11]. In Fig. 1.7 it is described summarily the microbial degradation pathway of PCP in aerobic and anaerobic condition.

The reductively dechlorination has been suggested as the primary PCP biodegradation mechanism [225, 226]. In this process, the chlorine atoms are sequentially replaced by

hydrogen atoms until it is completely transformed into phenol, benzoate, acetate, carbon dioxide and methane [227].

Under aerobic conditions, transformation of PCP by bacteria proceeds through sequential hydroxylation and reductive removal of chlorine substituents yielding poly-hydroxybenzene compounds that are eventually mineralized to CO_2 [218]. The methylation of PCP results in the production of volatile pentachloroanisole (PCA) [218]. The aerobic degradation of PCP by *Sphingobium chlorophenolicum* L-1 (formerly *Sphingomonas chlorophenolica* ATCC 39723) [228], Kocuria sp. CL2 [229], *Bacillus* sp. [230], *Novosphingobium lentum* [231], *Pseudomonas* sp. [232], *Sphingomonas* chlorophenolica [233, 234] and *Enterobacter* sp. SG1 has been observed [235]. These bacteria utilize PCP as a sole source of carbon and energy [11]. In the case of *S. chlorophenolicum* L - 1 bacteria, the enzyme PCP- 4 - monooxygenase (PcpB) catalyzes the conversion of PCP to tetrachloro - p - hydroquinone via the removal of chloride ions [11]. In the next step, tetrachloro - p-hydroquinone is sequentially dehalogenated to 2,3,6 - trichloro – p - hydroquinone by a tetrachloro – p - hydroquinone-reductive dehalogenase (PcpC). Further degradation of 2,6 - dichlorohydroquinone occurs via ring cleavage by the 2,6 – dichlorohydroquinone - 1,2 - dioxygenase (PcpA), leading to formation of 2-chloromaleylacetate that is further degraded via the Krebs cycle [11]. For *Mycobacterium chlorophenolicum* PCP - 1 and *Mycobacterium fortuitum* CG-2, PCP is hydroxylated to tetrachloro - p-hydroquinone by a membrane bound cytochrome P - 450 type enzyme [236, 237].

Under anaerobic conditions, the pathway for PCP transformation is the sequential replacement of chlorines by hydrogen (reductive dechlorination) leading to phenol benzoate, acetate, CO_2 and CH_4 [218]. A few anaerobic isolates having the capacity for reductive dechlorination of PCP have been discovered and these often gain energy by coupling this process to oxidative phosphorylation [218]. The anaerobic degradation and dechlorination of PCP are linked to sulfate reduction conditions [219, 238] methanogenic conditions [218] and denitrifying conditions [239]. Anaerobic degradation of PCP by *Actinomycetes* sp., *Streptacidiphilus* sp., *Desulfobacterium* aniline, and also aerobic *Rhodococus erythropolis*, *Amycolatopsis* and *Gordonia* has been observed [240].

In anaerobic conditions, the PCP is reductively dehalogenated to 2,3,4,5 - tetra - chlorophenol, 3,4,5 - triclorophenol, 3,5 -dichlorophenol and finally to phenol of which the further benzene ring is then broken to produce methane and carbon dioxide [241]. The incomplete reductive dechlorination process was also observed. For instance, *Desulfitobacterium frappieri* PCP-1 of the (phylum Firmicutes) dechlorinates PCP to 3 - chlorophenol [242], *Desulfitobacterium hafniense* dechlorinates PCP to 3,4,5 - trichlorophenol

[243, 244] and *Desulfomonile tirdjei* PCB-1 (phylum Proteobacteria) dechlorinates PCP to 2,4,6 - trichlorophenol [245].

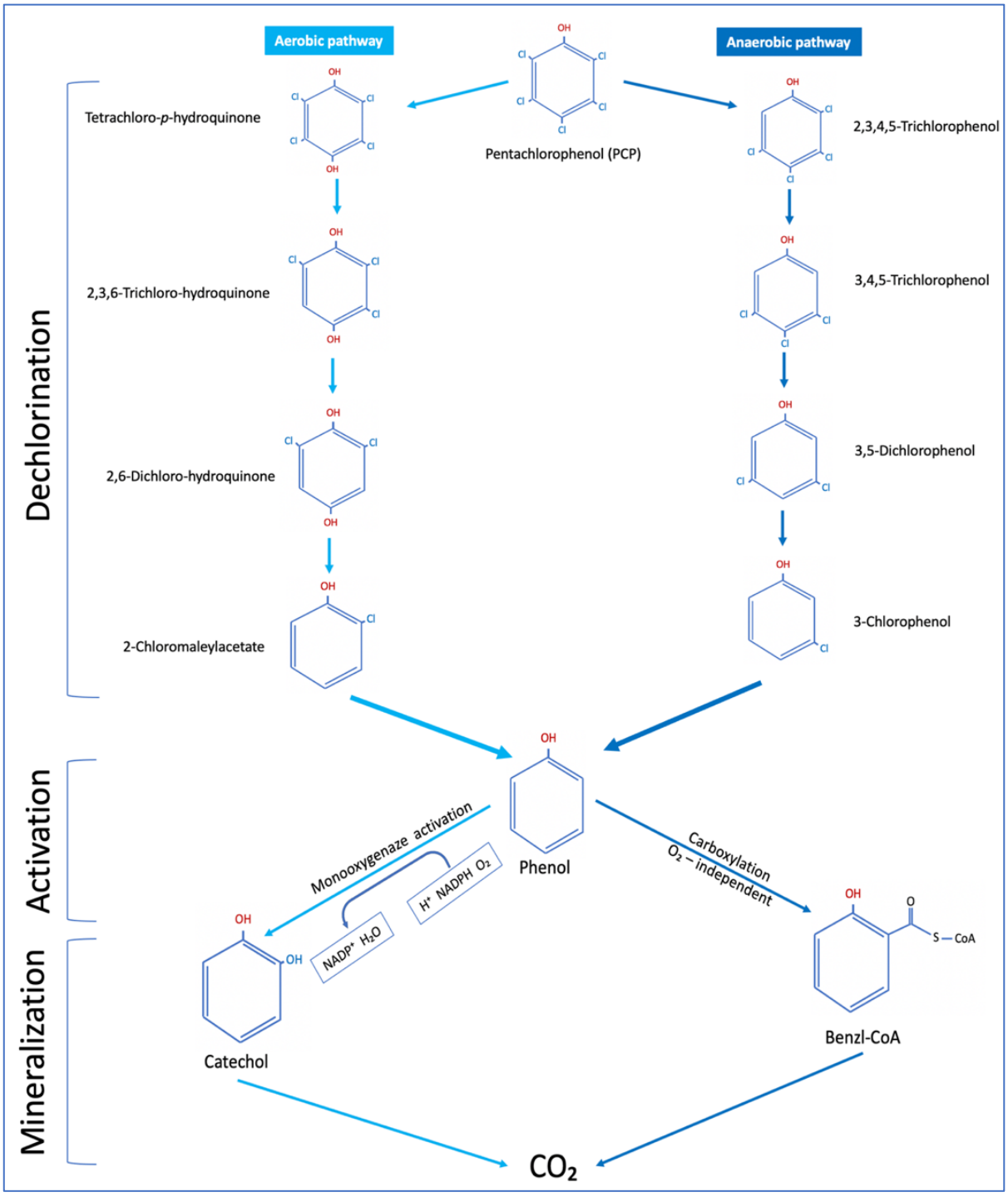


Fig. 1.7 - A simplified representation of reductive dechlorination pathway of PCP under aerobic and anaerobic conditions. PCP initially reductively dechlorinated to phenol which is then mineralized to CO_2 via monooxygenation in aerobic conditions or via carboxylation in anaerobic conditions (adapted from Chandra and Kumar [11] and long et al. [12]).

As shown, bacteria have potential for reductive dechlorination and mineralization of PCP [242, 246, 247] and also methanogenic archaea with syntrophic associations between

several archaea have this potential [248, 249], for instance, *Methanosaeta* and *Methanocella* genera (*Methanobacteria* class) [222], and *Methanosarcina* genus (class *Methanomicrobia*) [249–251]. The methanogens archaea can effectively metabolize PCP to produce CH_4 and the ring cleavage products were also further assimilated simultaneously by methanogens [222].

The ability for reductive dechlorination is likely from the high concentration of corrinoids, such as cobalamin, in methanogens which are needed for methanogenesis [252, 253]. Sometimes syntrophic growth between archaea and bacteria can be observed, for instance, *Dehalococcoides*, lack the ability to synthesize the needed corrinoids for reductive dehalogenation and instead have genes for corrinoid scavenging and import [254, 255]. Methanogens archaea may provide these key corrinoids for the organohalide-respiring bacteria (use halogenated hydrocarbons as terminal electrons acceptors) in these communities, though this role may be fulfilled by other corrinoids producing bacteria [256].

Considering the necessity to remove and recover environmental compartments contaminated with PCP, these bacterial strains should be explored to be used for PCP biodegradation.

1.2. Natural Wetlands

1.2.1. "*What is a wetland* ? "

Wetlands are more than just swamps, but wetlands are difficult to define and "*what exactly is and constitutes a wetland*" may not always be clear and remained an enigma to scientists. They are difficult to define precisely, not only because of their great geographical extent, but also because of the wide variety of hydrologic conditions in which they are found [13]. The Oxford English dictionary, define the word *wetland* - "(f. wet a. + land *sb*.) an area of land that is usually saturated with water, often a marsh or swamp" [257]. With this definition only lakes or rivers could be considered wetlands because it omits many seasonally flooded habitats that most ecologists would consider wetlands [257].

Many definitions of *wetland* have been developed and some of them have been changed over time in response to an increased understanding of wetland ecology and to political arguments [258]. Defining "*what exactly is and constitutes a wetland*" is especially important in terms of governmental administrative and legal organization, in this sense it is not surprising that governmental administrations began to develop the first comprehensive definitions [257].

An international definition for *wetland* was developed by the Ramsar Convention, an intergovernmental environmental agreement of more than 90 countries, in Iranian city of Ramsar in 1971 [259]. Under the Article 1.1. of the Ramsar Convention on Wetlands (Ramsar, Iron 1971) wetlands are defined as describe below:

"Wetlands are areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six meters".

According with the first article of the Ramsar Convention text, wetlands are characterized by having two or more of these attributes. The emphasis on soils and plant adaptations found in other definitions is absent from this one, possibly because it targets non-scientists who may not be familiar with what constitutes wetlands conditions, however, hydrology remains a focal tenet [257]. Many countries have adopted this definition to promote wetland conservation in their lands and to designate "wetlands of international importance" in accordance with the terms of the Ramsar Convention [260].

On the 30th of august 2021 the United Nation General Assembly adapted *Resolution* 75 / 317 that established the 2nd of february as World Wetlands Day, the day of the anniversary of the Ramsar Convention on Wetlands [261]. World Wetlands Day is celebrated each year to

raise awareness about wetlands. The theme for 2022 campaign is Wetlands Action for People and Nature (Fig. 1.8). The public campaign is an appeal to invest financial, human and political capital to save the world's wetlands from disappearing and to restore those that have been degraded.

Fig. 1.8 - The poster of 2022 campaign, a call to take action for wetlands and an appeal to invest financial, human and political capital to save the world's wetlands from disappearing and to restore those that are degraded.

1.2.2. Type of Wetlands

Wetlands cover at least 6 % of Earth's surface and include a large spectrum of habitats [262]. These habitats include from temporary shallow waterbodies such as playas or billabongs to marshes and swamps, from lake margins (littorals) to large river floodplains, from coastal beaches and salt marshes to mangroves, from peat bogs and fens to coral reefs and beds of marine algae or seagrasses [263]. The common factor to all these diverse kinds of habitats is that the land is so wet or under water for a part or whole of the year that the vegetation is quite distinct from that of the adjacent upland areas [263].

The Ramsar Convention has adopted a *Ramsar Classification of Wetland Type* which includes 42 types, grouped into three groups of wetlands: Inland Wetlands, Coastal and Marine Wetlands, and Human - made (Fig. 1.9) [259].

Inland wetlands are defined as those occurring inland from the marine strandline, and are not of estuarine and deltaic origin [264]. They can subdivided into riverine, lacustrine palustrine and geothermal [264]. These wetlands cannot be considered isolated wetlands, because they are usually connected hydrologically to groundwater and biological through the movement of many mobile organisms [13].

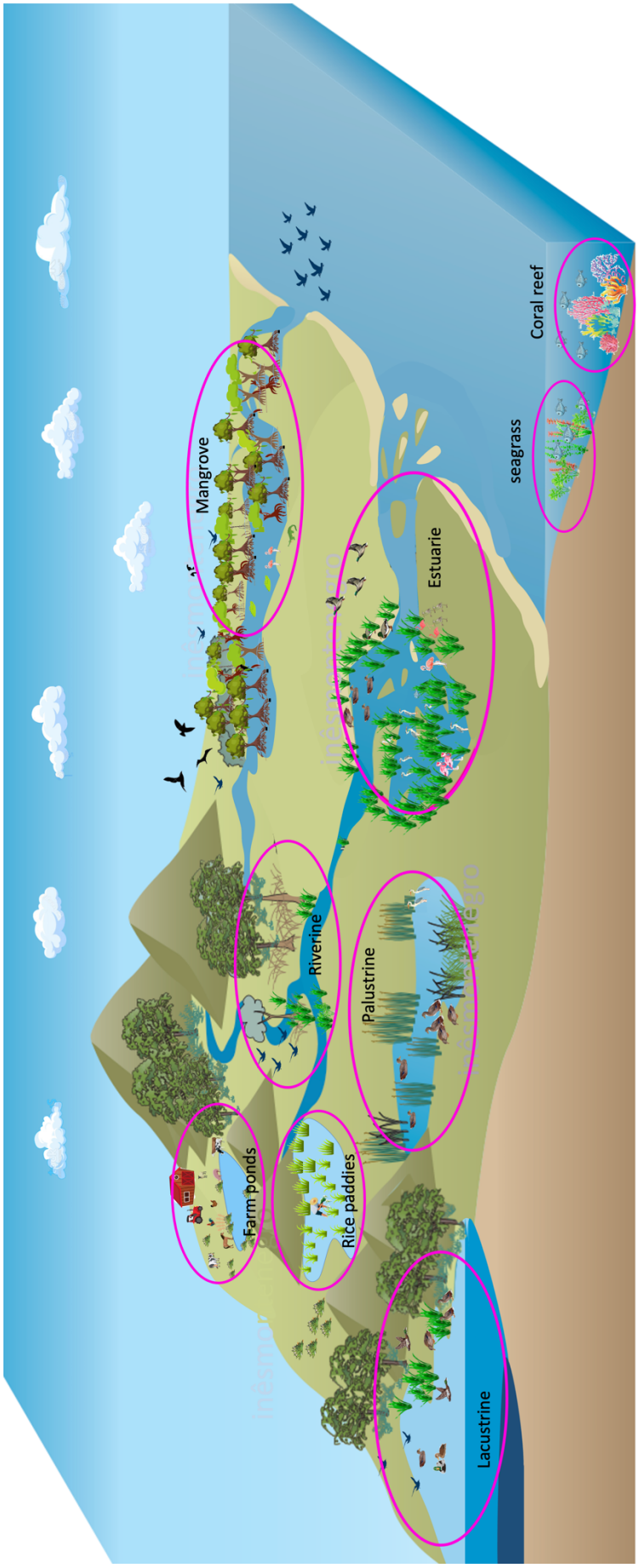


Fig. 1.9 - Representation in the landscape of different wetlands of each group of Ramsar classification: inland (riverine, lacustrine, palustrine), coastal (mangrove, salt marsh), marine (seagrass, coral reef) and human - made (farm ponds, rice paddies).

Coastal wetlands are defined as terrain that are subject to wetting by coastal processes, daily wetting of tidal flats by tides, wave swash and run - up, and storm surges and atmospheric-depression - induced high - water stands causing flooding of lowlands by incursion of estuarine water or marine water [265]. There are various types of coastal wetlands including, tidal freshwater marshes, tidal salt marshes, and mangroves (Fig. 1.9) [266].

Marine wetlands are defined as permanent shallow marine waters less than six metres deep at low tide, and includes sea bays and straits. Marine subtidal aquatic beds include kelp beds, seagrass beds and tropical marine meadows. Also coral reefs, rocky marine shores, rocky offshore islands and sea cliff are part of the group of marine wetlands (Fig. 1.9) [259]. Human - made wetlands are any type of wetland constructed or maintained by humans including fish and shrimp ponds, farm ponds, irrigated agricultural land as rice paddies, salt pans, dams, reservoirs, gravel pits, wastewater treatment ponds and canals (Fig. 1.9) [259].

Wetlands combine attributes of both aquatic and terrestrial ecosystems, but are neither, they have fallen between the cracks of the scientific disciplines of terrestrial and aquatic ecology [13].

Differences in the hydrological regime of diverse kinds of habitats, associated with other climatic and edaphic factors, create the diversity of wetlands with different structural and functional characteristics [263] and leads to considered, on a global scale, wetlands to be one of the most valuable resources per unit area [267, 268].

In 2006, the *Millennium Ecosystem Assessment* committee (MEA) described wetlands ecosystems as a complex of living communities (including human communities) and nonliving environment (Ecosystem Components) interacting (through Ecological Processes) as a functional unit which provides a variety of benefits to people (Ecosystems Services) [259].

From the point of view of *Ecosystems Services,* wetlands can be described as *provider*, due to the extensive food webs and the rich biological diversity that it sustains, being found even in very cold climate, and as *regulator* of air and water quality due to the great capacity of carbon sequestration from atmosphere and removal of pollutants / contaminants from water, with benefits within a climate change scenario mitigation [269].

1.2.3. Coastal Wetlands: estuarine salt marshes

Estuarine salt marshes occur on low - energy shorelines in temperate and high latitudes [264]. Most commonly, salt marshes occur at the heads of estuaries in areas where there is high sedimentation and little wave action [270]. The survival of a salt marsh depends primarily on the rate of sea level rise and change in the tidal range relative to movement of the shoreline, which can be stable, submerging or emerging [270]. Commonly, shorelines with

the largest salt marshes consist of mudflats or sand flats (known also as tidal flats), which are sustained primarily by sediment deposit from inflowing rivers and stream [270].

Where sediment supply is sufficient, then accommodation space for horizontal growth of the marsh along the shore becomes the determining factor for salt marsh survival and growth [270]. Salt marshes in coastal area differ from other wetlands because of the tidal flow, which floods the shoreline by a variable amount on a daily basis [271]. The amount of tidal inundation, which is ultimately determined by elevation, leads to differences in soil and water salinities, and in soil type and drainage patterns [271]. Tidal salt marshes are colonized by halophytic vegetation species, i.e., macrophytes adapted to complete their lifecycle in saline environments and flooded environments. The spatial distribution of halophytic vegetation (Fig. 1.10) over tidal salt marshes is not random nor spatially uncorrelated but is, on the contrary, organized in characteristic patches [272].

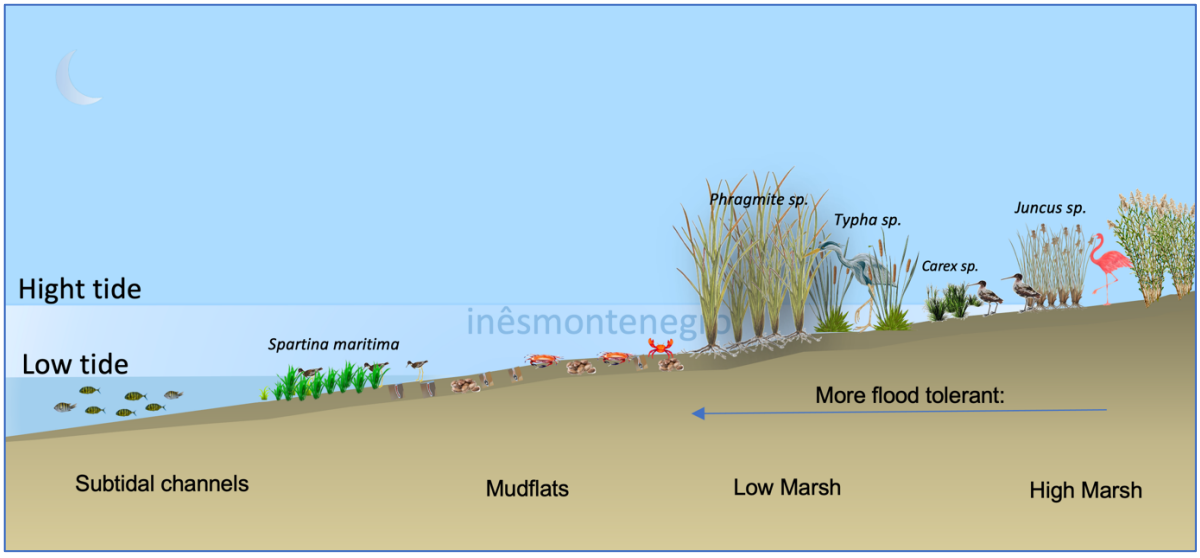


Fig. 1.10 - Schematic representation of plant zonation example in salt marsh wetland.

The high marsh has the lowest tidal inundation, which leads to low salinity in areas of high rainfall, soil runoff and river flow [271]. In the higher marsh zones, the plants species must tolerate periods of low nutrients availability, dryness and more interspecific competition [271]. In case of estuarine wetlands, the presence of sand and mud also determine the zonation because they can support totally different plants [273, 274]. Two examples of salt marsh in Portugal can be seen in Fig. 1.11 (Rio Lima estuary - Viana do Castelo) and Fig. 1.12 (Ria Formosa - Algarve).

At low marsh zones, with daily tidal inundation, the soil salinity levels are essentially the same as the seawater, with little seasonal change in evaporation and salt accumulation [271]. In general, the lower marsh zones have stable salinities and moisture levels and higher nutrients, but the plants require special adaptation to lower soil oxygen [271].

In the areas of regular inundation (e.g. subtidal channels) only resistant halophytes colonize it, but as marsh elevation increase, the plant diversity becomes greater [271]

Fig. 1.11 - Picture with salt marsh zonation at low tide, in *Rio Lima* estuary - Viana do Castelo.

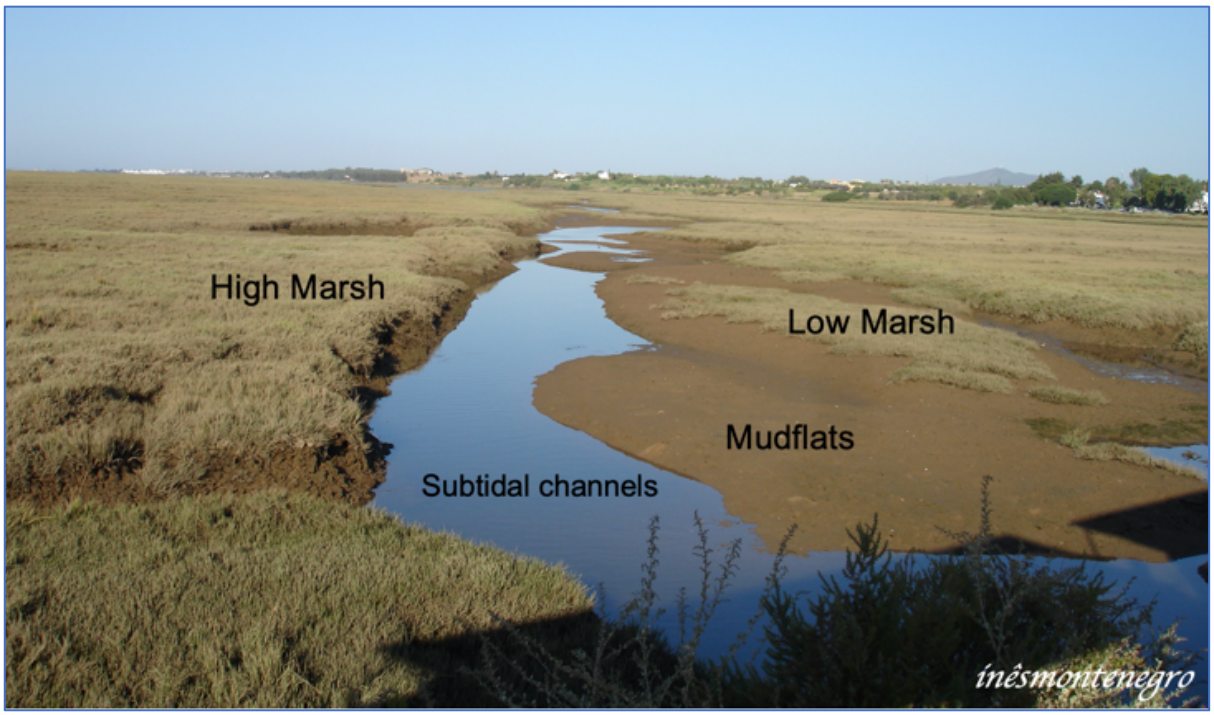


Fig. 1.12 - Picture with salt marsh zonation at low tide, in *Ria Formosa* - Algarve, Portugal.

Abiotic and biotic factors have been found associated to vegetation zonation in salt marsh wetland. For instance, Alvarez and Rogel et al. [275] observed that the discrimination of habitats by plant species can be associated with less or more tolerance to soil moisture, to salinity and to the distance to the shoreline. These authors observed that *Schoenus nigricans, Juncus maritimus, Limonium cossonianum, Sarcocornia fruticosa, Arthrocnemum macrostachyum, Sarcocornia fruticosa and Arthrocnemum macrostachyum* were detected in soil consistently more saline, soaked with a shallower water table, than the soil where *Crucianella maritima, Teucrium dunense, Ammophila arenaria and Lygeum spartum* were observed. In addition to salinity and soil hydrological regime, Lee et al. [276], observed that factors such as electronic conductivity, total nitrogen level, clay and sodium might play important roles in the formation of zonal plant communities of salt marshes.

1.2.3.1. Structural dynamics of Coastal Wetland

Defining wetlands precisely is even more complex than defining ecosystem, although, properly, most wetland definitions include both biotic (vegetation, animals, microorganisms) and abiotic (soils, hydrology) components [13]. In general, wetlands are shown to be a three components ecosystem, hydrology, physiochemical environment and biota (Fig. 1.13) [13].

The hydrology of the landscape influences and changes the physiochemical environment, which in turn, along with hydrology, determines the biotic communities that are found in the wetlands ecosystem [1]. The prevailing functions of the wetlands, or any landscape ecosystem for that matter include climate, which includes solar energy, temperature patterns and precipitation [13].

Ultimately, the climate associated with geomorphology of the landscape influence where and when water is present long enough to cause wetlands to exist [13].

The movement, distribution and quality of water is the primary factor influencing wetland structure and function [277]. To be classified as a wetland system, the presence of water must contribute to the formation of hydric soils, which are formed under flooded or saturated conditions persisting long enough for the development of anaerobic conditions during the growing season [278].

The hydrological regime in wetlands can vary tremendously with respect to the timing and duration of surface water inundation as well as seasonal patterns of inundation [277]. In coastal wetlands, tidal regime influence drives the movement and distribution of water and can range from permanent flooding in subtidal wetlands to less frequent flooding in others, with changes in water level occurring in daily or semi - daily regime [277].

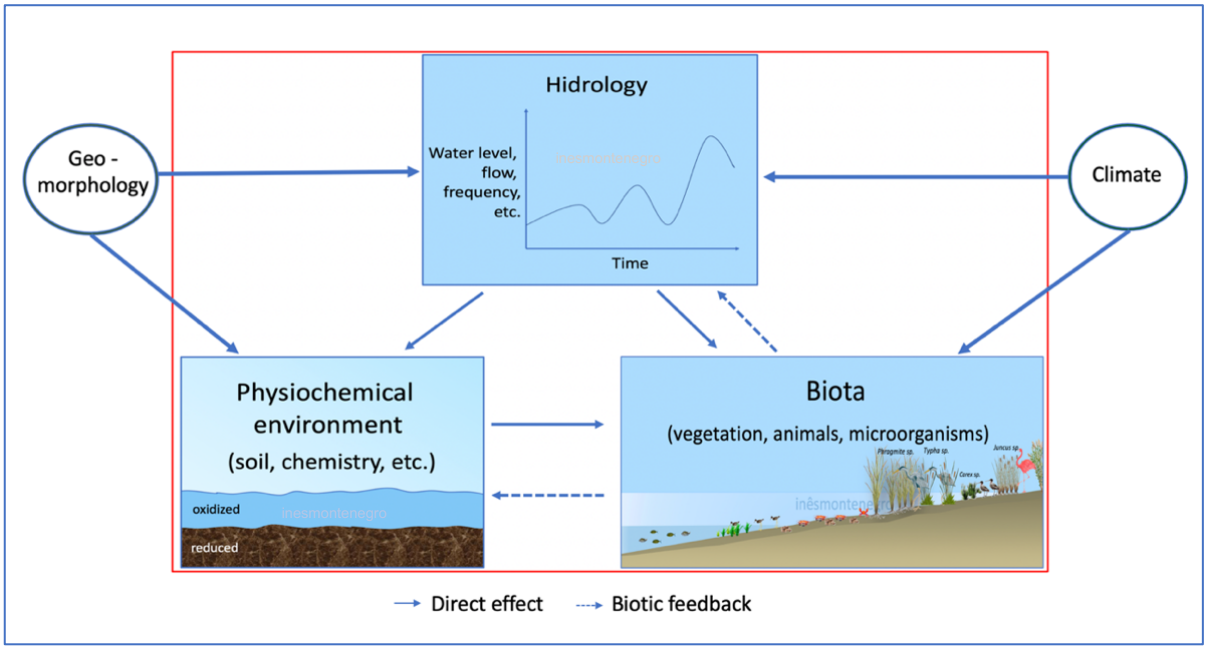


Fig. 1.13 - Conceptual model of wetland ecosystem, showing the three - component basic of wetland often used in wetlands definition, and the principal cause of wetlands which are climate and landscape geomorphology (modified from: Mitsch and Gosselink [13]).

The balance of water inflows (e.g. precipitation, surface flow, groundwater flow, tides) and outflows (e.g. evapotranspiration, surface flow, groundwater flow, tides), or the water budget (Fig. 1.14), as well as the geomorphology and soils will determine the timing, duration and patterns of flooding in the wetland [277].

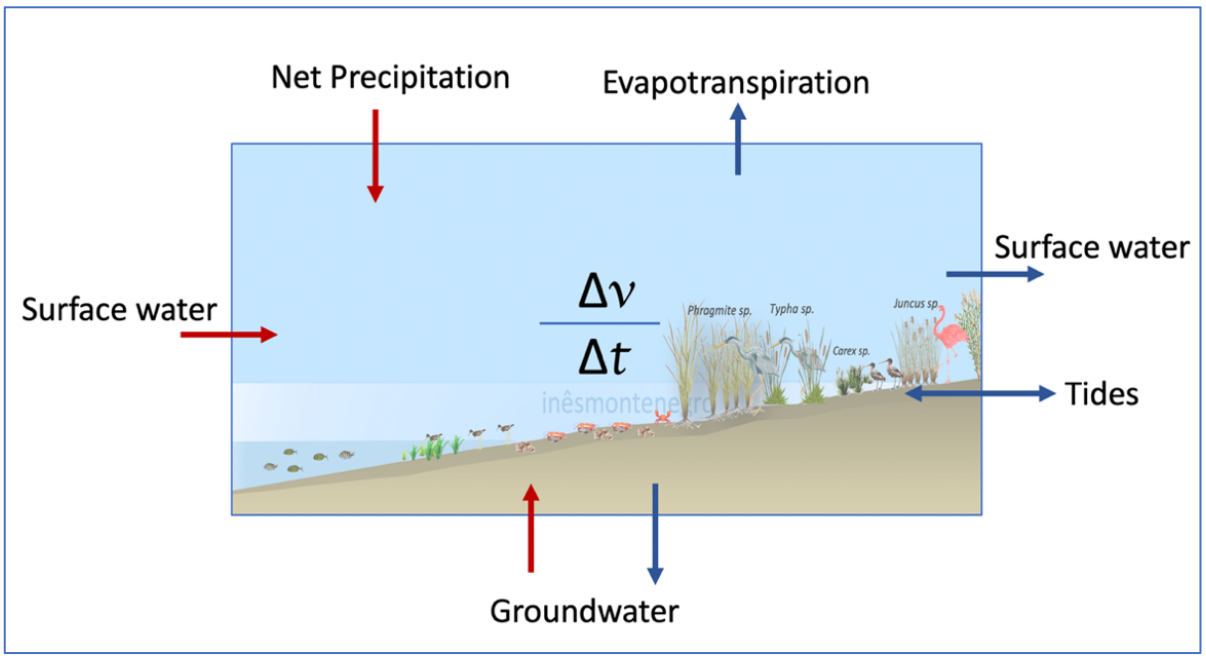


Fig. 1.14 - A wetland 's water balance describes its change in water volume over a given time period, and includes all sources of water inflows (surface water, groundwater, net precipitation and tides) and outflows (surface water, groundwater, evapotranspiration, tides) (modified from Mitsch and Gosselink [13]).

The hydrology is rarely stable, fluctuating over time and resulting in pulsing hydroperiods, being in coastal wetlands more visible [277]. Hydrologic pulses can alter productivity along a flooding gradient by altering the extent of flood subsidies and stresses in a wetland [14, 277]. Fig. 1.15 show that, when subsidies are high but stress is relatively low, hydrologic pulses can promote productivity by introducing water, sediments and nutrients while also removing waste materials and toxic substances [14, 277].

Flooding and drainage period can affect the physio - chemistry of wetlands in various ways. Water movement can introduce or remove sediment, salt, nutrients or other materials from wetlands, thereby influencing its soil composition and water chemistry [277].

The hydrological component also influences the structure and function of wetlands ecosystems through its influence on species richness, productivity, rates of organic matter accumulation, and nutrient cycling [277].

This abiotic factor may restrict plants species richness in an area subject to long - term flooding while enhancing it in areas with variable or pulsing hydroperiods (see plant zonation) [277]. Similarly, productivity is typically lower in permanently flooded, stagnant wetlands, or in drained wetlands than in slow - flowing or seasonally flooded wetlands [279]. The anaerobic conditions created under these inundated or flooded conditions often limit decomposition rates, thereby promoting organic matter accumulation in soils, and can alter redox reactions controlling nutrient transformation in wetlands soils [277].

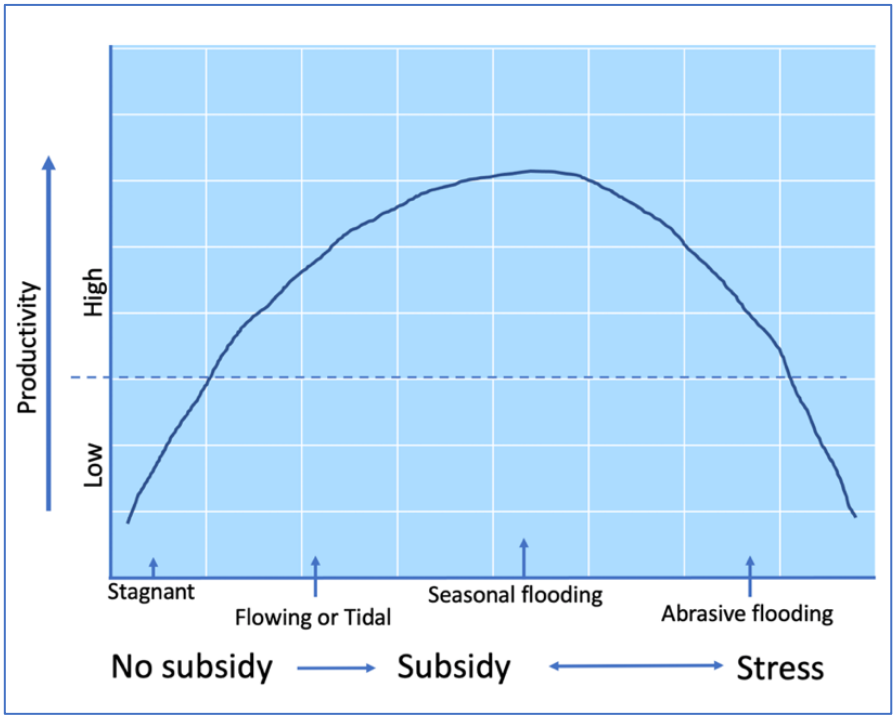


Fig. 1.15 - Graphic model illustrating the relationship between productivity and a flooding gradient in wetlands, shown as a subsidy - stress model (modified from Odum et al. [14]).

At a salt marsh ecosystem, the interaction at community level minimizes individual shortcomings. For instance, salt reduces the net primary productivity of vascular plants since they must invest more energy to exclude salt and sulfides, but at the community level this reduction may be compensated for by the contribution to productivity by marine algae, greater aeration of sediment due for instance to burrowing of fiddler crabs and the presence of more plants with C_4 photosynthetic mechanisms [14]. Most plants of the upper edge of salt marshes have C_3 (Calvin - cycle) photosynthetic pathways in their leaves [271]. The photosynthetic mechanisms of C_3 plant leaves is less efficient than C_4 mechanisms (found in *Spartina* and many tropical grasses) because it uses a slow enzyme system, denominated ribulose - 1,5 - bisfosfato carboxilase oxigenase (RuBisCO) in the leaf cells containing chlorophyll (mesophyll) to trigger the capture of carbon dioxide (CO_2) from openings stomata (Fig. 1.16a and b) [271]. In contrast, C_4 (Hatch - Slack pathway) plants have evolved a more efficient, less wasteful, two-step pathway in which the bundle sheath cells surrounding the food and water - transporting veins also have chlorophyll and store CO_2 up to a level that switches off the inefficient RuBisCO and turns on another enzyme, phosphoenolpyruvate carboxylase (PEP) that increases the efficiency of the RuBisCO [271].

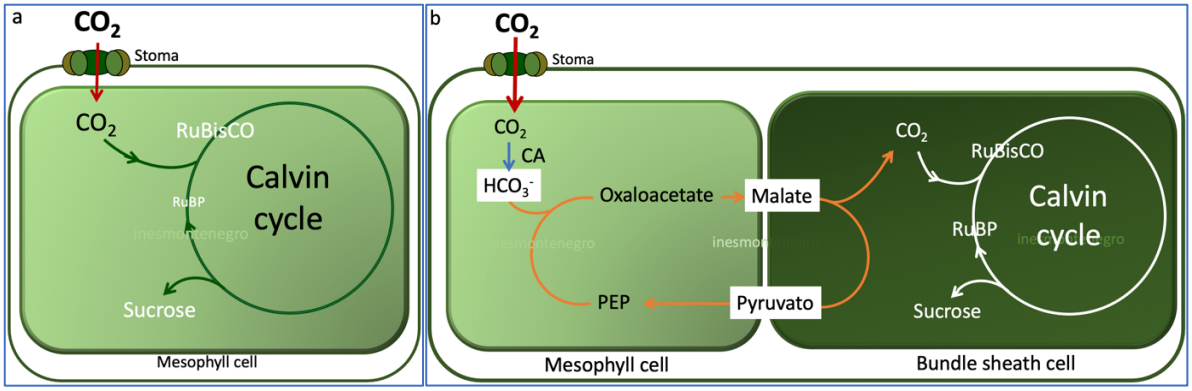


Fig. 1.16 - a) The C_3 and **b)** C_4 photosynthetic mechanisms.

The development and high productivity rates of salt marsh plant communities are associated with water depth flow rates, water chemistry, nutrient availability and soil and sediment characteristics [280]. The hydrologic regime can be thought of as a master variable with respect to all of these factors since it not only determines the hydroperiod but is also ultimately responsible for carrying nutrients and sediment, thus modifying soil type, into wetland ecosystems [280].

Molecular oxygen is rapidly used in salt marsh soils as an electron acceptor for microbial respiration and an oxidant for reduced chemical species. Many salt marsh plants including, *Spartina alterniflora*, *Spartina anglica*, and *Juncus roemerianus* can oxidize subsurface soils through root oxygen loss [15].

The subsurface redox environment is also further modified by macro - organisms (infauna) increased soil oxidation through bioirrigation, burrowing (e.g, burrowing of fiddler crabs crabs, Fig. 1.17), and physical mixing of the substrate [15]. Water movements caused by tidal infiltration and drainage, factors that vary over tidal to seasonal and interannual scales, also affect the redox environment [15]. Consequently, wetlands provide highly reactive surfaces that modify water quality [15].

Biofilms of salt marsh are the initial stage of more complex structures of multi - layered of microorganisms, also called microbial mats, that can form both within the salt marsh substrate (infauna) or on top of the marsh substrate (epifauna) [281]. The presence of benthic micro- and macro- organisms can change the edaphic factors. The first few millimeters of intertidal sediment can contain varied microbial and microphytobenthos biomass by organisms such as diatoms and cyanobacteria [281]. These organisms form secretions of a carbohydrate - rich exopolymer (also called extracellular polymeric substance), which surrounds the sediment matrix and may result in an increased interparticle cohesion that promote the stability of the slim sediment layers [281]. Beyond the physical alterations of the soil matrix, microbial mats can generate chemical changes in the sediment structure by trapping nutrients and forming minerals (i.e., apatite, carbonates) that are not regularly found in mudflats and marshes [281]. Usually, the microbial mats are better developed in mudflats than in marshes, due to presence of vascular plants and crab bioturbation that somewhat prevent extensive microbial mats formation [281].

Fig. 1.17 - Fiddler crabs and the burrowing in sediment of salt marsh at *Ria Formosa* - Algarve.

The high organic matter production rates, variable redox environments, and physically dynamic ecosystems that are regularly pulsed by tides are characteristic features of estuarine salt marshes [15]. In fact, it is generally accepted that the key to tidal wetlands function and structure is the pulsing water - flow regime, or the hydroperiod [14].

The presence of marsh plants modifies the hydrodynamics and suspended sediment concentrations, thus enabling the estabishment of the interrelationships between the vegetation characteristics and the sedimentation patterns [282, 283]. Vegetated soils of salt marshes differ considerably from nonvegetated soils in terms of overall autotrophic productivity and impacts of macrophyte root production on the cycling of macroelements [15].

The inundation or saturation of wetland soil by water leads to the formation of anaerobic condition as oxygen is depleted faster than it can be replaced by diffusion [277]. The rate of oxygen loss in flooded soils can vary depending on other soil conditions, such as temperature and rates of microbial community respiration. In most wetlands, small, oxidized layers of soils may persist on the surface or around the roots of vascular plants, but generally, anaerobic, or reduced, conditions prevail [277]. The prevalence of anaerobic condition in wetlands has a special impact on their biogeochemistry cycles, with important implications for carbon, nitrogen (Fig. 1.18), phosphorus, iron, manganese, and sulphur transformations [277].

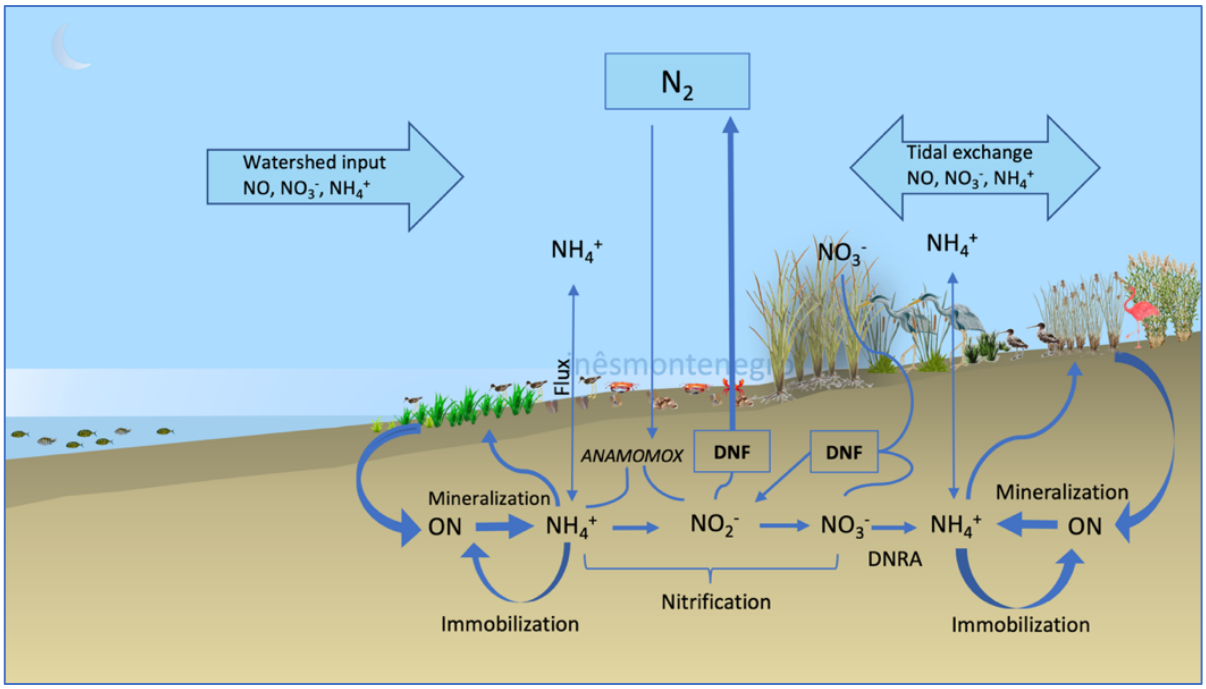


Fig. 1.18 - Summary of the major nitrogen cycling pathways in salt marshes (DNF - denitrification; DNRA - dissimilatory nitrate reduction to ammonium; ANAMOX - anaerobic ammonium oxidation). ANAMOX is an alternate pathway by which nitrogen can be exported from ecosystems. ANAMMOX uses ammonium to reduce nitrite to produce N_2. It is chemoautotrophic and, unlike denitrification, does not require organic carbon. The sizes of the arrows denote the relate magnitude of the processes (modified from Tobias et al.[15]).

While the nitrogen removal in wetlands is mainly attributed to microbial nitrification - denitrification and anammox processes the phosphorus removal is mainly due to physico - chemical processes such as precipitation, sedimentation and soil/sediment adsorption [284]. That mean, that removal processes of pollutants, varies with its type and with the interaction between the tree- component basic of wetland (hydrology - biota - environmental physiochemical conditions).

In wetlands, mainly in estuarine wetlands, nutrient availability is dramatically altered by agriculture or other practices the increase nutrient loading, contributing to change in ecosystem structure and function [277]. Through processes like denitrification and plant uptake, wetlands can help remove some of this excess nitrogen introduced to wetlands and aquatic ecosystems [277]. Wetlands can function effectively as sinks or transformers of pollutants, however their success depends on pollutants inflows/outflows and also the internal cycling rates [277].

Some of the interactions among the three basic component of wetlands, hydrology - biota - environmental physiochemical conditions, were mentioned, but many others were left unmentioned. Isolation of each component is not possible, as the interconnection and interdependence among them is clear.

1.2.3.2. Estuarine Wetlands contamination

Estuarine wetlands due to their natural beauty and specific localization, are the ecosystems with the most increasing pressure from human activities (e.g., extensive urban development [285, 286], intensive tourism [287, 288] and also concentration of anthropogenic activity such as industry, agriculture and nearby harbor activities [289]).

Estuarine areas can easily accumulate several types of contaminants, mainly derived from rivers or streams, storm - water run - off, sewage discharges, and atmospheric deposition, due to its unique properties such as high productivity, organic - rich matter scrap, fine grain of wetland soil, and anoxic environment [290, 291]. Several types of contamination, (e.g. PAHs, PCBs, organochlorine pesticide, PFASs, phenolic compounds, etc) have been detected in estuarine wetlands in different countries (Table 1.7).

Even though the concentrations are in the order of mg to ng per gram or liter, many of them can result in adverse effects to biological communities. So, it is important to remove these pollutants from the aquatic environment and wetlands promotion can be a solution.

To use the word contaminant and pollutant must be taken into account the concept of each of them. The contamination is simply the presence of a substance where it should not

be or at concentrations above background [292]. Pollution is contamination that result in or can results in adverse biological effect to resident communities [292]. All pollutants are contaminants but not all contaminants are pollutants. Also, differentiating pollution from contamination cannot be done only on the basis of chemical analysis because such analysis provides no information about bioavailability or on toxicity [292].

Table 1.7 - Organic pollutants concentrations detected in sediment and water of estuaries at different locations of the world reported in the last twelve years (2007 - 2020).

Organic Pollutants	Concentration	Estuarine area	Sampling year	Reference
PAHs	25 - 106 μg g^{-1} (d.w.) 38 - 60 ng L^{-1} (water)	Douro river estuary - Portugal	2011	Rocha et al. [293]
	0.076 - 8.76 ng g^{-1} (d.w.) 0.35 - 16.72 ng g^{-1} (d.w.) 0.13 - 18.81 ng g^{-1} (d.w.) 7.74 - 0.28 ng g^{-1} (d.w.) 0.30 - 34.82 ng g^{-1} (d.w.)	Portugal rivers estuaries: Aveiro estuary Guadiana estuary Mira estuary Sado estuary Tejo estuary	2009	Serafim et al. [294]
	45 - 7901 ng g^{-1} (d.w.)	Ría de Arouca - Spain	2011	Pérez - Fernandéz [289]
	49 - 1065 ng g^{-1} (d.w.)	Guadeloupe river - France	2008	Ramdine et al. [295]
	0.57 - 4.23 μg g^{-1} (d.w.)	Huveaune river estuary - France	2010 - 2011	Kanzari et al. [296]
	2.9 - 58.6 ng L^{-1} (water)	Partizanskaya estuary - Russia	2012	Chizhova et al. [297]
	2.2 - 159.6 ng L^{-1} (water)	Tumen estuary - Russia	2017	Chizhova et al. [297]
	3.3 - 630 ng g^{-1} (d.w.)	Hooghly river estuary - India	2013	Mitra et al. [298]
	146 - 249 ng g^{-1} (d.w.)	Paranaguá river estuary - Brazil	2016	Garcia et al. [286]
	<0.50 - 234 ng g^{-1} (d.w.)	Paranaguá estuarine system - Brazil	2017	Garcia and Martins [299]
	19.7 - 30054.5 ng g^{-1} (d.w.)	Bahía Blanca estuary - Argentina	2011 - 2013	Oliva et al. [300]
	12.54 - 21.20 ng g^{-1} (d.w.)	Sarawak estuary - Malaysia	2020	Asare et al. [301]
	N.D. - 24.91 μg L^{-1} (water) N.D. - 7792 μg g^{-1} (d.w.)	Buffalo River estuary - South Africa	2015 - 2016	Adeniji et al. [302]

(continued)

(Table 1.7 continued)

PAHs	163.3 - 9943 ng g^{-1}(d.w.)	Yangtze river estuary - China	2015 - 2016	Chen et al. [303]
	69.1 - 1297 ng g^{-1} (d.w.)	Pearl river delta - China	2015	Li et al. [304]
	29 - 438 ng g^{-1} (d.w.)	Nainliu mangrove - China	2010	Kaiser et al. [305]
Polychlorinated biphenyls (PCBs)	0.90 - 98.85 pg g^{-1} (d.w.)	Mondego river estuary - Portugal	2009	Nunes et al. [306]
	62.06 - 73.81 ng g^{-1} (d.w.)	Tiber river estuary - Italy	2014 - 2015	Montuori et al. [307]
	29.2 - 64.4 ng g^{-1} (d.w.)	Volturno estuary river - Italy	2017 - 2018	Montuori et al. [308]
	9 - 20 ng g^{-1} (d.w.)	Hythe salt marsh and Beaulieu estuary - England	2016	Celis - Hernandez et al. [309]
	0.28 - 7.7 ng g^{-1} (d.w.)	Hooghly river estuary - India	2013	Mitra et al. [298]
	N.D. - 0.43 ng g^{-1} (d.w.)	Samborombón estuary; Chiquita estuary; Quequén grande estuary - Argentina	2015	Díaz-Jaramillo et al. [310]
	1.83 - 36.68 ng g^{-1} (d.w.)	Shuangtaizi river estuary - China	2013	Yuan et al. [311]
	1.86 - 148.22 ng g^{-1} (d.w.)	Yangtze river estuary - China	2010 - 2011	Gao et al. [312]
	0.08 - 26 ng g^{-1} (d.w.)	Several Portuguese estuarine wetlands	2007 - 2008	Carvalho et al. [313]
	5.68 - 8.14 ng g^{-1} (d.w.)	Tiber river estuary - Italy	2014 - 2015	Montuori et al. [307]
	3.50 - 9.89 ng g^{-1} (d.w.)	Volturno river estuary - Italy	2017 - 2018	Montuori et al. [308]
	0.19 - 3.98 ng g^{-1}(d.w.)	Sarno river estuary - Italy	2008	Montuori et al. [314]
	1.8 - 8.5 ng g^{-1} (d.w.)	Mangroves of Hainan Island - South China	2014	Qiu et al. [315]
	< 0.2 - 41.0 ng g^{-1} (d.w.)	Nanliu mangrove - China	2010	Kaiser et al. [305]

(continued)

(Table 1.7 continued)

Polychlorinated biphenyls (PCBs)	0.14 - 18.6 ng g^{-1} (d.w.)	Hooghly river estuary - India	2013	Mitra et al. [298]
	120 - 911 ng g^{-1} (d.w.)	iSimangaliso Wetland Park - South Africa	2015 - 2016	Buah - Kwoflie [316]
	N.D. - 1.52 ng g^{-1} (d.w.)	Samborombón river estuary; Chiquita river estuary; Quequén Grande river estuary - Argentina	2015	Díaz-Jaramillo et al. [310]
	0.07 - 7.25 ng g^{-1} (d.w.)	Shuangtaizi estuary - China	2013	Yuan et al. [311]
TPHs	0.25 - 0.63 mg g^{-1} (d.w.)	Lima river estuary - Portugal	2010	Ribeiro et al. [317]
	5.4 - 17.6 mg g^{-1} (d.w.)	Lima river estuary - Portugal	2011	Almeida et al. [318]
	0.01 - 0.11 mg g^{-1} (d.w.)	Yangtze River estuary -China	2015	Wang et al. [319]
	2.0 - 19.1 µg L^{-1} (water) 2.1 - 16.8 µg g^{-1} (d.w.)	Kundalika river estuary - India	2014	Rao et al. [320]
	7.2 - 39.7 µg L^{-1} (water)	Amba river Estuary - India	2014	Rao et al. [321]
	0.01 - 0.02 mg g^{-1} (d.w.)	Arvand river estuary - Iran	2018	Moazampour et al. [322]
	7.65 - 477 µg L^{-1} (water) 0.013 - 1.10 mg g^{-1} (d.w.)	Buffalo river estuary- South Africa	2016	Adeniji et al. [323]
	33 - 846 µg g^{-1}(d.w.)	Mira river estuary - Colombia	2017	Ordóñez and Díaz [324]
Per- and Polyfluorinated substances (PFASs)	< 0.27 - 22.6 ng g^{-1} (d.w.) < 0.02 - 2775 ng L^{-1} (water)	Ebro Delta - Spain	2015 - 2016	Pignotti et al. [325]
	0.030 - 2.2 ng g^{-1} (d.w.)	Elbe Estuary - Germany	2016 - 2017	Joerss et al. [326]
	0.2 - 5.3 ng g^{-1}(d.w.)	Hythe salt marsh and Beaulieu estuary - England	2016	Celis-Hermandez et al. [309]
	0.02 - 19.2 pg g^{-1} (d.w.)	Charleston estuary - USA	2012	White et al. [327]
	0.60 - 11.32 ng g^{-1}	Ganges river estuary-India	2011	Corsolini et al. [328]
	1600 - 5300 pg g^{-1} (d.w.)	Nakivubo wetland - Uganda	2015	Dalahmeh et al. [329]
	7.5 - 84.2 pg g^{-1} (d.w.)	Pearl River estuary and Delta - China	2017 - 2018	Wang et al. [330]
	56.2 - 586.3 pg g^{-1} (d.w.)	Riverine and Coastal sediment - Beibu Gulf - China	2017	Pan et al. [331]
	1.7 - 12 ng L^{-1} (water)	Yangtze river estuary - China	2012	Zhao et al. [332]
	26.2 - 164.0 ng L^{-1} (water) 62.6 - 566.5 ng g^{-1} (d.w.)	Yangtze river estuary - China	2008	Pan and You [333]

(continued)

(Table 1.7 continued)

Phenolic endocrine disruptors	< 1.2 - 3700 ng L^{-1} (water)	Minho river estuary - Portugal	2012	Salgueiro - Gonçalves et al. [334]
	19 - 877 ng L^{-1} (water)	Lima river estuary - Portugal	2011	Rocha et al. [88]
	45 - 205 ng L^{-1} (water)	Ave river estuary - Portugal	2010	Rocha et al. [90]
	132 - 32554 ng L^{-1} (water)	Mondego river estuary - Portugal	2010	Rocha et al. [335]
	< 0.005 - 0.337µg L^{-1} (water)	Northwest coastal estuary - Spain	2011 - 2012	Salgueiro-González et al. [96]
	5.8 - 15.4 ng g^{-1} (d.w.)	Hythe salt marsh and Beaulieu estuary - England	2016	Celis-Hermandez et al. [309]
	< 1.0 - 120.6 ng dm^{-3} (water)	Gizdepka estuary - Poland	2011 - 2012	Staniszewska et al. [336]
	< 50 - 32000 ng g^{-1} (d.w.)	Auckland estuary - New Zealand	2008	Stewart et al. [337]
	94.59 - 4587 ng L^{-1} (water)	Hooghly river estuary - India	2016	Mukhopadhyay et al. [338]
	6.8 - 134 ng L^{-1} (water)	Liaohe river estuary - China	2009	Yang et al. [339]
	0.98 - 43.8 ng L^{-1} (water) N.D. - 7.87 ng g^{-1} (s.d.w)	Yangtze river estuary - China	2010	Shi et al. [340]
	<1 - 11.96 ng L^{-1} (water) N.D. - 13 ng g^{-1} (d.w.)	Yangtze river estuary - China	2011	Nie et al. [341]
	0.01 - 29 µg g^{-1} (d.w.)	Anzali wetland - Iron	2010	Mortazavi et al. [342]
	85.5 - 3366 ng L^{-1}(water) 0.4 - 3ng g^{-1} (d.w.)	Pearl river delta - China	2013	Chen et al. [343]

d.w.- dry weight of sediment; N.D. - not detected;

1.2.3.3. Estuarine salt marshes: a biological filter for the environment

Estuarine salt marshes can act as a water purifiers ("water living filters" [344]) of the natural environment due the functions they can perform in the hydrological and biogeochemical cycles [345]. This capacity has been used in different parts of the world since the 1950s [346]. The wetland ecosystems have special characteristics which make them particularly suitable to removal pollutants from the environment as a passive and natural-based solution:

1. The semi-aquatic systems, which normally contain large quantities of water, therefore the flooding caused by wastewater addition is a normal feature for these systems [346];
2. The oxic and anoxic soils condition, in which organic matter breakdown takes place through special pathways involving electron acceptors other than oxygen, e.g. nitrate,

sulphate and iron. As a result, nitrogen as well as phosphorus dynamics are very different from those in unpland ecosystems [346];

3. The high productivity condition, nutrients entering a wetland ecosystem are quickly assimilated because of the high productivity and demand of nutrients for emergent vegetation. This vegetation is capable of taking up large amounts of nutrients and responding to enrichment of nutrients with enhanced growth [346] and also with rapid microbial activities and turnover [290]. For instance, the turnover periods of nitrogen and phosphorus in mangrove ecosystems dominated by *Kandelia candel* (20 years old) were 7 and 10 years, respectively, which were shorter than that in *Bruguiera sexangula* community with 12 years turnover periods for both N and P [290, 347].
4. The architecture of roots in salt marsh plants with large root system providing a large rhizoplane surface area for sorption of pollutants (e.g., *P. australis,* Fig. 1.19 a) and b) [348].

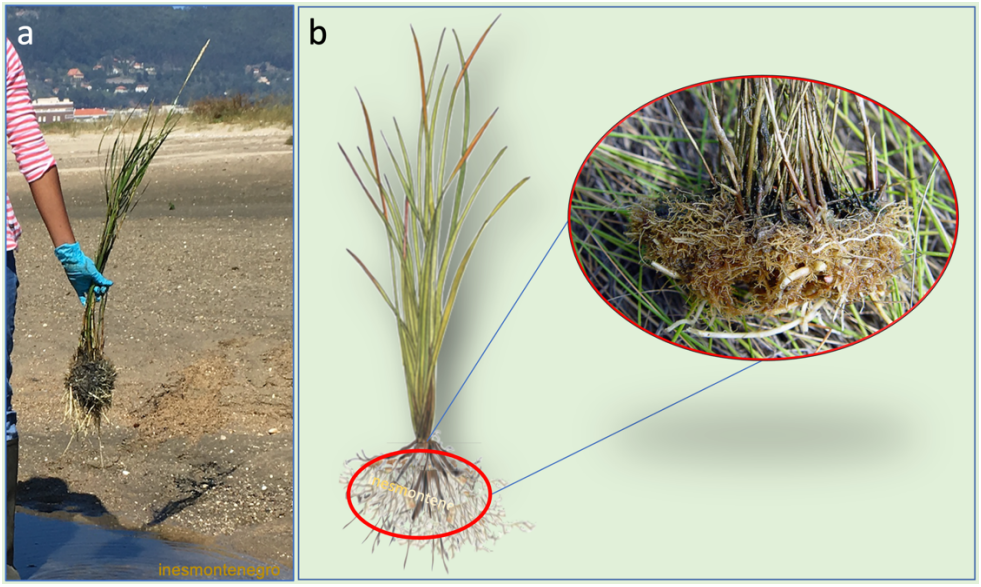


Fig. 1.19 - **a)** *P. australis* collected in the river *Lima* estuary; **b)** root zone of *P. australis.*

5. The texture of sediment. The fine grain of wetland soil can easily accumulate a variety of pollutants [290, 291].
6. The high rate of primary productivity in wetlands contributes to the great organic matter content of wetland soil [15]. As a consequence an increased in the accumulation capacity of pollutants into the soil matrix can be observed.
7. Wetland ecosystems increase the pollutants retention time. The pollutants retention rate is inversely related to river channel depth and positively correlated to water residence time [349]. For instance, when the flow increases, phosphorus removal

efficiency decreases due to less water retention time, while, increased inflow takes more phosphorus into the wetlands [350].

8. Salt marsh vegetation has specific adaptations to flooding and salinity stress, being in direct contact with the compounds that are in the aquatic matrix of coastal wetlands [351, 352]. For estuarine salt marsh vegetation to complete their life cycle in these environments (reproduction, germination and development) they are dependent on a number of morphological and physiological needs [271], to stressing factors [272]. The presence of water and subsequent lack of oxygen may create hydric (anaerobic) soils in which plants are adapted to flooding, ponding, or saturated conditions. Soils inundated or saturated by water at a rate sufficient to support a prevalence of vegetation typically adapted to living with oxygen deficit conditions. Systemically formation of adventitious roots (e.g. Fig. 1.20 *P. australis)*, aerenchyma (e.g. Fig. 1.21 a; *P. australis*), and root oxygen loss barriers (e.g. *P. australis* and *Glyceria maxima* [353]; *Juncus effusus* and *Juncus inflexus*) [354, 355] are some of these adaptations. Difference in anatomical structure of aerenchyma, between an aquatic plant (*P. australis)* and a terrestrial plant (*Zea mays L.*) are shown in Fig. 1.21 a) and b), respectively. More details about adaption to flood tolerance and anoxic conditions of vegetation are mentioned in section 3.2. Wetlands vegetation in Constructed Wetlands.

Fig. 1.20 - Adventitious roots in *P. australis.*

In the specific case of coastal wetlands, the vegetation has morphological, physiological and anatomical adaptations to salinity. For instance, the reduce plant size and increased vascularization of internal tissues (e.g. *Juncus roemerianus* [356]), the storing the salt in succulent leaves or stems which are later shed as dry tissues

(e.g. *Salicornia*, *Sarcocornia*, *Arthrocnemon*), salt glands (Fig. 1.22 a), e.g. *Spartina* spp., *Puccinellia*, *Distichlis, Limonium* spp., *Atriplex,* as well as some mangroves: *Avicennia*) [271] and salt bladders (Fig. 1.22 b), e.g. *Atriplex Prostala*, *Halimione Portulacoides* and *Atriplex halimus* [357]) that excrete excess salt, are another physiological adaptation to salinity conditions of salt marsh plants.

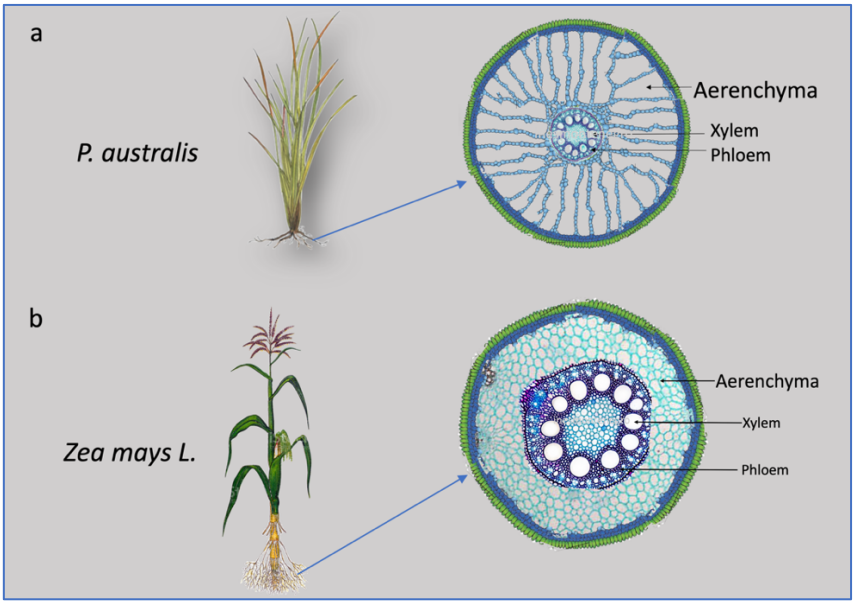


Fig. 1.21 - Difference in anatomical structure of aerenchyma, between an aquatic plant (*P. australis)* and a terrestrial plant (*Zea mays L.*); **a)** Root section of *P. australis,* the schematic representation of root section is reproduced from microscope image present in Fogli *et al.* [16] and Baldantoni *et al.* [17]; **b**) Root section of *Zea mays L.*, the schematic representation of root section is reproduced from microscope image present in Smith and Smet [18].

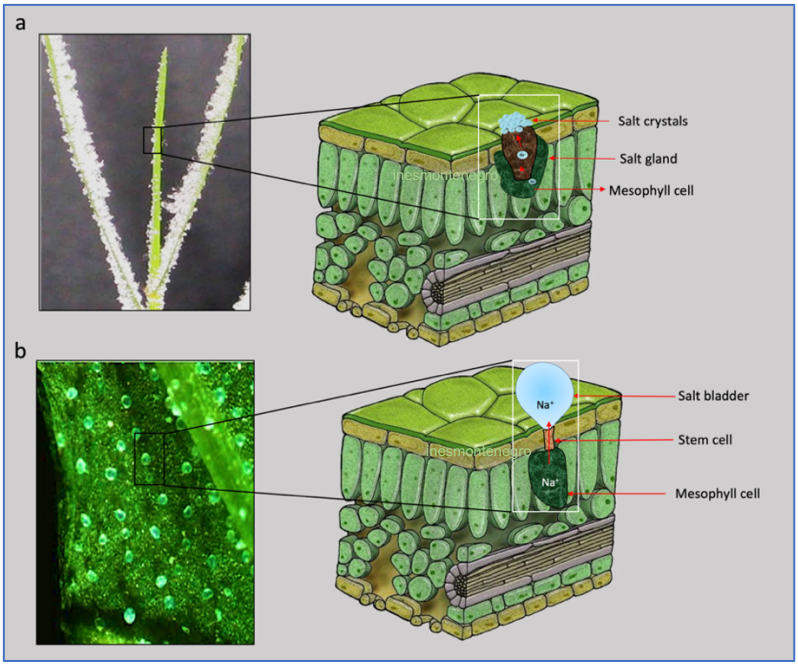


Fig. 1.22 - **a)** Representation of salt gland and **b)** salt bladder in halophytes plants (adapted from Natarajan et al. [19] and Yuan et al. [20]).

10. The environmental contamination promotes strong stress to vegetation [352], however wetlands plants have physiological and anatomical adaptations to response to this environmental stressor [358] and are particularly suitable for phytoremediation purposes [24, 25]. For instance, cell wall binding, chelation and sequestration into the cell vacuoles and the production of ROS (reactive oxygen species) – scavenging proteins are some of the adaptation strategies to live with toxic stress promoted by environmental contamination [352, 361, 362].
11. The salt marsh plants have a high capacity of compounds enzymatic degradation, transforming in less toxic compounds for the ecosystem, when compared with plants of freshwater wetlands. A good example of this capacity is shown in two works of Chu et al. [363, 364]. These authors compared accumulation, distribution and transformation process between *P. australis* and *Oryza sativa L*. (rise) and verify that both plants accumulated substantial quantities of DDT and PCB but with different degradation capacities. The enzyme systems in salt marsh plants, *P. australis,* were much more effective than those in rice in the degradation and transformation of the organic pollutants. Additionally, these authors also verify that the degradation and transformation of both compounds, DDT and PCBs, by *P. australis* enzymes were partly mediated by peroxidase and the plant P - 450 system.

All these characteristics shows the high potential for salt marsh estuarine plants for the remediation aquatic system, avoiding contamination propagation in the environment.

1.2.3.4. Salt marsh plants and the remediation of phenolic compounds

The role of plants in environment remediation contaminated with organic pollutants can be categorized in four stages: phyto - uptake, translocation, bioaccumulation and biodegradation. The degradation within plants is subjected to *in planta* detoxification mechanisms such as transformation, conjugation and compartmentalization [365]. The process involving remediation of organic compounds depends on the physicochemical characteristics of the compound, the plant species, the medium and the rhizosphere microbial community [352, 366].

The more relevant physicochemical characteristics of the compounds that can condition the phytoremediation processes are octanol – water partition coefficient (log k_{ow}), acidity constant (pk_a), aqueous solubility (S_w) and octanol solubility (S_o), as well as the

concentration of the pollutant [365]. Among all these characteristics, the role of log k_{ow} value is of significant concern due to its direct involvement in determining how hydrophobic or lipophilic the compound is. Usually, the compounds with lower log k_{ow} values (0.5 - 3.0) are easily taken up by plants as compared to those having higher log K_{ow} values [365].

Among compounds transported across plants tissues, volatiles ones can be released through the leaves to the atmosphere via transpiration [367]. In case of non - volatile compounds they are subject to sequestration or degradation processes in the internal structures of the plant (phytoextraction, phytodegradation), or may be degraded within the rhizosphere area, through the microbial community (phytostimulation or phytodegradation) [368]. The phytostabilisation is another remediation process, where the bioavailability of hydrophobic organic compounds from the substrate matrix is reduced [369]. In addition to biotic processes, several abiotic ones are involved in the removal of organics, with sorption to plant roots, substrate or biofilm in the rhizosphere considered as the major among them [352].

Wetlands, especially estuarine salt marsh have been suggested as promising tools for phenolic compounds phytoremediation (such as NP, OP and PCP) when present in aquatic environment due to their unique hydrology, physiochemical and biota conditions. Experiments in natural estuarine wetlands have shown that these ecosystems can effectively retain these contaminants. For instance, Yang et al. [339] observed that 94.3 % of NP and 96.3 % of OP were removed from water, i.e. they were retaining in estuarine wetlands red bed. Zhao et al. [370] observed high removal rates of PCP in sediment planted with *P. communis Trin* (90 %), *T. orientalis* (99 %) and *S. validus Vahl* (99 %) when compared with control (no plant) treatment (30 %).

The phenolic compounds can be accumulated into salt marsh plants body (bioaccumulation). Zhao et al. [370] observed the accumulation of PCP in roots of *P. communis* Trin, *T. orientalis* and *S. validus* Vahl, while in the above - ground parts PCP was undetectable.

However, phenolic compounds can be degraded into less toxic compounds by the enzymatic activity in the rhizosphere of salt marsh plants. For instance, Nejla et al. [371] when examined the phytoremediation potential of *P. australis* salt marsh plant grown in PCP and cadmium co-contaminated soils, observed a significant positive correlation between soil dehydrogenase activity (DHA) and PCP removal in planted soil. This observation imply that *P. australis* enhanced the biodegradation of PCP through the activities of enzymes and the microorganisms. Also, the microbial community isolated from salt marsh plants rhizosphere has the potential to degrade phenolic compounds. For instance, Toyama et al. [166] verify that three bacterial strains, *Stenotrophomonas* sp. strain IT-1 and *Sphingobium* spp. strains IT- 4 and IT- 5, isolated from *P. australis* rhizosphere, were able to use OP and NP as carbon

source. It has also been observed that the plant species exerts a significant influence on the composition of the microbial community and these changes can be plant - specific and associated to their root exudates [372, 373]. Work done by Cheema et al. [374] and Yang et al. [375] observed both microbial and enzymatic activities when influenced by the root exudates promoted the remediation of the environment, removing contaminants. Also, climate conditions influence the microbial activity. For instance, Yang et al. [339] observed a significant correlation between NP and OP removal rate and water temperature. These authors concluded that the high temperature benefits *P. australis* growth and microbial metabolism activity, and consequently favored the removal, by biodegradation, of phenolic compounds.

Current studies indicate that salt marshes can be suitable for the remediation of organic pollutants, namely phenolic compounds and their role in that degradation deserved investigation, either in natural or constructed wetlands.

1.3. Constructed Wetlands

1.3.1. Definition

CWs, also referred to as Artificial Wetlands, are man-made systems based in natural wetlands and are considered very promising technologies as water quality improvement. This "green treatment technology" uses the natural process to assist in water quality improvement and they are designed to take advantage of many of the same processes that occur in natural wetlands [376]. While natural wetlands are characterized by extreme variability in functional components, making it virtually impossible to predict responses to wastewater application [377], CWs are a controlled manner of treatment, more predictable and possible to manipulate.

These water treatment systems have been implemented with success in Europe, America, Australia and New Zealand [378, 379]. In general, it is consensual that CW are a reasonable option for treating wastewater, due to self - adaptive treatment processes [380], lower cost and less operation and maintenances requirements [381]. The main disadvantage of CWs systems is their relatively slow rate of operation in comparison to conventional wastewater treatment technology [382].

Over the past few decades, CWs have been proven functional in the treatment of various types of wastewater, such as domestic sewage, urban run-off, stormwater, industrial wastewater and landfill leachate, showing capacity for removing organic pollutants [22, 383–386] microcontaminants, such as endocrine disruptors [387], pharmaceutically active compounds and personal care products [384, 388], total suspended solids (TSS) [389, 390], nutrients [391], human pathogens [392], metals [393], [394]) and mitigation of algal bloom [395]. Aside from its effectiveness in pollutants removal, CWs have also displayed cost efficiency in wastewater treatment [383, 396]. More recently, CWs have been applied to deal with more specific pollutants, such as some types of organic pollutants that are more recalcitrant to conventional processes used in most wastewater treatment plants (WWTPs) [22, 369, 387]. In fact, 40 - 45% of persistent organic pollutants remain in the sewage of treatment plants, being the residues discharge to nearby rivers [397]. In this sense WWTPs effluents can be considered a critical source of contamination of chemical compounds, such as phenolic compounds, into watercourses (e.g. river and surface waters) [398], despite the science and engineering of wastewater treatment having significantly progressed in the last fifty years [399].

CWs systems may support a large spectrum of biogeochemical reactions and also several environmental conditions at the wetland system scale and this is the fundamental key for organic contaminant transformation [400]. The prevailing conditions of CWs generally determine both the thermodynamic feasibility of chemical reactions and the activity of

indigenous microbial community with enzymatic capacity to achieve the target biochemical reactions [400]. CWs are intricate biologic reactors, characterized by considerable fluxes of material and energy governing chemical reactions over spatial and temporal gradients [400]. Those fluxes of material and energy are particularly accentuated in certain zones, such as the rhizosphere, allowing the maintenance of thermodynamic non - equilibrium conditions and enabling the occurrence of various reactions with exergonic free energy changes [401]. The biogeochemical reactions in CWs affecting contaminant remediation mainly depend on types of processes simultaneously occurring at different scales: (1) the various and co-existing redox processes at the wetland system scale, and (2) the processes occurring at the rhizosphere scale [400].

CW systems consist of four main components: plants, substrate that includes sediment or soil, microbial biomass and an aqueous phase loaded with the chemicals (wastewater), and typically include beds filled with poorly drained graded medium and aquatic plants [400]. The diversity and complexity of these systems are principally the result of interactions between the main components [402]. For instance, the variation of hydrologic regime and composition of the substrate strongly influences the vegetation [402] but plants species also influence the structure of the microbial community in the system (e.g. Chaudhary et al. [403]). Additionally, also the environmental factors influence the structure of the microbial community [404]. Since restoring or creating wetlands is dependent on mimicking these components and their interactions, an understanding of the role of each one and the ways they influence each other is important for the correct design and selection of a CW. A simplified analysis of each compartment (Fig. 1.23) and their interactions will be described in the next paragraphs.

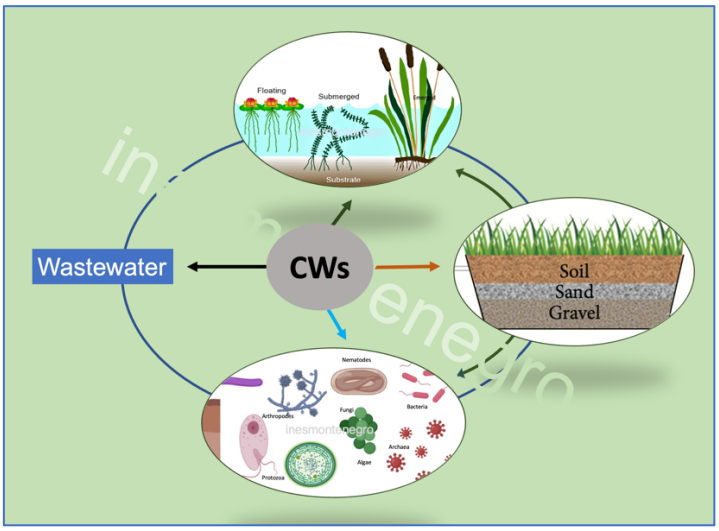


Fig. 1.23 - The main compartments of CW systems: plants (floating; submerged; emerged), substrate that can contain sand, gravel and soil or sediment, microbial biomass and an aqueous phase loaded with the chemicals (wastewater).

1.3.2. Wetlands vegetation in Constructed Wetlands

The aquatic plants growing in wetlands are usually called macrophytes. This term includes aquatic vascular plants (angiosperms and ferns), aquatic mosses and some algae that have tissues that are easily visible [405]. Macrophytes are photoautotrophic organisms that use the solar energy to assimilate inorganic carbon from the atmosphere to produce organic matter which subsequently provides the energy source for heterotrophs (such as bacteria) [405]. Macrophytes are morphologically adapted to growing in a water saturated substrate by virtue of large internal air spaces for transportation of oxygen to roots [406]. This oxygen leakage from roots creates oxidized conditions in the otherwise anoxic substrate and stimulates both aerobic decomposition of organic matter and growth of nitrifying bacteria [406]. According to their life form, macrophytes growing in wetland may be classified in the following three groups: leaved, emergent and submerged. Table 1.8, show some examples of aquatic macrophyte and the characteristics of each group used in constructed wetland [26]

Table 1.8 - Characteristics of the three major group of macrophytes present in wetlands [26]. Plant species examples are also indicated.

Group	Characteristics	Examples
Emergent	Growing within a water table range from 50 cm below the soil surface to a water depth of 150 cm or more Produce aerial stems and leaves and have an extensive root and rhizome-system Morphologically adapted to growing in a water-logged or submersed substrate by virtue of large internal air spaces for transportation of oxygen to roots and rhizomes	*P. australis* *Glyceria* spp. *Eleocharis* spp. *Typha* spp. *Iris* spp. *Zizania aquatica*
Submerged	The photosynthetic tissue is entirely submerged but usually the flowers are exposed to the atmosphere.	**Elodeid type:** *Elodea* *Myriophyllum* *Ceratophyllum* **Isoetid type**: *Isoetes* *Littorella* *Lobelia*
Floating - leaved	Includes species which are rooted in the substrate and freely floating on the water surface	**Rooted in the substrate type:** *Nymphaea* spp. *Nuphar* spp. *Potamogeton natans* *Hydrocotyle vulgaris*

(continued)

(Table 1.8 continued)

Floating - leaved	Freely floating species range from large plants with rosettes of aerial and/or floating leaves and well-developed submerged roots, to minute surface-floating plants with few roots or without roots	**Freely floating type:** *Eichhornia crassipes* *Pistia stratiotes* *Lemna* spp. *Spirodella* spp. *Eichhornia* *Trapa* *Hydrocharis* *Lemnaceae* *Azolla* *Salvinia*

The presence of macrophytes is one of the most prominent components of CWs applied to wastewater purification system. The plants selected for these systems, should (1) be tolerant to high organic and nutrient loadings, (2) have rich belowground organs (i.e. roots and rhizomes) in order to provide substrate for attached bacteria and oxygenation (even very limited) of areas adjacent to roots and rhizomes and (3) have high aboveground biomass for winter insulation in cold and temperate regions and nutrient removal via harvesting [407, 408].

Constructed wetlands can work with both vascular plants (higher plants) and nonvascular plants (e.g., algae [409]). Nevertheless, vascular plants are more used (Table 1.9) and just a few varieties of macrophytes are commonly utilized in developed wetlands: *Phragmites*, *Typha*, *Scirpus*, *Juncus*, *Eleocharis* [410]. One of the issues being evaluated is the difference in treatment efficiency between species (e.g., *Typha* sp. and *Scirpus* sp. for nitrogen removal [411]) or difference between one species only and a polyculture (e.g., *Canna indica*, *Colocasia esculenta*, *Hymenocallis littoralis* and *P. australis*) for nutrients removal [412].

Table 1.9 - Macrophytes used in CWs in microcosm and mesocosm studies.

Vegetation	CW type	Scale	Reference
Acorus calamus	HF[a] (baffled)	microcosm	Yuan et al. [413]
Cyprus papyrus	HF[a]	pilot	Khalifa et al. [414]
Hymenachne grumosa	VF[b]	pilot	Silveira et al. [415]
Iris pseudocarus	VF[b]	microcosm	Lu et al. [394]
P. australis;	VF[b]	microcosm	Mateus and Pinho [416]
	VF[b] - upflow	microcosm	Zhang et al. [417]
	HF[a]	microcosm	Hua et al. [418]
	VF[b] (tidal)	microcosm	Tan et al. [419]
	HF[a], VF[b], FWS[c]	microcosm	Liu et al. [420]
Typha sp.	HF[a]	pilot	Dires et al. [421]

(continued)

(Table 1.9 continued)

Cynodon sp.; Peninisetum purpureum	HF[a]	mesocosm	Saraiva et al. [422]
Cyperus alternifolius; Cynodon dactylon	VF[b]	microcosm	Mustapha et al. [423]
P. australis; Canna indica	Batch CW	microcosm	Liang et al. [424]
P. australis; Cyperus alternifolius	HF[a]	mesocosm	Anh et al. [425]
P. australis; Vetiveria zizanioides	VF[b] (tidal)	mesocosm	Saeed et al. [426]
Typha angustifolia; Pucinellia frigida	VF[b]	microcosm	San Miguel-Espinosa et al. [427]
Arundo donax; Cyperus alternifolius; Typha latifolia	VF[b]	mesocosm	Licata et al. [428]
Canna indica; Cyperus papyrus; Hedychium coronarium	HF[a]	microcosm	Zamora et al. [429]
P. australis; Juncus effusus; Iris pseudacorus	FCW[d]	mesocosm	West et al. [395]

[a]HF - Horizontal Flow; [b]VF - Vertical Flow; [c]FWS - Flow water surface; [d]FCW- Floating Constructed Wetlands.

The physical presence and metabolism of macrophytes can influence the treatment processes in CW. Macrophytes can distribute and reduce water/wastewater flow within the system and this can promote better conditions for sedimentation of suspended solids and increase contact time between the water/wastewater and the plant surface areas [405]. Moreover, the above ground part of macrophytes can reduce the wind velocity near water surface (compared to the velocities above the vegetation), which reduces resuspension of settled material and thereby improves the removal of suspended solids by sedimentation [405].

The above ground part of macrophytes also promotes sun light attenuation hindering the production of algae in the water below the vegetation cover [405]. The thermal insulation of soil is also observed when vegetation cover is present. For instance, in the winter, when the dead still-standing plant material is covered by snow it provides a perfect insulation and helps keep the soil free of frost [405]. This layer of the dead plant material helps protect the soil from freezing during winter but also keeps the soil cooler during spring [405].

The presence of plants is also important for stabilizing the soil surface in CWs, as their dense root systems within the filter medium prevent the formation of erosion channels, helps decompose organic matter and prevent clogging [405]. For instance, in the case of Vertical Flow Constructed Wetland (VFCW) the presence of macrophytes associated with an intermittent loading regime helps prevent clogging of the medium [405].

In CW with subsurface horizontal flow, the flow of water in the bed is intended to be largely subsurface through channels created by the living and dead roots and rhizomes as well as through soil pores [405]. In case of living roots and rhizomes the growth process disturbs and loosens the soil, but when roots and rhizomes die and decay, they may leave behind tubular pores channels (macro pores), which can increase and stabilize the hydraulic conductivity of the soil [405]. This structure of the macropore system can be very effective in channeling water through a bed of soil [405].

Plants also provide a good surface area for microbial growth. The stems and leaves of macrophytes that are submerged in the water column provide an extensive surface area for biofilms, but also roots and rhizomes that are buried in the wetland soil provide a substrate for attached growth of microorganisms. Moreover, the plant tissues can be colonized by dense communities of photosynthetic algae as well as by bacteria and protozoa, therefore, biofilms can be present on both the above and below ground tissues of macrophytes [405].

As mentioned, plants can have a significant role in CWs also in the removal of diverse pollutants. For instance, wetland plants require nutrients (e.g., nitrogen, phosphate) for their growth and the rooted macrophytes take up nutrients primarily through their root systems, but some uptake occurs also through immersed stems and leaves from the surrounding water/wastewater [405]. Wetland plants are very productive and considerable amounts of nutrients can be bound in the biomass, however, the majority of the nutrients that have been incorporated into the plant tissue can return to the water by decomposition processes [405].

On the other hand, aquatics macrophytes release oxygen from roots into the rhizosphere and that release has direct implications in the biogeochemical cycles occurring in CW substrate namely through the effects on its redox status [405]. The oxygen leakage from roots creates oxidized conditions in the otherwise anoxic substrate and promotes both aerobic decomposition of organic matter and growth of nitrifying bacteria [405, 406], which promotes also contaminants removal.

Oxygen release rates from roots depend on the internal oxygen concentration, the oxygen demand of the surrounding medium and the walls permeability of the roots [405]. Rates of oxygen leakage by roots are generally the highest in the subapical region (Fig. 1.24) and decrease with distance from the root-apex [405]. However, with tissues age a decreased oxygen leakage rates are observed, probably caused by cuticularization and hypodermal suberization and associated decreased of root walls permeability [405]. This decrease of root walls permeability minimizes radial leakage outward, allowing more oxygen to reach the apical meristem [405]. The oxygen leakage at the root (apical meristem) serves to oxidize and detoxify potentially harmful reducing substances in the rhizosphere [405].

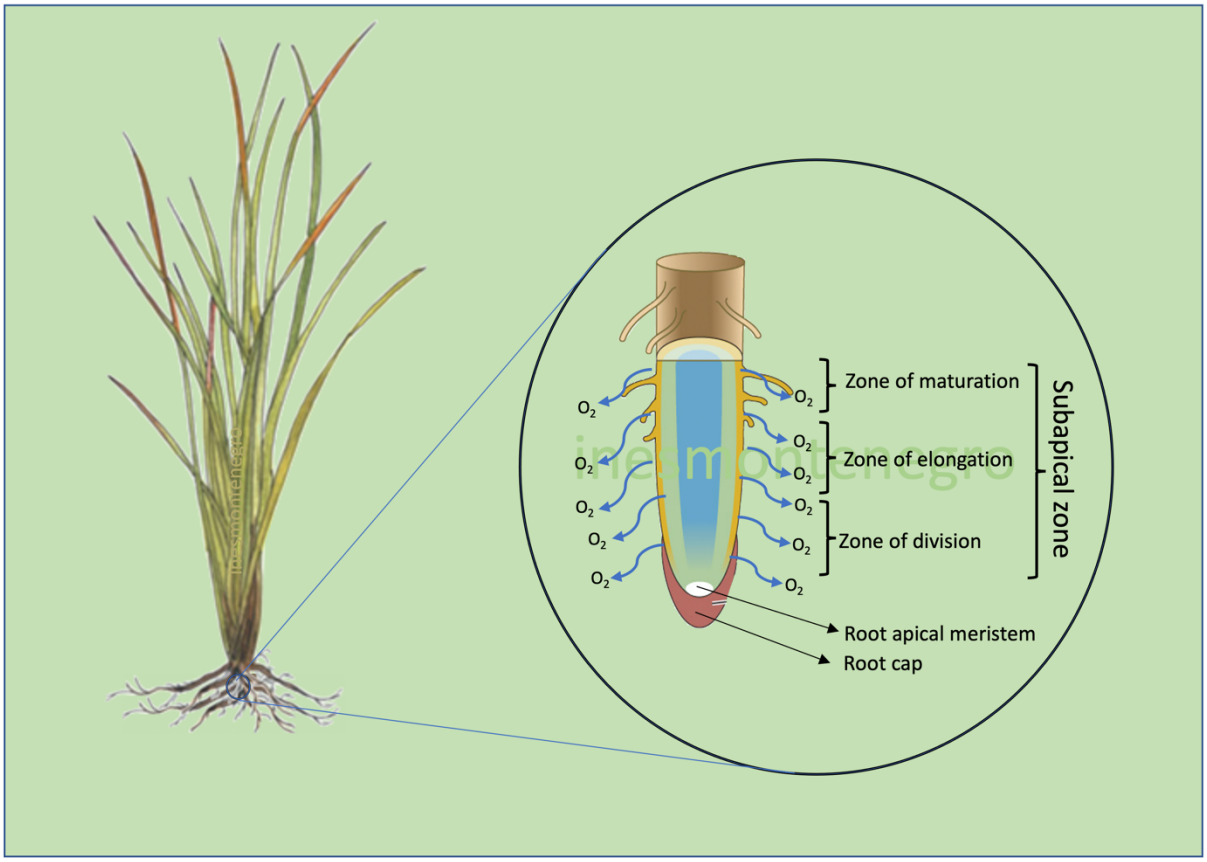


Fig. 1.24 - Representation of the subapical zone of root associated with release of oxygen.

The aquatic macrophytes, have an extensive system of gas spaces throughout the culm, rhizome and roots, through which atmospheric oxygen passes down to the extremities of the roots and to the rhizosphere and waste gases can pass back into the air [430]. The interconnecting nature of this system consists of aerenchyma channels and large pith cavities [430].

The aeration of the root system and underground buds and rhizome tips must be chiefly by diffusion, but the aeration of the culms and rhizomes can be by diffusion or by both diffusion and convection [430]. Wetland plants that transport gases only via diffusion are, among others, *Carex rostrata*, *Oryza sativa*, *Scirpus lacustris* and *Peltandra virginica* [431–433]. The gas transport via convective through - flow due to pressure gradient has been identified in *Typha latifolia*, *Typha angustifolia*, *Nymphaea odorata*, *Nuphar luteum*, *Nelumbo nucifera*, *Nymphoides peltata*, *P. australis* [21, 432, 434] and *Eleocharis sphacelata* [435]. These wetland plants have the potential to release more oxygen from their roots compared to species without convective flow [405].

The convective through - flow increases the rate of internal gas transport, enhancing oxygen concentrations in underground tissues and accelerating the ventilation of biogenic gases from sediment [21]. The transport of gas via convective through - flow is due to a pressure gradient. The internal pressurization in wetland plants is usually generated within

leaf and shoot tissues, induced by gradients in temperature and humidity between the internal gas of the plants and the surrounding atmosphere [436, 437], or wind - driven *Venturi* effects [430].

The humidity - induced pressure gradient within the leaf sheaths (the part of the leaf that encircles the stem) leads to an airflow from the leaf sheaths towards the rhizomes, which is vented via old and broken stems [436, 438, 439]. This transport mechanism is more than five times faster than diffusion and is regulated by the pores (stomata) in the leaf sheaths [431]. These stomata do not transport gas due to pressure differences, but allow gas transport by diffusion [431]. Due to higher humidity in the internal culm of macrophytes (e.g. *P. australis*) oxygen concentrations inside the plant are diluted [431]. Therefore, oxygen is transported along the concentration gradient from the atmosphere into the sheaths, increasing the internal pressure (Fig. 1.25 a) and b). Since this process depends on stomatal conductance, humidity induced convective flow starts after sunrise when the stomata open, reaches an optimum stage around noon when photosynthetic rates are highest and then decreases till sunset [431]. Like humidity, convection in the plant can be induced by the *Venturi* effect. *Venturi* effect is the reduction in pressure (and increase in speed) that a fluid experiences when it flows through a constricted section of a pipe. This phenomenon can explain through the continuity equation and the Bernoulli's equation.

The wind creates a suction drawing gases from these culms and the rhizomes system and this is replaced by air entering the rhizomes via the stubble which is sheltered from wind flow (Fig. 1.26 a) and b) [430]. The *Venturi* - induced convention is initiated by wind blowing across the top of tall dead culms, this being especially effective where the top of plants have been snapped off [430]. The *Venturi* - induce flow is directly proportional to the wind speed and will be influenced by resistances in the substrate [430].

Therefore, wetlands plants with gas transport via convective through - flow due to pressure gradient, have a greater potential for the removal of some pollutants in flooded soils. Root systems also release other substances besides oxygen, for instance *Seidel* showed that bulrush *Schoenoplectus* released antibiotics from roots, leading to the elimination of a range of bacteria (coliforms, Salmonella, Enterococci) from wastewater when in contact with this vegetation [405]. Plants also released a wide range of organic compounds through their roots. These organic compounds may act as a carbon source, for instance, for denitrification processes, thus increasing nitrate removal in CWs [405]. Wetland plants can also, for instance, produce and secrete substances, such as aliphatic low molecular weight organic acids (ALMWOAs) into their rhizosphere [440]. These compounds released by plant roots will also promote microbial growth.

So, all these features, make these plants a key player in wetlands, promoting pollutants removal / degradation.

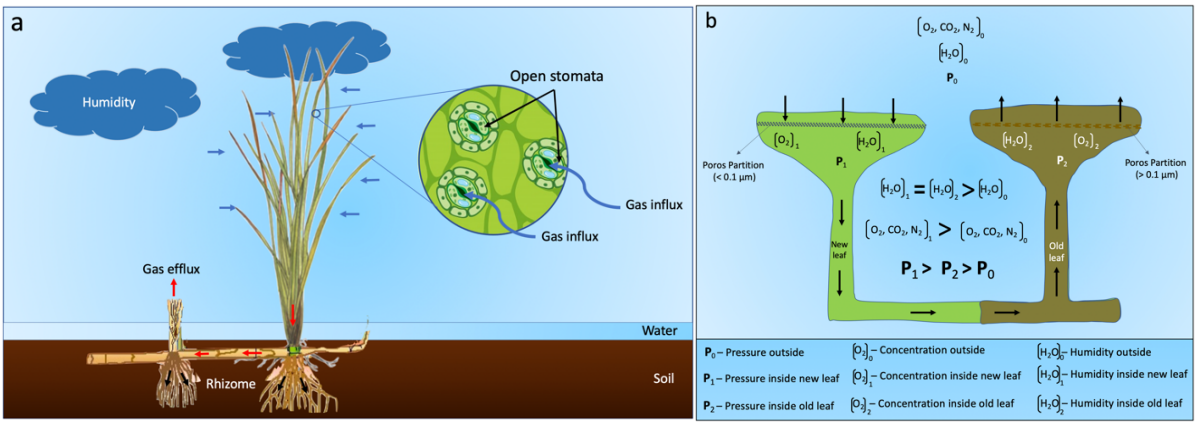


Fig. 1.25 - a) Schematic overview of humidity induce convention inside *P. australis* plants. The difference in humidity between the sub-stomatal gas space of the plant and the drier atmosphere favors the inward diffusion of oxygen through leaf sheath and nodal stomata. A pressurized flow of gases is induced through the rhizomes due to stomatal resistance to backflow. The throughflow results in increased diffusion of oxygen into root and rhizosphere (Adapted from: Brix et al. [21]). **b**) The physical process between *P. australis* and neighborhood.

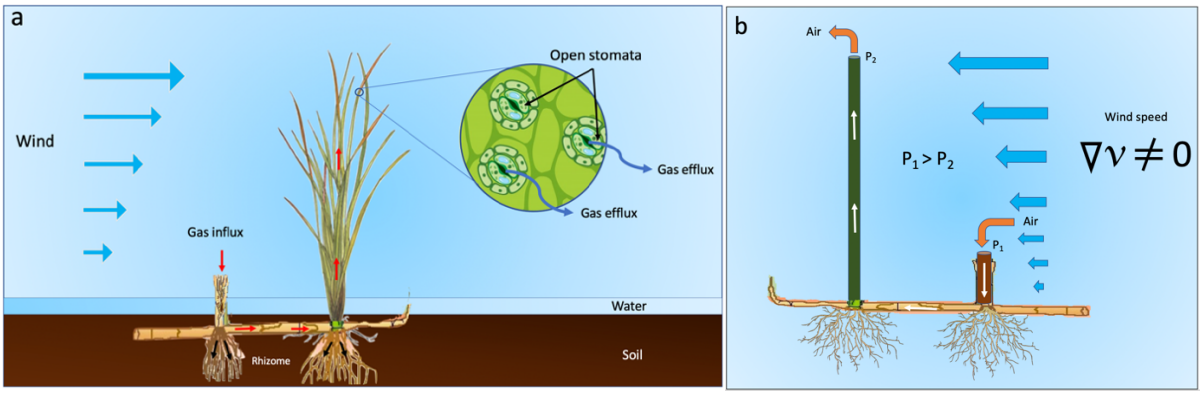


Fig. 1.26 - a) Schematic overview of the wind induced convention inside *P. australis* plants. A suction pressure ΔP (Pa) is created by the wind such that $\Delta P = (1/2).\rho V^2$, where ρ in the density of air and V is the wind velocity (ms^{-1}). The convective flow through the rhizomes results in an increased diffusion of oxygen to root and rhizosphere (Adapted from: Brix et al. [21]). **b)** Representation of the physical process between *P. australis* and neighborhood. The gradient relative to wind speed is different of zero ($\nabla v \neq 0$).

1.3.3. Substrate in Constructed Wetlands

Substrates are important component of CWs, and most of the physical, chemical and biological process take place in substrates [441]. The substrates provides a physical support for plants, an attachment surface for microbial populations and a considerable reactive surface area for contaminant removal [22, 442, 443]. The mechanisms for contaminants removal in the substrate include physical sedimentation and filtration [444], sorption and burial in

substrate matrix [445], complexation and precipitation by colloid composition [446], ion exchange [447], gas diffusion in the substrate gap [448], microbial degradation, transformation and bio-immobilization in the substrate [351], and uptake and metabolism by plant root in the substrate [405].

A variety of materials with different properties could be used as substrates in CWs (Table 1.10), being classified into natural material, industrial by-products, and artificial / man-made products [399, 449–451]. Soil, sand and gravel are the three most common substrates used, due to their abundance, low-cost, and their retention potential for pollutants. However, a special focus has been given to a wide variety on newly emerged substrates as shown in Table 1.10 [441].

The correct selection of substrates can lead to significant enhancements of CW efficiencies. In general, cost and local availability are the two fundamental factors determining the selection [369], however, to optimize the system performance the physical (e.g., particle size, porosity, hydraulic and electrical conductivities, specific surface area, mechanical strength), chemical (e.g., surface charge, toxicity, and chemical stability), and biological (e.g., electron donors / acceptors) characteristics of the substrates must be considered [441].

Table 1.10 - Some types of substrates used in CWs.

Group	Material	Reference
Natural material	Bentonite	Xu et al. [452]
	Calcite	Ann et al. [453]
	Charcoal	Scholz and Xu [454]
	Clay	Calheiros et al. [455]
	Coal cinder	Xiong et al. [456]
	Dolomite	Ann et al. [453]
	Gravel	Mustapha et al. [423]
	Laterite	Wu et al. [457]
	Lava rock	Santos et al. [458]
	Limestone	Tao et al. [459]
	Maerl	Seo et al. [460]
	Marble	Arias et al. [461]
	Medical stone	Wu et al. [457]
	Peat	Saeed et al. [462]
	Vermiculite	Arias et al. [461]

(continued)

(Table 1.10 continued)

Natural material	Sand	Saeed et al. [463]
	Shale	Saeed et al. [462]
	Shell	Seo et al. [460]
	Slag	Xiong et al. [456]
	Shuck	Wu et al. [457]
	Tobermorite	Liu et al. [464]
	Wollastonite	Brooks et al. [465]
	Zeolite	Bruch et al. [466]
Industrial by products	Alum sludge	Babatunde et al. [467]
	Bast furnace slag - based	Korkusuz et al. [468]
	Coal cinder	Ren et al. [469]
	Fly ash	Xu et al. [452]
	Hollow brick crubs	Ren et al. [469]
	Moleanos limostone	Mateus et al. [470]
	Oil palm shell	Chong et al. [471]
	Slag	Cui et al. [472]
	Wollastonite tailings	Hill et al. [473]
Artificial products	Active carbon	Ren et al. [469]
	Calcium silicate hydrate	Li et al. [474]
	Ceramsite	Li et al. [474]
	Compost	Saeed et al. [462]
	Light weight aggregates	Saeed et al. [462]
Others	Alum sludge	Liu et al. [475]
	Apatite material	Harouiya et al. [476]
	Bauxite	Cherukumilli et al. [477]
	Biochar	Zhou et al. [478]
	Construction wastes	Shi et al. [479]
	Tire chips	Reddy et al. [480]
	Oyster shell	Park et al. [481]
	Filtralite	Albuquerque et al. [482]
	Polyethylene terephthalate	Tatoulis et al. [483]
	PHBV[a] and PLA[b] blend	Yang et al. [484]
	Rice straw	Cao et al. [485]
	Wood mulch	Saeed et al. [486]

[a]PHBV - poly - 3 - hydro - xybutyrate-co-3-hydroxyvalerate; [b]PLA - polyacetic acid.

1.3.4. Microbial community in Constructed Wetlands

Soil is the most important factor for plants and their associated microorganisms. But plants and microorganisms have a crucial role in the modification of soil [487] and in the modification of contaminant substances to obtain nutrients or energy needed for their life cycles [488]. The microbial community also has an important role in the protection of plants from abiotic stresses and phytopathogens [489].

The effectiveness of CWs for wastewater treatment is dependent on developing and maintaining optimal environments for desirable microbial population [443]. In this environment, the microorganisms (bacteria, archaea, fungi and algae) are rarely encountered as single-species populations, and an enormous biodiversity and abundance can be observed in a small quantity of soil sample [490]. Additionally, microorganisms with different metabolic requirement (e.g. photoautotrophic, photoheterotrophic, chemoautotrophic chemoheterotrophic) and with different respiratory process (e.g., aerobic, anaerobic) can be detected in CW systems influencing treatment efficiency. For instance, in the case of the removal of nitrogen from wastewater, previous studies have suggested that the diversity of microorganisms involved in the N - cycle in these systems, e.g. archaeal nitrifiers, denitrifying fungi, aerobic denitrifying bacteria and heterotrophic nitrifying microorganisms, may play an important role in nitrogen transformations in CWs [491].

The microbial communities composition is significantly associated to the type of vegetation (e.g., *Typha latifolia* and *P. australis* [492]), wastewater flow (e.g. horizontal flow and vertical flow [493]), oxygen concentration (e.g., [494, 495]) and soil properties (e.g., pH [496], soil texture [497]).

Most of the contaminant transformations conducted by microorganisms are controlled by enzymes, genetically - specific proteins that catalyze chemical reactions [498]. The metabolism of microorganisms includes the use of enzymes to break apart complex organic compounds into simpler compounds with the release of energy (catabolism) or the synthesis of organic compounds (anabolism) by the use of chemically stored energy [498].

The microbial metabolism not only depends on the presence of appropriate enzymes but also on suitable environmental conditions such as temperature, dissolved oxygen (DO), and pH [498]. Besides, the contaminant concentration is of primary importance in determining the reaction rates [498].

1.3.5. Water column / wastewater

The flow and storage volume determine the length of time that water spends in the wetland and thus the opportunity for interactions between compounds present in the water and the wetland ecosystem [499]. Natural wetlands are formed on substrates that are fully or partially submerged in water, where a relatively impermeable subsurface layer prevents the surface water from seeping into the ground [1, 2].

Hydrological requirement is an important factor to be considered in constructed wetlands. This factor can determine the success or failure of most constructed wetlands [500], because, a small change in water regime can have fairly significant repercussion on a wetland's functionality and its treatment effectiveness and efficiency [500]. For instance, the most frequent cause of vegetation problems in CWs is inappropriate water supply to wetlands plant [499].The duration and depth of flooding affects plant physiology because of soil oxygen concentration, soil pH, dissolved and chelated macronutrients and micronutrients, and toxic chemical concentration [499]. Therefore, predicting and controlling the water regime of a treatment wetland is required [499]. It is important to stress that the term water regime refers to the combination of water depth and flooding duration [24].

The combined loss of water by evaporation from the water surface and loss through transpiration by plants and the density of the vegetation of a wetland strongly affects the constructed wetlands hydrology [500]. This can be verified through the obstruction of water flow paths as the water finds its way through the network of stems, leaves, roots, and rhizomes, and it can also occur through the blockage of exposure to wind and sun [7, 24, 25].

Water always acts as a vehicle for delivering the pollutants to the system and also for discharging the still remaining pollutants away from the system [24]. Detention time is an important features of wetlands hydrology from the standpoint of treatment efficiency, because this time will determine the duration of interaction between water and biota (plants and microbial community) and the proximity of waterborne substances to the sites of biological and physical activity [499].

With the objective of intensifying and optimizing removal processes in CWs different water flows have established. In this sense, based on water flow CW systems can be divided in two broad categories: free water surface flow constructed wetland (FWSCW) and subsurface flow constructed wetland (SSFCW)) which will affect the treatment efficiency. Based on water flow direction SSFCW can be divided in two types: horizontal flow constructed wetland (HFCW) and vertical flow constructed wetland (VFCW) [442, 501] (Fig. 1.27). In the next sections FWSCW, HFCW and VFCW are analyzed with more detail.

Although a single type of CW can be used, different ones can be combined. The hybrid or combined systems, consisting in the combination of various types of CWs, aim to achieving a more efficient treatment with the specific advantages of the different systems [502]. The hybrid systems were first introduced by Seidel in Germany in the 1960s [503] but at that time their use did not spread too much [504]. The design of hybrid systems generally consists of two stages of several parallel CWs in series, such as VF - HF CWs, HF - VF CWs, HF - FWS CWs and FWS - HF CWs [504]. In the case of multi - stage CWs, three or more stages CWs are used [442].

Different types of CWs have emerged over the years, but the main objective is to have a designed that emphasizes specific characteristics of natural wetland ecosystems to improve treatment efficiency [442]. Currently, numerous studies have focused on the design, development, and performance of CWs with the purpose of increasing their efficiency for the removal of various pollutants (organic matter, nutrients, trace elements, pharmaceutical contaminants, pathogens, etc) from wastewater [22, 462, 472]. In recent years, with the objective of intensifying and optimizing removal processes in CWs, several techniques have been proposed. For instance: artificial aerated CWs, baffled flow CWs, hybrid towery CWs, step feeding CWs and circular flow corridor CWs [22].

Despite all different designs and improvements, the aim of CWs remains the same: to treat contaminated water by effectively removing pollutants.

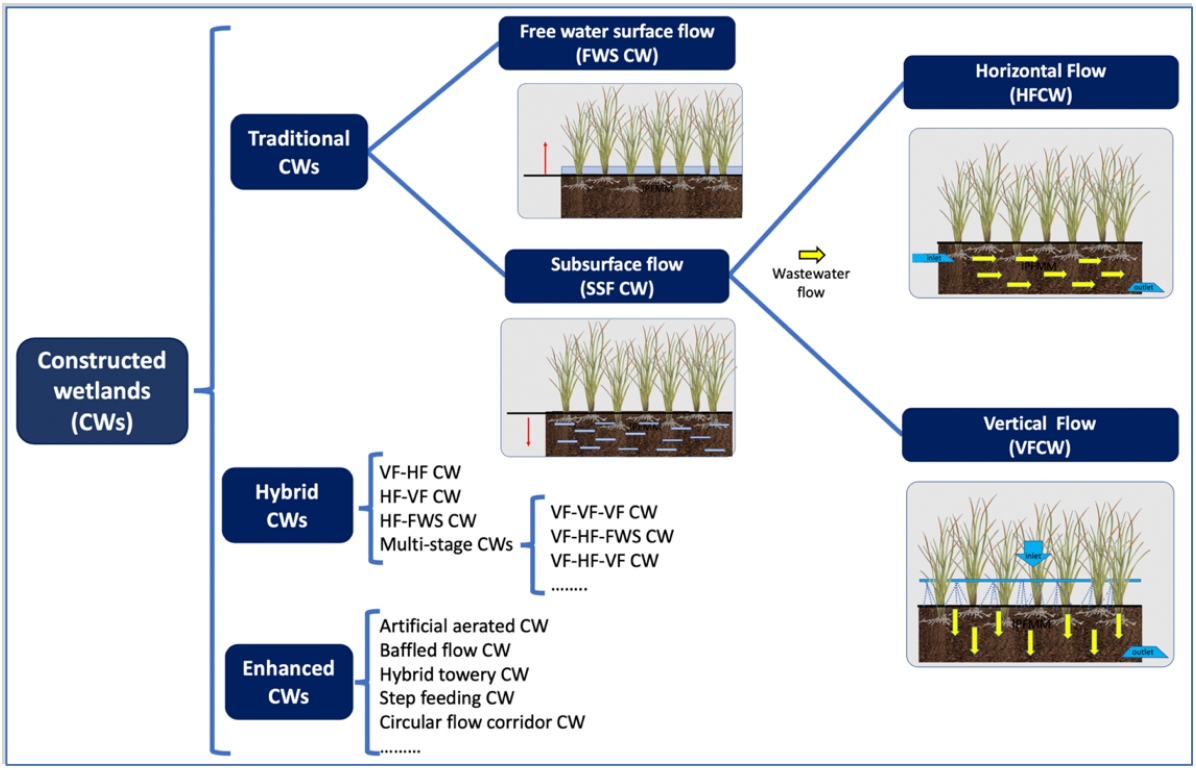


Fig. 1.27 - The classification of types of CWs used in wastewater treatment (Adapted from Wu et al. [22]).

1.3.6. Types of Constructed Wetlands

1.3.6.1. Free Water Surface Flow Constructed Wetlands

The Free water surface flow Constructed Wetlands (FWSCWs) consists of open water system with floating and emergent vegetation where the water flows over a vegetated soil surface from an inlet point to an outlet point [505]. Most natural wetlands are FWSCW systems, for instance, bogs (primary vegetation mosses), swamps (primary vegetation trees), and marshes (primary vegetation grasses and emergent macrophytes) [27]. The verification of water quality improvement in these natural systems for many years led to the development of CWs) [27]. The FWSCW typically consist of one or more shallow basins or channels with a barrier to prevent seepage to sensitive ground waters and a submerged soil layer to support the roots of the selected emergent macrophyte vegetation [27, 442]. Emergent macrophytes but also submerged or floating are used in these type of systems [442]. The plants have an import role, for instance, the plant canopy shades the water surface preventing the growth of algae and reduces wind - induced turbulence in the water flowing through the system, while the submerged portions of living plants provide the periphytic-attached growth organisms responsible for much of the biological treatment in the system [27].

In FWSCW the water flows nearly horizontally and at low velocity above the soil layer along the system (Fig. 1.28), creating a water column depth of 20 - 40 cm [24] or even up to 80 cm [506, 507], consequently the water is exposed to the atmosphere and to partial sunlight [23]. The water level in these systems is maintained with an appropriate outlet level control arrangement [23]. The water flows through the wetland bed and comes into contact with the soil grains and plant parts, thus enabling a series of physical, biological, and chemical processes to occur [23]. These processes contribute to the degradation and removal of several types of pollutants, however, nearly standing water increases the possibility of mosquito breeding [23].

The most commonly used design and operational characteristics of FWSCW are: (a) 0.2 - 0.3 m of rooting soil; (b) water depths between 0.2 - 0.6 m; (c) hydraulic loading rates between 12 - 50 mm d^{-1}; (d) organic loading rates between 1 - 11 g BOD_5 mM^{-1}, and (e) hydraulic residence time (HRT) between 5 - 15 days [377]. It is common opinion, that this CW have a good performance in all climates, including cold habitat, however, they are considered more sustainable in warm climates (> 10 °C) [508]. In this system the dissolved oxygen (DO) concentration in the water column varies from high (near the surface) to practically zero (near the bottom) [508]. The removal of suspended solid (SS), organic matter (OM), nitrogen (N),

pathogens, and other pollutants (e.g. metals) is high, while phosphorus (P) removal is limited [391, 508–511].

Fig. 1.28 - Schematic representation of FWSCWs with floating and emergent vegetation. Adapted from Stefanakis [23].

Because of the potential risk for health human when expose to pathogens, this type of system is commonly used for advanced treatment of effluent from secondary or tertiary treatment [504], in other words, before treating wastewater, these systems are usually equipped with a pre-treatment stage. At the same time, these systems have been employed for urban agricultural and industrial rainwater treatment due to their ability to work with pulse flows and changing water levels [504].

This type of CWs has an important advantage, because the presence of a water surface, these systems tend to better resemble natural wetlands, and thus, provide more wildlife habitat benefits [448]. This type of systems has several advantages (Table 1.11) but also some limitations such as the water flows above the soil / sediment substrate limiting its contact with CW substrate and exposition of the water to the atmosphere [505].

Usually, FWSCWs demand higher surface area when compared to other CW types for the same wastewater flow and characteristics, since the porous media and the plant roots in SFCW systems provide a greater contact specific area [27, 28, 510, 512]. On the other hand, according with Tsihrintzis et al. [512] the SFCWs tend to demand higher initial investment.

Table 1.11 - Some of advantages and disadvantages of FWSCW (Source: USEPA [27]):

Free Water Surface Constructed Wetland (FWSCW)	
Advantages	Disadvantages
FWSCW offer effective treatment in a passive manner, minimizing mechanical equipment energy and skilled operator requirements.	The land area required for FWSCW can be large, especially if nitrogen or phosphorus removal are required.
FWSCW may be less expensive to construct and are less costly to operate and maintain than conventional mechanical treatment systems.	The removal of BOD, COD, and nitrogen are biological processes and essentially continuously renewable. The phosphorus, metals and some persistent organics compounds removed by the systems are bound in the wetland sediments and accumulate over time.
Year-round operation for secondary treatment is possible in all but the coldest climate. Year-round operation for advanced or tertiary treatment is possible in warm to moderately temperate climates.	In cold climates the low winter temperatures reduce the rate of removal for BOD and the biological reactions responsible for nitrification and denitrification. An increased detention time can compensate for this, but the increased wetland area required in extremely cold climates may not be cost effective or technically feasible.
Wetland systems provide a valuable addition to the "green space" in a community and include the incorporation of wildlife habitat and public recreational spaces.	The bulk water in most FWSCW is essentially anoxic, limiting the potential for rapid biological nitrification of ammonia. Increasing the wetland area and, therefore, the detention time, may compensate for this, but may not be cost effective. Alternate methods for nitrification in combination with a FWSCW have performed successfully.
Wetland systems produce no residual biosolids or sludges requiring subsequent treatment and disposal.	Mosquitoes and other insect vectors can be a problem, for instance, to human health.
The removal of BOD, TSS, COD, metals and persistent organics in municipal wastewaters can be very effective with a reasonable detention time. The removal of nitrogen and phosphorus can also be effective with a significantly longer detention time.	The bird population that lives in a FWSCW can have adverse impacts if an airport is nearby.
	FWSCW can remove fecal coliforms by at least one log form typical municipal wastewaters. This may not be sufficient to meet discharge limits in all locations and supplemental disinfection may be required. The situation is further problematic because birds and other wildlife in the wetland produce fecal coliforms.

1.3.6.2. Subsurface Flow Constructed Wetlands

Subsurface flow (SSF) refers to any flow below the surface of the ground with low flow (base flow) and quick flow (subsurface stormflow). A subsurface flow constructed wetland (SSFCW) consists of a sealed basin with a porous substrate of rock or gravel [513]. The water level in SSFCW is designed to remain below the top of the substrate [513]. Due to the hydraulic constraints imposed by the substrate, SSFCW are best suited to treat wastewaters with relatively low solids concentrations and under relatively uniform flow conditions [513].

In the SSFCW systems the available submerged substrate includes the plant roots growing in the media, and the surfaces of the media themselves [513]. The media surface area in a SSFCW can far exceed the available substrate in a FWSCW, as a result, the microbial reaction rates in a SSFCW can be higher than in a FWSCW for most contaminants [28]. In this sense, for the same flow rate, SSFCW need a smaller area for treatment than FWSCW.

The advantages and disadvantages of this type of systems is compiled in Table 1.12. The SSFCWs may be of two types according to the flow direction and level and duration of saturation of the substrate: horizontal flow constructed wetlands (HFCWs) and vertical flow constructed wetland (VFCWs) [399].

Table 1.12 - Some of advantages and disadvantages of SSFCW (Source: USEPA [28]):

Subsurface Flow Constructed Wetland (SSFCW)	
Advantages	Disadvantages
SSFCW provide effective treatment in a passive manner and minimize mechanical equipment energy, and skilled operator attention.	A SSFCW will require a large land area compared to conventional mechanical treatment processes.
SSFCW can be lee expensive to construct and are usually less expensive to operate and maintain as compared to mechanical treatment processes designed to produce the same effluent quality.	The removal of BOD, COD, and nitrogen in SSFCW are continuously renewable processes. The phosphorus, metals, and some persistent organics removed the system are bound in the wethand sediments and accumulate over time.
Year-round operation secondary treatment is possible in all but the coldest climates.	In cold climates the low winter temperatures resuce the rate of removal for BOD, NH_3 and NO_3. An increased detention time can compensate for these reduced rates but the increased CW size in extremely cold climates may not be cost effective or technically possible.
Year-round operation for advanced or tertiary treatment is possible in warm to moderately temperate climates. The SSFCW configuration provides more thermal protection than the FWSCW type.	Most of the water contained in the ssfcw is anoxic and this limits the potential for nitrification of wastewater ammonia. Increasing the CW size and detention time will compensate , but this may not be cost effective. Alternative methods for nitrification in combination with SSFCW and have been successful. SSFCW cannot be designed for complete removal, for instance, organic compouds, TSS, nitrogen and coliforms. The natura ecological cycles in these CW produce "background" concentrations of these substances in the system effluent.
SSFCW produce no residual biosolids or sludges requiring subsequent treatment and disposal.	SSFCW can typically remove fecal coliforms by at least one log. This in not always sufficient to meet discharge limits in all locations and post disinfection may be required. UV disinfection has been successfully used in a number of applications.
SSFCW is very effective and reliable for removal of BOD, COD, TSS, metals, and some persistant organics in municipal wastewaters. The removal of nitrogen and phosphorus to low levels is also possible but requires a much longer detention time.	Although SSFCW can be smaller than FWSCW for the removal of most contituents, the high cost of the gravel subtrate in the SSFCW can result in higher construction costs for SSFCW larger than about 227.100 liters per day (60.000 gallons per day).
Mosquitoes and similar insect vectors are not a problem with SSFCW as long the system is properly operated and a subsurface water level maintained. The risk of contact by children and pets with partially treated wastewater is also eliminated.	

1.3.6.3. Horizontal Flow Constructed Wetlands

In horizontal flow (HF) the wastewater is fed through the inlet, continues its way under the surface of the bed in a more or less horizontal path, reaching the outlet zone where it is collected and discharged [514] (Fig. 1.29). The water flows horizontally along the bed below the substrate surface through the pores of the porous media and the plant roots [448]. In the filtration beds of horizontal flow constructed wetlands (HFCWs), pollutants are removed by plant removal and microbial degradation and chemical and physical processes in a network of aerobic, anoxic, anaerobic zoned with aerobic zones being restricted to the areas adjacent to plant roots where oxygen leaks to the substrate [376].

The typical design of HFCWs consist of a rectangular bed with substrate (usually is gravel or a mixture of sand and gravel) and planted with wetland vegetation and lined with an impermeable membrane [24, 442]. In general, the wastewater enters in the distribution zone filled with large stones, is filtered through the substrate (filtration medium with a smaller porosity and grain size) and is collected in the collection zone filled also with large stones, before discharge via level control arrangement at the outlet [24].

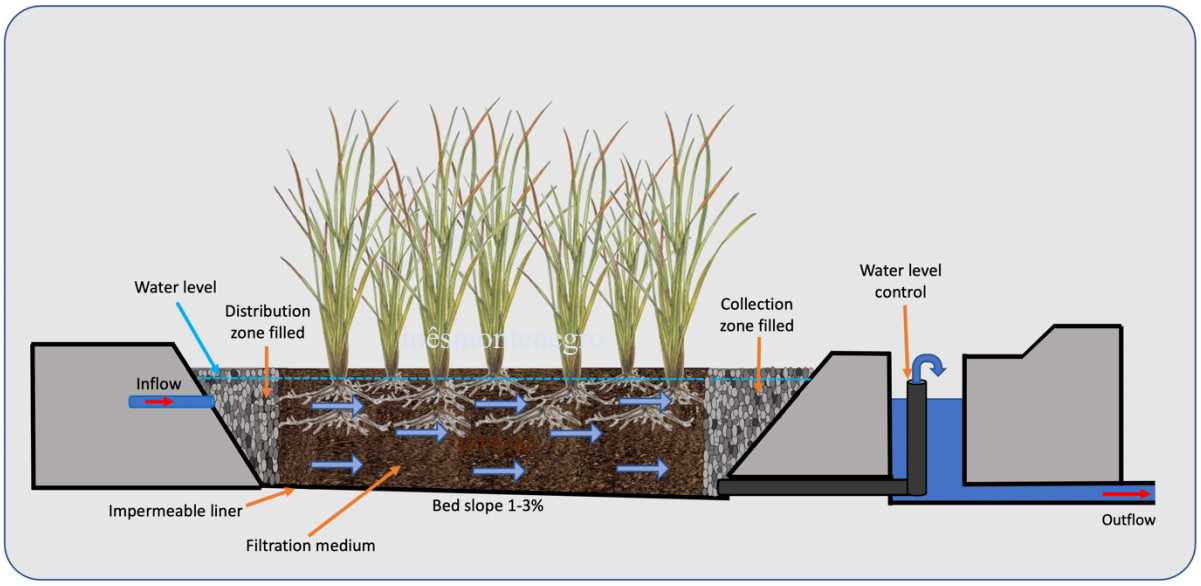


Fig. 1.29 - Schematic representation of CW with HFCWs. The arrows indicate only a general flow pattern. Adapted from Vymazal [24] and Stefanakis et al. [23].

The substrate supports the growth of the wetland vegetation and the depth of this substrate varies between 30 and 80 cm, depending on plant root depth [23]. The bottom of the bed is covered by an impermeable geo-membrane and a small slope (1 - 3 %) of the bottom is usually applied to promote gravitational water flow [442, 515]. This type of system is considered well designed when no surface flow is visible and the water level in maintained about 5 - 15 cm below the top of the substrate with an appropriate outlet structure [24].

In the 1950s in Germany, HFCW was developed by *Käthe Seidel* who designed the HFCWs using coarse materials as the rooting medium [376]. Ten years later, *Reinhold Kickuth* suggested soil media with clay content and named it “Root Zone Method” [376]. In the early 80s HFCWs technology was introduced into Denmark and in 1987 nearly 100 soil-based systems were put in operation [516]. Still in the 80s, the HFCWs technology was also introduced into other countries, such as Austria and the United Kingdom [377]. In the 1990s, this technology was applied into most European countries and also in North America, Australia, Asia and Africa [376]. Currently, this type of system is most commonly used in the United States and Europe [24].

HSFCW are effective in municipal wastewater treatment [23]. At present this type of system are also used to treat many other types of wastewater including industrial and agricultural, landfill leachate and runoff waters [376].

In HFCWs the presence of plant roots and porous media favors the development of the biofilm, which enhance the removal of OM and SS, while the removal of other type of nutrient is usually lower [24, 442, 508, 511, 517]. The SS removal efficiency is usually very high, because the SS are retained predominantly by filtration and sedimentation [377]. The

main removal mechanism for nitrogen in HFCWs is denitrification while removal of ammonia is limited due to lack of oxygen in the filtration bed as a resulting of permanent waterlogged state [391].

In the case of phosphorus, the HFCW do not usually remove substantial amounts of this nutrient because (a) the root biomass fills the pore spaces of the substrate (gravel), directing wastewater flow to deeper wetland media [518], (b) the litter formed by decomposing vegetation remains on the surface of the substrate and thus does not interact with the wastewater, (c) the substrate media usually used does not contain sufficient concentrations of Ca^{2+}, Fe^{3+}, or Al^{3+} to actively adsorb phosphorus [519, 520]. Phosphorus is removed primarily by ligand exchange reactions, where phosphate displaces water or hydroxyls from the surface of iron and aluminum hydrous oxide, therefore, unless special materials are used, removal of phosphorus is usually low in HFCWs [391].

According with Kyambadde et al. [521] the effective nutrient removal mechanisms including root zone oxidation, adsorption and plant uptake therefore require adequate interaction of wastewater with the treatment media. In this sense, on last few years the improving performance of HFCW has enabled research efforts, in order to determine possible modifications toward further efficiency enhancement, e.g., wastewater step-feeding [522, 523], effluent recirculation and outlet water level raising [397, 515].

1.3.6.4. Vertical Flow Constructed Wetlands

At the early years of wetlands technology, FWSCW and HFCW were the most used due mainly to low costs of construction and operation when compared with Vertical Flow Constructed Wetlands (VFCWs) [524]. The interest in build VFCWs gradually increased with time, especially when the higher oxygen transfer capacity of these system was realized compared to the other type of systems [524]. The main advantage of this type of systems is their higher oxygen transfer capacity due to the feeding regime of nearly instantaneously flooding the bed surface of the VFCW [23]. Due to the aeration capability, the VFCW are very effective in organic matter and ammonia nitrogen removal [23, 391, 442]. Comparatively, phosphorus removal remains limited and alternative modifications has been proposed for the improvement of CW performance, e.g., an additional stage with gravity filters containing bauxite, zeolite or other reactive material for wastewater treatment [525, 526]. Their overall effectiveness has enabled the used of VFCW for the treatment of different wastewaters, e.g., domestic, municipal [527] urban [528], and industrial, agro - industrial landfill leachate [22, 23, 529]. Generally, when compared with HFCW (usually 5 - 10 m^2 / person equivalent (PE)), VFCWs possess smaller area demands (up to 2 m^2 / PE), consequently also implies lower

construction costs [448, 501]. Currently, VF systems are mainly used in Europe, more specifically in Denmark, Austria, Germany, France and the United Kingdom, but also in the United States [442, 530].

The most commonly used configuration of VFCWs includes a bed filled with gravel/sand layers with increasing gradation from top to the bottom (Fig. 1.30) [24].

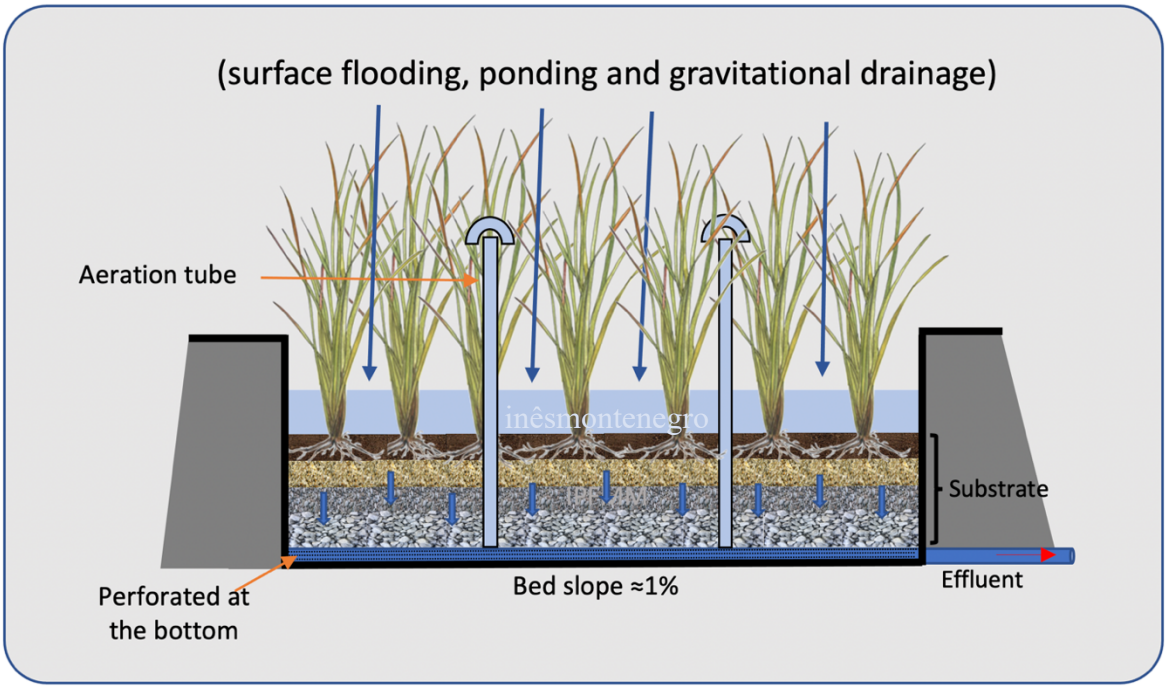


Fig. 1.30 - Schematic representation of VFCWs. Adapted from Stefanakis et al. [23].

Usually, the bed depth varies between 0.45 to 1.20 m and the top layer of bed contains sand and the bottom of the bed contains a small slope (1 - 2 %), which allows collection of treated wastewater and drainage out of the CW [23]. The bottom of these CWs is covered by a geo-membrane / geo - textile material or may be made of reinforced concrete layers [23]. For the better aeration of the deeper parts of the bed, the VFCWs may contain perforated vertical aeration tubes, which are connected at the bottom of the bed with the drainage collection pipeline systems [526].

Intermittent loading is the most commonly operating mode used is VFCWs. Wastewater is applied uniformly across the entire surface area of the bed in large batches, flooding the entire surface and creating for a short time water ponding of 3 - 5 cm. Then, the wastewater is percolated through the substrate and drained vertically by gravitational acceleration [23, 525, 526]. With this mode of operation, the wastewater spreads on the entire CW surface and moves downward by gravity force, pushing out the trapped air and sucking fresh air into the bed, this is crucial for thus enhancing aeration [23]. The *Phragmites australis*, is the emergent macrophyte more often used, and are planted at the top of bed [23]. Based

on the water flow direction along the vertical axis and the level and duration of saturation, VF systems can be divided in the following variants: VFCWS with intermittent loading (downflow), recirculating VFCWs, tidal flow CWs, saturated vertical upflow CWs, saturated vertical downflow CWs and integrated VFCWs (downflow and upflow) [23].

1.4. Constructed Wetlands and phenolic compounds removal

In recent years CWs have been applied successfully for the removal of nutrients, organic matter and diverse trace inorganic and organic contaminants [531], including endocrine disrupting compounds (EDCs) [532–537].

This section provides an overview of studies that have been carried out recently regarding NP, OP and PCP removal in different types of CWs at microcosm scale (e.g., Belmont and Metcalfe [538]), mesocosm scale (e.g., Papaevangelou et al. [535]) and large scale (e.g., Hsieh et al. [539]).

1.4.1. Nonylphenol

Belmont and Metcalfe [538] used SSFCWs built in a greenhouse with temperature between 14 - 20 ºC and 16 h of natural / artificial light per day. The CW substrates consisted of a 30 cm layer of crushed rock with 3 - 5 cm diameter and the porosity of the media was 0.35 cm. The flow rate was adjusted for two different experimental phase, 27 mL min^{-1} for a hydraulic retention time of 2 days and 55.5 mL min^{-1} for a hydraulic retention time of 1 day. The water level was kept at 5 cm below the gravel surface. Four of the six systems were planted in interval of 15 cm with *Calla lily*. The water samples were collected three times a week over the experiments. The study showed that the SSFCWs reduced appreciable the concentration of NP in water, and no significant difference was observed in NP reduction between CW planted (57 (± 13) %) and unplanted CW (54 (± 7) %), indicating that in this case the plants had no influence on system performance.

Papaevangelou et al. [535] compared the removal efficiency of EDCs present in municipal wastewater in two types of CWs (HFCW and VFCW) planted with *P. australis* or with *Typha latifolia*. The HF systems were constructed in rectangular tanks with dimensions 3 x 0.75 x 1 m (long x wide x deep). Each tank contained medium gravel (D_{50} = 15.0 mm, range 4 - 25 mm) as porous media at a thickness of 45 cm. The VFCWs were assembled in cylindrical plastic tanks of 0.82 m diameter and 1.5 m height. The substrate included from bottom to top: (a) a drainage layer of cobbles, 15 cm thick (D_{50} = 90 mm, range 30 - 180 mm); (b) a 10 cm thick layer of medium gravel (D_{50} = 24.4 mm, range 10.0 - 35.0 mm), (c) a 15 cm thick layer of fine gravel (D_{50} = 6 mm, range 2.5 - 16.0 mm), and (d) a 10 cm thick layer of sand (D_{50} = 0.5 mm, range 0.2 - 1.5 mm), with a total thickness of 50.0 cm. This drainage layer also contained PVC aeration tubes (50 mm in diameter) perforated only within the cobble drainage layer.

The HFCWs were assembled with three different porous media i.e., medium gravel (D_{50} = 15.0 mm, range 4 - 25 mm), fine gravel (D_{50} = 6 mm, range 0.25 - 16.0 mm) and cobbles (D_{50} = 90 mm, range 30 -180 mm). The hydraulic residence times in the HFCWs were 14 and 20 days and the hydraulic loading rate ranged from 15.8 to 28.6 L / day. In VFCWs two feeding strategies were used: loading period (wet) of 2 days for both and resting periods (dry) of 6 days (hydraulic loading rate of 0.209 m / day) or 4 days (hydraulic loading rate of 0.283 m / day). The influent wastewater was introduced in the systems every 8 h each day. The study showed 96.2 % and 95.6 % of NP removal in VFCWs planted with *P. australis* and *Typha latifolia*, respectively. In unplanted VFCWs only 12.5 % of NP removal was observed and in HFCWs no NP removal were reported. So, this study shows the contribution of plants for NP removal as well as the need of an appropriate CW design.

Toro-Velez et al. [534] investigated over eight weeks (one grab sample at the inlet and outlet collected once per week) the removal efficiency of EDCs (bisphenol A (BPA) and NP) in 3 HFCWs (planted with *P. australis*, planted with *Heliconia psitacorum* and unplanted). The HFCW (surface area of 27 m^2 and 0.6 m of depth), were composed by gravel diameter 20 mm with 40 % of porosity media. The hydraulic retention time was 1.8 day and 0.13 m / day of surface loading rate. In this comparatively study, the biggest NP removal efficiency was observed in CWs planted (*Heliconia psitacorum* 63 (± 20) % and *P. australis* 52 (± 37) %) while the lower removal efficiency was detected in unplanted CW 25 (± 37 %), showing again plants contribution.

Dai et al. [540] studied over two months the removal efficiency from secondary effluent of EDCs (NP; OP; triclosan; estrone; BPA), in an assemble biofilter and in a planted hybrid system (VFCW - HFCW). *Canna glauca, Thalia dealbata, Canna indica, and Typha angustifolia* were planted in VFCW units and *Cyperus alternifolius*, *Arundo donax*, *Acorus tatarinowii*, and *Desmodium styracifolium* were planted in the HFCW units. One single species was planted in each subunit and the influent flowed sequentially through the VFCW and HFCW.

The VFCW units operated under unsaturated conditions and the HFCW units operated under saturated condition. The areas for the distribution and collection of water in the wetland bed were filled with coarse gravel (3 - 5 cm diameter) to avoid clogging, and 1 - 3 cm of diameter gravel was used in the other parts of the system. The assemble biofilter, operating under unsaturated conditions, was designed to provide the optimum oxygen enrichment. This biofilter was divided into four layers of equal thickness (0.4 m per layer) and the air space between adjacent layers was 0.2 m. The upper two layers were filled with blast furnace slag, and the subsequent two layers were filled with gravel. These authors observed that the assemble biofilter showed higher (45 (± 12) % for NP) removal efficiency better than planted

hybrid system (32 (± 21) % of NP) and concluded that aerobic conditions are more effective for the degradation of NP.

Hsieh et al. [539] evaluated the removal efficiencies of selected compounds (veterinary antibiotics, alkylphenolic and estrogens) in CW from wastewater containing concentrated animal feeding (livestock operations and aquaculture farms). The study was conducted in one of the largest wetland systems in southern Taiwan (Wuluo CWs). This wetland system combined filter beds, lotus ponds, and three free flow water CWs (FWCWs). The predominant plant species in lotus ponds, and FWCWs substrate, include native cattail, *Cyperus*, *Phragmites*, and *Vetiveria*.

The FWCWs system consisted of three basins with an impermeable bottom soil and shallow water depth. After 18 months of monitoring, 89.7 % of NP removal was observed and NP removal was higher in the lotus pond than in filter beds and FWSCW units, reflecting uptake by aquatic macrophytes according with authors.

Hsieh et al. [541] examined the distribution and removal efficiencies of phenolic EDCs (nonylphenol diethoxylates (NP_2EO), nonylphenol monoethoxylates (NP_1EO), NP and OP) from wastewater in six CWs (mesocosm systems) in Taiwan: three estuarine CWs system in Dapeng Bay National Scenic Area (DBNSA) and three freshwater CWs system along the Dahan River located in the south and north of Taiwan, respectively. Removal varied from 15.8 to 78.4 % for NP in the estuarine CW system and 41.2 % in freshwater CWs system along the Dahan River. According with authors the concentration of NP decreased in areas with greater plant coverage, indicating that plants in these CWs facilitated adsorption and degradation processes of the compounds.

Although studies in general show significant reductions of NP in CW effluents, increase in the NP concentration in CW effluent when compared with influent was also observed. Carranza-Diaz et al. [542] investigated, over a twelve month period in HSFCWs, the role of *P. australis* on the removal process of organic micropollutants, such as NP, from municipal wastewater. In this work, HSFCWs with density of 5 plants per square meter, granular medium (predominantly gravel) with 8 - 16 mm size and in influent and effluent zones coarse gravel with 16 - 32 mm were used. The systems were dosed with the municipal wastewater at a rate of 5 L every 30 min, leading to an average inflow rate of 0.18 m^3 per day. The authors observed that the effluent concentrations of NP were significantly higher than the influent concentrations in both planted and unplanted system. Authors associate this increased concentration to the biodegradation of alkylphenol polyethoxylate that was also present in the wastewater (e.g. municipal wastewater [543]) that resulted in the formation of NP.

1.4.2. Octylphenol

Dai et al. [540] studied over two months EDCs removal efficiency from secondary effluent, in an assemble biofilter and in planted VFCW - HFCW. The assemble biofilter showed higher (58 (± 19) % for OP) removal efficiency than planted hybrid system (51 (± 25) % of OP), concluding that aerobic conditions were more effective for the degradation of OP.

Hsieh et al. [539] evaluated the removal efficiencies of selected compounds (veterinary antibiotics, alkylphenolic and estrogens) in a wetland system (that included filter beds, lotus ponds, and three free FWCWs) receiving wastewater with concentrated animal feeding. After 18 months of monitoring, a higher removal efficiency for OP (85.1 %) in FWSCW units was observed, showing the influence of plants and CW design.

Hsieh et al. [541] examined the distribution and removal efficiencies of phenolic EDCs (NP_2EO, NP_1EO, NP and OP) in wastewater treated in three estuarine CWs system in DBNSA and three freshwater CWs system along the Dahan River. The estuarine CWs received domestic and aquaculture wastewater and freshwater CWs only received domestic wastewater. When the inflow concentration was compared with outflow, an OP removal efficiency from 38.4 % to 42.7 % was observed in the estuarine CW system and of 89.4 % in freshwater CWs system along the Dahan River, indicating that environmental conditions can affect system performance.

Nguyen et al. [544] evaluated the effect of combining aerobic and anoxic HSFCW on nutrient, organic, metals and OP removal. Two HSFCWs were constructed, the first with only three aerobic compartments ($HSFCW_1$) and the second with two combined aerobic compartments separated by anoxic compartments ($HSFCW_2$). The two HSFCWs had the same design parameters (filters, plants), except oxygen conditions. Both HSFCW systems were planted with *P. australis*, except the anoxic compartment), filter materials were 50 cm thick, including 5 cm gravel (D = 30 - 50 mm), 20 cm small gravel (D = 5 - 8 mm), 15 cm quartz sand (D = 1 - 2 mm) and 10 cm rock (D = 12 - 15 mm) in the order from the bottom to the HSFCW surface. Results showed that OP removal was higher in the $HSFCW_1$ (aerobic condition and planted in all compartments) than in $HSFCW_2$ (combined aerobic and planted with anoxic and unplanted compartments), the removal efficiencies being 68 % and 57 % respectively. The authors hypothesized that lower oxygen and lack of vegetation decreased removal efficiencies. So, combined aerobic and anoxic condition may decrease by 11 % the efficiency of OP treatment.

1.4.3. Pentachlorophenol

Matamoros et al. [204] tested the removal of different emergent compounds, including PCP, in eight SSFCW systems planted with *P. australis*. The systems were characterized by an average water depth of 0.3 m, a surface area of 55 m^2, an average granular medium size of 3.5 mm (granitic gravel) and a hydraulic loading rate at the time of the experiments of 36 mm / day. Authors observed that 94 % of PCP was effectively removed from the system but only 23 % of the spiked PCP was retained in the gravel, indicating that gravel bed accumulation was not a major removal pathway in the wetland.

Choudhary et al. [545] used SSFCW composed by fine sand and gravel (layer up to 0.28 m) with particle size of 0 - 10 mm, 0.27 mm of porosity media, planted in interval of 30 cm with *Canna indica* and uniform distribution of wastewater across the system. With this system configuration, these authors observed removal efficiency of chlorophenolic compounds between 66.7 % and 96 %. In the case of PCP a removal efficiency of 89.1 % was observed. These authors also observed that between 36.5 % to 77.9 % of the chlorophenolic compounds were removed by sorption processes and removal processes were more effective for highly chlorinated chlorophenolics as compared to lower chlorinated chlorophenolics [545]. Also, microbial degradation accounted for removals of 19.9 % to 28.8 % after 48 h and 30.6 % to 43.3 % after 96 h. The extent of microbial degradation or transformation of organic compounds within a CW is also expected to strongly depend on the physicochemical properties (e.g., water solubility and concentration) of the contaminant [400].

Levesque *et al*. [546] evaluated the performances of horizontal sub - surface flow hybrid constructed wetland (HSSF - HCW) followed by a willow planted filter (WPF) in wastewater treatment contaminated by wood pole preservatives (PCP and chromated copper arsenate). The HSSF - HCW received 1 m^3 / d and are composed of four sequential sections, one wetland and three filters, separated by empty permeable plastic sampling boxes. The first section was filled with sand (52 %), blond peat (40 %) and zero valent iron powder (ZVI; 25 µm; 8 % v) and was maintained under anaerobic conditions. The second section was filled with sand (75 %), black peat (25 %) and calcined clay (5 %), equipped with forced aeration, and planted with *P. australis*. The third section was filled with electric arc furnace steel slag to remove metals, especially ZVI, escaping from the first section. The fourth section was made of 100 % blond peat to neutralize the high pH arising from the steel slag section. The effluent of HSSF - HCW was pumped into WPF for polishing. The WPF, consisting in 48 m^2, 1.5 m deep and filled with sand (80 %) and brown peat (20 %) and planted with *Salix miyabeana* SX67. More than 98.6 % of PCP was removed by the hybrid CW system. These authors suggest that PCP leachate would be treated, first sent to a system composed of anaerobic

and aerobic zone CWs followed by underground retention basins upstream of a total evapotranspiration WPF.

All studies presented have shown the potentialities of CWs for the removal of NP, OP and PCP indicating that all CWs components can have a significant role in compounds removal. But more studies are needed to fully understand the role of plants, microorganisms, substrate and CWs design in this removal to optimize and increase CWs performance and applicability.

Moreover, currently, available data of NP, OP or PCP effect on CWs performance, including on CW microbial community, are still limited and more research is needed to fully assess their removal potentialities.

1.5. Objectives of this work

This study aimed to investigate the effect of two different groups of phenolic compounds on CWs performance, including on organic matter, nutrients and contaminants removal efficiency, and on microbial community structure in the plant bed substrate, improving the knowledge regarding the processes occurring in CWs to be able to promote the implementation and good operation of these green systems.

Specifically, it was intended to study:

1) The performance of CW systems for the removal of phenolic compounds, namely alkylphenols (nonylphenol (NP) and octylphenol (OP)) and chlorophenols (pentachlorophenol (PCP)) from contaminated water;

2) The possible influence of phenolic contaminants on CWs performance to remove organic matter and nutrients;

3) Characterization, at phylum, genus and class level, of the microbial community of *P. australis* rhizosphere in CW treating water contaminated with alkylphenols (nonylphenol (NP) and octylphenol (OP)) or chlorophenols (pentachlorophenol (PCP));

4) To assess the response of the microbial community (richness, diversity (*Shannon* index) and dominance (*Berger-Parker* index) in CW substrate to the presence of alkylphenols (nonylphenol (NP) and octylphenol (OP)) or chlorophenols (pentachlorophenol (PCP));

5) To evaluate if the enzymatic machinery necessary for the degradation process of phenolic compounds is present in CW microbial community.

2.Experimental Part

2.1. Materials and Reagents

Methanol of HPLC Grade (CH_4O; CAS-No:67-56-1; MW = 32.04 g mol^{-1}), supplied by Fisher Scientific (Loughborough, UK), was used. Ethanol absolute PA (C_2H_6O; CAS-No:64-17-5 MW = 46.07 g mol^{-1}) and sodium carbonate anhydre (Na_2CO_3; MW = 105.99 g mol^{-1}) were supplied by Panreac (Barcelona, Spain). Acetic anhydride extra pure ($C_4H_6O_3$; MW = 102.09 g mol^{-1}), sodium chloride for analysis (NaCl; CAS-No:7647-14-5; MW = 58,44 g mol^{-1}) and Sulfuric acid (H_2SO_4; CAS-No:7664-93-9; MW = 98.08 g mol^{-1}) were supplied by Merck (Darmstadt, Germany).

Standards of 4-tert-Octylphenol (cot # MKBT8005V; CAS:140-66-9; MW = 206.32 g moL^{-1}) and 4-Nonylphenol (cot # SZBD273XV; CAS:104-40-5; MW = 220.35 g mol^{-1}) Sigma ALDRICH (Steinhein, Germany), were obtained from Supelco. The stock solutions of 4-tert-Octylphenol (OP) and 4-Nonylphenol (NP) at 1.0 g L^{-1} were prepared in methanol and stored at 4 °C. Working standard mixture of OP with NP (defined as OP_NP) were prepared at 1.0 mg L^{-1} and stored at 4 °C for a maximum of one week. Standard of Pentachlorophenol (Lot#G1411588; CAS: 87-86-5; MW = 266.32 g mol^{-1}) Dr Ehrenstorfer GmBH (Augsburg, Germany). The stock solutions of PCP at 1.0 g L^{-1} were prepared in ethanol and stored at 4 °C.

Gel agarose of grade standard and low electroendosmosis (GRS Agarose LE; CAS: 9012-36-6) was supplied by Grisp (Porto, Portugal). The TAE buffer used in electrophoresis was supplied by Thermo Fisher Scientific (Altrincham, UK) and SYBR® safe was supplied by invitrogen (Carlsbad, USA). All products used in DNA extraction were supplied by Qiagen (Hilden, Germany) and all product used in DNA quantification in Qubit® fluorometer kit were supplied by (Thermo Fisher Scientific; Carlsbad, EUA).

All material used was properly decontaminated by washing with deionized water, immersion in a 20 % nitric acid solution overnight, washing with deionized water and dried in an oven at 30 °C.

2.2. Constructed Wetlands experiments

2.2.1. Microcosm experiments

Phragmites australis (*P. australis*) was the emergent macrophyte chosen for this work due to its anatomical characteristics, presented a fibrous and dense root system and resistance when exposed to contaminated media. All plants (*P. australis*) (Fig. 2.1. a), sediments and sand used to assemble the CW systems of this work were collected in *Lima River* estuary (Lat: 41º 41'21.5" N and Long: 8º48'56.4" W, north of Portugal). Only young plants (plants without a senescent appearance) and with similar size were collected with the sediment attached to roots (rhizosediment) in cubes with 20 cm of edge (Fig. 2.1 b) and c). Simultaneously, sand was collected within the estuarine area, ca. 3 m from the site where plants were collected. Sediment attached to belowground structures of *P. australis* was removed and discarded. The sampling process in estuary of *Lima River* is illustrated in Fig. 2.2.

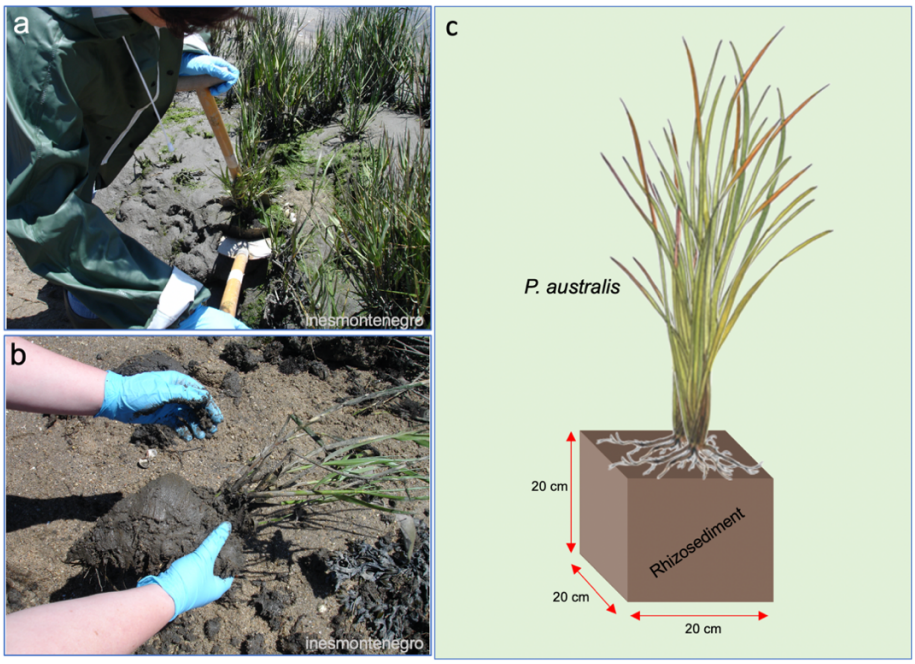


Fig. 2.1 - a) Image of collected *P. australis* in *Lima River* estuary; b) Image of *P. australis* with rhizosediment; **c)** Schematic illustration of the dimension of rhizosediment cubes collected in sampling held in the *Lima River* Estuary.

Six CWs were assembled at laboratory scale in vertical flow configuration (vertical flow constructed wetland; VFCW), as described by Carvalho et al. [547], and adapted to automatically operate with a recirculating water system (flow rate ca. 10 mL min^{-1}) as described in Gorito et al. [548]. Water level was maintained just above the substrate surface, i.e., the CWs substrate were kept at their water saturation level.

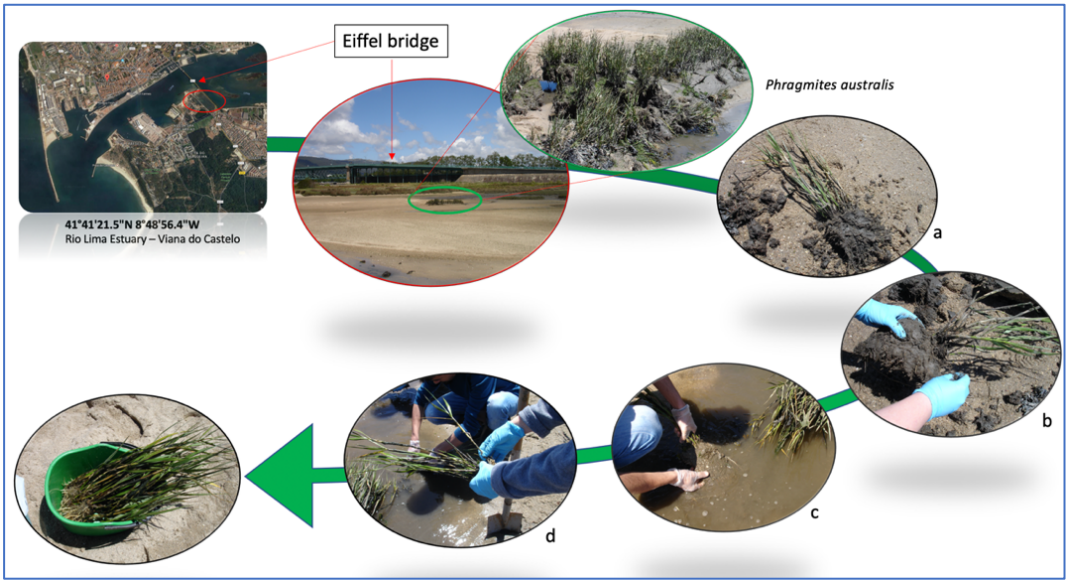


Fig. 2.2 - Schematic illustration of step by step of the sampling process of *P. australis* at low tide in *Lima River* Estuary. **a**) *P. australis* with rhizosediment cubes collected in *Lima River* Estuary. **b)** Removal from part of the sediment from the root system of *P. australis*. **c**) Washing of *P. australis* root system. **d)** Removal of residues (biological and non - biological) from the above - ground part of the plant.

Each microcosm was set up using plastic boxes (56 x 39 x 28 cm). Each box had three layers and for simulating the dark natural condition of soil and also to preventing photodegradation of the compounds, each box was involved in aluminum foil. Regarding the layers: main layer was the roots bed substrate on top (16 cm), the intermediate layer is lava rock (2 cm) and drainage layer is gravel on bottom (4 cm) (Fig. 2.3 a) b) and c). With the objective to increase the substrate porosity the main layer is composed by sediment and sand mixture in a proportion of 1:2. The main layer is a physical support for *P. australis* rhizomes and roots maintaining in rhizosphere all the native microbial community activity (Fig. 2.3 d). About forty plants with the same length stems and in good physiological condition were placed in each microcosm. The lava rock layer was used with the purpose to provide a support for the microbial community (capable of degrading the compounds under study) to form a biofilm and also a physical structure for retaining (through sorption) the target compounds (Fig. 2.3 b). The gravel layer was used to promote good drainage of the system (Fig. 2.3 a) and also prevent possible blockages of the system.

The six CW systems were kept under “greenhouse” conditions (Fig. 2.4 a) and b), namely in nature light cycle. The temperature was recorded weekly during the experimental period. In experiment #1 the min. temperature ranged between 10 - 13 °C and max. temperature between 22 - 30 °C, while, in experiment #2 the min. temperature ranged between 15 - 22 °C and max temperature between 23 - 27 °C. The CWs was under saturated water conditions and with an effluent recirculation system. The effluents recirculation period was interrupted by 12 h of non-recirculation period.

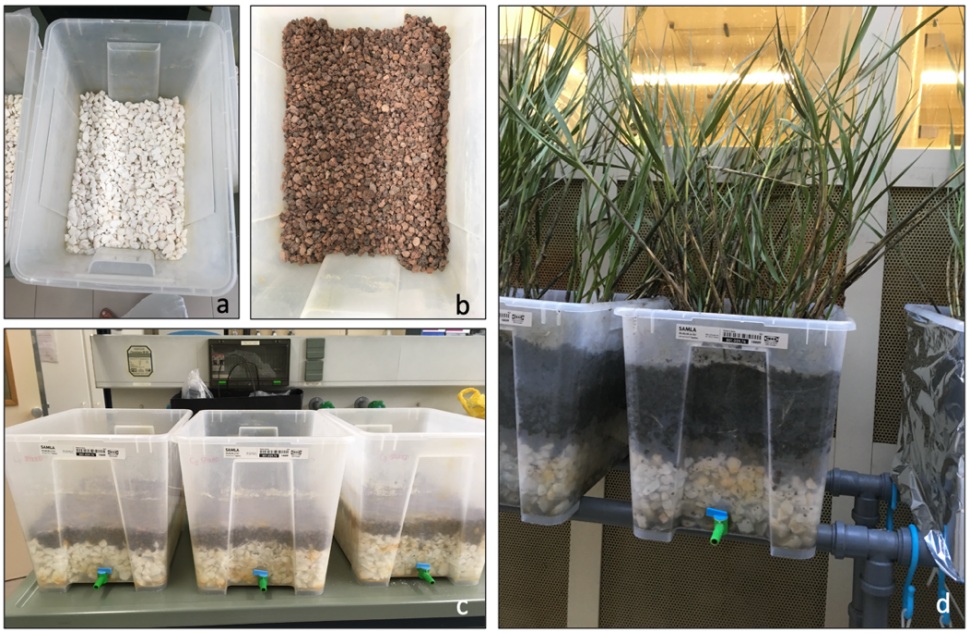


Fig. 2.3 - Schematic illustration of step by step of CW assembly in the laboratory. (**a**) Layer of gravel. (**b**) Layer of lava rock. (**c**) CW with two layers (gravels stones and lava rock). (**d**) CW with 3 different layers (gravel, lava rock and sediment plus sand) and plants (*P. australis*), before each box was covered with aluminum foil.

Fig. 2.4 - **a)** The six CWs with recirculation water system in "greenhouse" conditions. **b)** A view of space where the CWs were during the experimental period under "greenhouse" conditions.

The schematic representation of VFCW with a recirculating water system is shown in Fig. 2.5. Before the beginning of the experiment period, systems were acclimated for one week with Hoagland nutrient solution in order to provide suitable nutritional conditions to the system.

After this first acclimatization period followed a second acclimatization period. In this acclimatization period (two weeks) the six CWs systems were weekly irrigated with 1.5 L of water of *Ribeira de Joane*. The water from *Ribeira de Joane* used in the experiments was collected weekly from the mouth located in the *Praia do Paraíso* (Lat: 41°13'33.5"N; and Long: 8°43'00.4"W) (Fig. 2.6).

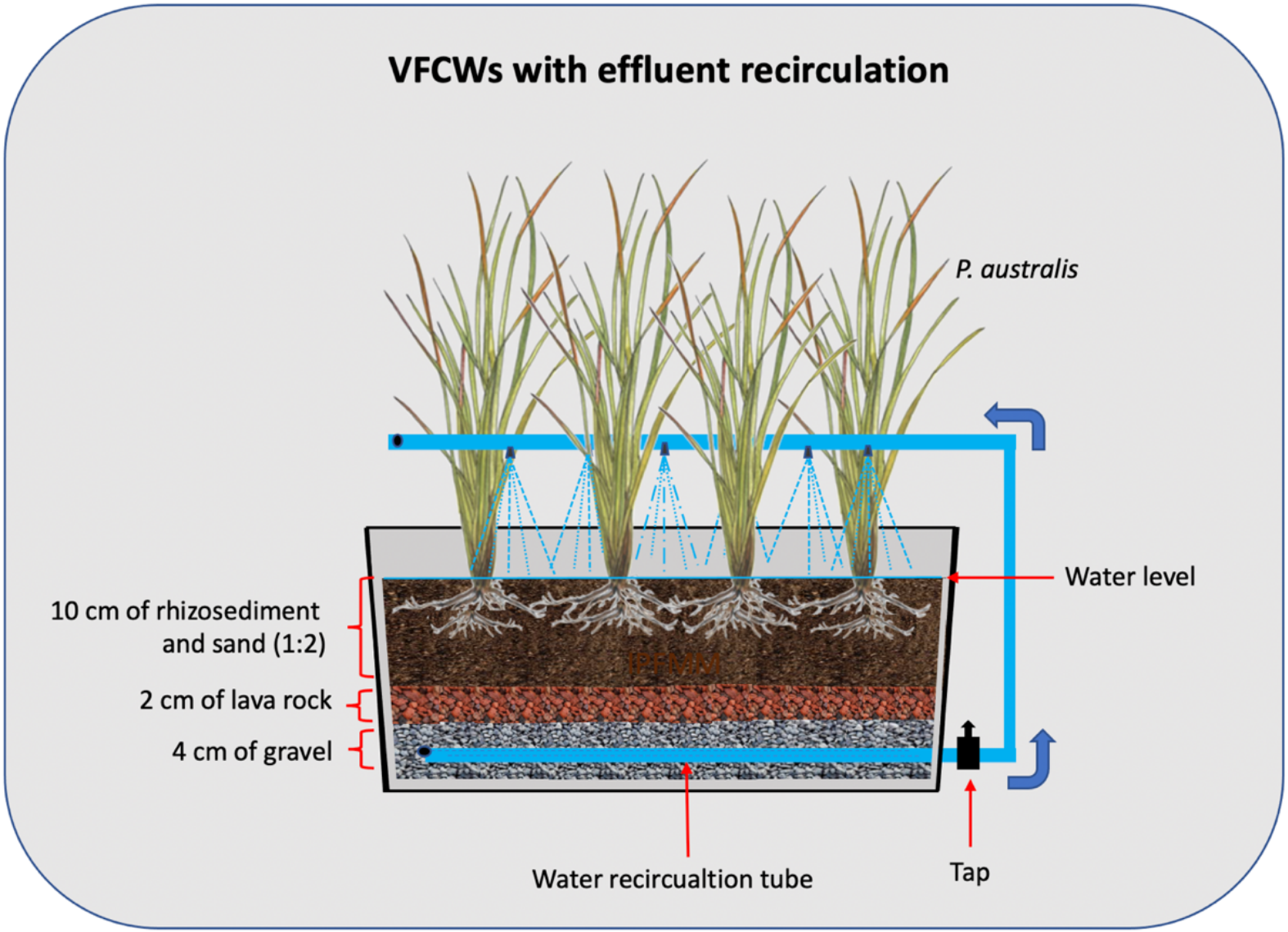


Fig. 2.5 - Schematic representation of CW with a recirculating water system containing an air pump attached to a timer. The water recirculation is done through a tube with 2 cm of diameter. The water drained at the bottom of the CW is released through the tube into the top of CW. The tap in CW was used for water weekly sampling.

The *Ribeira de Joane*, runs 3.7 km through a very urbanized area and its main run is canalized and can be defined as an urban stream that receives some untreated wastewater water as well as storm water. The *Ribeira de Joane* water was a crucial element in the rural activity located along of stream banks.

This experimental work was performed in two different experiments (experiment #1 and experiment #2). The same experimental procedure was used for testing two different group of phenolic compounds. In experiment #1, it was tested NP and OP removal and occurred during spring of 2018, while in experiment #2, it was tested PCP removal and occurred during spring of 2019.

Fig. 2.6 - Images of the weekly sampling performed during the experimental time in the *Ribeira de Joane* in *Praia Paraíso* (Matosinhos, Portugal).

The experimental period ran for eight weeks. In this period, three of the CWs (defined as CWs doped) were weekly irrigated with 1.5 L of *Ribeira de Joane* water doped with 100 µg L^{-1} each target compound (NP and OP in experiment #1 and with PCP in experiment #2). The doping concentration was based in concentrations previously used in other works. The other three CWs, (defined as CWs control) were irrigated only with 1.5 L of *Ribeira de Joane* water, each one. The water from the *Ribeira de Joane* is defined as CWs influents and the water that is removed from the CWs is defined as effluent in this work. The influents and effluents CWs were monitored for eight weeks, and the water losses were readjusted by adding deionized water to each CW system.

2.2.2. Sampling

2.2.2.1. Water sampling

Before and after of the eight days in recirculation process in the CWs system, *Ribeira de Joane* water was collected for analysis. The schematic representation of this process is shown in Fig. 2.7.

Weekly during the experimental period (eight weeks) water from influent and effluent of all CWs were collected for analysis of organic matter (through COD measurement), carbon organic total (TOC) only in experiment # 2, nutrients (ammonia, nitrate, nitrite and phosphates) and target compounds (NP_OP in experiment #1 and PCP in experiment #2).

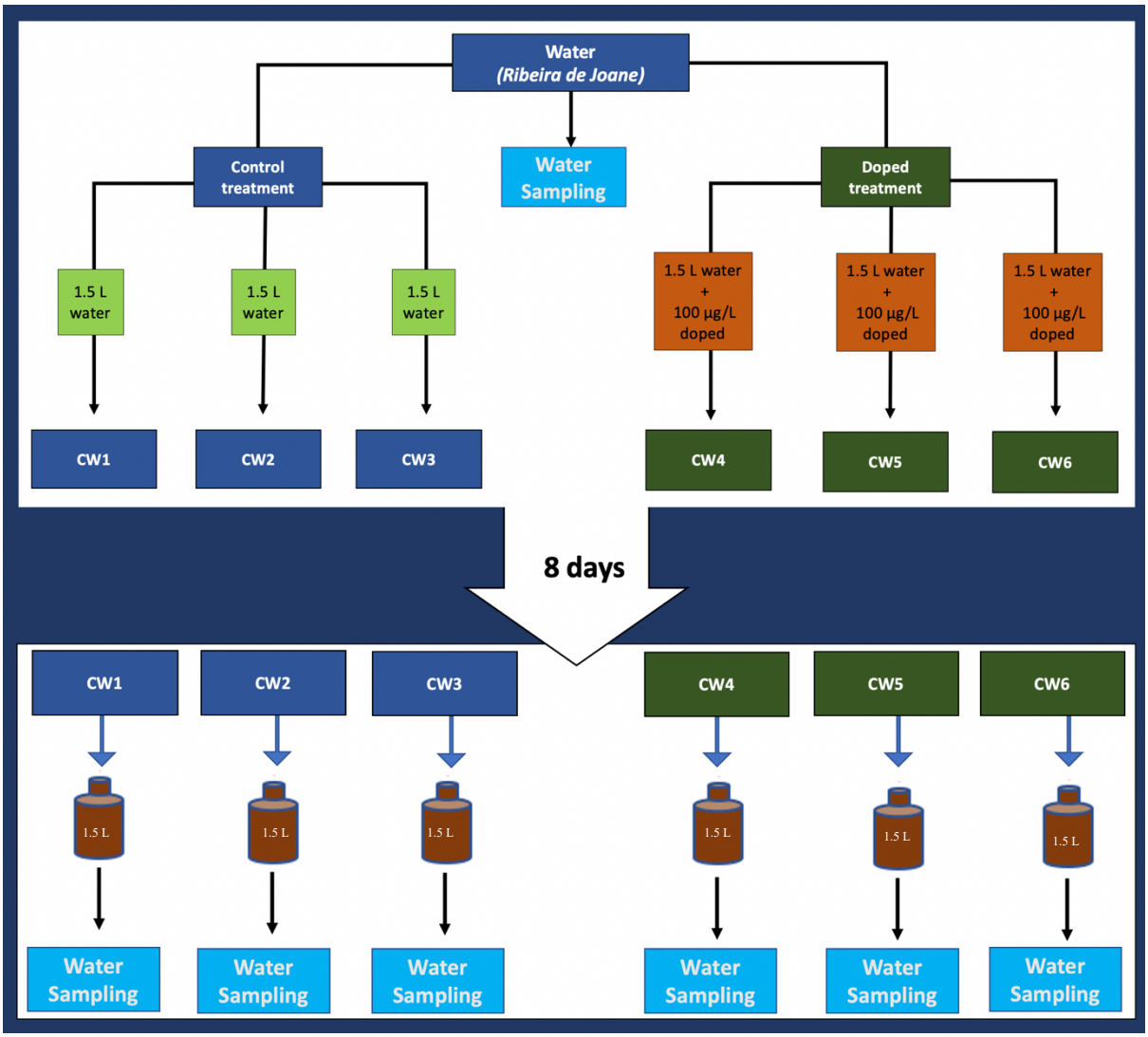


Fig. 2.7 - The experimental design of each week of the experimental period (eight weeks). The flow chart represents the irrigation process of CWs with water of *Ribeira de Joane* not doped in CWs of control treatment and doped with target compounds in CWs of doped treatment. The flow chart also contains the sampling water process.

From each CWs, 1.5 L of effluent was collected, deionized water has been added when this volume was not reached. In Fig. 2.8 a) is shown the CW effluent collection process to a bottle previously descontaminated.

The preparation and storage of the water samples replicas for the various analyzes was carried out right after the collection from the CW Fig. 2.8 b) and c) from *Ribeira de Joane*.

The water samples for nutrients analyses were stored in plastic tubes of 15 mL while the water samples for target compounds chemical analyses were stored in glass tubes of 20 mL. The COD measurements were always made on the day of collection, the remaining water samples (properly labeled) were stored at -20 ºC until analysis. Before the analysis, each water sample was previously acclimatized at room temperature in the laboratory.

Fig. 2.8 - a) CWs effluent collection for analysis in bottle previously decontaminated in an acid bath; **b)** Preparation of sample replicated in the laboratory; **c)** Samples labels for storage at -20 ºC.

2.2.2.2. Sediment sampling

Sediment samples were collected from CW systems at time T0 (week before the experimental period start) and time T8 (in the last week of experiment period) to be used in chemical analyses of target compounds and in molecular biology analyses of microbial community (Fig. 2.9 a) and b).

Approximately 20 g of sediment (up to 5 - 8 cm below surface sediment) were collected from each CW and homogenized (Fig 2.10 a) and b). All stones and plant tissues were removed.

According to the type of analysis, the sediment was stored in different recipients, in aluminum foil for chemical analysis and in eppendorf tubes (1.5 mL) for biological analysis. All sediment samples were immediately stored at - 20 ºC until it is analyzed.

Before chemical sediment analysis, the samples were exposed to a lyophilization process. The lyophilization technology is described in some detail in Annex A3. In the case of biological analysis, the samples were exposed to room temperature until thawed (about 2 hours).

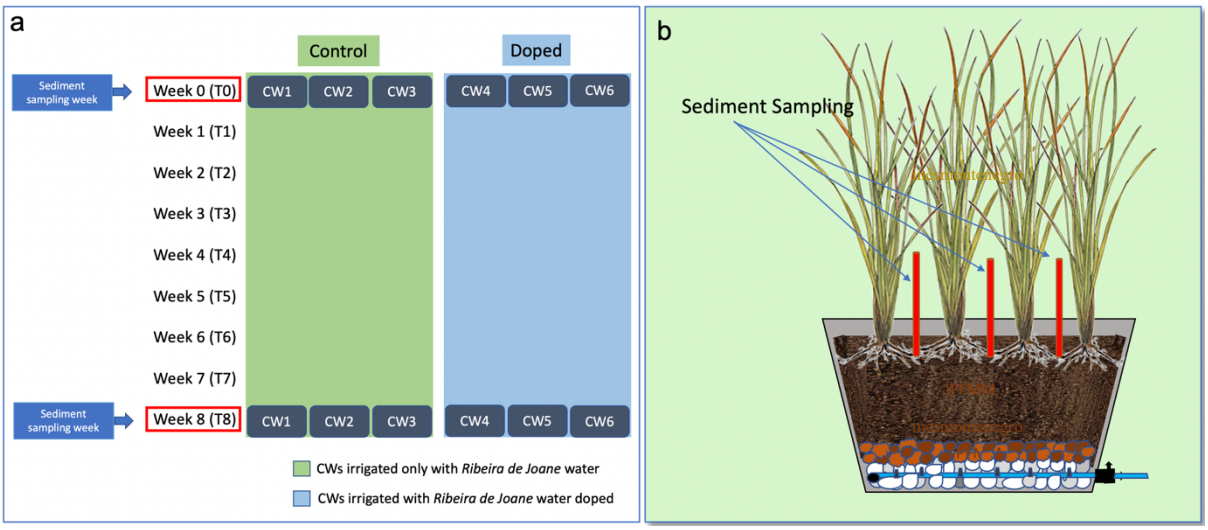


Fig. 2.9 - a) The experimental schematics of sediment sampling; **b)** schematic representation of sediment sampling in each CW.

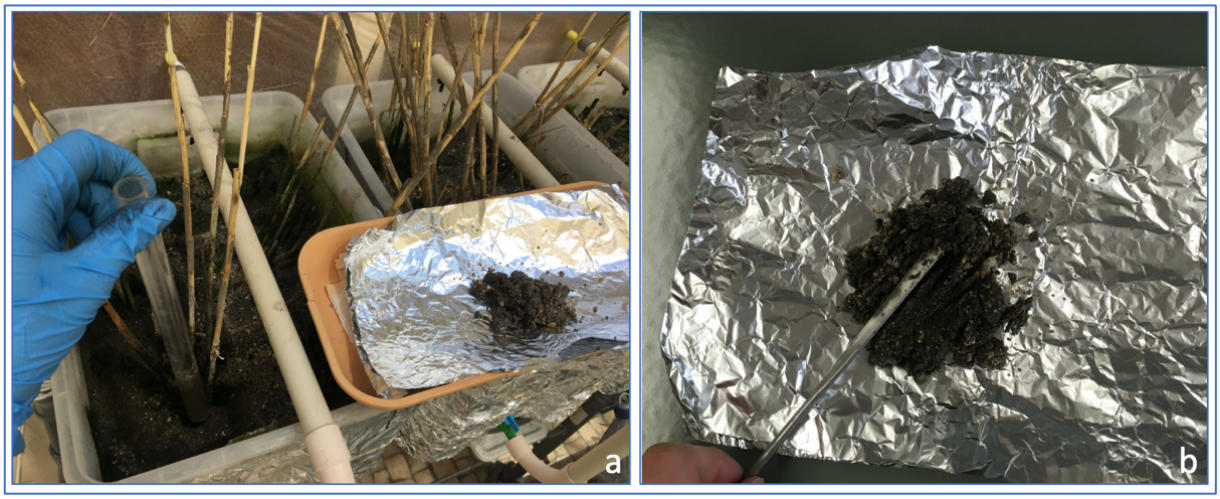


Fig. 2.10 - a) Sediment sampling in each CW; **b)** homogenization process of each sediment sampling.

2.3. Analytical methodologies

2.3.1. Nutrient analysis

The analysis of nutrients were carried out in the water collected weekly in *Ribeira de Joane* and in CWs effluents. Orthophosphate (PO_4^{3-}), nitrite (NO_2^-) and ammonium (NH_4^+) ions were quantified using methods described in Grasshoff [549]. Nitrate ion (NO_3^-) was measured using an adaptation of the spongy cadmium reduction technique [550], with the nitrite value subtracted from the total. In Fig. 2.11 a) and b) was shown detail of steps of nutrient analyzes performed in the laboratory. Analysis was carried out by ultraviolet and Visible Absorption Spectroscopy (UV - Vis; V-1200 Spectrophotometer) supplied by Perkin Elmer (Waltham, EUA) (Fig. 2.12). Specific wavelengths used were: 540 nm for NO_2^- and NO_3^-, 630 nm for NH_4^+ and 880 nm for PO_4^{3-}. Details about the spectrophotometer apparatus are included in Annex A4.

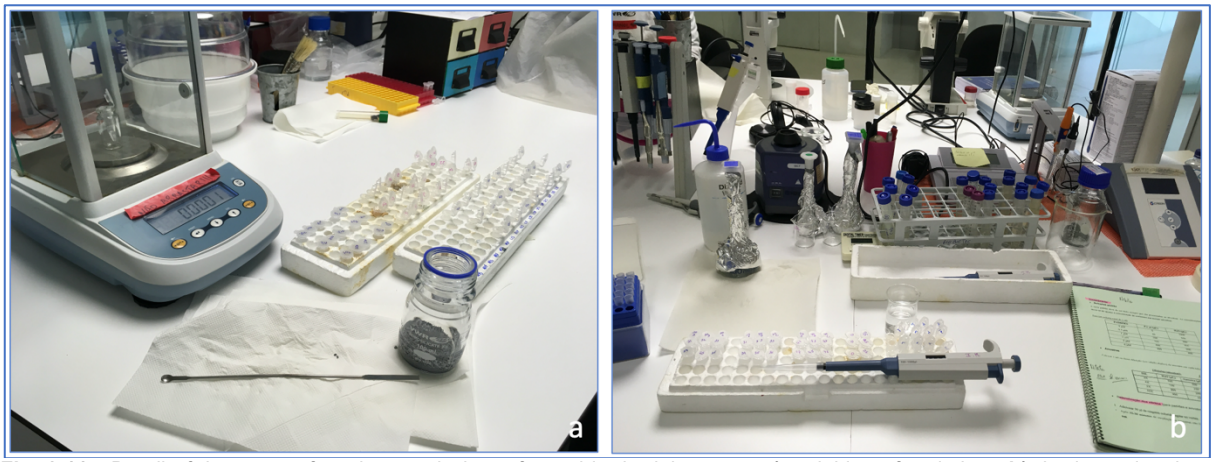


Fig. 2.11 - Detail of the steps of nutrient analysis performed in the laboratory; **a)** weighing of cadmium; **b)** the long pipetting process of the specific reagents and water samples in the eppendorf tubes.

Fig. 2.12 - Spectrophotometer used in nutrients quantification (equipment brand: V-1200 Spectrophotometer).

2.3.2. Chemical Oxygen Demand analysis

Chemical Oxygen Demand (COD) was measured using a kit with a reagent tube with barcode for COD test, provided by HANNA instruments (Fig. 2.13 a). Before the COD measurement in a photometer equipment (brand: HANNA instruments - HI83214 Multiparameter Bench Photometer; supplied by HANNA instruments (Leighton Buzzard, UK)), the reagent tubes with 2 mL of water sample each, were placed into a heater block (brand: HANNA instruments - HI 839800; supplied by HANNA instruments (Leighton Buzzard, UK)) at 150 °C during two hours for the oxidation reaction to occur (Fig. 2.13 b) and c).

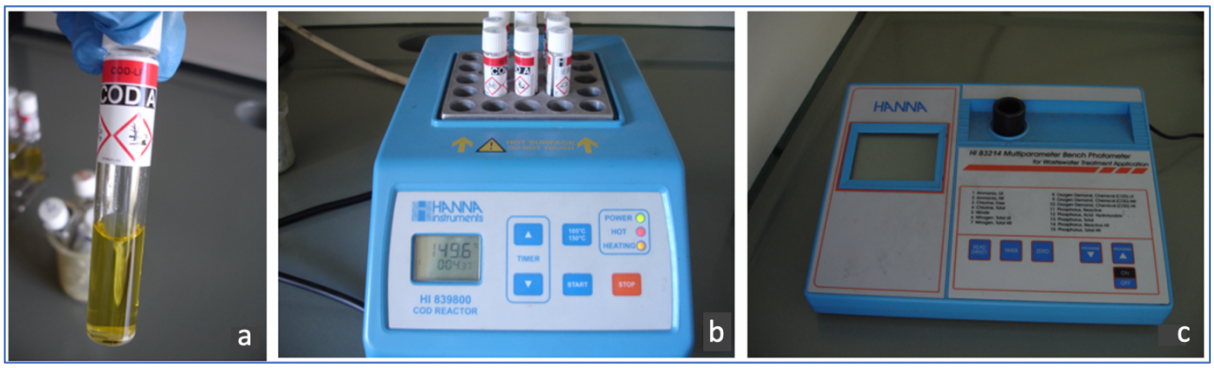


Fig. 2.13 - a) Test tube containing a mixture of sulfuric acid and the dichromate ion; **b)** Heater block (equipment brand: HANNA instruments - HI 839800-COD REATOR); **c)** Spectrophotometer used for COD quantification (equipment brand: HANNA instruments-HI 83214 Multiparameter Bench Photometer).

2.3.3. Total Organic Carbon analysis

The water samples were acidified (pH = 2) with a solution of H_2SO_4 (1.0 mol L^{-1}) and maintain at 4 º C until they were analyzed. The Total Organic Carbon (TOC) was measured by the catalytic carbon oxidation process, at 680 ºC and the CO_2 formed is measured by infra-red (IR). For measurement, SHIMADZU TOC-L/TNM-L apparatus was used. These analyzes were made through an external service provided by the Engineering Faculty of the University of Porto.

2.3.4. pH analysis

The pH measurement was made weekly in water from *Ribeira de Joane* and in water of all CW. pH was measured with a Crison Micro pH 2002 meter supplied by Mettler Toledo

(Greifensee, Switzerland) Potentiometric were carried out with a PC - controlled system assembled with a Crison Micro pH 2002 pH meter (Fig. 2.14). An Orion 90-02-00 (double junction AgCl/Ag) reference electrode and a Russel SWL/S7 glass electrode.

Fig. 2.14 - Measurement of pH in each water samples using a Crison Micro pH 2002 meter.

2.3.5. Phenolic compounds measurements by GC - MS

The detection of the selected phenolic compounds in the influent and effluent of CWs was determined using an analytical methodology that couples solid phase microextraction with gas chromatography tandem mass spectrometry (SPME–GC-MS) analysis. The same analytical methodology was used in the detection of the selected compounds in sediments samples, which were first subjected to an extraction process.

2.3.5.1. Extraction methods used for sediments

Frozen samples (- 20 °C) were previously exposed to a lyophilization process (Bench Top Pro manifold lyophilizers with Omnitronics control – SP Scientific). Details about the lyophilizer (apparatus) and lyophilization process are included in supplementary material, attachments, Annex A3.

In both experiments the target compounds (NP and OP, experiment #1) and (PCP, Eperiment #2) were extracted from sediments with methanol in an ultrasonic bath (Transsonic

- 460/H, with a working frequency 40kHz (1000W)) for 15 min. Prior to that, one gram of dry sediment samples was weighted and mixed with 5 mL of methanol. Each sample was prepared in triplicate. After the extraction, the solution was centrifuged (5 min at 2500 rpm). The extract was collected to a new vial and 5.0 mL of methanol were added to the sediment sample and subject again to 15 min. of extraction in the ultrasonic bath. Details about the ultrasonic bath (apparatus) and ultrasonic extraction process are included in supplementary material, attachments, Annex A2. After that, the solution was centrifuged (5 min at 2500 rpm), and the extract was combined with the previous one. The 10.0 mL of extract was stored at -20 °C in the dark until analysis. Schematics of the experimental procedure flow chart is shown in Fig. 2.15. Details about the method development for extraction phenolic compounds, used in this work, are included in supplementary material, attachments, Annex B.

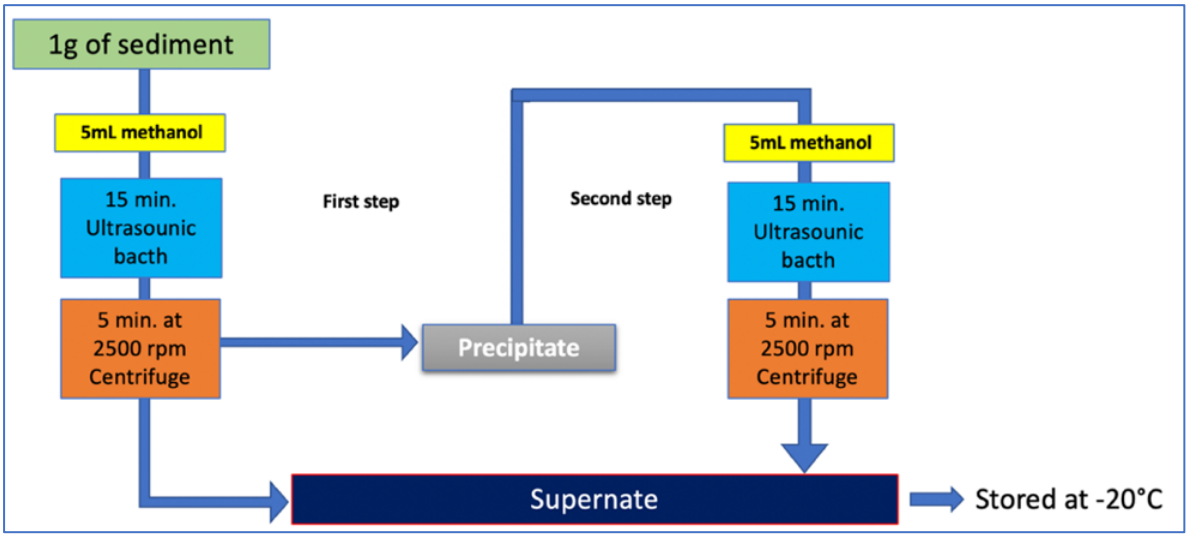


Fig. 2.15 - Flow chart of experimental procedure of sequential extraction of target compounds from sediment for analysis of phenolic compounds.

2.3.5.2. Chromatographic water samples and sediment extracts preparation

2.3.5.2.1. Salting - out technique

The salting - out technique, addition of the sodium chloride (g) to a water sample (10.0 mL), was used to enhance the extraction process of these organic compounds from water samples to the headspace. In the preparation of samples for phenolic compounds analysis, a salting out agent, sodium chloride (NaCl), was added to each sample. Fig. 2.16 represent a

simplistic representation of the salting - out process. In this process, dissolved anions of high charge density (PO_4^{3-} ; SO_4^{2-} ; $S_2O_3^{2-}$; CO_3^{2-}; OH^-; F^-; Cl^-) cause salting out through a combination of electronic repulsion [551, 552] and enhancement of the hydrophobic effect [553–557].

In pure water, the hydrophobic effect causes solute aggregation to minimize the entropy penalty associated with highly ordered structure at the solute-water interface [558]. In the presence of salts of high charge density the analogous surface contacts are more ordered and promoting an even larger entropic penalty [556, 559–562]. Therefore, fully water solvated states induce aggregation [556, 560, 562] and causes passing of the compound from the aqueous phase to the organic phase.

In the headspace (HS) - SPME of aqueous or suspended /solubilized samples, the efficiency of the extraction is generally improved by using a salting out agent able to increase the concentration of the polar analytes in the HS, and thus into the SPME fiber coating, because this increases the ionic strength of the solution and leads to decreased solubility of these compounds in the solution [563]. This was the approach followed in this work, addition of 3.0 g of NaCl per 10.0 mL of each water sample or 200 µL of extract sample in case experiment #1 and 1.0 g of NaCl per 9.4 mL of each water sample or 200 µL of extract sample in experiment #2.

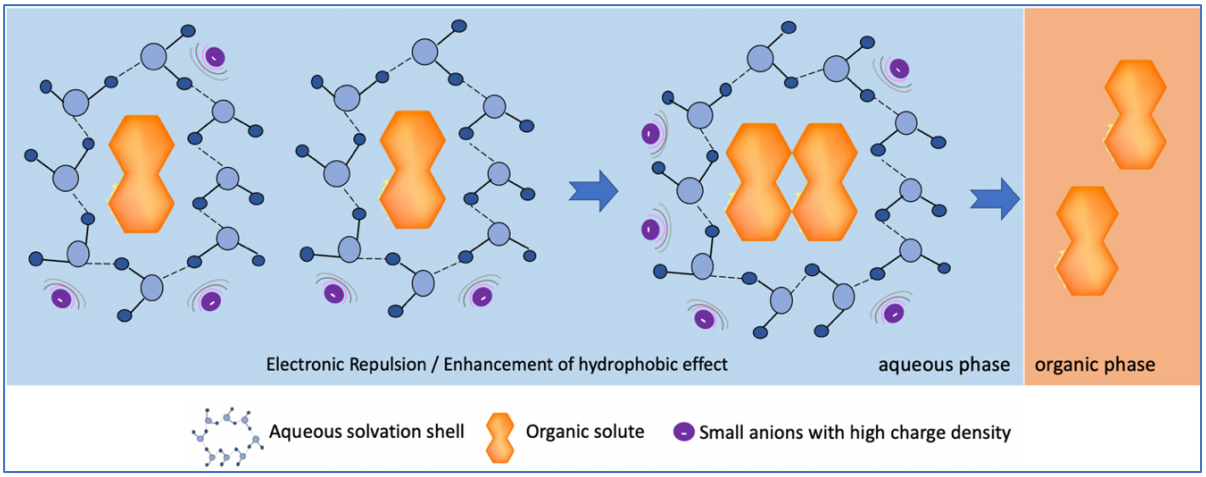


Fig. 2.16 - Molecular forces that dictate the aqueous solubility of organic solutes. Image adapted from Hyde et al. [25].

2.3.5.2.2. Derivatization reaction

Only the water and sediment samples of experiment #2 were exposed to a derivatization reaction. Acetic anhydride was used as derivatizing reagent to convert PCP into nonpolar ester compounds according to the acylation reaction [564]. Derivatization reaction is

shown in Fig. 2.17. Before GC - MS analysis, the water samples and extract of sediment samples, in experiment #2, were derivatized with acetic anhydride ($C_4H_6O_3$) in sodium carbonate anhydride (Na_2CO_3) buffer media. This was the approach followed in this work, addition of 0.5 mL of Na_2CO_3 and 0.1 mL $C_4H_6O_3$ per 9.4 mL of water sample or 200 µL of extract sample.

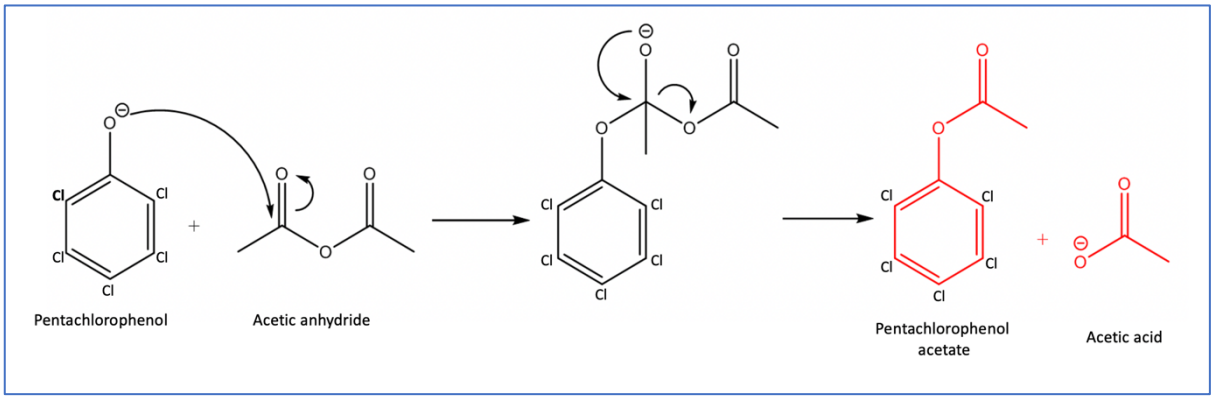


Fig. 2.17 - Derivatization reaction of PCP with acetic anhydride.

2.3.5.3. Preparation of samples for HS - SPME

The HS - SPME method was used to extract and to pre-concentrate the target compounds from the samples before GC - MS analysis using a methodology previously optimized in the laboratory by de Morais et al. [565]. The compounds measured are volatile organic compounds therefore, a headspace (HS) sampling system was used for extracting these compounds. HS - SPME procedure followed by GC coupled with MS was used as a sensitive and selective tool for the determination of target compounds.

In the case of NP and OP measurements, HS - SPME was carried out in 20 mL headspace vials closed with Teflon - lined septa (Supelco) to which 3.0 g of NaCl and 10.0 mL of water sample (in case of water analysis) or 200 µL of extract plus 9.8 mL of deionized water (in case of sediment analysis) were added.

In the case of PCP measurements, HS - SPME was carried out in 20 mL headspace vials closed with Teflon - lined septa (Supelco) to which 1.0 g of NaCl, 0.5 mL of daily prepared solution of Na_2CO_3 in deionized water (20 % W/V) and 0.1 mL acetic anhydride and 9.4 mL of water of sample (in the case of water analysis) or 200 µL of extract plus 9.2 mL deionized water (in the case of sediment analysis) were added.

The obtained solution was pre-equilibrated at 70 ºC for 10 min and shaken at 500 rotations per minute (rpm), after which, the microextraction process occurred at the same temperature for 60 min and 250 rpm. HS - SPME was performed by an automatic sampler

CTC Analytic, CombiPal model (Fig. 2.18 a); San Diego - USA), using 60 µm polyethylene glycol (PEG) fiber from Supelco in the case of NP and OP analysis, and 65 µm polydimethylsiloxane /divinylbenzene (PDMS / DVB) fiber from Supelco in the case of PCP analysis). Each new fiber was conditioned to the manufacturer's specifications, and for 30 min daily at the same conditions.

2.3.5.4. GC - MS analysis

The gas chromatography (GC) equipment (VARIAN 3900) used in separation processes of mixtures of substances in this work (Fig. 2.18 b) was equipped with a Varian Capillary Column; CP-Sil 5 CB LOW BLEED/MS; Length - 60 m; Internal Diameter - 0.25 mm; Stationary phase film thickness - 0.25 µm.

The temperature gradient of the column oven was as follow: initial temperature of 40 °C for 300 second, then a linear gradient of 20 °C / min up to 235 °C and gradient of 30 °C / min up to 300 °C; the column flow rate, 1 mL / min (constant); the flow pressure 10 Psi (constant) and the injector temperature, 260 °C (constant). Split sample injection of the organic phase was employed; the initial split ratio 10, between 0.6 second and 420 second split state is off, after that is again on with split ratio of 10. The carrier gas was helium with 99.9995 % of purity.

Varian 3900 GC coupled to an ion - trap mass spectrometer (MS; Varian MS - Saturn 2000) was used with previously optimised conditions. The MS was operated in electronic ionization (EI) and the ion trap analysis was used in selected - ion - storage (SIS) mode. The Fig. 2.18 b) shows a joint view of the ion trap and the location of ion detector - electron multiplier. In Table 2.1 the operation conditions of the MS in order to perform SIS method for the phenolic compounds analysis in this study are shown.

The MS system temperatures of the transferline was set at 220 °C, the ion trap was set at 200 °C and the manifold at 40 °C. A schematic of the main components of GC - MS is shown in Fig. 2.19. More details about the GC - MS (apparatus) and operational process are included in supplementary material, attachments (Annex A1). A standard autotune with perfluorotributylamine (PFTBA) was performed on each day of operation. The control charts of GC - MS over the operation day is shown in supplementary material (Annex C).

The calibration curves were obtained for each of the NP and OP compounds by analyzing standard solutions with concentrations of 1; 5; 10; 15; 30; 45 and 60 µg L^{-1} of each compound. For PCP analysis, calibration curves were obtained by analyzing standard solutions with concentrations of 0; 0.01; 0.02; 0.03; 0.05; 0.07; 0.1; 0.2; 0.5; 0.7;1 and 1.5 µg L^{-1}. The measured values were handled by the GC - MS solution software, Version 6.6.

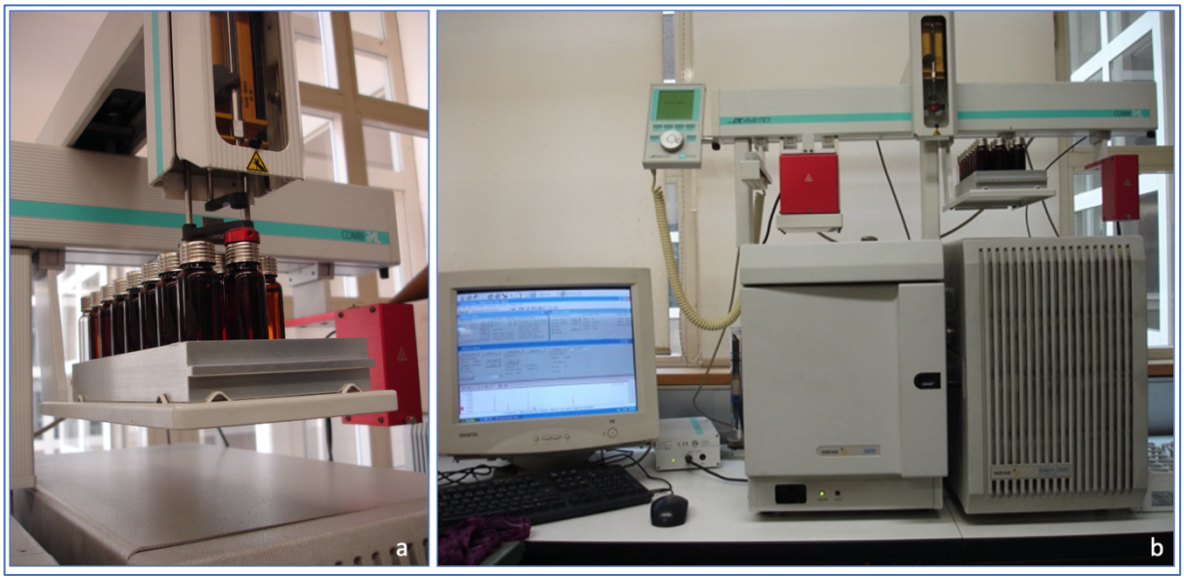


Fig. 2.18 - a) GC-MS apparatus (Varian 3900 GC coupled to Varian MS-Saturn 2000) used in this work; **b)** Automatic sampler CTC Analytic, CombiPal model, where the HS-SPME occurred.

Table 2.1 - Operation conditions of the MS in order to perform SIS method for the phenolic compounds analysis: scan time, multiplier offset, SIS mass range and quantification ions.

Compounds (segment)	Scan (s / scan)	Multiplier offset (V)	SIS mass range (m / z)	Monitored ions (m / z)
NP	0.85	± 200	100 - 250	107 + 108 + 115 + 220
OP	0.85	± 200	35 - 215	41 + 57 + 97 + 105 + 135 + 206
PCP	0.85	± 200	260 - 275	265.9 + 266.8 + 268.4 + 270.0

2.3.5.5. LOD calculation

The LOD was calculated using the following equation:

$$LOD = (3 \times Noise\ signal) \times (Concentration \div Peak\ height) \quad (1)$$

In experiment #1 the value of LOD in water was 0.25 (± 0.02) μg L^{-1} for OP and 0.33 (± 0.09) μg L^{-1} for NP and in sediment it was 0.12 (± 0.06) μg g^{-1} for NP and OP. In experiment #2 the LOD in water was 4.0 x 10^{-5} μg L^{-1} and in sediment was 0.030 ng g^{-1}.

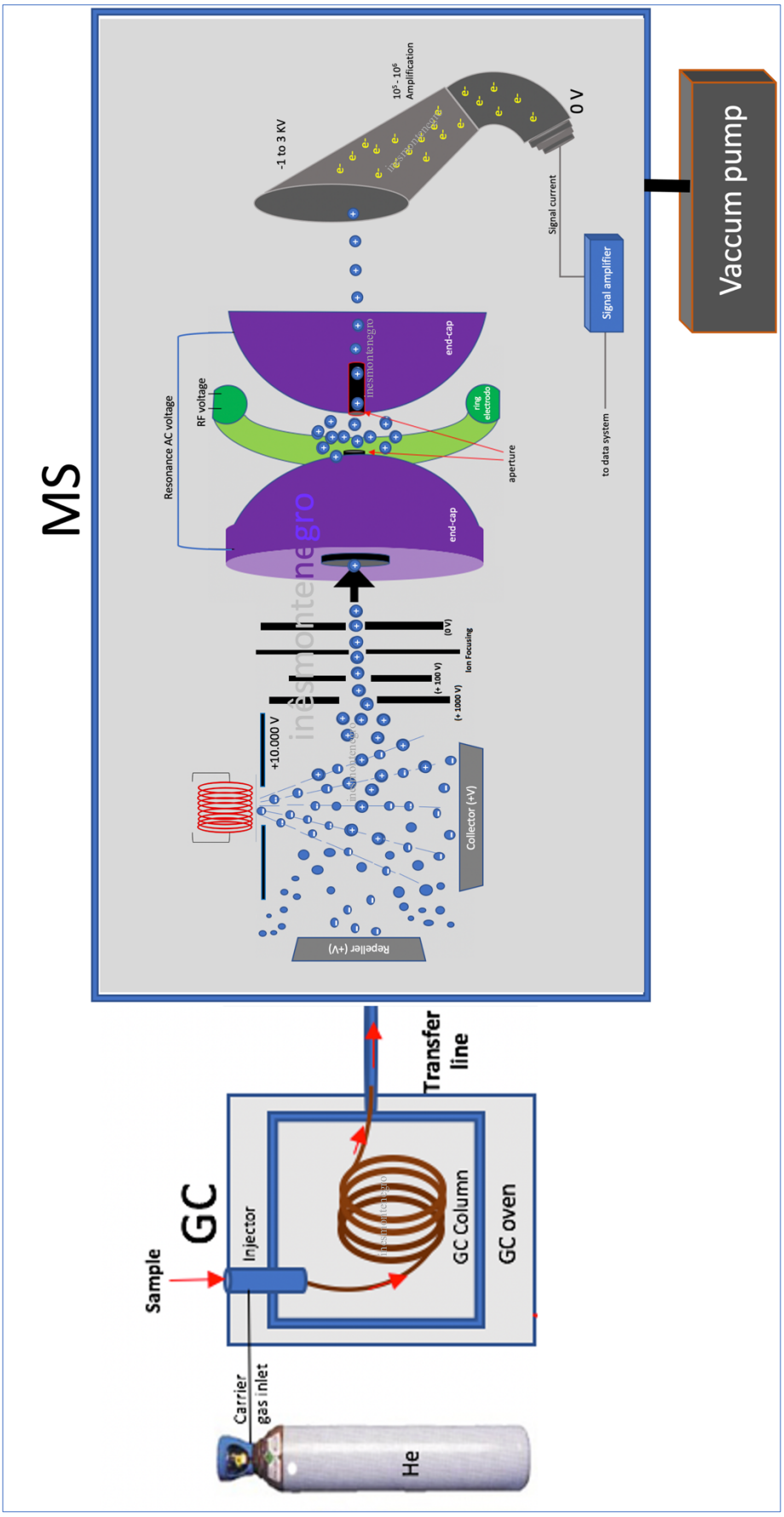


Fig. 2.19 - Schematic representation of the main components of the GC-MS, more details in attachments (Annex A1).

2.3.6. Calculation of removal efficiency

The effect of different conditions on CW performance was evaluated on the basis of percent removal. The percentage removal (removal efficiency) was calculated as follows:

$$\textit{removal efficiency (\%)} = \frac{C_{in} - C_{out}}{C_{in}} \times 100 \quad (2)$$

Where C_{in} and C_{out} is the influent and effluent concentration, respectively.

2.3.7. Statistical analysis

To evaluate statistically significant differences ($p < 0.05$) in the performances of CWs treatments for the removal of each compound, a parametric one - way analysis of variance (ANOVA) was applied. The presence of significant differences was detected by a multiple *Tukey* comparison test.

2.3.8. Microbial communities sampling and characterization

2.3.8.1. DNA extraction, quantification, qualification and sequencing

Environmental DNA extraction

Total DNA extraction from sediment sample of each CW was performed using the DNeasy PowerSoil kit (Fig. 2.20 a) of the Mo BIO Power Soil DNA Isolation (Quichen, MerckKgaA, Darmstadt, Germany) following the manufacturer 's instruction protocol. In this standard kit protocol, the DNA extraction procedure involved several steps with different types of special solutions to isolated and increase the DNA purity in each sample. Sediment (0.5 g) was loaded into PowerBead tube. Each PowerBead tube contains a buffer that will help disperse the sediment particles and dissolve humic acids and protect nucleic acids from degradation. After mixing in the vortex, a solution that contains SDS and others disruption agents for complete cell lysis was added to each tube.

For complete homogenization and cell lysis the vortexing step was carried out in MO BIO Vortex adapter that keeps the PowerBead tube in a safe horizontal position (Fig. 2.20 b).

With the aim of increasing the DNA purity in each sediment sample, it was added a solution that contains a reagent to precipitate non-DNA organic and inorganic material including humic substances, cell debris and proteins.

A special filter with silica membrane was used to selected DNA from other biological material. Since DNA binds tightly to silica at high salt concentration, a high concentration salt solution was used, this adjusted the DNA solution salt concentration to allow binding of DNA, but not non-DNA organic and inorganic material that could be present at low levels.

An ethanol wash solution was used to removed residual salt, humic acid and other contaminants while allowing the DNA to stay bound to the silica membrane. With the aim to complete release DNA from the silica membrane an elution buffer was used. DNA that was bound in the presence of high salt is selectively released by elution buffer which lacks salt.

Fig. 2.20 - a) PowerSoil DNA Isolation Kit (MO BIO Laboratories); **b)** The MO BIO Vortex adapter that keeps the PowerBead tube in a safe horizontal position.

DNA quantification

DNA samples were quantified by fluorometry method using Qubit™ 4 fluorometer (Q32854, Invitrogen, Thermo Fisher Scientific). The Qubit Fluorometers detect fluorescence of the fluorescent dye bound to the biomolecule (DNA, RNA, etc), with sensitivity to accurately measure low concentration (detection range 0.2 -100 ng), measuring absorbance at 260 nm. Details about the fluorometry (apparatus) and fluorescence spectroscopy technique are included in supplementary material, attachments (Annex A5).

The samples preparation for the quantification was made using the Qubit™ dsDNA High Sensibility Assay kit supped by Invitrogen (Thermo Fisher Scientific Inc., Waltham, MA,

USA), according to the manufacturer 's protocol. This standard kit protocol indicated the preparation of a “working solution” that contains a Quibit® reagent and a Qubit® buffer available in the quantification standard kit. The specific chemical composition of the Quibit® reagent and Quibit® buffer was not provided by manufacturer.

DNA amplification

The DNA was first amplified by Polymerase Chain Reaction (PCR). The V3-V5 region of the bacterial 16S rRNA[1] gene was amplified by Polymerase Chain Reaction (PCR) with the forward primer 515YF (5 '-GTGYCAGCMGCCGCGGTAA-3') and reverse primer Y926R (5 '-CCGYCAATTYMTTTRAGTTT-3'). For the PCR it was used the DreamTaq DNA Polymerase Master Mix (5U μL^{-1}) supplied by Thermofisher (Waltham, EUA). The DreamTaq PCR MasterMix, is a ready-to-use solution containing DreamTaq DNA polymerase, optimized with DreamTaq buffer $MgCl_2$ and dNTPS. Each 10 µL PCR reaction mixture contains 5 µL of Dream Taq, 1 µL forward primer and 1 µL indexed reverse primer. The PCR reaction included 3 µL of DNA in a total volume of 10 µL. The PCR protocol includes a 180 seconds denaturation step at 95 °C, followed by 35 cycles at 95 °C for 45 seconds 52 °C for 45 seconds and 68 °C for 90 seconds, and a final extension step at 68 °C for 300 seconds. Amplified products were subjected to electrophoresis using an agarose gel. Negative controls were included in all PCR amplification procedures.

Gel electrophoresis

DNA samples (3 µL each) were loaded on 1.5 % agarose gel (GRS Agarose LE) supplemented with (0.5 µL) of SYBR® Safe stain (Invitrogen™, Carlsbad, USA). The 1x TAE buffer (Tris Acetate - EDTA, 10x concentrate; SIGMA – Aldrich) used in manufacture of agarose gel was supplied by Merck KGaA (Darmstadt, Germany). The electrophoresis was performed at 150 V for 1800 second. The GRS Ladder 1 Kb (Invitrogen, Carlsbad, USA) was used as a molecular marker. To evaluated DNA quality, the gel was visualized using Gel Documentation System (Gel Doc XR + System; BioRad, USA). In Fig. 2.21 it is shown the flow chart of several experimental steps of the molecular analysis.

Sequencing method

The extracted rRNA was then used for 16S rRNA gene sequencing and metabarcoding analysis targeting prokaryotic communities. The sequencing method includes the amplification

of the 16S rRNA gene fragment (hypervarable V3-V5 region, ≈412bp) using the forward primer 515YF(5'-GTGYCAGCMGCCGCGGTAA-3') and reverse primer Y926R (5 'CCGYCAATTYMTTTRAGTTT-3') [566] and the paired-end sequencing was carried out by an Illumina MiSeq® platform with the V3 chemistry, according to manufacturer's instructions (Illumina, Inc., San Diego, CA, United States) at Genoinseq facilities (Cantanhede, Portugal).

Raw reads were extracted from Illumina MiSeq® System in fastq format and quality - filtered with PRINSEQ version 0.20.4 [567] to remove sequencing adapters, reads with less than 100 bases and trim bases with an average quality lower than Q25 in a window of five bases. The forward and reverse reads were merged by overlapping paired - end reads with Adapter Removal version 2.1.5 [568] using default parameters. Then filtered merged amplicons received from Genoinseq in fastq format were converted into fasta format by Mothur software (mothur v.1.43.0; [569]) and processed by the automatic pipeline SILVA rRNA gene database project (SILVAngs 1.3). Fig. 2.21 represent a simplistic representation of the sequence of steps after DNA extraction.

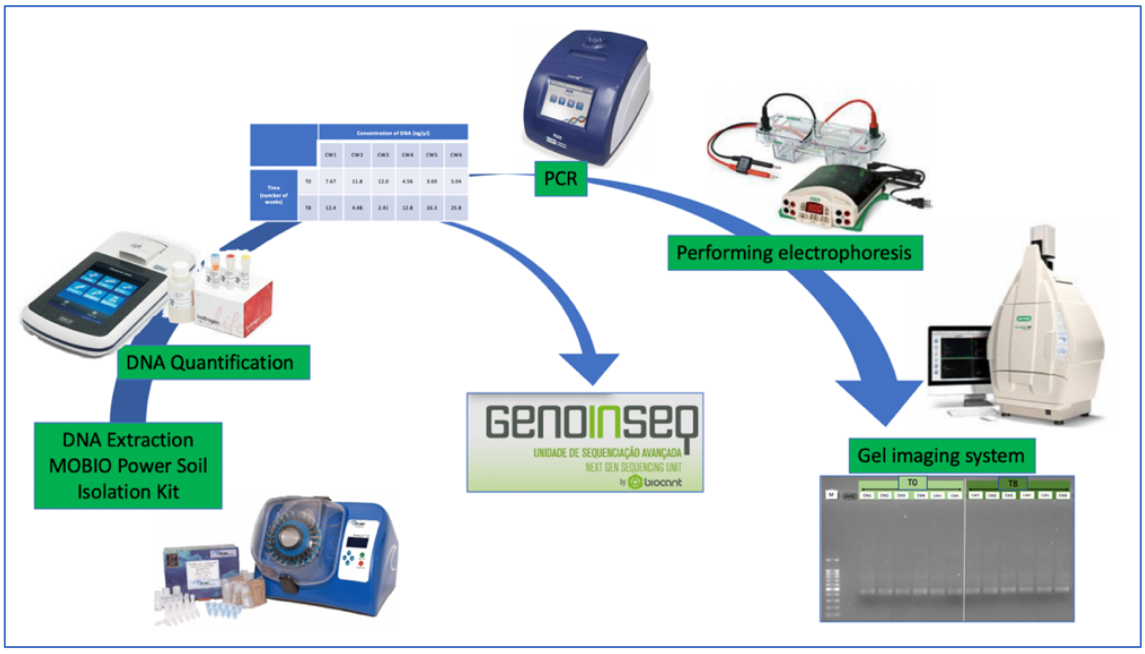


Fig. 2.21 - Flow chart of the experimental procedure: DNA extraction, DNA quantification, DNA amplification by polymerase chain reaction (PCR), run of RNA in horizontal agarose gel electrophoresis system and visualization of gel electrophoresis in an imaging system.

2.3.8.2. Bioinformatic analysis

For the taxonomic classification the merged reads were further uploaded and processed for analysis by the automatic pipeline SILVAngs (Silva Next Generation Sequencing, https://ngs.arb-silva.de/silvangs/; last accessed 30 may 2020) of the SILVA rRNA

gene database project (SILVAngs 1.3) [570]. In each sediment CW samples, the different taxonomic levels of microbial community, was determinate using pipeline default setting. Specifically, reads were aligned using the SILVA Incremental Aligner (SINA v1.2.10 for ARB SVN (revision 21008)) against the SILVA SSU rRNA SEED. The control of quality was done according to the following quality filtering parameters: max ambiguity 2 %, max homopolymers 2 %, min seq. quality 30 %, min length 200 bp, min align. identity 50 %. After quality checked steps, reads were dereplicated and clustered at 98 % of similarity to generate operational taxonomic units (OTUs); the classification was done against SILVA SSU Ref dataset release 132 using blastn (2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi; last accessed 30 july 2020) with standard settings [571]. Reads without any BLAST hits or reads with weak BLAST hits ((% of sequence identity + % of alignment coverage / 2) < 93), remained unclassified and were labeled as 'no relative'. Rarefaction curves were extrapolated by calculating 100 data points [570]. Protocol detailed description is given in Bragança et al. [572].

Abundance tables at different taxonomic levels: higher (phylum) and lower level (genus) have been produced to show the relative abundance of each taxonomic group within each sample (the total number of sequences assigned to each taxonomic path).

Undesirable lineages, 'chloroplast', 'mitochondria', and 'eukaryota' were excluded from the dataset.

Taxonomic abundance tables obtained from SILVAngs analysis were imported into R environment [573] to perform the downstream analysis that included the taxonomic profile, alpha and beta diversity of the microbial community. Alpha diversity was estimated using the "vegan" R-package [574] and included the following estimators: OTU richness (Observed unique OTUs in each sample), Shannon-index, Berger Parker-index. Constructed box plots were used to explore diversity among times and treatments using the function "ggboxplot" in the "ggplot2" R package [575].

Beta diversity analysis included Nonmetric Multidimensional Scaling (NMDS) based on Bray - Curtis dissimilarity matrix calculated using phyloseq R-package [576], and by hierarchical clustering constructed using the Hellinger transformation that reduces the asymmetry of heavily shewed abundance data (using "decostand" function in the "vegan" R package).

Hierarchical clustering of samples into groups was based on Hellinger dissimilarity calculated by the "vegdist" function and "hclust" function in the "vegan" R package. The function "vegdist" was used to find the dissimilarities between samples and "hclust" function was used to complete linkage method for hierarchical clustering. R package "phyloseq" was also used to plot the taxonomic profile of the prokaryotic community across the samples.

2.3.8.3. Statistical analysis

For microbial communities, statistical analysis was conducted using the R software [573]. In alpha diversity analysis, differences were considered significant at the level of α = 5 % and estimated using Kruskal-Wallis statistical methods. Non-metric multivariate scaling (NMDS) analysis of data was transformed by “phyloseq” function and the *Bray - Curtis* dissimilarity matrix was used to ordinate. The “clamtest” function from “vegan” R package and described in [577] was used for statistical approach for classifying generalists and specialists in two distinct treatments.

3. Results

3.1. Results of chemical analysis

3.1.1. Experiment #1 - Removal of a Mixture of Alkylphenol Compounds

During the experimental time period, the plants of all systems showed vitality and even new plant shoots appeared. The CWs influents and effluents were monitored during the eight weeks. Evaluated parameters were organic matter (through COD) and nutrients (NH_4^+; NO_2^-; NO_3^-; and PO_4^{3-} ions), aiming to assess CWs ability to remove organic matter and nutrients from *Ribeira de Joane* water and the effect of the alkylphenol compounds presence in CWs removal capacity of these parameters. Alkylphenol compounds removals by CWs were also assessed as well as their possible impact on CWs substrate microbial community.

3.1.1.1. Removal efficiency of Chemical Oxygen Demand

COD is a measure of the oxygen equivalent to the organic matter content in a water sample that is susceptible to oxidation by a strong chemical oxidant. Table 3.1 describes the concentration of COD (mg of oxygen per litter of water; mg L^{-1}) in *Ribeira de Joane* water and in CWs effluent (water collected after CW treatment) and the percentage of removal along eight weeks of the experiment. Influent soluble COD varied markedly (15 to 83 mg L^{-1}) along the experimental period. COD removal values also varied significantly (Table 3.1) ranging from non-removal and 73.0 (± 0.1) % in control systems (*Ribeira de Joane* water not doped) and from non-removal and 83 (± 15) % in doped systems, the ones where the *Ribeira de Joane* water was doped with NP_OP. However, different behaviours between treatments were observed: when the highest COD concentration in influent (83 mg L^{-1}) was registered it was observed the lowest COD removal in doped systems whereas in control systems no COD removal occurred. The highest COD removal (73.0 (± 0.1) %) was observed when the influent concentration registered the lowest concentration (15 mg L^{-1}).

In doped CWs there was an apparent tendency for COD removal percentages to increase over experimental period, with a high variability among replicate microcosms in the

four first weeks, showing a probable adaptation of the system to the doped water. On the other hand, in control CWs, when COD was removed, values were more or less identical over time.

In five of the eight weeks of the experimental period (week number 1, 3, 5, 7 and 8) no significant difference in COD removal percentages were observed ($p > 0.05$) between control and doped treatments. The results show there are no clear effect of NP and OP presence in COD removal but in most part of the experimental period the presence of NP_OP did not interfere with removal efficiency of CWs systems.

Table 3.1 - Concentration of COD (mg O_2 L^{-1}) in CWs influents and effluent (mean ± standard deviation, n = 3) and percentage of removal (%, mean ± standard deviation, n = 3) of CWs irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP. "n.r." indicates that there was no COD removal in CWs system.

COD					
Week	**Influent (mg O_2 L^{-1})**	**Effluent**			
		Control (mg O_2 L^{-1})	Removal %	Doped (mg O_2 L^{-1})	Removal %
1	45	114 ± 7	n.r.	112 ± 12	n.r.
2	83	86 ± 42	n.r.	70 ± 61	15 ± 74
3	43	18 ± 8	58 ± 18	36 ± 42	16 ± 97
4	59	23 ± 10	61 ± 18	61 ± 20	n.r.
5	15	4.0 ± 0.1	73.0 ± 0.1	7 ± 5	53 ± 35
6	27	13 ± 2	52 ± 7	6 ± 1	78 ± 4
7	34	95 ± 12	n.r	64 ± 13	n.r.
8	46	14 ± 2	69 ± 5	8 ± 7	83 ± 15

3.1.1.2. Nitrogen removal

Normally, nitrogen in municipal or domestic wastewater is present in five main forms: ammonium (NH_4^+) ion, nitrite (NO_2^-) ion, nitrate (NO_3^-) ion, nitrous oxide (N_2O), and dissolved elemental nitrogen or dinitrogen gas (N_2). But nitrogen can also be present in other organic forms (e.g. amino acids, urea and uric acid, purines and pyrimidines). In this work, in the water from *Ribeira de Joane* only three nitrogen forms were considered, NH_4^+, NO_2^-, NO_3^- ions, being, the removal balance of nitrogen based on these ion concentrations in CWs influent and effluent.

Ammonia ion removal

During the eight weeks of the experiment NH_4^+ ion concentrations in CWs influent varied between 34 (± 2) and 163 (± 23) µmol L^{-1}, being the highest value observed in week five and the lowest in week six (Table 3.2). Regarding CW effluents, NH_4^+ ion concentrations varied between 3.9 (± 0.9) µmol L^{-1} and 12 (± 4) µmol L^{-1} in control treatment and between 5 (± 3) µmol L^{-1} and 11 (± 2) µmol L^{-1} in doped treatment. In both treatments, the lowest and highest NH_4^+ ion concentrations were observed in the same weeks, in week one for lowest concentration and in week seven for the highest concentration.

As shown in Fig. 3.1, in general, removal of NH_4^+ ions were always higher than 86 %. The only exception was week 6 (72 (± 4) % in control; 76 (± 3) %) in doped), probably do the low influent concentration (34 (± 2) µmol L^{-1}), as NH_4^+ ion concentration in CWs effluents was in general identical over time. The vertical flow in CW system demonstrated to be effective in removing NH_4^+ ion from CW influent. Moreover, the presence of both contaminants, NP_OP, in the water did not interfere with the efficient removal of NH_4^+ ions by CWs. In other words, no significant differences ($p > 0.05$) between control and doped treatments were observed over the experimental time.

Nitrite ion removal

As shown in Table 3.3, CWs influent concentration of NO_2^- ion varied between 6.3 (± 0.2) and 53 (± 4) µmol L^{-1}. The lowest concentration value was observed in week three and the highest value was observed in week five. Regarding CWs effluent, the average concentration in control treatment varied between no detection (0.25 µmol L^{-1}) and 1.6 (± 0.2) µmol L^{-1}. In the case of doped treatment, the concentration of NO_2^- ion varied between no detection (0.25 µmol L^{-1}) and 1.7 (± 0.1) µmol L^{-1}. The highest concentration of NO_2^- ion was observed in the first week in both treatments. In the remaining weeks, values were below or very close to the detection limit. During the eight weeks of the experimental period the removal percentage of NO_2^- ions was always higher than 96 % (Fig. 3.2). The results indicate that there was an effective removal of NO_2^- ions by CWs throughout the experience. No significant differences ($p > 0.05$) between removal percentages in both treatments (Control and Doped) over the experimental period. The presence of the target contaminants (NP_OP) did not interfere in NO_2^- ion removal efficiency of CWs.

Nitrate ion removal

The concentration of NO_3^- ion, during the eight weeks of experiment varied between 211 (± 39) and 655 (± 60) µmol L^{-1} in CWs influent (Table 3.4). The low concentration value was observed in week three while the highest concentration value was observed in week two. The NO_3^- ion concentration in CWs effluent in control treatment varied between 3 (± 1) µmol L^{-1} and 39 (± 51) µmol L^{-1} and in the case of the doped treatment the variations were between no detection (2.5 µmol L^{-1}) and 24 (± 5) µmol L^{-1}. The lowest concentration value was observed in week three in both treatments, and the highest concentration value was observed in the fourth week in case of control and in second week in case of doped treatment.

As shown in Fig. 3.3 during the eight weeks of the experimental period the removal percentage of NO_3^- ions were always higher than 88 %. In the control treatment removals varied between 88 (± 16) % and 99 (± 1) % and in the doped treatment the removals varied between 96 (± 3) % and 99 (± 1) %. The results indicate that there was an effective removal of NO_3^- by CWs throughout the experience. With the exception of week two, there were no significant differences ($p > 0.05$) between percentage of removal of both treatment (Control and Doped), therefore, the presence of added contaminants did not interfere with NO_3^- removal efficiency of CWs.

Table 3.2 - Concentration of ammonia ion (NH_4^+; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP.

NH_4^+ (µmolL^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	108 ± 14	3.9 ± 0.9	5 ± 3
2	90 ± 6	10 ± 1	9 ± 2
3	118 ± 9	7 ± 3	5.6 ± 0.9
4	95 ± 16	8 ± 2	6.3 ± 0.2
5	163 ± 23	8 ± 2	8 ± 1
6	34 ± 2	9 ± 1	8 ± 1
7	135 ± 2	12 ± 4	11 ± 2
8	63 ± 4	7.1 ± 0.5	9 ± 1

Table 3.3 - Concentration of nitrite ion (NO_2^-; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP. (*) NO_2^- concentration below the detection limit (LOD = 0.25 µmol L^{-1}).

NO_2^- (µmol L^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	43 ± 6	1.6 ± 0.2	1.7 ± 0.1
2	29.0 ± 0.7	< 0.25*	< 0.25*
3	6.3 ± 0.2	< 0.25*	< 0.25*
4	23 ± 2	< 0.25*	< 0.25*
5	53 ± 4	0.3 ±0.1	0.3 ± 0.1
6	45 ± 2	< 0.25*	< 0.25*
7	51 ± 3	0.3 ± 0.1	0.3 ± 0.1
8	20.9 ± 0.9	< 0.25*	0.3 ± 0.1

Table 3.4 - Concentration of nitrate ion (NO_3^-; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP. (*) NO_3^- concentration below the detection limit (LOD = 2.5 µmol L^{-1}).

NO_3^- (µmol L^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	490 ± 113	20 ± 25	3.9 ± 0.7
2	655 ± 60	10 ± 5	24 ± 5
3	211 ± 39	3 ± 1	< 2.5*
4	634 ± 127	39 ± 51	4 ± 2
5	303 ± 163	4 ± 2	9 ± 6
6	213 ± 9	25 ± 34	9 ± 2
7	382 ± 21	5 ± 1	5 ± 4
8	253 ± 26	4 ± 2	6 ± 5

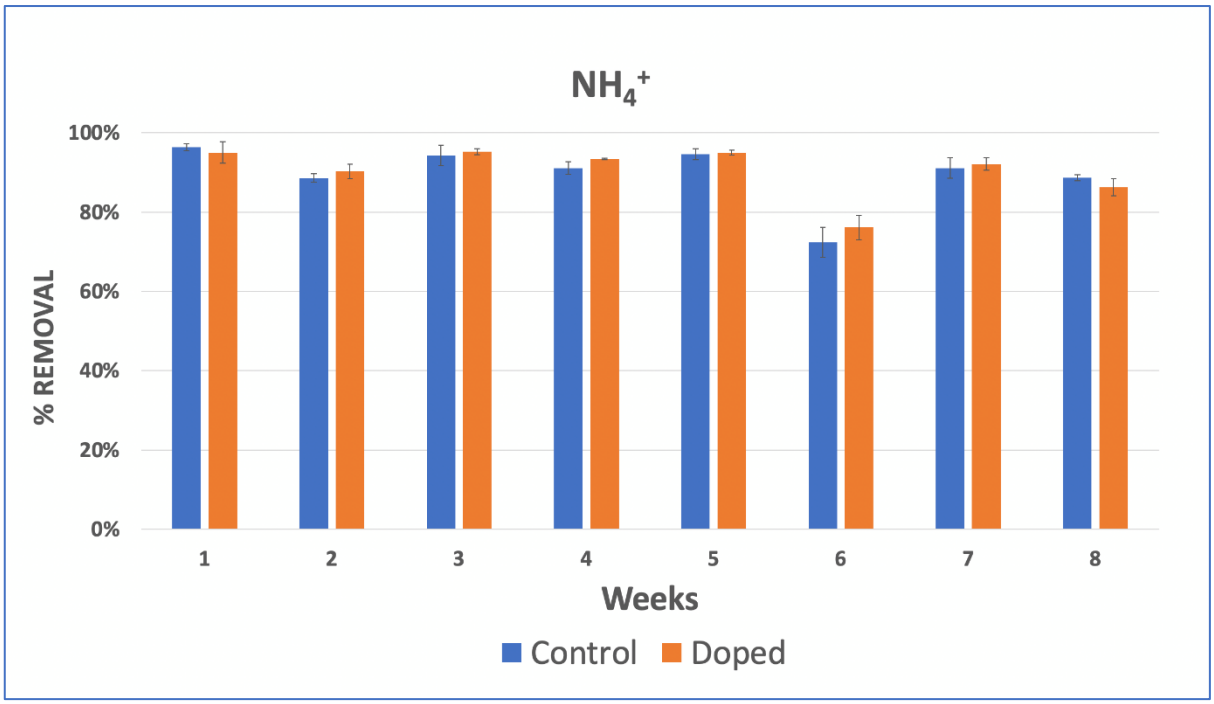


Fig. 3.1 - Removal percentage (%, mean and standard deviation, n = 3) of ammonia ion (NH_4^+(µmol L^{-1}) in CWs irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP.

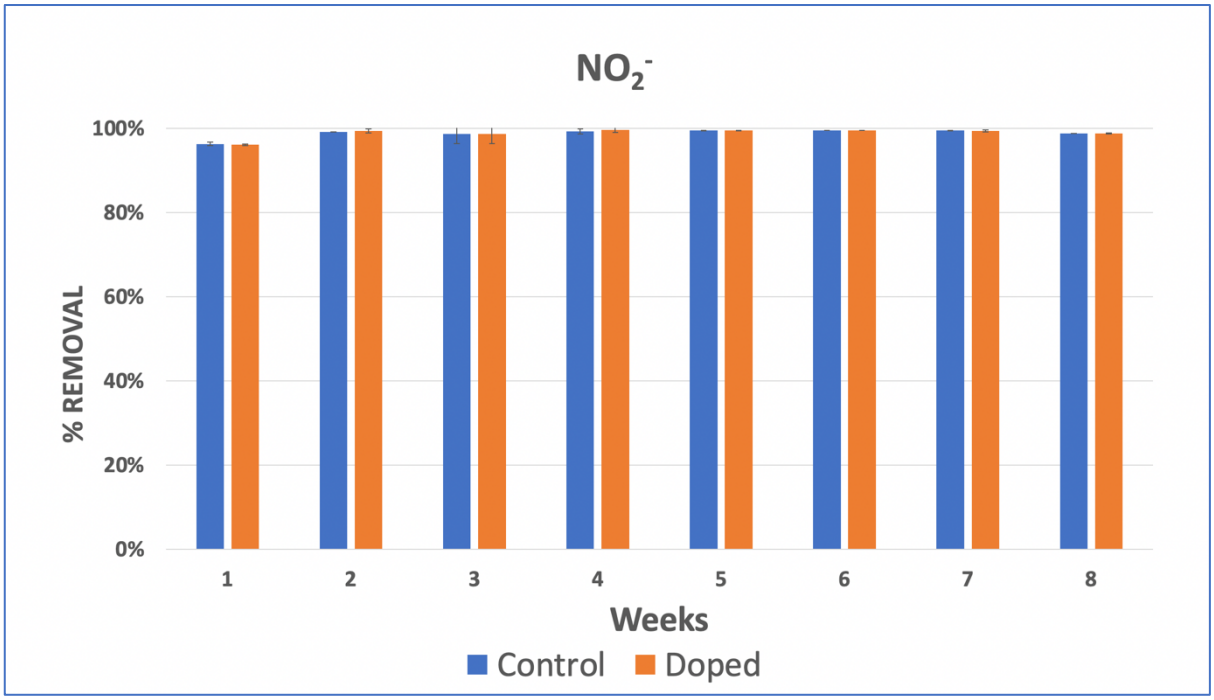


Fig. 3.2 - Removal percentage (%, mean and standard deviation, n = 3) of nitrite ion (NO_2^-) in CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP.

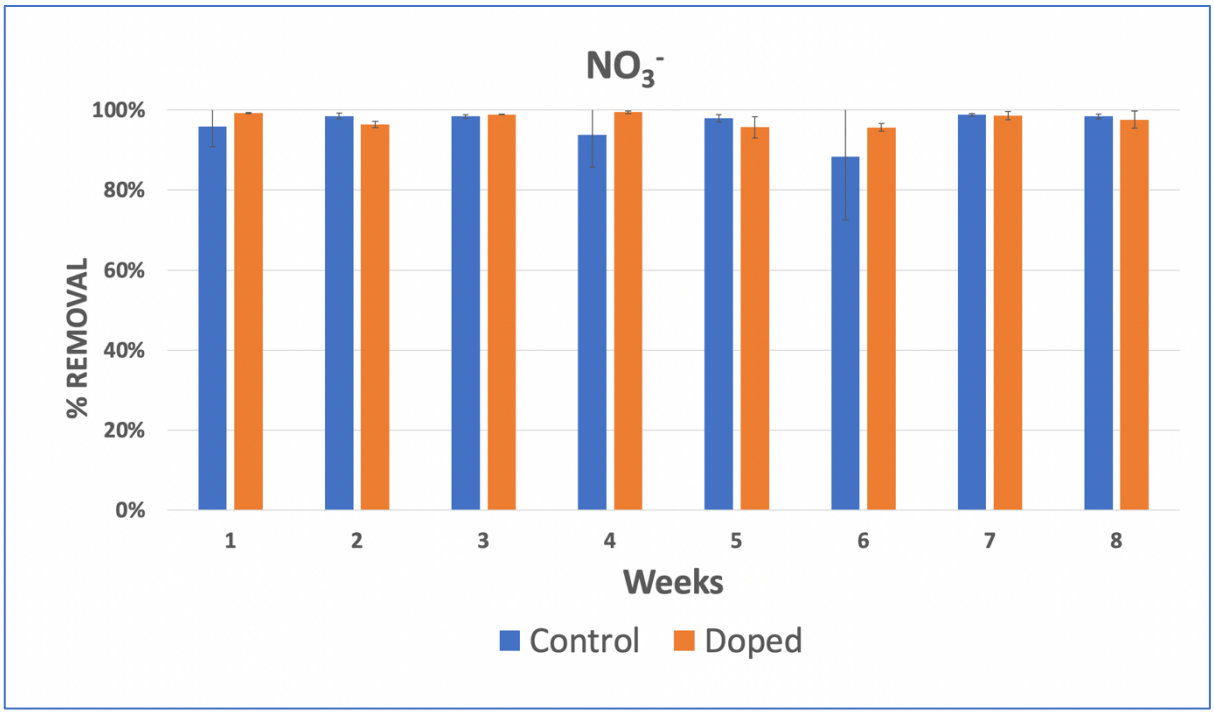


Fig. 3.3 - Removal percentage (%, mean and standard deviation, n = 3) of nitrate ion (NO_3^-) in CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP.

3.1.1.3. Phosphates removal

As observed in Table 3.5 the concentration of PO_4^{3-} ions in CW influent varied between 2.0 (± 0.1) and 20 (± 2) µmol L^{-1}. The lowest concentration value was observed in week four and the highest concentration value was observed in week one. Regarding CWs effluent, in control treatment the average of PO_4^{3-} ion concentration varied between 1.5 (± 0.9) µmol L^{-1} and 9 (± 4) µmol L^{-1} and in doped treatment it varied between non detection (< 1.0 µmol L^{-1}) and 6 (± 1) µmol L^{-1}. The lowest concentration value was observed on week two in control treatment and weeks one to three in doped treatment. The highest concentration was observed in week seven, for both treatments.

Fig. 3.4 shows PO_4^{3-} ions removal in CWs systems during the eight weeks. Results showed a large variation in removal percentages (removals between 0 - 95 %). Moreover, in week seven (in control treatment) the CW did not remove any PO_4^{3-} ions. These results indicate that PO_4^{3-} ions removal in CW was more variable than nitrogen removal over the experimental period. As noted in Fig. 3.4, it is evident that in week five the presence of target contaminants enhanced the PO_4^{3-} ions removal in CWs systems. With the exception, of this week, in remaining weeks the presence of NP_OP did not interfere with the PO_4^{3-} removal,

with removal percentages being statically identical ($p > 0.05$) between control and doped treatments.

Table 3.5 - Concentration of anion phosphate (PO_4^{3-}; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP. (*) PO_4^{3-} ion concentration below the detection limit (LOD = 1.0 µmol L^{-1}).

PO_4^{3-} (µmol L^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	20 ± 2	2.1 ± 1	< 1.0*
2	5.2 ± 0.4	1.5 ± 0.9	< 1.0*
3	4.0 ± 0.4	1.9 ± 0.9	< 1.0*
4	2.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.6
5	14.0 ± 0.4	5 ± 2	1 ± 1
6	15.0 ± 0.7	4 ± 2	2.1 ± 0.3
7	8.7 ± 0.3	9 ± 4	6 ± 1
8	9.0 ± 0.4	6 ± 3	5 ± 1

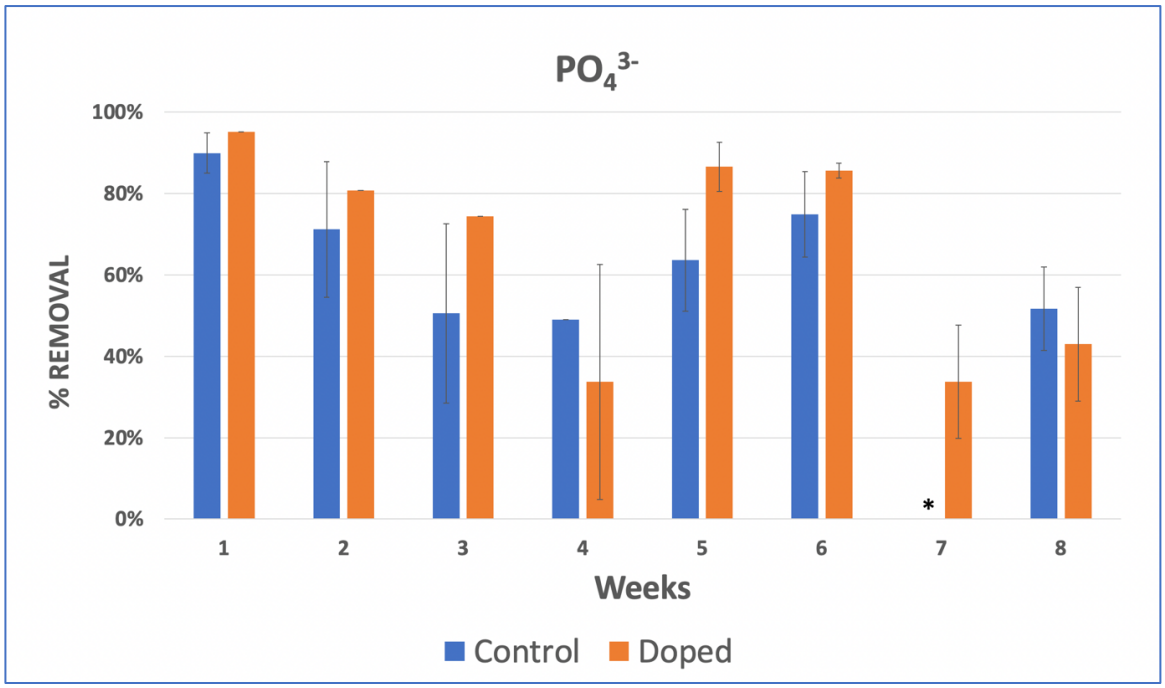


Fig. 3.4 - Removal percentages (%, mean and standard deviation, n = 3) of phosphate anion (PO_4^{3-}) in CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP_OP. (*) In week seven of control treatment there was no PO_4^{3-} ion removal.

3.1.1.4. Alkylphenols removal

In the water of *Ribeira de Joane* (influent of CWs) and in CWs effluents, the NP and OP concentrations were always below the limit of detection, i.e., below the value 0.25 (± 0.02) μg L^{-1} for OP and 0.33 (± 0.09) μg L^{-1} for NP (Table 3.6).

When the *Ribeira de Joane* water was doped with 100 μg L^{-1} of NP and OP, the concentrations of the compounds decreased drastically from influent to effluent, the removal rates of OP and NP being always > 99 %. The VFCW was efficient in alkylphenol removal.

Table 3.6 – The LOD value of OP and NP (μg L^{-1}) (mean and standard deviation, n = 5) in water from *Ribeira de Joane* and effluent of CWs irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with OP and NP.

LOD (μg L^{-1})	
OP	**NP**
0.25 ± 0.02	0.33 ± 0.09

In the plant roots bed substrate collected from each CW at the beginning of the experiment (T0) and at the end of the experiment (T8), no NP was detected. NP concentration in sediment of all CWs was always below de LOD value (0.12 (± 0.06) μg g^{-1}), indicating that the compound was totally removed from the system.

Relatively to OP, the compound was detected in both T0 e T8 plant roots bed substrate in both control and doped treatments, all values being statistically identical ($p > 0.05$) (Table 3.7).

The compound was detected in the sediment of estuary of *Lima River* collected to prepare the plant roots bed substrate (data not shown), being this the source of the compound. Results indicate that only OP added to CW systems was removed/degraded probably because it was in a more bioavailable form.

The CWs were able to remove the new OP contamination but failed relatively to the old contamination, probably because this fraction of contaminant established strong connections with sediment structure which may have made it difficult the access to microbial community and subsequent degradation.

Table 3.7 - Concentration of OP in sediments of CWs irrigated with not doped water of *Ribeira de Joane* and in sediments of CWs irrigated with water of *Ribeira de Joane* doped weekly with 100 µg L^{-1} of both NP_OP. Mean and standard deviation (µg g^{-1}, n = 3) at experimental initial period (T0) and at the end of the experiment period (T8).

OP (µg g^{-1})		
T0	**T8**	
	Control	Doped
0.44 ± 0.01	0.46 ± 0.01	0.47 ± 0.01

3.1.2. Experiment #2 - Removal Pentachlorophenol

3.1.2.1. Removal efficiency of Chemical Oxygen Demand

COD measures both biodegradable and non-biodegradable organic matter components, estimating oxygen usage. The use of COD will result in overestimated biological oxygen consumption since not all of the removed COD is necessarily aerobically biodegraded [578]. According to Platzer to estimate only the biodegradable component of COD a factor of 0.7 is generally applied to COD values [578].

The COD removal percentages observed in CWs over the experimental time are presented in Table 3.8. COD concentrations in water of *Ribeira de Joane* varied between 19 and 55 mg L^{-1}. The lowest and the highest concentration were recorded in week eight and three, respectively.

The water of *Ribeira de Joane* is a stormwater runoff. When it rains with a certain intensity the volume of water increased substantially and the water appears cloudy on Praia do Paraíso. After week five it was observed the increased of water turbidity and a decrease COD concentration in influent (see Table 3.8). A plausible explanation for these records could be the heavy rains that occurred in the region during the sampling period. These rains promoted the dragging of soil particles that remained in the water column and the dilution effect of organic matter concentration in water of *Ribeira de Joane*.

Regarding CWs effluents, the COD concentrations averaged between 16 (± 2) and 39 (± 5) mg L^{-1} in control treatment, the lowest concentration being recorded in week seven and the highest in week four. Meanwhile in doped treatment the COD concentration varied

between 15 (± 3) and 39 (± 1) mg L^{-1} and the lowest and highest COD concentrations were recorded in consecutive weeks, weeks one and two, respectively.

The average COD removal percentages varied between non removal and 35 (± 6) % in control treatment and between non removal and 48 (± 2) % in doped treatment. In week one of control treatment the lowest COD removal percentage was recorded while the highest COD removal percentage was recorded in week three. Regarding doped treatment, the lowest and highest COD removal percentages were recorded in weeks two and three, respectively. As observed in Table 3.8, the highest COD removal percentage was observed in the same week for both control and doped treatments and coincided with the highest COD input into CWs. COD removal percentages were variable through time but, in general, no significant difference in COD removal percentage were observed ($p > 0.05$) between control and doped treatments. Therefore, the presence of PCP did not interfere with removal efficiency of CWs systems.

Table 3.8 - Concentration of COD (mg O_2 L^{-1} water) in CWs influents and effluent (mean ± standard deviation, n = 3) and percentage of removal (%, mean ± standard deviation, n = 3) along eight weeks. "n.r." means that there was no COD removal in the systems.

COD					
Week	**Influent** (mg L^{-1})	**Effluent**			
		Control (mg L^{-1})	Removal %	Doped (mg L^{-1})	Removal %
1	23	22 ± 6	3 ± 25	15 ± 3	35 ± 15
2	42	35 ± 1	16 ± 3	39 ± 1	8 ± 2
3	55	36 ± 4	35 ± 6	29 ± 1	48 ± 2
4	46	39 ± 5	16 ± 10	36 ± 4	22 ± 8
5	28	21 ± 5	26 ± 18	21 ± 2	24 ± 5
6	22	27 ± 3	n.r.	30 ± 1	n.r.
7	22	16 ± 2	29 ± 9	19 ± 7	14 ± 32
8	19	24 ± 4	n.r.	26 ± 8	n.r.

3.1.2.2. Removal efficiency of Total Organic Carbon

One measure of water quality is TOC analysis. This parameter is one of the most important composite parameters in the assessment of the organic pollution of water. TOC is a measurement method for the content of carbon of dissolved and undissolved organic substance in the water sample. TOC concentration (mg of carbon per liter of water; mg L^{-1}) in

influents and effluents of CWs and removal performances of CWs over the experimental time is presented in Table 3.9. The concentration (mean and standard deviation) of carbon in influents of CWs varied between 6.6 mg L^{-1} and 6.2 mg L^{-1}. The highest TOC concentration was recorded in fourth week and the lowest in the last week of the experience.

Table 3.9 - Concentration of TOC (mg L^{-1}) in CWs influents and effluent (mean ± standard deviation, n = 3) and percentage of removal (%, mean values ± standard deviation, n = 3) in two specific weeks, at half and final of the experimental time.

TOC					
Week	**CW influent** (mg L^{-1})	**CW effluent**			
		Control (mg L^{-1})	Removal %	Doped (mg L^{-1})	Removal %
4	6.6	4.6 ± 0.6	30 ± 8	5.1 ± 0.1	23 ± 1
8	6.2	3.7 ± 0.2	40 ± 2	4.9 ± 0.4	22 ± 6

In CWs effluents the carbon concentration varied between 3.7 (± 0.2) and 4.6 (± 0.6) mg L^{-1} in the control treatment and between 4.9 (± 0.4) and 5.1 (± 0.1) mg L^{-1} in the doped treatment. Regarding the removal percentage, in control treatment it varied between 30 (± 8) % and 40 (± 2) %, while in doped treatment removal between 22 (± 6) % and 23 (± 1) %. Unlike what happened in week four, in week number eight the differences were significant ($p < 0.05$) in removal efficient of CWs between treatment. Therefore, the presence of PCP interfered with carbon removal process from water.

3.1.2.3. Nitrogen removal

Ammonia ion removal

The NH_4^+ ion concentrations, average and standard deviation in influents and effluents of CWs and removal performances of CWs over the experimental period are presented in Table 3.10 and in Fig. 3.5. In CWs influent NH_4^+ ion concentrations varied between 18 (± 2) and 84 (± 2) µmol L^{-1}. The lowest value was observed in weeks one and two and the highest value in week four. For CWs effluents of control treatment the NH_{4^+} concentration varied between non detection (1.4 µmol L^{-1}) and 6 (± 7) µmol L^{-1}. The lowest values of NH_4^+ concentrations were recorded in weeks three, four and seven and highest value in first week. In CWs effluents of doped treatment NH_4^+ ion concentration varied between non detection (1.4

μmol L^{-1}) and 3 (± 2) μmol L^{-1}, the lowest NH_4^+ concentrations being recorded in weeks two, three and five and the highest in week number eight (Table 3.10).

The NH_4^+ removal percentage in control and doped treatment were above 85 %, except in week one of control with a removal percentage of 68 (± 37) %. There were no significant differences between control and doped treatment removal percentages ($p > 0.05$). The presence of PCP did not interfere with the removal capacity of CWs for this nutrient.

In both treatments two CWs removal behavior patterns were observed. When NH_4^+ influent concentration value was above 38 μmol L^{-1} the removal percentage of CWs was ≥ 95 %, while for NH_4^+ influent concentration below 23 μmol L^{-1} the removal percentage of CWs varied from 68 % to 94 % (see Table 3.10 and Fig. 3.5).

Nitrite ion removal

The NO_2^- concentrations, average and standard deviation in influents and effluents of CWs and removal performances of CWs over the experimental period are presented in Table 3.11 and in Fig. 3.6. The NO_2^- concentrations varied between 2.2 (± 0.2) and 26.8 (± 0.4) μmol L^{-1} in influent of CWs.

The values of effluent NO_2^- concentration varied between non detection (0.26 μmol L^{-1}) and 0.3 (± 0.1) μmol L^{-1} in control treatment. In doped treatment NO_2^- concentrations were always below detection limit (LOD = 0.26 μmol L^{-1}) over the experimental period (Table 3.11). In both treatments, the removal percentages were always above 85 % over the experimental period. No significant differences between treatments ($p > 0.05$) were observed, therefore, the presence of PCP did not interfere with the removal efficiency of NO_2^- (Fig. 3.6).

Nitrate ion removal

The NO_3^- ion concentrations, average and standard deviation in influents and effluents of CWs and removal performances of CWs over the experimental time are presented in Table 3.13 and in Fig. 3.25. In influent of CWs the NO_3^- concentrations varied between 32 (± 3) and 938 (± 1) μmol L^{-1}. The lowest concentration was recorded in week seven and the highest concentration was recorded in week two. The range of NO_3^- ion concentrations in CWs effluent was higher in the control treatment then in the doped treatment (Table 3.12).

In CWs effluent the NO_3^- ion concentrations varied between 9 (± 3) and 371 (± 98) μmol L^{-1} in control treatment and between 15 (± 10) and 76 (± 36) μmol L^{-1} in doped treatment. The lowest and highest NO_3^- effluent concentrations were recorded in the same week in both

treatments, weeks eight and two, respectively. Along weeks a high variation was observed in NO_3^- removal percentages for both treatments (Fig. 3.7).

In control treatment the NO_3^- removal percentages varied between 33 (± 29) % and 93 (± 2) % in weeks four and eight, respectively. Also was observed no NO_3^- removal in week number tree of control treatment. In doped treatment the NO_3^- removal percentages varied between 28 (± 58) % and 94 (± 4) % in weeks seven and five, respectively.

Up to week five it appears that the presence of PCP promoted the removal of NO_3^-. There was no relationship between influent concentration and removal percentage over time in both treatments.

Table 3.10 - Concentration of ammonia ion (NH_4^+; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. (*) NH_4^+ concentration below the detection limit (LOD = 1.4 µmol L^{-1}).

NH_4^+ (µmol L^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	18 ± 2	6 ± 7	2.3 ± 8
2	18 ± 2	2 ± 2	< 1.4*
3	52 ± 4	< 1.4*	< 1.4*
4	84 ± 2	< 1.4*	1.5 ± 0.2
5	48 ± 2	2.2 ± 0.9	< 1.4*
6	38 ± 2	2.1 ± 0.6	1.6 ± 0.3
7	22.6 ± 0.3	<1.4*	2.6 ± 0.6
8	21 ± 1	1.5 ± 0.1	3 ± 2

Table 3.11 - Concentration of nitrite ion (NO_2^-; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. (*) NO_2^- concentration below the detection limit (LOD = 0.26 µmol L^{-1}).

NO_2^- (µmol L^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	5.5 ± 0.1	< 0.26*	< 0.26*
2	8.7 ± 0.3	0.3 ± 0.1	< 0.26*
3	18.9 ± 0.4	< 0.26*	< 0.26*
4	18.6 ± 0.1	< 0.26*	< 0.26*
5	26.8 ± 0.4	< 0.26*	< 0.26*
6	2.3 ± 0.1	< 0.26*	< 0.26*
7	2.2 ± 0.2	< 0.26*	< 0.26*
8	4.2 ± 0.1	< 0.26*	< 0.26*

Table 3.12 - Concentration of nitrate ion (NO_3^-; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP.

NO_3^- (µmol L^{-1})			
Week	**Influent**	**Effluent**	
		Control	Doped
1	394 ± 3	239 ± 44	56 ± 34
2	938 ± 1	371 ± 98	76 ± 36
3	118 ± 4	187 ± 62	49 ± 44
4	92 ± 5	62 ± 27	30 ± 10
5	573 ± 35	189 ± 98	32 ± 22
6	78 ± 1	35 ± 45	26 ± 23
7	32 ± 3	12 ± 9	23 ± 18
8	119 ± 1	9 ± 3	15 ± 10

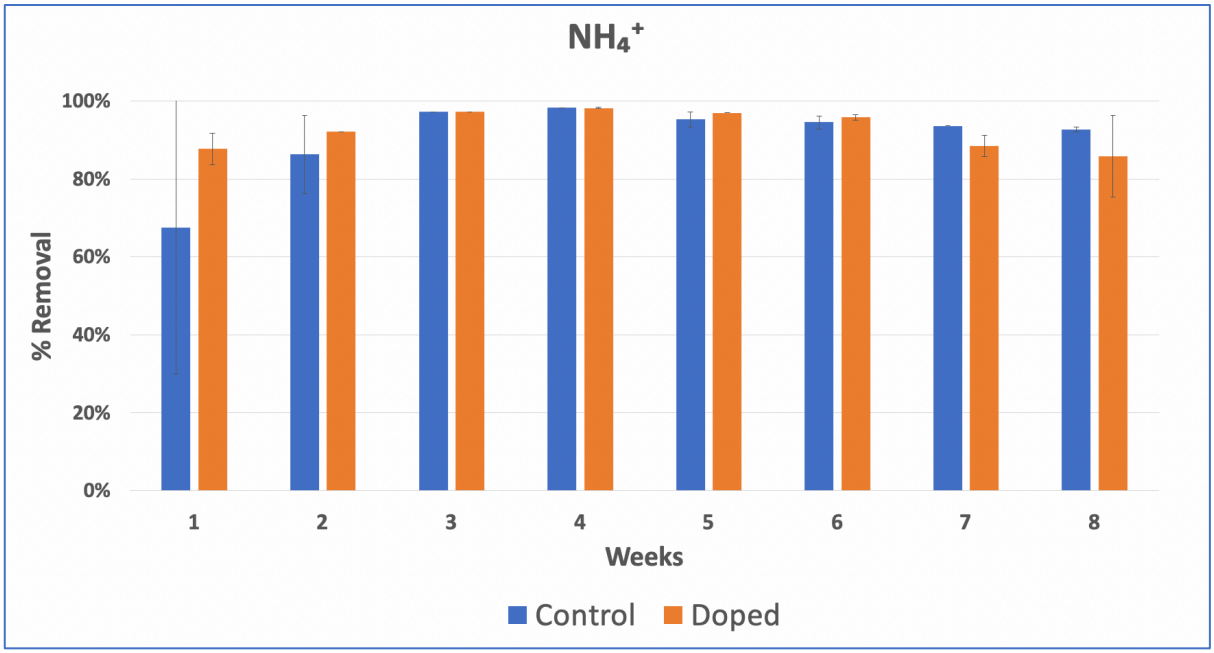


Fig. 3.5 - Removal percentage (%, mean and standard deviation, n = 3) of ammonia ion (NH_4^+) in CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP.

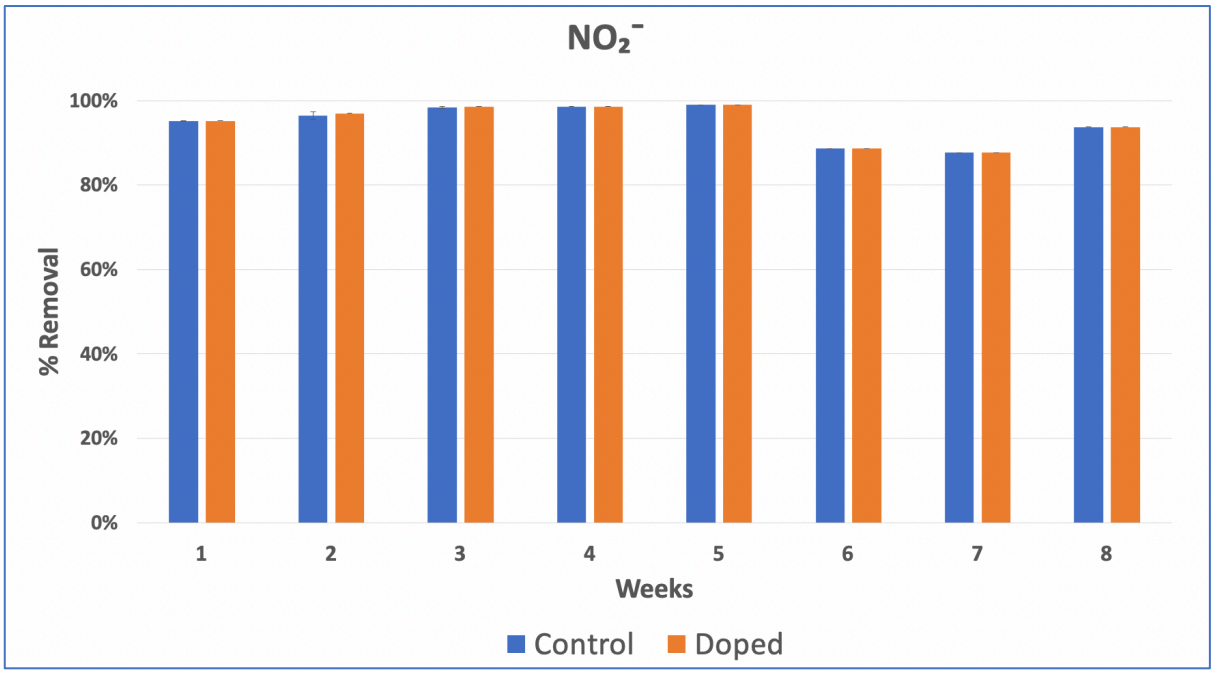


Fig. 3.6 - Removal percentage (%, mean and standard deviation, n = 3) of nitrite ion (NO_2^-) in CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP.

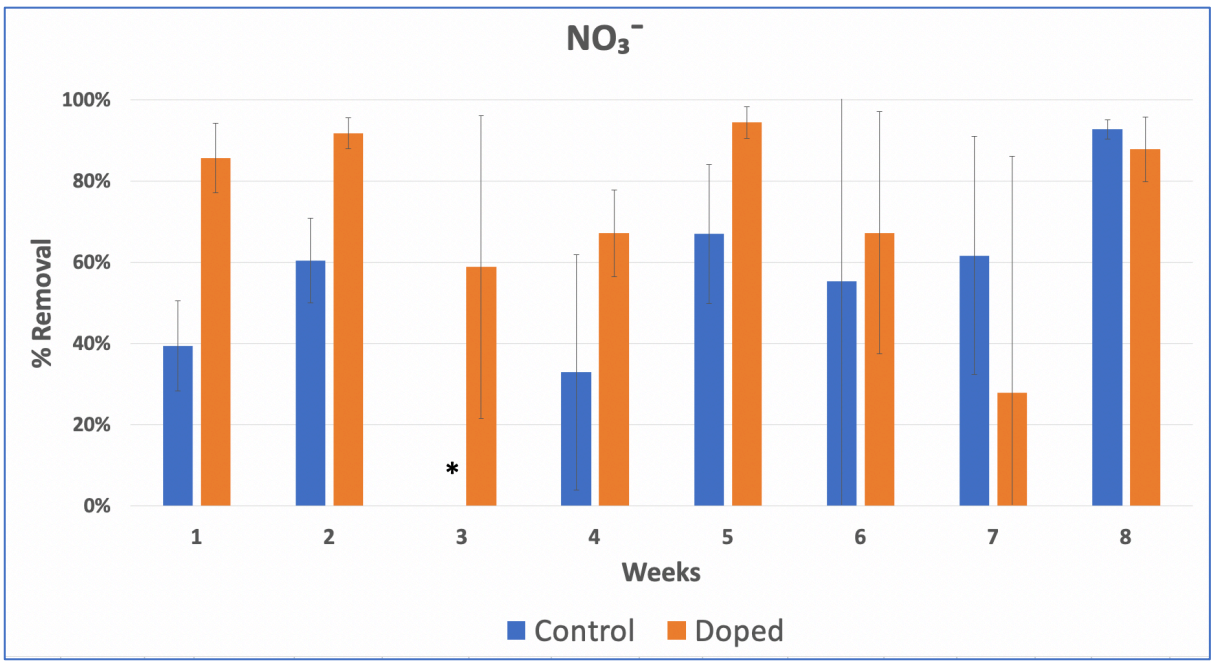


Fig. 3.7 - Removal percentage (%, mean and standard deviation, n = 3) of nitrate ion (NO_3^-) in CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. * no NO_3^- removal in CWs.

3.1.2.4. Phosphates removal

The PO_4^{3-} ions concentrations, average and standard deviation in influents and effluents of CWs and removal performances of CWs over the experimental period are presented in Table 3.13. The PO_4^{3-} concentration varied between 2.7 (± 0.1) and 8.4 (± 0.2) µmol L^{-1} in influent of CWs. The lowest and highest PO_4^{3-} concentration value was registered in weeks seven and five, respectively.

Regarding CWs effluent the PO_4^{3-} concentration varied between 11 (± 3) and 26 (± 20) µmol L^{-1} in control treatment and between 9 (± 3) and 12 (± 3) µmol L^{-1} in doped treatment. No significant differences between treatments were observed ($p > 0.05$). The lowest and highest PO_4^{3-} concentration were observed in weeks five and two, respectively.

As observed in Table 3.13, in week five the lowest value of PO_4^{3-} concentration in CWs effluent was registered of both treatments and highest concentration in CWs influent.

Throughout the experimental time, there were no removals of PO_4^{3-} by CWs in both treatments, with the concentrations slightly increasing in CWs effluents.

Table 3.13 - Concentration of phosphate ions (PO_4^{3-} ; µmol L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP.

PO_4^{3-} (µmol L^{-1})			
Weeks	**Influent**	**Effluent**	
		Control	Doped
1	2.8 ± 0.1	15 ± 2	12 ± 2
2	3.1 ± 0.1	26 ± 20	12 ± 3
3	5.6 ± 0.1	13.1 ± 0.7	10 ± 3
4	8.2 ± 0.4	12 ± 4	10 ± 3
5	8.4 ± 0.2	11 ± 3	9 ± 3
6	4.5 ± 0.1	14 ± 2	11 ± 3
7	2.7 ± 0.1	13 ± 4	12 ± 3
8	4.6 ± 0.2	11 ± 2	11 ± 2

3.1.2.5. Chlorophenols removal

PCP concentration in water from *Ribeira de Joane* and CWs effluents of control treatment was always below detection limit (4 E-05 µg L^{-1}). In CW effluents of the doped

treatments PCP was always detected, concentrations varying between 2.5 (± 0.5) and 13 µg L^{-1} (Table 3.14). Removals varied between 87 (± 2) % and 98 (± 1) % (Fig. 3.8). Over the experimental period the highest PCP removal value in doped CWs was observed in the first week, followed by a downward trend until a further increase was observed in the fifth week. After that, a further decrease was observed following an increase in the last experimental week. These oscillations in PCP removal can be due to the matrix substrate saturation effect. Because of PCP hydrophobicity, there is a tendency for this compound to stay sorb to the substrate matrix, which could difficult PCP removal by biologic processes.

Table 3.14 - Concentration of PCP (µg L^{-1}) in influent and effluent of CWs (mean and standard deviation, n = 3), irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with 100 µg L^{-1} PCP per week. *PCP concentration below the detection limit (LOD = 4 E-05 µg L^{-1}).

Week	Influent (µg L^{-1})	Effluent (µg L^{-1})	
		Control	Doped
1	< 4E-05 *	< 4E-05 *	2.5 ± 0.5
2			3.1 ± 0.6
3			7 ± 2
4			11 ± 4
5			8 ± 2
6			13 ± 2
7			13 ± 1
8			9 ± 4

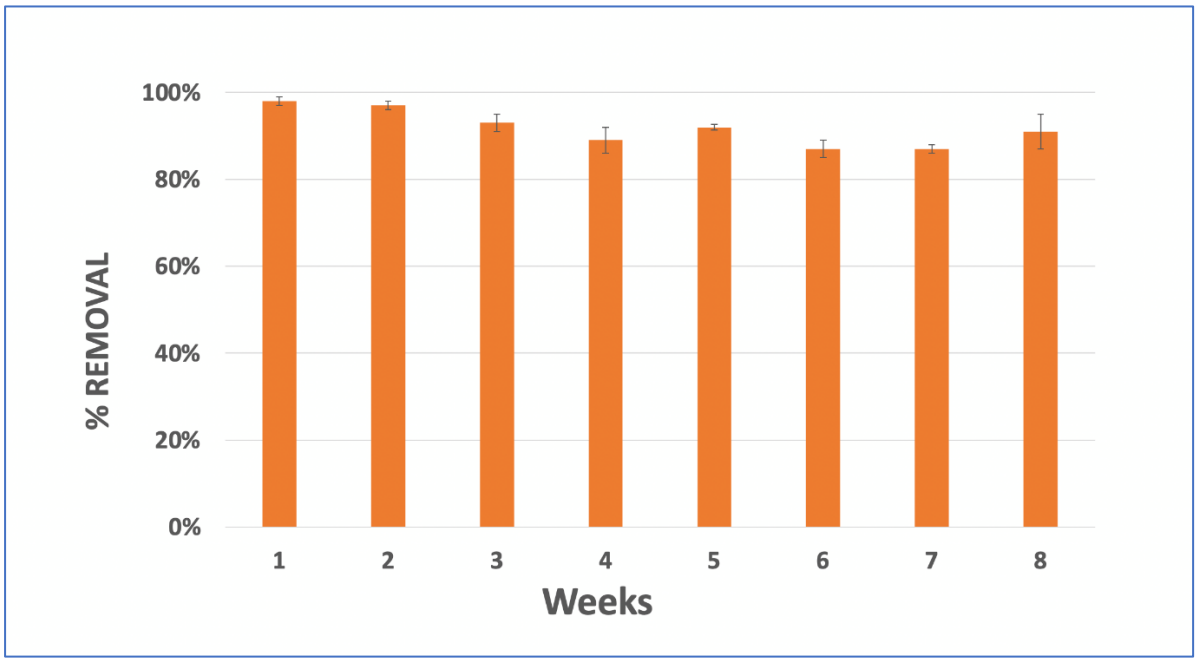


Fig. 3.8 - Removal percentage (%, mean and standard deviation, n = 3) of PCP in CWs, irrigated along eight weeks with *Ribeira de Joane* water doped with 100 µg L^{-1} of PCP per week.

No PCP was detected in initial plant roots bed substrate (T0) nor in plant roots bed substrate from control treatment at the end of the experiment (T8), PCP concentration being below detection limit (0.030 ng g^{-1}) (Table 3.15).

In plant roots bed substrate from doped treatment, PCP was detected at the end of the experiment period (T8) indicating that the compound was retained in the CW systems not being degraded.

Table 3.15 - Concentration of PCP in sediments of CWs irrigated with not doped water of *Ribeira de Joane* (control) and in sediments of CWs irrigated with water of *Ribeira de Joane* doped weekly with 100 µg L^{-1} of PCP. Mean and standard deviation (ng g^{-1}, n = 3) at experimental initial period (T0) and at the end of the experimental period (T8). *PCP concentration below the detection limit (LOD = 0.030 ng g^{-1}).

PCP (ng g^{-1})		
T0	**T8**	
	Control	Doped
< 0.030*	< 0.030*	20 ± 9

3.2. Results of biological analysis

3.2.1. Impact of a alkylphenol compounds mixture on Constructed Wetlands substrate microbial community

The 12 sediment samples in experiment #1 (six samples from T0; doped (n = 3) and non - doped (n = 3)) and the other six from T8, doped (n = 3) and not - doped (n = 3)). After samples preparation using the Qubit™ dsDNA High Sensibility Assay kit the DNA samples were quantified by fluorometry, these values are shown in the table of Annex D1. In Annex D2 can be seen the image obtained in Gel Documentation System for evaluated the DNA quality.

For the 12 collected samples, a total of 877881 of V4–V5 16S rRNA gene sequences were generated by Illumina MiSeq which decreased to a total of 870156 after the quality filtering performed by the sequencing company. A total of 767470 quality checked merged reads (around 88 % of the total dataset) were then submitted and processed by SILVAngs automatic pipeline that clustered into 172430 OTUs and classified them using the SILVA

reference database. The total number of sequences per sample ranged from 28965 to 9696 with an average of 63956 ± 17540 reads per sample.

The number of OTUs per sample ranged from 7400 to 21254 with an average of 14369 ± 3380 sequence variants per sample (see details in Table 3.16). Bacteria domain represented around the 96 % of the total dataset, Archaea domain represented around 1 % while the remained 3 % was classified into "No Relative" group (without any close relatives).

Table 3.16 - Summary statistics of 16S rRNA gene amplicon sequencing for experiment #1.

Sample ID	Number of sequences	OTUs[a]	Classified %	No Relative %	Rejected %
T0_C_1	59369	14182	74.41	2.18	23.41
T0_C_2	62416	14116	76.27	1.94	21.79
T0_C_3	66747	14931	77.68	1.87	20.45
T0_DP_1	92696	21254	74.83	2.10	23.06
T0_DP_2	69956	16244	78.28	2.00	19.72
T0_DP_3	80982	17006	75.26	2.10	22.64
T8_C_1	75430	15043	75.88	2.60	21.52
T8_C_2	28965	7400	77.97	2.79	19.23
T8_C_3	45287	11377	78.49	1.89	19.61
T8_DP_1	55609	13017	78.00	2.32	19.68
T8_DP_2	79581	15830	78.56	2.39	19.03
T8_DP_3	50432	12030	78.98	1.82	19.20
Total	767470	172430			
Average ± stdev	63956 ± 17540	14369 ± 3380			

The alpha diversity varied between control and doped CWs systems (Fig. 3.9), but no significant differences (*Kruskal-Wallis*, $p > 0.05$) were observed in terms of richness (number of observed OTUs), diversity (*Shannon* diversity index) or dominance (*Berger - Parker* index).

The analyses of the cluster dendrogram (Fig. 3.10) showed two groups according with similarity, the group of T0 (beginning of the experiment) and the group of T8 (end of the experiment) samples. The samples in T0 (samples from plant root bed before being subjected to doped stream water) were more similar to each other than to the T8 samples.

The same is observed based on NMDS analysis (Fig. 3.11), two groups are formed in function of the experimental time (T0 and T8). The existence of similarity between initial samples was expected as plant roots bed origin was the same. Over the experimental time the experimental conditions influenced the microbial community structure, which showed a tendency towards greater specificity.

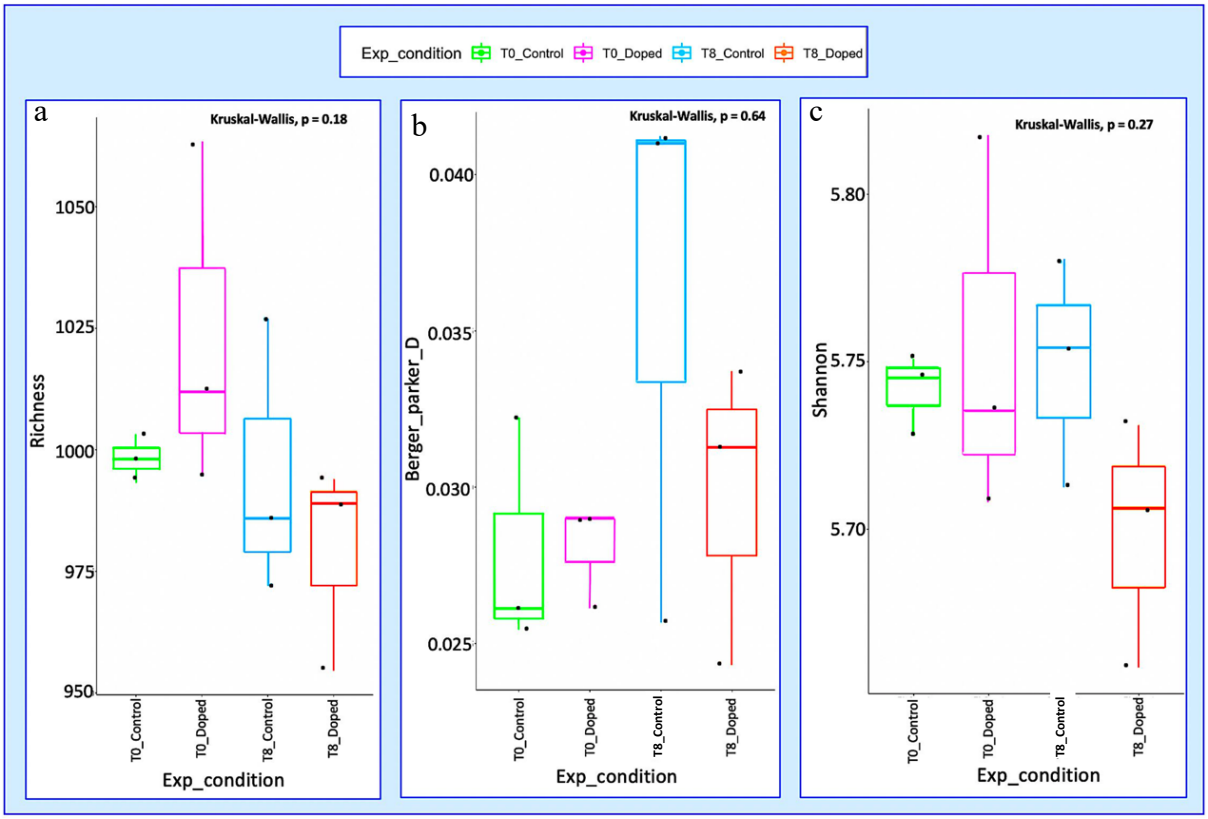


Fig. 3.9 - Alfa diversity index of the microbial community in the different CWs systems, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). **a)** Richness (number of observed OTUs); **b)** dominance (*Berger-Parker* index); **c)** diversity (*Shannon* diversity index).

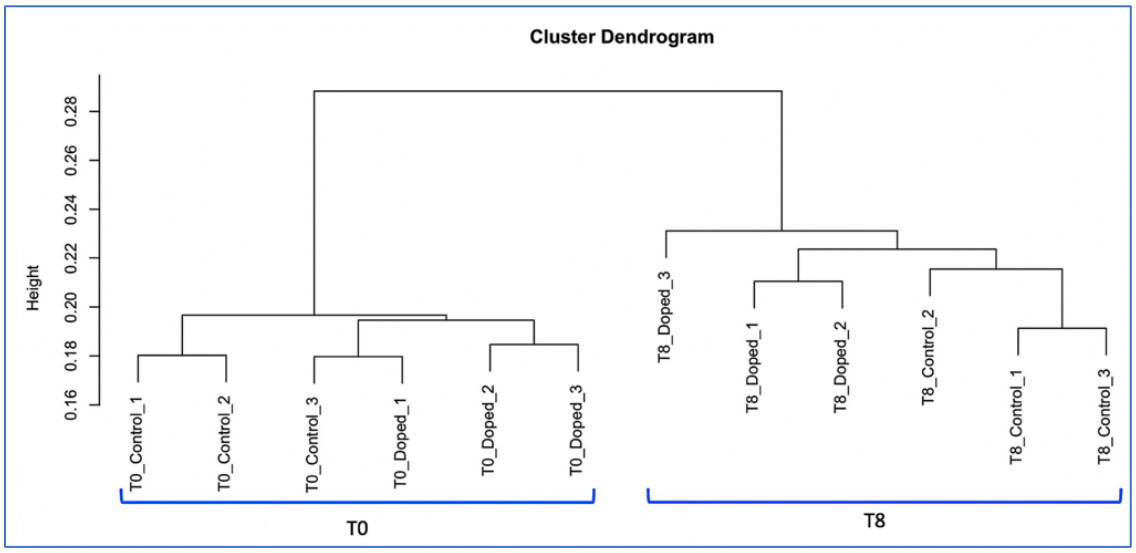


Fig. 3.10 - Cluster dendrogram analysis of Hellinger transformation using the *Bray - Curtis* dissimilarity matrix to ordinate microbial community composition of the different CWs systems, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3).

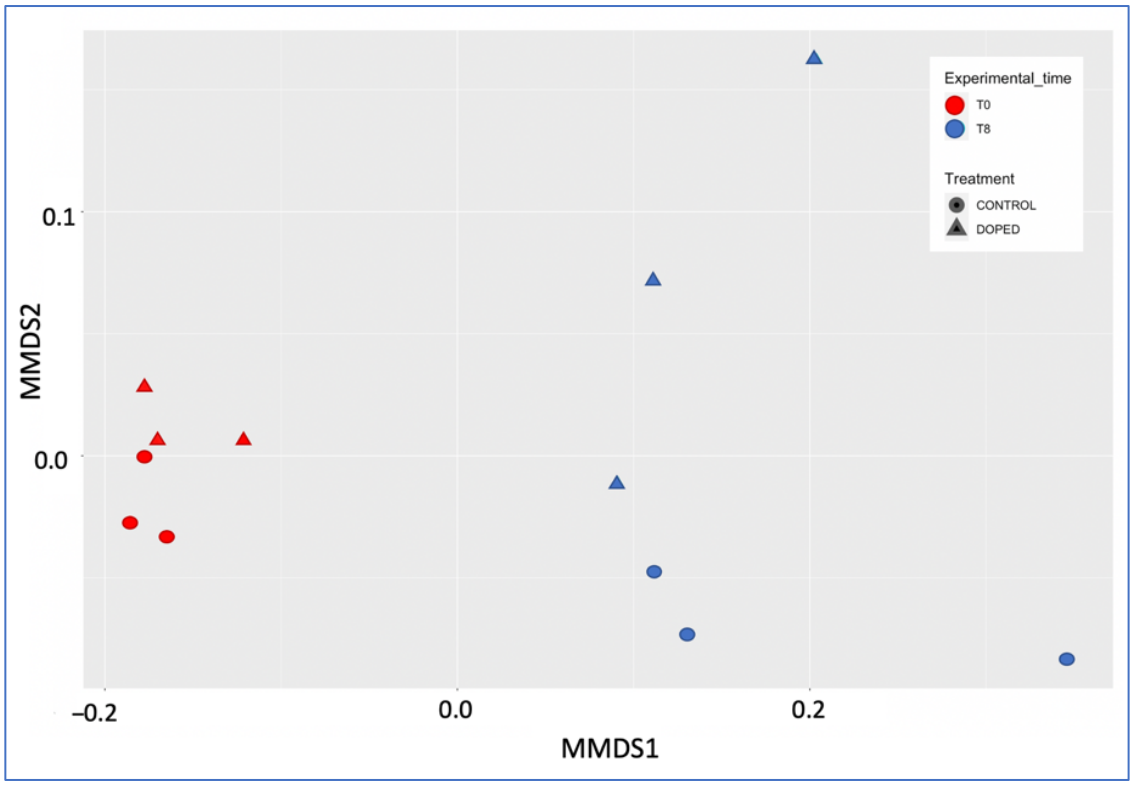


Fig. 3.11 - MDS analysis of data transformed by "phyloseq" function and based on *Bray - Curtis* dissimilarity matrix to ordinate microbial community composition of the different CWs systems, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). The *Kruskal - Wallis* test was conducted to examine the difference between microbial communities of each sample.

Taxonomic profile of the bacteria communities was performed at phylum, class and genus level. At phylum level, the three most abundant taxa were *Proteobacteria* (abundance up to 41 %), *Bacteroidetes* (abundance up to 21 %) and *Planctomycetes* (abundance up to 10 %) (Fig. 3.12), being very similar among samples.

Relatively to class level, four classes represented more than 50 % of those with abundance > 1 %. Three of those classes belong to *Proteobacteria* phylum (classes *Gammaproteobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria*) and the other class to the *Acteroidetes* phylum (class *Bacteridia*) (Fig. 3.13). When the analysis was made at genus level, the fifteen genera more abundance across the samples represented up to 23 % of genera community (Fig. 3.14) (average total number of genera per sample 453 ± 51, cut - off of 0.90 %).

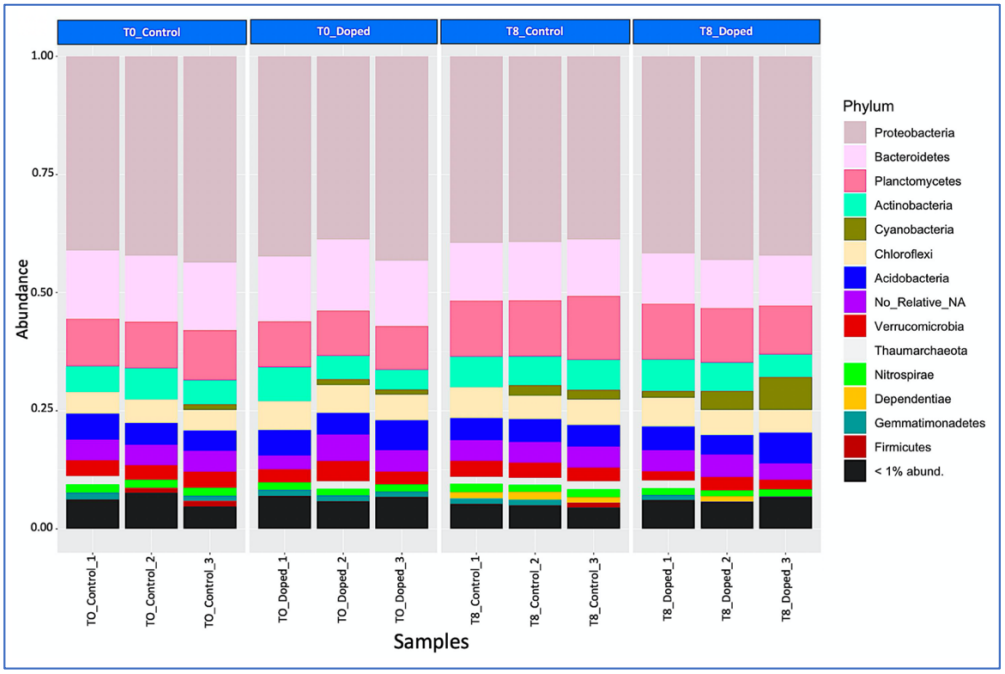


Fig. 3.12 - Abundance of the phyla of domain bacteria, in the substrates of different CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). < 1 % abund. represent the groups of the phyla with abundance lower than 1 %; "No_Relative_NA" groups the phyla without any close relatives.

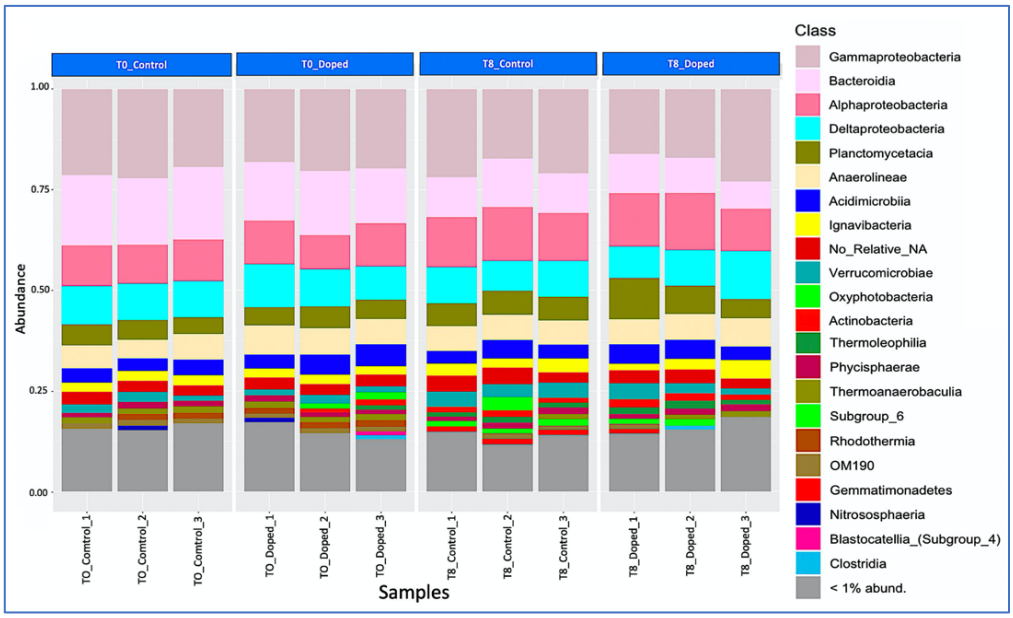


Fig. 3.13 - Classes of domain bacteria with abundance > 1% across the different CWs systems substrates, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). <1 % abund.: groups the phyla with abundance lower than 1 %; "No_Relative_NA" groups the phyla without any close relatives.

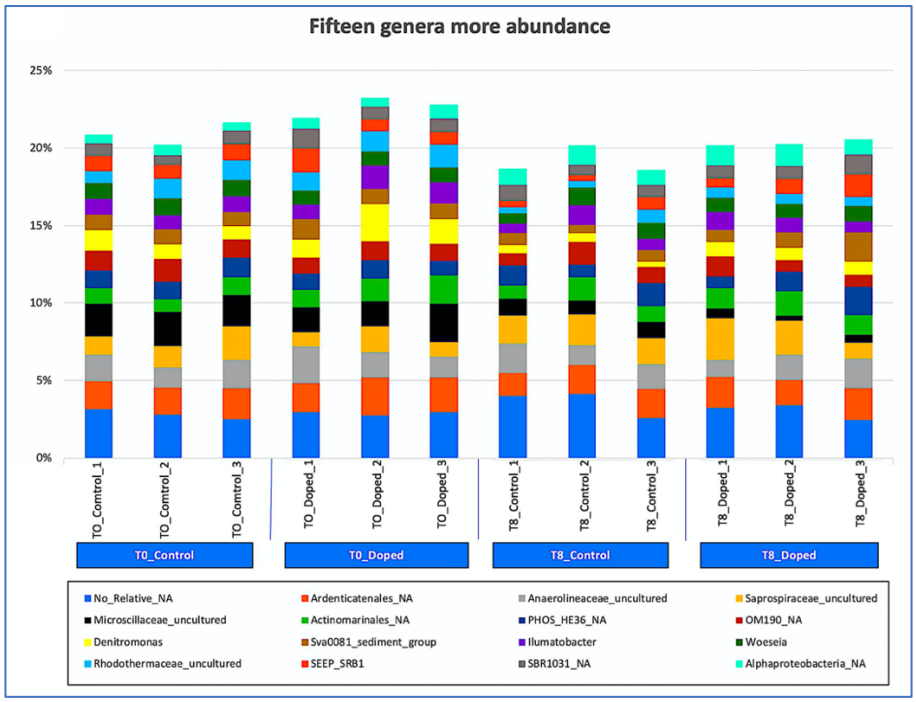


Fig. 3.14 - Genera more abundant of bacteria across the different CWs systems substrates, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). < 1 % abund.: groups the phyla with abundance lower than 1 %; "No_Relative_NA" groups the phyla without any close relatives.

Multinominal Species Classification Method (CLAM) analysis was performed to identify the potential specialists taxa present in T8 doped when compared with T8 control (see Fig. 3.15 a) and b). CLAM uses a multinominal model based of relative abundance of estimated OTUs for two distinct conditions ("generalist" and "specialist").

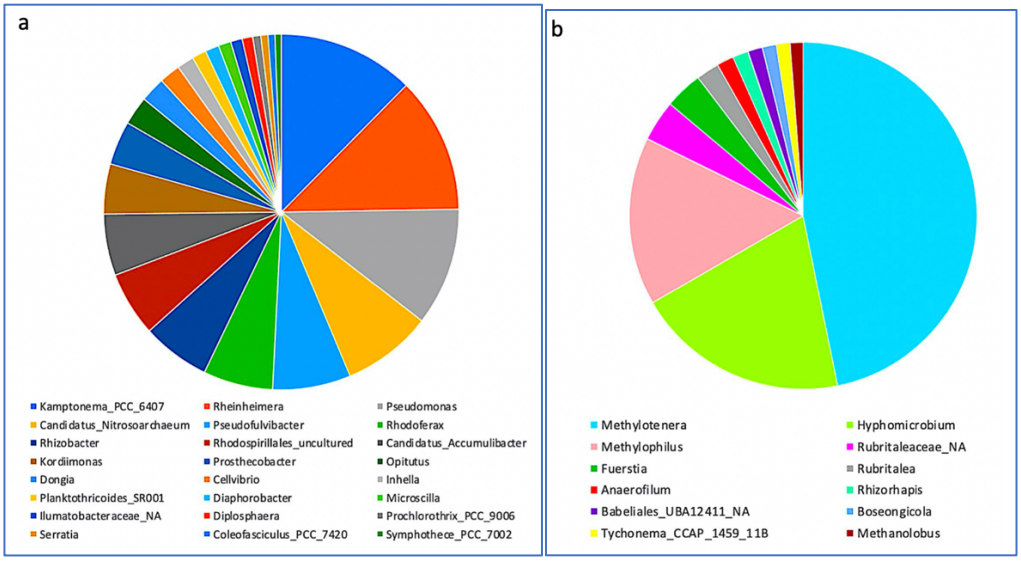


Fig. 3.15 - Relative abundance of the specialist genera in plant roots bed substrate samples; **(a)** in control CWs and **(b)** in doped CWs at the end of the experiment (after 8 weeks). Systems irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Only genera with abundance > 1 % are shown.

Looking more specifically at samples collected at the end of the experiment, in samples from control CWs 25 different genera specialists were observed while in samples from doped CWs only 11 different genera specialists were observed.

The most abundant specialist genera in control CWs (Fig. 3.15 a) were *Kamptonema*_PCC_6407 (13 %), *Rheinheimera* (13 %) and Pseudomonas (11 %), while the doped CWs (Fig. 3.15 b) were dominated by *Methylotenera* (43 %), *Hyphomicrobium* (19 %) and *Methylophilus* (14 %).

Regarding archaea communities (which represented only ca. 1 % of the entire community), the taxonomic profile showed they were represented by seven phyla (*Thaumarchaeota, Crenarchaeota, Nanoarchaeaeota, Euryarchaeota, Asgardaeota, Altiarchaeota, Diapherotrites*). The most abundant taxa across all the samples, ca. 87 % of the total community, were *Thaumarchaeote* (34 - 72 %), *Crenarchaeota* (9 - 35 %) and *Nanoarchaeaeota* (7 - 18 %) (Fig. 3.16).

At class level of archaea community, the three most abundant taxa across all the samples were *Nitrososphaeria* (34 - 72 %), *Bathyarchaeia* (9 - 35 %) and *Woesearchaeia* (6 - 18 %) (Fig. 3.17). The presence of the mixture of NP and OP caused a significant decreased in *Thaumarchaeote* phylum (*Nitrososphaeria* class) and a significant increase in *Crenarchaeota* phylum (*Bathyarchaea* class).

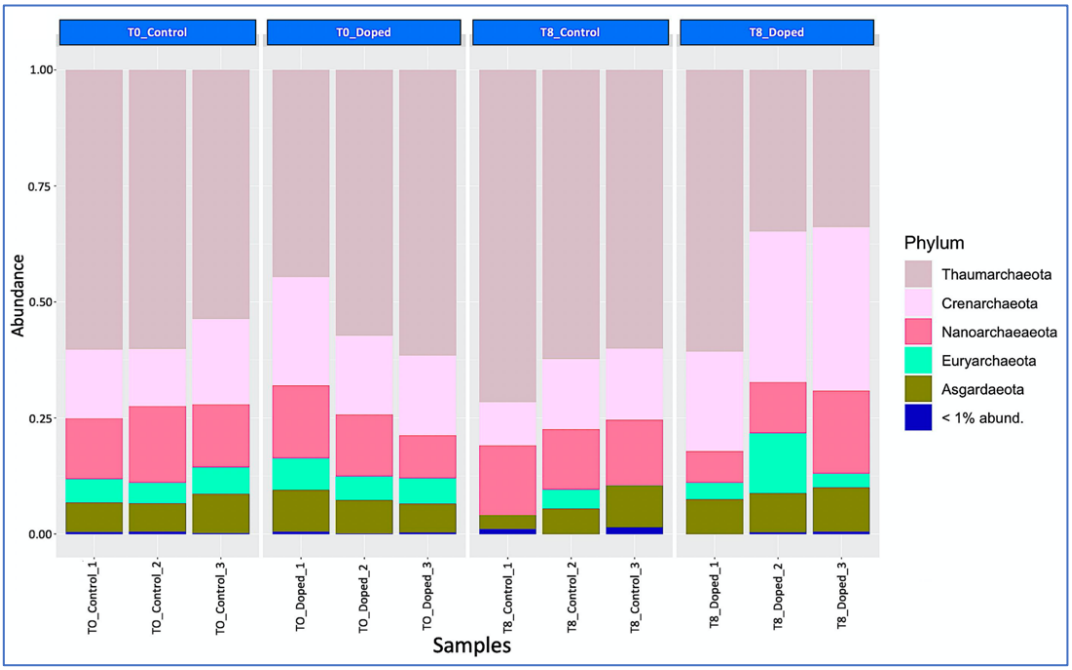


Fig. 3.16 - Phyla of domain archaea in different CWs substrates, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). "< 1 % abund." represent the group of the phyla with abundance lower than 1 %.

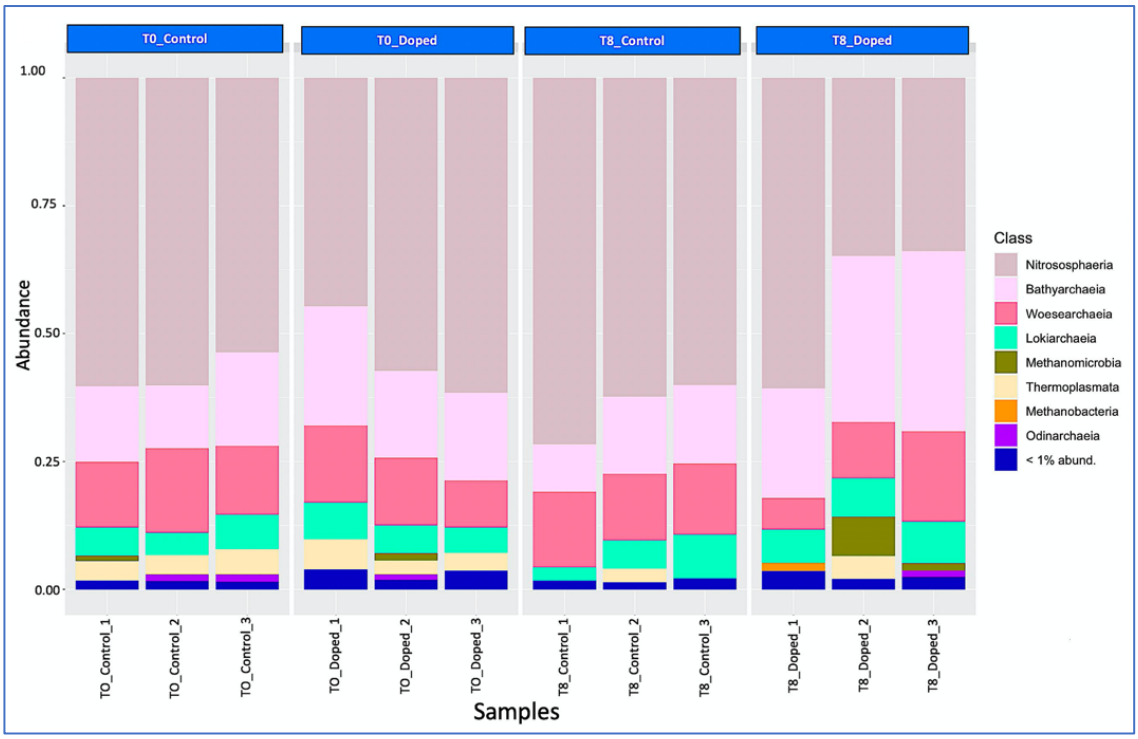


Fig. 3.17 - Classes of domain archaea in different CWs substrates, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with NP and OP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). "< 1 % abund." represent the class group with abundance lower than 1 %.

3.2.2. Impact of PCP on Constructed Wetlands substrate microbial community

The 12 sediment samples in experiment #2 (six samples from T0; doped (n = 3) and non - doped (n = 3)) and the other six from T8, doped (n = 3) and not - doped (n = 3)), After samples preparation using the Qubit™ dsDNA High Sensibility Assay kit the DNA samples were quantified by fluorometry, the values of these quantify as shown in table of Annex D3.

For the 12 collected samples, a total of 887135 of V4 - V5 16S rRNA gene sequences were generated by Illumina MiSeq which decreased to a total of 883920 after the quality filtering performed by the sequencing company. A total of 789722 quality checked merged reads (around 89 % of the total dataset) were then submitted and processed by SILVAngs automatic pipeline that clustered into 185748 OTUs and classified them using the SILVA reference database. The total number of sequences per sample ranged from 52216 to 95767 with an average of 65810 (± 13681) reads per sample. The number of OTUs per sample range from 11316 to 20710 with an average of 15479 (± 2847) sequence variants per sample (see details in Table 3.17). Bacteria domain represented around the 94 % of the total dataset,

Archaea domain represented around 2 % while the remained 4 % was classified into “No Relative” group (without any close relatives).

Table 3.17 - Summary statistics of 16S rRNA gene amplicon sequencing for experiment #2.

Sample ID	Number of sequences	OTUs[a]	Classified %	No Relative %	Rejected %
T0_C_1	95767	20710	91.92	3.75	4.32
T0_C_2	69693	16520	92.34	3.43	4.23
T0_C_3	56353	13527	92.59	3.58	3.83
T0_DP_1	88032	20212	94.85	2.31	2.84
T0_DP_2	69792	17386	91.05	4.43	4.52
T0_DP_3	68302	15787	92.02	3.49	4.49
T8_C_1	59040	13752	91.03	3.69	5.27
T8_C_2	52216	13789	91.42	3.74	4.84
T8_C_3	62916	15673	90.81	3.84	5.35
T8_DP_1	54021	13434	91.53	3.73	4.74
T8_DP_2	57251	11316	91.39	3.87	3.74
T8_DP_3	56339	13642	91.61	2.90	5.48
Total	789722	185748			
Average ± stdev	65810 ± 13681	15479 ± 2847			

The alpha diversity varied between systems (Fig. 3.18), but no significant differences (*Kruskal-Wallis*, $p > 0.05$) were observed in terms of richness (number of observed OTUs) or dominance (*Berger-Parker* index). In terms of diversity (*Shannon* diversity index), significant differences were observed among samples from control and from doped CWs systems at the end of the experiment (*Kruskal-Wallis*; $p < 0.05$), showing that the presence of PCP lead to a decrease in the microbial species diversity and an increase in number of dominance species.

The analyses of the cluster dendrogram (Fig. 3.19) showed two groups according with similarity, the group of T0 (beginning of the experiment) and the group of T8 (end of the experiment) samples. The samples in T8 were more similar to each other than to the T0 samples. T0 samples refer to plant root bed before being subjected to doped stream water. The same is observed based on NMDS analysis (Fig. 3.20), two groups are formed in function of the experimental time (T0 and T8). The existence of similarity between initial samples was expected as plant roots bed origin was the same. Over the experimental time the experimental conditions influenced similarly the microbial community, which showed a tendency towards greater specificity.

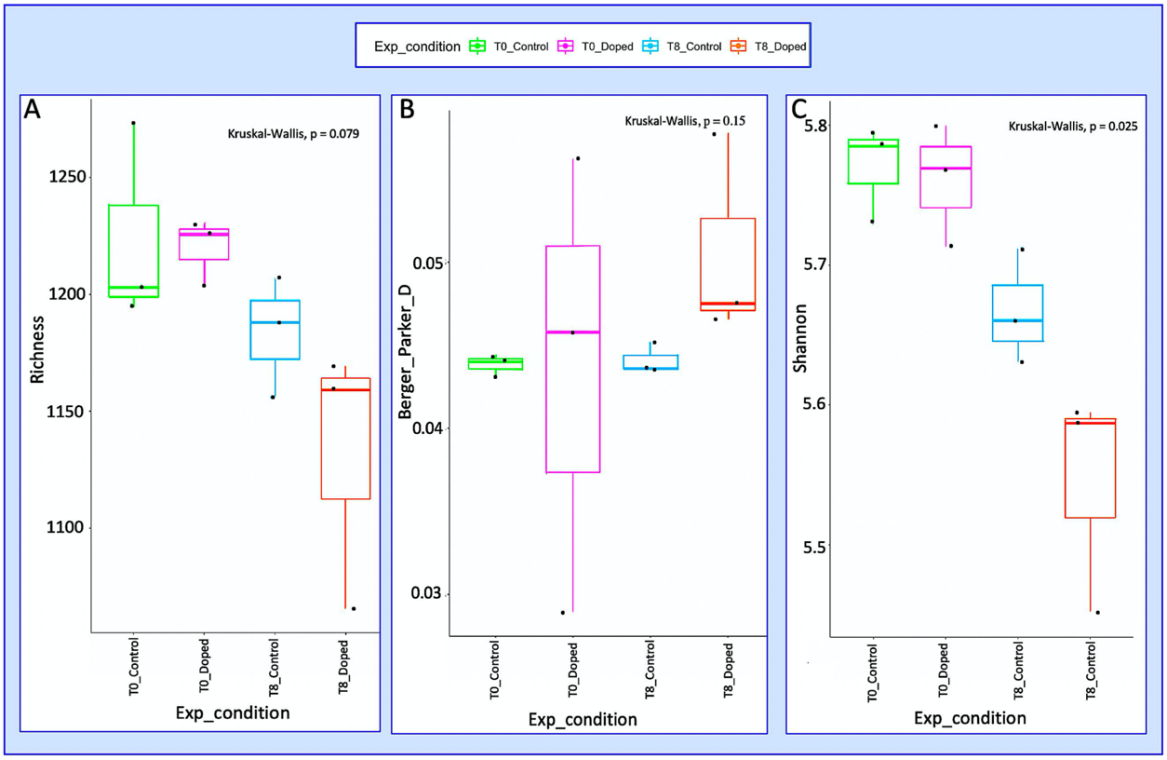


Fig. 3.18 - Alfa diversity index of the microbial community in the different CWs systems, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). **a) -** Richness (number of observed OTUs); **b) -** dominance (*Berger-Parker* index); **c) -** diversity (*Shannon* diversity index).

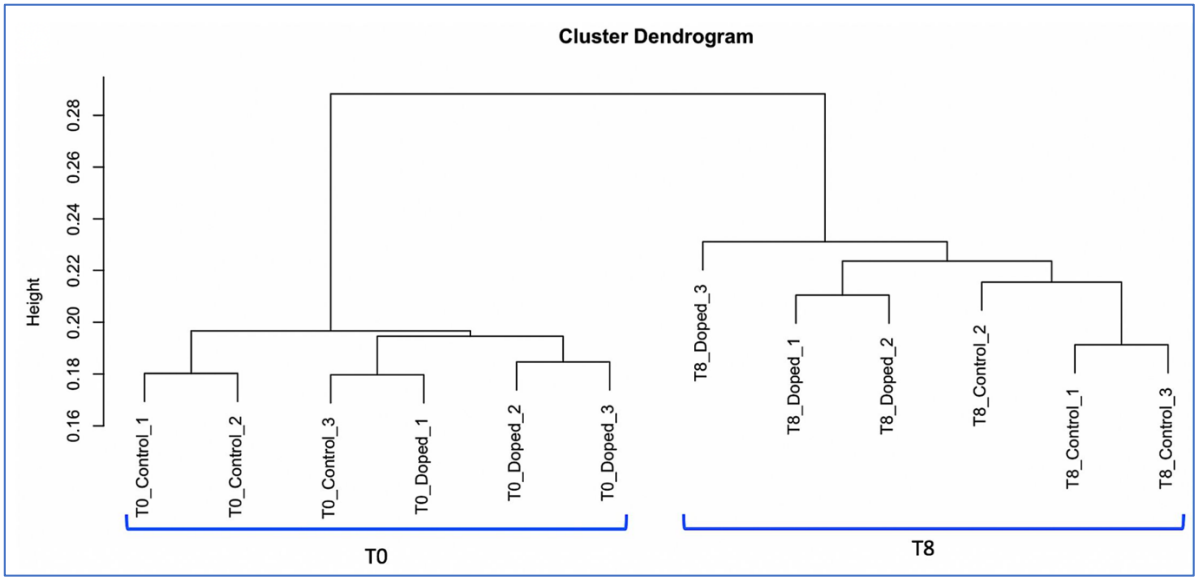


Fig. 3.19 - Cluster dendrogram analysis of *Hellinger* transformation using the *Bray - Curtis* dissimilarity matrix to ordinate microbial community composition of the different CWs systems, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment time (T8) (n = 3).

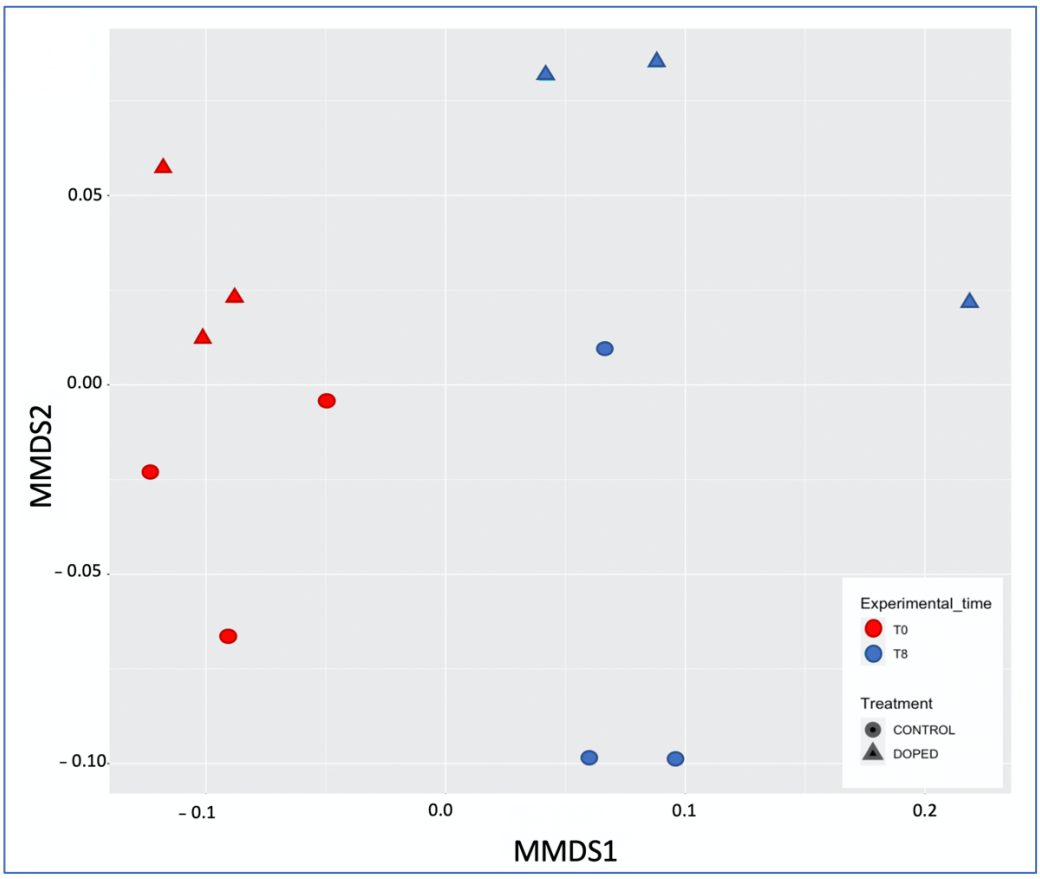


Fig. 3.20 - NMDS analysis of data transformed by "phyloseq" function and based on *Bray - Curtis* dissimilarity matrix to ordinate microbial community composition of the different CWs systems, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment time (T8) (n = 3). The *Kruskal - Wallis* test was conducted to examine the difference between microbial communities of each sample.

For the Bacteria domain, the three most abundant phyla were the same (Fig. 3.21): *Proteobacteria*, *Bacteroidetes* and *Planctomycetes*, being the percentage composition very similar among samples.

Relatively to class level, four classes represented more than 50 % of the group with abundance > 1 %. Three of those classes belong to *Proteobacteria* phylum (classes *Gammaproteobacteria*, *Alphaproteobacteria* and *Deltaproteobacteria*) and the other to *Acteroidetes* phylum (class *Bacteridia*) (Fig. 3.22).

When the analysis was made at genus level, the fifteen genera more abundance represented between 20 to 26 % of the total number of genera (Fig. 3.23) (average total number of genera per sample 799 (± 61), cut-off of 0.95 %).

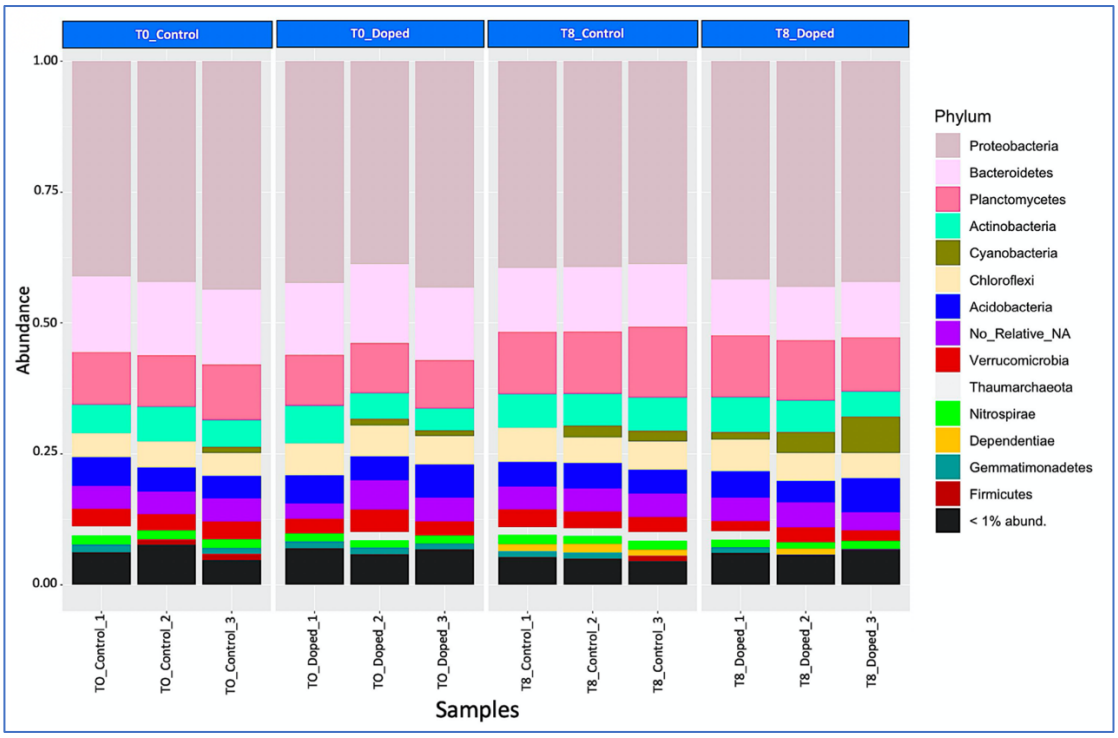


Fig. 3.21 - Abundance of the major phyla of bacteria, in the red bed substrates of different CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). < 1 % abund. groups the phyla with abundance lower than 1 %; and "No_Relative_NA" groups the phyla without any close relatives.

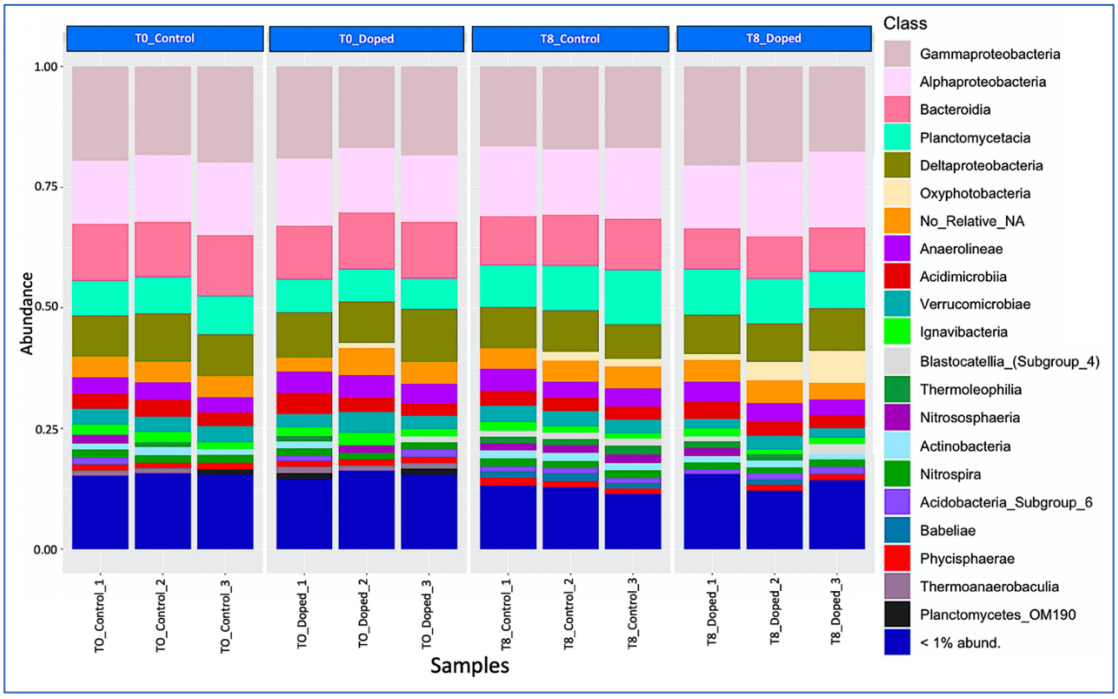


Fig. 3.22 - Classes of bacteria with abundance > 1 % in the red bed substrates of different CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). < 1 % abund. groups the class with abundance lower than 1 %; and "No_Relative_NA" groups the class without any close relatives

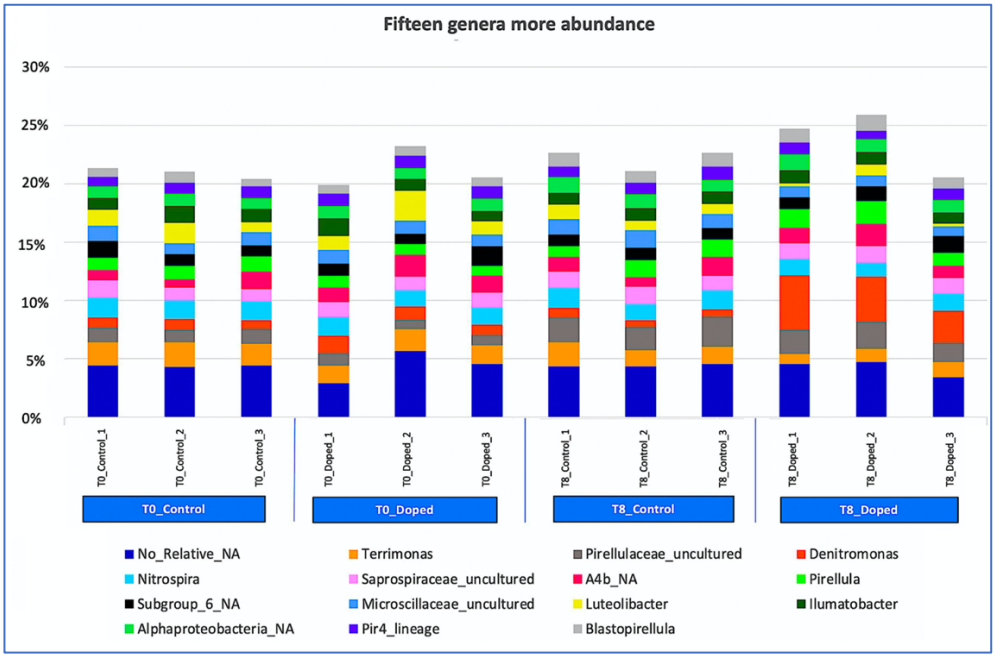


Fig. 3.23 - Genera more abundant of bacteria present in the red bed substrates at different CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3).

Looking more specifically at samples collected at the end of the experiment (T8), in samples from control CWs, 25 different genera specialists were observed while in samples from doped CWs only 22 different genera specialists were observed, Fig. 3.24 a) and b) respectively. The most abundant specialist genera in control CWs were *Arenomonas* (23 %) and *Limniphilus* (10 %) while the doped CWs were dominated by *Denitromonas* (40 %) and *Xenococcus* (33 %).

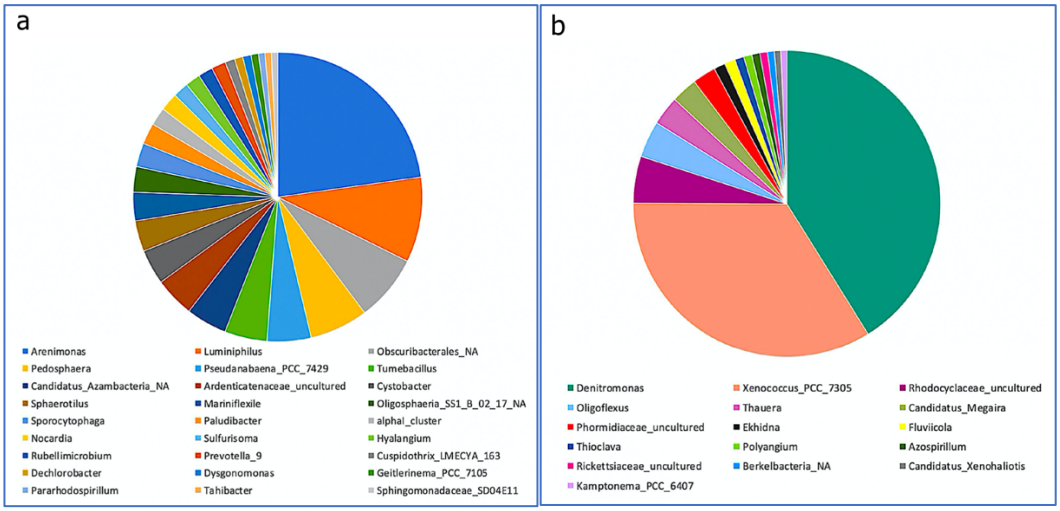


Fig. 3.24 - Relative abundance of the specialist genera of Bacteria in plant roots bed substrate samples, **a)** in control CWs and **b)** in doped CWs collected at the end of the experiment (after 8 weeks). Systems irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Only genera with abundance > 1 % are shown.

Taxonomic profile of the archaea community (which represent ca. 2 % of the total community) showed that they were represented by seven phyla (*Altiarchaeota*, *Asgardaeota*, *Crenarchaeota*, *Diapherotrites Euryarchaeota*, *Nanoarchaeaeota*, *Thaumarchaeota*).

At phylum level, the three most abundant taxa, representing more than 80 % of the total community, across all the samples were *Thaumarchaeote* (abundance between 60 - 87 %), *Crenarchaeota* (6 - 19 %) and *Asgardaeota* (3 - 10 %) (Fig. 3.25).

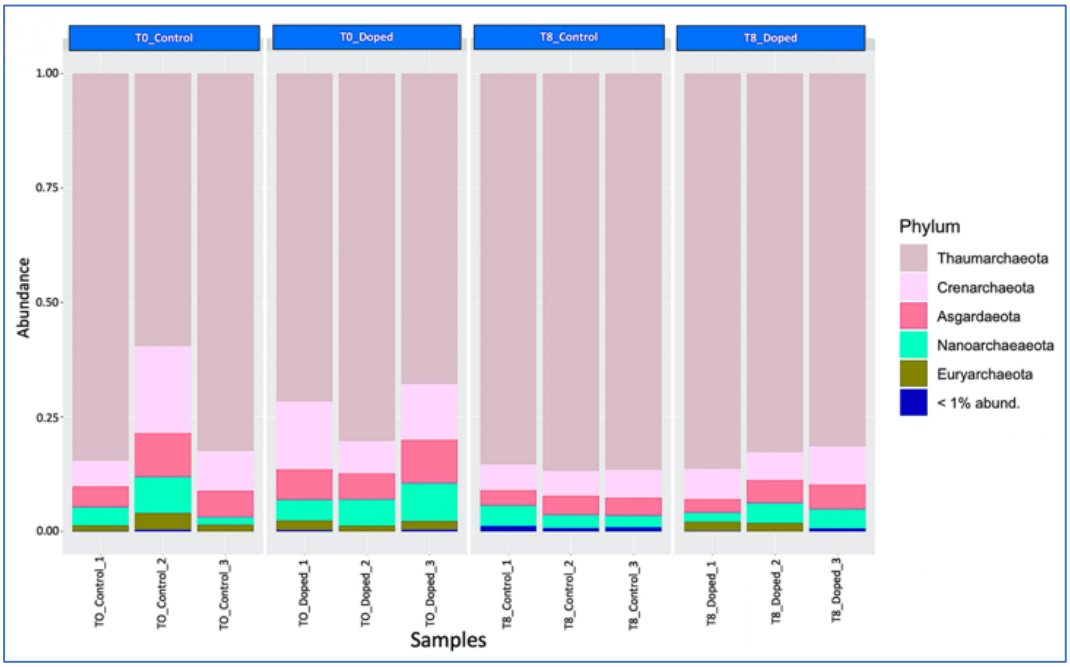


Fig. 3.25 - Abundance of the major phyla of archaea, in the red bed substrates of different CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). < 1 % abund. groups the phyla with abundance lower than 1 %.

At class level, the most abundant taxa across all the samples were Nitrososphaeria (abundance between 55 - 85 %), Bathyarchaeia (5 - 18 %), Woesearchaeia (1 - 7 %) and Lokiaechaeia (2 - 8 %).

The presence of PCP did not interfere with the Archaea community, however, over time the experimental conditions influenced the Archaea community, with a slight increase of the dominant phylum *Thaumarchaeota* (*Nitrososphaeria* class) (Fig. 3.26).

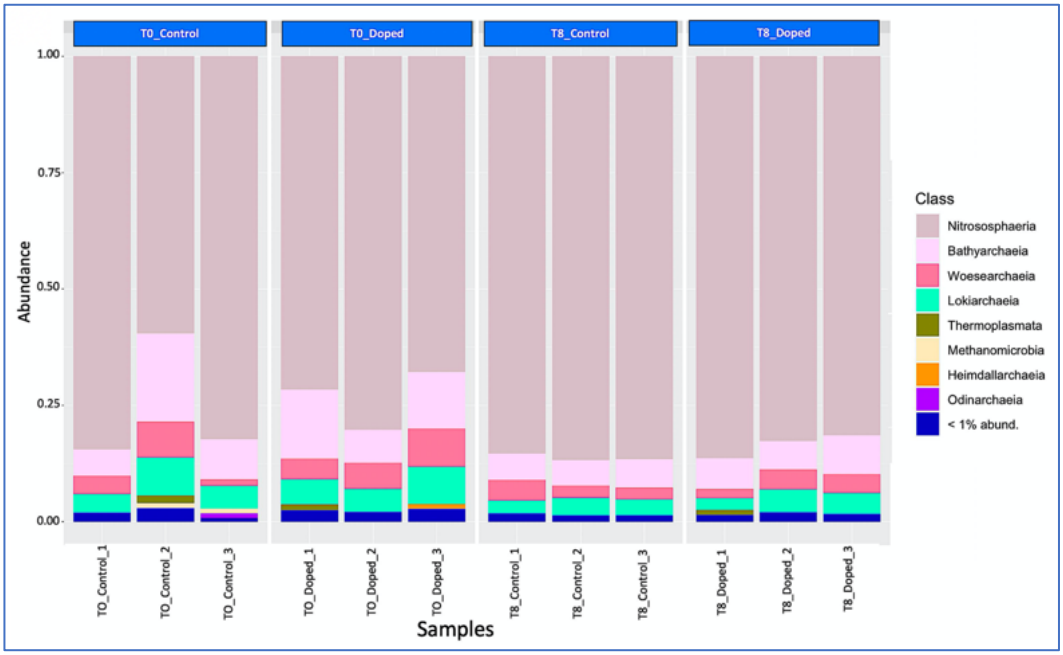


Fig. 3.26 - Classes of archaea present in the red bed substrates of different CWs, irrigated along eight weeks with *Ribeira de Joane* water not doped (control) or doped with PCP. Samples collected at the beginning (T0) and at the end of the experiment (T8) (n = 3). < 1% abund. groups the class with abundance lower than 1 %.

4. Discussion

4.1. Removal of conventional water quality parameters

4.1.1. Removal efficiency of Chemical Oxygen Demand

Organic matter is a major pollutant, especially in municipal wastewater, and is expressed by the biodegradable part (normally measured through biochemical oxygen demand (BOD_5)) and the total organic matter, that can be measured through COD [23]. The CW with vertical flow system can achieve full COD removal because of high amount of oxygen transfer capability through the reed bed [501]. Higher oxygen content in this CW is required for bacteria to remove organic and nutrient pollutants. For instance, Cooper [501] reported a high capacity of VFCW in removing COD from residual waters, with removal rates of 78 % for COD. With the same type of CW, Gikas and Tsihrintzis [579] reported removal efficiencies of 94 % from domestic wastewater and for domestic and dairy wastewater Rousseau et al. [580] reported the same removal efficiencies value (94 %). In the experiment #1 of this study, COD removal occurred after system acclimation and reached a value of 73 % in control treatment and 83 % in doped treatment, similar to the mentioned studies (Table 3.1 experiment #1). Other authors, however, reported lower removal efficiency values of COD by CWs, as observed in some weeks of experiment #1 and in general of experiment #2 of this study, where the range of COD removal was between 3 % and 35 % at control treatment and between 8 % and 48 % at doped treatments. For instance, Liu et al. [581] who studied the performance of three parallel subsurface horizontal flow CWs for surface water quality improvement, reported monthly average removal rates of COD from 15 - 40 % and Li et al. [582] who studied the performance of three parallel units of pilot-scale CWs (i.e., vertical subsurface flow, horizontal subsurface flow and free water surface flow) for the same purpose reported values from 17 - 40 % for COD removal. These authors justify the results with the relatively low influent COD concentrations, i.e., the low organic loads resulted in the lower COD removal efficiencies within the CWs. In Liu et al. [581] and Li et al. [582] the influent concentration of COD ranged from 4.5 to 7.5 mg L^{-1} and from 5.4 to 10.3 mg L^{-1} over the experimental period, respectively. Korkusuz et al. [468], state that it was more difficult to reduce COD concentrations below 50 mg L^{-1} in CWs. In fact, in experiment #2 the highest COD removal values in both treatments were obtained when the concentration in CWs influents was above 50 mg L^{-1}. Nevertheless,

in our study despite the generally low COD influent concentration, higher removals were observed.

For instance, in case of 15 mg O_2 L^{-1} in influent of week five (Table 3.1 experiment #1), the removal efficiency was 73 % in control treatment and 53 % in doped treatment. And in week six, with COD concentration of 27 mg O_2 L^{-1} in CW influent, removal efficiency was 52 % and 78 % in control and doped treatment respectively.

COD concentration in influent of experiment #1 was lower than the concentration in experiment #2 influent was, and these concentration values were still lower (22 and 19 mg L^{-1}) in the last weeks, probably due to a dilution effect caused by the heavy rains that occur in the region and consequent effect in the *Ribeira de Joane* flow.

In CWs effluents of both experiments a great variation in COD concentrations was observed over the experimental period. For instance, in experiment #1, taking into account the similar input COD concentration into the system, in the control treatment the COD concentration in week number one was 114 (± 7) mg L^{-1}, but in the week number three the value was already 18 (± 8) mg L^{-1}. Liu et al. [583], observed the same variation in COD concentration. These authors associated this variation with other oxygen - consuming reactions such as nitrification of ammonia, that requires dissolved oxygen [583]. Moreover, these authors, associate the increase in the production of organic matter with plant age. As vegetation dies in the system, the dead tissue acts as COD, consuming oxygen and therefore causing the negative COD removal rate [584]. Indeed, in the week number 7 at experiment #2, the week after the aged vegetable tissues removal, coincided with the lowest COD concentration in CWs effluent (16 (± 2) mg L^{-1} in control treatment; 19 (± 7) mg L^{-1} in doped treatment) (Table 3.8 experiment #2). Additionally, the same result was observed in experiment #1 after removal from substrate surface CWs of aged vegetable tissue. In this case the reduction in effluents COD concentration of both treatments (4.0 (± 0.1) and 13 (± 2) mg L^{-1} in control treatment; 7 (± 5) and 6 (± 1) mg O_2 L^{-1} in doped treatment) was observed in the two following weeks after this removal. This removal process of vegetable tissues may have avoided the organic matter load and consequent dissolved oxygen depleted in the CWs systems. Thereby, only the organic matter that adsorbed to the substrate from the earlier influent water was released and biodegraded in CW system during the next period. However, after aged vegetable tissue removal the reduction of COD concentration in effluents was more expressive in experiment #1 than in experiment #2, maybe because, the plants in experiment #2

were more developed and mature than plants in experiment #1 and according with Liu et al. [583], the roots from plants more developed and mature produced more organic matter.

Herouvim et al. [585] verify that COD bacterial removal is temperature dependent, which implies that organic matter removal is mostly a result of aerobic and anaerobic activity when temperature rises [586–588]. In experiment #1, that occurred along eight weeks during the spring/summer (between may and june), the air temperature in greenhouse was in the range of 10 - 30 °C. In experiment #2, that occurred along eight weeks during the spring / summer (between march and april), the air temperature in greenhouse was in the range of 15 - 27 °C. These temperatures during the experimental period were good conditions for microbial activity, the reduction of COD concentration may have resulted from microbial degradation, for instance in the case of experiment #1 where COD removal rate was as high as 50 % in most of the weeks in both treatments. For instance, Jing et al. [584] observed that the COD removal rate was as high as 51 % between april and october and was lowest between november and march, with COD concentrations sometimes actually higher in the effluent than in the influent during the winter season. Although the thermal amplitudes are not very different between experiments, the highest COD removal values were observed in experiment #1 and the lowest in experiment #2. In this last experiment the lowest values may be related to other biotic and abiotic factors earlier discussed.

Several studies indicate that vertical flow system in CW should not be used in the COD removal (and mineral forms of nitrogen) due to possible clogging the VFCWs, with sewage uniformly spread over the beds surface, gradual clogging of the surface of the media bed can take place [501, 589–591]. According to Tuszyńska and Obarska - Pempkowiak [592] good effectiveness of pollutants removal in the VFCWs results from additional reaeration in the intervals between sewage discharge. The effective air flow is only possible if the upper layers have good hydraulic conductivity and if the bed surface of VFCW is well drained between the inflow of subsequent sewage doses [593]. Based on these studies, to avoid possible clogging the VFCWs used in this study have drainage layer of 4 cm of gravel at the bottom.

To improve the removal efficiency of VF systems, the recirculation of effluent is often used for treatment of wastewaters from different sources [594–599]. The recirculation process provides two major effects in VFCW, improvement of the oxygenation of the wastewater,

because water is repeatedly pumped back onto the surface, and also potentiate purification processes through longer contact time between wastewater and microorganisms attached to rhyzosediment and biofilter material [597]. For instance, effluent recirculation applied to VFCW enhanced the removal of organic matter (TSS and COD) and the removal of TSS and COD were up to 95 % and 90 %, respectively [505]. Another author, Gross et al. [598] obtained high efficiency in removing COD (81 %) when using a recirculating in VFCW bed perforated bottom. Effluent recirculation was found to enhance the oxygen consumption by microorganisms for organic matter degradation [23]. In this sense, in both experiments of this work, a recirculation water system was used which may have contributed to improving COD removal efficiency. In this case, the recirculating water system (flow rate ca. 10 $mL.min^{-1}$) is an effluent recirculation and is an operational modification to improve the effluent quality of CWs. The concept of this method (effluent recirculation) consists of extracting a part of the effluent and transferring it back to the inflow of the systems. The main goal of effluent recirculation is to enhance aerobic activity through the enhance interactions between pollutants and microorganisms, which are in the rhizosphere and on the substrate surface, by allowing a longer retention time without significant alterations in the system operation [600].

Treatment efficiency of the CWs for the removal process of organic matter is generally highly dependent on the oxygen concentration in the bed, the wetland design, treatment conditions and the characteristics of the filling medium [448, 601]. Organic compounds are degraded both aerobically and anaerobically by the heterotrophic microorganisms in the wetland systems depending on the oxygen concentration in the bed [601]. Suitable to the wetland design, the oxygen required for aerobic degradation can be supplied by diffusion, convection and oxygen leakage from macrophyte roots into the rhizosphere [468]. According with Tang et al. [602] biological degradation is one of the most important COD removal routes in CWs. The removal of COD in CWs depends on the aerobic respiration of the microorganisms and the degradation of organic matter in CWs is correlated with the change of DO concentration in the system [603]. According with experimental results obtained by Chang et al. [604], COD removal presented relatively high correlation (R^2 = 0.523) with DO concentration. The roots systems promote the aerobic environment in rhizosphere, i.e., the oxygen produced through photosynthesis is transfers and diffuses into root surroundings [605]. Besides in an aerobic environment in the rhizosphere, the presence of plant stimulates the microbial community density and activity by providing root surface for microbial community growth and a source of carbon compounds through root exudates [606]. Because of the special physiology and ecological characteristics of *P. australis*, i.e., its rooting system is very developed, consequently, more oxygen was provided to CWs by the roots, which led to an enhanced degradation of organic matter in the systems [603]. For instance, Dan et al. [533]

observed that in presence of *P. australis* in CWs the removed COD is more efficiently (76 – 96 %) than in unplanted CWs (51 - 91 %). In this sense, in both experiment of this work the presence of *P. australis* had an important role in COD remove rate.

In both experiments the presence of target compounds (NP_OP in experiment #1 and PCP in experiment #2) did not interfere in COD removal efficiency. The same results were observed by Jin et al. [607] in the COD removal, from sludge (in reactor) in presence of PAHs (naphthalene and phenanthrene), while Mrowiec and Suschka [608] observed decrease in COD removal from municipal sewage in the presence of aromatic hydrocarbons. Zhao et al. [609] also observed toxicity effected and consequence decreased in COD removal however only in initial stage after which the microbial community adapted to the presence of 4-clorophenol (4-CP) in wastewater treatment. Since the removal of organic matter is a microbial degradation process (essentially), in this work, the presence of phenolic compounds did not change the ability of the microbial community to degrade organic matter, that is, what we observe is that the systems with and without the addition of phenolic compounds removed the same amount of organic matter (measured through COD). Therefore, these compounds were not toxic to this microbial community and the microbial community has maintained the degradation functions of compounds. If the compounds were toxic, the community would perform worse and remove less organic matter.

When compared the control and doped treatment of experiment #1 the influence of the presence of target contaminants (NP_OP) demonstrated that there is an increase over the experimental period in the removal efficiency of COD. This is demonstrative that the performance of VFCWs was stable during the short period experimental time (2 months) and show tendency. For instance, Merlin et al. [610] verify a very stable HFCW performance for an extended period experimental of time (6 years).

4.1.2. Removal efficiency of nitrogen

Nitrogen concentration is often of concern because of its potential to cause adverse effects in receiving water systems [611, 612]. Besides that, is a risk to human health, especially as a possible cause of infant methaemoglobinaemia [613]. Among various nitrogen groups, dissolve inorganic nitrogen species like nitrate ion (NO_3^-) nitrite ion (NO_2^-) and ammonia (NH_3) or ammonium ion (NH_4^+), rather than particular organic nitrogen, have the greatest impact on aquatic systems, because they are easily available for the microorganisms uptake [614]. Nitrate leaching is often concern load removal mechanism with potential negative consequences for groundwater quality [615]. According to the classical

transformation and removal routes of nitrogen, nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) coupled with canonical denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) was the major pathway of nitrogen removal [462, 616].

Depending on the redox status of the CW, several different forms of nitrogen can exist simultaneously at different and varying concentrations over time, including organic nitrogen, ammonium ion (NH_4^+), nitrite ion (NO_2^-), nitrate ion (NO_3^-), and nitrogen gas. Nitrogen in wastewater is in two main forms: organic N and NH_4^+.

It is important to remove nitrogen from wastewater, as they are among the principal constituents of concern in wastewater because of their role in eutrophication process, their effect on the oxygen content of receiving waters, and their toxicity to aquatic invertebrate and vertebrate species.

All pathways of nitrogen cycle removal are active in treatment wetlands [617]. These pathways can be divided in two groups: transfer processes and transformations processes. The transfer processes cover particulate settling and resuspension, diffusion of dissolve forms, plant translocation litterfall, ammonia volatilization and sorption of soluble nitrogen on substrate. Transformation processes comprise microbial conversion like ammonification (mineralization), nitrification, denitrification, assimilation and decomposition [442].

According with Vymazal [391] the main mechanisms for nitrogen removal in CWs are plant uptake, mineralization, nitrification, denitrification, volatilization, adsorption (organic nitrogen and nitrogen adsorbed to particles) and sedimentation. When wastewater flows into the CWs, the NH_{4^+} can be absorbed by plants and adsorbed into substrate, oxidized to NO_2^-, and further oxidized to NO_3^- by nitrifying bacteria [618]. Afterward, the generated NO_3^- can be reduced to NO_2^- and converted into gas by denitrifying bacteria [618]. Meanwhile, there is also a non - traditional microbial nitrogen removal pathway known as anammox, in which NH_4^+ is directly oxidized to N_2 under anaerobic condition [619].

Nitrification process

CW with vertical flow system for wastewater treatment have been widely researched in last decades [501, 525, 620, 621]. The special advantage of these systems over classical horizontal flow constructed wetlands is their higher oxygen transfer rate, and consequently their ability to achieve higher levels of organic matter removal and nitrification [591, 622].

According with Vymazal [623] not all CW systems types can perform high N removal efficiency, for instance, VFCWs are more advantageous to the nitrification process, while

HSSF can only provide suitable conditions for denitrification. VFCWs are popular when the nitrogen form contained in wastewater have to be nitrified [502, 624, 625].

In this sense, in general in both experiments of this study, the VFCW presented an NH_4^+ and NO_2^- removal efficiency between 80 - 100 %. These results demonstrate that VFCW provided nitrification favourable environmental condition (present later in the text) in bed red substrate. Similar results in nitrification process into VFCWs used for treatment of domestic wastewater, were reported by other authors. For instance, Wu et al. [392] reported 98 %, Brix and Arias [525] reported 90 % and Korkusuz et al. [626] reported 88 % removal efficiency of NH_4^+.

The result of week six in experiment #1 shows that probably the nitrification process was limited when compared to the remaining weeks (Fig. 1 experiment #1). According with Decezaro et al. [627] the nitrification is limited by the nitrogen adsorption capacity in the VFCW. During the VFCW feeding periods, the NH_4^+ ions are adsorbed by the negatively charged filter material surface. During the drainage, the oxygen goes through the material pores and the nitrification process of the adsorbed NH_4^+ occurs [627]. In this study the constant recirculation flow promoted the oxygenation of substrate, but sometimes clogging of CW system occurred, which may have caused reduction of the oxygen level for nitrification process to occur. It is believed that nitrification occurs when oxygen is present in a high enough concentration to support the growth of strictly nitrifying bacteria [628] and some CWs systems are unable to supply oxygen requirements for the nitrification process. According with Li et al. [629], nitrogen removal rates depend in part on the complete oxidation of ammonia.

Another hypothesis, for low removal NH_4^+ rate in CWs in the week six of experiment #1, could be the low NH_4^+ concentration in influent. The low NH_4^+ concentration in influent probably resulted in the low removal efficiencies of the CWs, by analogy with COD removal in experiment #2. In fact, the lowest removal percentages (72 (± 4) % in control and 76 (± 3) % in doped treatment) were recorded in the week with lowest concentration of NH_4^+ in CWs influents (34 (± 2) µmol L^{-1}). However, when NH_4^+ concentrations were above 63 (± 4) µmol L^{-1} in CWs influents at experiment #1, NH_4^+ removal range between 89 - 96 % in control treatments and between 86 - 95 % in doped treatments (Table 3. 2 and Fig. 3.1 in experiment #1). These results show no linear relationship between the influent concentrations and the removal percentages. The same CWs behaviour was observed over the experimental period at experiment #2, i.e., the percentage of NH_4^+ removal by CWs was independent of the NH_4^+ influent concentrations. With exception of control treatment in week number one, the range of percentage removal was between 86 - 98 % in control treatment and between 88 and 98 % in doped treatment despite the range of variation of NH_4^+ concentration being between 18 (± 2) µmol L^{-1} and 84 (± 2) µmol L^{-1} (Table 3.10 and Fig. 3.5 experiment #2). These results are in

accordance with those observed by Green et al. [630]. These authors observed a saturation effect in VFCWs system, i.e., for NH_4^+ concentration value above 65.7 mg L^{-1} there was not a gradual increase in NH_4^+ percentage removal with gradual increase of influent NH_4^+ concentration, see Fig. 7 in Green et al. [630]. Nevertheless, these authors verify a linear relationship between NH_4^+ influent concentrations and NH_4^+ removal for values above 65.7 mg L^{-1} in NH_4^+ influent concentrations.

In both experiments, in general the presence of target contaminants (NP_OP and PCP) did not interfere in nitrification process. A different result was observed by Kang et al. [631]. These authors observed a decrease in NH_4^+ removal process of CWs in presence of PAHs. According with these authors the presence of PAHs in wastewater inhibited the abundance and activity of nitrifying bacteria.

Several studies regarding municipal and agricultural wastewater treatment have shown that effluent recirculation increases the performance of wetlands, e.g. [595, 597, 632–634]. According with Sharma et al. [635], Sklarz et al. [599] and Platzer [636] the nitrification process in VFCW can be improved by effluent recirculation. For instance, Sharma eta al. [635] reported before recirculation value of NH_4^+ removal between 13 - 57 % that reached 25 - 75 % after recirculation. In this work, in general, NH_4^+ removal was above 85 % and NO_2^- removal was between 85 - 96 %. By similarity with other studies, the effluent recirculation probably had an important role in the success of nitrification process in both experiments of this study, because the recirculation can increase the oxygen dissolved rate into substrate and also prolonged wastewater – biofilm contact. This prolonged contact is important because nitrifying bacteria are autotrophic microorganisms that multiply slowly and need time to convert ammonium into nitrite and nitrate [637, 638].

Denitrification process

According with Matheson and Sukias [639], denitrification, plant assimilation, immobilization and assimilative nitrate reduction (ANR) are the main processes involved in nitrogen removal of nitrate-laden wastewater in CWs, which could account for 77 %, 9 %, 11 % and 2 % respectively, of total nitrogen removal. While immobilization process is just a transient storage for nitrogen, denitrification is generally regarded as the permanent and predominant removal process for nitrate in nitrate-laden wastewater after mass balance assessment [411, 640, 641], especially above a nitrate loading rate of 0.33 $gm^{-2}d^{-1}$ [442]. In the case of VFCWs, denitrification process can happen in anaerobic microsites and during the loading phase of beds, making it in combination with nitrification, a countable source of N_2O emissions [642].

There are key environmental factors that influence the denitrification process in CWs, namely, temperature, carbon source, loading rate as well as dissolved oxygen [391, 640, 643, 644] moreover, these factors may be closely related [645]. Temperature can influence the activity of denitrifying bacteria, these authors observed denitrifying bacteria in wetland sediments to be more abundant in spring and summer compared to fall and winter [646]. Subsequently, Stober et al. [647] observed an overall nitrate removal rate significantly higher in summer than in winter. It is well known that most suitable temperature range for denitrifying bacteria was between 20 to 40 °C [648]. Sirivedhin and Gray [645] indicated that denitrification slows down below 15 °C and almost ceases below 5 °C. In this study air temperature condition during the experimental period range between 11.5 °C - 26 °C and between 16.5 °C - 25 °C, in case of experiment #1 and experiment #2, respectively. Thus, the air temperature did not justify the difference observed between experiments in denitrification process. Other factors that interfere with heterotrophic bacteria in denitrification process, as observed in experiments #1 and #2, need to be investigated.

Additionally, the fluctuation in inflows NO_3^- concentration can be associated with fertilizer usage and precipitation patterns which can cause temporal variation of denitrification rates in wetlands [54]. Many researchers have found that high NO_3^- concentrations improve the denitrification rates in wetlands [649, 650]. In fact, in this study the NO_3^- influent concentrations probably had an important role in denitrification rate, since the highest removal percentages were observed in the experiment (Table 3.4 experiment #1) for the highest influent NO_3^- concentrations (between 211 (± 39) μmol L^{-1} and 655 (± 60) μmol L^{-1}). The same effect was observed in doped treatments of experiment #2, the highest removal percentages coinciding with the influent nitrate concentration above 119 (± 1) μmol L^{-1} (weeks 1, 2, 5 and 8). But the same effect was not observed in control treatments of experiment #2 except in the week number 8 (Table 3.12 experiment #2). This suggests that other factors must be considered.

In general, in experiment #2 limited NO_3^- removal in CWs systems was observed when compared with experiment #1. Differences were probably associated to limited available of organic carbon source in the CW systems. According with literature, while nitrification depends on the dissolved oxygen concentrations of aerated mode [651] the process of denitrification depends on the sources of carbon, and this carbon sources can provide electron donors for this process [652]. Therefore, carbon source is a controlling factor in the process of denitrification [652].

In fact, the TOC concentration in effluent water of experiments #2 ranged between 3.7 and 4.6 mg L^{-1} in control and between 4.9 and 5.1 mg L^{-1} in doped treatment (Table 3.10

experiment #2), this concentration may have restricted the process of denitrification. This result is in line with the one observed by Lin et al. [653] which verified, that when the TOC effluent concentration was below 6 $mg_C\ L^{-1}$ a limited nitrate removal from wastewater in CWs occurs. However, in the doped treatment of experiment #2 the limited availability of a carbon source was probably compensated with presence of PCP. In fact, in the week prior to the start of doping no NO_3^- removal in doped CW was observed (data not shown). Therefore, the presence of PCP probably had an important role in denitrification process. Similar result was observed by Kang et al. [631]. These authors observed a significantly enhance NO_3^- removal from wastewater treatment in CWs when polycyclic aromatic hydrocarbon (PAHs) was present. Additionally, these authors also observed a decline in the dissolved oxygen concentration with PAHs presence. According to these authors, PAHs addition was a carbon source additional for organic matter microbial activity leading to higher oxygen consumption, which was highly beneficial for denitrification and NO_3^- removal. But according with some authors the organic compounds can be also used by denitrifying bacteria. For instance, previous studies showed that denitrifying bacteria from genus *Azoarcus* could use many aromatic hydrocarbons as carbon sources during denitrification processes [654–656]. Several studies demonstrate that the carbon source external addition to the system can enhance the denitrification efficiencies in CWs. For instance, Songliu et al. [648] observed that additional carbon source (glucose) can increased the NO_3^- removal rate from 20 % to more than 50 % in CW systems.

In experiment #2 the increased of the NO_3^- removal percentage observed in week 8 in control, it could probably be explained by an additional carbon source into the CWs systems that may have come from *Ribeira de Joane* wastewater (Table 3.12 experiment #2).

In general, NO_3^- removal by plant uptake is low comparatively to denitrification process, nevertheless the presence of plants is important in NO_3^- removal from CWs. The plants growing in wetlands automatically convert atmospheric CO_2 into biomass (organic C) through photosynthesis [657]. This endogenously derived organic C can then become available to denitrifying bacteria through a number of pathways, including secretion of root exudates death and decomposition of plant litter [658, 659]. Diverse studies have checked that actively growing plants released a cocktail of low - molecular weight organic C compounds into the soil [660–662]. For instance, Huett et al. [663] observed that removal NO_3^- was not affected by carbon source addition (methanol) when the *P. australis* are present, but the removal increased to upper 97 % with methanol addition to CWs unplanted. Farther, these authors also observed removal efficiency for NO_3^- from wastewater up to 96 % in CWs planted with *P. australis* while in unplanted removal varied from 40 % to 80 %. Similar result was observed in experiment #1, NO_3^- removal rate reached 94 %, so the present of *P. australis* in CWs probably

had an important role NO_3^- removal. Nevertheless, at experiment #2 the low performance of *P. australis* may also contributed to general low NO_3^- removed in control treatment (between 33 % and 67 %) (Fig. 3.7 experiment #2). The rate of root exudation can be affected by several factors including photosynthesis, temperature, light-regime, nutrient availability and the plant species [664, 665]. Based in literature, carbon released from living plant roots to soil have been reported to account for 15 - 25 % of below-ground carbon ([666] and represent 10 - 40 % of the net primary production of the plants [667]. According with Valé et al. [668] the organic C released by roots stimulates the microbial activity in the rhizhosphere. These authors found that the microbial activity in the soil was positively correlated with the DOC release rate from plants. Hence, in anoxic wetland soils, the root exudates are likely to stimulate denitrifying bacteria if NO_3^- is available [657].

Nitrification and denitrification occur simultaneously, while nitrification takes place in aerobic region, denitrification occurs with anaerobic/anoxic condition [442]. Conventional biological denitrification requires hypoxic conditions with dissolved oxygen concentration less than 0.2 mg L^{-1} [669]. Since it was first discovered in the 1980s [670] “aerobic denitrification” (co - respiration of nitrate or nitrite and oxygen) at dissolved oxygen levels of 5.0 - 6.0 mg L^{-1} has attracted much attention because of its potential to overcome the disadvantages of conventional biological denitrification [671–673]. In this sense, the oxygen availability in CWs might favour aerobic chemo - autotrophic microbial process of nitrification [674, 675], however, the higher oxygen supply into CW system, it could reduce the anoxic environment for denitrification to take place. This could for instance explain why NO_3^- concentration effluent increased in control treatment at week 3 of the experiment #2. Similar result was observed by Wu et al. [392] when tested the dynamic transformation of nitrogen in intermittently aerated VFCWs with different aeration times at constant airflow rate. These authors observed that NO_3^- increased when the aeration time increased from 6 h d^{-1} to 10 h d^{-1} due to excessive aeration in the CWs system.

4.1.3. Removal efficiency of phosphorus

Although phosphorus represents an essential nutrient for biomass growth in several ecosystems (such as rivers, lakes or lagoons), an excessive input of phosphorus, together with nitrogen, into water bodies causes eutrophication, which favours an abnormal growth of algae and aquatic plants resulting in the depletion of the oxygen amount in the water column with negative impact on aquatic organisms [676]. Phosphorus in aquatic environment may be

derived naturally from phosphorus in soils, though significant amounts may also be contributed to aquatic systems by anthropogenic sources such as fertilized fields, animal wastes, wastewater treatment plants effluents, and industries that use phosphorus in cleaning processes [677, 678]. Nitrogen removal, for example is mainly attributed to microbial nitrification-denitrification and anammox processes [679] whereas phosphorus removal occurs mainly due to physico - chemical processes such as precipitation, sedimentation and substrate adsorption [641].

Adsorption and precipitation are considered as coupled processes and generally, no differentiation is reported among them in most the studies [680]. Although with limitation, CWs are effective in removing phosphorus from wastewater. When evaluating phosphorus retention capacities five fundamental mechanisms in CWs can be observe. First mechanism is the adsorption to the substrate; second is formation of complexes with organic matter; third is precipitation with aluminium (Al), iron (Fe), calcium (Ca) and mineral constituents of the sediment; fourth is uptake by plants and the fifth is bacterial immobilization [391, 681].

The CWs from both experiments in this study have the same composition of substrate and same species of plant (*P. australis*) nevertheless in general in experiment #1 PO_4^{3-} removal was observed, whereas in experiment #2 PO_4^{3-} was not removed and its concentration in CW effluent increased. Similar results were observed by Singh et al. [682]. According with these authors, the increased effluent PO_4^{3-} concentration maybe related with intermittently fed wastewater, which could not be effective because the oxygenation of bed may cause desorption and subsequent release of phosphorus. In this sense, in experiment #2, the oxygenation of red substrate promoted by effluent recirculation may have cause desorption and subsequent release of PO_4^{3-}. But phosphate can also be released by aboveground parts of *P. australis*. This phosphorus released from biomass to wetland ecosystem occurs after the plant decay [391]. In fact, some plants of CWs in experiment #2 showed signs of aging during experimental period.

In experiment #1, the highest PO_4^{3-} removal (90 % in control and 95 % in doped treatment) coincided with the highest influent PO_4^{3-} concentration (20 (± 2) µmol L^{-1}), as previously observed for NO_3^- in experiment #2 and NH_4^+ in both experiments. The same results were observed by Martín et al. [683] treating urban wastewater in VFCW. These authors observed that the removal efficiency of PO_4^{3-} depended strongly on input concentration, i.e., for input concentration higher than 0.5 $mg_P\ L^{-1}$, removal was higher than 70 % but at lower input concentration the efficiency dropped dramatically. However, different result was observed by Ni et al. [684], these authors observed that influent load increase had no remarkable effect on PO_4^{3-} removal in eutrophic water treatment in CWs. Another

interpretation can be given for removal percentage in first week, this high removal rate may be due to the sorption on the "fresh" substrate of CWs. Similar result was observed by Prochaska and Zoubouli [685]. These authors observed 45 % of PO_4^{3-} removal and a decreased in PO_4^{3-} removal only after the first month of operation when using sand and dolomite in VFCW.

According to Vymazal [391], PO_4^{3-} removal in CWs varies between 40 and 60 %. The efficiencies of PO_4^{3-} removal obtained in the experiment #1, between 49 and 90 (± 5) % in control treatment (with exception of week 7 with no PO_4^{3-} removal) and between 34 and 95 % in doped treatment (Fig. 3.4 experiment #1), were considered satisfactory since according to the same author, phosphorus removal in all CWs is considered low, unless when using specific substrates with high adsorption capacity together with support medium. Additionally, also a substrate saturation trend was observed after the first week, between second and fourth weeks and between seventh and eighth weeks. These results show an insufficient high capacity to bind phosphorus for a prolonged period. Although PO_4^{3-} removal rates were not high, removal of PO_4^{3-} is generally low when substrates in VFCW usually do not contain high concentration of zeolite, gravel, sand or dolomite [686–688]. For instance, Fisher [689] reported a 20 % phosphorus removal using gravel in substrate, Singh et al. [682] reported 37 % phosphorus removal using zeolite-gravel in substrate and 55 % when using gravel in substrate and Andrés et al. [690] reported phosphorus removal between 20 and 50 % when using zeolite in substrate.

In experiment #1, the results indicated that the presence of NP and OP increase PO_4^{3-} removal capacity by CWs, (the difference between treatment ($p < 0.05$) were significant in weeks 3, 5 and 7) and a stable period in removal efficiency (between 86 (± 2) % and 87 (± 6) % was observed in week 5 and 6. NP and OP could be probably a source of energy for the biological absorption process of PO_4^{3-}, leading to an increase in PO_4^{3-} removal. These results also show that the NP and OP concentration used in CWs doping had no inhibitory effect on the microbial community. In literature, there is reference to inhibitory effect promote by quinolone antibiotic group, but no inhibitory effect was promoted by alkylphenols (NP and OP) has been reported. For instance, Xu et al. [691] found that norfloxacin induced a significant inhibitory effect on the uptake of carbon source (acetate) by phosphorus accumulating organisms (PAO), which resulted in low PO_4^{3-} removal efficiency. The PAO are bacteria that might influence PO_4^{3-} removal. Zhang et al. [692] observed the same inhibitory effect in PO_4^{3-} removal when ciprofloxacin was present. Additionally, Xu et al. [691] and Zhang et al. [692] relate the inhibitory effect with the antibiotic concentration. Jin et al. [693], also verify removal efficiency of PO_4^{3-} declined with PAHs addition (naphthalene (NAP) and phenanthrene (PHE)). These authors associated this result, to PAO bacteria community decreased and glycogen

accumulating organisms (GAO) community used the carbon source of PAO, acetate, and not retain the PO_4^{3-}. PAO bacteria community used acetate to accumulate the PO_4^{3-}.

4.2. Phenolic compounds removal in Constructed Wetlands

Several phenolic emerging contaminants defined as EDCs have been detected in effluent from industrial and municipal wastewater treatment plants [694, 695] environmental waters [696, 697] and landfill leachates. Due to their persistence and ability to bioaccumulate these contaminants must be removed to reduce the adverse effects they cause to aquatic ecosystems.

In this study, during two months of CW treatment (water doped with 100 μg L^{-1} of NP and OP per week) both alkylphenols NP and OP were completely removed, indicating that CWs were able to effectively remove these alkylphenols compounds from the wastewater. To our knowledge no studies have reported the removal of NP and OP in CW with vertical flow. But, for instance, Belmont et al. [538] observed a 54 % NP removal percentage in horizontal flow CW. Dai et al. [540] observed 58 % for OP removal and 32 % for NP removal from discharge of sewage treatment plants in a hybrid system CW. Hsieh et al. [539] observed high removal rates, 89.7 % for NP and 85.1 % for OP in free water surfaces CW. In these studies, the removal rates were lower than those observed in this present work. These results showed a good performance of VFCW in removing alkylphenols from wastewaters. However, in the treatment of wastewater doped with PCP by CWs, slightly lower removal rates were observed (between 87 – 98 %). Choudhary et al. [545] also observed 89 % of PCP removal from pulp and paper mill wastewater through VFCW planted with *Canna indica*.

In CW systems, alkylphenols and chlorophenol compounds may be removed from wastewater by a number of processes, including adsorption and absorption, microbial interactions and uptake by plants [540, 545]. The high log K_{ow} (4.5, 5.3, and 5.0 for NP, OP and PCP, respectively) and low solubility (7 mg L^{-1}, 5 mg L^{-1} and 14 mg L^{-1} for NP, OP and PCP, respectively), are indicators of compounds high adsorption / absorption capacity to the substrate [698–700]. Based on the physicochemical properties of these compounds, a considerable adsorption / absorption to substrate would be expected. For instance, Choudhary et al. [545] observed that 78 % of PCP CW removal from aqueous solution with initial concentration of 9.96 μg L^{-1} of PCP was by adsorption / absorption to the soil. However, at the end of the experiment, NP was not detected in CW substrate, whereas a small amount of PCP was detected, indicating that if adsorption occurred other processes, e.g. microbial degradation, lead to compound removal. OP was detected in CW substrate of both treatments

(not doped and doped) and with no significant differences between them. This indicates that the presence of OP in CW substrate was probably not related to water doping done weekly but associated to aging contamination. Sediment collected in the estuary of *Lima River* - *Viana do Castelo* and used as substrate in CW after mixing with sand, was already contaminated with OP (0.53 (± 0.03) μg g^{-1}) but not with NP (NP below detection limit). This result indicates that probably the new OP contamination (OP added to *Ribeira de Joane* water) was more easily available than the old contamination, the compound being more strongly bound due to significant diffusion into particle inner structure. A similar behaviour of OP desorption from sediment was observed by Zhou [701]. Similar results have been also reported for desorption of lindane from subsurface particles by Miller and Pedit [702] and for desorption of naphthalene from soil by Connaughton et al. [703]. These results indicate that in the long - term OP and similar compounds can become more persistent.

Ying et al. [704] show that branching degree of the alkyl substituent of alkylphenols interferes with degradation process. These authors verify that NP is more amenable to biodegradation than OP under aerobic condition due to its straight side chain, nevertheless in this work the same efficiency in NP and OP degradation was observed. This results can probably be explained by co - metabolic processes as noted by Gabriel et al. [159] with NP isomers.

The deposition of contaminant sorbed to the solid phase can also lead to long-term sources of contamination in soils and sediments, though, the adsorption and desorption could be a hysteresis process and never the totality of adsorbed contaminant could be desorbed. This irreversible process was observed by Struck and Schwuger [705] in sediment contaminated with naphthalene and phenanthrene. Connaughton et al. [703] attributed the difference between slower release from aged than from freshly contaminated soil, to the difference sediment - water partitioning ($K_{p(sw)}$) value between aged and freshly contaminated soils, and the compartmentation of sorbed molecules in soil matrix, leading to nonlabile vs labile sorbed fraction.

Moreover, Kukkonen et al. [706] found in the case of PAHs and polychlorinated biphenyl (PCBs), that the properties of sediments were also important in the desorption from sediment, as shown by a positive correlation between desorption flux and the amount of pigments and lipids in sediment [702]. And Xie et al. [707], observed in their experiments with atrazine that the most irreversible sorbed atrazine was in the bound humic acid and mineral components of humin.

According with Lee et al. [708] biodegradation may be limited due to slow desorption kinetics especially when dealing with aged sediments. The aging of contamination results from chemical reactions sequestering contaminants into organic matter, diffusion into very small

pores or inclusion of non-aqueous phase liquids into semi - rigid films [709, 710]. Several studies verify that bioavailability of aged contaminants in soil can be considerably lower than in spiked soil e.g. [711–713]. For Langdon [714] it is clear that when compounds are present in soil as an indigenous component of soil (aged contaminants), there is a fraction that is recalcitrant, whereas when the compound was spiked into the soil there is no recalcitrant fraction [714]. On the other hand, Sjöström et al. [117] suppose that the recalcitrant fraction of NP (mixture of isomers) in sediment may consist only of branched isomers resistant to biodegradation. In this work, the branched structure of OP may have made stronger the linkage to sediment matrix, nevertheless, the branched structure is not enough explanation because the fraction resulting from doping apparently did not present resistant to biodegradation as it was not detected in CW substrate or treated wastewater.

The behaviour of recalcitrant fractions of compounds in soil have also been observed e.g. [117, 715, 716], however, is unclear what mechanism is responsible for recalcitrant fractions in soil and several hypotheses have been considered. According with Zhou [701] the recalcitrant fractions in sediments is highly dependent on the duration of its storage in sediments, this suggested that sorption by soil not only involves surface adsorption but also slowly fuses through the particle. A possible explanation for existence of the compounds recalcitrant fractions may be associated to the presence of anaerobic zones within the aggregates of soil and may result in delayed degradation due to limited oxygen availability [715]. For instance, in the case of alkylphenols (NP and OP) can rapidly degrade in the soil under aerobic conditions but persist under anaerobic condition [704], therefore the oxygen availability is a limiting factor and can influence the degradation rates of these compounds [715]. A second possible explanation is associated with non-reversible sorption of the compounds to the soil matrix [716]. The affinity of chemical species for soil compounds are associated with manifest non-singularity or hysteresis [717]. This hysteresis can be associated with formation of metastable states of adsorbate in the fixed mesopores and irreversible deformation of the sorbent (soil) by the sorbate (sometimes called “low-pressure hysteresis”) [717]. In both cases, sorption is “irreversible” i.e., the pathway for uptake and release it is not the same. Several studies [718–722] support irreversible deformation as the cause of hysteresis in flexible solids, such as natural organic matter and organoclays. The slow and /or nonreversible desorption affects the mobility and degradation of the compounds in the environment [716].

The concentration of organic matter in CW substrate might also affect adsorption process. Chien et al. [723], observed higher PCP adsorption after organic matter addition, due to the formation of additional adsorption sites. Therefore, in the present study, the low input of organic matter might not promote PCP adsorption to CWs substrate. In fact, COD

concentration in experiment #2 (15 (± 3) and 36 (± 4) mg O_2 dm^{-3}) was lower than in experiment #1 (6 (± 1) and 112 (± 12) mg O_2 dm^{-3}). In this sense, other edaphic factors, will have to be taken into account in adsorption process of PCP to CWs substrate, observed in the experiment #2. For instance, Diagboya et al. [182] observed that soils minerals (iron oxides) played greater roles in PCP sorption then organic matter, and according with Howard et al. [724], the PCP adsorption to oxidized sediment is higher than to reduced sediment [724].

Additionally, soil pH and physicochemical properties like acidity, alkalinity, polarity and polarization of organic contaminants also affect the adsorption behaviour of charged substances. According with Hamker and Thompsom [725], slightly acidic organic contaminants dissociate to form anionic substances repulsive to negative charges on soil surface in an alkaline environment resulting in the poor adsorption of the contaminants by soil. For instance, Shimizu et al. [212] observed in aquatic environment at pH = 3, 80 % of the PCP was sorbed to a natural solid mix (consisting of organic matter, clay minerals and metal oxides) while at pH = 12, only 30 % of the PCP was absorbed. Based in this work, we will be able to affirm, that the neutral-slightly alkaline environment in CWs systems of experiment #2 (pH = 7 - 8), might not favour PCP (dissociated form; pK_a 4.74) adsorption rate to CWs sediment. In fact, only 20 (± 9) ng g^{-1} was detected in CWs sediment doped week with 100 µg L^{-1} of PCP (Table 3.15).

The adsorption / absorption process suggests transfer of the compound from the water column to the substrate which is not a definitive elimination as in the future the residues adsorbed could become bioavailable and be a source of toxicity to aquatic environments. For instance, Chen et al. [726] observed that sorption-desorption process of PCP to sediment exhibit hysteresis behaviour and most likely attributable to irreversible binding to specific sorption sites, slow rates of desorption, and sorbing molecule entrapment. In this sense, the degradation and/or transformation in a less toxic compound need to take place for a permanent elimination.

In the present work the undetected and low concentration detected of target compounds in effluent of CW doped, showed that beyond the adsorption/absorption process other biologic processes promoted by plants and/or microbial community (like uptake, accumulation and degradation and/or transformation) probably took place in CWs systems.

Wetland macrophytes have shown great potential for the phytoremediation of organic contamination [534, 727–729]. According with Zhang et al. [730] when the molecular mass of an organic pollutant is below 1000, it is easily taken up by plant roots. Based in this information, in this present work the uptake NP (220 g mol^{-1}), OP (206 g mol^{-1}) and PCP (266.3 g mol^{-1}) by *P. australis* might have occurred. For instance, Zhao et al. [370] observed that emergent macrophytes (*Phragmites communis* Trin*, Typha orientalis, Scripus Validus* Vahl)

can uptake and accumulated PCP in their belowground part. The difficulty in the transport from root tissues to upper part tissue of plant observed by Zhao et al. [370] was attributed to the high hydrophobicity of PCP (log K_{ow} 5.01). High log K_{ow} indicate a limited ability of compounds to be taken up through the plant cell membrane [731], but can imply ease in absorption by root epidermis [730]. In this sense, the high hydrophobicity of the NP (log K_{ow} 5.76) and OP (log K_{ow} 4.8) might have promoted the accumulation of these compounds in belowground part of *P. australis* in this present work. Furthermore, hydrophobic chemicals have affinity to be retained in the lipids of the root epidermis and surrounding mucilage [732]. The higher root density of *P. australis* permits large proportion of inflow to intimate contact with root surface and associated biological films facilitates microbial degradation and possibly plant uptake of estrogenic compounds [536].

Numerous studies have shown that plants promote microbial degradation of organic pollutants [733, 734] by an increase in microbial numbers, enhancement of microbial activity and/or modifications of the microbial community structure in the rhizosphere [735]. For instance, Toro-Vélez et al. [534] observed clear increased in removal percentage of NP in the presence of plants. These authors also observed that *Heliconia* showed a higher efficiency NP removal than *P. australis*, possibly due to microenvironments associated to the Heliconia sp. rhizosphere. In case of PCP similar results was observed by Hechmi et al. [371], with PCP removal rate increasing significantly (between 15 - 20 %) in the presence of *P. australis*. Additionally, these authors also observed a significant positive correlation between indicators of soil microbial activity (dehydrogenase activity - DHA) and removal of PCP in planted soil, which implied that plant root exudates promoted the rhizosphere microorganisms and enzyme activity, thereby improving biodegradation of PCP. Additionally, Hechmi et al. [736] observed that *P. australis* rhizosphere soil compared to the other terrestrial plants also showed a greater PCP biodegradation efficiency.

According with Huang et al. [737] the definitive removal of compounds from sediments can be the combined effects of plants and microorganisms, and sometimes the effect of microorganisms may contribute the most. Rhizodegradation of alkylphenols specifically NP and OP occurs in aerobic conditions in various environments [51, 738, 739]. Some microorganisms possess the ability to degraded alkylphenols use as a sole carbon and energy source [157, 740] and Toyama et al. [741] isolated bacteria with ability to degrade NP and OP from *P. australis* rhizosphere. Ying et al. [704] show that branching degree of the alkyl substituent of alkylphenols interferes with degradation process. These authors verify that NP is more amenable to biodegradation than OP under aerobic condition due to its straight side chain, nevertheless in this work was observed the same efficiency in NP and OP degradation of doping fraction. This result can be explained by co-metabolic process as noted by Gabriel

et al. [159] with NP isomers. Additionally, the OP fraction detected in sediment suggests that solubilization was the limiting factor in its degradation. It has already been suggested in literature that dissolution rates of water-insoluble organic compounds (e.g. biphenyls, naphthalene, phenanthrene) can be one of the factors that limited the rates of biodegradation [164]. It is plausible that the bacterium utilized only dissolved fraction of OP and the adsorbed fraction are unavailable for mineralization.

In the case of PCP, D' Angelo and Reddy, [218] observed that under laboratory conditions no PCP degradation occurred in wetland soil which had been sterilized but the compound was degraded in nonsterile soil. These authors suggest that PCP degradation was attributed to biological activity and that most wetland soils contain microorganisms with capacity to transform PCP despite no known history of contamination. However, these authors consider that expression of this soil capacity was regulated by contamination level, type of electron acceptors and donors and microbial biomass. Therefore, current results indicate a clear effect of degradation potential of target compounds by microbial communities present in red bed substrate, with CW system providing chemical and biological conditions for this transformation process to occur.

Wetland plants represent a group of plants with an immense potential for removal / degradation of a variety of contaminants [742] due to their faster growth, production of more biomass and relative higher ability of pollutant uptake [743]. However, toxic effects of organic pollutants, on wetlands plant growth have been observed. According with Zhang et al. [744] these toxic effects were related to the pyrene concentration to which the plant is exposed. These authors observed a biomass reduction of *Scripus triqueter* in relatively high pyrene concentration (80 and 160 mg kg^{-1}) and also Hechmi et al. [371] observed a reduction of 20 % and 40 % in biomass of *P. australis* in both PCP concentration tested, 50 and 250 mg kg^{-1}. On the other hand, Zhao et al. [370] did not detect toxic effects or growth inhibition on wetland plant when exposed to low PCP concentration in sediment (2 μg Kg^{-1} dry weight). These authors observed no significant effect on the plant height of *Phragmites communis* Trin*, Typha orientalis* and *Scripus Validus* Vahl at 60 days of experimental period. Additionally, Zhao et al. [370] observed from 60 to 90 days, that this concentration of PCP in the sediments stimulated the growth of *Typha orientalis* and *Scripus Validus* Vahl. The effect of organic pollutants on wetland plant growth could be species specific regardless of pollutant types and media [745], and may also be associated with extension of the time period that wetland plant was exposed to the contaminant.

4.3. Microbial Communities' Characterization

Alpha diversity analysis showed that the alkylphenol compounds (NP and OP, experiment #1) did not significantly impact the microbial community, whereas the presence of PCP (experiment #2) leaded to a decrease of the microbial diversity and incresed the dominance species, in CWs substrate at the end of the experiment. The impact of PCP in microbial diversity has been already observed in compost bacterial community [746], in aerobic granular sludge doped with PCP [747] and in soil acclimated with PCP [748]. In addition, Xu et al. [749] observed that the microbial community of a mangrove soil was greatly influenced by PCP, with the preferential growth of bacterial groups that were active during PCP transformation, which might inhibit and compete with other bacteria for the limited living space and resources. Therefore, CWs microbial communities adapted to the presence of the contaminants, probably selecting the communities with capability to resist and degrade the compounds, leading also to the compounds biodegradation observed in CWs systems. However, microbial community structure showed that there was also an adaptation of the microbial community to the CW system conditions and not only to the presence of each contaminant. In both experiments, the "initial" microbial community (in T0) was different from the microbial community at the end of the experiment (in T8) independently of the exposure to non-doped or doped stream water. Over time, a natural species selection in the microbial community of each CW microcosms was observed, showing an adaptation to the experimental conditions.

In the current study, the following bacterial phyla with abundance above 1 % in CW substrate were detected: *Acidobacteria, Actinobacteria, Bacteroidetes, Dependentiae, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Thaumarchaeota, Nitrospirae, Planctomycetes, Proteobacteria and Verrucomicrobia*. With the exception of the phylum Dependentiae that was identified only in microbial community of experiment #2 (exposure to PCP), the remaining bacterial phyla were identified in CWs substrate from both experiments. A similar bacterial community with capacity to degraded NP was detected in river sediment: *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Nitrospirae, Planctomycetes, Proteobacteria and Verrucomicrobia* [750].

Most of the phyla identified in this study have been discussed in the literature on their contribution in CWs treating wastewater, e.g. [751–754], and on their contribution to the biodegradation or biotransformation of different types of emerging contaminants [751, 755, 756].

Plant species-specificity, with similar rhizobacterial communities, can be found in different environments where the same plant is present [757, 758]. For instance, Calheiros et

al. [759] have studied the microbial community of the same plant used in this study (*P. australis*) but retrieved from substrate samples of the CWs treating industrial wastewater and reported several phyla similar to those observed in this work, such as *Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria*.

The microbial degradation is reported to be the more important process by which organic compounds are biodegraded / biotransformed into less toxic, persistent and complex metabolites [760]. In this study, the *Proteobacteria, Bacteroidetes and Planctomycetes* were the three more abundant phyla in both experiments with relative abundance 58 – 72 %. Due to their diversity and marked adaptability, these dominant phyla are able to degrade a variety of environmental organic contaminants, and also dominate the microbial community of the CW rhizosphere in wastewater treatment, e.g., [751, 753, 754]. Previous studies reported that *Proteobacteria and Bacteroidetes* are part of the dominant phyla group in NP anaerobic degradation in river sediments [750, 761], *Proteobacteria and Firmicutes* [762] are important in OP biodegradation in granular sludge and Proteobacteria, *Bacteroidetes* and *Firmicutes* can be the predominant phyla responsible for the dechlorination of chlorinated compounds like PCP [763, 764]. Therefore, in this work, the dominant phyla present in *P. australis* rhizosphere of CWs may have contributed to the effective biodegradation/biotransformation of NP, OP and PCP. Moreover, Xu et al. [749] reported that the abundance of *Bacteroidetes, Firmicutes, Proteobacteria and Euyarchaeota* varied greatly during PCP dechlorination process.

The impact of OP to bacterial community of CWs substrate remains poorly understood. On the other hand, a few previous studies indicated that NP addition showed no or little impact on aquatic bacterial community structure [765–767]. In contrast, Stenrød et al. [768] and Wang et al. [156] found that NP affected soil bacterial community structure. Moreover, Wang et al. [750] verify that NP presence and biodegradation altered the sediment bacterial community structure at the phylogenetic levels of phylum, class and genus. Nevertheless, compounds concentrations can affect microbial communities' response. Domene et al. [769] indicated that soil microbial community did not change significantly at NP concentration below 90 μg g^{-1}, but changed at 270 μg g^{-1}. Wang et al. [750] reported that an NP concentration of 100 μg g^{-1} had a strong impact on sediment bacterial community structure at phylogenetic levels of phylum, class and genus. Regarding OP toxicity, Liu et al. [762] reported a toxicity effect for degrading microorganisms present in granular sludge at a concentration of 220 μg L^{-1}, but not for concentrations of 120 and 30 μg L^{-1}, the toxicity affecting the degradation rate of OP.

Comparison of substrate exposed to doped stream water with that of control systems showed that several specialist genera were enriched following exposure to the alkylphenol compounds, suggesting a growth benefit for target compounds biodegradation. In doped

systems substrate, the three more abundant specialist genera were *Methylotenera* (Methylophilaceae family), *Hyphomicrobium* (Hyphomicrobiaceae family) and Methylophilus (Methylophilaceae family). The genera *Methylotenera* and *Methylophilus* are from the same family, the Methylophilaceae. Members of this family have been previously described to utilize some organic pollutants as an energy source [770], degrading benazolin - ethyl from activated sludge [771], methanol present in industrial sewage [109] and PAH [772]. Lu et al. [773] have suggested that this family might have the ability to use ring - hydroxylating - dioxygenase (RHDα) genes in pollutants degradation. These genes encode for dioxygenases and monooxygenases enzymes, that are involved in aerobic degradation pathways [774]. According to Tuan et al. [6], the hydroxylases and ring-cleavage dioxygenase are involved in long-chain alkylphenol degradation pathways. This same study identified in *Methylobacterium* genus, from Methylophilaceae family, the presence of the genes that encode these key enzymes in alkylphenol degradation pathways. Moreover, *Methylobacillus* genus from this family has been linked to degradation of a variety of phenolic compounds [775]. Considering the great representation that the members of Methylophilaceae family have in groups of "specialist genera" in the presence of NP and OP, members of Methylophilaceae family probably had an important role in the degradation of NP and OP in the present study. The genus *Hyphomicrobium*, from Hyphomicrobiaceae family are linked to degradation of a variety of phenolic compounds [775] more specifically to NP degradation in laboratory scale column soil [776] and in sludge at laboratory condition [777]. Based in these reports and in the presence of this genus among the "specialist genera" in systems doped with NP and OP in the present study, this genus probably played an important role in the NP and OP degradation.

As previous mention, PCP can affect the structure of the microbial community. For instance, Liu et al. [747] reported changes in aerobic granular sludge microbial community but only at PCP concentrations between 100 and 200 μg g^{-1}, with no changes below 100 μg g^{-1}. In the present study, the comparison of substrate exposed to doped stream water with that of control systems, indicated that several specialist genera were enriched following exposure to the chlorophenol compound, suggesting a growth benefit for target compounds biodegradation. The three more abundance genera in doped samples were: *Denitromonas* (Rhodocyclaceae family), *Xenococcus*_PCC_7305 (Xenococcaceae family) and *Rhodocyclaceae*_uncultured (Rhodocyclaceae family). Members of the Rhodocyclaceae family have been described to use organic pollutants as a food source [770] and have a prominent role in the degradation of aromatic compounds such as toluene [778], benzene [779], quinoline [780] and PAHs [781, 782], including pyrene [783] and phenanthrene [784]. Important genes in PAH biodegradation, PAH - specific dioxygenase genes, are found in this bacterial family [784], showing anaerobic pathway for aromatic degradation that includes the

benzylsuccinate synthase genes and central benzoly coenzyme A (benzoyl - CoA) reductase in *Dechloromonas* genus [785]. This benzoyl - CoA reductase is an important enzyme in pathway PCP degradation [786, 787]. According to Tong et al. [222], the *Dechloromonas* genus is associated with PCP anaerobic degradation process. The biodegradation of PCP can occur by different processes: hydroxylation, oxygenolysis and reductive dichlorination [10, 245]. Moreover, the intermediates of PCP metabolism indicated that carbon ring-cleavage had taken place after dichlorination process [222]. In the PCP degradation, two important steps for ring-cleavage, including hydroxylation and carboxylation, can occur [788]. Microorganisms belonging to the family of Rhodocyclaceae, i.e., *Azoarcus* or *Thauera* genus [779, 789] can act as the degraders into these two steps [222]. Therefore, the *Denitromonas* genus may have had an important role in degradation of PCP in this work. The *Xenococcus*_PCC_7305 from Xenococcaceae family is the cyanobacteria species with higher proportion in the group of "specialist genera". There is no information about the Xenococcaceae family (*Nostocales* order) being associated to degradation of organic compounds. Sometimes cyanobacteria do not have a direct role in the degradation process, but cyanobacteria exudates can provide an endogenous source of growth substrates to bacteria [790], promoting degradation of contaminants.

The role of low abundance bacteria and the genes in degradation of phenolic compound

The correlation between more abundance phyla (or other taxonomic level) and the respective importance role in biodegradation / biotransformation processes can not be directly established, because sometimes the bacteria with low abundance can have a very important role in the ecosystem processes. According, with Dawson et al. [791], 91 % of microbe - plant interaction are run by bacteria with < 0.1 % relative abundance. And Pester et al. [792], observed that bacteria species which constitutes only 0.006 % in total microbial community 16S rRNA genes amplicon library are important drivers in all sulfate reduction process. In this sense, in this work it was observed that *Stenotrophomonas* and *Sphingobium* genera indicated by Toyama et al. [166] as using NP and OP as a sole carbon source via type II ipso - substitution, are present with relative abundance of only 0.0038 % and 0.0964 % respectively in total microbial community in experiment #1 (data not show). Also, *Pseudomonas* genus that was been described as involved in 4-NP and 4-OP degradation [6, 154] appears with 0.2438 % of relative abundance in total microbial community in experiment #1.

In literature, many other bacterial genera were described as being involved in NP biodegradation [156, 793], and appears in this work with low abundance, for instance: *Acidovorax* (0.0865 %), *Bacillus* (0.0433 %), *Sphingomonas* (0.0394 %) and *Bacillus* genus,

previously associated with OP degradation by Yi et al. [762], with 0.0433 %. In case of PCP, the genera with an important role in biodegradation process (Li et al. [794] and reference) appear with very low percentage: *Arthrobacter* (0.00385 %), *Pseudomonas* (0.48118 %), *Mycobacterium* (0.14375 %), *Serratia* (0.00062 %), *Desulfitobacterium* (0.00015 %), and *Rhodococcus* (0.07334 %) in the experiment #2 of this work (data not show).

Functional genes and environmental factors

Another important question is the fact that the analysis was performed using DNA or rRNA, but the presence of DNA or rRNA does not mean that in the relevant proteins are being expressed or are catalytically active. We can identify bacteria species that can even degrade the target compounds, but these bacteria species can have the functional genes in a non-active or in reduce activity phase. The identification is not enough to confirm an active role in the degradation of the target compounds.

Yang et al. [795] verified that gene abundance showed significant positive correlations with the levels of total organic carbon and aromatic hydrocarbons. However, these authors also verified that in some samples showed a large difference in the gene density, regardless of their similar levels of total organic carbon and aromatic hydrocarbons. These authors conclude that other unknown environmental factors might affect the spatial distribution of gene density in the polluted region. According with, Gomes et al. [796] the long - term impact of pollution, together with the specific environmental conditions at each site, might affect the abundance and diversity of functional genes (in case of PAH; naphthalene dioxygenase genes) in the environment.

However, the links between environmental parameters and abundance and diversity of metabolic genes should be taken into account. For instance, the saline and pH conditions can affect the the efficiency of microbial function. For instance, Jimenez et al. [797] found that salinity could affect the growth of PAH degraders. Wu et al. [798] suggested that pH was one of the factors affecting the distribution of PAH - degrading genes in sediments of the Pearl River estuary. da Silva et al. [799] found that one PAH - degrading dioxygenase could have high activity under alkaline conditions. Additionally, Sorokin et al. [800] verify that some microorganisms can function efficiently under alkaline (pH value above 9) and saline conditions (salinity above 1.5 mol L^{-1}).

The multiple stresses factors on soil microbial community might induce the enrichment of specific degraders and degrading genes that were adapted to the unique habitats. Therefore, it is not convenient directly link metabolic function and phylogenetic identity for microbial community.

The role of Archaea community in degradation of phenolic compound

Regarding Archaea, which represented only 1 - 2 % of the global microbial community, the phylum with more representation was *Thaumarchaeota* (*Nitrososphaeria* class). The *Thaumarchaeota* encompasses all known ammonia-oxidizing archaea [801] and metagenomics studies indicate that *Thaumarchaeota* is one of the most abundant archaea on the planet with a broad environmental distribution (soils, aquatic habitats and engineered environments) [801–803]. Certain lineages of *Thaumarchaeota* play a major role in nitrification, preferably at low NH_4^+ concentration [790, 803, 804]. The *Thaumarchaeota* have acquired capacity associated to electron transport and energy production, to facilitate the transition from anaerobic to aerobic lifestyle [805]. *Thaumarchaeota* are associated to aerobically degrade of crude oil [789]. However, no information is available regarding their possible role in alkylphenol and / or chlorophenol compounds degradation.

The role of cobalamin has been associated with reductive dechlorination of halogenated and play an essential role as electron transfer mediator [806]. Cobalamin is synthesized only by a subset of prokaryotes [807] and according with Lu et al. [808] soil cobalamin production can be associated to archaeal group *Thaumarchaeota*. The ability of *Thaumarchaeota* to cobalamin synthesis suggests that this archaea group may grow in a sustainable syntrophic association with bacteria community that are involved in the reductive dechlorination of PCP, like syntrophic association observed by Men et al. [256] between methanogen archaea and bacteria genus *Dehalococcoides* in reductive dechlorination of chloroethene. *Dehalococcoides* genus, lack the ability to synthesize needed corrinoids for reductive dehalogenation, and instead have genes for corrinoid scavenging and import [254, 255]. But, methanogens archaea in these cultures may provide cobalamin for the organohalide-respiring bacteria in these communities, though this role may be fulfilled by other corrinoid-producing bacteria [256]. The ability of methanogens to dechlorinate suggests that these Archaea may contribute to dechlorination activities even in systems dominated by organohalide respirering bacteria [809].

Various strain of methanogenic archaea were found to be involved in reductive decholination of PCP [251], as previously mentioned, as the ability to reductive decholination was associated to presence of corrinoids, such as cobalamin [252, 253]. In phylogenetic

analyse made by Tong et al. [222] *Methanomicrobia* class and *Methanobacteria* class (*Euyarchaeota* phylum) and *Thermoprotei* class (*Crenarchaeota* phylum) dominated the archaeal community in PCP degradation microcosms experiment. In the experiment #2 of this work, other class of the same phyla was detected in phylogenetic analyse. In case of *Crenarchaeota* phylum (*Bathyarchaeia* class) are present in both treatment (control and doped with PCP), it was also observed that over time there was a decrease in abundance, with an eventual involvement of this class of archaea in PCP degradation. In case of *Euyarchaeota* phylum the class *Methanomicrobia* and *Termoplasmata* are present but only in residual fraction in T0 of both treatment and in T8_doped (in one of the triplicates) Fig 3.26.

Relatively to the role of archaea community in alkylphenol biodegradation process, Wang et al. [156] verify that ammonia - oxidizing archaea are associated to degradation of the NP. In this work, the more abundant archaea class *Nitrososphaeria*, (phylum *Thaumarchaeota*), detected in rhyzosediment of the CWs doped with NP_OP, experiment #1, are considered ammonia - oxidizing archaea [810].

Crenarchaeota phylum is the other phylum of archaea domain more abundant in experiment #1 (Fig. 3.16). The *Crenarchaeota* are widely distributed and abundant on the earth, consisting of sulfur - dependent, extreme thermophiles [811], and ammonia - oxidizing archaea [812] like *Thaumarchaeota* phylum. Kuzikova et al. [813] verify that the number of *Crenarchaeota* archaea represented by the *Candidatus nitrososphaera* genus, increased by 40 %, day after the application of 30 mg NP/kg to an absolutely dry soil, when compared with control soil samples. This suggested that some genus of *Crenarchaeota* archaea have nonylphenol biodegradation capacity. However, no reference was found in the literature that associated the alkylphenols compounds degradation with the presence of *Bathyarchaeia* class (*Crenarchaeota* phylum).

5. Conclusion and Future Directions

The vertical flow CW systems with recirculation used in this study were capable of removing compounds from two different groups of phenolic families (alkylphenol and chlorophenols), when in mixture (NP_OP) in experiment #1 or individually (PCP) in experiment #2 from a contaminated water.

In both experiments, the CW systems were effective in the removal of organic matter and nutrients from the contaminated water and the presence of the target compounds did not interfere with this removal process. However, at the beginning of either experiments, two exceptions were observed. In experiment #1, the presence of the target compounds showed some initial negative impact in COD removal efficiency, the system being able to overcome this difficulty afterwards. In experiment #2, the target compound promoted the removal of NO_3^- in the first five weeks of the experimental time.

The microbial community in CWs substrate was composed mostly by bacteria, 96 % in experiment #1 and 94 % in experiment #2. The archaea community represented a residual fraction of the microbial community, 1 % and 2 % in experiment #1 and experiment #2 respectively.

The alpha diversity analysis evidenced that the presence of the target compounds in experiment #1 did not promote significant changes in terms of richness, diversity or dominance of the microbial community. However, in experiment #2 the presence of the target compound, PCP, interfered with the diversity of the microbial community, in other words, promoted a decrease in diversity.

In both experiments, over the experimental time the experimental conditions also influenced similarly the microbial community, which showed a tendency towards greater specificity.

The phyla *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* were the three most abundance phyla in both experiments. According with some authors, these phyla groups are involved in the degradation of the target compounds used in work. In this sense we can conclude that undetected or low detected concentration of the target compounds in effluent and substrate of CWs was probably due to the degradation process that occurred in red bed substrate promoted by the microbial community.

OP was not detected in water, but was detected in sediment of *Lima River*, showing an old contamination of the estuary area with this compound. In experiment #1, we found that microbial community was unable to degrade the old contamination with origin in estuary sediment, perhaps due to the type of bonds that the OP established with certain sediment particles of CW substrate.

Unlike what happened in experiment #1, that all doped compounds were removed from the effluent and substrate of CW, in experiment #2, it was observed sorption of the added PCP to sediment. Taking in account that the temporal window was the same in both experiments, the detection of PCP may be due to physicochemical characteristics or/and composition of substrate, for instance, the organic carbon content or/and percentage of lipid in the total organic matter of the sediment.

Future work should address in some detail the composition of sediment and CW substrates and their implication in adsorption processes of these compounds and also the time factor in adsorption processes. It would be interesting in future works to discuss the role of mixed contamination in the removal of compounds (for example, will the presence of the OP benefit the removal of the NP?), and the rule of *memory effect* of the microbial community (that have been previously exposed to OP), in the removal processes of OP and NP.

This study clearly shows the potential of vertical flow CWs for the removal of phenolic compounds, a still little explored subject, contributing to promote the use of CWs as nature-based solutions to remediate water contaminated with different families of persistent and/or emergent contaminants.

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Attachments

Annex A - Apparatus

Annex A1 -

Gas Chromatography - Mass Spectrometry (GC-MS)

Source information:

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W.H. Freeman and Company (Ed); New York; 771pp.

Haffmann, E., and Stroobant, V., 2007. Mass Spectrometry- principles and applications, 3rd Edition, Wiley (Ed); England; 489pp.

Hubschmann, H.-J., 2009. Handbook of GC/MS - Fundamentals and Applications. Wiley-VCH Verlag, (Ed.); Weinheim; 693pp.

Pavia, D.L., Lampman, G.M., Kriz Jr., G.S., 1979. Introduction to spectroscopy: A guide for students of organic chemistry. Saunders College Publishing. Harcourt Brace Jovanovich College Publishers (Ed); Fort Worth, Philadelphia, San Diego, New-York, Orlando, Austin, San Antonio, Toronto, Montreal, London Sydney, Tokyo; 367pp.

Scott, R.P.W., 2003. Principles and Practice of Chromatography, Library four Science. Chrom-Ed Book Series; 100pp.

Smith, R.M., 2004. Understanding Mass spectra. A basic Approach. 2nd edition. Wiley (Ed), Hoboken; New Jersey; Canada; 353pp.

Sparkman, O.D., Penton, Z.E., Kitson, F.G., 2011. Gas chromatography and Mass spectrometry - A practical Guide, 2nd edition. Elsevier (Ed); Amsterdam, Boston, Heidelberg, London, New York, Oxford, Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo; 589pp.

1. Gas Chromatography

Gas Chromatography - Mass Spectrometry (GC-MS) is the most ubiquitous analytical technique for the identification and quantification of organic substance in complex matrices. GC-MS is the synergistic combination of two powerful microanalytical techniques. The gas chromatograph separates the components of the mixture in time and mass spectrometer provide information that aids in the structural identification of each component (Fig. 1).

Chromatography, although primarily a separation techniques, is mostly employed in chemical analysis. In a single step process it can separate a mixture into its individual components and simultaneously provide an quantitative estimate of each constituent. Samples may be gaseous, liquid or solid in nature.

Chromatography has been defined as separation process that is achieved by distributing the components of a mixture between two phases. Those components held preferentially in the stationary phase are retained longer in the system than those that are distributed selectively in the mobile phase. As a consequence, solutes are eluted from the system as local concentrations in the mobile phase in order of their increasing distribution coefficients with respect to the stationary phase; that is a separation is achieved.

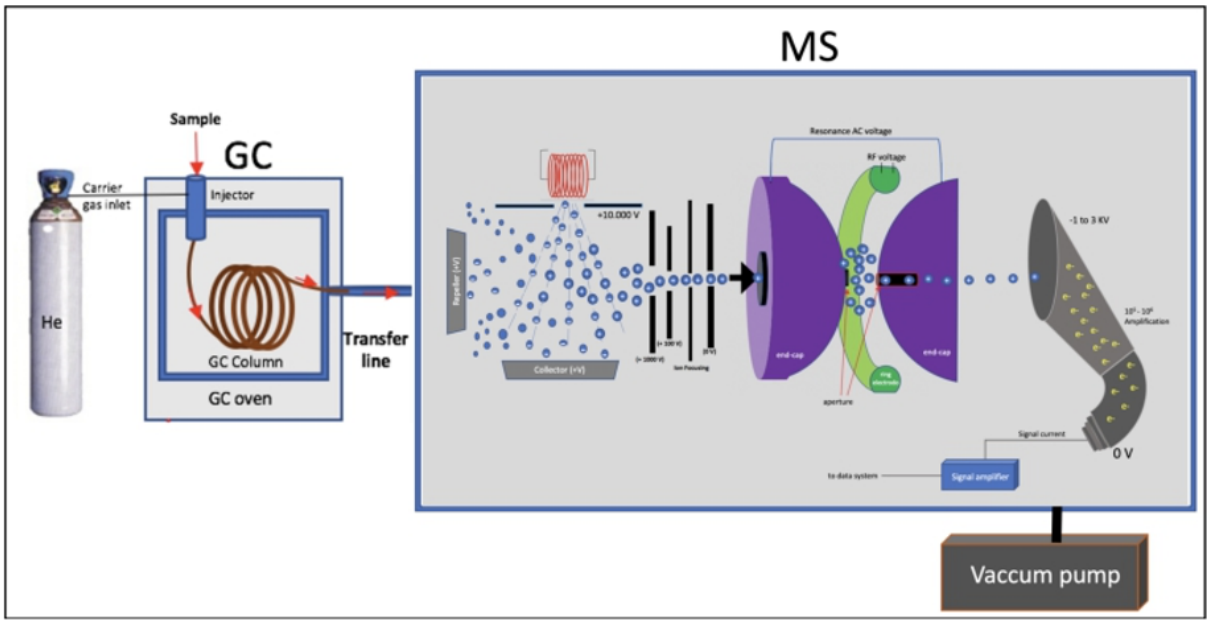


Fig. 1 - Gas Chromatography (GC) and Mass Spectrometry (MS).

1.1. Split / Splitless injectors

The oldest and most commonly used injector for capillary columns is the heated split/splitless injector. The injector can operate in two modes: split or splitless. Selection of either the split or the splitless mode depends on the concentration of the analytes in the sample.

In the **split mode** the injected sample is vaporized into the stream of carrier gas, and a small portion of the sample and solvent, if any, is directed onto the head of the GC column. The remainder of the sample is vented for a splitter vent opened (Fig. 2).

In the **splitless** mode is another way to use a split/splitless injector, in this mode the sample is injected with the splitter vent closed (Fig. 3).

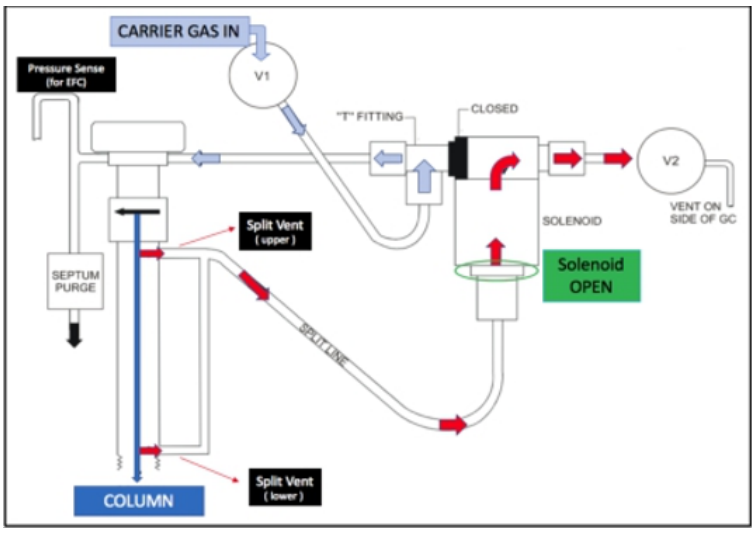


Fig. 2 Split injectors (adapted from: Sparkman *et al*., 2011).

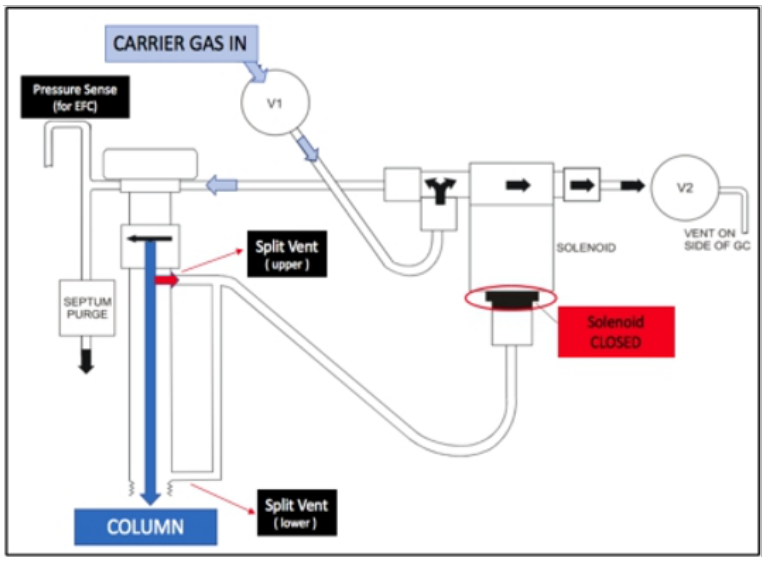


Fig. 3 Splitless injectors (adapted from: Sparkman *et al*., 2011).

1.2. GC Column

Fluid entering the column is called **eluent** and the fluid emerging from the end of the column is called **eluate** Fig. 4. The process of passing liquid or gas through a chromatography column is called **elution**.

Fig. 4 - The elution process in GC column (adapted from: Daniel and Lucy, 2016).

The speed of the mobile phase passing through a chromatography column is expressed either as a **volume flow rate** or as a **linear flow rate**. The volume flow rate such as 0.30 mL / min tells how many milliliters of solvent per minute travel through the column. And the **linear flow rate** tells how many centimeters are traveled in 1 min. by the solvent.

A chromatogram is a graph showing the detector response as a function of elution time (Fig. 5).

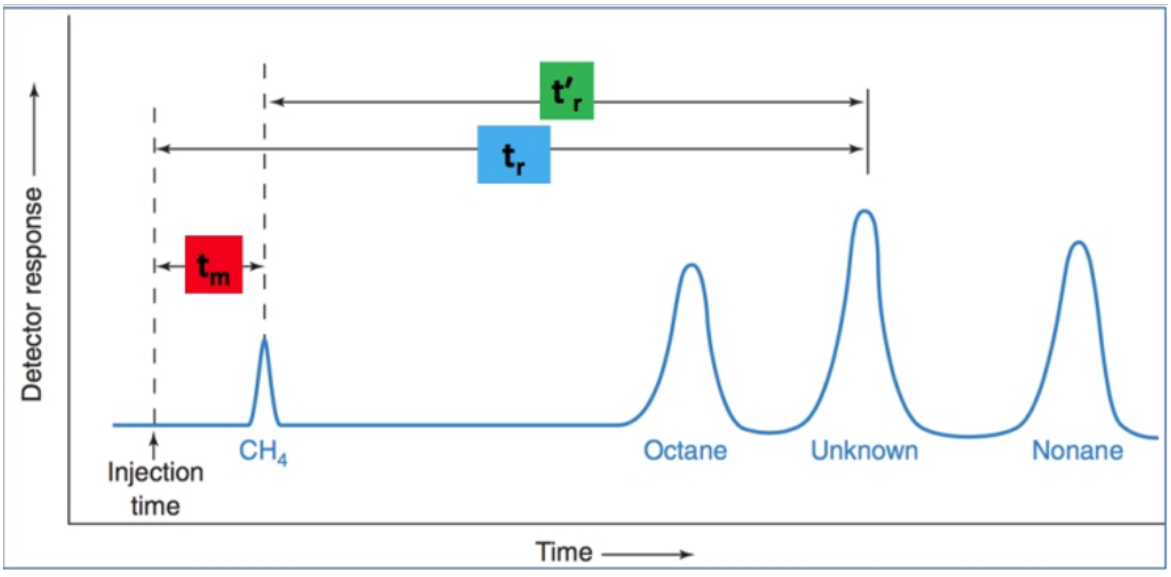


Fig. 5 - Schematic gas chromatogram showing measurement of retention times; t_r - retention time; t_m - unretained mobile phase; t'_r - adjusted retention time (Adapted from: Daniel and Lucy, 2016).

Retention time (designated by t_r) for each component is the time needed after injection of the mixture onto the column until that component reaches the detector (Fig. 5).

Unretained mobile phase travels through the column in the minimum possible time, designated by t_m.

The **adjusted retention time** (t'_r) for a solute is the additional time required for solute to travel the length of the column beyond the time required by unretained solvent:

$$t'_r = t_r - t_m$$

We can define the ***Capacity factor*** (k') as:

$$k' = \frac{t_r - t_m}{t_m}$$

The longer a component is retained by the column, the greater is the capacity factor. The capacity factor is equivalent to:

$$k' = \frac{\text{Time solute spends in stationary phase}}{\text{Time solute spends in mobile phase}}$$

For instance, if capacity factor equal to zero the solute spends no time in stationary phase. Suppose that solute spends equal time in the stationary phase and mobile phase capacity factor will be equal to one, and so one.

The last equation is equivalent to:

$$\frac{\text{Time solute spends in stationary phase}}{\text{Time solute spends in mobile phase}} = \frac{\text{Moles of solute in stationary phase}}{\text{Moles of solute in mobile phase}}$$

The **C_S** is the concentration and **V_S** is the volume of the stationary phase and **C_m** is the concentration and **V_m** is the volume of the mobile phase.

$$k' = \frac{C_s - V_s}{C_m - V_m}$$

Being:

$$Partition\ Coefficient\ (K) = \frac{C_s}{C_m}$$

$$k' = K\frac{V_s}{V_m} = \frac{t'_r}{t_m}$$

Define relative retention **(α):**

$$\alpha = \frac{K_2}{K_1}$$

That is, the relative retention of two solutes is proportional to the ration of their partition coefficients. This is the physical basis of chromatography.

Physical basis of chromatography: the greater the ratio of partition coefficients between mobile and stationary phases, the greater the separation between two components of the mixture.

2. Transfer line (TL)

Gaseous compounds with a high diffusion degree such as He tend to flow make straight angles at the output of a column (blue dots in Fig. 6). The less diffusion compounds have a

more straight trajectory, that is, they will be pushed to the source ion (black dots in Fig. 6). The temperature of the TL is equal to the maximum temperature of the column to keep the sample in its gaseous state without deteriorating thermally.

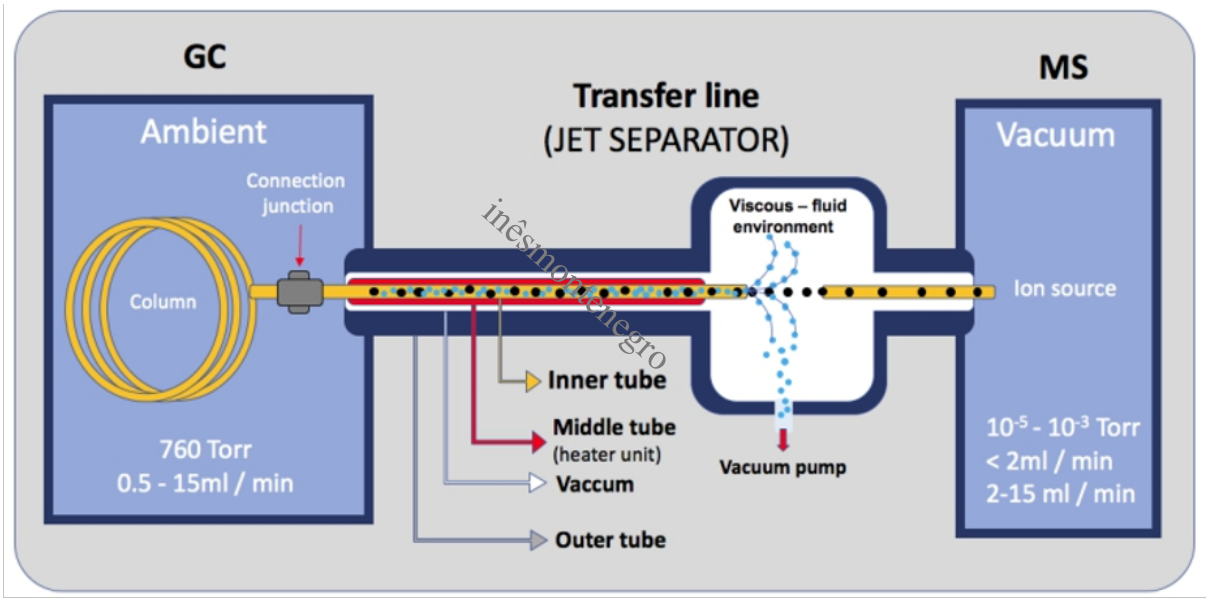
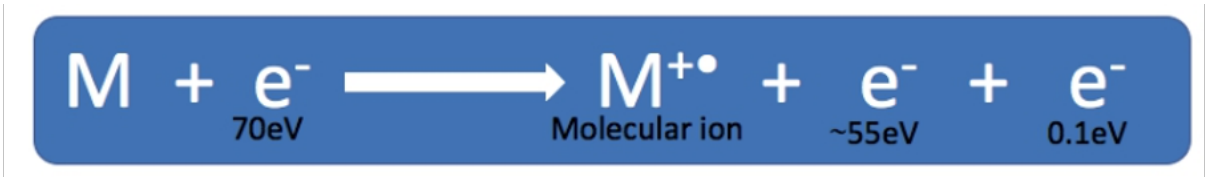


Fig. 6 - GC - MS and transfer line.

3. Mass Spectrometry

3.1. Electron Ionization source

The electron ionization (EI) source is most commonly a small chamber about 1 cm^3 in volume, in which analyte molecules interact with a beam of highly energetic electrons that have typically been accelerated thought a potential difference of 50-70 volts (V) across the volume of the ion source (50-70 electron volts (eV); 1 eV =23kcal; 1eV= 96,5kJ/mol).



This electron beam is produced by boiling electrons off a narrow strip or coil of wire made of a tungsten-rhenium alloy. Between the filament and the center of the ion source is a metal plate with a slit called the electron aperture. This slit limits the size of the electron beam and confines ionization to a small volume within the center of the ion source. Opposite the filament is the collector, a metal plate held at a positive electrical potential (+V in Fig. 7) that attracts and intercepts the electron beam after it has passed through the source (Fig. 7).

What happens during ionization?

Ionization is a complex process, it is naive to view electrons as literally smashing into sample molecules and knocking electrons out of orbitals. Instead, when an energetic electron approaches the electrons density field of the molecule closely enough that sufficient energy is transferred quantum mechanically to overcome the ionization potential of the molecule, one electron is ejected from one of the bonding or nonbonding orbitals of the molecule. Molecule are made up mostly of open space with an orderly dispersion of atomic nuclei and electrons, and the electrons in the molecule are in motion within certain limits.

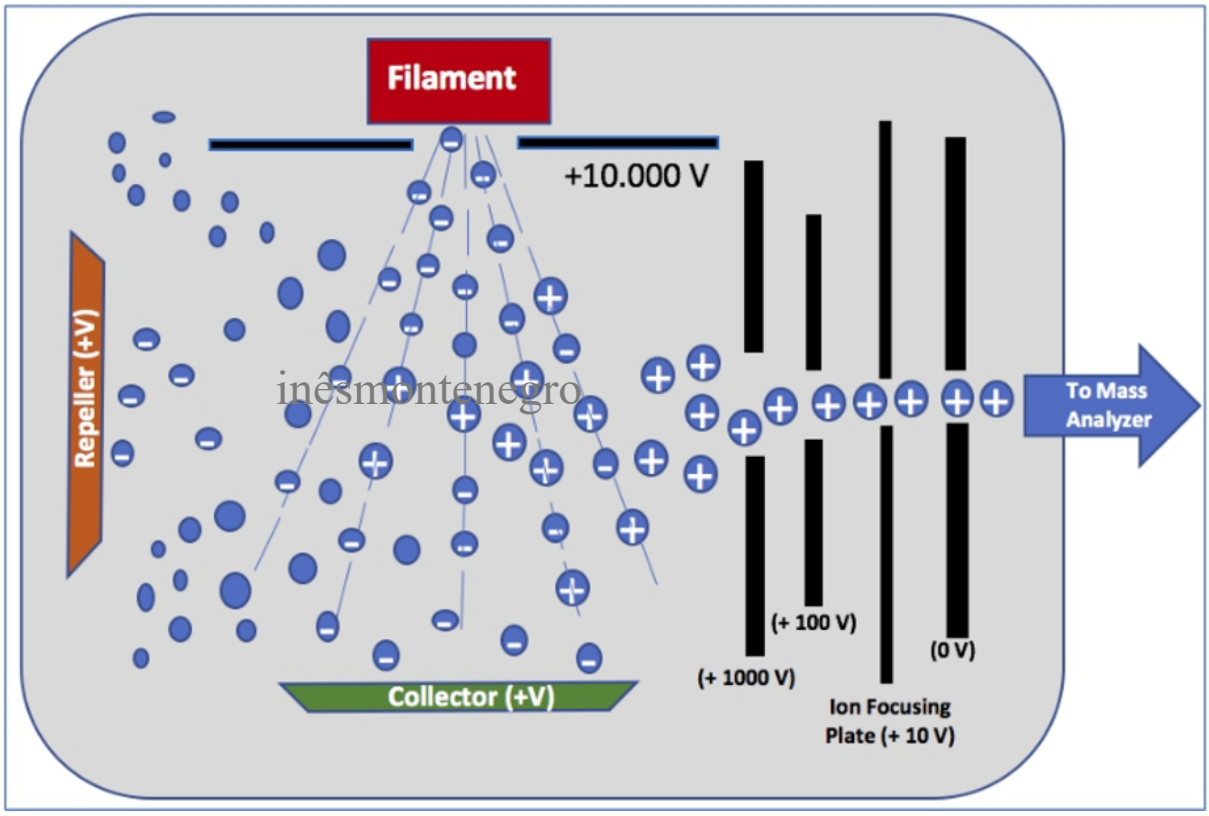


Fig. 7 The electron ionization (EI) source is most commonly a small chamber about 1 cm^3. Adapted from: Smith *et al.* 2004.

Ionization energies (**IE**) for most organic compounds range from about 5-15 eV. Bond dissociation energies are even smaller, so this method of ionization not only causes molecules to expel one or more electrons (Fig. 8), it also provides enough energy for substantial fragmentation of the first-formed ion (the molecular ion, $M^{+\bullet}$). In Fig. 9 as shown a detail of that occur in ionization chamber at the atomic level.

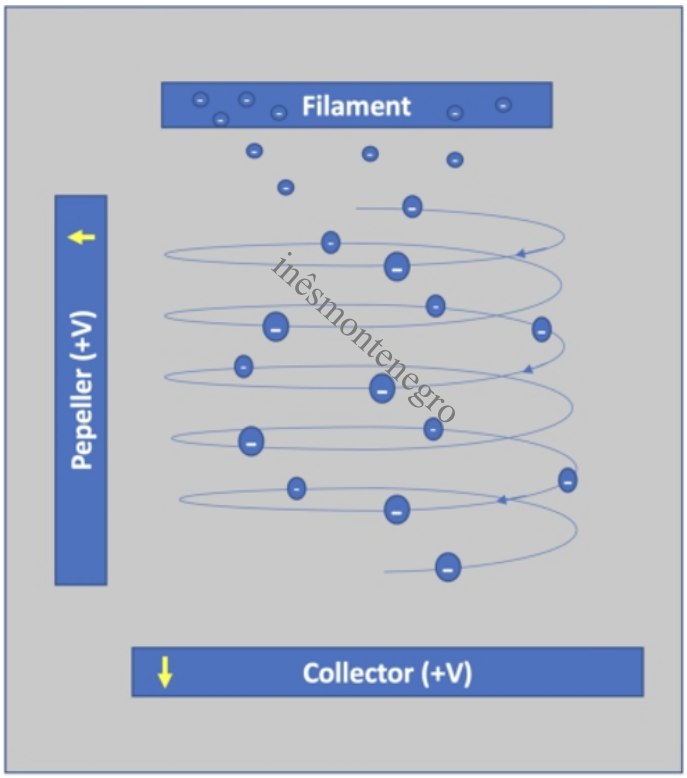


Fig. 8 Interaction between the particles and the electrons released by the filament in the ionization chamber.

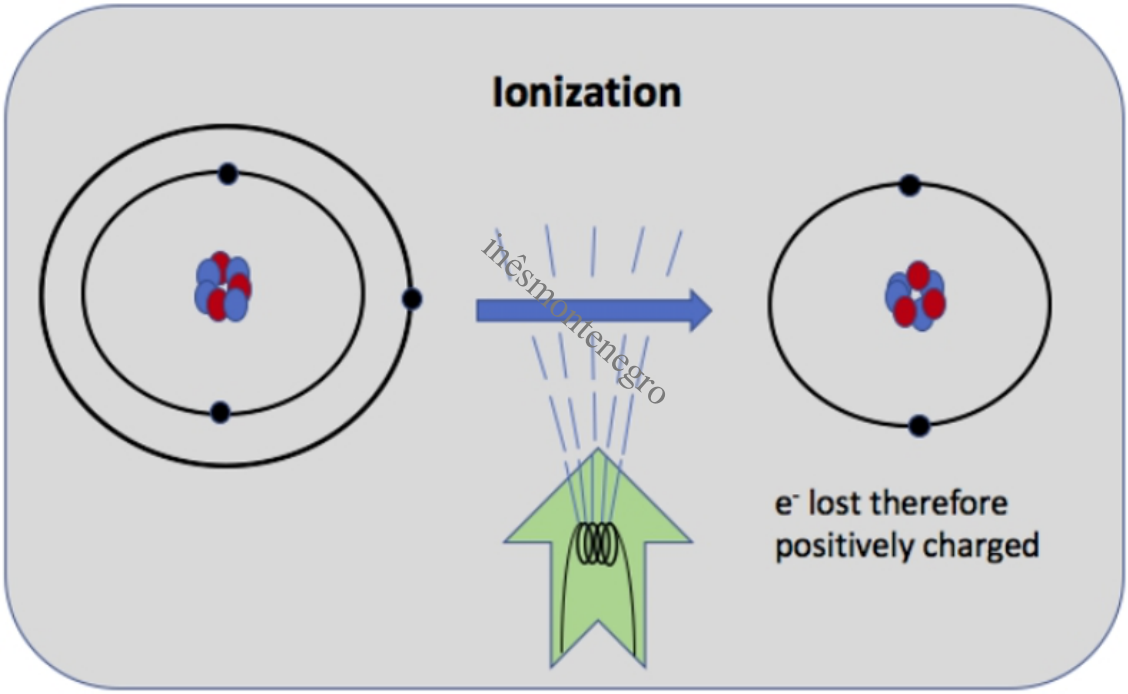


Fig. 9 - Details of the ionization process in ion source at the atomic level.

EI is a very energetic process resulting in molecular ion ($M^{+\bullet}$) that have a great deal of internal energy. These ions want to get to a lower energy state and will fragment (Fig. 10)

resulting in the multiple-peak mass spectra commonly associated with mass spectrometry. It is possible that the ionizing electron may interact with more than a single analyte molecule.

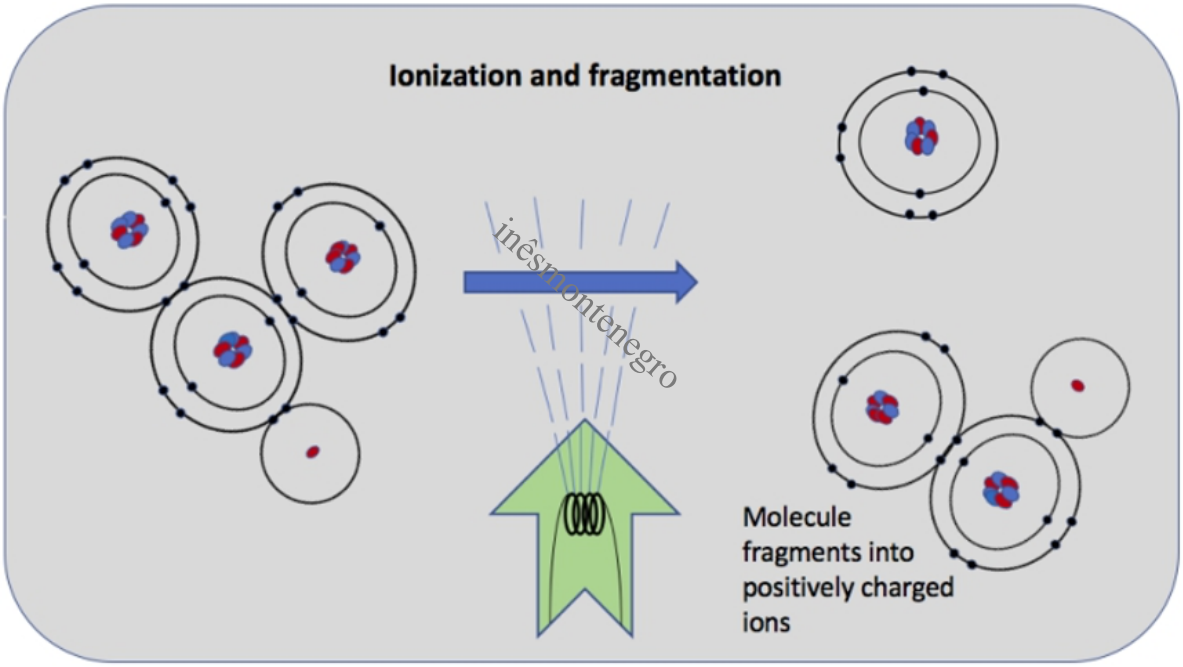


Fig. 10 - The ionization and fragmentation process in ion source.

Excited molecules can return to their neutral ground state through thermal vibrations or the emission of light, and because no ions are formed in the process, they are simply pumped away from the ion source by the vacuum system.

This molecular ($M^{+\bullet}$) is very important because it has virtually the same mass as that of the analyte molecule (the small mass of the lost electron can be ignored). Indeed, mass spectrometry is one of the few analytical tools available for determining the molecular mass of a compound. That is why the spectrum must be read from right to left.

The complex mixture of ionic and neutral products formed by any ionization method (Fig. 11) must be separated so that positive ion products travel in the direction of the m/z analyzer, and negative ions and neutral products ate left behind. Neutral products are removed by the vacuum system, because the electric and magnetic fields present in the ion source have no effect on their motion.

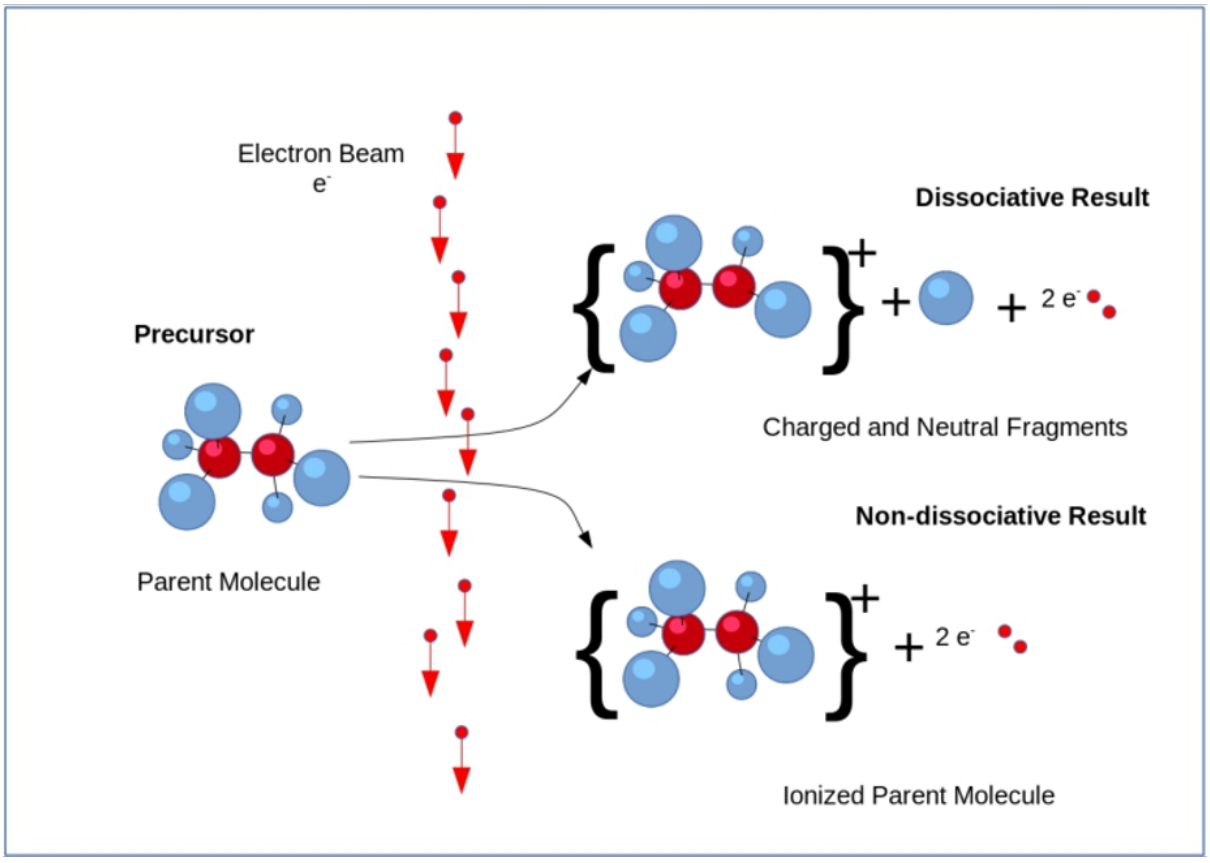


Fig. 11 - The ionization and fragmentation process in ion source.

3.2. Mass Analyser

The Ion-Trap Mass Spectrometer Analyzer consists of two end caps and a central ring electrode. In on sence, an ion trap is aptly named because, acts as a mass filter, it can "trap" ions in a 3D space for relatively long periods of time, with important consequences. The simplest use of the trapped ions is to sequentially eject them to a detector, producing a conventional mass spectrum.

The ion trap generally consists of three electrodes, one ring electrode with a hyperbolic inner surface and two hyperbolic end cap electrodes at either end. The ring electrode is operated with a sinusoidal radio frequency field while the endcap electrodes are operated in one of three modes. The endcap may be operated at ground potential, or with either a DC or a AC voltage (Fig. 12 and 13).

In other mass analyzers, only a small fraction of ions reach the detector. With an ion trap, half of the ions of all m/z values reach the detector, giving to this spectrometer 10-100 times more sensitivity than others. In this case ions are confined in three- dimensional space.

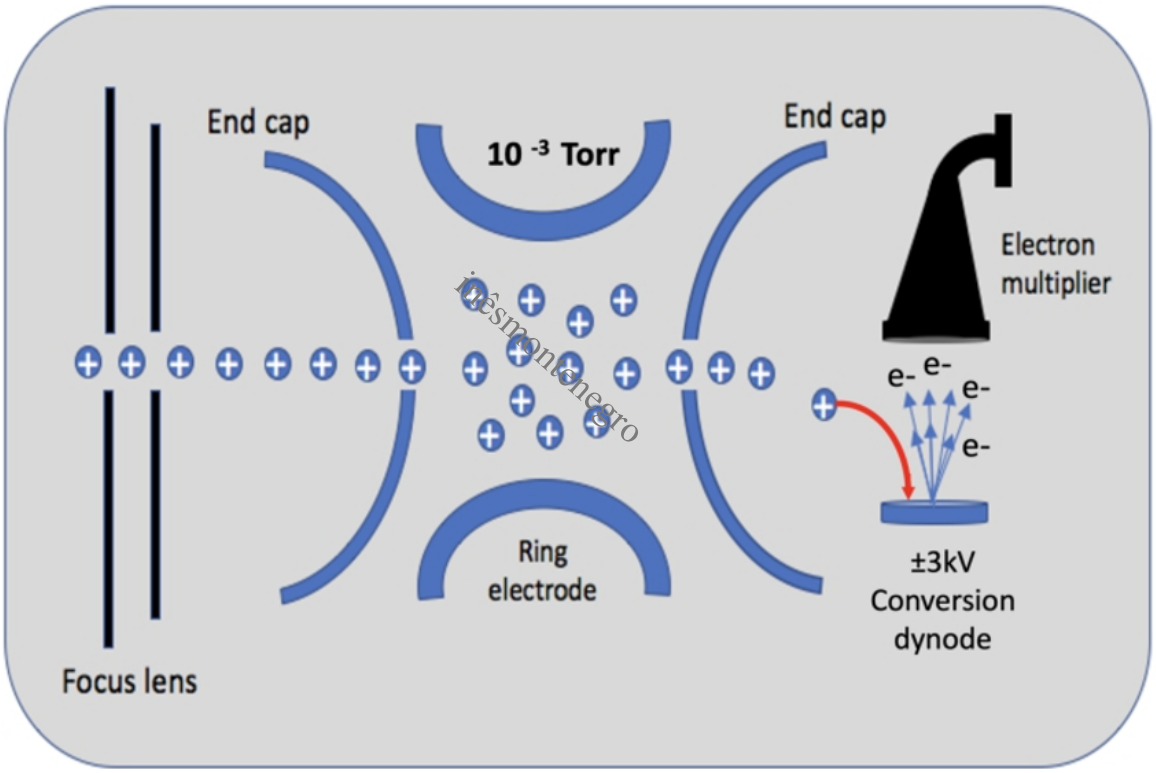


Fig. 12 - Schematic diagram of Ion -Trap Mass Spectrometer. The mass analyzer consists of two end caps (left and right) and central ring electrode. Adapted from: Sparkman *et al.* 2011.

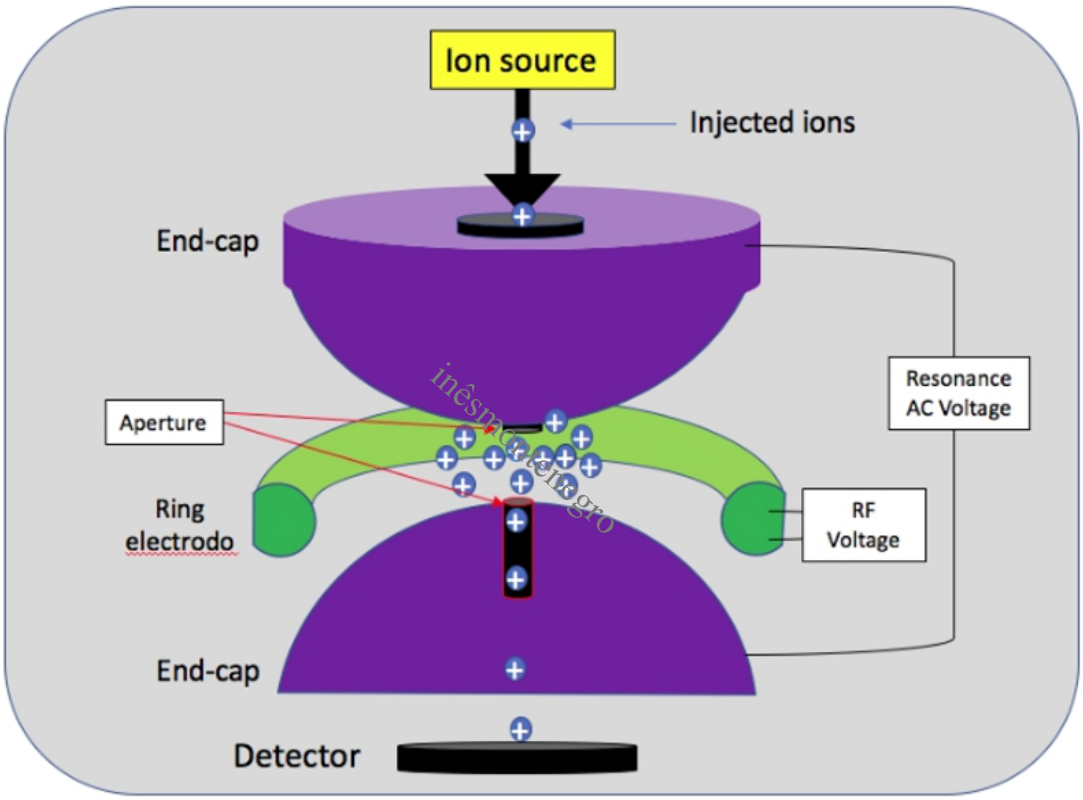


Fig. 13 - Schematic diagram of Ion -Trap Mass Spectrometer. The mass analyzer consists of two end caps (left and right) and central ring electrode. Adapted from: Sparkman *et al.* 2011.

3.3. Ion Detection/Electron multiplier

Because the **EI** process is inefficient, the number of positive ions that actually reach the detector is very small. An electron multiplier (**EM)** detector restores this lost sensitivity by exploiting the ability of surfaces that contain glass doped with about 10-20% lead oxide to expel more than one electron when a charged particle collides with it. The Fig. 14 shows a diagram of a continuous dynode electron multiplier, in which the entire surface of the multiplier is physically and electrically continuous. Other types of **EMs** may have discrete dynodes or stages that are physically distinct but electrically connected to one another.

Until recently, **EM** surfaces have been sensitive to air and especially sensitive to high concentrations of ions, so that venting the vacuum system when the **EM** is on, or leaving the filament on while the solvent is eluting during a GC run, could seriously damage in the **EM**. With GC/MS, it is desirable not to measure ion current below m/z 10 because of the high concentration of He (m/z 4) or O_2 (m/z 2) in the ion source. Many users routinely do not acquire spectra below m/z 35 to avoid ions due to air and water background.

The interior surface of the **EM** that is located near the entrance is held at a highly negative potential (usually -1.2 to - 3 kV); the exist end is referenced to ground (0.0 V). As each incoming ion collides with the multiplier surface approximately two electrons are ejected of the multiplier surface.

Most multiplier provide about a 10^5 to 10^6 fold increase in signal about 18 - 20 collisions (Fig. 14). Throughout its life, the voltage applied to the **EM** must be increased to maintain this level of gain. A voltage is eventually reached where the *noise* produced is so great that the signal at low levels is no longer distinguishable from the noise, this is when the **EM** has reached the end of its life.

Electrons generated in the last collisions with the multiplier surface constitute the signal current output of the multiplier. This current is sent to an external electronic signal amplification circuit and finally to the data system.

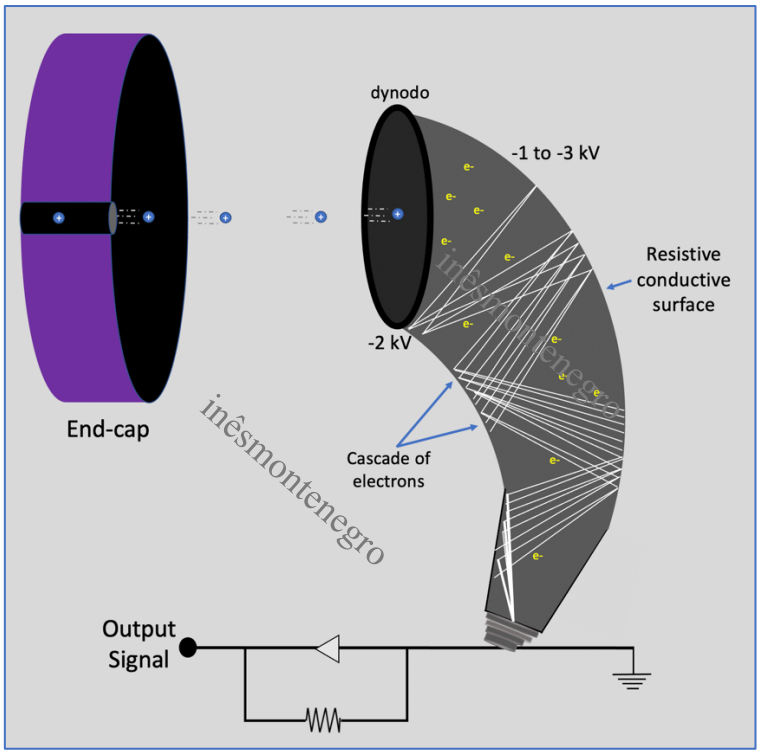


Fig. 14 Output ions from end cap of ion trap and consequent collision into dynodo (ion multiplier). Adapted from: Smith *et al.* 2004.

In Fig. 15 are shown, step by step of MS. From ion source, passing by ion trap to ion multiplier.

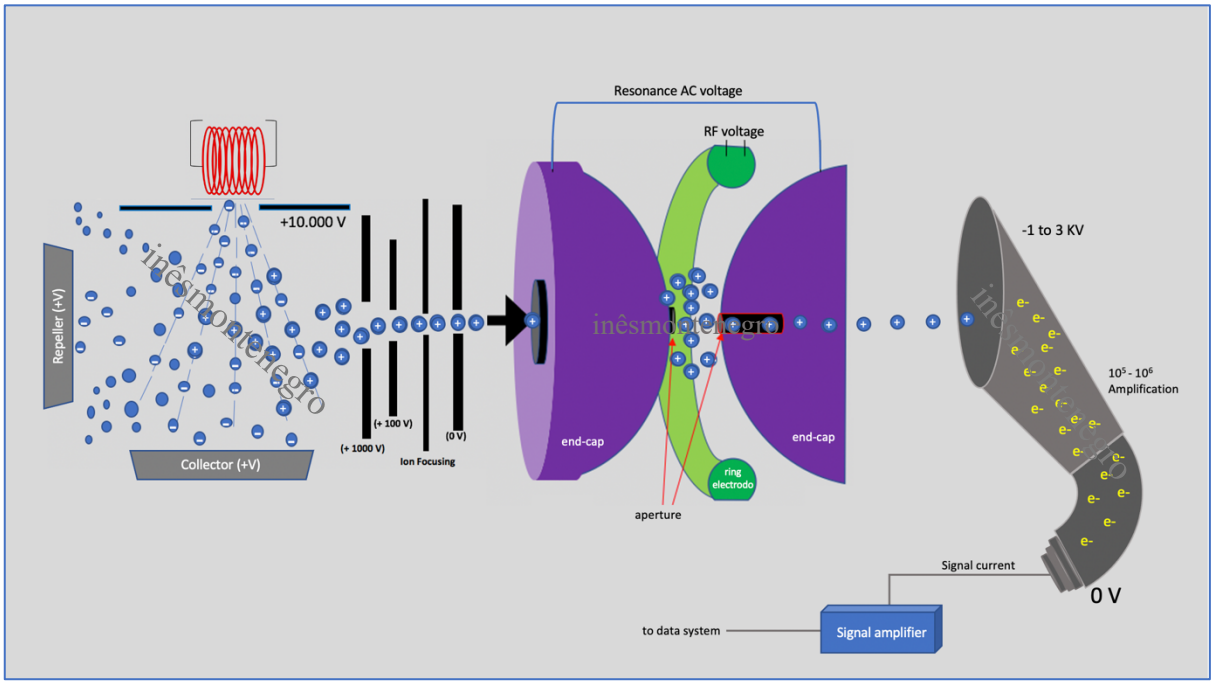


Fig. 15 - All the path in MS by ions. Adapted from Smith *et al.* 2004.

3.4. Vacuum pumps

As the ions travel from the ion source through the m/z analyzer into the detector they are in free motion. Their path and direction of travel must be determined only by the electrical or magnetic fields used to separate them according to their individual m/z value. This is why the mass spectrometer must be operated at a very low pressure, i.e., a vacuum. The ions must not collide with any other matter which could cause their direction to change or cause them to break apart. The MS is operated at very low pressures on the order of 10^{-5} torr or less. The GC - MS is a dynamic system with a continuous introduction of gas (the GC mobile phase). In order to maintain the required operating pressure, the introduced gas must be continually removed.

The function of the vacuum system is to remove the entering gas at the same time the pressure is being maintained at the desired operating level. The vacuum pump system is primarily comprised of two separate components: the low-vacuum ≈ 10^{-3} torr) component known as the *fore pump* or the roughing pump and the high-vacuum system that brings the pressure down to ≈ 10^{-5} torr or less. The pressure in the ion source itself is ≈ 10^{-3} torr for **EI**. The Low - Vacuum Component for most GC-MS systems is an in-line rotary vane pump, schematically illustrated in Fig. 16.

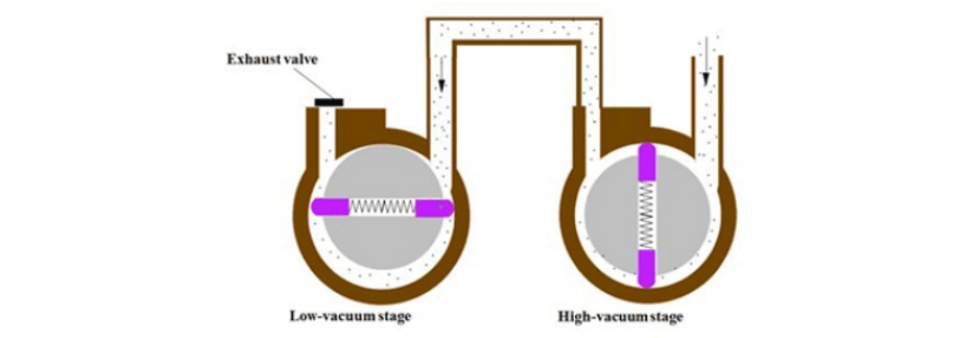


Fig. 16 - Schematically illustrated of Low - Vacuum pump and High - Vacuum pump, the fundamental component of the MS.

The exhaust of the mechanical pump as well as the split vent of the gas chromatograph must be filtered or vented to a chemical hood. The most of the sample introduced into a mass spectrometer ends up in the mechanical pump exhaust or the pump oil. This oil can be the source of chemical background an needs to be changed on a regular basis. It is important to monitor the color and turbidity of the oil to determine the proper changing interval.

The High - Vacuum Component is a turbo-molecular pump (Fig. 17). This pump must be evacuated to pressure of 10^{-3} torr before it begins to operate properly this is another function of the mechanical pump.

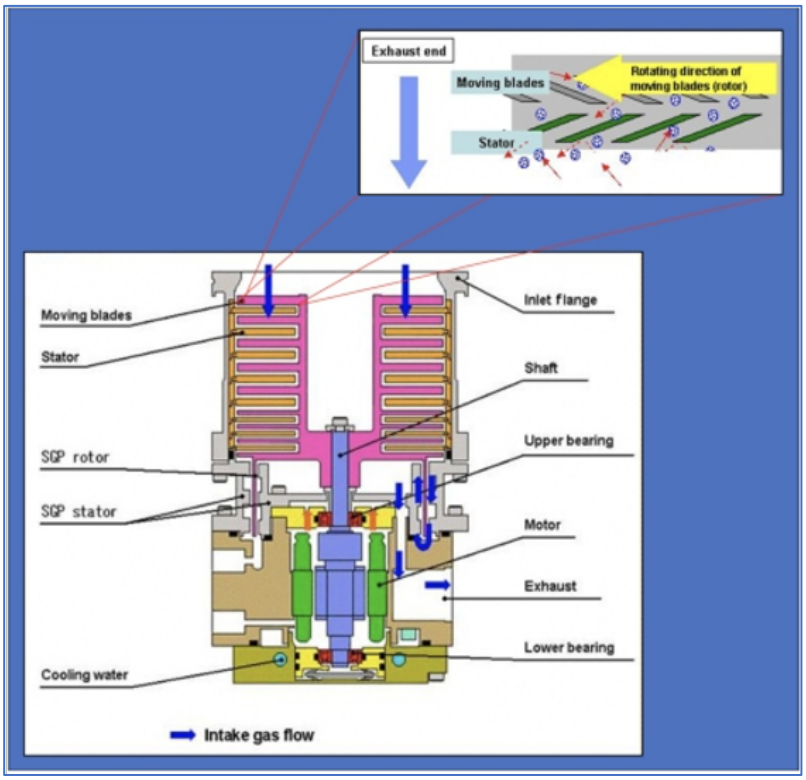


Fig. 17 High-Vacuum scheme of turbo-molecular pump.

The pressures are read by a *Pirani Gauge* and *Penning Gauge*. The Pirani gauge is a robust thermal conductivity gauge used for the measurement of the pressures in vacuum systems. The *Pirani Gauge* detected thermal conductivity of the gas by a change in temperature, this temperature is sensed by the change in resistance of the heated wire (Fig. 18).

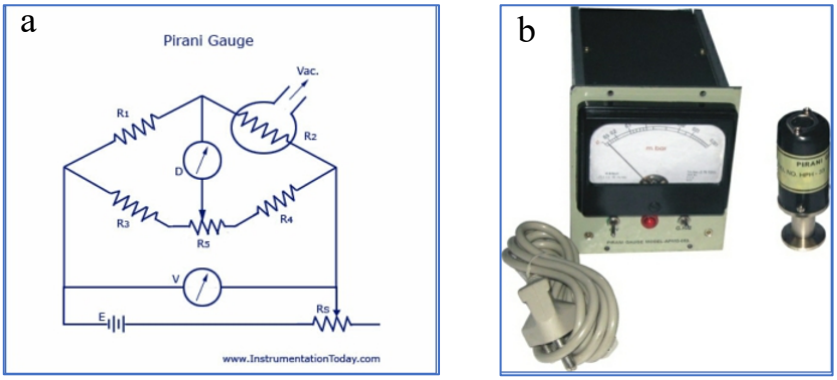


Fig. 18 - (a) Scheme of *Pirani Gauge*; (b) reader and structure containing *Pirani Gauge*.

The *Penning* Gauge is also known as cathode gauge which uses an ion current to measure pressure. In this gauge, a much higher voltage (2 kV) is used but there is no heating to the cathode. The gauge is formed from two cathode plates with a wire loop anode halfway between the two (Fig. 19). An electron emitted from the cathode is accelerated towards the positive anode and due to the magnetic field it also accelerated perpendicular to its direction of motion. This result is a helix trajectory of the electron and can have a length of several meters. In Fig. 20 are shown the vacuum system sequency and the respectively vaccum gauges (Penning gauge and Pirani gauge).

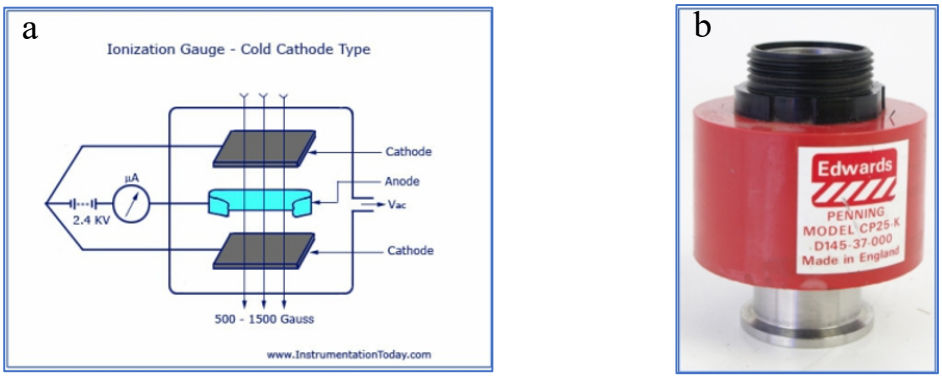


Fig. 19 - **(a)** Scheme of the *Penning* Gauge; (**b**) structure containing the *Penning* Gauge.

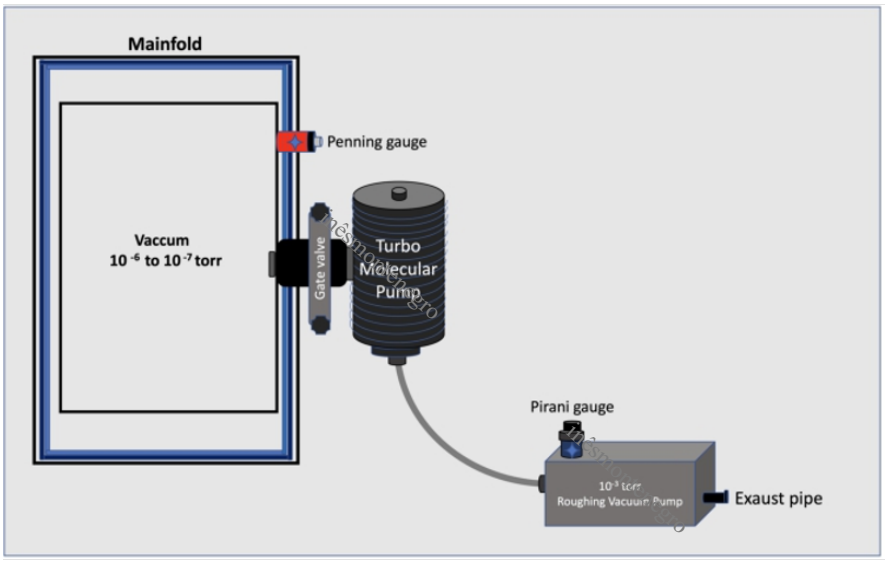


Fig. 20 - The complete vacuum system.

There is a **Tygon**® tube that connect the high - vacuum system to the mechanical pump (Fig. 21). This tube usually has a spiral wire inside of it to keep the tube from collapsing under vacuum. It will be noticed that this tube becomes discolored over time. This is from oil back- streaming and from un - ionized sample passing into the mechanical pump. This material contributes to the chemical background of the systems. When that background begins to

interfere with the desired performance of the instruments, replace this tube. At some point in the time, a peak at m/z 149 will begin to appear in all the spectra. This peak represents a fragment ion of phthalate plasticizers. A good potential source of unwanted plasticizers in the mass spectrometer is in the tube connecting the high-vacuum system and the mechanical pump.

Air leaks are another source of trouble in the mass spectrometer. A simple method of leak detection is to squirt a small volume of acetone or isopropanol on flanges and other areas where leaks could occur. A second way to test for small leaks is to tune in the mass spectrometer to m/z 40 and to use argon to test for leaks. The m/z 40 peak will increase if the argon enters the source. The Helium (He; m/z 4) is a better choice, except when He carrier gas is used in conjunction with the gas chromatograph.

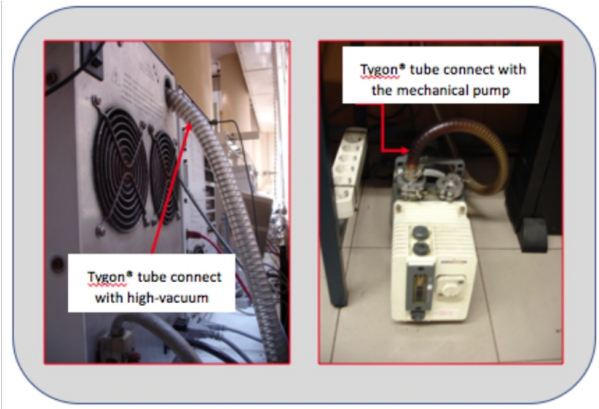


Fig. 21 - **Tygon®** tube, is a special type of tube that connect the high - vacuum system to the mechanical pump.

4. Contaminated carrier gas

Specified high - purity He is supposed to be contaminant free. Argon (Ar) contamination is usually not a problem in less than six-nines He gas. An acquisition should be run at m/z 10 to check to air leaks (peaks at m/z 18, H_2O; m/z 28, N_2; m/z 32, O_2; m/z 44, CO_2) and for Ar, m/z 40. Air leaks are bad for the GC column stationary phase and the MS. Anytime they are found they should be corrected.

Calibration is another source of potential problems. In the positive-ion mode, calibration is most frequently performed using perfluorotributylamina. Calibration of m/z scale is usually a part of the **autotune** procedure.

Annex A2-

Ultrasound assisted extraction (UAE)

Ultrasonic extraction is the use of ultrasonic radiation pressure produced by strong cavitation effect, the disturbance effect, high acceleration, and breaking and mixing function of multistage effect, which increase material molecular motion frequency and speed and solvent penetration, increasing the target component into the solvent and promoting extraction.

Ultrasonic is an elastic mechanical vibration wave, essentially different from the electromagnetic wave. Electromagnetic waves can travel through a vacuum, while ultrasonic transmission travel must be through the media. The ultrasonic transmission including expansion and compression form in the movement of fluid. In the compression zone, air-pocket soon collapse and produce a large number of micro bubbles, they can be used as a new gas nucleus. The main reason to apply this mechanical method on chemical reactions, is due that the micro bubble in grew up suddenly burst can produce strong shock waves.

In an ultrasonic tank we actually create what is called *cavitation*. Cavitation is known as the repeated formation of vapor bubbles due to the low pressure and violent collapse of bubbles due to the high pressure in a liquid flow, which generates very large hydrodynamic stresses. *Cavitation* occurs mainly when the static pressure becomes smaller than the liquid vapor pressure.

Vapor pressure

The concept of vapor pressure is best considered from the viewpoint of classical thermodynamics [814]. In the phase diagram of water (Fig.1a), the curve from the *triple point* to the *critical point* separates the liquid and vapor phases [814]. Crossing that line is representative of a reversible transformation under equilibrium conditions, i.e. evaporation or condensation of the fluid at pressure p_v, defined as the vapor pressure [814]. In the case of *cavitation* in a liquid can be made occur by lowering the pressure at an approximately constant temperature, as often happens locally in real flows (Fig.1b). In summary, can be said that *cavitation* thus appears similar to boiling, except that the driving mechanisms is not a temperature change but a pressure change, generally controlled by the flow dynamics [814].

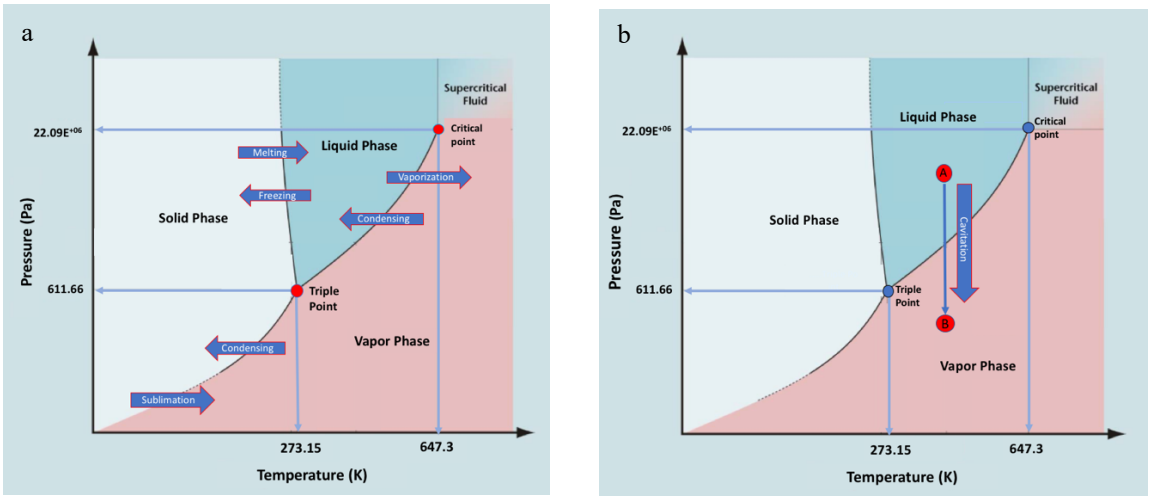


Fig. 1 a) - Phase diagram of water; **b)** Phase diagram of water and the process cavitation, from A to B (adapted from Franc and Michel [814]).

Transducers

Ultrasonic baths use *transducers*, placed at the bottom of the tank to generate the ultrasonic waves [815]. The bottom of the tank vibrates at an ultrasonic frequency using *transducers*. These *transducers* convert an electrical ultrasonic signal into mechanical vibrations again at that ultrasonic frequency that the equipment is operating. So, when we turn on, the ultrasonic baths, we are actually going to vibrate the tank bottom of 40000 cycles a second (40 KHz) in this particular system, what we worked in the laboratory. The sound that occurred when the ultrasonic bath was turned on, is means that the resonant transducers were activated and the mechanical vibrations that is going on.

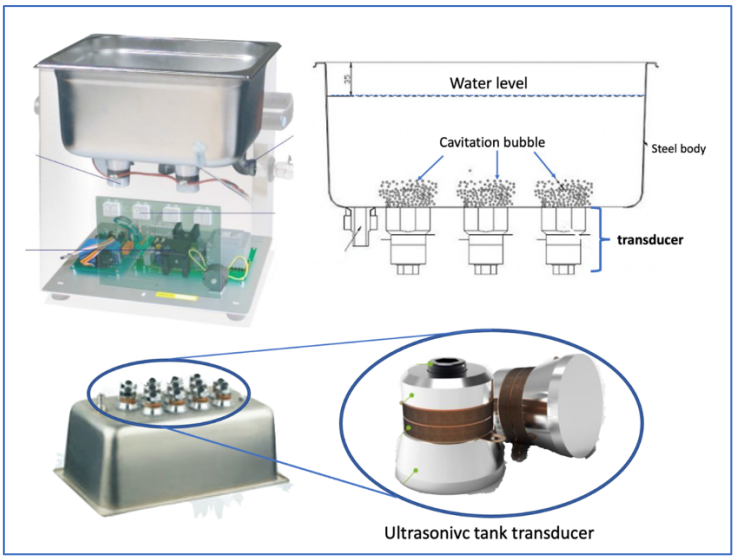


Fig. 2 - Picture of an ultrasonic bath tank and transducers (adapted from: http://www.clangsonic.com/?cur_lang=en).

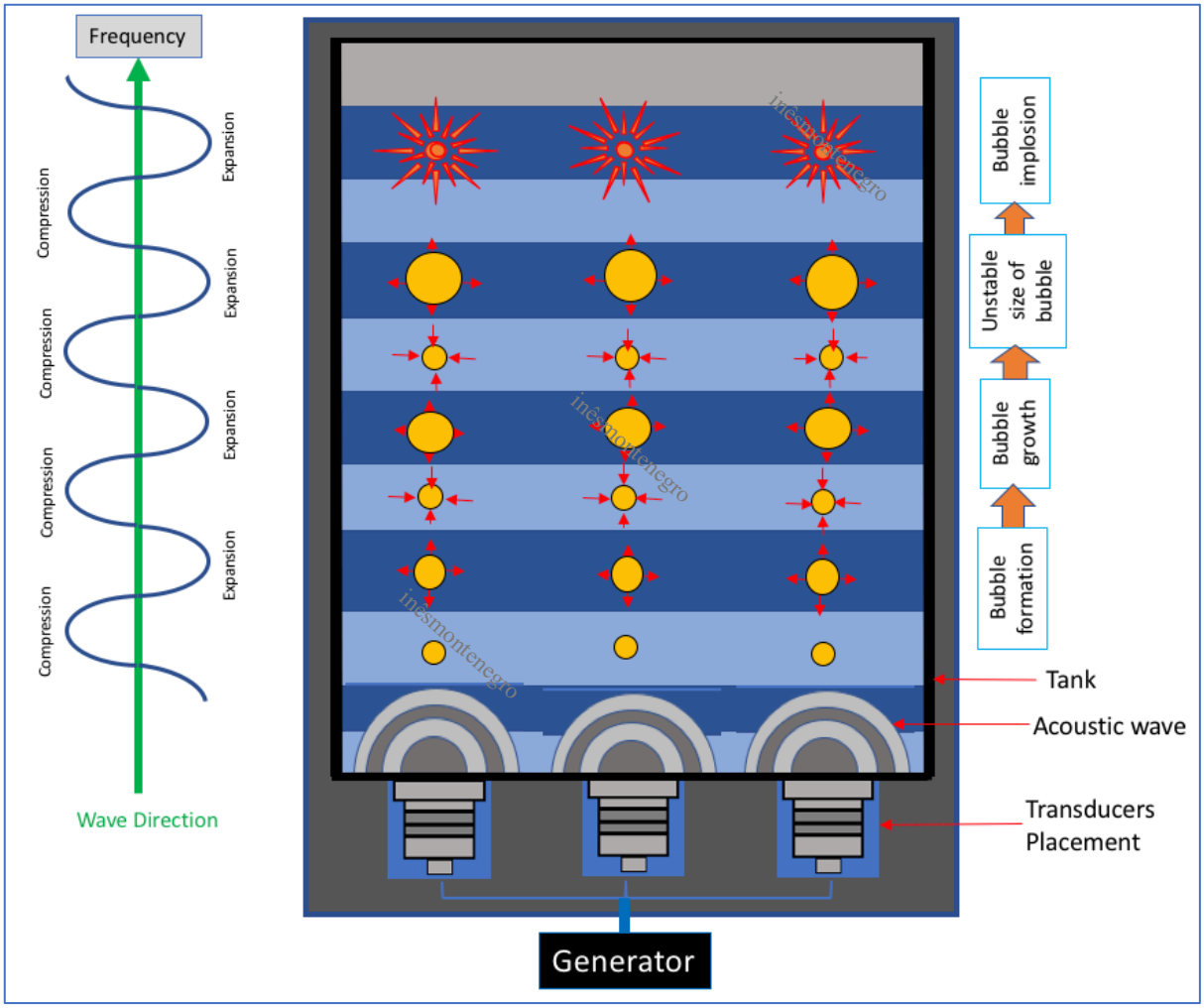


Fig. 3 - Schematic tank with ultrasonic systems (adapted from: Leong *et al*. [816]).

By using an electrical generator that puts out a high frequency signal the transducer rapidly induces compression and rarefaction waves in the liquid. During the rarefaction cycle the liquid is torn apart, this creates a vacuum cavity within the liquid. These cavities will grow larger and smaller as the compression waves are continued. When the cavity reaches a certain size (based on the frequency and the voltage of the signal) the cavity can no longer retain its shape. The cavity collapses violently and creates a temperature of 5000 °C and a microjet of water that impacts against whatever object is in the tank. There are millions of these bubbles created and collapsing every second in an ultrasonic tank (Fig. 3).

The ultrasonic transducers are excited by electric current, leading to ultrasonic cleaner to vibrate at ultrasonic frequencies, that cause the bottom (and sides as the case can also be) of the tank to vibrate and thus serve as a membrane (Fig. 3). This vibration forms the vacuum bubbles that implode (not explode) on contact with items in the ultrasonic tank.

When the bubbles collapse they emit shock waves and a microjet of water, the force of the implosion of a cavitation bubble releases a lot of energy [817]. Cavitation bubble implosions are ideally suited for creating micro-agitation at the solvent/contaminant interface (Fig. 4).

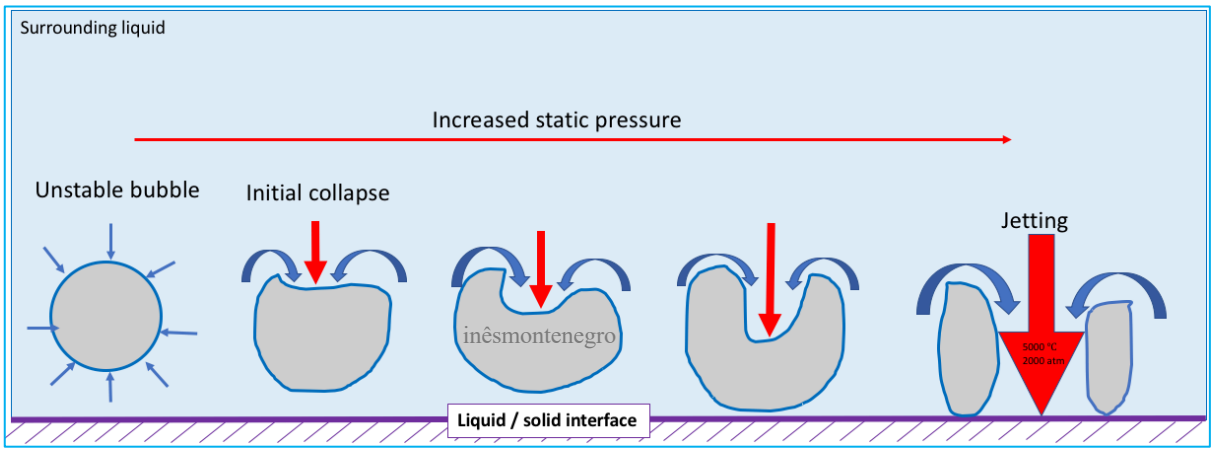


Fig. 4 - Schematic of cavitation bubbles that implode and releases a lot of energy. Cavitation bubble imploding close to a fixe surface generate a jet of the surrounding liquid. (Adapted from: Acoustical Society of America [817]).

Annex A3 -

Lyophilization technology

Lyophilization (also known as freeze drying) is an important process in sample preparation that is widely used to improve the stability and long-term storage stability of labile compounds [818]. Sample lyophilization not only has the advantage of better stability but also provide easy handling (shipping and storage) [818].

The main principle involved in lyophilization process is a phenomenon called *sublimation*. Sublimation is a phase transition where water passes directly from solid phase (ice) to the vapor phase, without passing through the liquid phase, as shown in Fig. 1a) and b). Sublimation of water can take place at pressures and temperatures below triple point, i.e., 611.657 Pa (0.006 atm; 6.11657 mbar) and 273.160K (0.0099 °C; 32.02 °F). Just like evaporation, sublimation occurs when a molecule gains enough energy to break free from the molecules around it [819].

The lyophilization process involves three stages, (Fig. 1b). It starts with a freezing process, by reducing temperature at constant pressure (stage A-B), followed by reducing pressure at constant temperature, the pressure below the triple point (stage B - C). Finally, at constant pressure, heat is provided for sublimation of the ice in the primary drying (stage C - D).

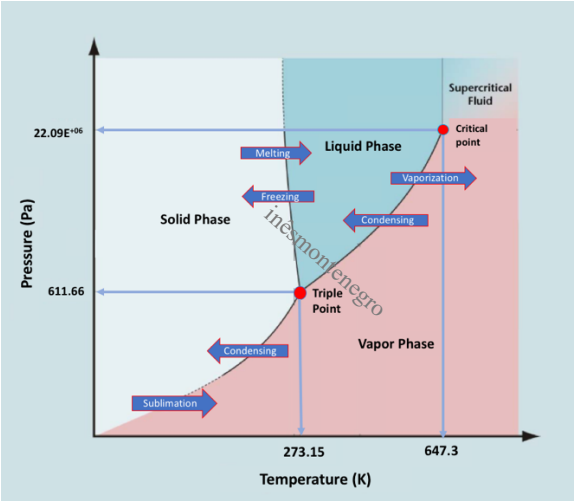


Fig. 1a) A typical water phase diagram.

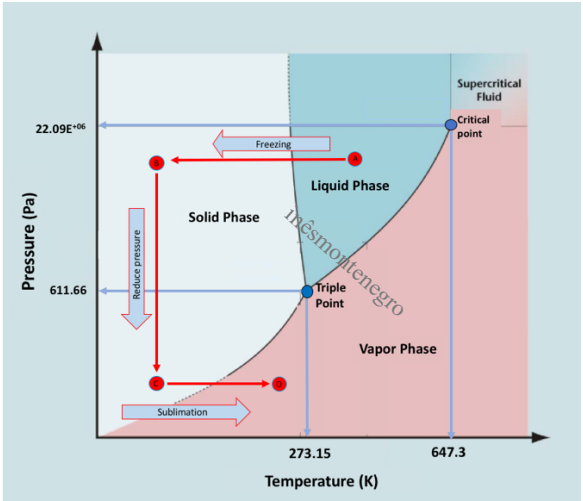


Fig. 1b) Typical lyophilization cycle in water phase diagram.

Typical stages in lyophilization process

1. Freezing
2. Primary drying
3. Secondary drying

1.Freezing

The first stage of the lyophilization process can be described simply as freezing, an important step in the lyophilization process [820]. In this stage the sample is completely frozen, this provide the necessary condition for low temperature drying [821]. The principal function of the freezing process is to separate the solvent from the solutes, minimise thermal degradation within the dispensed product, and immobilise the components in the solution, keeping their concentration during drying, and finally, prevent product foaming when vacuum is applied [820].

In case of aqueous system the water will form ice crystals and solutes will be confined to the interstitial region between the ice crystals [820]. The temperature necessary to achieve complete freezing of the formulation will depend on the nature of the solvent [820]. Ideally pre-freezing process should result in the complete crystallisation of both the water (solvent usually) and the solutes within the solution [820]. The sample to be subjected to the lyophilization process must first be adequately subjected to a pre-frozen process. The method of pre-freezing and the final temperature of the frozen sample can affect the ability to successfully lyophilize the material [822]. Rapid cooling results in small ice crystals, useful in preserving structures to be examined microscopically, but resulting in a product that is more difficult to freeze dry, in opposition, slower cooling results in larger ice crystals and less restrictive channels in the matrix during the drying process [822].

2. Primary drying

The second stage of the lyophilization process can be described as a primary drying. Once the sample has reached a completely frozen state, this sample is placed under a deep vacuum, i.e., the pressure in the freeze-dryer is reduced and heat is applied to the sample to initiate sublimation of the ice crystals, being important that the pressure is well below the triple point of water (see fig 1b) [820]. This enables the frozen solvent in the sample to vaporize without passing through the liquid phase, a process known as sublimation.

The application of vacuum allows the free migration of water vapour from the frozen mass. The migration of water vapour from the product may be regarded basically as a diffusion process and this will help to appreciate why lyophilization is a relatively slow process. The completion of the primary drying process occurs when all of the ice crystals have been removed from the sample [820].

3. Second drying

After the completion of primary drying there will still be some water adsorbed onto the surface of the sample. The second drying, is called *isothermal desorption* process, because bound water is removed by a process known as *desorption* at constant temperature [820]. Desorption process involves removing water molecules that cling to the surface of the compounds by means of Van der Waals forces. This process is much more energy intensive than sublimation process. Because the process is non-absorbent and the vacuum should be as low as possible (no elevated pressure) and the collector temperature as cold as can be attained [822].

Typically, the remaining water removal is usually accomplished by increasing the temperature of the product and reducing the partial pressure of water vapor in the container. The shelf temperature is raised 20 or 30 °C above the final primary drying temperature (Fig. 2). By the end of the primary drying, most sample have lost about 95% water content, however the remaining 5% is often enough to still keep the biological or chemical activity that causes degradation of the compounds to be analysed, in the future. Secondary drying is usually carried out for approximately 1/3 to 1/2 the time required for primary drying (Fig. 2) [820].

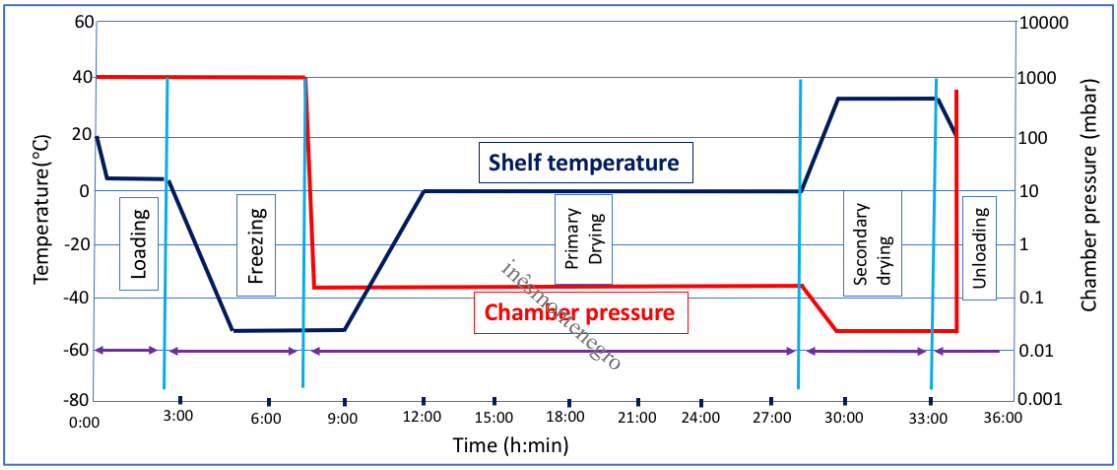


Fig. 2 - Typical freezedrying cycle. Variation of temperature and pressure over the time. (Modified from: Empower Pharmacy [823]).

Freeze dryer design

There are essentially three types of freeze-dryer: manifold freeze-dryer, rotary freeze-dryer and the tray style freeze-dryer [821].

There are two components common to all types of freeze-dryers: a vacuum pump (used to reduce the pressure in a vessel containing the sample to be dried) and a condenser (used to remove the moisture by condensation on a surface cooled to -40 to -80 °C) [821].

The manifold, rotary and tray type freeze-dryers differ in the method by which the dried substance is interfaced with a condenser [821].

In the case of the manifold freeze-dryers a short usually circular tube is used to connect multiple containers with the dried product to a condenser (section A in Fig. 3) and in the case of rotary and tray freeze-dryers they have a single large reservoir for the substance to dry [821].

Manifold freeze-dryers are normally used when drying liquid substances are in small containers and when the product will be used in a short period of time [824].

A manifold dryer will dry the sample to less than 5 % moisture content, this is the unbound water, and the process occurring is the primary drying without the heat supply.

To remove the remaining water, a heater must be added for secondary drying, which removes the bound water and will produce lower moisture content [821].

The design of the freeze-drying process consist in: sample are placed in containers (such as glass vials) that are placed on the shelves within the *vacuum chamber* (also known sample chamber) (Section A, Fig. 3) [818].

Cooling elements within the shelves freeze the sample, once the sample is frozen, the *vacuum pump* (section C in Fig. 3) evacuates the *sample chamber* (section A in Fig. 3) and the sample is heated, heat is transferred by thermal conduction from the self, through the vial, and ultimately into the sample) [818].

The basic concept for the sublimation process involves the absorption of heat by the frozen sample in order to vaporize the ice and the use of a vacuum pump to enhance the removal of water vapor from the surface of the sample. Then, the transfer of water vapor to a collector and the removal of heat by the collector in order to condense the water vapor. In summary, the freeze dry process is a balance between the heat absorbed by the sample to vaporize the ice and the heat removed from the collector to convert the water vapor into ice.

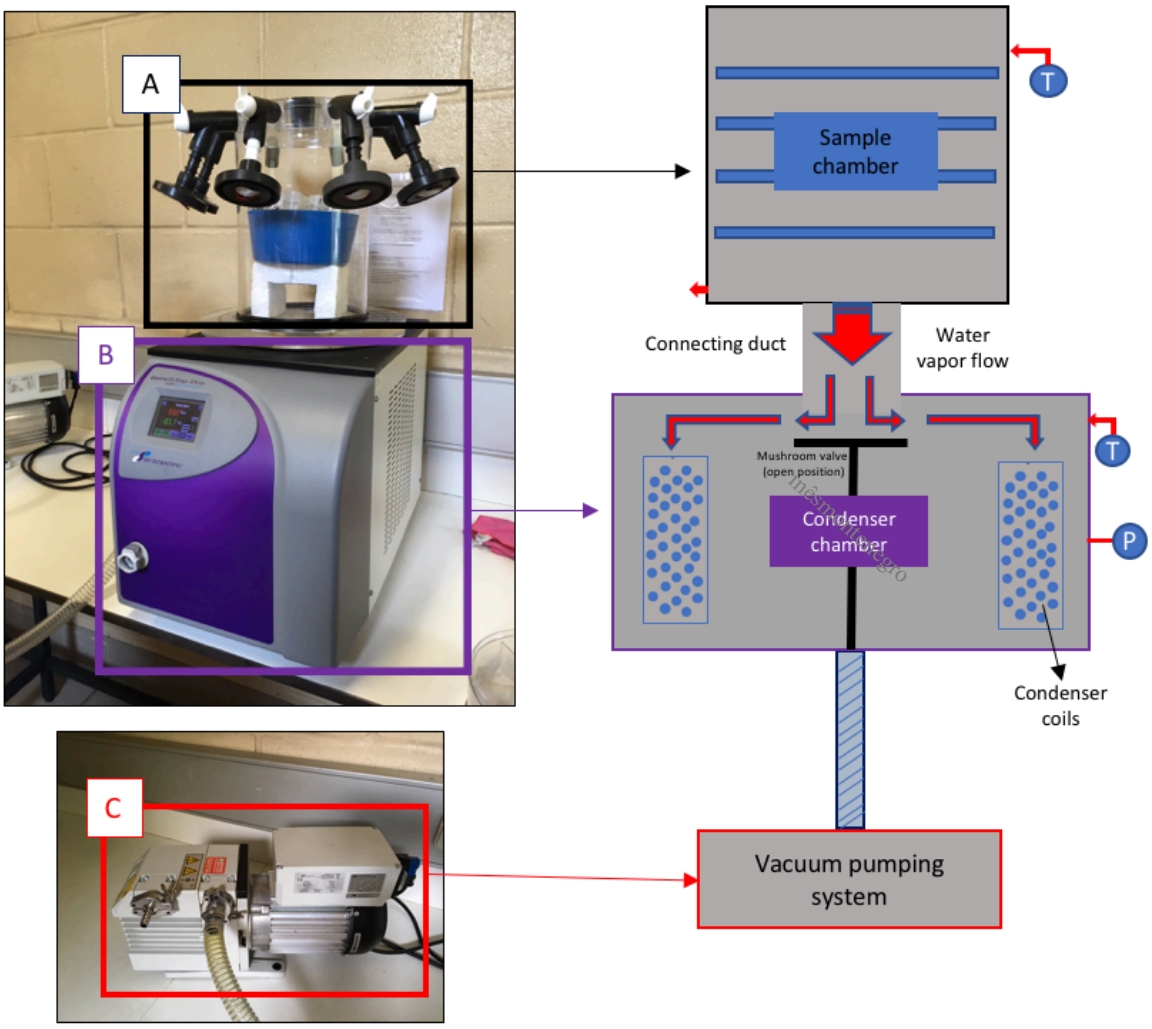


Fig. 3 - The apparatus present in the laboratory (Bench Top Pro Freeze Dryer - SP SCIENTIFIC) used in lyophilization process and a schematic representation of the same apparatus. The apparatus consists of a vacuum chamber (section A in Fig 3) that contains shelves capable of cooling and heating containers and their contents, a vacuum pump (section C in Fig 3), a refrigeration unit (section B in Fig 3) and associated controls connected to the vacuum chamber. The schematic representation of apparatus was modified from: Empower Pharmacy [823].

The apparatus used in this work was a *Bench Top Pro Freeze Dryer - SP SCIENTIFIC* with 8 liters.

Table 1 the performance specifications are presented. This information was obtained from the official website https://www.spscientific.com.

Table 1 - Specification of the *Bench Top Pro Freeze Dryer - SP SCIENTIFIC*. Modified table sfrom: https://www.spscientific.com/VirTis_BTP_-_BenchTop_Pro_Freeze_Dryers/

Performance specifications* (8 Liters)								
Lowest Condenser Temperature (°C) (50Hz / 60 Hz)	Maximum Condenser Capacity (L)	Maximum Ice Condensing Capacity in 24 hours (L)	Maximum Deposition Rate (L / hour)	Number of Compressores	Compressor Horsepower	System Refrigerant	Avarege Vacuum Time to 100 Militorr (minutes)	Lowest system Vacuum (mT)
-102 / -105	8	3	0.13	2	1/3, 3/8	M089/R1150	18	≤20 (≤30 for ES models)

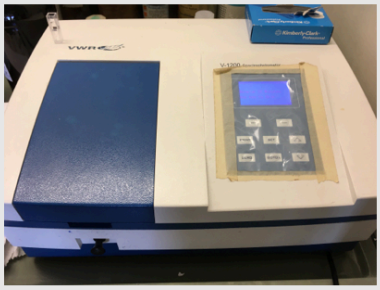
***Note**: Performance specifications are based on SP Scientific test data from units operating at an ambient room temperature of approximately 20°C. SP Scientific recommends an operating range of 15-25°C (59-77°F).

Annex A4 -

Absorption spectroscopy

The spectrophotometer was used to quantify nutrients using wavelengths previously set by the protocol (880 nm for phosphate ion; 630 nm for ammonium ion and 540 nm for nitrate and nitrite ions). The device used was a Visible Spectrophotometer V-1200 from VWR supplier. In Table 1 are given the specifications of the apparatus used.

Table 1 - Specification of Visible spectrophotometer V-1200.



V-1200 spectrophotometer
Supplier: VWR Collection

Specifications V-1200 of spectrophotometer	
Absorbance range*	- 0.3.....+ 0.3 A
Optical design*	Grating monochromator
Absorbance accuracy*	± 0.002 A / h @500 nm
Photometric accuracy*	± 0.5 % T
Photometric range*	0 - 200 % T
Wavelength range*	325 – 1000 nm
Wavelength repeatability*	≤ 0.8 nm
Wavelength accuracy*	± 2 nm
Stray light*	≤ 0.3 % T
Spectral bandwidth*	4 nm
Light source*	Tungsten
Detector*	1200 lines / nm silicon photodiode

Source: *https://us.vwr.com/store/product/12243123/vwr-spectrophotometers-basic-vis-or-uv-vis-v-1200-uv-1600pc

How does a spectrophotometer work?

There are four fundamental components in the spectrophotometer, light source, sample compartment and detector (Fig. 1).

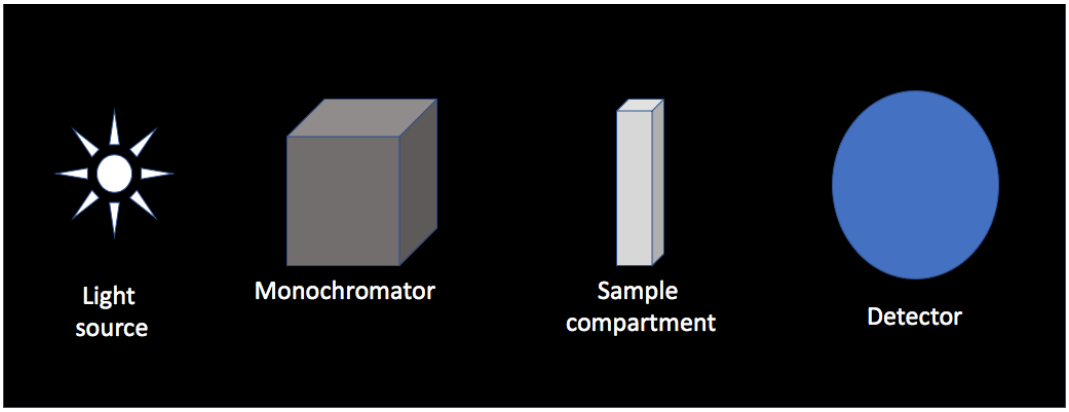


Fig. 1 - The four basic components of a spectrophotometer.

In Fig. 2 is represented the path of the light beam through the four these basic components of spectrophotometer.

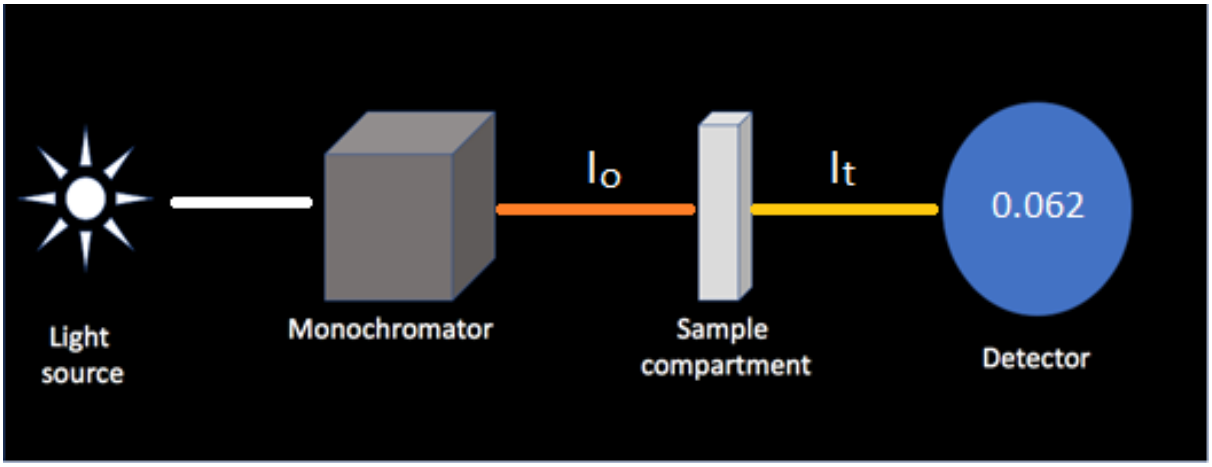


Fig. 2 - Path of the light beam through the four basic components of a spectrophotometer (source [825]).

A lamp provides the source of light. The beam of light strikes the diffraction grating which works like a prism and separates the light into its component wavelengths [825]. The grating is rotated so that only a specific wavelength of light reaches the exit slit. Then the light interacts with the sample [825]. From this point, the photometer detects the amount of photons that is absorbed and then sends a signal to a galvanometer or a digital display, i.e. the detector measures the transmittance and absorbance of the sample solution [825]. Transmittance refers to the amount of light that passes completely though the sample and strikes the detector [825]. Absorbance is a measurement of light that is absorbed by the sample. The detector senses the light being transmitted though the sample and converts this information into a digital display.

What is UV / VIS spectroscopy?

In a simple way the spectroscopy is the study of matter using electromagnetic radiation. It this part of this work we will be examining various aspects of spectroscopy that make it a scientific tool.

Light and matter

What happens when matter is irradiated by light? You might have a quantum mechanical of light absorption, which emphasizes that light energy comes in quantized units,

that call photon, and that a molecule is energy, that also comes in quantized units or “quanta”, so when a molecule absorbs a *photon* it takes up the photon energy to reach an excited state of some sort (see Fig. 2 a and b). The figures (2a and b) shown the energy conservation principle. The *photon* energy does indeed get turned into molecular energy.

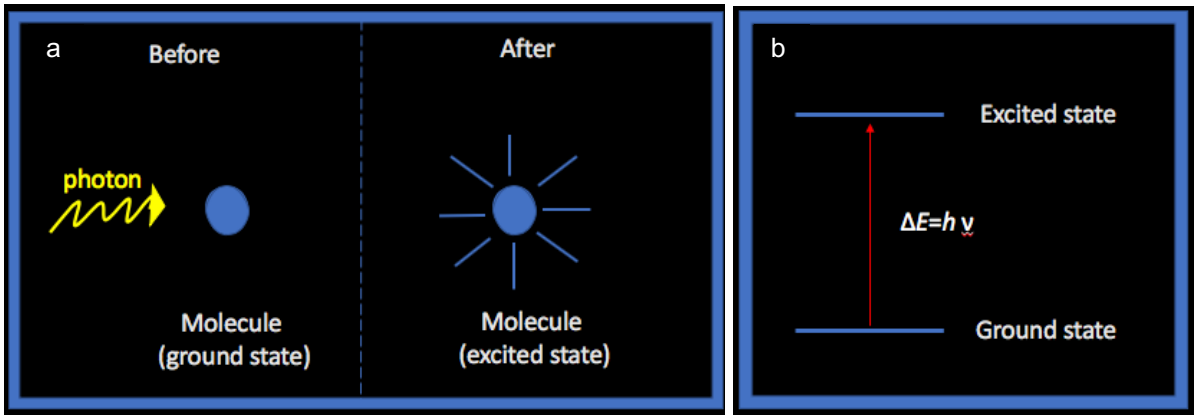


Fig.2 a) Optical absorption **b**) quantum representation of the emission (adapted from M.I.T. [826]).

Fig. 3 is another graphic representation of light absorption. This figure is very different from Fig. 2. Light is now represented as a propagating wave not as a particle as depicted in Fig. 2a.

The molecule in now shown as a classical harmonic oscillator, suggesting any possibility vibration amplitude, rather than only a set of discrete states. However, both depicted of interaction light and matter are correlated.

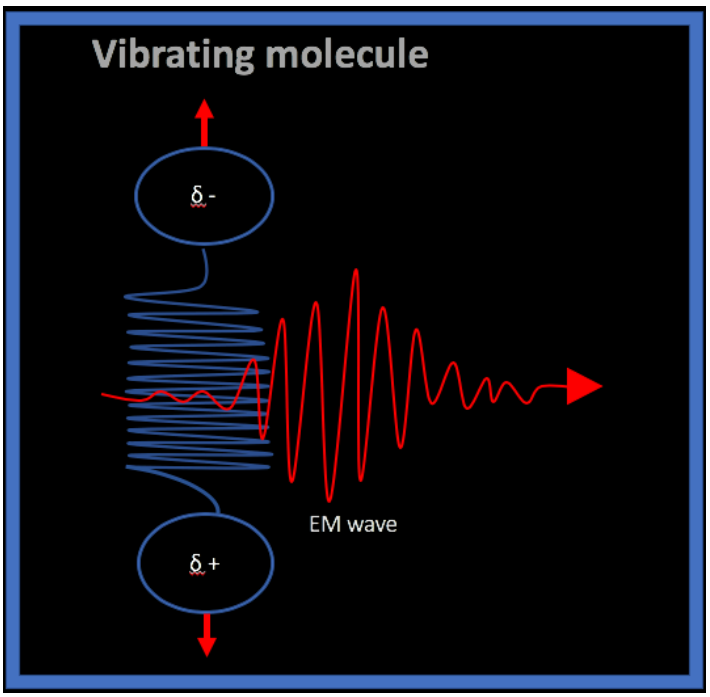


Fig. 3 - Optical absorption (adapted from M.I.T. [826]).

Light

To better understand the UV/VIS spectroscopy it is convenient to describe light in terms of both particles and waves. Light waves consist of perpendicular, oscillating electric and magnetic fields. The plane-polarized wave (Fig. 4) is composed by the electric field in the *xy* plane and in plane *xz* the magnetic field.

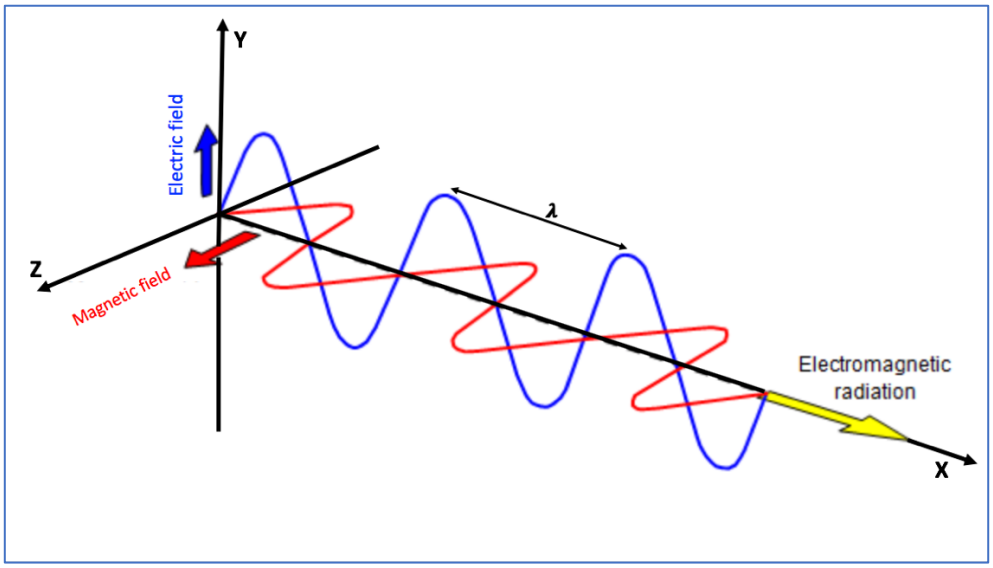


Fig. 4 - Plane -polarized electromagnetic radiation of wavelength λ, propagating along the x-axis. The electric field is in the *xy* plane, and the magnetic field is in the xz plane (adapted from M.I.T. [826]).

Two important definitions must consider, the ***wavelength*** (λ; m) is the distance between waves and ***frequency*** (ν; Hz) is the number of complete oscillations that the wave makes each second. According to the equation one (1) the frequency is inversely proportional to wavelength. This inversely proportional relation between frequency and wavelength, result that the speed of light in vacuum (c) be a constant.

$$\nu = \frac{c}{\lambda} \qquad c = 2.998\,X\,10^{8}\,ms^{-1} \qquad (1)$$

Light is energy and it is more suitable to think of light as photon and each photon carries energy (*E*). According with equation 2 and 3, this energy is proportional to *frequency* and inversely proportional to *wavelength*. The eq. 3 is the combination of eq.1 whit eq. 2.

$$E = h\nu \qquad (2) \qquad\qquad E = \frac{hc}{\lambda} \qquad (3)$$

In the electromagnetic spectrum of visible light (see fig c) and following what was said earlier, the red light has a longer wavelength than blue light, being less energetic than blue

light. The electromagnetic spectrum is the group of waves that are created by disturbances in electric and magnetic fields. This spectrum from left to right are arranged in order of decreasing frequency and increasing wavelength. The electromagnetic spectrum of visible light ranges from approximately 390 nm up to approximately 780 nm (Fig. 5). Into visible spectrum each color has a specific wavelength, for instance, red light has a wavelength of 660 nm, while green has a wavelength of 520 nm. The white light can be separated into its component colors (dispersion), and the different components are characterized by a specific wavelength.

The spectrophotometer is composed by a source of light, a *tungsten* or *deuterium* lamp depending on the range of wavelength that pertains (VIS or UV). The *tungsten* lamp is an excellent source of continuous visible and near-infrared radiation whereas ultraviolet spectroscopy normally employs a *deuterium* arc lamp [827]. In a typical UV-VIS spectrophotometer, a switch is made between deuterium and tungsten lamps when passing through 360 nm [827]. In visible spectrophotometer (V-1200) used in this work, and according with the specifics provided by the supplier VWR, the light source is a *tungsten* lamp (see Table 1). A typical *tungsten* lamp filament operates at a temperature near 3000 K and produce useful radiation in the range 320 to 2500 nm, consequently this range covers the entire visible region and parts of the infrared and a very narrow part of the ultraviolet region [827]. According with the specificity of V - 1200 spectrophotometry the wavelength range is between 325 and 1000 nm (see Table 1) i.e. a visible spectrophotometer.

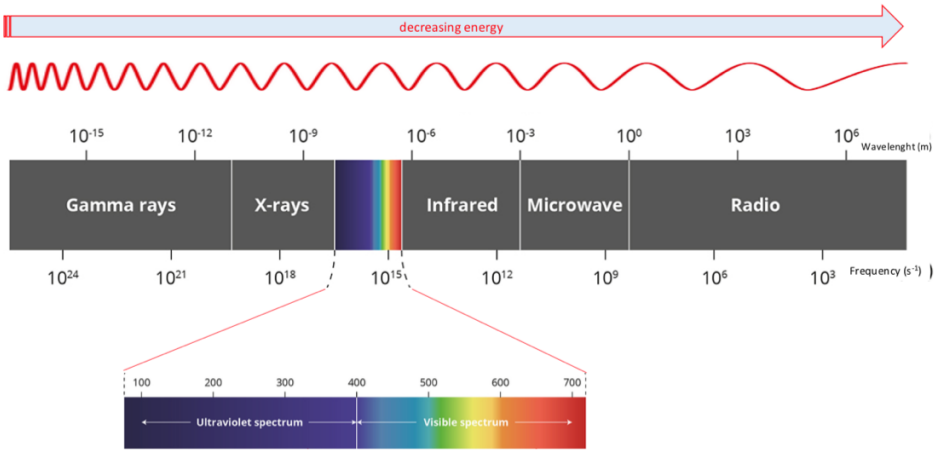


Fig. 5 - The electromagnetic spectrum. There are seven main segments of the electromagnetic, gamma rays, *X* - rays, infrared rays, ultraviolet rays (UV), visible light, microwaves and radio waves.

Monochromators

A monochromator disperse light into its component wavelengths and selects a narrow band of wavelengths to pass on to the sample or detector. The monochromator consists of entrance and exit slits, mirrors and a *grating* or prims to disperse the light (Fig. 6).

In the older spectrophotometers the *Prisms* were used instead of gratings in the monochromator (Fig. 7) [827, 828]. According with the specificity of V-1200 spectrophotometry the monochromator has a *grating* to disperse the light (see Table 1).

A *grating* is a reflective or transmissive optical component present into the spectrophotometer with a series of closely ruled lines [827, 828]. When light is reflected from or transmitted through the grating, each line behaves as a separate source of radiation, therefore, different wavelengths of light are reflected or transmitted at different angles from the *grating* [827, 828].

The bending of light rays by a *grating* is called diffraction whereas bending of light ray by a *prism* or lens, called refraction [827]. Whereas the refraction is when the light passes from one medium into another (from air into *prism*), in case of diffraction no change of transmission medium occurs (Fig. 6).

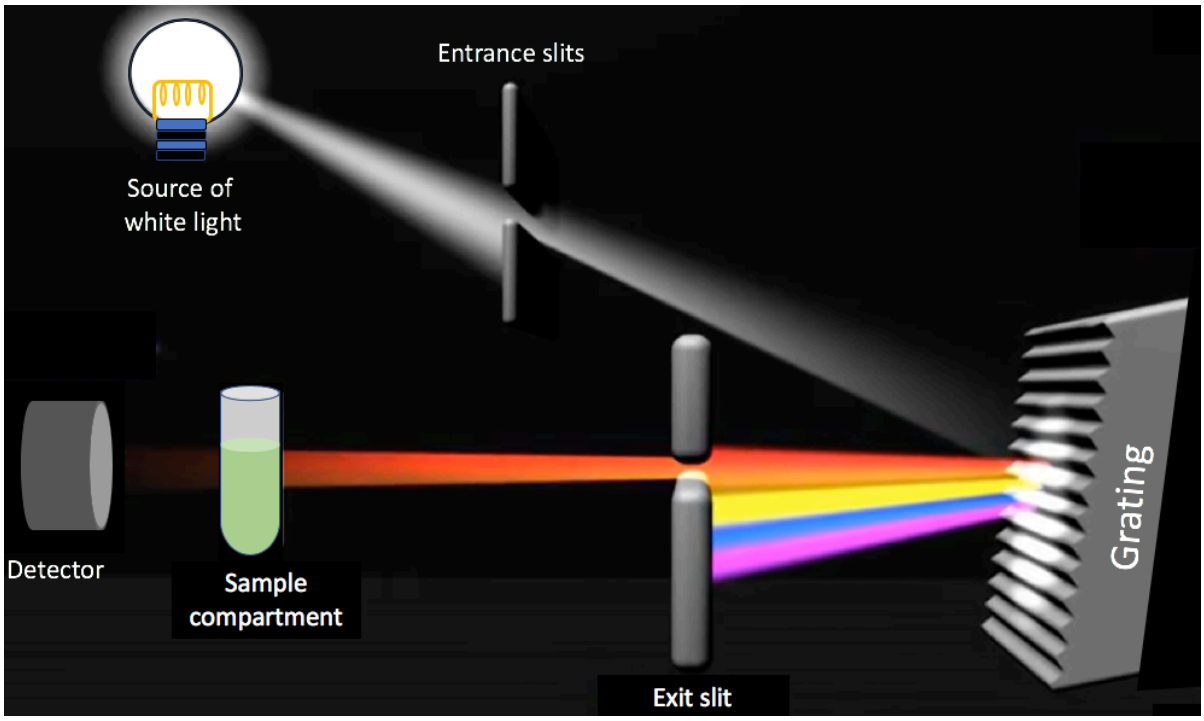


Fig. 6 - The spectrophotometer with a *Grating* as a monochromator.

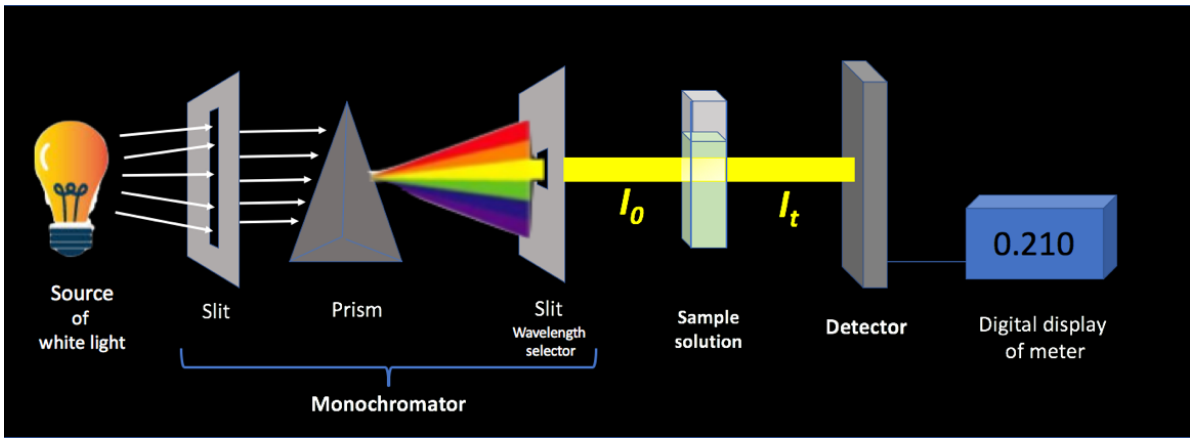


Fig. 7 - The older instruments with *Prism* as a monochromator.

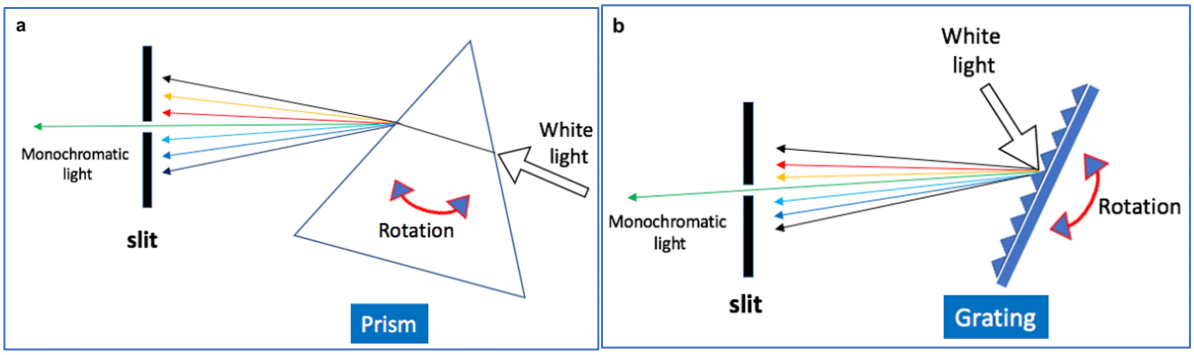


Fig. 8 - Difference between the monochromator disperses light: **a)** refraction process light passes from one medium into another (from air into *prism*) **b**) case of diffraction *grating* no change of transmission medium (adapted from Harris [827]).

How does work a grating monochromator?

In the spectrophotometer composed by grating monochromator, the polychromatic radiation from the entrance slit is collimated (made into a beam of parallel rays) by a concave mirror. These rays fall on a reflection grating, whereupon different wavelength are diffracted at different angles [827]. The light strikes a second concave mirror, which focuses each wavelength at a different point on the focal plane [827]. The orientation of the reflection grating directs only one narrow band of wavelength to the exit slit of the monochromator and rotation of the grating allows different wavelengths to pass through the exit slit [827]. The grating is coating with aluminum to make it reflective and the reflection grating is ruled with a series of closely spaced, parallel grooves with a repeat distance *d* [827]. When light is reflected from the grating monochromator, each groove behaves as a source of radiation. When adjacent light rays are in phase, they reinforce one another, but when they are not in phase, they partially or completely cancel one another [827].

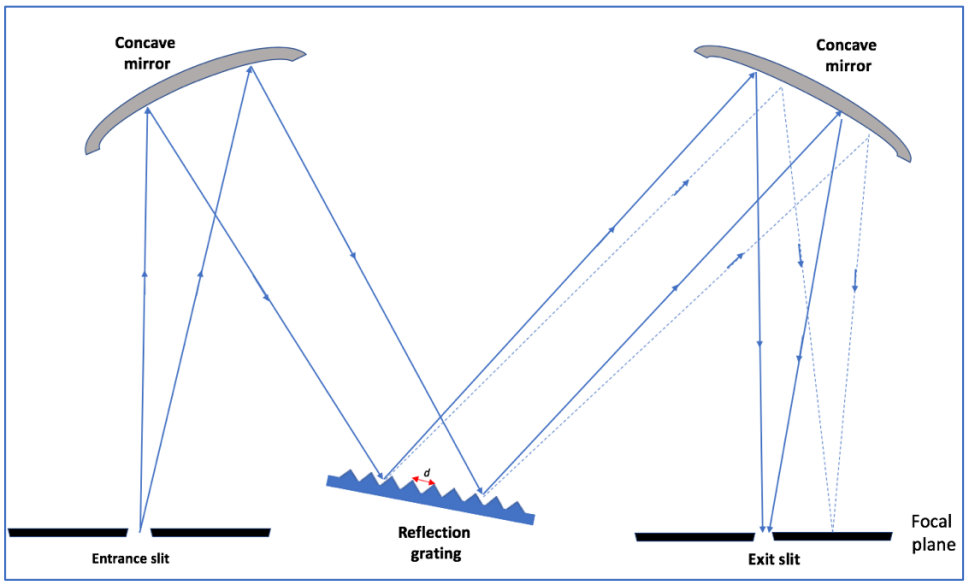


Fig. 9 - *Czerny -Turner* grating monochromator **(**adapted from Harris [827]).

Detectors

The detector is a (photo) diode therefore this component produces an electric signal when it is struck by photon. The detector can receive all wavelengths of white light or to receive only one wavelength [828]. In a *dispersive* spectrometer or *scanning* spectrophotometer with a monochromator (Fig. 10 a), only one narrow band of wavelengths reaches the detector at any time while, in a *photodiode array* spectrophotometer (Fig. 10 b) different wavelength reach different parts of the detector array [827, 828]. In this work the *dispersive spectrometer* was use and the detector is a silicon photodiode composed by 1200 lines nm^{-1}.

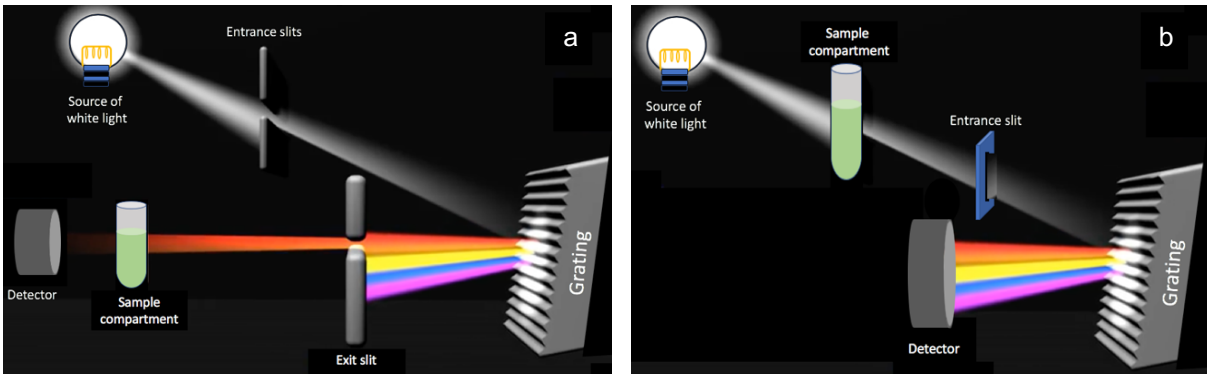


Fig. 10 - UV/VIS spectrophotometers can be classified according to the geometry of the components building up the optical system for the recording of spectra and two configurations are generally used (**a**) Scanning spectrophotometer; (**b**) Diode Array spectrophotometer.

Photodiode is a semiconductor device that convert light into electric current. When radiation (*photon*) strikes the photodiode, free electrons and holes are created and migrate to regions of opposite charge, partially discharging the capacitor [827, 829, 830]. It can be said that more radiation that strikes each diode, the less charge remains at the end of the measurement [829, 830]. The longer the array is irradiated between readings, the more each capacity is discharged and the state of each capacitor is determined at the end of the cycle by measuring the current needed to recharge the capacitor [827, 829].

What is photodiode?

A photodiode is a *p-n junction* or *pin* semiconductor device that consumes light energy (photon) to generate electric current. It is also sometimes referred as photodetector, photo-sensor or light detector [829–831]. Photodiodes are specially designed to operate in reverse bias condition. Reverse bias means that the *p* side of the photodiode is connected to the negative terminal of the battery and *n* side is connected to the positive terminal of the battery [829–831]. The symbol of photodiode is like to normal PN junction diode except that it contains arrows tracking the diode [829–831]. The arrows tracking of the diode represent light or photons and the photodiode has two terminals a cathode and an anode [829–831].

The photodiode is a junction diode specially constructed to allow the use of light as a determining factor in the control of electric current (Fig. 11). When the *junction* is inversely polarized in the absence of light incident on it, the current is practically zero [829–831]. A normal PN junction diode allows a small amount of electric current under reverse bias condition. To increase the electric current under reverse bias conditions we need to generate more minority carriers [829–831].The external reverse voltage applied to the p-n junction diode will supply energy to the minority carriers but not increase the population of minority carriers [829–831]. However, a small number of minority carriers are generated duo to external reverse bias voltage. The minority carries generated at *n* site or *p* sit will recombine in the same material before they cross the junction [829–831]. As a result, no electric current flows due to the charge carriers. For example, the minority carriers generated in the p-type material experience a repulsive force from the external voltage and try to move towards n-side [829–831]. However, before crossing the junction the free electrons recombine with the holes within the same material. As a result, no electric current flows, to overcome this problem we need to apply external energy directly to the depletion region to generate more charge carriers. For

this effect a special type of diode called photodiode is designed to generate more number of charge carriers in depletion region.

In photodiode we use light or photos as an external energy to generate charge carriers into depletion region.

The operation of the photodiode is based on the fact, that the photons that collide with the junction produce electron-holes pairs because they yield their energy, facilitating the electric current (see Fig.12). The light beam incident on the junction has a similar effect to that of the base current in a conventional transistor. When light falls exactly on the junction, the photo diode response is increased, rapidly decreasing on either side of the junction as moves away from it photodiodes respond very quickly to light variations.

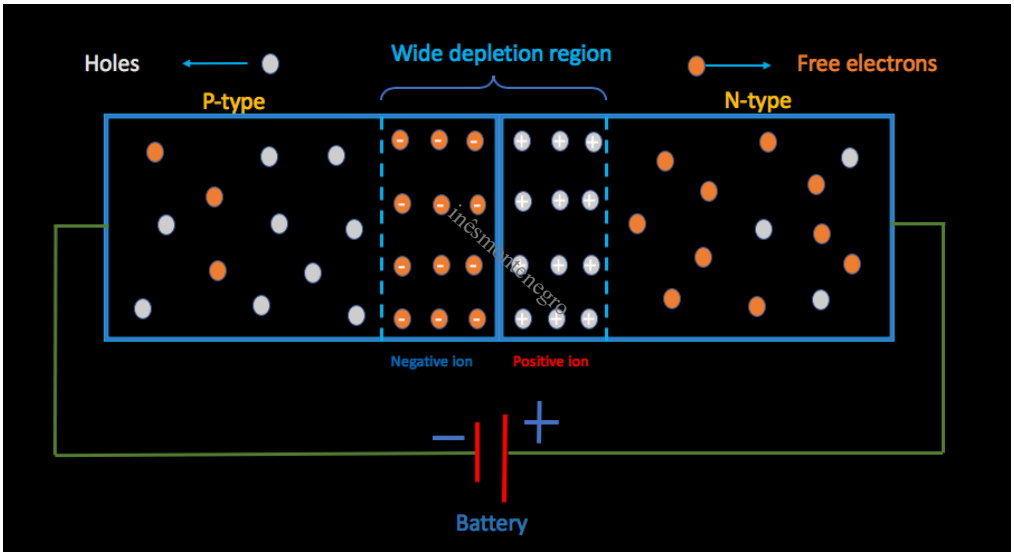


Fig. 11 - Photodiode inversely polarized, electric balance between the depletion region and power supply (zero current *i*=0).

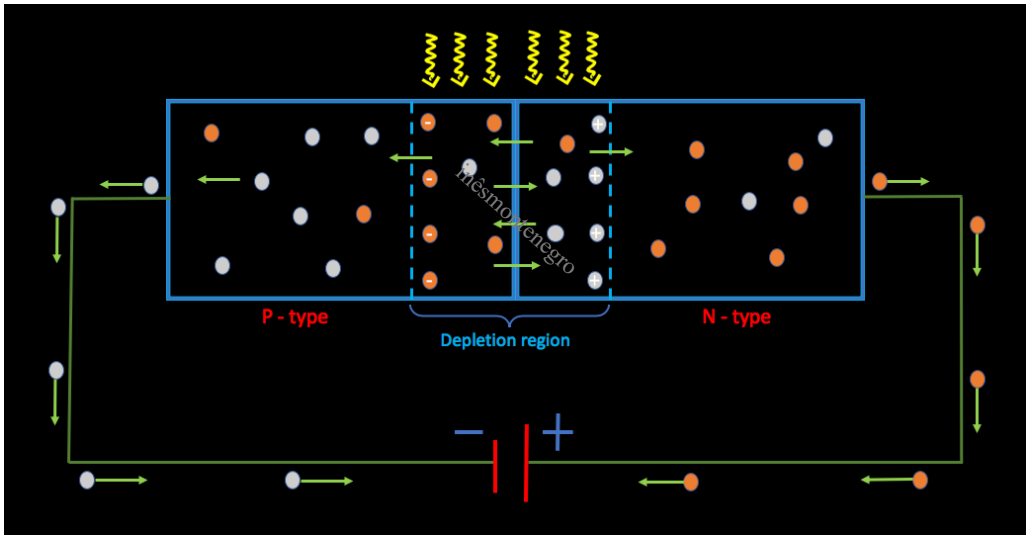


Fig. 12 - Photodiode inversely polarized after photons collision in the region of depletion ($i \neq 0$, no electric balance between the depletion region and power supply).

What happens in sample compartment?

The physical element of the spectrophotometer after the monochromator is where the samples are placed (*cuvette*). Light passes through a monochromator (prim or grating) to select one wavelength and becomes monochromatic light because is a light with a narrow range of wavelengths [832]. This monochromatic light, with irradiance I_0 strikes the *cuvette* of length *b,* and the irradiance of the beam emerging from the other side the *cuvette* is *I* [832].

Two things can happen when the light strikes the cuvette with the sample (see Fig. 13): (a) light is not absorbed by the sample so $I_0 = I$ or (b) light is absorbed by the sample so $I \leq I_0$.

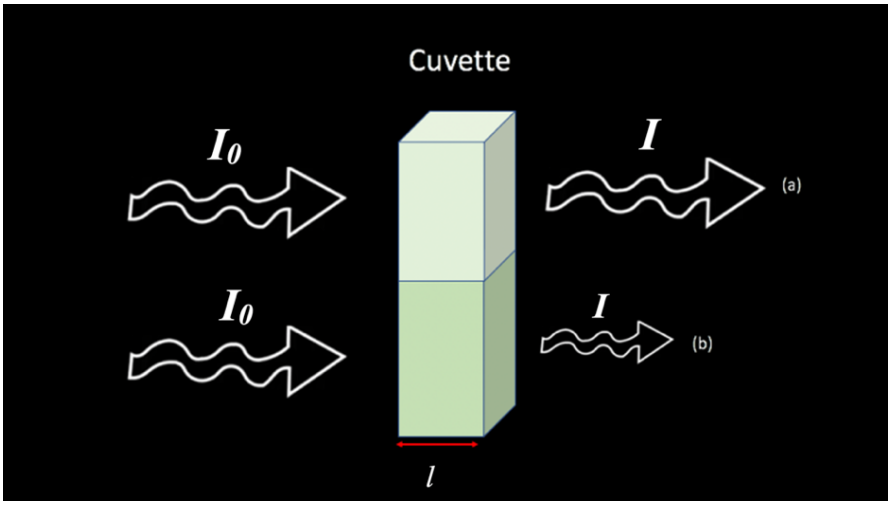


Fig. 13 - The photon strikes the cuvette with *l* of thickness. Percentage or totality of photon is not absorbed by the sample. According with *Beer - Lamber* law the concentration of sample is directly proportional to the absorbance.

The fraction of the original light that passes through the sample is defined by *transmittance* (T) and range 0 to 1.

$$T = \frac{I}{I_0} \qquad (4)$$

The *absorbance* is an indirect measure because it is calculated from *transmittance*:

$$A = -\log T \qquad (5)$$

When $P = P_0$ is because light is not absorbed by sample, 100 % of light is transmitted and the value of absorbance is null ($A = 0$). In the case of 90 % of the light is absorbed by sample, 10% is transmitted and the absorbance is 1. If only 1 % of the light is transmitted by the sample the absorbance is 2 [828, 832].

To calculate the concentration of sample it is necessary to have the absorbance value. This value is important because it is directly proportional to the concentration of the light - absorbing species in the sample

$$A = \varepsilon l (\textit{Beer - Lambert law}) \qquad (6)$$

The quantity ε (epsilon) is called the molar absorptivity and is the light absorbed at particular wavelength by a particular substance. The unit of ε (M^{-1} cm^{-1}) make de absorbance dimensionless and is the cuvette thickness [828, 832].

Spectrophotometric determination of nitrate and nitrite

(EPA Method 353.4)

Nitrate ion in the sample is reduced to nitrite ion in a buffer solution. The nitrite is then determined by diazotizing with sulfanilamide and coupling with N – 1(-naphthyl)-ethylenediamine dihydrochoride to form a colored azo dye. The absorbance measured at 540 nm is linearly proportional to the concentration of nitrite and nitrate ions in the sample. The nitrate ion concentrations are obtained by subtracting nitrite values, which have been separately determined without the cadmium reduction procedure. Cadmium reduces any nitrate ion present in the sample into nitrite ion.

Spectrophotometric determination of phosphates

In this experimental work, orthophosphate ion reacts with ammonium heptamolybdate to form a phosphomolybdic acid. The complex formed is reduced by ascorbic acid in the presence of potassium antimony tartrate, forming molybdenum blue [833]. The measured concentration of molybdenum blue by spectrophotometry determines the concentration of orthophosphate ion in the water sample. The intensity of blue color is proportional to the amount of phosphate ion in the sample [833].

Spectrophotometric determination of ammonium

The spectrophotometric method used in this work for ammonium ion determination was the *Koroleff* method. The reaction between ammonium ion and phenol in the presence of chlorine forms indophenol blue. The blue colour of indophenol is formed by phenol and hypochlorite in the presence of ammonium ion.

Annex A5 -

Fluorescence spectroscopy

The fluorescence spectroscopy (also defined fluorimetry or spectrofluorimetry) is a technique to detect and analyze the fluorescence in the sample [834]). Fluorescence is the emission of light by a substance (fluor) that has absorbed light or other electromagnetic radiation. In this emission phenomenon of quantum physics, a beam of light (usually UV light) excites the electron in a molecule which moves from ground state to higher energy excited state [835].

In Fig. 14 (a and b) is a simplified picture that gives an overview of absorption and fluorescence processes. In the fluorescence process an excited dye molecule returns to its ground state of energy and emission of a (fluorescence) photon occur, while in the absorption process a photon hits a dye molecule in its ground state and the dye may be brought into its excited state and the photon is absorbed during this process [835].

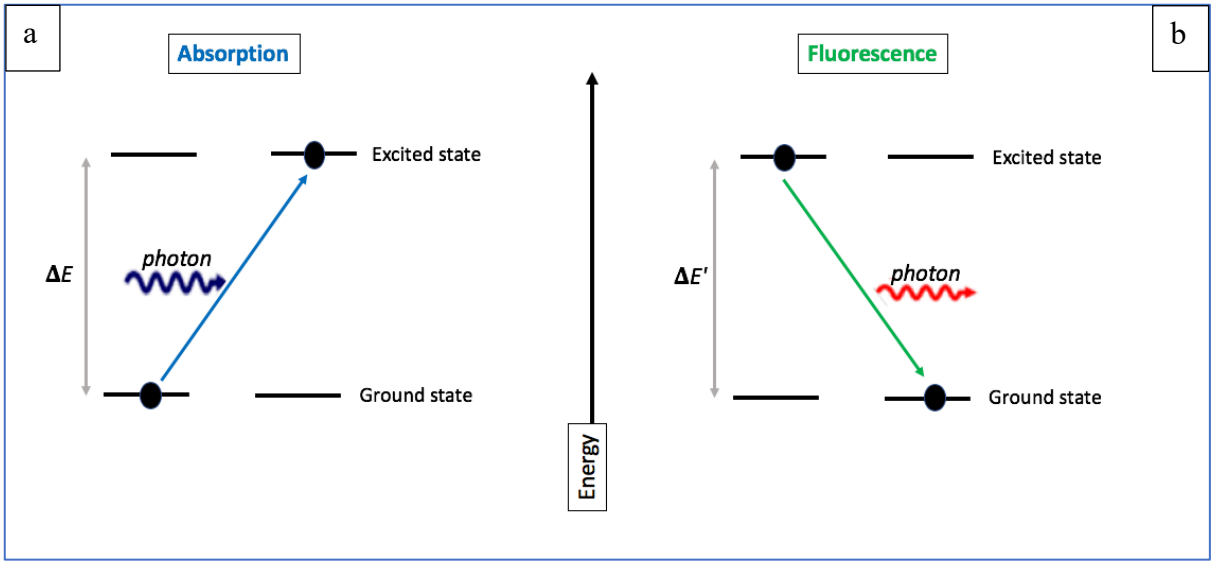


Fig. 14 - a) After energy absorption the molecule goes to higher energy level. **b**) After molecule lowers the energy level that same amount of energy is released but in another form of energy (e.g. light energy) (adapted from: [832]).

Fluorescence spectroscopy is mainly concerned with electronic (ground state and excited state) and vibrational states [836]. When the electron falls back to the ground state, it emits fluorescence (Fig. 15). The most electrons will occupy the ground state and lowest vibrational level (S_0V_0) at room temperature, but they are elevated to the high energy excitation

state (e.g., S_1 or S_2 excited level) by the absorption of photons provided by the electromagnetic radiation. In fluorescence emission measurement, the excitation wavelength is fixed but the detection wavelength varies but, in case of fluorescence excitation measurement, the detection is fixed but the emission wavelength varies [834].

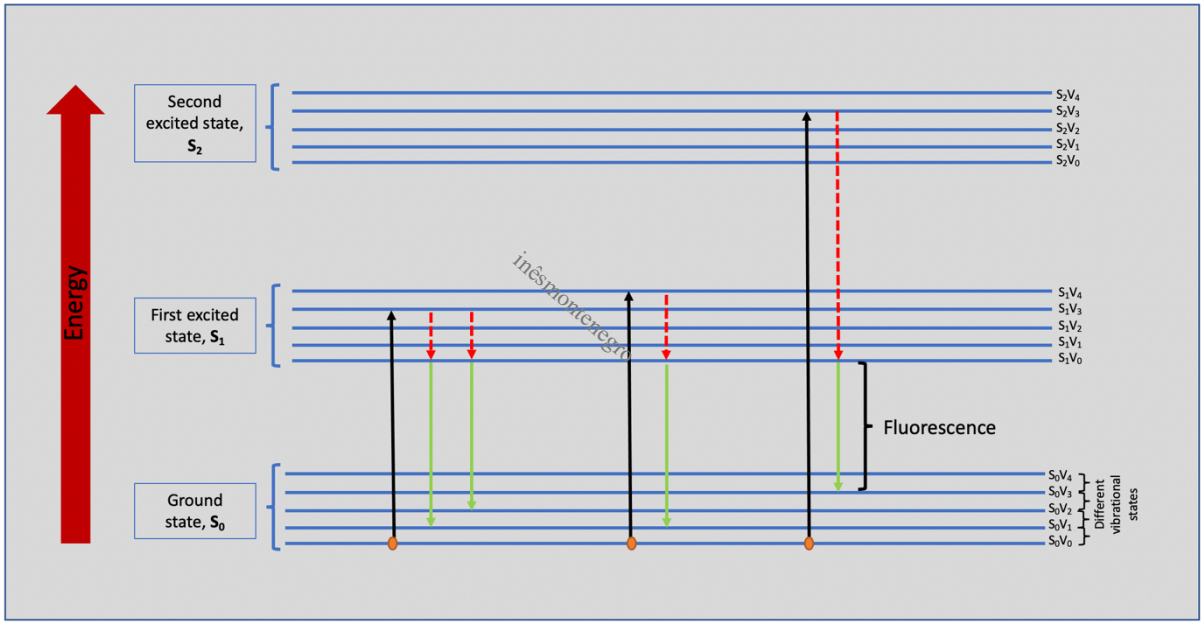


Fig. 15 - *Jablonski* Diagram of molecular absorbance and fluorescence. Representation of energy stage of electron in particle, the low stage (ground state) to eight energy stage (e.g. S_1 or S_2 excited level) after photon absorption provided by the electromagnetic radiation. Only the radiation associated with emission in fluorescence wavelength is detected and quantified (adapted from [834]). The red dotted arrows represent vibrational relaxation from the emission of light by decay to the ground state.

Instrumentation design

Light source, it's composed by lamp that emits near peak wavelengths. For instance, in case of the Xenon arc exhibits continuous emission spectrum with constant intensity, between 300 - 800 nm, but can also be used for just above 200 nm [834].

Before the light reaches the sample, the light goes through an excitation filter (excitation monochromators), and before the light hits the detector the radiation goes through emission filter (emission monochromator) [834], see Fig. 16.

The first monochromator (excitation monochromator) is used for the selection of the excitation wavelength from incident beam. Fluorescent sample will emit the fluorescence in all the directions. The angle of 90° is chosen for emitted florescence and the second monochromator (emission monochromator) is used for determination the fluorescence spectrum [834]. The excitation wavelengths which are frequently being selected are in the

ultraviolet region and the emission wavelength is in the visible region [834].

Detector can be a single-channeled (detects the one wavelength at a time) or multi-channeled (detects all emitted wavelength) both having advantages and disadvantages. Detector is a sensitive photocell, (e.g. red sensitive photomultiplier for wavelengths greater than 500nm) [834].

Fluorophores absorb light of a distinct excitation wavelength and emit, or fluoresce, light of reduced energy thus a longer wavelength.

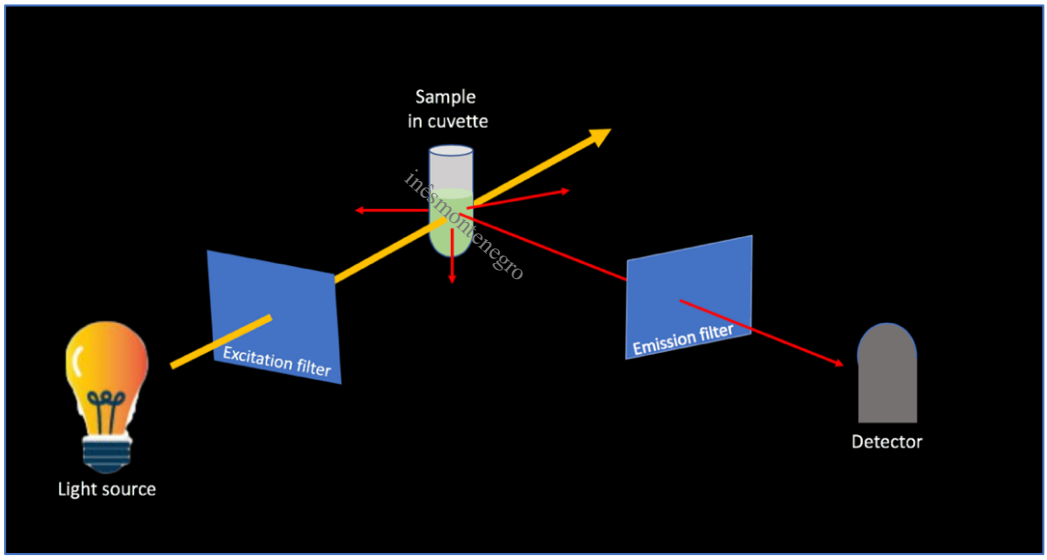


Fig. 16 - The design of fluorometer (adapted from [834]).

Applications of fluorimetry

Fluorometers are used for precise quantitation of biological molecules including nucleic acids and proteins in sub-picogram concentrations. They can measure the concentration of double - and single – stranded DNA (dsDNA and ssDNA) ribonucleic acid (RNA) and proteins in solution with demonstrable applications in a range of biochemical fields. In Table 2 are shown the specification of fluorometer used to DNA quantification in this work.

Table 2 - Specification of fluorometer used to DNA quantification.

Qubit 3
Fluorometer

Specifications Qubit 3 fluorometer	
Instrument type*	Benchtop fluorometer
Light sources*	Blue LED (max ≈ 470 nm) Red LED (max ≈ 635 nm)
Excitation filters*	Blue 430-495 nm Red 600-645 nm
Emission filters*	Green 510-580 nm Red 665-720 nm
Detectors*	Photodiodes - measurement capability from 300 – 1000 nm
Tube type*	0.5ml Real Time PCR (polypropylene) tubes

Source: *Qubit ® 3.0 Fluorometer – user guide – Catalog number Q33216 – Publication number MAN0010866- Revision A:0 ; Life technologies

How does Qubit fluorometer work?

Fluorometer quantify biological analytes as a function of fluorescence. This process requires the sample to be bound to a specific fluorescent agent and loaded into the instrument by a polypropylene tube. In this experimental work the dye was provided as a solution in dimethyl sulfoxide (DMSO) by the Qubit dsDNA HS Assay Kits. A fluorescent dye was added to a solution of DNA. Once added to a solution, the DNA dye binds to the DNA. The dye has extremely low fluorescence until bound to their target molecule. The amount of fluorescence signal from this mixture is directly proportional to the concentration of DNA in the solution.

Annex B -

Extraction of phenolic compounds from sediments

Abstract

Phenolic compounds are present in different matrix. This work, aimed to investigate the levels of phenolic compounds, more specifically NP and OP, in estuarine sediments. For that propose, an extraction method was optimized for the determination of NP and OP in sediment.

The proposed analytical methodology was validated, recoveries and overall method reproducibility were determined by the analysis of sediment fortified with a known amount of the compounds (5 µg / g).

Good recoveries (between 80 -130 %) were obtained with different polarity of the solvent. Methanol was chosen as extraction solvent, due to the good recovery for both phenolic compounds, the simplicity in sample preparation (only one solvent) and also due to the accuracy and precision of recovery values, 96 % (6 % RSD; n = 3) and 99 % (2 % RSD; n = 3), for OP and NP, respectively.

Better recoveries were observed with two sequential extractions with methanol (96 % (7 % RSD; n = 3) and 77 % (5 % RSD; n = 3) than with single extraction (86 % (4 % RSD; n=3) and 69 % (2 % RSD; n = 3) for OP and NP, respectively.

The methodology developed (1 g sediment, 10 mL methanol and two sequential extractions), showed to be suitable for determination of phenolic compounds in complex matrix such as sediments, due to accuracy and precision value, 77 % (5 % RSD; n = 3) and 96% (7 % RSD; n = 3), for OP and NP respectively.

Matrix effect was observed in NP quantification (recovery: 40 (± 10) %), and should be taken into account that the values obtained in real samples are under-quantified. Contrary, no matrix effect observed in the case of OP quantification.

This methodology developed was applied to investigate the level of contamination with alkylphenols (NP and OP) in sediment samples from river Lima and also CWs (influent of CWs was previously doped).

Introduction

Alkylphenols (such as nonylphenol (NP) and octylphenol (OP)) are a metabolic intermediate from microbial transformation of widely used nonionic surfactants, with high toxicity even at extreme low concentrations (e.g. NP; 0.022 - 220 ng L^{-1} [837]). NP and OP has been detected in different ecosystem matrix in the order of ng to µg (e.g. water [96, 336, 338, 340, 341, 343, 838] and estuarine sediment [309, 337, 340–343]). Since these organic compounds appear in the environment at trace and even ultra - trace levels, systems of detection and quantification sufficiently sensitive and selective are needed.

The presence of alkylphenols in different and complex environmental matrices has generated the development of analytical methods for the determination of these substances in various liquid and solid matrices. During the past decade, a lot of extraction techniques have been developed to isolate these analytes in solid samples, to reduce the organic solvent consumption and to increase the speed of the process [839]. In Table 1 are shown with some detail methodologies for extraction and analysis of alkylphenols in complex matrix, found in the literature.

Extraction methodologies for NP and OP in solid samples include conventional methods such as the Soxhlet extraction and advance extraction techniques such as ultrasonic assisted extraction, microwaves assisted extraction (MAE) or pressurized liquid extraction (PLE).

Ultrasonic extraction has been presented as an attractive alternative to the conventional extraction process in solid matrix. Although sonication is faster than Soxhlet extraction, it also requires relative large volumes of toxic and expensive organic solvents [839].

According with Table 1, the solvents more commonly used in the extraction process of NP and OP employing ultrasonic irradiation are methanol, acetone, hexane, dichloromethane and mixtures of them in different proportions.

Different recovery percentages can be observed due, for instance, to the physical state of the matrix material (solid or liquid) or to the solvent group used (e.g., polar / nonpolar and aprotic / protic), etc. Some studies associated the success of recoveries with the capacity of solvents to participate in hydrogen bonding, namely protic solvents (e.g. 2 - propanol [840]).

Based on Table 1 analysis, the recovery values or NP range from 71 to 106 % in sediment, from 70 to 100 % on sewage sludge, from 72 to 85 % on wastewater and from ~50 % to 103 % in water. In the case of OP the recovery values in sediment range from 67 % to 92 %, from 63 to 100 % on sewage sludge, from 98 - 113 % on wastewater and from ~50 % to 110 % in water.

Table 1. Literature survey of extraction and identification techniques used to analyze NP and OP in environmental samples.

Matrix	Substance	Solvent (v : v)	Solvent volume	Sample mass /	Extraction Process	Elution process	Recoveries (%)	Detection limit (LOD)	Analytical technique	Reference
Sewage sludge	NP	Hexane - Acetone (1 : 1)	20 mL	0.03 - 0.3 g	MAE - SPE		91.4 % (4.69 % RSD; n = 6)	2.86 μg g^{-1}	HPLC - FLD	Fountoulakis et al. [841]
Soil	NP	Water - Methanol (3 : 7)	5 mL	2 g	Ultrasonic extraction		84.2 % (14.1 % RSD; n = 5) 84.9 % (5.3 % RSD; n = 5)	0.06 μg g^{-1}	HPLC - FLD	Núñez et al. [842]
Sludge	NP	Dichloromethane - Methanol (7 : 3)	20 mL	2 g	Ultrasonic extraction + SPE (C_{18})		73.5 % (7.8 % RSD; n = 3)	5 μg kg^{-1}	HPLC / ESI - MS	Petrovic et al. [843]
	OP						75.7 % (7.1 % RSD; n = 3)			
Sediment	NP			5 g			90.4 % (3.8 % RSD; n = 3)	2 μg kg^{-1}		
	OP						78.5 % (7.1 % RSD; n = 3)			
Sediment	NP	Methanol - Dichloromethane (7 : 3)	20 mL	5 g	Ultrasonic extraction + SPE (C_{18})		81 % (10 % RSD; n = 3)	7.5 μg kg^{-1}	HPLC - MS	Petrovic and Barceló [844]
	OP						75 % (8 % RSD; n = 3)	5 μg kg^{-1}		
Sediment	NP	Acetone	15 mL	1 g	MAE - SPE		100 % (30 % RSD; n = 3)	100 ng g^{-1}	GC - MS	Bartolomé et al. [845]
Particulate material	OP			500 mL	SPE (C_{18})	Methanol - Dichloromethane (9 : 1)	47 % (≤ 6 % RSD; n = 3) NP 55 % (≤ 6 % RSD; n = 3) OP	0.06 μg L^{-1} OP 0.07 μg L^{-1} NP	HPLC - MS	Céspedes et al. [846]
Wastewater	NP						85 % (≤ 6 % RSD; n = 3) NP 113 % (≤ 6 % RSD; n = 3) OP			
Surface water							87 % (≤ 6 % RSD; n = 3) OP 103 % (≤ 6 % RSD; n = 3) NP			
Sediment	NP	Methanol	10 mL	5 g	PLE		106 % (5% RSD, n=3)	0.01 μg g^{-1}	GC - MS	Ding and Fann [847]
			150 mL	5 g	Soxhlet extraction		85 % (6 % RSD, n=3)	0.01 μg g^{-1}		

(continued)

Table 1 (continued)

Sediment	NP	Methanol	150 mL	5 g	Ultrasonic extraction		82 % (5 % RSD, n = 3)	0.01 μg g^{-1}	GC - MS	Ding and Fann [847]
Sediment	NP	Methanol	400 mL	6 g	PLE		85 % (6 % RSD; n = 3)	0.08 μg g^{-1}	HPLC	Valsecchi et al. [848]
Sediment	NP	Hexane: 2-propanol (7 : 3)	3.5 mL	1 g	Ultrasonic extraction		82.8 % (5.4 % RSD; n = 12)	0.2 ng g^{-1}	HPLC - ESI / MS	Ferguson et al. [79]
Water	NP			1 L	SPE	Acetone	78 % (4 % RSD; n = 12)	0.92 ng L^{-1}	HPLC - ESI / MS	Ferguson et al. [79]
Sediment	NP	Methanol	5 mL	0.2 g	Ultrasonic extraction		95 % (14 % RSD; n = 18)	21.5 ng g^{-1}	HPLC	Ferguson et al. [849]
Sediment	NP	Ethyl acetate	15 mL	1 g	Ultrasonic extraction		109 % (n = 8)	0.1 μg g^{-1}	GC - MS	Blackburn et al. [850]
	OP						92 % (n = 8)			
Wastewater	NP			200 mL	SPE (C_{18})	Water : Methanol (7 : 3)	72 % (8 % RSD; n = 3)	0.04 ng L^{-1}	LC -MS/MS	Sódre et al. [851]
	OP						98 % (7 % RSD; n = 3)			
Soil	NP	Acetone - Hexane (5 : 5)	40 mL	5 g	PLE - SPE (C_{18})		97 % (19 % RSD; n = 3)	30 μg Kg^{-1}	LC–APCI-MS	Andreu et al. [852]
	OP						91 % (15 % RSD; n = 3)			
Water	NP			1 L	SPME	*n*-Hexane : Acetone (6.5 : 3.5)	6 % RSD; n = 5	2.4 μg L^{-1}	GC - MS	Braun et al. [853]
Water	OP		60 mL	300 mL	Liquid - liquid extractions	Dichloromethane	80 % (7.6 % RSD; n = 1 4)	0.17 ng L^{-1}	GC - MS / SIM	Jin et al. [854]
	NP						111 % (1.6 % RSD; n = 14)	0.73 ng L^{-1}		
Water	OP			25 mL	BAμE		99.8 % (4.0 % RSD; n = 3)	0.8 μg L^{-1}	HPLC - DAD	Neng and Nogueira [855]
	NP						96.2 % (4.5 % RSD; n = 3)			
Water	OP			~4 L	Solid - phase extraction	Dichloromethane - Methanol - Acetone	110 %	0.04 - 3 ng L^{-1}	LC - MS /MS	Loyo - Rosales et al. [856]
	NP						95 %			

continued)

Table 1 (continued)

Sediment	OP	Acetone - Hexane (5 : 5)	0.5 mL	1 g	ASE - SPE		67 % (2 % RSD; n = 2)	0.2 - 13 ng g^{-1}	LC - MS / MS	Loyo - Rosales et al. [856]
	NP						95 % (4 % RSD; n = 2)			
Sewage sludges	NP	Methanol - Acetone (1 : 1)	10 mL	0.2 g	SPE (C_{18})		70 - 73 % (5 % RSD; n = 3)	11 µg Kg^{-1}	LC / ESI - MS / MS	Koh et al. [857]
	OP						63 % (5 % RSD; n = 3)	34 µg Kg^{-1}		
Water	NP			400 mL	SPE	Methanol / Acetone / Ethylacetate (2:2:1)	~ 50 %	10 ng L^{-1}	HPLC - MS / MS	Loos et al. [858]
	OP							5 ng L^{-1}		
Water	OP			500 mL	SPE (C_{18})	Acetone	85 - 110 % (6 - 13 % RSD; n = 5)	0.06 ng m L^{-1}	GC - MS	Yang et al. [859]
Water	OP			1 L	SPE (ENV+)	Ethylacetate	99 % (8% RSD; n = 5)	0.01 ng L^{-1}	GC - MS	Bolz et al. [860]
Water	NP				SPE (ENV+)	Acetone / Methanol / Water (6 / 10 / 5 mL)	100 % (7 %; n=3)	< 10 - 50 ng L^{-1}	GC - MS	Bolz et al. [861]
	OP						110 % (4 %; n=3)			
Sediment	NP	Methanol : Diethylether (10 : 1) + 0.1 % (v / v) HCl		10 mg	Soxhlet extraction + SPE (ENV+)	Acetone (5 mL)	71 % (9 % RSD; n = 3)	≤ 0.5 mg kg^{-1}	GC - MS	
	OP						70% (21 % RSD; n = 3)			
Sewage sludge	NP	Methanol : Diethylether (10 : 1) + 0.1 % (v / v) HCl		3 g	Soxhlet extraction + SPE (ENV+)	Acetone (5 mL)	100 % (2 - 34 % RSD; n = 4)	< 0.5 - 19 mg kg^{-1}	GC - MS	
	OP									
Soil	NP	Methanol	10 mL	0.5 - 2 g	MAE		98 % (4.5 - 5.9 % RSD; n = 11)	0.5 ng Kg^{-1}	GC - MS	Azzourz and Ballesteros [862]
	OP						98 % (6.0 - 6.8 % RSD; n = 11)	1 ng Kg^{-1}		

SPE - Solid phase extraction; **PLE** - Pressurized Liquid Extraction; **APCI** - Atmospheric Pressure Chemical Ionization; **MS** - Mass Spectrometry; **HPLC** - High Performance Liquid Chromatography; **SPME** - Solid Phase Microextraction; **SIM** - selected ion monitoring; **BaµE** - Bar adsorption microextraction; **DAD** - Diode Array Detection; **ASE** - Acelerated Solvent Extraction; **RSD** - Relative Standard Deviation; **(ENV+)** - polystyrene copolymer phase ; **C_{18}** - Reversed Phase C18 SPE Cartridges; **MSD** - Mass Selective Detector ; **MAE** - Microwave Assisted Extraction; **FLD** - Fluorescence detector

Different extraction and quantification methodologies have been used. In liquid sample, extraction methodology ranged from classical liquid - liquid extraction to advanced extraction and preconcentration techniques such as solid phase extraction (SPE), solid phase microextraction (SPME) or bar adsorptive microextraction (BAμE). Given the low polarity of NP and OP molecules, the most widely used analytical methodologies for the determination of trace levels of these compounds is chromatographic separation, high - performance liquid chromatography (HPLC) or gas chromatography (GC). Electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) are the processes most used in the ionization step however, ionization process is not always referred in the studies found. For most cases, independently of the matrix, mass spectrometry (MS or in tandem (MS / MS)) was used for the detection of alkylphenols as observed in Table 1, but other detectors such as fluorescence detector (FLD) have been used. According with Reemtsma [863] the MS may provide further structural and quantitative information, facilitating the correct and unambiguous identification of each analyte and an increase in sensitivity. The major weakness of "traditional" methodologies for phenolic compounds, such as NP or OP, analysis can be solved by coupling LC to MS, particularly to a tandem mass spectrometer (LC / MS / MS).

In order to optimize a methodology for determination of phenolic compounds, nonylphenol and octylphenol, in sediment samples, effective parameters such as type of the most suitable extraction solvents was investigated. Several solvents and solvent mixtures were tested: hexane - acetone (1:1 *v/v*), water - methanol (3:7 *v/v*), methanol - dichloromethane (7:3 *v/v*), acetone - methanol (1:1 *v/v*), acetone and methanol.

Materials and Methods

Reagents and chemicals

The following alkylphenols were analytical grade: 4-Nonylphenol (Lot # SZBD273XV; CAS 104-40-5; $C_{15}H_{24}O$; MW: 220.35 g mol^{-1}) standards of 99.8 % (w / w) purity, purchased from Sigma-Aldrich (Germany) and 4-ter-Octylphenol (Lot # MKBT8005V CAS 140-66-9 MW: 206.32 g mol^{-1}) purchased from Supelco.

Individual stock standard solutions (1mg mL^{-1}) of each compound were prepared in methanol and stored at 4º C in the dark.

The following solvents were HPLC grade and purchased from SIGMA - ALDRICH (Steinheim, Germany): acetone (Lot # SZBD014SV; CAS 67-64-1; C_3H_6O; MW: 58.08 g mol^{-1}), *n*-hexane (Lot # 9187S; CAS 110-54-3; C_6H_{14}; MW 86.18 g mol^{-1}) and dichloromethane (Lot # 00556EE; CAS 75-09-2; CH_2Cl_2; MW 84.93 g mol^{-1}). In case of methanol (Lot # 1678910; CAS 67-56-1; CH_4O; MW: 32.04 g mol^{-1}) also HPLC grade, was purchased from Fisher Scientific (Loughborough, UK).

The deionized water (was properly deionized through an ion exchange system resin membrane) with conductivity <0.1 µS cm^{-1} was used in this experiment.

All glass material used was properly decontaminated by washing with deionized water, immersion in a 20 % (*v* / *v*) nitric acid solution overnight, washing with deionized water and dried in an oven at 30 °C.

Sediment fortification process

Sediments (from *Lima River* estuary) used for fortification experiments were air - dried and sieved through 0.2 mm mesh sieve. A total of 200 g of sediments were mixed with acetone (100 mL) until the sediment were completely soaked to form a slurry. This slurry sediment was spiked with an appropriate volume of the standard solution to achieve 5 µg g^{-1} levels of each analyte. The contents were mixed well with a glass rod for over 1 h. To avoid photodegradation of the phenolic compounds the glass beaker with sediment was wrapped in aluminum foil. The sample was left for three days in a hotte to dry completely and aged about one week at room temperature. The prepared sediment was store in aluminum foil in a dry place until analysis.

Extraction process

To test the solvent extraction, 1 g of subsamples (spiked sediment) was ultrasonicated for 15 min in ultrasonic bath with one solvent or solvent mixture (hexane - acetone (1:1 *v*/*v*); water - methanol (3:7 *v*/*v*); methanol - dichloromethane (7:3 *v*/*v*); acetone - methanol (1:1 *v*/*v*); acetone; methanol). The extract was separated by centrifugation at 2500 rpm for 5 min. The extraction was repeated two times with fresh solvent. The extracts were stored in precleaned glass at 4º C until analyzed. The flow diagram depicting the overall protocol for test the best solvent in spiked sediment are shown in Fig. 1.

Analysis of the extracts

The analysis of the phenolic compounds was carried on a GC - MS system, Saturn 2000 model from Varian brand, with a gas chromatograph (3900), injector (1177), mass spectrometer detector (Saturn 2000) and equipped with a Autosampler (CombiPal model). The instrument operational conditions are described in section 3.5.4.

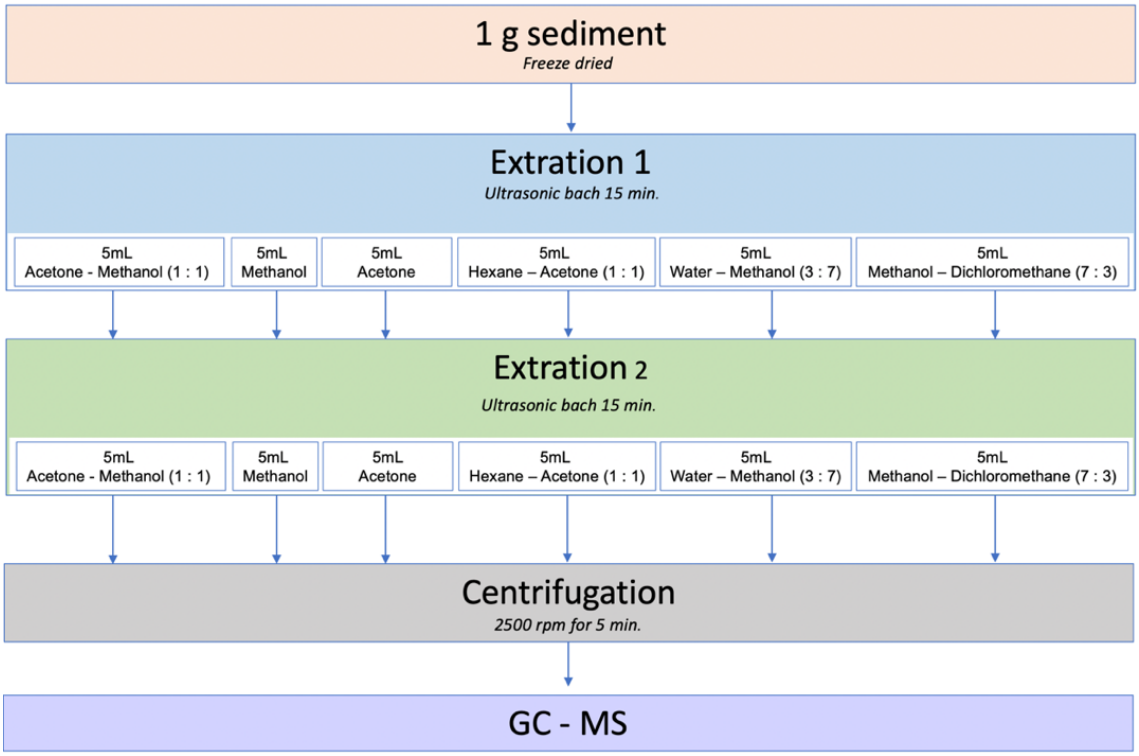


Fig. 1 - flow diagram depicting the overall protocol for test the best solvent in spiked sediment.

Linearity, precision and detection limits

For every set of samples, a procedural blank and a standard solution consisting of all the reagents were run to check for interferences and cross - contamination. The external standards were used for calibration. Calibration curves were established with standard solutions of six concentration levels (1, 5, 10, 15, 30 and 60 μg mL^{-1}) for each compound NP and OP).

Each calibration standard was measured tree times. The correlation coefficients of the calibration curves were all above 0.99. LOD, calculated as the minimum amount of a compound present in a sample that produced a signal to noise ratio of 3, according to the IUPAC recommended methods as described in Long and Winefordner [864].

Recoveries

For each solvent (isolated or mixed) three fortified sediment samples were measured, and the obtained recovery of each analyte was the average value of the three trials.
For calculating the % of recovery:

$$\%\, recovery = \frac{[C_s]}{[C_p]} \times 100 \quad (1)$$

where, $[C_s]$ is the concentration of the analyte in the fortified matrix extract and $[C_p]$ is the concentration of analyte used in fortified sediment (5 μg g^{-1} dw.)

Data acquisition and processing

Data acquisition and processing were conducted using V*arian Star Chromatography WorkStation 6.30* software. Statistical studies were performed using one one - way analysis of variance (ANOVA) and significant differences was detected by a multiple *Tukey* comparison test.

Results and discussion

Solvent selection

To optimize the method used for determination of phenolic compounds in sediment samples, effective parameters such as kind of extraction solvents was studied (Fig. 1). Two phenolic compounds were selected, NP and OP. Solvents and solvent mixtures were tested namely acetone, methanol, hexane - acetone (1:1 v/v), water - methanol (3:7 v/v), methanol - dichloromethane (7:3 v/v), acetone - methanol (1:1 v/v).

The low value of LOD, LOQ and linearity (R^2) of a standard calibration curve (> 0.993) were taken into account in the analytical procedures to selected the best extraction solvent (Table 2).

Recoveries of the target phenolic compounds for each extraction solvent are show in Fig. 2. Lower recoveries (81 % for NP and 84 % for OP) were obtained by the use of the solvent mixture with very different polarities (acetone - hexane). But the recoveries increased

(104 % for NP and 105 % for OP) in the case of the mixture with similar polarities (acetone - methanol).

Table 2 Parameters that have been taken into account in th analytical procedures for solvent selection.

	NP	OP
LOD (µg L^{-1})	0.076	0.040
LOQ (µg L^{-1})	0.253	0.134
R^2	0.9949, n = 5	0.9939, n = 5

Recovery was high than 100 % for OP (134 %) and NP (124 %) when a solvent mixture with similar polarity (water - methanol) and solvents with different polarities (methanol - dichloromethane), respectively, were tested.

Two solvents with different polarities were used alone, acetone (slightly polar) and methanol (high polar). Both showed good recoveries. Recoveries were within the range to be taken into account for a good recovery (80 % - 120 %), however the RSD when methanol was used (2 % and 6 % RSD, n=3) was lower than in the case of acetone. Therefore, methanol was the solvent of choice for sediment analysis.

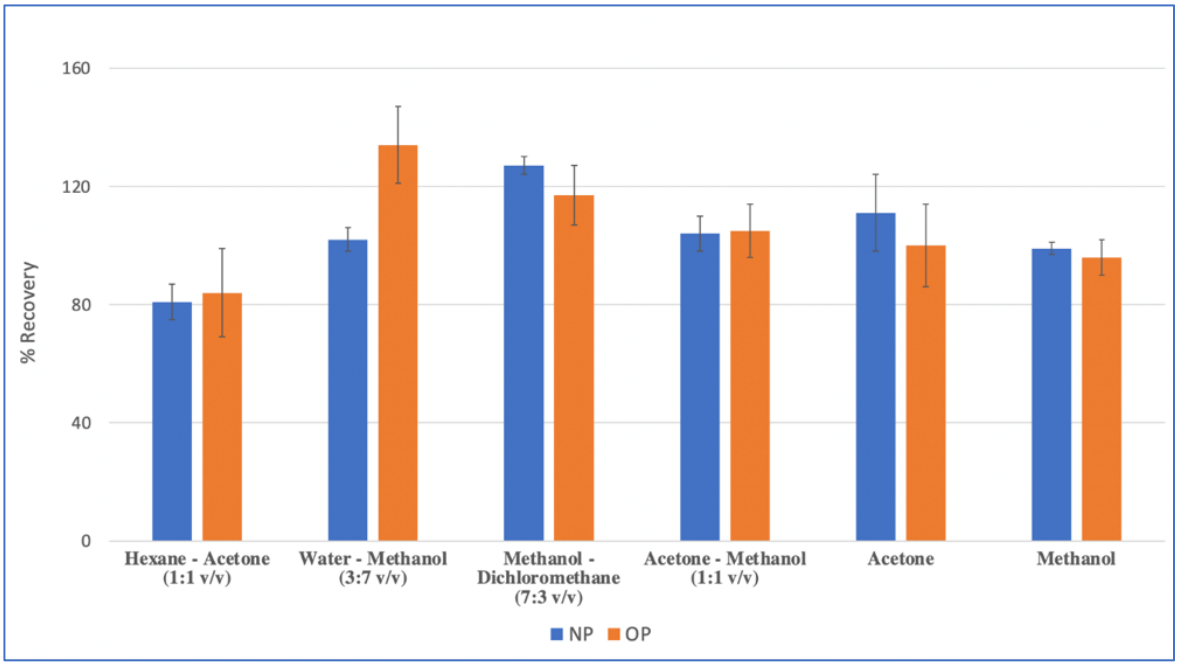


Fig. 2 - Percentage of recovery of phenolic compounds for each solvent tested (n=3).

Comparison between sequential extraction and single extraction

After the choice of the more suitable solvent, i.e. methanol, the extraction single and double was tested. The same steps were used in both group of extractions, as described in the flow diagram in Fig. 3.

The analytical method was carefully optimized and their characteristics precision, accuracy, limits of detection (LOD), limits of quantification (LOQ), correlation coefficient (R^2) and matrix interference were assessed. Calibration curves were constructed for NP and OP from 1 to 60 µg L^{-1}. These curves were evaluated by analysing standard solutions prepared in Milli-Q water in triplicate at six different concentration levels. Excellent linearity was achieved in these concentration ranges, for which correlation coefficients higher than 0.99 were calculated for all validation batches.

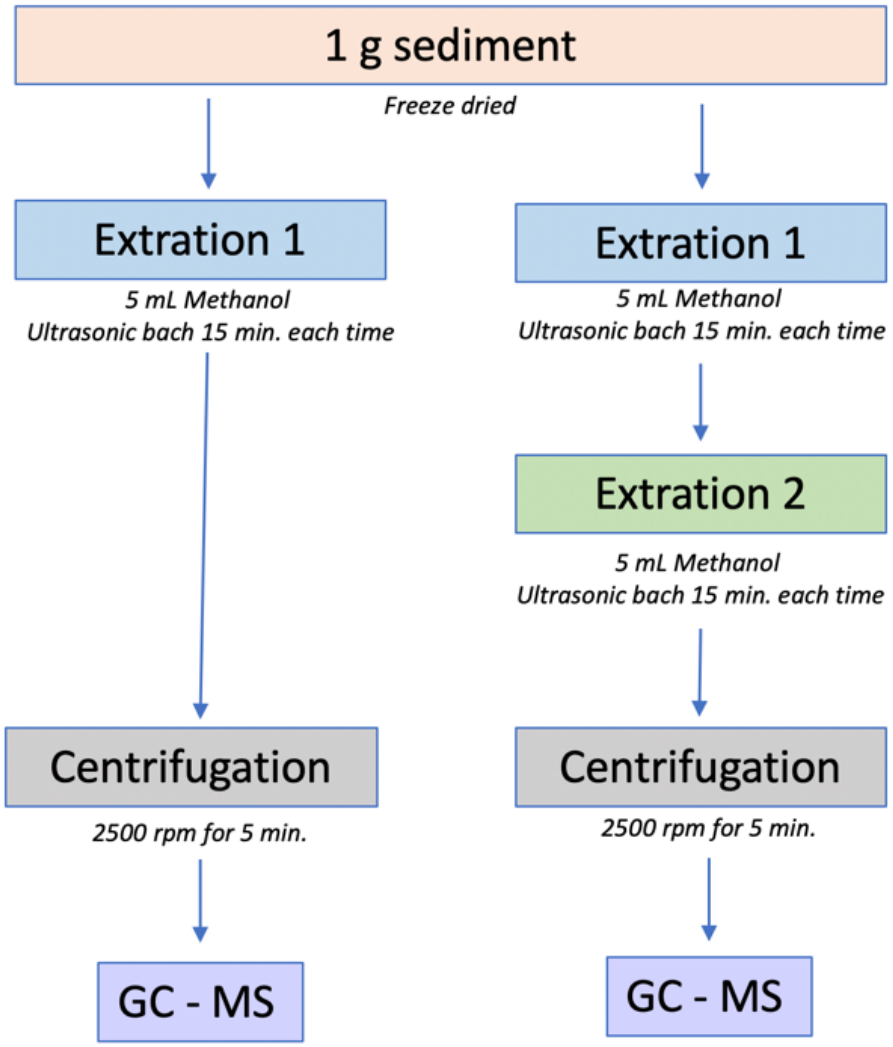


Fig. 3 - Flow diagram depicting the overall protocol for the determination of alkylphenols in sediment sample.

Replicate analyses were performed to determine the recovery (n = 3) of the proposed method. The recovery calculations were done in triplicate of sediment fortified with all of the analytes under study at 5 μg g^{-1}. Precision was expressed as the relative standard deviation (% RSD), for which values of 5 % (OP) and 7 % (NP) were obtained. Accuracy was always expressed as recovery (spiked samples). Recoveries achieved for each analyte are displayed in Table 3. The results for sequential extraction with methanol showed a higher recovery for both compounds NP (96 %) and OP (77 %) than the single extraction. Therefore, two sequential extractions, was chosen based in these results.

Evaluation of matrix effect

An important consideration has to be given to the potential problems that may arise from matrix interference, especially when dealing with complex matrices, such as sediment or sludge. Matrix effects can be observed with any analytical tool, are not associated with the sensitivity of the device, because of insufficient co-extractive removal during the sample preparation stage. The matrix interference on the recovery of compounds was evaluated for sediment based on 100 μL of standard addition (10 μL L^{-1}) to 100 μL of extract (fortified sediment) and a blank using 100 μL methanol addition to 100 μL of extract (fortified sediment).

The signal suppression was derived using the following equation:

$$\frac{[C_{ap}]-[C_b]}{[C_p]} \times 100 \qquad (2)$$

where, [C_{ap}] is the concentration of the analyte in solution with sediment extracted and addition of standard solution; [C_b] is the concentration of the analyte in solution with sediment extracted without addition of standard solution; [C_p] is the concentration of the analyte in standard solution used.

Greater matrix effect was observed in NP quantification. Only 40 % of the concentration of fortified sediment extract was recovered. That mean that the result observed in real samples are under quantified. In the case of OP quantification, the recovery of the analyte was higher (84 (± 14) %) the matrix did not seem to interfere with the quantification.

There are several recognized analytical approaches that can be assessed for reducing potential matrix interferences [865, 866], for instance the calibration standards made up in the matrix material, and calibration based on standard additions.

Table 3 - Recovery of single and two sequential extractions for NP and OP (average ± standard deviation; n = 3) and the parameters that have been taken into account in optimized method.

		NP	OP
Type of extraction (average ± stdev; n = 3)	**Single**	86 ± 4 %	69 ± 2 %
	Double	96 ± 7 %	77 ± 5 %
LOD (µg L^{-1})		0.093	0.041
LOQ (µg L^{-1})		0.309	0.137
R^2		0.998	0.999
Matrix effect (average ± stdev; n = 3)		40 ± 10 %	84 ± 14 %

Conclusions

The results observed in this work, demonstrate that a suitable methodology was optimized that allowed to determined OP and NP from sediments. The methodology used was based on two sequential extractions with methanol, the recovery showing acceptable values. The matrix effect was observed in the case of quantification of NP in sediment.

Annex C - Control chart

In this work, the “control chart” was used to control how the GC-MS behavior over the experimental measurement time. Data were plotted in the time order. A control chart always has a central line for the average, an upper line for the upper control limit and a lower line for the lower control limit. In our case was used three upper / lower control limit (±1σ; ±2 σ; ±3σ). It was used de average and standard deviation in control chart construction.

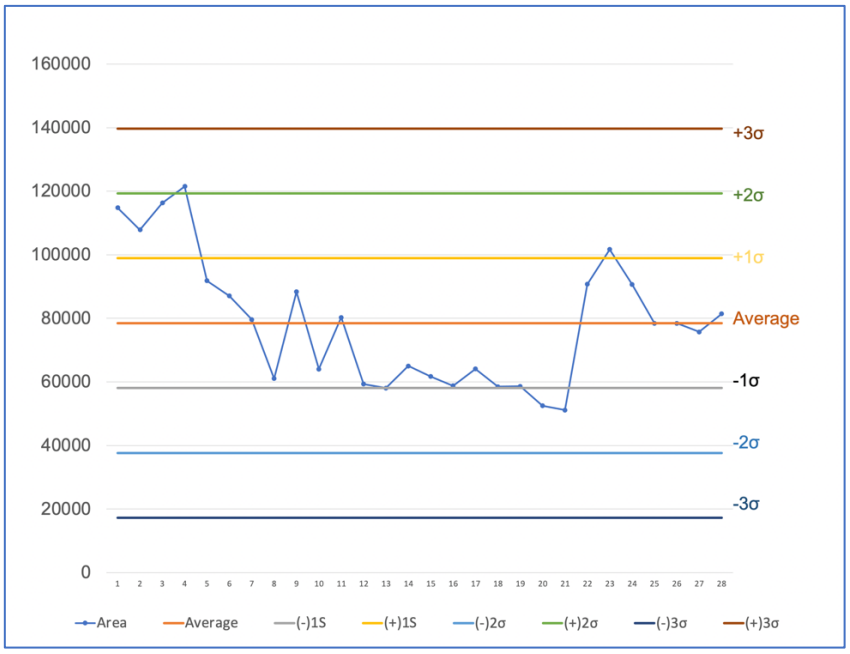


Fig. 1 - Control chart of the lower concentration (5µL) of straight line, over the experimental measurement time of OP.

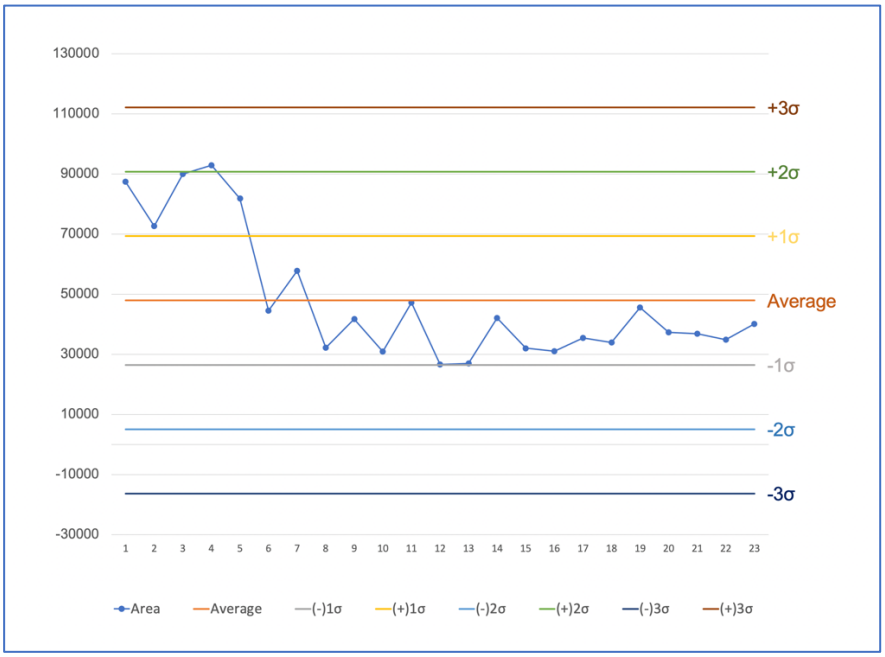


Fig. 2 - Control chart of the lower concentration (5 µL of straight line, over the experimental measurement time of NP.

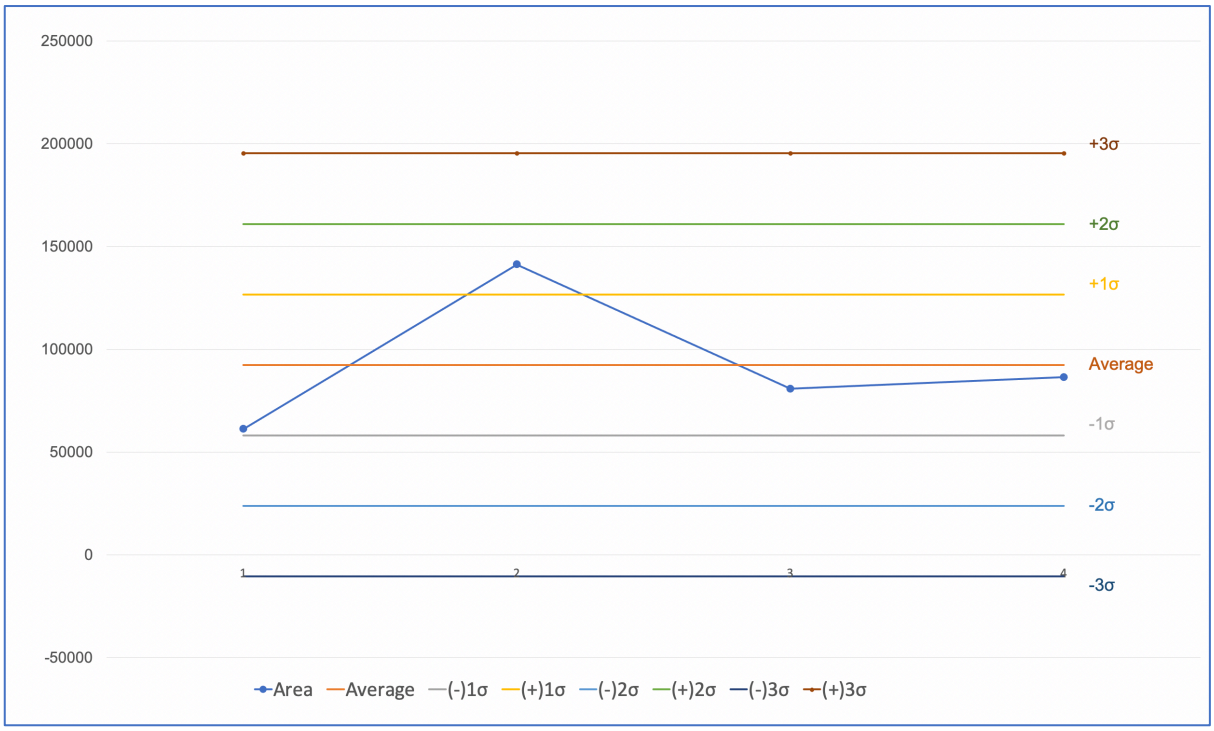


Fig. 3 - Control chart of the lower concentration (10 µL) of straight line, over the experimental measurement time of PCP.

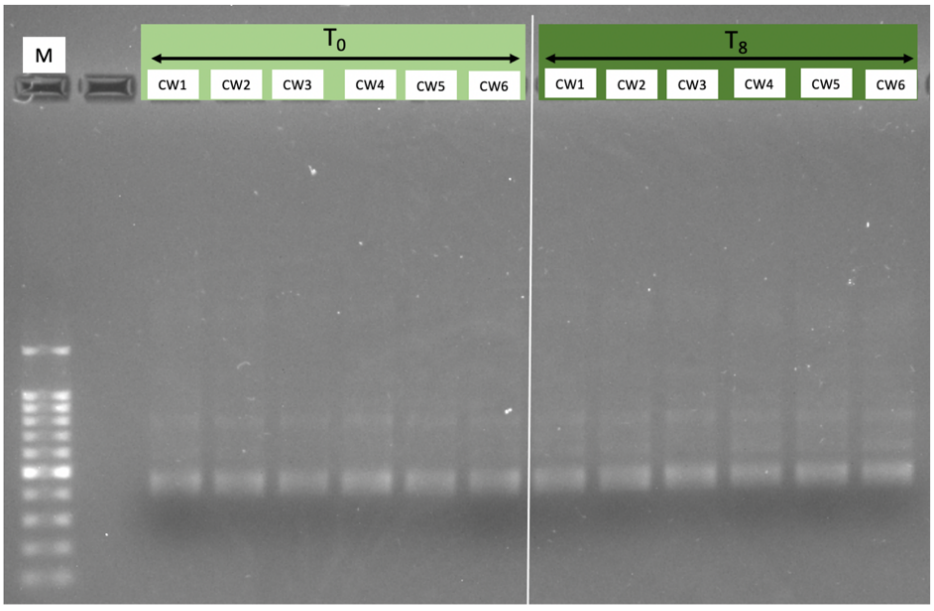
Annex D -

Results from biological analysis

Annex D1 - DNA quantification in experiment #1.

DNA quantification				
Sample			**Sediment (g)**	**DNA (ng / µL)**
T0	Control	1	0.517	7.67
		2	0.612	11.8
		3	0.689	12.0
	Doped	1	0.526	4.56
		2	0.556	3.69
		3	0.558	5.04
T8	Control	1	0.509	12.4
		2	0.527	4.48
		3	0.730	2.41
	Doped	1	1.047	12.8
		2	1.318	26.3
		3	0.566	25.8

Annex D2 - Gel eletroforesis in experiment #1.



T_0 - initial time
T_8 - final time
CW_1 - not doped
CW_2 - not doped
CW_3 - not doped
CW_4 - doped
CW_5 - doped
CW_6 - doped

Annex D3 - DNA quantification in experiment #2.

DNA quantification				
Sample			**Sediment (g)**	**DNA (ng / µL)**
T0	Control	1	0.508	11.7
		2	0.502	12.7
		3	0.503	10.7
	Doped	1	0.501	6.56
		2	0.523	9.97
		3	0.505	15.6
T8	Control	1	0.503	14.2
		2	0.510	14.3
		3	0.509	9.8
	Doped	1	0.511	12.2
		2	0.508	15.9
		3	0.516	22.4