Ph. D. thesis

Bio- and phyto-remediation of an historically PCB-contaminated soil

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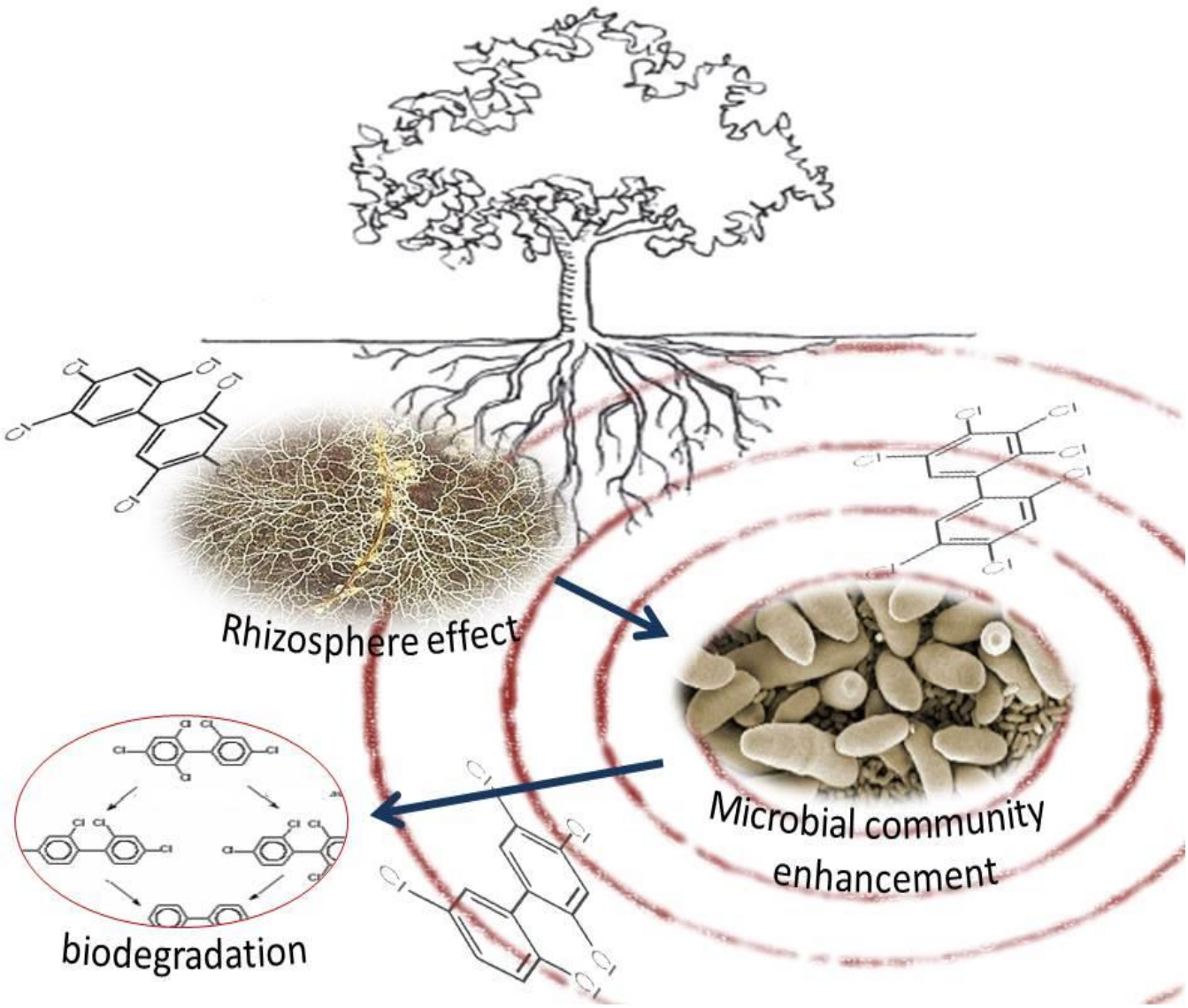
Academic year 2014-2015



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Bio- and phyto-remediation of an historically PCB-contaminated soil

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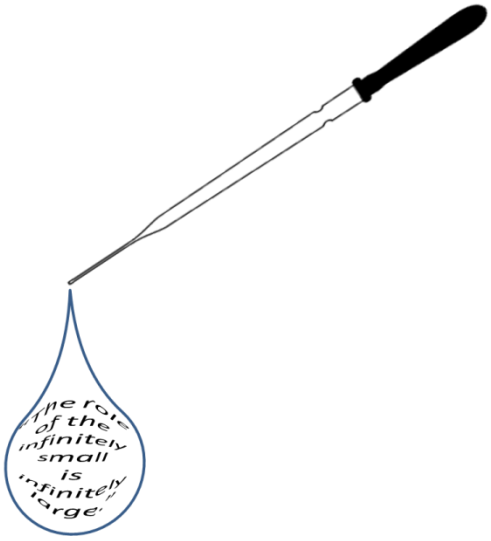

Louis Pasteur (1862)

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1. General introduction

The homeostasis concept, typical of natural ecosystems, is at the base of phytoremediation. We can exploit such ability to maintain a biological equilibrium for the restoration of contaminated environments. Phytoremediation approach uses plants to create ecosystems that stabilize or reduce contamination in environmental matrices such as air, soil, sediments, surface water, or ground water. However the plants are not the sole player in the phytoremediation processes; in most of cases the whole rhizosphere ecosystem (the ensemble of roots and microorganisms inhabiting the volume influenced by roots) contribute to attenuate the pollution. Phytotechnologies allow us to operate *in situ*, at relatively low costs, also improving the aesthetic nature of the site.

This Ph. D. thesis originated from the need to remediate a soil contaminated with polychlorinated biphenyls (PCBs) and heavy metals using phytoremediation. The purpose of the present work is to find the best conditions in order to optimize the phytoremediation treatment; to this aim we proceeded along the following steps:

- to gather and organize information and data about the plant species used in phytoremediation
- to review the scientific literature about phytoremediation and bioremediation of PCBs
- to draw-up an analytical protocol for the extraction, clean-up and detection of PCBs in soil
- to test different treatment conditions in a microcosm trial and select the best ones.

The reviewing activity of the present study has not been restricted to first period of the Ph. D. program but developed along the entire course of the project, which led to the publication of a book, a review article and of a section of the IBAF (CNR) web-site.

This Ph.D. thesis is divided in 3 parts; each of them is organized as monothematic chapter, with an identical internal partition: Introduction, Materials and Methods, Results, Discussion and Conclusion. The three parts are the following:

PART I. "*Phytoremediation potentially useful plants: the selection of the appropriate species for each specific situation of environmental pollution*". The evolution and the problems of phytotechnologies are pointed out, the book and the web-site projects are introduced and drafting rules are described. At the end of this section some excerpt from the edited book and of the realised web-site are quoted.

PART II. "*Analytical methods for extraction, clean-up and detection of PCBs in environmental samples*" All the experimented analytical methods are briefly described; advantages and applicability of the selected protocol of analysis are discussed.

PART III. "*Plant-assisted bio-remediation study using historically PCB contaminated soil*", the current state of knowledge and the research perspectives on bio- and phyto-remediation of polychlorinated biphenyls (PCBs) are reviewed, experimental design and analytical methods employed are described and results obtained are discussed.

During the experimentation in microcosms, the study of PCBs degradation has been supported by microbiological analysis performed by a research group of Water Research Institute (IRSA)- CNR. The experimental phase has been held in the greenhouse of the Institute of Agro-Environmental and Forest Biology (IBAF-CNR, Roma); chemical analyses have been carried out in the Institute for Atmospheric Pollution (IIA-CNR, Roma).

PART I Phytoremediation potentially useful plants: the selection of the appropriate species for each specific situation of environmental pollution.

1 Abstract

The infinite variability of biodiversity offers a wide spectrum of tools to remediate water and soils by using plants. Phytoremediation systems are site dependent: each remediation project must be conceived individually, depending on the soil and the climatic conditions, on the type and the history of the contamination. In this context, the choice of the plant species and varieties is of crucial importance in order to optimize each remediation process.

We pointed out the lack of a global database gathering the botanical and ecological information finalized to remediation purposes and describing the potentialities of each species in phytoremediation. Although those topics have been object of many scientific researches, the knowledge about it is scarcely available to both the general public as well as the specialised audiences.

Furthermore some botanical and ecological information are site-specific, i.e., data on the life cycle, the plant habit or the invasive potential are not generalizable to all the geographical regions. While there are several publication dealing with the autochthonous flora used for phytoremediation purposes in England, France and U.S., this type of information is lacking for the italian territory.

Two projects arose from the above observations:

- to develop a global phytoremediation plant database on the web-site of the Institute of Agro-Environmental and Forest Biology (IBAF, CNR).

- to produce a publication describing the botany and the ecology of italian plant species used in water remediation, gathering hands-on experiences and tested knowledge from different applications of water phytoremediation.

2. Introduction: the phytoremediation technology and the plant selection criteria

In this section phytoremediation will be introduced with a special focus on evolution and problems of phytotechnologies management.

Phytoremediation, recently supplanted by the term “phytotechnologies”, is a clean-up technology that use plants to remediate or contain contaminants in air, soil, groundwater, surface water, or sediments. This technology makes use of the naturally occurring processes by which plants and their microbial rhizosphere flora degrade and sequester organic and inorganic pollutants. It have become attractive alternative to conventional clean-up technologies due to relatively low costs and the inherently aesthetic nature of planted sites. Typical organic contaminants (“organics”) that can be addressed using this technology include petroleum hydrocarbons, crude oil, chlorinated compounds, pesticides, and explosive compounds. Typical inorganic contaminants (“inorganics”) include salts (salinity), heavy metals, metalloids, and radioactive materials.

2.1 Why phytotechnologies have still not been largely applied

After more than two decades of scientific development in phytoremediation, recent reviews still consider phytotechnologies as an emerging tool, showing that the reliability of those techniques, even inside the scientific community, has not yet been achieved (Conesa, Evangelou et al. 2012). There are differences between Europe and North America in the commercial management of phytotechnologies. North America has higher private investment in phytotechnologies, and this has resulted in a larger number of profitable private companies. In contrast, Europe is more focused on solving fundamental issues and in describing biological mechanisms. Consequently, North America is far ahead of Europe in the application and commercialisation of phytotechnologies (Marmiroli, Marmiroli et al. 2006, Conesa, Evangelou et al. 2012).

European normative and public opinion are still precautionary in the use of phytoremediation; in particular in Italy the real application of phytotechnologies seem to be blocked. The following constraints have been evidenced:

- the lack of venture capital and thus the lack of innovative remediation companies dedicated to phytoremediation services or diversified speciality companies involved also in phytotechnologies. The research team may be involved but usually lacks adequate business experience for commercialization

- during the last years the Italian government, a major client in clean-up, had limited resources, which are rarely employed for the remediation purposes

- field trials or commercial operations that demonstrate successful phytoremediation are still lacking; uncertainties in long-term effectiveness and difficulties in the transfer of particular metabolic pathways to productive and widely available plants make the investments in phytotechnologies less safe and attractive (Mench, Lepp et al. 2010)

- lack of transparency and efficiency in the management of polluted sites

All of these factors contribute to lags in development of phytotechnologies in Italy.

Informing and communicating remain key aspects however, due to the relatively limited awareness (and indeed scepticism) of phytotechnologies as practical site solutions in Europe. This is an area where demonstrator sites can make a significant contribution to stakeholder engagement via providing evidence on the effectiveness of phytotechnologies under varying site contexts and conditions, and data for economic and other assessments (e.g. LCA) (Cundy, Bardos et al. 2013).

Furthermore to establish phytoremediation as a reliable clean-up method, documented accurate assessment of the post-harvest strategies is also necessary. Disposal and transformation of harvested material is rarely taken into consideration; up to the present post-harvest improvements seem to have not developed consistently with pre-harvest approaches (McCUTCHEON and Schnoor 2004).

Anyway, as pointed out by Conesa *et al*., one of the main barriers for the application of phytotechnologies is the absence of economic studies, cost evaluations and mathematical models to calculate the performance of phytoremediation systems (Conesa, Evangelou et al. 2012).

2.2 The core of phytoremediation processes: the rhizosphere

Most of the phytoremediation processes take place in the rhizosphere, the area around a

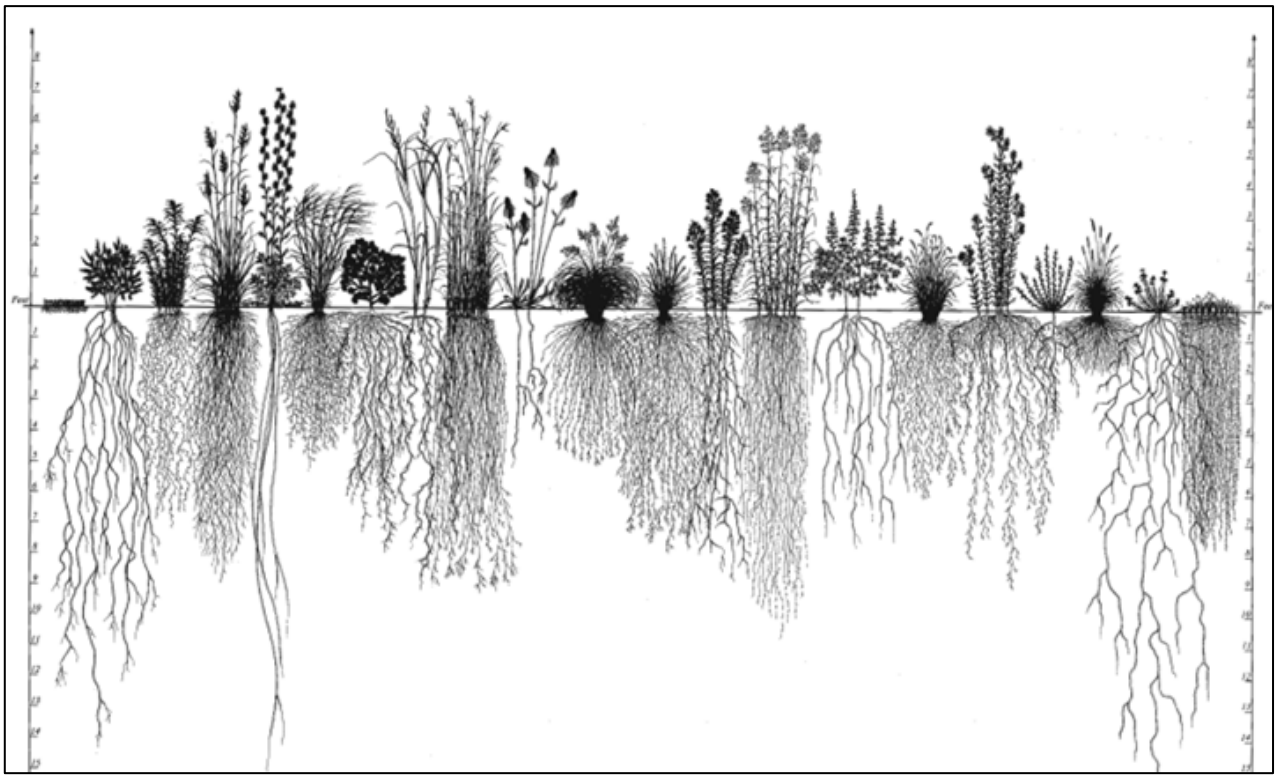

Figure 1. Image showing the diversity of root system architecture in prairie plants. Source: Conservation Research Institute, Wisconsin, USA.

plant root that is inhabited by a unique population of microorganisms influenced by the chemicals released from plant roots, as described for the first time by Lorenz Hiltner in 1904. As might be expected because of the inherent complexity and diversity of plant root systems (Figure 1), the rhizosphere is not a region of definable size or shape, but instead, consists of a gradient in chemical,

biological and physical properties which change both radially and longitudinally along the root. Rhizodeposits make the rhizosphere a desirable niche for microbial communities to proliferate.

One teaspoon of bare or tilled soil contains more microorganisms than there are people on Earth, however, the rhizosphere can have 1000-2000 times that number (1010-1012 cells per gram rhizosphere soil) making it a pretty crowded place (McNear Jr 2013). The plant root-soil interface is therefore a dynamic region in which numerous biogeochemical processes take place driven by the physical activity, and the diversity of chemicals released by the plant root.

2.3. Mechanisms involved in phytoremediation

In the rhizosphere the pollutant is susceptible to uptake, translocation, chelation or degradation, depending mainly on its chemical nature and on the involved vegetal species. The specific phytoremediation mechanism used to address a contaminant is dependent also on the remediation goals; typical goals include containment, stabilization, sequestration and degradation. To achieve these goals, the proper phytoremediation system must be designed using detailed knowledge of the site layout, soil characteristics, hydrology, climate conditions, analytical needs, operations and maintenance requirements, economics, public perception, and regulatory environment (ITRC, 2009).

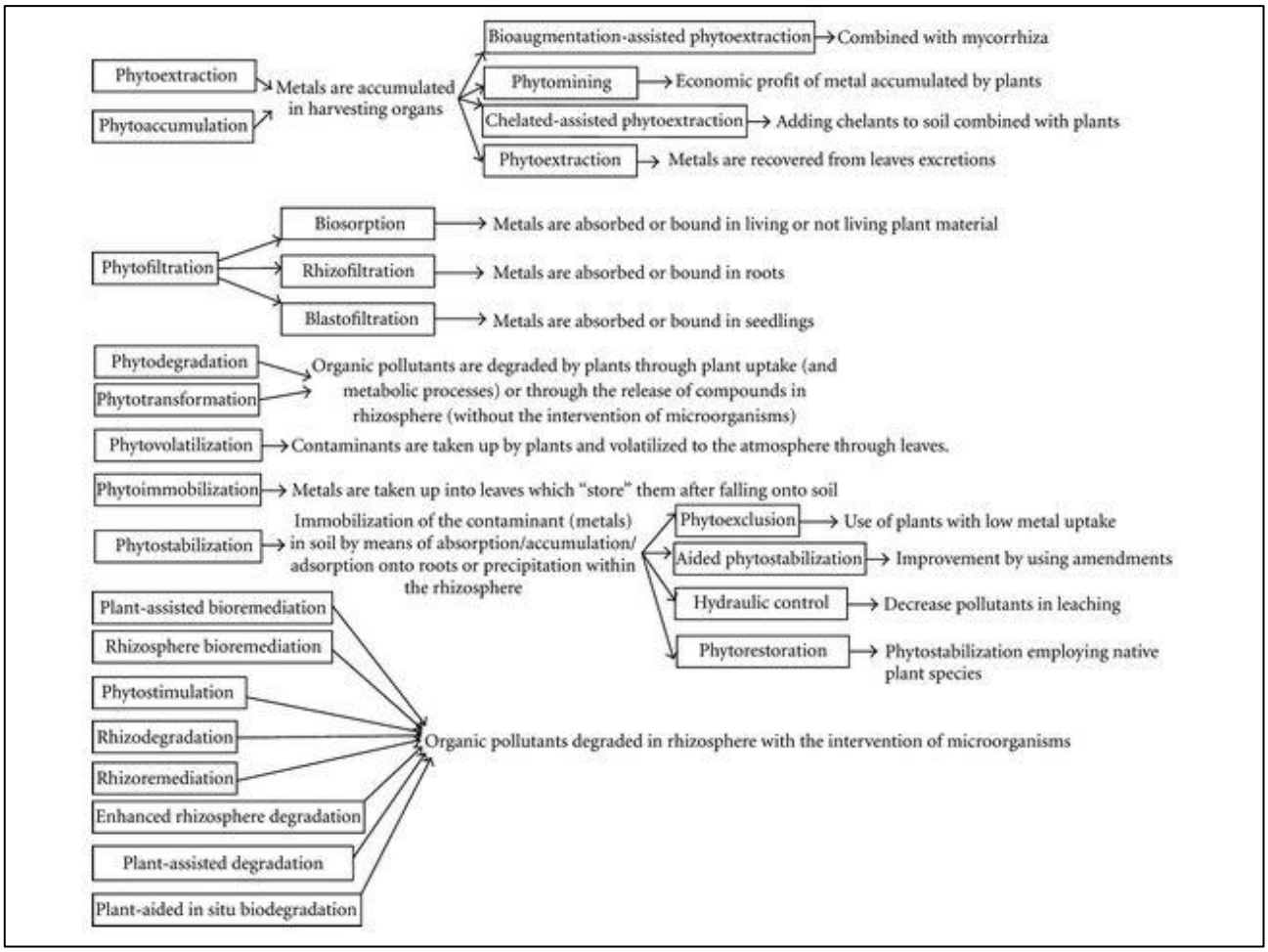

Figure 2. Current classification of most frequently used phytotechnologies for soil remediation. Source: (Conesa, Evangelou et al. 2012)

The number of concepts/processes in phytotechnologies has recently increased with the development of new research domains, and consequently new terms have been introduced to describe new techniques and findings in addition to the renaming of existing techniques (Figure 2). The explosion of new terms may bring an additional problem for the commercial development of phytotechnologies because it may confuse nonspecialized stakeholders who are not familiar with these fields. There is an imperative for researchers to clarify these concepts (Conesa, Evangelou et al. 2012).

A series of six phytotechnology mechanisms have been identified in the last update of "Phytotechnology technical and regulatory guidance and decision tree" by the Phytotechnologies Team of the Interstate Technology and Regulatory Council (ITRC, 2009): phytosequestration, rhyzodegradation, phytohydraulics, phytoaccumulation, phytodegradation, phytovolatilization.

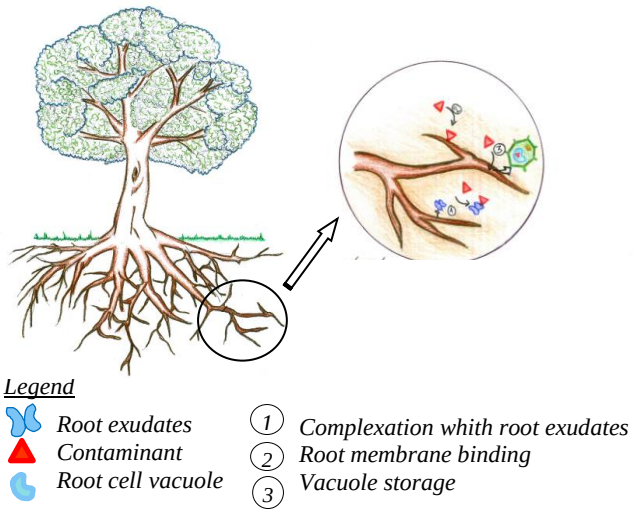

Figure 3. Phytosequestration process. Illustration by L. Donatsch

The term **phytosequestration**, also called phytostabilisation**,** denotes the ability of plants to sequester certain contaminants in the rhizosphere through exudation of

phytochemicals and on the root through root membrane binding or vacuole storage (Figure 3). This process leads to contain pollutants in soil, sediments or sludges.

Plants can reduce the contaminant mobility and avoid its migration into the soil, groundwater or air by preventing erosion, leaching or runoff or by converting the pollutant to less bioavailable forms (i.e. chelation or precipitation of metal ions). This process is mostly applied to face heavy metals contamination; most plants growing on metalliferous soils are not hyperaccumulators, but excluders of heavy metals. The use of excluders is the base of phytosequestration.

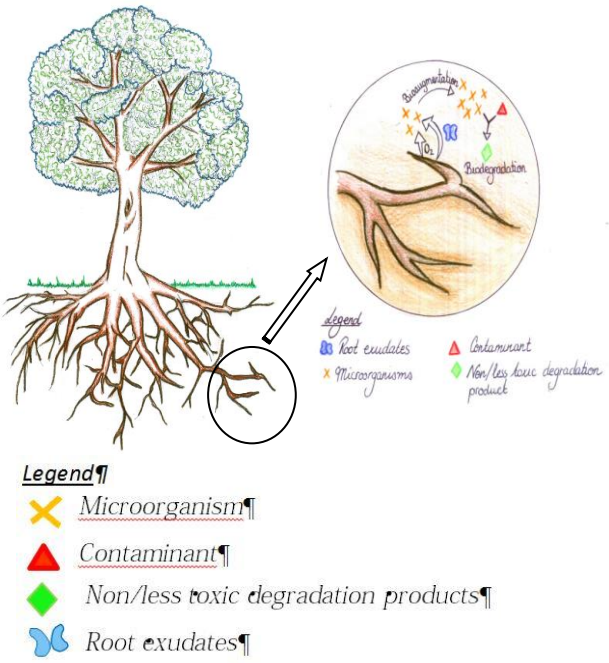

Figure 4. Rhizodegradation process. Illustration by L. Donatsch

In the presence of a soil polluted with organic compounds (TPH, PAHs, pesticides, chlorinated solvents, PCBs), it is suitable to exploit the bioactivity of rhizosphere organisms (bacteria, yeast and fungi) trough **rhizodegradation** (Figure 4), also known as plant-assisted bioremediation (Zhuang, Chen et al. 2007, Wenzel 2009). The plant sustain and enhance the soil microbial population that eat or breathe the contaminant, leading to the breakdown of the target molecules with the production of non-toxic or less-toxic compounds.

Evapotranspiration process is at the base of **phytohydraulics** (Figure 5); certain plant species as poplars and willows have a great evapo-transpiration capacity that can be exploited to pump groundwater, restraining the contaminant plume migration through hydraulic control.

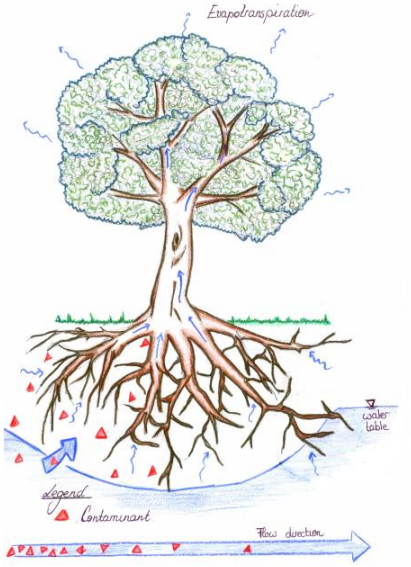

Figure 5. Phytohydraulics process. Illustration by L. Donatsch

Some species of plant can translocate and concentrate certain contaminants into the roots and aboveground shoots or leaves; this process, known as **phytoaccumulation** (Figure 6), acts mainly on heavy metals and radionuclides dispersed in soil, sludges, sediments or water. Phytoextraction involves repeated cropping of plants in contaminate substrate until the metal concentration drops to an acceptable level. Each crop is removed from the site; this leads to accumulation of huge quantities of hazardous biomass, which

must be stored or disposed appropriately to minimize environmental risk. Incineration or composting can be a treatment option to

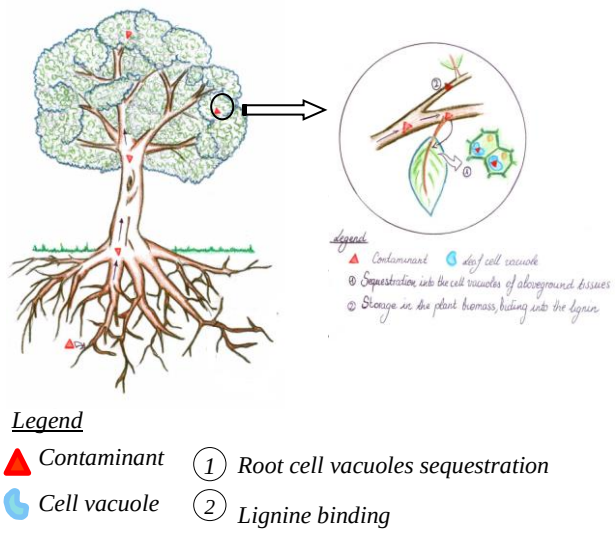

Figure 6. Phytoaccumulation process. Illustration by L. Donatsch

further concentrate the contaminant; those treatments can be combined with energy recovery by combustion, pyrolysis or gasification (Padmavathiamma and Li 2007); by combusting metal enriched wood, metals

may be dissipated to the environment through ash recycling; the use of industrial or collective boilers, equipped with efficient filters, is thus required to minimize air pollution (Delplanque, Collet et al. 2013). The accumulation of waste in the plants may present a problem with contaminants entering the food chain; the relative concentrations of contaminants in the plant tissue must be determined and a consequent assessment and monitoring should be made. The concerns regarding the contamination of other compartments of the food chain caused a progressive shift away from phytoextraction towards phytostabilisation (Conesa, Evangelou et al. 2012).

Phytodegradation (Figure 7), which is also called phytotransformation, is applied for organic compounds that are degraded, either

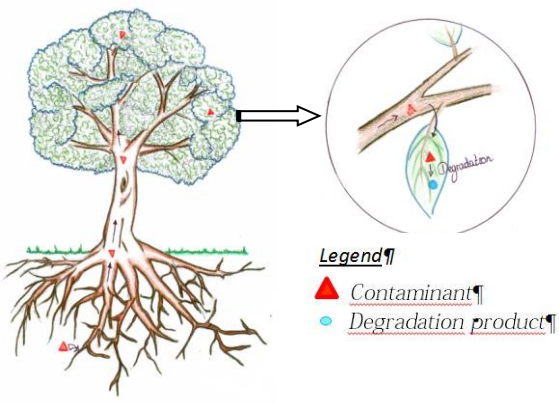

Figure 7. Phytodegradation process. Illustration by L. Donatsch

within the plants or through enzymes released into the rhizosphere but without the intervention of microorganisms. This process acts on certain organic compounds as chlorinated solvents, BTEX chemicals, pesticides and phenols.

If the contaminant is up-taken by the plant, possibly modified, and released by the leaves

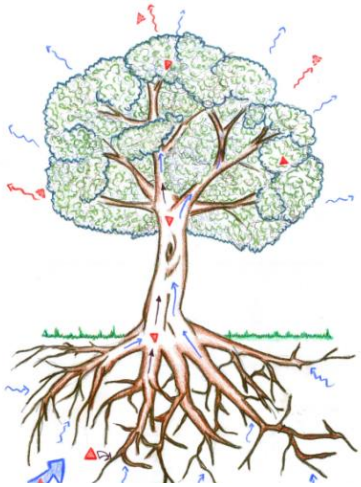

Figure 8. Phytovolatilization process. Illustration by L. Donatsch

into the atmosphere through the transpiration process, then we talk about **phytovolatilization** (Figure 8); typical contaminants that can be volatilized are Se, Ag, As, chlorinated solvents and MTBE. .

2.4 Applications in plant-based water treatment

The mechanisms described in the previous section can be exploited for wastewater treatment in specific design applications, depending on the constituents of concern and the remedial goals.

Constructed wetlands - engineered systems (e.g. subsurface flow systems, free water surface systems or floating treatment wetlands) that have been designed and constructed to utilize the natural processes involving wetland vegetation, soils, and their associated microbial assemblages to assist in secondary or tertiary water treatment. Water trickling through the reed bed is cleaned mostly by microorganisms living on the rhizosphere; adsorption, UV degradation and precipitation are other mechanisms participating to the depurative process. Typical treated contaminants are nutrients, like nitrate and phosphate, and also heavy metals, petroleum hydrocarbons and biological pathogens.

Hydraulic barriers – a stand of deep-rooted, high-transpiring trees, as poplar and willows, exploited to pump groundwater, restraining the contaminant plume migration through hydraulic control. Typical treated contaminants are dissolved organics and inorganics compounds (metals, salts, petroleum product and chlorinated hydrocarbons).

Vegetated buffers-strips- riparian areas that protect adjacent water resources from nonpoint source pollution; they can intercept the runoff and contain fertilizers, pesticides, and animal waste from agriculture, road salts, automotive fluids, and other urban chemicals from roadways.

Hydroponic systems – in those systems hygrophytes plant species (e.g. poplar, willows, eucalyptus or hemp) are grown in water solution, commonly supported by an artificial inert soil as vermiculite or perlite. The contaminated water is passed through the system and is then subject to rhizofiltration process.

Vegetated stands for lagooning – large tree-planted areas, vegetated river dead arms or side arms where polluted water are deviated where trough evapotranspiration, phytovolatilization, phytodegradation and rhizodegradation the water quality is improved.

Natural swimming pools - systems consisting of a constructed body of water in which no chemicals or devices that disinfect water are used, and all purifying of the water is achieved through biological filters and plants. Generally natural swimming pools are divided in to two areas: the swimming zone and the regeneration zone, a lined overflow pool filled with specific filtration substrate and flora. The biological processes that clean the water take place in this zone.

2.5 Plant selection criteria

The infinite variability of biodiversity offers a wide spectrum of tools to remediate water and soils using plants. Phytotechnologies systems are site dependent: each remediation project must be conceived individually, depending on the soil and the climatic conditions, on the type and the history of the contamination. In this context, it is of crucial importance the choice of the plant species and varieties in order to optimize each remediation process. Generally the first criteria of selection is the plant adaptation to the site pedo-climatic conditions, therefore the choice will be restricted to autochthonous species and oriented towards the species

currently growing at the site; among them, the screening process must consider the following aspects:

-root extension and shape against the site hydrology and the contaminant distribution

-growth rate

-evapotranspiration potential

-life span and leaf drops (perennial, annual, biennial, deciduous, evergreen)

-specific tolerances towards the pollutant

-fate of the contaminant in the selected plant species (exclusion or phytodegradation capacity, bioaccumulation rates, bioaccumulation organs)

The final selection of plants includes more practical considerations such as commercial availability and plant stocks. Using locally available stock is highly recommended since it is acclimated to local conditions; those plants will have a higher rate of survival and will require less maintenance.

2.6 Phytotechnology database: an important tool for plant species selection

Information on phytoremediation suitable plants can be gathered from local, or state environmental agencies, from universities and from botanical literature. Internet resources also are available that provide botanical information, such as the Plants Database of the USDA Natural Resources Conservation Service (http://plants.usda.gov/about_plants.html) about the flora of U.S., the Integrated Taxonomic Information System (ITIS http://www.itis.gov) regarding regions of North America, eFlora (http://www.tela-botanica.org) source of information about the flora of France and Acta Plantarum Project (http://www.actaplantarum.org) about wild plants in Italy. For particular types of contaminants, documents and databases have been compiled to identify plants capable of adapting to certain levels of toxicity and containing or mitigating contaminants, like as PhytoPet : a database of plants that play a role in the phytoremediation of petroleum hydrocarbons (http://inis.iaea.org)). All of the above listed web-sites provides local botanical information or data about specific contaminants.

Therefore we pointed out the lack of a global database gathering the botanical and ecological information finalized to remediation purposes, and describing the potentialities of each species in phytoremediation. Although those topics have been object of many scientific researches, the knowledge about it is scarcely available to both the general public as well as the specialised audiences.

3 Materials and methods

The editing criteria and the tools used to compile the internet database and the book will be described separately.

3.1 Implementation of an accessible online database

After having defined a data-sheet template including all the information useful for the screening of plant candidates in phytoremediation projects, we proceeded to complete a pre-existent plant-database (previously redacted by IBAF). We referred

mainly to the following bibliographic sources:

-fact sheets and plant guides from the United States Department of Agriculture (USDA) web-site: they provide information about conservation plants that are commonly used to improve the land

-data-sheets edited by Centro Sperimentale per il Vivaismo: synthetic description of ornamental garden plants, they include information about uses and cultivation

-taxonomic Information System, created by a partnership of U.S., Canadian, and Mexican agencies (ITIS-North America) and taxonomic specialists

-data-sheets edited by Acta Plantarum Project: an open database, organised by quoted botanists, reporting ecological and botanical information of Italian flora

Each data-sheet, in addition to the botanical and ecological entries, includes a part dedicated to the most relevant phytoremediation experimentation involving the plant concerned, as reported in scientific literature.

A web-site section has been developed to support and introduce the database; it has been setup with Powerpoint and published in internet as interactive Pdf. At the moment the IBAF web-site is going to be implemented and the section devoted to phytoremediation will be incorporated in a web format.

3.2 Book editing

The book has been developed by four authors, (M.C. Grandi, L. Passatore, A. Massacci and F. Romagnolli) each of them has contributed according his own employment field: natural swimming-pools, phytoremediation and constructed wetlands, respectively. The work has been published by "Editrice il Campo, Bologna" in February 2014. An e-book version of the work is being implemented.

There follows the editing rules adopted for the fact-sheet drafting, related to each entries.

Name of the species and family – at the beginning of each fact-sheet we reported the scientific name of the plant as follows: the Latin binomial followed by the name, generally abbreviated, of the author who first described and named the species. In the case of uncertainty or confusion about the plant name we have chosen that-one reported in the latest checklist of Italian vascular flora (2005, updated 2007). Consecutively the most widely used synonyms and common names (in Italian and in English) are reported. Finally the name of the botanical family is indicated. For some species there is confusion about the family of belonging because of the continuous updates resulting from the last phylogenetic researches. In the text we take as reference the most recent source commonly accepted by the scientific community: APG III (third version of the classification published by the Angiosperm Phylogeny Group, 2009).

General description – the plant is introduced: its structure, its growth habit, his natural environment and its possible uses are described. An interpretation on the etymology of the species name is also provided.

Ecological type - This entry defines the aquatic plants growth habit from an ecological point of view. The hygrophytes, plants growing in moist habitats, usually not submerged. The helophytes, related to the

shallow water areas, at the edge of the water bodies, and hydrophytes, related to deeper waters. The hydrophytes can be further divided in sub-categories according to the position occupied in the water column (rooted on the bottom and not emergent, rooted on the bottom with floating leaves, not rooted and free floating). Five ecological types are thus recognised: hygrophytes, helophytes, hydrophytes rhizophytes, natant rhizophytes, pleustophytes rhizophytes.

Maximum height (m) - the height reached by the plant at the end of his life cycle; this parameter is expressed as an interval, indicating the heights generally reached by plants subject to the environmental factors characteristic of the Italian environment.

Root system –the root structure is described; indicating the length and, where possible, the depth reached by underground organs. This data are essential to predict the phytoremediation potential of the plant.

Leaves, flowers, fruits - the morphology of each part of the plant is illustrated. This section is not aimed at the determination of particular biological characters for the systematic recognition of the species; we instead opted for a generic description that summarizes the more evident features, avoiding strictly botanical names, that could not be understood by lectors.

Life span and leaf drops - the biological cycle of the plant (perennial, annual or biennial) and his habit during the colder season are reported. Both information are related to plants growing in Italy and are not generalizable in the presence of different climates.

Growth rate - this indication includes not only the development speed of a single plant, but also the aptitude of the species to reproduce by seed or to vegetatively propagate.

Temperature (° C) – the optimal temperature range within the plant accomplish all stages of his annual growth (phenological stages) is reported.

Light requirements- optimal light conditions.

Water depth (m) – optimal depth range.

Resistance / tolerance – degree of resistance and tolerance to edaphic, climatic and physical factors (eg, salinity, temperature, water flow, drought).

Trophic conditions- nutrients availability in soil or water, typical of the environments in which the species grows. The information has been obtained from the values assigned to each species by the macrophitic index GIS (Haury, Peltre et al. 1996), and from the values defined by Ellenberg indices, as modified by Sandro Pignatti (Pignatti, 2005).

Plant density - the recommended number of plants per square metre is a relevant data in the planning stage of constructed wetlands, natural swimming-pools and phytoremediation systems.

Gamic reproduction – sexual reproductive strategies adopted by the plant for pollination and seed dispersal processes. The number and the fertility of seeds are also indicated.

Agamic reproduction – vegetative or asexual reproduction strategies (through cuttings, tubers, bulbs, runners, sprouts or roots), leading to the formation of plant clones.

Origin – geographical area where the species originated.

Distribution – geographical area where the species grows wild in the world. This indication is based on the chorotypes reported by Pignatti (Pignatti and Anzalone 1982) and updated by the earlier biogeographic literature.

Naturalisation – we pointed out if the species is autochthonous or allochthonous in Italy, and eventually if it is present in self-sustaining populations (naturalized) or only as cultivated plant. We referred to the last checklist of the Italian vascular flora (2005, updated in 2007) and, where possible, we relied upon the indications of a group of experts belonging to "Acta Plantarum" open-source project (www.actaplantarum.org).

Invasive potential – it indicates the reproduction and colonisation rates of the species; the data is strictly referred to plants growing on the Italian territory; therefore it cannot be applied to plants of the same species growing in other areas. The invasive potential is high for the species having a fast vegetative reproduction rate.

Plant passport – a document accompanying the plant and attesting compliance with Community plant-health rules is needed for certain species both for movements within and between EU member states. This rule has been ratified by Legislative Decree No . 214/2005 enacted to implement EU directive No 2002/89/CE on protective measures against the" introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community". The passport, issued by growers who are registered and authorised for the purpose, shall indicates the place where the plant has been cultivated and shall certifies that the plant is free from quarantine organisms (bacteria, fungi, insects and virus).

Related species, subspecies, cultivars and varieties common in Italy – we briefly describe the relevant congener species and other intraspecific entities (subspecies, cultivars and varieties). Of course the list does not include all the existent botanical entities. We described only the most common ones, the ones particularly decorative and useful for phytoremediation purposes and the species that may be confused with the plant object of the datasheet.

Application - the plant potentialities in phytoremediation projects, constructed wetlands and natural swimming-pools are summarized.

Each data-sheet includes also 4 graphs illustrating: i) the **flowering period** (data refer to a temperate Mediterranean climate, it is not applicable to plant of the same species growing under different climate conditions), ii) the **regional distribution** in Italy (the Italian regions where the species grows spontaneously are highlighted, data refers to the last checklist of the Italian vascular flora of 2005, updated in 2007), iii) the **altitudinal distribution** (data refer to a temperate Mediterranean climate, it is not applicable to plant of the same species growing under different climate conditions), iv) **Ellenberg bioindicator values**

The data reported as Ellenberg bioindicator values come from the database compiled by S. Pignatti for Italian vascular flora (Pignatti et al., 2005). The values applies a 9 or 12 point scale for each of six gradients: soil acidity, soil productivity or fertility, soil humidity, climatic continentality, light availability and soil salinity. The last indicator has not been reported on the data sheets due to the limited availability of this information. These values represent the

realized ecological niche of each species, as developed by the observation of the plant growth conditions in his natural environment, they are thus also influenced by the competition with the other species. For example, if we observe *Carex paniculata* in an alpine wetland (reaction value of the substrate = 9), we can reasonably assume that the soil is alkaline, and if we find *Carex pauciflora* (reaction value of the substrate = 1) we could expect the presence of an acid soil. However, it does not necessarily mean that these soils are most suitable substrates for these species.

Photographs – In each data sheet we reported the images of the plant as a whole and of the plant details (leaf, flower, fruit, seed, and root system). In some cases we also included photos of the congeneric species, subspecies, varieties, or cultivars described. The name of the photograph has been reported under each photos.

4 Results

There follows a description of the internet database structure and of the book contents

4.1 The Phytotechnologies Plant Database

The internet database is accessible from the IBAF web-site (www.ibaf.cnr.it) and it is available in English and Italian languages.

An introductory section provides a review of phytoremediation processes dynamics and discusses phytotechnologies advantages and limits, with a special focus on rhizosphere and rhizoremediation (figure 9).

From the homepage it is possible to enable

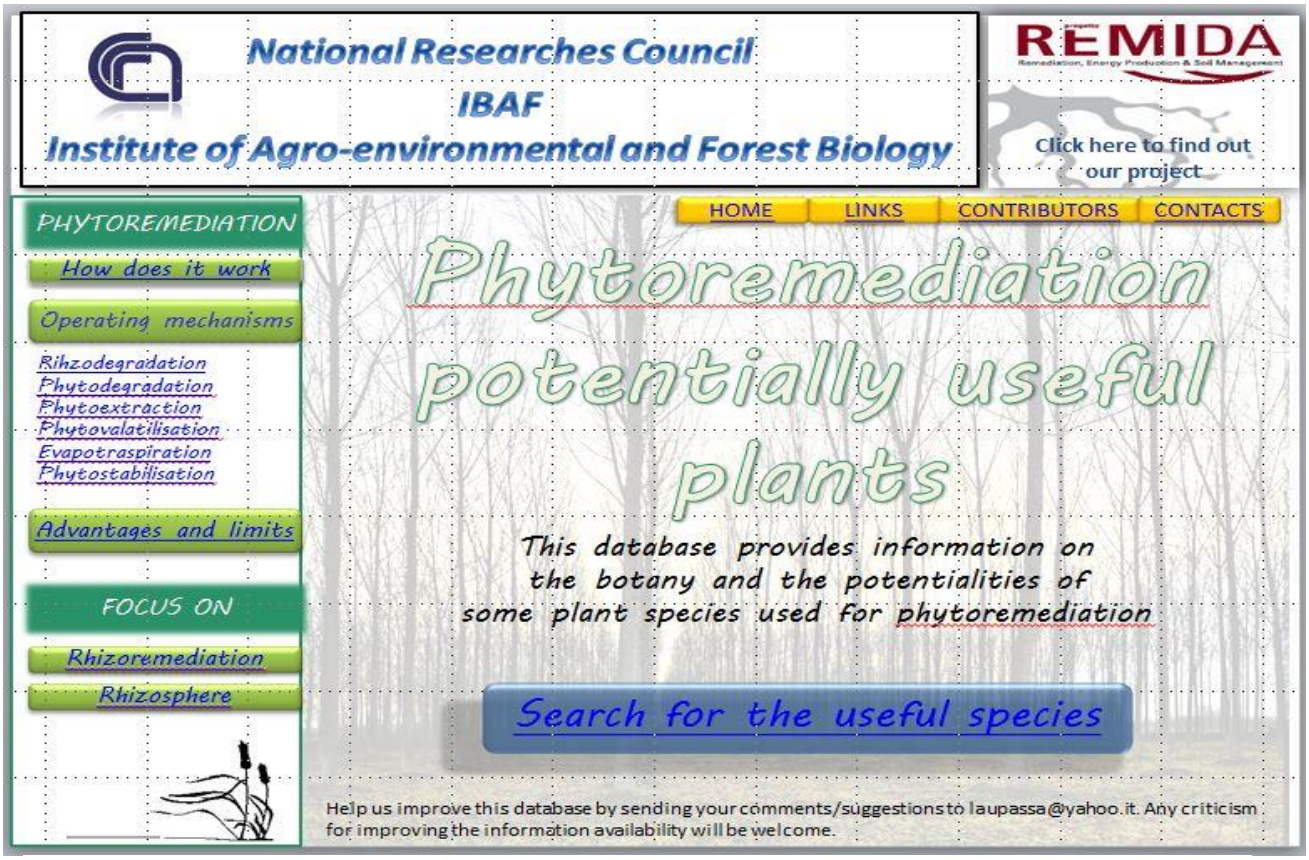

Figure 9. Homepage of the Phytoremediation Plant Database

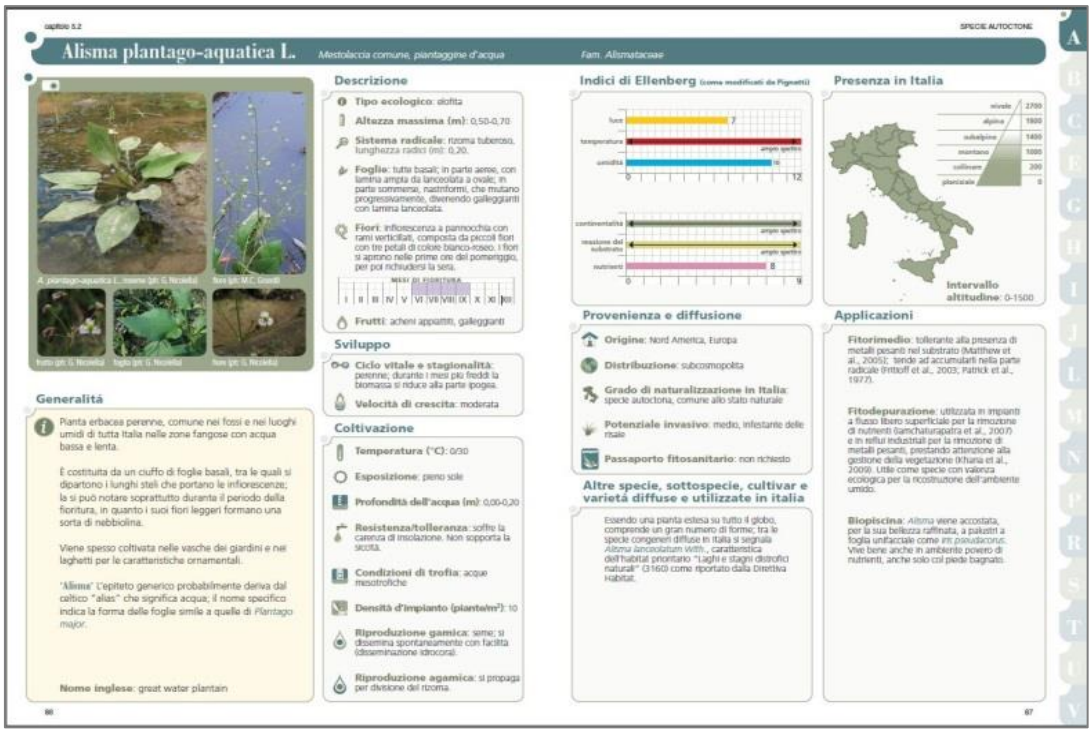

Figure 10. An example of plant profile extracted from the book "Le piante che depurano l'acqua"

the search for the plant data-sheet of interest, accessing a web-page that permit to choose between two different plant searching criteria: by the botanical name or by the pollutant to treat. In this way the user can access the datasheet of each plant species useful in phytoremediation. To date 71 datasheets have been published, providing information on the botany and the remediation potentialities of the species. A special section at the end of each datasheet resumes the most relevant scientific paper concerning phytoremediation investigations on the species at issue. The system is easily implementable, adding new plant profiles or updating the scientific literature reported in each datasheet.

4.2 The book on water purifying plants: "Le Piante che depurano l'acqua"

After an introduction about the ecology of aquatic plants, their cultivation and maintenance, the book presents tree main technical application of plant-based water treatment: constructed wetlands, phytoremediation (hydraulic control, hydroponic systems, lagooning systems, vegetated buffer-strips) and natural swimming pools, as described in paragraph 2.4. Even if "constructed wetlands" are commonly ascribed to "phytoremediation systems", we kept those concept separated in the book, since usually in Italian language the word "fitodepurazione" is not assimilated to the word "fitorimedio".

The fifth chapter represents the core of the book, where 60 datasheets describe the botanical, ecological and phenological aspects of native, not-native aquatic plants and hygrophytes. In each profile there are descriptive photos of the main parts of the plant and the description of the species, organized in easily consultable sections (figure 10). Last section of each datasheet presents the possible applications and the functionalities of the plant in constructed wetlands, phytoremediation systems and natural swimming pools.

PART II Analytical methods for extraction, clean-up and detection of PCBs in environmental samples

1 Abstract

The aim of the present study is to delineate an analytical method for PCB analysis allowing us to follow the time course of the bio-degradation taking place in a polychlorinated biphenyls-contaminated soil and the possible contaminant transport into the tissues of the plants growing on it. We compared several methods of drying techniques, extraction and clean-up, whose efficiency, accuracy and repeatability had already been proved in the scientific literature. Environmental studies require the analysis of a large number of samples therefore, among the tested techniques, we selected the best compromise between ease of procedure and degree of automation, with consideration of the cost and the analytical instrumentation availability.

2 Introduction

Analysis of polychlorinated biphenyls (PCBs) in environmental matrices usually necessitates of an extraction step followed by gas chromatographic (GC) analysis. Natural samples can rarely be used in their raw state; their nature must be compatible with the detection method. Different steps of pre-treatment, extraction and purification are necessary before the determination and the quantification of individual compounds. The principal step of the sample treatment is the extraction of analytes from matrix, which consists of transfer of the target compounds into a solvent. Extraction is rarely selective. The raw extract is then constituted of studied compounds at trace level but also of co-extracted compounds (endogenous compounds, macromolecules, other contaminants...) which can interfere during the analysis. It is necessary to purify extracts, to reconcentrate or to dilute (Letellier and Budzinski 1999).

The analysis of PCBs for environmental purposes can be described with the following steps:

-Pre-treatment of the sample (homogenization, grinding, sieving, drying)

- Extraction

- Clean-up

-Chromatographic separation (GC), identification and quantification (ECD or MS).

In the following paragraphs PCB molecules will be introduced, and the most common techniques for each PCB analysis step will be presented and briefly discussed. Most of the analytical operations described below can be carried out in the same way regardless for the sample type (soil, sludge, sediment or biomass matrices).

2.1 Polychlorinated Biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a class of persistent organic pollutants (POPs) differing in the number of chlorine atoms (1–10) attached to their biphenyl rings (Figure 1).Because of their high molecular stability, low solubility in water and high tendency to adsorb on particulate phase, PCBs are extremely hard to remove from soil and sediment matrices. Their hydrophobicity and lipophilicity make them poorly taken up by plants but susceptible to bioaccumulate in animals, mainly in adipose tissue and breast milk.

Physical states (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	oil, viscous liquid, sticky resin
Water solubility (mg/L) (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	0,0027 (Aroclor 1260)- 0,59 (Aroclor 1221)
Octanol-Water Partition Coefficient (Log k_{ow}) (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	4,7 (Aroclor 1221) - 6,8 (Aroclor 1260)
Bioconcentration factors (BCFs) in aquatic organisms (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	60 (penta-and esa-CBs in Ciliate *protozoa*)-274000 (tri- and tetra-CBs in the freshwater fish Feated minnow)
Toxic equivalency factor (TEF) for mammals (Van den Berg, Birnbaum et al. 1998)	0.00001 (2,3',4,4',5,5'-HexaCB)-0,1 (3,3',4,4',5-PentaCB)

Table 1 *Properties of the most common PCB commercial mixtures and single congeners*

Depending on chlorines number and position, PCB congeners differs in chemical, physical and toxicological properties. (Table 1) summarizes some characteristics that are significant for predicting the environmental fate and impact of PCBs.

Chlorine substitution further stabilizes the aromatic structure by electron displacement and an increase in oxidation state. As a general rule, the more the molecule is chlorinated, the less water soluble and volatile it is (Campanella, Bock et al. 2002). The octanol-water partition coefficient (log K_{ow}) allows to predict the mobility of PCBs in the environment, i.e., higher-chlorinated PCBs, with log K_{ow} above 6, are associated with particulate matter in the atmosphere, soils and sediments, while lower-chlorinated congeners exist in the gaseous phase. As a consequence, soils generally contain higher proportion of higher-chlorinated congeners, while air is dominated by lower-chlorinated fractions (Aken, Correa et al. 2009).

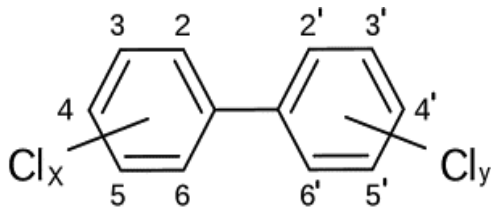

Figure 1. General chemical structure of chlorinated biphenyls. The chemical formula is: $C_{12}\ H_{10-x-y}\ Cl_{x+y}$ *where* x *and* y *are between 1 and 5.*

The sorption properties of PCBs make those compounds subject to sorption on surfaces capable of adsorbing lipophilic molecules. Thus, the choice of polymeric materials for use in laboratory experiments with PCBs needs careful circumspection to avoid errors. According to experiments of Cseh et al (Cseh, Sanschagrin et al. 1989), plastics and rubbers must be avoided, including silicone, Norprene, and Tygon tubing in peristaltic pumps. The use of hard plastic materials is advisable only when the contact time with materials contaminated with PCBs is short. Teflon appears to be the only safe material able to handle PCBs in aqueous media

without significant losses. When PCBs are being studied and the use of plastics cannot be avoided, a mass balance must always be established.

2.2 Sample pre-treatment: drying step

Among the different drying techniques thermal drying is certainly the most common one; nevertheless PCBs are subject to volatilization when drying temperature exceeds 40°C Therefore, drying soil samples between 25 °C and 40°C for several days might be a good choice.(Berset, Ejem et al. 1999). Owing to its efficiency freeze-drying has become a popular drying technique and is recommended for the analysis of nonpolar micropollutants Sometimes the two drying techniques described above are still too laborious, especially when results of PCBs contaminated soils have to be delivered very quickly. In such a case chemical drying might be an alternative. The soil sample is mixed with anhydrous sodium sulfate. Advantages of this method are: water is trapped through binding to sodium sulfate (i), soil is exposed very shortly to laboratory air (ii), the microbial activity is stopped due to the high salt concentration (iii) and samples treated in this way might be stored for a longer period of time as the risk of degradation of the pollutants is minimized (iv). The main disadvantage is the dilution of sample due to addition of the salt (Berset, Ejem et al. 1999).

2.3 PCB extraction

Extractions are traditionally performed by means of Soxhlet or sonication, as prescribed by two official EPA methods (EPA 3540, 1996; EPA 3550C, 2007). Unfortunately, these techniques demand long extraction times and large volumes of highly purified and hazardous organic solvents, generating dirty extracts that involve extensive clean-up steps before analysis; therefore the analysis of numerous samples in environmental studies is often limited by the extraction step. The cold-liquid extraction combined with mechanical shaking is still a well-established method due to its easy handling; however it shows the largest analysis error compared to other techniques (Sułkowski and Rosińska 1999). As a consequence there has been an increasing demand for new techniques such as, microwave-assisted extraction (MAE) (Lopez-Avila, Benedicto et al. 1995, Pastor, Vázquez et al. 1997, Letellier and Budzinski 1999, Parera, Santos et al. 2004), supercritical fluid extraction (SFE) (Silva, Pietri et al. 2012) and pressurised liquid extraction (PLE, also known as accelerated solvent extraction or pressurised fluid extraction) (Björklund, Nilsson et al. 2000), that overcome some of the previous problems (Gomez-Ariza, Bujalance et al. 2002, Sporring, Bøwadt et al. 2005).

PCB percent recoveries for a specific extraction method are dependent on the specific PCB compound, on his concentration, on the age of the contamination and on the matrix type. However it is possible to have a general idea about the different percent recoveries from the graphic below (Sporring, Bøwadt et al. 2005) (figure 2), where average PCB recovery is reported for high-level and low-level contaminated soils, and compared using different extraction techniques (classic Soxhlet extraction, Soxtec extraction, ultrasonication extraction, supercritical fluid extraction, microwave assisted extraction and accelerated solvent extraction).

Sparr Eskilsson and Bjorklund (Sparr Eskilsson and Björklund 2000) compared traditional and recent PCB extraction techniques summarizing their advantages and drawbacks (Table 2).

2.4 Clean-up

Clean-up has to be used if compounds are present that can interfere with the PCB congeners of interest in the gas chromatogram or if those compounds can influence the GC-procedure (i.e. contamination of the chromatographic system).

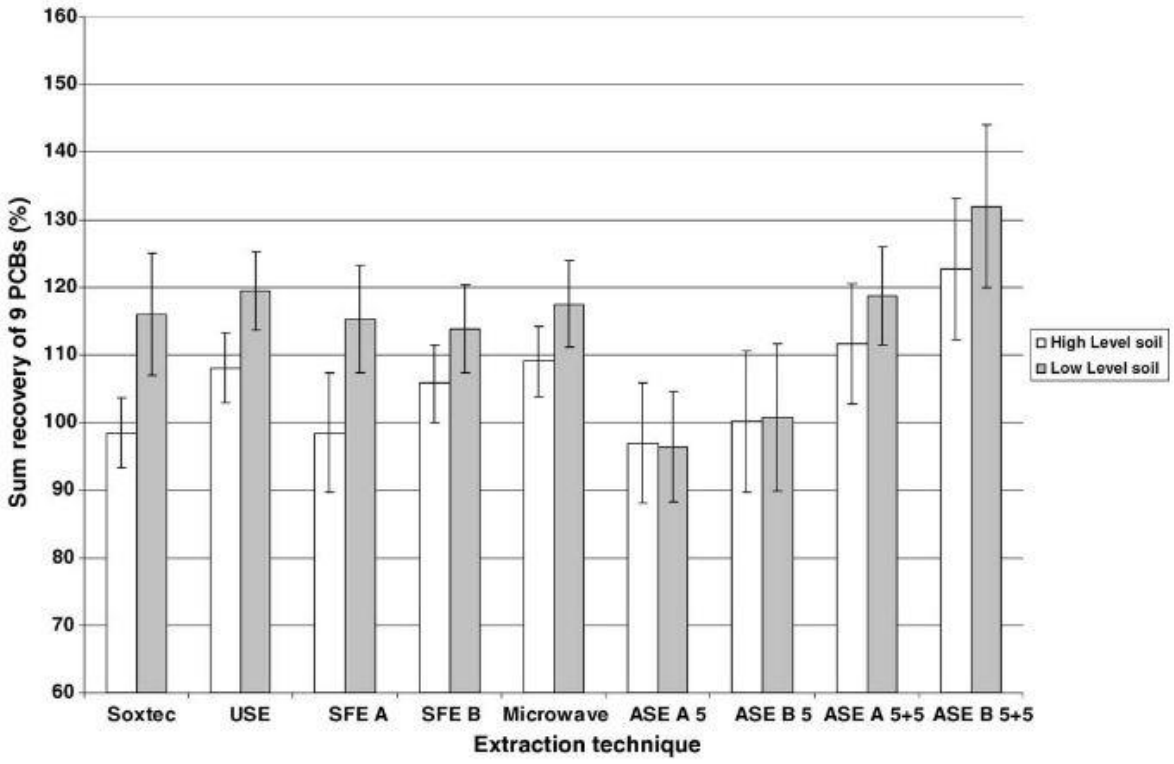

Figure 2. Average PCB recovery for all investigated PCB in the high contaminated soils (High level) and low contaminated soils (Low level) for different techniques expressed as percentage of Soxhlet recoveries. The authors compared Soxtec, ultrasonication extraction (USE), supercritical fluid extraction (SFE), microwave-assisted extraction (microwave), accelerated solvent extraction with hexane/acetone 1:1 (ASE A) or toluene (ASE B), in one 5 minute step (ASE 5) or two 5 minutes steps (ASE 5+5). (Sporring, Bøwadt et al. 2005).

A draft of European standard (prEN 15308) drawn up in 2005 describes eight possible clean-up methods, depending on the compounds needing to remove : Aluminium oxide, silica gel, Gelpermeation, Florisil, silica H2SO4/silica NaOH and Benzenesulfonic acid/sulfuric acid methods for polar compounds and lipids , DMF/n-hexane partitioning for aliphatic hydrocarbons removal, concentrated Sulphuric acid, TBA-sulfite reagent, Clean-up using pyrogenic copper and AgNO3/Silica to remove elemental sulphur and some other organic sulphur compounds.

In order to reduce time and cost of analysis, a new pressurized liquid extraction method has been proposed (Ezzell, Richter et al. 1996, Björklund, Müller et al. 2001, Gomez-Ariza, Bujalance et al. 2002) achieving very

	Extraction technique					
	MAE	FMASE	PLE	SFE	Soxhlet	Sonication
Brief description	Sample is immersed in a microwave-absorbing solvent in a closed vessel and irradiated with microwave energy.	Sample is immersed in a microwave-absorbing solvent in an open vessel and irradiated with microwave energy.	Sample and solvent are heated and pressurized in an extraction vessel. When the extraction is finished, the extract is automatically transferred into a vial.	Sample is loaded in a high pressure vessel and extracted with supercritical fluid (most commonly carbon dioxide at pressures of 150–450 bar and temperatures of 40–150°C). The analytes are collected in a small volume of solvent or onto a solid-phase trap, which is rinsed with solvent in a subsequent step	Sample is placed in a glass fibre thimble and, by using a Soxhlet extractor, the sample is repeatedly percolated with condensed vapours of the solvent.	Sample is immersed in solvent in a vessel and placed in an ultrasonication bath.
Extraction time	3–30 min	10–60 min	5–30 min	10–60 min	3–48 hrs	10–60 min
Sample size	1–10 g	1–30 g	1–30 g	1–5 g	1–30 g	1–30 g
Solvent usage	10–40 ml	10–150 ml	10–100 ml	2–5 ml (solid trap) 5–20 ml (liquid trap)	100–500 ml	30–200 ml
Investment	Moderate	Moderate	High	High	Low	Low
Advantages	• Fast and multiple extractions • Low solvent volumes • Elevated temperatures	• Fast extractions • Low solvent volumes	• Fast extractions • Low solvent volumes • Elevated temperatures • No filtration required • Automated systems	• Fast extractions • Minimal solvent volumes • Elevated temperatures • Relatively selective towards matrix interferences • No clean-up or filtration required • Concentrated extracts • Automated systems	• No filtration required	• Multiple extractions
Drawbacks	• Extraction solvent must be able to absorb microwaves • Clean-up step needed • Waiting time for the vessels to cool down	• Extraction solvent must be able to absorb microwaves • Clean-up step needed • Waiting time for the vessels to cool down	• Clean-up step needed	• Many parameters to optimize, especially analyte collection.	• Long extraction times • Large solvent volumes • Clean-up step needed	• Large solvent volumes • Repeated extractions may be required • Clean-up step needed

Table 2 Comparison of traditional and recent extraction techniques: microwave-assisted extraction (MAE), focused micro-wave assisted extraction (FMASE), supercritical fluid extraction (SFE) pressurised liquid extraction (PLE, Dionex trade name ASE), soxhlet and sonication (Sparr Eskilsson and Björklund 2000).

selective extraction of PCBs, without the need of a subsequent clean-up step. The method consist of on-line clean-up by inclusion of sorbents in the extraction cell. Alumina was successfully used as a retainer sorbent in a Dionex Application Note (Dionex 1996). Accordingly to Björklund (Björklund, Müller et al. 2001) et al., the use of sulphuric acid impregnated silica gel inside the cell was superior in some extent to the use of Florisil (Gomez-Ariza, Bujalance et al. 2002).

2.5 Chromatographic separation (GC), identification and quantification (ECD or MS)

Before to discuss the available analytical detection methods for PCB analysis, it is of primary importance to understand which PCB measure is the most suitable depending on regulatory requirements and project needs.

With regard to this, the detection of PCB can be expressed as concentration of specific commercial mixtures (e.g. Aroclors), concentration of individual congeners (e.g. the indicator congeners), or can be estimated as the sum of PCBs (total PCBs). When the regulatory requirements are based on specific commercial mixture concentration (e.g. Aroclor concentration), then the analysis of the whole concentration of the commercial mixture is appropriate. As an alternative, the congener method is of particular value in determining weathered Aroclors; in fact, the environmental degradation (weathering) of commercial multicomponent mixtures may results in differences of peak patterns compared to the original mixture. Caution must be exercised when using ∑PCB levels to study the biogeochemical cycling of PCBs. Using the sum approach, the widest possible range of individual PCB congeners must be measured and if, for example the environmental persistence of PCBs as an overall class of compounds is not to be underestimated. Anyway this approach can cause some problem in the comparison of total PCB concentrations obtained from different laboratories, as the congener measurements are limited to those congeners for which the laboratory possess analytical standards(Ayris, Currado et al. 1997).

Presently, the most widely practised techniques for the determination of PCBs are capillary GC-ECD (electron capture detector), GC-MS (single quadrupole mass spectrometer) or GC-MS/MS (triple quadrupole mass spectrometer). Although ECD has many attributes, it is unable to differentiate between coeluting PCBs Furthermore, its ability to identify individual PCBs relies on retention time alone. As a result, the provision of a genuine ∑PCB measurement (with retaining congener-specific information) using GC-ECD requires the use of a reference standard comprising all PCBs present in the environment. By comparison, the use of mass spectrometric detectors not only enhance selectivity, but by the use of selected ion monitoring mode (SIM or SIR) and isotope ratios, provides qualitative information to supplement that supplied by GC retention time (Ayris, Currado et al. 1997) and a major sensitivity. The chemical ionisation (CI) in positive ions leads to the formation of the protonate molecular ion , whit low or no fragmentation, and can be useful in the presence of interfering compounds.

Internal standards have to be used. If MS-detection is used, isotopes have to be used; at least one for every level of chlorination (CEN/TC292-308, 2003). For ECD—detection PCB30 and PCB 209 are recommended by the European Standard

CEN/TC292 and 308, PCB 30 and 204 were used by Jeremiason et al (Jeremiason, Hornbuckle et al. 1994); these PCB congeners, as also PCB14, PCB65 or PCB 166, are not normally found in environmental samples, are not degradation products of the congener tested and can be used as a surrogate for the whole suite of 209 PCB congeners based on their physic chemical properties. Frame (Frame 1997) used decachlorobiphenyl (PCB 209) and 2-fluorobiphenyl as internal standards; Flanagan et al. injected samples with 4-fluorobiphenyl (Flanagan and May 1993). Internal and surrogate standards prescribed in EPA method 8082A are decachlorobiphenyl, tetrachloro-m-xylene, or hexabromobiphenyl (EPA, 2007). Ottonello et al. performed the PCB quantification in fish samples with GC-MS using PCB 155 (hexa-CB) for congeners with a level of chlorination up to six and PCB 198 (octa-CB) for the heptachloroderivatives, that proved to be an effective alternative to the more expensive labelled compounds.

3 Materials and methods

3.1 Sampling and pre-treatment

Soil samples were taken from a PCB and metal multi-contaminated area situated in the South of Italy (Taranto). The physical and chemical characterization of the soil is reported in the table below (table 3).

The levels of contamination for the parameters exceeding the Italian legal limits are summarized in the table 4.

Textural class	Sandy-loam
pH	7,86
Organic carbon (g/Kg)	14,94
Organic matter (g/Kg)	25,75
Total Nitrogen (g/Kg)	0,20

Table 3 Physical and chemical characterisation of the sampling area, data provided by IRSA-CNR (Bari).

Plant biomass samples consists in tree roots which developed in the PCB-polluted soil described above.

All the solvents and reagents employed in this study were of pesticide quality being purchased acetone, hexane, dichlorometane, tetrachloro-m-xylene, Na_2SO_4, Aroclor standards, silica gel and silica SPE cartridges from Chebios S. r. l (Italy) and mass-labelled PCB standards from Wellington Laboratories Inc. (Canada).

Parameter	Italian legal limit (D.Lgs 152/2006) (mg/Kg)	Soil content (mg/Kg)
Vanadium (V)	90	127,4
Chromium (Cr)	150	179,5
Nickel (Ni)	120	156,2
Zinc (Zn)	150	181,3
Selenium (Se)	3	10,4
Tin (Sn)	1	6,0
Total PCBs	0,06	0,271

Table 4 Levels of contamination in the sampling area, data provided by IRSA-CNR (Bari).

The pre-treatment operations have been performed with caution in order to avoid loss of the more volatile analytes. Samples and extracts have been processed carefully, in order to avoid a long contact with plastic materials, potentially realising phthalates that could interfere in analysis, and/or capable of adsorbing lipophilic molecules as PCBs.

Soil samples were air dried and mixed in order to homogenize the material, any foreign objects was discarded (stiks, roks), and then the samples were grinded in a mortar and passed through a 2-mm sieve.

Roots were washed from soil particles accordingly with the method proposed by Barillot et al. (Barillot, Sarde et al. 2013): hand-shacked vigorously for 10 minutes in air and for a further 10 minutes in NaCl solution (0,9%). Then the samples were air-dried and grinded into a fine powder in a Moulinex mill.

In order to avoid PCB volatilization freeze-drying was preferred to oven-drying. Soil and roots where lyophilized for 25 hours in a Virtis Freezmobile 12ES. Samples were stored in glass bottles and kept dry in a vacuum desiccator until the time of analysis.

3.2 Soxhlet extraction, analytical procedure

The Soxhlet extraction was performed according to the EPA guidelines (U.S. EPA, 1996). An aliquot of 10 g dry biomass or soil samples was mixed with 5 g of NaSO4 and 10 g of hydromatrix, and transferred in a Soxhlet thimble (figure 3).

Figure 3 Soxhlet extraction. laboratories of the institute of atmospheric pollution research , IIA-CNR (Rome).

The extraction was performed for 24 h using toluene as the extraction solvent (approximately 250 ml). A 50 µl portion of labelled standard (PCB LCS-H, Wellington Lab, 20 pg/µl).) was added as extraction standard.

One analytical duplicate for each sample, and one analytical blank (Ottawa sand) for every nine samples were also extracted and processed.

The Soxhlet extraction has been performed in the laboratories of the institute of atmospheric pollution research , IIA-CNR (Rome).

3.3 Microwave extraction, analytical procedure

Accurately weighted samples of soil and biomass (approximately 3g) were loaded into the Teflon extraction vessels with 20 ml of acetone/hexane 1:1 and processed in a microwave system Excel (EU Microwave Chemistry Technology Instruments Co.) (figure 4), according to the following conditions:

Temperature: 115 °C
Pressure: 50-150 psi
Time at temperature: 10 min
Static time: 15 min
Power:1000 W
Cooling: to room temperature

Figure 4 Microwave extraction system (Excel , EU Microwave Chemistry Technology Instruments Co.), laboratories of the Institute of agro-environmental and forest biology, IBAF-CNR (Rome).

One analytical duplicate for each sample was extracted and subsequently processed; for every nine samples one analytical blank (Ottawa sand) and one control sample (a blank sample spiked with a known amount, 10 mg/l, of Aroclor 1260) were also extracted and processed. Tetrachloro-m-xylene was added to each sample as extraction standard.

The microwave extraction process have been performed in the laboratories of the agro-environmental and forest biology institute , IBAF-CNR (Rome).

Following microwave extraction, sample extract were decanted and centrifuged twice, in order to separate the liquid phase from the solid phase.

3.4 Accelerated solvent extraction (ASE), analytical procedure

A 33-mL stainless cell was prepared by inserting one cellulose filter at the outlet end, and by adding 10 g of sample mixed with 10 g of hydromatrix and 5 g of $NaNO_4$; hydromatrix was added to fill the cell.

Figure 5 Accelerate solvent extraction (ASE 200, Dionex), laboratories of the institute of atmospheric pollution research , IIA-CNR (Rome)

The extraction was performed in ASE 200 from Dionex (figure 5), according to the following conditions:

Extraction Solvent: Toluene
Pressure: 2000 psi
Temperature: 180 °C
Static Time: 5 min
Static Cycles: 3
Flush: 60%
Purge: 90 s

One analytical duplicate for each sample and one analytical blank (Hydromatrix) for every five samples were also extracted and processed. Tetrachloro-m-xylene, tetrachloronaphtalene and octachloronaphtalene, were alternatively added to each sample as extraction standards.

The accelerated solvent extractions have been performed in the laboratories of the institute of atmospheric pollution research , IIA-CNR (Rome).

3.5 Selective pressurized liquid extraction (SPLE), analytical procedure

The selective pressurized liquid extraction was performed coupling the accelerated solvent extraction (ASE) with the in-line clean-up, according to the guidelines of the Dionex Technical Note 210. A 33-mL cell was prepared as shown in figure 6, by inserting two cellulose filters at the outlet end of the extraction cell and by adding a layer of activated silica gel (1 g) and then a layer of the acid-impregnated silica gel (10 g) with a cellulose filter on top.

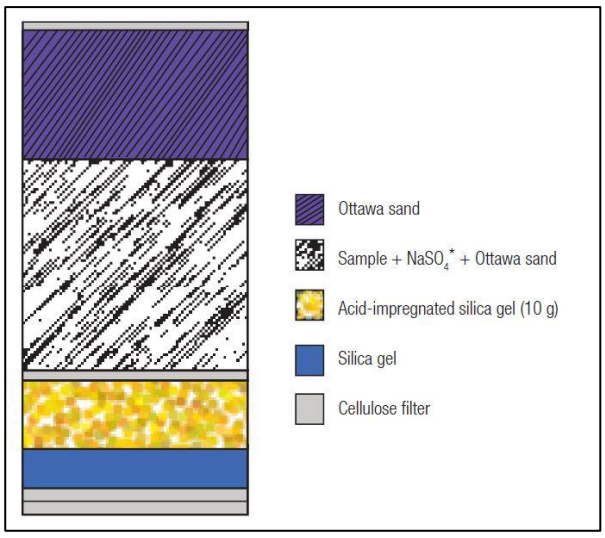

Figure 6 Preparation of the extraction cell for selective extraction (Dionex Technical Note, 210).

The sample was prepared mixing 2 g of hydromatrix with 5 g of sample and 2 g of $NaNO_4$; any remaining cell volume was filled with Hydromatrix.

The extraction was performed in ASE 200 from Dionex (figure 5), according to the following conditions:

Extraction Solvent: n-Hexane
Pressure: 1500 psi
Temperature: 100 °C
Static Time: 5 min
Static Cycles: 2
Flush: 60%
Purge: 90 s

One analytical duplicate for each sample and one analytical blank (Hydromatrix) for every five samples were also extracted and processed. Tetrachloro-m-xylene, tetrachloronaphtalene and octachloronaphtalene, were alternatively added to each sample as extraction standards.

The accelerated solvent extraction coupled with in-line clean-up have been performed in the laboratories of the institute of atmospheric pollution research , IIA-CNR, of Rome.

3.6 Silica SPE cartridge clean-up, analytical procedure

The solid-phase extraction (SPE) was performed according to the EPA guidelines (EPA, 1996).

Except for extraction by Soxhlet, the concentrated extracts (1 ml) were purified in SPE cartridges. The silica cartridges (6 mL serological-grade polypropylene tubes, with the 1 g of silica held between two polyethylene frits with 20 μm pores) were arranged on a CNW vacuum manifold, with 12 ports permitting a multi-processing of the extracts, and conditioned with 4 ml of hexane (Figure 7) . The extract was transferred into the cartridge and passed through it at approximately 2 mL/minute. The volumes of 2,5 ml of hexane and of 5 ml of diethyl-ether:hexane (1:1) were passed through the cartridge one after the other, and collected in two different fractions.

Figure 7 Solid-phase extraction (SPE), laboratories of the Water Research Institute (IRSA-CNR), Rome.

The extracts obtained were concentrated under a gentle stream of nitrogen and transferred in vials with 50 µl of Deca-chlorobiphenyl (2 ng/µl) as internal standard.

The extraction has been performed in the laboratories of the water Research Institute (IRSA-CNR), Rome

3.7 Silica H_2SO_4 clean-up, analytical procedure

Extracts from Soxhlet were reduced to a volume of 1 mL prior to clean-up.

The glass column (200 mm x 15 mm i.d.) has been filled (from bottom to top) with:

-2 g of anhydrous sodium sulphate,

-2 g of activated silica gel,

-2 g of $AgNO_3$ (10%),

-0,5 g of activated silica gel,

-10 g of acid-impregnated silica gel (H_2SO_4 44%)

-2 g of activated silica gel

-2 g of anhydrous sodium sulphate.

After adding 100 ml of hexane to the column, extract was loaded onto the surface by means of a Pasteur pipette (Figure 8).

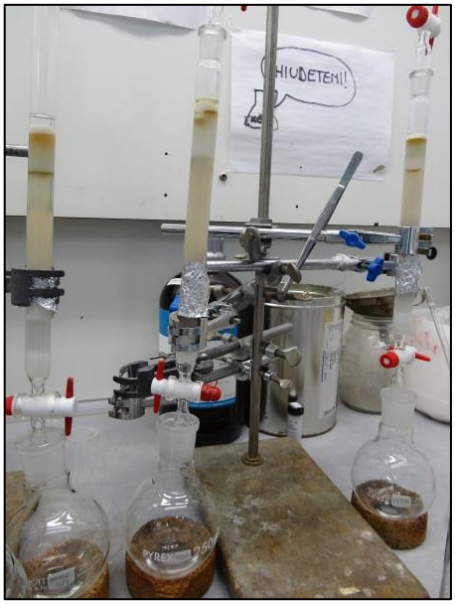

Figure 8 Silica H_2SO_4 clean-up, laboratories of the institute of atmospheric pollution research , IIA-CNR (Rome)

Final elution of the extracts occurred with a total volume of 150 ml of hexane. The elute was concentrated and transferred to a vial with 50 µl of labelled standard (PCB-ISS-H, Wellington Lab, 20 pg/µl) as internal standard.

The extraction has been performed in the laboratories of the institute of atmospheric pollution research , IIA-CNR (Rome).

3.8 Mass spectrometric detection CG-MS and GC-MS/MS, analytical procedure

Extracts were analysed on a Thermo Scientific TRACE Ultra Gas Chromatograph, TSQ Quantum mass spectrometer. The gas chromatograph was equipped with a TG-XLB fused silica capillary column, (60 m x 0,25 mm i.d. x 0,25 µm f.t.) from Thermo

Scientific. The carrier gas was helium at a flow-rate of 1 ml/min.

A sample volume of 2,0 µl was injected into a split-splitless injector operating in the splitless mode, at a temperature of 290°C. The temperature of the GC-MS transfer line was 280°C. The oven temperature program started at 140°C for 1min. Subsequently, the temperature was increased to 200°C at 60°C/min., held for 5 min and to 245°C at 6°C/min., held for 10 min, finally to 325 °C at 6°C/min and held for 7 min (Figure 9).

In order to lower the detection limits , the option MS-MS was used, with electron ionisation, under the following conditions: source temperature 250°C, source electron energy 70 eV, source emission current 50 µA, MS collision gas pressure 1,5 mTorr (Ar), MS collision energy 29 V.

The quantitation of the individual PCB congeners was performed by internal standard multi-point calibration using 6 calibration standard solutions (P48-M, Wellington Laboratories) in three replicates, from 0,1 pg/µl to 5 ng/µl. The compounds are quantified using the ratio of the analyte and internal standard response (peak area). The instrumental limit of quantitation (LOQ), the concentration at which quantitative results can be reported with a high degree of confidence, has been determined with the

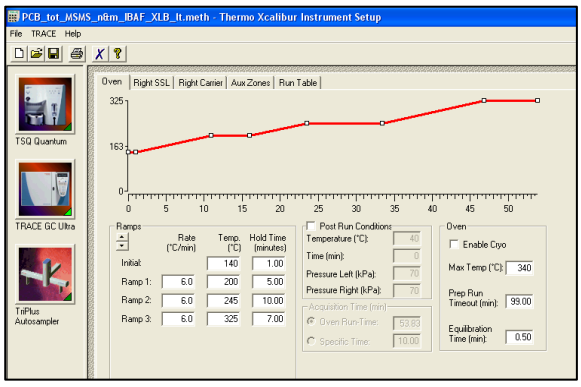

Figure 9 GC oven settings

PCB congener IUPAC number	LOQ (pg)
28	43,73
52	25,46
101	11,00
153	26,59
138	29,65
180	27,97

Table 5, Limits of quantitation for PCB indicators congeners

approach based on parameters from the analytical curve (Ribani, Collins et al. 2007). LOQ values, measured for each PCB indicator, are reported in table 5.

The GC-MS determination has been achieved with the support and the analytical instrumentation of the institute of atmospheric pollution research , IIA-CNR (Rome).

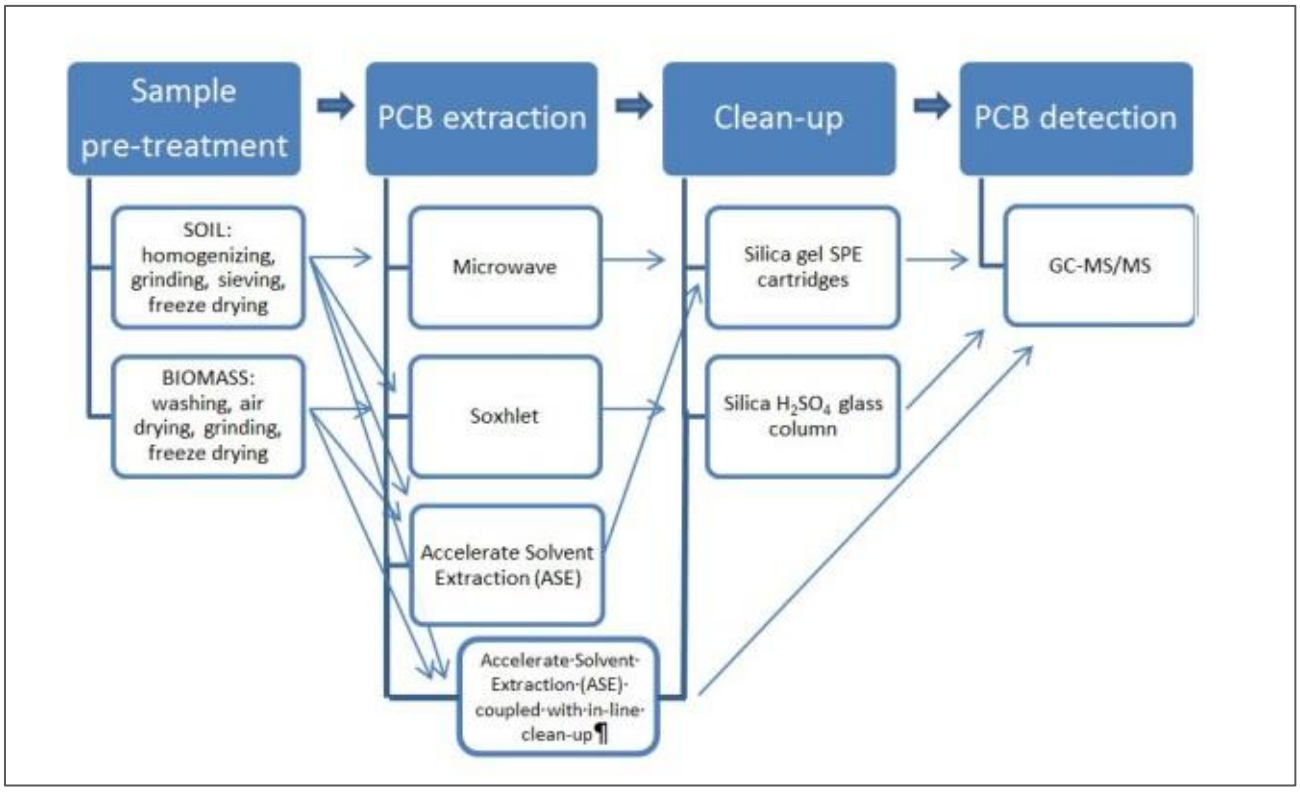

Figure 10 Analytical techniques adopted for the analysis of PCBs in soil and biomass samples; light blue arrows connect the different steps followed within the present study.

4 Results and discussion

Soil and biomass samples have been processed following different well established procedures as summarized in the diagram on figure 10.

Testing different techniques we could select the most suitable procedure for the PCB bio-degradative process monitoring, considering the ease of procedure, the cost and the analytical instrumentation availability.

In the following paragraphs the advantages and the disadvantage of each tested technique will be presented and discussed.

4.1 Sample pre-treatment

The soil sample homogenization is a critical step for PCB analysis due to the typical irregular dispersion of the PCB pollution in the soil matrix related to the oil spill slicks. Therefore, in order to collect representative sub-samples it is important to grind and accurately mix the sample before to take a portion of it for the analysis.

PCB were unexpectedly found in root tissues at concentrations even higher than in soil, ranging from 0,3 to 89,5 µg/Kg for each PCB congener. Modelling for plant uptake predicts that being hydrophobic molecules such as PCBs (log k_{ow}>3.5) too strongly sorbed to soil particles, roots arenot able to translocate them within plants to any significant extent (Whitfield Åslund, Zeeb et

al. 2007); reports indicate that plants absorb PCBs, but in very low amounts (Iwata, Gunther et al. 1974, Strek and Weber 1982)

For these reasons the presence of PCBs that we observed in root samples could be due to the adsorption to the root exterior surface rather than to an active absorption into root tissues. PCBs in plant tissues follow the general trend of increasing their concentration with decreasing their chlorination; consequently lower chlorinated PCB congeners are taken up much more readily than the higher chlorinated ones (Iwata, Gunther et al. 1974, Strek and Weber 1982) In our experiment PCBs congener pattern in root samples is similar to that-one found in soil. According to the literature above mentioned, this result shows that the presence of PCBs in root tissues is due to the adsorption to the root exterior surface. In order to make a clear distinction between adsorbed and absorbed PCBs, a further washing step of the root material with a surfactant (e.g. Tween) would be necessary (Barillot, Sarde et al. 2013). In this case the freezing of root samples before the washing step has to be avoided, in order to prevent the damage of the cell wall and the potential penetration of the surfactant into the root tissues; surfactants may yield interferences to sample analysis.

Accordingly to the results, roots act as bioconcentrator of PCB. This is probably due to the realising of root exudates and to the root turnover, these processes lead both to an increase in organic carbon around the root surface, increasing the adsorption of PCB molecules on the organic matrix (Haque, Schmedding et al. 1974, Choi, Nfodzo et al. 2012). For this reason, it is important to pay attention during the soil sampling in order to avoid the presence of roots; even a small root segment could distort the exact estimation of the PCB soil concentration.

In order to avoid the loss of analytes, occurring when temperature exceeds 40°C (Berset, Ejem et al. 1999), freeze-drying soil and biomass samples might be a good choice, rather than drying samples at low temperatures for several days which could cause the contamination of the sample and of the surrounding environment (oven or laboratory). In addition the air-drying technique does not lead to the complete dehydration of the sample requiring a more intensive drying of at least a sample aliquot in order to estimate the dry weight. The freeze drying resulted therefore as the most suitable technique for the preparation of soil and biomass samples for the PCB analysis; in this case attention should be given in putting samples in different vacuum chambers in order to prevent sample cross-contamination due to the most fine particles volatilisation.

4.2 PCB extraction

Soxhlet extraction is the most ordinarily applied technique for the extraction of PCBs; the necessary apparatus is commonly present in the laboratories for the analysis of micropollutants; despite this, Soxhlet extraction required large amounts of solvent (approximately 250 ml) and demanded long extraction times (24 h).

Microwave procedure has been accomplished with a lower solvent consumption (20 ml). The time required for the extraction step has been reduced too (25 min for the simultaneous extraction of 10 samples); however this method involves the vessel cooling time and two additional steps of

centrifugation to separate the liquid phase from the solid phase, thus limiting the time-saving advantage. Since the largest volume that can be loaded in each microwave extraction vessel is 3g, the method is not the optimal solution in presence of low PCB pollution (< 0,20 µg/Kg for a single PCB congener). When the concentration of the target PCB congener is lower than 0,20 ppb, the extraction of each sample in two different vessel would be required, collecting the two extracts in a single vial before the analysis. Since the microwave system is equipped of a turntable containing 10 vessels (including the method blank, the matrix spike sample and the replicate samples), the advantage of the process automation is thus limited in the case of low contaminated matrices.

ASE was performed with approximately 40 ml of solvent, in 3 extraction cycles, requiring a total extraction time of 25 min for each sample; this resulted in a time and solvent saving compared to Soxhlet extraction. The instrument used allows for the automated extraction of up to 24 samples. The only drawback of ASE is that the possibility of extracting samples is dependent on the proper functioning of the instrument and on its availability (often this type of instrument are shared between different research teams or used for various purposes), compared to the more simple and reliable Soxhlet apparatus.

Among the tested extraction standards (tetrachloronaphtalene and octachloronaphtalene, tetrachloro-m-xylene, and labelled PCB LCS-H), tetrachloro-m-xylene and tetrachloronaphtalene resulted to have a retention time too short compared to PCB congeners, octachloronaphtalene resulted to be valuable in quantifying high chlorinated PCBs; the labelled standard was obviously the most effective, against his high cost.

4.3 Clean-up

Even if the solid-phase extraction with polypropylene tubes packed with silica gel is time saving technique and it requires low amounts of solvent (12 ml for each sample), this method has proven to be not suitable for clean-up in PCB analysis. A high peak of an interfering compounds (iso-octyl phthalate) appeared on the chromatograms of samples cleaned up with SPE cartridges (figure 11). It is probably due to the contamination of the extract with phthalates released by the polypropylene tubes; additionally as polypropylene polymer is a very efficient PCB adsorber, its use should be avoided in laboratory experiments with PCBs, or at least the contact time of the contaminated solution with this material must be short (Cseh, Sanschagrin et al. 1989).

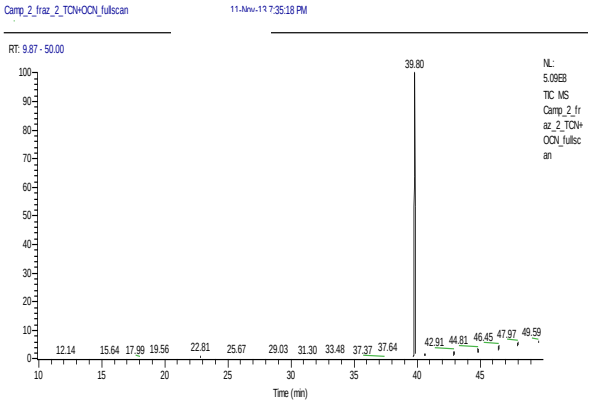

Figure 11 Gas chromatogram in full scan mode of a soil extract cleaned-up with SPE cartridges, the out of scale peak has been identified as iso-octyl phthalate.

Furthermore, when the cartridges have been arranged in a manifold system connected to a vacuum pump, a specific difficulty was

found in controlling the flow rates by opening and closing cartridge valves; in fact the valve regulation was not sensible enough to fix a reproducible elution flow rate.

These problems were overcame using standard glass chromatographic columns, however the required volume of solvent (250 ml) was higher and the clean-up process was longer and more laborious compared with SPE cartridges technique.

Selective pressurized liquid extraction (SPLE) offered several advantages compared to the techniques described above:

- low solvent consumption (20 ml for extraction and clean-up steps)

- time saving (the treatment of each sample in ASE system requires 25 min)

- automatable technique

- the single-step extraction and clean-up of the sample allows to reduce the loss of analyte due to extract transfer operations

- low operating costs

However the capital cost of the SPLE technique is much higher than that for PCB column clean-up, furthermore SPLE requires instruments more complex and thus more subjected to technical complications then other clean-up methods.

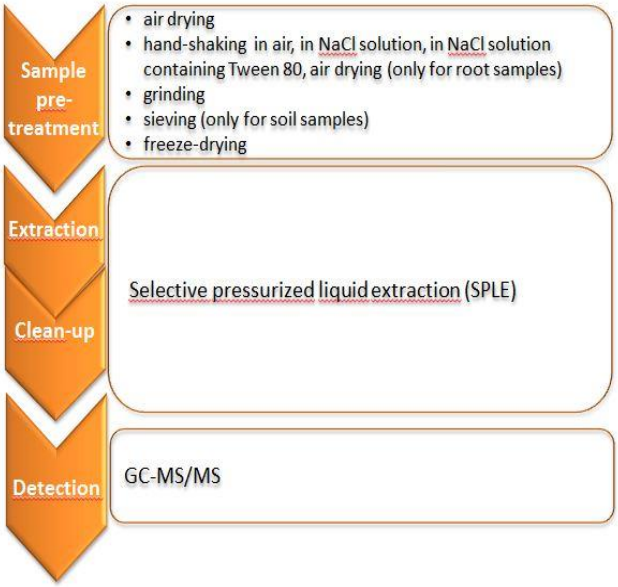

Figure 12 Analytical protocol for the determination of PCB in soil and biomass samples

consideration of the cost and the analytical instrumentation availability. A simple, rapid and accurate protocol for the determination of PCBs in soil and biomass samples has been delineated as pointed out in the figure 12.

The method has been optimised for environmental studies which generally require the analysis of a large number of samples, this protocol allows to follow the time course of the bio-degradation taking place in a polychlorinated biphenyls-contaminated soil or sediment.

5 Conclusions

Among the tested techniques of sample pre-treatment, extraction and clean-up, we selected the best compromise between ease of procedure and degree of automation, with

PART III Plant-assisted bioremediation study using long-term PCB-contaminated soil.

1 Abstract

Field and greenhouse investigations on rhizoremediation of a PCB historically contaminated soil have been performed for one year, evaluating the role of *Populus* species (clone Monviso) and of the autochthonous microbial community.
The greenhouse experimental set-up consisted of pots filled with the contaminated soil and poplar cuttings planted in it, under the following conditions: microbiologically active soil (TMA), previously sterilized soil (TS), microbiologically active soil in hypoxia (TMAA).
PCB concentrations in soil samples and plant roots were analysed 6 months and 12 months after the start of the experiments. At the same time plant growth, biomass production and plant stress indicators (i.e. chlorophyll content, leaf fluorescence, antioxidant in plant tissues) were investigated together with cell abundance and viability of soil microorganisms under the different growing conditions.
The overall results show the influence of hypoxic/aerobic conditions and soil sterilization on PCB degradation and poplar physiology. At month 6 and month 12 different effects were observable.
The field investigation is still in progress, however, preliminary results suggest the capability of the poplar clone Monviso to thrive in PCB soil. The present study is part of a wider Project involving several CNR Institutes: the Water Research Institute of Rome and Bari (IRSA-CNR), the Agro-Environmental and Forest Biology Institute (IBAF-CNR) of Rome and the Institute of Atmospheric Pollution (IIA-CNR) of Rome.

2 Introduction: Phytoremediation and bioremediation of polychlorinated biphenyls (PCBs): state of knowledge and research perspectives

2.1 Background

Polychlorinated biphenyls (PCBs) are a class of Persistent Organic Pollutants (POPs) differing in the number of chlorine atoms (1-10) attached to their biphenyl rings (figure 1).

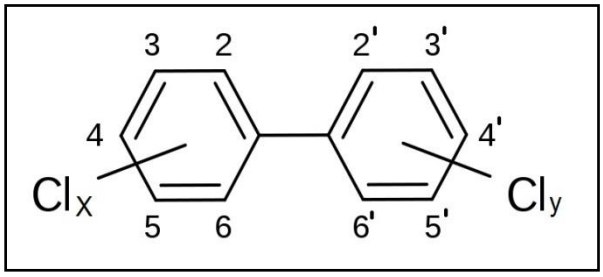

Figure. 1. General chemical structure of chlorinated biphenyls. The chemical formula is: $C_{12}\ H_{10-x-y}\ Cl_{x+y}$ *where x and y are between 1 and 5*

Mixtures of PCB congeners were widely manufactured throughout 40 years, from 1930 to the 1970s, being used as coolants in transformer oil, dielectric fluids and lubricants. While PCBs were manufactured and sold with different commercial names (e.g., Delor, Askarel, Aroclor, Fenclor), the most common was the Aroclor series. Each type of Aroclor has a distinguishing suffix number that indicates the degree of chlorination: the last two digits indicate the percentage of chlorine by mass in the mixture. By the late 1970's most governments banned PCB production but an extensive environmental contamination still persists as a consequence of accidental spills

and leaks due to improper transport, storage and disposal. To date PCBs still represent a global problem since they are persistent and bioaccumulate. Due to atmospheric transport, they have become environmental pollutants having ubiquitous distribution, including polar regions and depth of oceans (Beyer and Biziuk 2009) .

2.1.1 Physical-chemical properties of PCBs

Because of their high molecular stability, low solubility in water and high tendency to adsorb on particulate phase, PCBs are extremely hard to remove from soil and sediment matrices. Their hydrophobicity and lipophilicity make them poorly taken up by plants but susceptible to bioaccumulate in animals, mainly in adipose tissue and breast milk. Depending on chlorines number and position, PCB congeners differs in chemical, physical and toxicological properties. Table 1 summarizes some characteristics that are significant for predicting the environmental fate and impact of PCBs.

Chlorine substitution further stabilizes the aromatic structure by electron displacement and an increase in oxidation state. As a general rule, the more the molecule is chlorinated, the less water soluble and volatile it is (Campanella, Bock et al. 2002). The octanol-water partition coefficient (log K_{ow}) allows to predict the mobility of PCBs in the environment, i.e., higher-chlorinated PCBs, with log K_{ow} above 6, are associated with particulate matter in the atmosphere, soils and sediments, while lower-chlorinated congeners exist in the gaseous phase. As a consequence, soils generally contain higher proportion of higher-chlorinated congeners, while air is dominated by lower-chlorinated fractions

2.1.2 Toxicological properties of PCBs: epidemiological studies

At the present time PCBs still represents a global problem. They pose a threat to the environment due to their high adsorption capacity to soil, to their low water solubility, to their considerable stability both in environment and in human tissues, and to their toxicity. As a result of atmospheric transport, they have become ubiquitous environmental pollutants, found also in places where they have never been used, such as polar regions and deep oceans (Aken, Correa et al. 2009, Lauby-Secretan, Loomis et al. 2013). PCBs are lipophilic chemicals that enter lipophilic body membranes,

Physical states (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	oil, viscous liquid, sticky resin
Water solubility (mg/L) (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	0,0027 (Aroclor 1260)- 0,59 (Aroclor 1221)
Octanol-Water Partition Coefficient (Log k_{ow}) (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	4,7 (Aroclor 1221) - 6,8 (Aroclor 1260)
Bioconcentration factors (BCFs) in aquatic organisms (Agency for Toxic Substances and Disease Registry (ATSDR). Atlanta 2000)	60 (penta-and esa-CBs in Ciliate *protozoa*)-274000 (tri- and tetra-CBs in the freshwater fish Feated minnow)
Toxic equivalency factor (TEF) for mammals (Van den Berg, Birnbaum et al. 1998)	0.00001 (2,3',4,4',5,5'-HexaCB)-0,1 (3,3',4,4',5-PentaCB)

thereby promoting the absorption of toxic hydrophilic chemicals that would not be able to penetrate lipophilic membranes. Due to their ability to bioaccumulate, today they are present in the whole food production chain. PCBs were detected in almost all the selected food samples: the samples of animal origin as eggs, milk, shell powder and fish oil (also used to feed animals) showed the highest concentrations, while ingredients from vegetable origin ranged from non-detectable to small doses (Fernández-González, Yebra-Pimentel et al. 2013, Malisch and Kotz 2014). Besides dietary exposure, PCBs affect humans through dust ingestion and inhalation (Wang, Huang et al. 2013).

Given the widespread exposure to these compounds, the potential public health consequences of even small effects on human health are relevant. PCBs proved to cause neurological, reproductive, endocrine, cutaneous diseasesas, as well as many different infections ascribable to the reduction of immunitary responses. Epidemiological studies suggest that most of these effects result from chronic exposure to PCBs.

The relevance of these pollutants to human health is evidenced by the hundreds of papers published in the last couple of years, the most commonly reported effects are summarized below. Both epidemiological and experimental investigations strongly suggest or prove the correlation between exposure to these pollutants and a number of metabolic diseases, such as type 2 diabetes (Taylor, Novak et al. 2013, Zeliger 2013), obesity (Tang-Péronard, Andersen et al. 2011), metabolic syndrome and nonalcoholic fatty liver disease (Wahlang, Falkner et al. 2013). In particular, the worldwide increase of type 2 diabetes, in pandemic-like numbers, has been considered by Taylor *et al.* (Taylor, Novak et al. 2013) to be, at least in part, an environmental illness.

In addition, the potential neurotoxicity of polychlorinated biphenyls (PCBs) has been extensively investigated in humans and in animals. Exposure to some organochlorine compounds, including PCBs, may produce cognitive and motor deficits (Wigle, Arbuckle et al. 2008), in particular their involvement in attention and concentration problems (Forns, Torrent et al. 2012, Polańska, Jurewicz et al. 2013) has been suggested, as well as in inattentive and hyperactive/impulsive symptomatology during pre-adolescence, and in some impairment of early motor development (Berghuis, Soechitram et al. 2013).

Some of the above mentioned neural effects may result from the disruption of thyroid hormone function, which is one of the hormonal actions exerted by these pollutants (Leijs, ten Tusscher et al. 2012, Wise, Parham et al. 2012). These motor-cognitive effects, together with the changes in the levels of some brain neurotransmitters observed in PCB-exposed experimental animals (Faroon, Jones et al. 2000), induced Sioen *et al.* (Sioen, Den Hond et al. 2013) to warn that environmental contaminants might influence the mental health of the next generation.

Prenatal exposure to PCBs and dioxins may also decrease immunitary responses, thus increasing the susceptibility to infectious diseases in early childhood (Stølevik, Nygaard et al. 2013). Clear signs of immune dysregulation are indicated by the strong association between the non-Hodgkin lymphoma (a tumor associated with immunitary cells) and PCB exposure (Freeman and Kohles 2012). Recently a Working Group gathered by the International Agency for Research on Cancer (IARC) classified PCBs as carcinogenic to humans

(Lauby-Secretan, Loomis et al. 2013). The skin disease known as chloracne is the typical effect of exposure to PCBs.
Finally, it must be underlined that in most cases PCB contamination of soil and air is associated with the presence of other pollutants (e.g., heavy metals and dioxins); the study of potential co-exposure to chemicals with a common mechanism of toxicity is a crucial issue. Further research is required to establish the potential cumulative effects.

2.1.3 Actual PCB remediation strategies

The critical factor affecting PCB remediation is the strong sorption of these molecules on soil and sediments. The ability to desorb these contaminants determines, in most cases, the effectiveness of remediation technologies (Gomes, Dias-Ferreira et al. 2013).
PCB concentrations in the polluted soils/sediments are usually relatively low, being generally less than 1% of the contaminated mass, even for the heavily contaminated sites (Zhou, Anitescu et al. 2004). Most of traditional remediation techniques for PCB polluted soil and sediments, such as solvent extraction, landfarming, thermal desorption or thermal alkaline degradation, are not appropriate to the treatment of large volumes. These methods require expensive operations for the excavation and transport of contaminated materials for *ex situ* treatment and/or imply high energy expenditure. An alternative *in situ* technology, thermal treatment using microwave energy, is limited to the characteristic of the polluted site and requires high costs (Gomes, Dias-Ferreira et al. 2013). The current concern is therefore focusing on developing cost-effective processes to deal with the pollution of large volumes of slightly contaminated soil/sediment. Technologies based on biological remediation capability of plants and microorganisms meet this need. These biological methods, although requiring more time, are far cheaper than the physical and chemical ones (the cost of biological techniques ranges between 1/10 and 1/2 of the others) (Campanella, Bock et al. 2002, Aken, Correa et al. 2009).

2.1.4 Comments on global remediation market

The application of bio- and phyto-remediation technologies is expanding rapidly. Bioremediation is a cost effective and environmentally sound alternative which has high public acceptance as it offers to destroy or render less harmful contaminants using biological activity (Vidali 2001). It is very difficult to quantify the value of emerging remediation market owing to the lack of comprehensive catalogues of worldwide contaminated sites. Literature indicates that the global market for remediation of contaminated sites is in the range of U.S.$30–35 billion (Singh, Kuhad et al. 2009). According to an estimate, the global demand of these bioremediation technologies is valued around $1.5 billion per year and has a ready market in countries such as Canada, U.S., Japan, Western European countries and Australia. Eastern European, Asian and Latin American countries represent emerging markets for the remediation sector. A report of United Nation Industrial Development Organization (UNIDO) prepared by Li and Mohamed (Li, Eng et al. 2007) listed the cost of few established bioremediation technologies about U.S.$55 and 360/m3 for remediation of chlorinated POP-contaminated sites. The cost of phytoremediation of chlorinated POPs

including PCBs has also been estimated in the range of U.S.$150-630/m3. However these figures may vary with respect to various environmental and legal factors. Nonetheless, the global market for remediation of contaminated sites is undergoing a process of transformation and apparently will achieve market maturity and stability.

2.1.5 Environmental degradation

Since 1987 the observation of gas chromatographic peak distribution of PCBs in environmental samples showed altered PCB congener distribution patterns, as compared to original commercial mixtures (Quensen, Tiedje et al. 1988), and differences depending on the sampling location. The analysis of PCB congener concentration in the sediments of Hudson River (NY, U.S.) provided the first evidence of PCB degradation processes naturally occurring in anaerobic ecosystems. Such degradation has been interpreted as being the result of reductive dechlorination mediated by different populations of anaerobic bacteria, each species with its own pattern of PCB congener selectivity (Brown, Feng et al. 1987, Quensen, Tiedje et al. 1988).

Studies on chiral PCB congeners further highlighted the presence of biotransformation processes occurring in soil and sediments, in fact the observation of the enantiomeric enrichment of chiral PCBs following the initial racemic release into the environment, proved the existence of an enantioselective microbial metabolism taking place under both aerobic (Singer, Wong et al. 2002) and anaerobic (Pakdeesusuk, Jones et al. 2003) conditions. Chiral PCBs have been used as molecular probes of biological metabolic processes also in studies on animal and plant species, showing that investigated organisms can either biotransform or accumulate PCBs (Zhai, Hu et al. 2011).

It is therefore evident that PCB molecules, despite their high chemical stability and their reduced bioavailability, are naturally undergoing biological degradation processes. The degradation processes involving bacteria, fungi and plant (aerobic and anaerobic bio-degradation, phyto-degradation and rhizodegradation) will be here presented. The prominent methods proposed in recent years for the stimulation and the acceleration of such biological processes aimed at assessing bio- and phyto-remediation treatments in contaminated soil and sediments will be also summarized.

2.2 Bio-remediation of PCBs

2.2.1 Anaerobic and aerobic biodegradation

As described in figure 2, bio-degradation of PCBs proceeds through numerous steps, like a metabolic-chain involving different bacterial and/or fungal strains. PCB-degradation system includes two major microbial metabolic steps:

- anaerobic reductive dechlorination, an energy-yielding process where PCBs serve as electron acceptor thus being turned into less chlorinated congeners (Vasilyeva and Strijakova 2007, Field and Sierra-Alvarez 2008, Aken, Correa et al. 2009).
- aerobic breakdown of the biphenyl structure that only works on lower-halogenated congeners (< five chlorine) through an oxidation reaction, resulting in chloro-HOPDA (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) and chlorobenzoic acids formation, ring opening and potentially complete mineralization (Pieper 2005).

Although the reductive dechlorination of commercial mixtures does not decrease the

molar concentration of PCBs, this process reduces their dioxin-like toxicity (Tiedje, Quensen III et al. 1993) and makes them more readily degradable by aerobic bacteria (Quensen, Tiedje et al. 1988).

However, the complete mineralization of higher-chlorinated PCBs can only occur under sequential anaerobic-aerobic treatment. The anaerobic reductive dechlorination can follow different alternative pathways, depending on physical, chemical and biological site-specific characteristics; each process is mediated by a different population of anaerobic bacteria with its own distinctive pattern of PCB congener selectivity. Brown and co-workers (Brown, Feng et al. 1987) characterized eight of these processes and gave each of them a designation letter (M, Q, H, H', P N, LP and T). Figure 2 shows only some example of those routes. As reported by Wu et al. (Wiegel and Wu 2000), temperature and pH have a strong influence in determining which route of microbial dechlorination is going to take place since, depending on temperature and pH, specific bacterial strains prevail over the others, with consequent diverse degrading skills (Wu, Bedard et al. 1997).

Environmental degradation of PCBs is very slow, especially with regard to the reductive dechlorination process. Half-life time in soil and sediment differs depending on the congeners; it has been estimated to vary from 3 to 37 years for tri-, tetra-, penta-, hexa- and hepta-CB, increasing according to chlorination degree (Sinkkonen and Paasivirta 2000). The rates of biodegradation depend on the characteristics of PCB contamination and are affected by many environmental site-specific variables (Sinkkonen and Paasivirta 2000). Some of those factors are summarized below.

PCB concentrations: under experimental condition the most rapid dechlorination was observed at the highest concentrations used, 800 mg/Kg (Quensen, Tiedje et al. 1988, Bedard 2008). At concentrations lower than 50 mg/kg the process substantially decelerates, due both to the lack of induction of the degradative enzyme and to the impossibility in sustaining the growth of the competent organisms. On the other hand, concentrations of PCBs above 1000 mg/kg may cause toxicity in bacterial communities (Borja, Taleon et al. 2005, Vasilyeva and Strijakova 2007).

Pollution age: with the progressing of months subsequent to the contamination event, PCB molecules are adsorbed on soil or sediment matrices, becoming progressively less bioavailable. Thus, freshly added PCBs are degraded faster than the preexisting ones (Alder, Haggblom et al. 1993, Choi, Nfodzo et al. 2012)

Number of chlorines: biodegradability decreases with increased number of chlorine atoms in the PCB molecule (Furukawa 2000).

PCB structure: non-planar congeners (di- to tetra-ortho substituted) are poorly degraded, presumably due to steric hindrance of the responsible enzyme; congeners with chlorines located on only one of the rings are much more easily degraded than those with chlorines on both rings (Furukawa 2000).

pH and temperature: the influence of these factors is complex because they affect: i. the specific bacterial communities involved in the degradation process, ii. the equilibrium between the relative proportion of PCBs that are dissolved and those that are adsorbed on organic matter, thus influencing the bioavailability of PCBs in soil (Wiegel and Wu 2000).

Site-specific microbial populations: microbial history of different areas plays a substantial role on the rate and the extent of the PCB biodegradation processes.

Vasileyeva and Strijakova (Vasilyeva and Strijakova 2007) highlighted the occurrence of a lag period ranging from one to several months before dechlorination in freshly spiked PCBs begins. This time is necessary to activate the preexisting anaerobic bacterial microflora that is scarce in PCB-non-contaminated sites (Wiegel and Wu 2000). Furthermore, even in aged contaminated matrices, the rate and the extent of the dechlorination may vary according to the existing microbial strains and activity, depending for instance on the interactions occurring between the dehalogenating and non-dehalogenating bacteria (e.g., the competition for substrates and other electron donors and acceptors) (Wiegel and Wu 2000).

Soil and sediment properties: as the organic carbon and clay content increases, so increases the adsorption of PCB molecules on the soil or sediment matrices, thus PCB bioavailability decreases (Haque, Schmedding et al. 1974, Choi, Nfodzo et al. 2012); the strong sorption may render PCBs more resistant to degradation and leaching from soils (Man, Lopez et al. 2011). Furthermore, the activity of degrading microorganisms depends on the disposal of macro- and micro-elements, as well as on the presence of organic carbon and oxygen in the contaminated matrix.

2.2.2 Microbial and fungal enzymes

As highlighted by Pieper and Seeger (Borja, Taleon et al. 2005) metabolism of biphenyl and PCBs should not be regarded as a simple linear pathway, but as a complex interplay between different catabolic gene modules.

Little is known about the dehalogenases responsible for the reductive dechlorination of PCBs; as far as we could verify, no enzyme involved in this process has been isolated or characterized (Bedard 2008, Aken, Correa et al. 2009)

Enzymatic pathways for bacterial oxidative PCB degradation are instead well documented as showed in figure 2 (Furukawa 2006, Pieper and Seeger 2008). Four enzymes are involved in the biphenyl upper pathway: a multi-component dioxygenase (bph A, E, F and G), a dehydrogenase (bph B), a second dioxygenase (bph C) and an hydrolase (bph D). These enzymes induce the insertion of two oxygen atoms into the aromatic ring, dehydrogenate the compound and cleave the dihydroxylated ring in ortho- or in meta-position. Initially Furukawa and co-workers (Furukawa, Tomizuka et al. 1979) described the oxidation pathway via 2,3-dioxygenase (as observed in cultures of *Acinetobacter* sp. and *Alcaligenes* sp. strains); successively

Bedard and co-workers (Bedard, Haberl et al. 1987) found a novel mechanism of PCB degradation, via 3,4-dioxygenase (as observed in *Alcaligenes eutrophus* H850 metabolism). Similar genes encoding enzymes involved in the upper pathway (termed *bph* A, B C and D), typically organized in an operon, were found on chromosomes, plasmids and transposons of different strains (Pieper 2005).

The enzymes involved in the biphenyl lower pathway and their encoding genes are not given an extended treatment in the literature. However, the process that degrades the dienoates leading to acetyl-CoA has been associated to the activity of three enzymes: bphH, bphI and bphJ, i.e., a hydratase, a dehydrogenase and an aldolase, respectively, encoded by genes typically organized in a bphHJI cluster (Pieper and Seeger 2008).

The fungal PCB degradation pathway has still not been completely determined; a non-specific, extracellular enzyme system is

known to be involved in PCB degradation by white rot fungi. Those enzymes evolved to degrade lignin, composed by structural analogues of PCB molecule, and fall into lignin-peroxidase, manganese-peroxidase and laccase groups (Koh, Park et al. 2000, Campanella, Bock et al. 2002). Recent studies indicate that intracellular fungal enzymes, as exemplified by cytochrome P450 monooxygenases, are also important in the degradation of a number of the chloroorganic pollutants (Marco-Urrea and Reddy 2012).

Natural substances, such as lignin and secondary plant metabolites (terpenoids and flavonoids), have an important role in the evolution of numerous PCB-degrading enzymes found in nature; this is the result of millions of years of plant-microbe interactions and constitute a real opportunity to harness and direct the catabolic activity of microorganisms (Singer, Crowley et al. 2003).

2.2.3 Dead-end metabolites and inhibition effect

The metabolism of certain PCB congeners can lead to several bottlenecks, starting with the accumulation of most recalcitrant ortho-chlorinated PCB, to the occurrence of dead-end metabolites resulting from aromatic ring oxidation, such as dihydrodiols, dihydroxybiphenyls (and 3,4-dihydroxylated derivatives), chlorinated hydroxyl-oxo-phenyl-hexa-dienotes (HOPDAs) or chloroacetophenones (Seah, Labbé et al. 2000, Pieper 2005, Furukawa 2006). Recent work focused on the role of bphD, which is considered a key determinant for the complete mineralization of PCBs due to substrate specificity of those enzymes and to their potential inhibition by certain metabolites (Seah, Labbé et al. 2000, Dai, Vaillancourt et al. 2002).

Interesting findings identified some PCBs dead-end metabolites inhibiting PCB degradation processes: Blasco *et al.* (Blasco, Mallavarapu et al. 1997) reported that the protoanemonin, a product of chlorobenzoate (CBA) metabolism, is toxic to PCB-degrading microorganisms. In others studies 4-CBA and 2-CBA are reported to inhibit the growth of Burkholderia LB400 strain (Martínez, Agulló et al. 2007). In addition ortho-chlorinated PCB metabolites seem to promote the suicidal inactivation of an hydroxybiphenyldioxygenase that catalyzes aromatic ring cleavage (Dai, Vaillancourt et al. 2002). Also dihydrodiols and dihydroxy-biphenyls revealed to be very toxic metabolites for bacteria, affecting cell viability (Cámara, Herrera et al. 2004). Some of the described toxic compounds are marked with asterisks in figure 2.

Thus the PCB recalcitrance to biodegradation and the low kinetic of the aerobic degradation process may be partly explained by the toxicity of certain metabolites generated during the oxidation process.

Efforts have been recently directed at developing strategies to overcome or decrease the inhibition induced by dead-end metabolites. Some research focuses on the synergic action of different degrading organisms, for instance the microbial consortium of PCB-degrading and CBA-mineralizing bacteria (Hickey 1996, Nielsen, Tolker-Nielsen et al. 2000, Pieper 2005) has been used. Other studies led to the construction of genetically modified bacterial strains with new catabolic abilities for mineralizing PCBs (Saavedra, Acevedo et al. 2010), or used certain fungi and plants to further degrade the main metabolites on the PCB pathway (e.g., ligninolytic *fungi* or *Salix* spp.) (Deavers, Macek et al. 2010,

Čvančarová, Křesinová et al. 2012). Ponce *et al*. (Ponce, Latorre et al. 2011) proposed that the addition of antioxidant compounds can have a cell protective effect against the oxidative stress induced in bacteria by the reactive oxygen species generated during PCB degradation

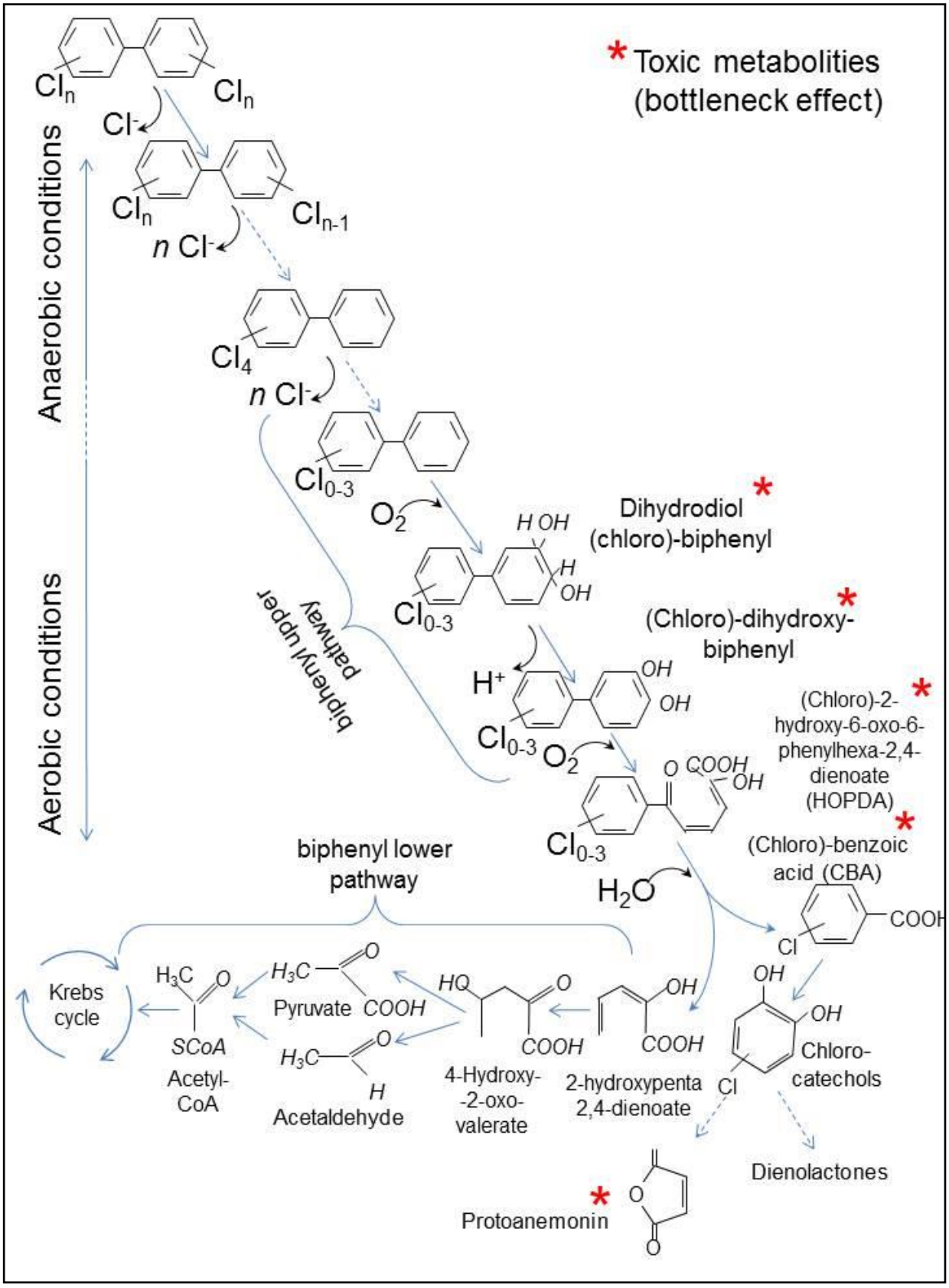

*Figure. 2. Representation of the some of the main pathways described for the PCB biodegradation. Some toxic metabolites, which can potentially cause pathway bottlenecks, are marked by an asterisk (*) (Pieper 2005, Furukawa 2006, Pieper and Seeger 2008).*

2.2.4 Bacterial strains

Several habitats have been investigated in order to find indigenous PCB-degrading microorganism, these studies proved that microbial communities able to metabolize PCBs are ubiquitously distributed in the environment. Numerous aerobic bacteria strains have been isolated from soil, sewage effluents, dredged spoils and even from the intestine of termites. Anaerobic strains are harder to isolate but their existence in contaminated soil and sediment (freshwater, estuarine and marine deposits) has been amply proved both by the observation of changes in PCB concentration during laboratory incubation assays and by culture-independent community fingerprinting techniques (Yan, LaPara et al. 2006, Adrian, Dudková et al. 2009, Zanaroli, Balloi et al. 2012, Wang and He 2013).

Each microorganism strain exhibits a particular activity spectrum with regard to the type and the extent of PCB metabolized (Pieper and Seeger 2008), the degradation of commercial mixtures of PCBs is thus attributed to the action of multiple degrading strains. The table SA in *Annex 1* lists, although not exhaustively, some bacterial strains found to have a role in PCBs degradation. Such table includes microorganisms with PCB anaerobic metabolism, with aerobic co-metabolism and some strains with PCB aerobic metabolism, in the last case the pollutant acts as the sole carbon and energy source (Boyle, Silvin et al. 1992, Arensdorf and Focht 1995, Sierra, Valera et al. 2003, Francova, Mackova et al. 2004, Adebusoye, Ilori et al. 2008, Tu, Teng et al. 2011, Ganesh-Kumar, Kalimuthu et al. 2013). Among the listed bacteria, the most efficient PCB degraders belong to *Burkholderia xenovorans* and *Ralstonia eutropha* strains, displaying the broadest substrate specificity.

Research on anaerobic dechlorinating populations has been hindered by the difficulties in isolating the bacterial strains, since they depend on the presence of both solid substrates in the cultures and of other microorganisms providing growth factors (Sowers and May 2013). However several experiments in laboratory slurry microcosms, selective enrichment sediment cultures and a few sediment-free cultures (Cutter, Sowers et al. 1998, Bedard 2008, Adrian, Dudková et al. 2009, Dudková, Demnerová et al. 2012) led to the identification of bacterial strains involved in the reductive dechlorination of PCBs, belonging mostly to *Chloroflexi* group and including some *Dehalococcoides* strains.

2.2.5 Fungal strains

Even though little is known about the PCB biodegradation mechanism in fungi, they seem to play an important role in the transformation of PCBs. The fungal degradation shows several advantages, as compared to bacterial activity: the involved enzymes have a free radical attack system with low specificity and their action occurs mainly in the extracellular environment, thus they can operate on a broad range of aromatic compounds and are less constrained by the low bioavailability of PCBs bound to the soil matrix. Moreover, several fungal strain can live in symbiosis with roots (endo- and ecto-mychorrizae); this association provides a survival advantage for fungi and makes them more resistant, thus increasing the performances of the *inocula* in contaminated sites (Donnelly and Fletcher 1995).

The majority of the investigated fungal strains seems to be most effective against the low chlorinated PCBs (Hickey 1996, Field

and Sierra-Alvarez 2008); however, at variance with bacteria, some strains are able to metabolize high chlorinated congeners also under aerobic conditions (Ruiz-Aguilar, Fernández-Sánchez et al. 2002). *Fungi* have been documented to further degrade the main metabolites (i.e., CBAs which represent the crucial recalcitrant bacterial PCB metabolites) simultaneously with PCBs (Čvančarová, Křesinová et al. 2012). However the fungal action seems to be limited by the sensitivity of some strains to high concentrations of PCBs, an increase in the initial PCB concentration led in some cases to a decrease in the pollutant degradation (Murado, Tejedor et al. 1976, Ruiz-Aguilar, Fernández-Sánchez et al. 2002).

Table SB in *Annex 1* offers an overview of the fungal strains involved in PCB degradation process, most of them belonging to the white rot fungi group that evolved their enzymes to metabolize lignin molecules, structurally similar to PCB compounds and to their metabolites.

2.2.6 From research to applicability: field study, full-scale applications and patents

To our knowledge, there are only a few reports in literature regarding *in situ* experiments on PCB biodegradation. Field studies results show generally less degradation than in laboratory investigations. The first experiment has been carried out on Moreau dredged sediments (NY, U.S.) (Rhee, Bush et al. 1989) incubated *in situ* in a polystyrene box, 1 m below the surface in anaerobic conditions. Seven month later the analysis showed little change in congener concentrations, which suggests on the basis of their experimental protocol, that temperature was the relevant factor. In 1993 Harkness *et al.* (Harkness, McDermott et al. 1993) reported a 73-day field study of *in situ* aerobic biodegradation in the Hudson River (NY, U.S.). Another study in New York (NY, U.S.) attempted to stimulate the process by repeatedly adding *Pseudomonas* strain over a designed area for a period of four months; many di- and tri-chlorobiphenyls were degraded, amounting to 20% of the total PCBs (Mondello 2002). Researchers anchored six large adjacent cylinders in shallow areas of the river; 37 to 55 percent loss of PCBs occurred with the addition of inorganic nutrients, biphenyl and oxygen (Harkness, McDermott et al. 1993). There are several reports of field application, mostly concentrated in United States, as reported by the U.S. Environmental Protection Agency in periodic reviews of site cleanup; in the majority of the such treatments plants were employed to enhance PCB biodegradation. A number of patents have been deposited in this field, describing improved processes for anaerobic and aerobic microbial biodegradation, mainly based on employing stimulatory agents, novel isolated microorganisms or recombinant bacteria (DeWeerd and Bedard 1996, Sarkar, De et al. 2003, Sowers and May 2008, Pfeiffer, Salinas et al. 2011).

2.3 Phytoremediation of PCBs

Numerous studies show an increase in PCB degradation, concerning mostly the low-halogenated congeners, in vegetated soil as compared with non-vegetated soil (Singer, Crowley et al. 2003, Chekol, Vough et al. 2004, Leigh, Prouzová et al. 2006, Mackova, Prouzova et al. 2009, Xu, Teng et al. 2010, Tu, Teng et al. 2011, Meggo and Schnoor 2013, Meggo, Schnoor et al. 2013, Secher, Lollier et al. 2013).

Two main processes are involved in plant action on PCBs:

- Rhizodegradation deals with the breakdown of organic contaminants by soil microbial activity which is enhanced by the rhizosphere's presence.
- Uptake of the contaminant from soil and translocation from roots to shoots.

Because of the low water solubility of these molecules (high log k_{ow} rates, 5-7), the uptake and translocation through the transpiration stream seem to be less significant than rhizodegradation on the fate of PCBs in soil; thus the plant role in PCB removal is mainly an indirect action, which consists in sustaining the contaminant degradation activity of soil microflora (Aken, Correa et al. 2009, Xu, Teng et al. 2010, Li, Liu et al. 2011, Meggo, Schnoor et al. 2013).
Additionally the biomass of some vegetal species, rich in flavonoids and terpenes, has been used as amendment to promote PCB degradation in soil, as described in chapter 4.1.

2.3.1. Rhizodegradation

A multitude of microbial processes is activated by rhizosphere. In particular, the degradation of specific pollutants can be promoted by the interactions between plant roots and soil microorganisms, in a process commonly defined as "rhizodegradation", "rhizoremediation" or "plant-assisted biodegradation" (Zhuang, Chen et al. 2007, Wenzel 2009). Many plant species are capable of thriving on PCB contaminated soils and certain species can promote indigenous populations of PCB-degrading microorganisms that harbor strong degradative abilities, as found from field investigations of a PCB-contaminated site in the Czech Republic (Leigh 2003, Leigh, Prouzová et al. 2006). Plants sustain fungal and bacterial degrading activity through the following processes:

- oxygen diffusion in soil, improving aerobic bacterial metabolism. Soil aeration is improved by root turnover, through the formation of air channels created when roots die and decay (Leigh, Fletcher et al. 2002) and by direct root oxygen release (Smith, Schwab et al. 2007, Meggo, Schnoor et al. 2013)
- organic carbon supply from root exudates and root turnover. Sugar, alcohols and organic acids can sustain bacterial communities acting as readily utilizable sources of energy (aerobic metabolism) or as electron donors (anaerobic metabolism)
- structural analogs of PCB contained in root exudates, such as phenolic compounds and terpenes, can induce the enzymes for PCB catabolism (inducers). Some of these compounds can enhance the growth of degrader organisms population to such a size that it becomes effective for metabolizing the contaminant, otherwise present at a too low level to support the growth of the degrader (co-metabolites) (Donnelly, Hegde et al. 1994, Crowley, Alvey et al. 1997, Chaudhry, Blom-Zandstra et al. 2005, Toussaint, Pham et al. 2012)
- biogenic surfactant release in root exudates. These molecules (e.g., quillaya saponin, a triterpenoid glycoside) enhance pollutants mobility, making them more bioavailable (Fava and Di Gioia 1998).

Distributed network of plant roots could serve as a natural injection system capable of capillary dispersion of oxygen and exudates in the soil volume explored (Donnelly, Hegde et al. 1994). In such a way plant roots can sustain large micro-organism communities and improve the establishment of new fungal and bacterial strains. Numerous studies demonstrated an increase of microbial density, activity and diversity in the rhizosphere as compared to bulk soil (Singer, Smith et al. 2003, Chekol, Vough et

al. 2004, Mackova, Prouzova et al. 2009, Tu, Teng et al. 2011, Qin, Brookes et al. 2014). It is relevant to highlight that even dead roots have a great potential in enhancing phytoremediation (Leigh, Fletcher et al. 2002, Slater, Gouin et al. 2011, Meggo and Schnoor 2013).

Furthermore root filaments create microzones with different redox conditions, allowing at the same time the anaerobic and aerobic steps of PCB biodegradation pathway (Smith, Schwab et al. 2007, Meggo, Schnoor et al. 2013), as discussed in chapter 5.

The presence of mycorrhizal hyphae network can increase the extension of the root system, the ability of the roots to penetrate relatively small soil pores (Leigh, Fletcher et al. 2002), root exudation and carbon translocation in underground organs. Thus mycorrhizal hyphae may have great potential in enhancing phytoremediation (Grayston, Vaughan et al. 1997, Teng, Luo et al. 2010). In addition some mycorrhizal fungi, such as *Radiigera atrogleba* and *Gautiera crispa*, are known to directly degrade PCBs (Donnelly and Fletcher 1995).

Obviously the size of a root system and its release capacity determine the sphere of influence of a plant species. Beyond the rooting zone the microbial growth-stimulating effects of exudates seem to diminish rapidly (Chaudhry, Blom-Zandstra et al. 2005, Ding, Guo et al. 2009). In soil rhizoremediation activities it is therefore preferred to use species with a well-developed root system, such as poplars or willows (figure 3).

Although rhizoremediation occurs naturally, it can be optimized by deliberate manipulation of the rhizosphere. This can be accomplished by using suitable plant-microbe pairs, possibly activated via seed-inoculation (Donnelly, Hegde et al. 1994, Gerhardt, Huang et al. 2009). Mackova *et al.* (Mackova, Prouzova et al. 2009) demonstrated how much the efficiency of PCB removal is dependent on the plant species used, since they influence the composition of consortia microorganisms in the soil surrounding the roots. Generally the use of indigenous plants and microbial species is preferred since they are already adapted to the remediation-site conditions, being naturally selected to be contaminant-tolerant and possibly to utilize the contaminant for their metabolism. In addition, the use of native-biota minimizes the risk of ecosystem disequilibrium.

Figure. 3. Selection of species and clones for phytoremediation purposes in the greenhouse. of the Institute Agro-environmental and Forest Biology (IBAF), National Research Council (CNR), Rome.

2.3.2 PCB uptake by plants

PCB uptake has been initially theoretically estimated, based on information on chemical properties (log k_{ow}) and plant physiology (Ryan, Bell et al. 1988). On the base of these studies PCBs would not be expected to be present in significant amounts in above-ground plant tissues, unless they are deposited from the air on the plant surface. Indeed only low chlorinated compounds would be taken up inside plant tissues, as PCB water solubility decreases with the degree of chlorination.

Later experimental studies are addressed to PCB plant uptake in order to assess the risks associated with land contamination, in particular its potential to contaminate the food chain. Differences among crops are reported, for instance higher concentrations were found in carrot peels, as compared with cabbage and corn (Webber, Pietz et al. 1994). In general, these studies (Suzuki, Aizawa et al. 1977, Strek, Weber et al. 1981, Webber, Pietz et al. 1994) confirmed the low translocation capability of plants toward PCBs, showing very low bioaccumulation factors in shoots (shoot BAF= 0,0015-0,042, where BAF=(PCB plant)/(PCB soil)). Experimental trials also confirmed that the translocation rates of PCB isomers differ, depending mostly on their different water solubility rather than on their selective absorption by sprouts (Suzuki, Aizawa et al. 1977) and that there is generally a lack of active transport of PCBs through the plant xylem system (Ye, Puri et al. 1992).
However, exceptions to the general low translocation rates have been identified by a Canadian research group in some weed species (*Vicia cracca, Polygonum persicaria*, etc.) and in zucchini (*Cucurbita pepo*) with BAF >1 (Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008, Ficko, Rutter et al. 2010). Hülster (Huelster, Mueller et al. 1994) reported first the presence of a molecule that is capable of increasing the bioavailability of a dioxin (hydrophobic compound, like PCB) in both tissues and root exudates of zucchini. Thus the ability of zucchini to absorb PCBs could be explained supposing that a molecule produced by the plant and released through its root exudates binds to the pollutant; this complex would then become hydrophilic enough to be absorbed by the roots and carried to all plant parts (Campanellaand and Paul 2000). Fan *et al.* (Fan, Cui et al. 2009) investigated the interspecies variance of dioxin-like polychlorinated biphenyls in the plants from a polluted wetland; the concentrations and ratios of congeners in plants varied greatly among species, according to morphological and physiological plant characteristics. Some relevant shoot BAF is reported in table SC.

In addition to uptake and translocation of PCBs, it seems that some plant species are able to partially metabolize these molecules. Several studies aimed at describing plant metabolism of PCBs are performed on *in vitro* plant cultures (Wilken, Bock et al. 1995, Kučerová, Macková et al. 2000, Mackova, Barriault et al. 2006, Rezek, Macek et al. 2007, Mackova, Prouzova et al. 2009). Important factors controlling this process seem to be the substitution pattern of chlorine atoms and the degree of chlorination. In general, most of the tetra-chlorinated and lower congeners can be metabolized, lesser chlorinated congeners more rapidly than those with higher number of chlorine atoms, with some exceptions due to the position of chlorine substitution. The metabolism is strongly dependent on the plant species, however a first phase of hydroxylation of PCB molecule (substitution of chlorine atoms with hydroxyl group to form more soluble hydroxyderivatives) has been described by several authors (Wilken, Bock et al. 1995, Kučerová, Macková et al. 2000, Mackova, Barriault et al. 2006, Rezek, Macek et al. 2007, Mackova, Prouzova et al. 2009). Cytochrome P450 monooxygenase system and peroxidases seem to be the enzymes responsible for PCB transformation in plants (Mackova, Barriault et al. 2006).
Table SC in *Annex 1* summarizes the involvement of some plant species in phytoremediation of PCB- contaminated soil and sediments, as reported in the references.

From the evaluated studies, plants phytoextraction potential appears to be limited and this may sustain the phytoremediation applications since vegetation constitutes the main route of entry of PCBs in the food web. However, the role of plants in the remediation of PCB contaminated soils and sediments still takes a lively interest, since plants can act as bacterial activity enhancer.

It is also possible to link the PCB metabolism of plant and bacteria along a single metabolic chain; Mackova and her group (Mackova, Barriault et al. 2006, Deavers, Macek et al. 2010) directed their interest to the ability of both plants to metabolize bacterial PCB degradation products and of bacteria to transform plant primary metabolites. Their study proved that certain species are able to degrade some chlorobenzoic acids entering the environment, due to the microbial PCB degradation.

2.4 PCB remediation enhancement

The most important limitations in PCB bioremediation are:

i) the low bioavailability of PCB molecules due to their tendency to bind tightly to soil organic matter, ii) the slowness of biological degradation kinetic due to low expression of catabolic genes

Numerous techniques have been proposed to overcome these limitations. The following paragraphs summarize the amendments recommended to enhance PCB biodegradation.

2.4.1. Inducers employed in aerobic PCB degradation

Biphenyl has been widely used as inducer of bph genes in cell cultures or microcosms (Brunner, Sutherland et al. 1985); however, the use of biphenyl in field applications is not acceptable due to its high cost, low water solubility and notably because of its possible toxicity to plants and soil organisms (European Union Regulation No 1272/2008). To find an alternative inducing dioxygenase gene expression it is necessary to consider the origins of the enzyme systems. As a result of thousands of years of plant-microbe interaction, xenobiotic metabolizing genes have in fact developed into microorganisms aimed at metabolizing some plant chemical derivatives that share structural similarities with PCBs (Singer, Crowley et al. 2003). Those plant compounds, structural analogs to xenobiotics, could provide an alternative to biphenyl, enhancing the rate of PCB aerobic removal. A number of such naturally occurring compounds have been tested as cosubstrates in aerobic PCB biodegradation with promising results, which also opened the way to further research (Donnelly, Hegde et al. 1994).

Plant terpenes such as carvone and limonene, as well as flavonoids and salicylic acid have been shown to enhance aerobic PCB biodegradation (Gilbert and Crowley 1997, Hernandez, Koh et al. 1997, Gilbert and Crowley 1998, Koh, Park et al. 2000, Singer, Gilbert et al. 2000, Master and Mohn 2001, Tandlich, Brežná et al. 2001, Oh, KOH et al. 2003).

However it has not been clarified as yet whether these plant derivatives serve as inducers of biphenyldeoxygenase or rather as biostimulators by representing an additional carbon source for bacteria (Dudášová, Lukáčová et al. 2012). These compounds can be introduced in the soil either as vegetal biomass (e.g., crushed willow roots, orange peels, ivy leaves, pine needles, eucalyptus leaves) or as living plants that secrete such compounds through root exudates

(Hernandez, Koh et al. 1997, Dzantor and Woolston 2001, Gerhardt, Huang et al. 2009, Dudášová, Lukáčová et al. 2012, Federici, Giubilei et al. 2012, Lehtinen, Mikkonen et al. 2013).

Not all the above mentioned inducers are efficient on a given bacterial strain; some of them, even biphenyl, have been reported to decrease the rate of PCB degradation, depending both on the bacterial culture on which they are introduced (Hernandez, Koh et al. 1997, Master and Mohn 2001, Sierra, Valera et al. 2003, Luo, D'Angelo et al. 2007) and on the available organic carbon substrates (Tandlich, Brežná et al. 2001, Luo and Hu 2013).

2.4.. Primers employed in anaerobic PCB dechlorination

Several authors highlighted the concept of "priming" the PCB anaerobic biodegradation process; they showed that certain compounds, added at elevated concentrations, are essential to activate the dehalogenation system in the relevant bacteria (Wiegel and Wu 2000). Bedard *et al.* (Bedard, Van Dort et al. 1998) hypothesized that PCB degrading bacteria communities, being stimulated and supported by these primer compounds used as electron acceptors, can fortuitously dehalogenate PCBs. An additional stimulation of the dehalogenation through the induction of dehalogenating enzymes is possible, even though such mechanism is presently regarded as having a poor role (Wiegel and Wu 2000). It was formerly found that some PCB congeners could act as primers, each-one activating a specific dechlorination pattern (DeWeerd and Bedard 1999), later alternative substrates, such as halobenzoates and certain bromobiphenyls were reported to have a similar effect in stimulating anaerobic dechlorination (Bedard, Van Dort et al. 1998, DeWeerd and Bedard 1999). Halobenzoates could offer an advantage over halogenated biphenyls because, once dehalogenated, they are easily mineralized (DeWeerd and Bedard 1999, Abraham, Nogales et al. 2002).

Several investigations were conducted in the presence of nonspecific inducers (common electron acceptors for anaerobic microorganisms). Results were heterogeneous and suggested that reductive PCB dechlorination can occur under methanogenic, sulfidogenic, iron (III) reducing and denitrifying conditions, the influence on PCB degradation depending mainly on the indigenous microflora of a given environment (Rhee, Bush et al. 1989, Alder, Haggblom et al. 1993, Sokol, Bethoney et al. 1994, Zwiernik, Quensen et al. 1998, Wiegel and Wu 2000, Zanaroli, Balloi et al. 2012).

2.4.3. Carbon sources

As PCBs usually do not provide the microorganism with energy or nutritive gains, the degradation can be enhanced by adding to cell cultures or microcosms specific organic substrates that provide dechlorinator microorganisms with a suitable carbon source. Examples of such substrates that have been proved to enhance the extent or/and the rate of dechlorination in several laboratory experiments are the following: biphenyl, glycerol, xylose, glucose, sorbitan trioleate, fatty acids (e.g., acetate, propionate, butyrate), pyruvate, malate, methanol and acetone (Nies and Vogel 1990, Alder, Haggblom et al. 1993, Singer, Gilbert et al. 2000, Tandlich, Brežná et al. 2001, Luo and Hu 2013).

The effects of the addition of a carbon source are complex, due to the numerous interactions with the other culture-

components. The same organic substrate can act differently on the PCB degrading abilities, depending on the involved bacterial communities (Alder, Haggblom et al. 1993). In some instance, the addition could inhibit PCB dechlorination by supplying a substrate to non-PCB dechlorinators or by competing with other substances that stimulate PCB degradation (Wiegel and Wu 2000). For example, experiments indicate that in some case glucose can inhibit PCB biodegradation. Tandlich *et al.* (Tandlich, Brežná et al. 2001) hypothesized that, since glucose is a readily utilizable substrate, it is not suitable to create selection pressure at bioaugmentation. Luo *et al.* (Luo and Hu 2013) showed that the interaction between salicylic acid (an inducer) and glucose limited anaerobic PCB removal; they hypothesized that bacteria used glucose rather than salicylic acid as a carbon source, in such a way the induction effect of the salicylic acid would be irrelevant.

Some of the tested carbon sources showed to have a dual role on the degradation process, serving also as electron source under anaerobic conditions, or as surfactant agent for the mobilization of PCBs (e.g., sorbitan trioleate) (Singer, Gilbert et al. 2000), or as an inducer in aerobic PCB biodegradation (e.g., biphenyl) (Tandlich, Brežná et al. 2001).

PCB degraders seem to thrive even in some substrate with low organic carbon concentrations, as shown by LuČnsdorf *et al.* (LuČnsdorf, Erb et al. 2000). They proposed an interesting mechanism explaining how, in a sandy clay soil poor in carbon, PCB degrading bacteria can proliferate in aggregates formed by clay leaflets arranged in the form of 'houses of cards'; PCB adsorbed by the clay colloids becomes an important carbon source for these microbial communities (Abraham, Nogales et al. 2002).

2.4.4 Electron donors (in anaerobic PCB dechlorination)

Chlororespiration implicates polichlorobiphenyl molecules as electron acceptors, involving the loss of a chlorine substituent and its replacement by hydrogen. The source of electrons might be the reduced substrates provided for microbial growth (e.g., acetate, methanol or organic acids), as well as zero-valence iron and hydrogen (Nies and Vogel 1990, Zanaroli, Balloi et al. 2012). Different electron donors have been employed to better understand the conditions that control both PCB dechlorination and the metabolism of dehalogenating bacteria. Experimental results show heterogeneous responses, indicating that the effects of a certain amendment depend on the origin of PCB dechlorinating culture, thus on the composition of the involved microorganisms community (Alder, Haggblom et al. 1993, Wiegel and Wu 2000, Zanaroli, Balloi et al. 2012). An innovative bioelectrochemical approach has been proposed by Chun *et al.* (Chun, Payne et al. 2013): the application of an electric current to sediment in microcosms, providing electron-donors to PCB dechlorinating microorganisms, led to significant dechlorination of weathered PCBs in sediment.

2.4.5 Mobilizing agents

It is well established that bioavailability is one of the most relevant limiting factors in PCB bioremediation; the problem could be overcome by adding some mobilizing agent in the polluted soil or sediment. Several substances have been successfully tested, such as plant oils (e.g., soybean oil), synthetic surfactants (e.g., Triton X-100,) or biogenic surfactants (e.g., cyclodextrins and rhamnolipids produced by microorganisms,

or soya lecithin and saponins of plant origin) (Fava and Di Gioia 1998, Fava and Di Gioia 2001, Fava and Ciccotosto 2002, Berselli, Milone et al. 2004, Federici, Giubilei et al. 2012, Luo and Hu 2013). Surfactants form PCB-water soluble aggregates, as a consequence PCB molecules can be released from soil particles. Even though several of them are non-toxic and biodegradable, they could act on PCB degrader microorganisms by either enhancing their activity or by exerting a detrimental effect. Cyclodextrins, for example, may also be utilized as carbon and energy sources by microbial populations; thus cyclodextrins, depending on the existing microflora and available substrates, could enrich for PCB degraders and non-degraders and eventually restrict the synthesis of PCB degradation enzymes (Fava and Di Gioia 1998, Berselli, Milone et al. 2004, Luo and Hu 2013). The effect of these amendments must be evaluated on a case by case basis, with small scale experimental systems, considering that different concentrations of the surfactant may produce opposite effects (Billingsley, Backus et al. 1999). The surfactant release by root exudates seems to be an interesting tool since radical system provides diffusion of surfactants in the whole rhizosphere volume.

A promising approach is the selection and engineering of plants and microbial strains able to modify solubility and transport of organic pollutants *in situ*, through exudation of biosurfactants. However, though promising, the applicability of this approach has not yet been demonstrated (Wenzel 2009).

2.4.6 Bioaugmentation

The addition of exogenous microorganisms in PCB contaminated substrates seems to be a promising technique for the remediation of a PCB polluted site. However, since most of the experiments were carried out on microcosms or bioreactors, there is still uncertainty regarding both the conditions of inoculation treatment and the feasibility of the application for *in situ* treatments

Several attempts to inoculate exogenous strains in weathered PCB contaminated soil and sediments failed to improve biodegradative activity, likely due to nutrient limitations and to the influence of the indigenous microflora (Rhee, Bush et al. 1989, Harkness, McDermott et al. 1993, Donnelly, Hegde et al. 1994, Fava and Di Gioia 2001, Mackova, Prouzova et al. 2009, Saavedra, Acevedo et al. 2010). Since the cultures capable of PCB dechlorination tend to lose their activities when transferred to another matrix (Evans, Dudley et al. 1996), the repeated inundation of the soil with an active inoculum has been suggested as a strategy for improving the efficacy of bioaugmentation. However this technique must be supported by a technology allowing to deliver large quantities of active microorganisms to contaminated soils (Gilbert and Crowley 1998, Singer, Gilbert et al. 2000).

Among the cases in which PCB-degrading microorganisms have been successfully transferred to a non-native matrix, the bioaugmentation treatment mainly led to a reduced time lag, to increased extents of dechlorination and/or to alterations of the specific pathways of PCB dechlorination (Brunner, Sutherland et al. 1985, Rhee, Bush et al. 1989, Evans, Dudley et al. 1996, Hickey 1996, Singer, Gilbert et al. 2000, Singer, Smith et al. 2003, Fagervold, Watts et al. 2011, Payne, May et al. 2011, Ponce, Latorre et al. 2011, Egorova, Demakov et al. 2013, Secher, Lollier et al. 2013).

To our knowledge, there are very few reports in literature regarding *in situ* experiments of

PCB-degrader bioaugmentation (Harkness, McDermott et al. 1993, Zhai, Hu et al. 2011). The most important factors affecting the feasibility of large scale applications seem to be:

- the practical impossibility to transfer PCB degrader microorganisms in the polluted soil using water as carrier, since PCBs molecules are hydrophobic
- the low level of contamination that generally characterizes PCB polluted sites is not sufficient to sustain microbial PCB-degrader communities

As reported by Sowers *et al.* (Sowers and May 2013), the inoculation of cells by means of a solid substrate, such as microbial granules, clay or granulated activated carbon (GAC) particles , offers a possible solution for dispersing cells in the field (Natarajan, Wang et al. 1995, Payne, May et al. 2011). Singer *et al.* (Singer, Smith et al. 2003) suggested that plant rhizosphere can contribute to enhance inoculum penetration into the soil profile.

Concerning the limitation in PCB molecules sustaining the growth of the competent organisms in field trials, several inducers or primers mimicking the behavior of PCB molecules have been proposed, as reported in the previous paragraphs.

In several PCB pollution situations the natural microflora, if adequately stimulated, was able to efficiently catalyze the degradation process (Harkness, McDermott et al. 1993, Fava and Di Gioia 2001, Tartakovsky, Michotte et al. 2001, Secher, Lollier et al. 2013); however the addition of exogenous strains in a polluted site has to be carefully evaluated, considering also the environmental risks related to the introduction of an alien microorganism in the ecosystem.

Several studies focused on the genetic manipulation of plants and microorganisms in order to enhance the rate and the extent of PCB bioremediation technologies. However we prefer to leave this issue to more specific reviews (1, 140, 141).

2.5. Surprises in store: simultaneous aerobic/anaerobic degradation of PCBs

It is interesting how aerobic and anaerobic PCB biodegradation could occur at the same time in the same contaminated matrix. A number of studies focused on the spatial heterogeneity of redox potential at small scale; due to different oxygen conditions, it is possible that aerobic and anaerobic processes take place simultaneously in the range of few centimeters, giving rise to a metabolic chain where the anaerobic dechlorination products become the substrate for the aerobic biodegradation steps. In such a way a near-complete mineralization of PCB congeners could be achieved in a single-stage system, as demonstrated by Tartakovsky *et al.* (Tartakovsky, Michotte et al. 2001) in a coupled aerobic/anaerobic bioreactor. A similar process is assumed to take place, at smaller scale, in biofilms and microbial separated microcolonies (Vasilyeva and Strijakova 2007). Kjellerup *et al.* (Kjellerup, Paul et al. 2012) detected both aerobic and anaerobic PCB transforming bacteria in the same soil samples and related the observed bacterial heterogeneity with the formation of microniches, as a consequence of the spatial and temporal variation of parameters such as oxygen, pH, redox, nutrients and moisture content. The presence of plants can greatly influence gas diffusion into a soil profile and play an important role in establishing aerobic and anaerobic zones in the rhizosphere (Singer, Smith et al. 2003). High-transpiring plants like *Salicaceae* (*Populus* spp. or *Salix*

spp.) and wetland species can thrive in anoxic flooded substrates by means of an internal gas transport system that allows the plant to transport the oxygen from the atmosphere to the roots (Armstrong 1979, Field and Sierra-Alvarez 2008). This ability has been exploited in up-flow constructed wetlands for the treatment of contaminants requiring the combination of aerobic and anaerobic degradation processes (Ong, Uchiyama et al. 2009). Meggo *et al.* (Meggo, Schnoor et al. 2013) reported PCB biotransformation in the rhizosphere of poplars, showing that reductive dechlorination can occur even in marginally aerobic soils.

2.6 Critical overview

The first main limitation to the study of PCB phytoremediation and bioremediation is the peculiarity of this class of pollutants. We deal with a mix of different molecules analytically hard to detect in gas chromatograms. In addition, each PCB congener follows a different degradation pathway, and intermediate products of degradation are often the same congeners present in the original polluted matrix, as a consequence the degradation process is difficult to be monitored.

A second limitation, which applies to most of bioremediation studies, is the lack of available data referred to *in situ* trials. Most of the studies have been held in confined systems, such as microcosms, mesocosms, bioreactors, hydroponics and greenhouse. Since all such systems are less complex then soil, their application leaves numerous untested factors, for example small confined reactors cannot be representative of the redox fluctuations and oxygen fluxes naturally occurring in soil. Only a few investigations have been attempted on large scale trials (Rhee, Bush et al. 1989, Harkness, McDermott et al. 1993, Whitfield Åslund, Zeeb et al. 2007, Mackova, Prouzova et al. 2009, Teng, Luo et al. 2010, Xu, Teng et al. 2010). Reports of full-scale application of these technologies are mostly concentrated in the United States and mainly address to the rhizoremediation process (as reported by the U.S. Environmental Protection Agency in periodic reviews of site cleanups). However, due to the long time span required for PCB phyto- and bio-remediation, many of those projects have not been completed as yet and we need some time before the results are available.

Furthermore, with regard to the artificial contamination created for experimental purposes, it must be underlined that it exhibits some difference, as compared with weathered PCBs in soil or sediments. In general, in historically contaminated substrates, the PCB molecules are more strongly bound to the particles of soil or sediment, therefore they are less bioavailable and more recalcitrant to biodegradation, as compared to freshly added PCBs in a laboratory spiked matrix (Alder, Haggblom et al. 1993, Vasilyeva and Strijakova 2007). Also, the concentrations employed in the experimental spikes are generally in the order of 100-1000 ppm (Quensen, Tiedje et al. 1988, Koh, Park et al. 2000, Chekol, Vough et al. 2004, Smith, Schwab et al. 2007, Fagervold, Watts et al. 2011, Zanaroli, Balloi et al. 2012, Meggo and Schnoor 2013), the aim being to accelerate the degradation kinetic and to achieve significant results in a time span of 6 to 24 months. PCB concentrations in historically polluted sites are often lower, therefore the dechlorination rates expected *in situ* are lower than those reported for laboratory experiments (Magar, Johnson et al. 2005), in some cases natural PCB concentrations are reported as being

even too low to activate the degradation process (Vasilyeva and Strijakova 2007).
Last but not least, since PCBs in the environment are frequently associated with other pollutants, such as heavy metals and dioxins, a deeper knowledge would be necessary concerning the effects exerted by these co-contaminants on microbial and vegetal species that have been proposed for PCB remediation (Wang and He 2013).
In conclusion, further research is needed to better understand potentialities and limits of these environmental biotechnologies, to assess the best conditions leading to the optimization of each PCB-remediation treatment, and to approach the co-contamination issue. New insights seem to emerge from recent studies, in particular concerning both the rhizosphere potential in PCB bioremediation and the implementation of simultaneous aerobic and anaerobic biodegradation processes.
Although PCB production has been banned in most countries, an extensive environmental contamination has taken place and still persists. What is at stake is human health, in particular the hazard that medical literature is reporting, as a result of chronic exposure to this pollutant.

2.7 Aims and objectives of the study

This study was aimed at assessing the impact of different soil conditions on the biodegradation of PCBs in an historically contaminated soil and at selecting a plant species capable to sustain the degradative bacteria activity. Specific objectives of the study were: i) to compare the effects of PCB degradation in rhizosphere under aerobic and hypoxic conditions, ii) to estimate the degradative role of the native bacterial community which developed in PCB polluted soils iii) to assess the feasibility of the plant-assisted biodegradation technique in the contaminated site of Taranto (Italy), iv) to select a plant species capable to thrive in the above-mentioned area stimulating PCB bio-degradation.

3 Materials and methods

3.1 Site description

The contaminated area selected for this study is is close to Taranto (Southern Italy), Figure 1.

The site, covering approximately 5000 square meters, has been used as an uncontrolled disposal of different kinds of waste and the original chalky soil has been mixed with heterogeneous coarse material.

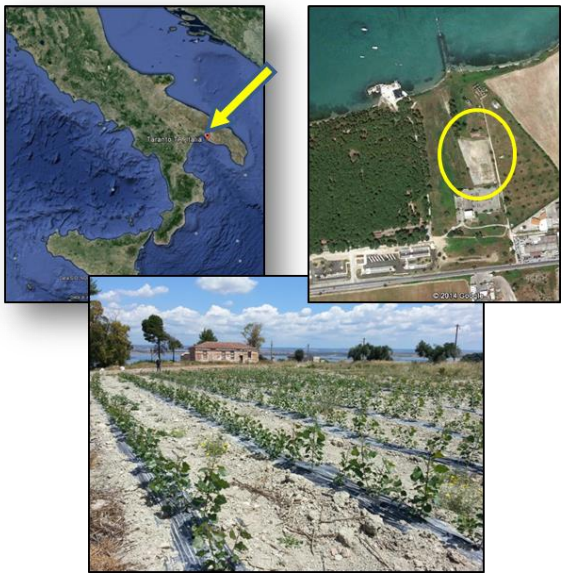

Figure 1 Location of the contaminated area in Southern Italy (Taranto).

The physical and chemical characterization of the soil is reported in the table below (table 1).

Sand (%)	15-74
Silt (%)	13-66
Clay (%)	6-58
pH	7,9-8,4
Total limestone (g/Kg)	242-614
Total organic carbon (g/Kg)	14-17
Organic matter (g/Kg)	24-30
Total Nitrogen (g/Kg)	0,07-0,35

Table 1 Main physical and chemical characteristics of the soil in the polluted site of Taranto, data provided by IRSA-CNR (Bari).

The levels of contamination in the soil from the polluted site are summarized in the table 2.

Parameter	Italian legal limit (D.Lgs 152/2006) (mg/Kg)	Soil content (mg/Kg)
Berillium (Be)	2	0,9-4,7
Vanadium (V)	90	55,6-336,1
Chromium (Cr)	150	34,4-283,7
Nickel (Ni)	120	25,9-156,2
Zinc (Zn)	150	0-556,1
Arsenic (As)	20	8,1-36,3
Selenium (Se)	3	0,9-13,1
Cadmium (Cd)	2	0,1-11,5
Tin (Sn)	1	1,5-167,4
Lead (Pb)	100	10,5-794,5
Total PCBs	0,06	0,045-0,308

Table 2 Levels of contamination in the soil in the polluted site of Taranto, data provided by IRSA-CNR (Bari).

The site has previously been employed as armoury of the Italian Navy; such use caused the high heavy metal concentration into the soil. Since a power plant is situated in the area next to the site, the PCB pollution is probably the result of some transformer oil spillage.
Metal and PCB pollution has a typical irregular dispersion into the soil matrix, related to the localized metal release from weapons and to the oil spill slicks.

3.2 Experimental set up

3.2.1 In situ trial

In spring 2013 part of the site has been organized in two experimental plots, planted with an hybrid poplar genotype (*P. generosa* X *P. nigra*, Monviso clone) and with *Tamarix gallica*, respectively. The poplar clone has been selected for his hardness, his tolerance towards organic contaminants in soil and for his proved effectiveness in the remediation of soils contaminated by organics through rhizodegradation (Bianconi et al., De Paolis et al., 2010). Tamarisk species has been chosen as it is autochthonous of Mediterranean regions, it is well adapted to chalky soil and is salt spray resistant; moreover tamarisk has a good tolerance towards heavy metals in soil (Manousaki, Kadukova et al. 2008). Two plant profiles in Annex 2, provide information on the botany and the remediation potentialities of Monviso poplar clone and Tamarisk. Both species have a well-developed root system, particularly suited to stimulate bacterial proliferation.

Prior to planting the coarse waste material was eliminated, the soil was turned, and compost was added (25,78 t/ha).

Poplar clones were planted as unrooted 20 cm long cuttings; whereas tamarisks were planted as rooted plants, approximately 80 cm high. Plantlets and cuttings were protected with a dark plastic film in order to inhibit the spread of weeds and to protect against sunlight. The 608 poplar cuttings were planted with a distance of 2.00 m

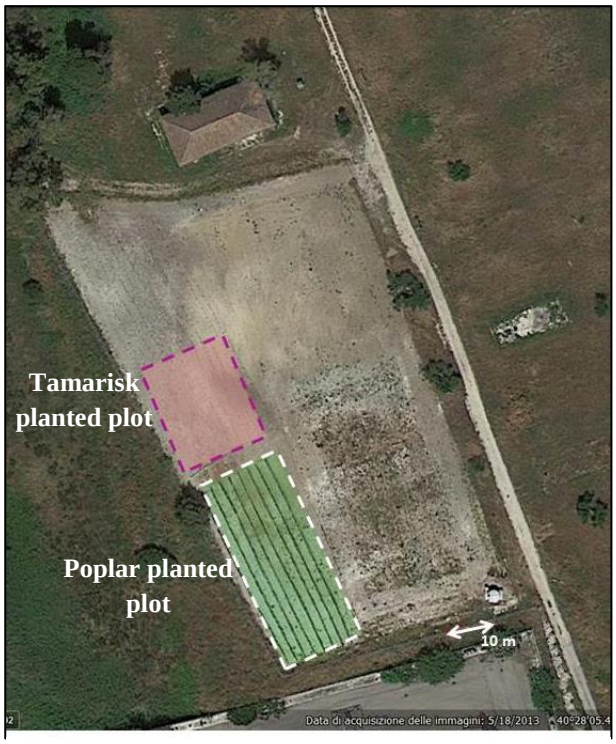

Figure 2 Experimental field design. The plots were planted in April 2013.

between rows and 0.50 m within rows (Short Rotation Coppice plantation); the 80 tamarisks where planted with a distance of 2.00 m between rows and 2 m within rows. The rows of plants were grown in the North-South direction along the long side of the field (Figure 2).

The plots have been equipped with an automatic irrigation system which delivers water every day.

An initial characterization of the site has been accomplished in 2012 by IRSA Institute (CNR) which included soil characterization, PCB and heavy metals chemical analysis and microbiological investigations. Further analysis have been carried out 1 year after the plots set-up, the results are still in the processing phase and will be not discussed in the present manuscript.

The field activity was conducted by IRSA Institute of Bari and Rome and by the participants to an employment assistance project coordinated by the non-profit

Figure 3 The location of the soil sampling points used for microcosms preparation.

organisation CEM (Centro Educativo Murialdo).

3.2.2 Ex-situ trial

A portion of the contaminated soil from the site described above was used to prepare 12 microcosms. It was collected from the two sampling points where PCB concentration was higher (figure 3).

Sand (%)	58
Silt (%)	27
Clay (%)	15
Textural class	Sandy-loam
pH	7,86
Total limestone (g/Kg)	360
Total organic carbon (g/Kg)	14,94
Organic matter (g/Kg)	25,75
Total Nitrogen (g/Kg)	0,20

Table 3 Main physical and chemical characteristics of the soil used for microcosm preparation, data provided by IRSA-CNR (Bari).

The sampled soil was air-dried, sieved (2,0 mm), mixed together in equal portions and homogenized. Its main properties and contaminant concentrations are reported in Table 3 and 4.

Pollutant	Italian legal limit (D.Lgs 152/2006) (mg/Kg)	Soil content (mg/Kg)
Vanadium (V)	90	127,4
Chromium (Cr)	150	179,5
Nickel (Ni)	120	156,2
Zinc (Zn)	150	181,3
Selenium (Se)	3	10,4
Tin (Sn)	1	6,0
Total PCBs	0,06	0,271

Table 4 Levels of contamination in the soil from the polluted site which has been used for microcosm preparation, data provided by IRSA-CNR (Bari).

The resulting soil mixture was employed to fill 12 polyethylene 3 litre pots, treated under 3 different conditions, in 4 replicated experiments each one.

The experimental set-up included the following treatments (figure 4):

- Microbiologically active treatment (TMA): consisting in historically polluted soil with a poplar plant growing on it.

- Sterilized treatment (TS): consisting in historically polluted soil previously sterilized by 2 cycles of autoclaving (at 121°C, lasting 30 min. each), in order to eliminate the native microorganisms, with a poplar plant growing on it.

- Microbiologically active treatment under hypoxic conditions (TMAA): in this case the pot, filled with the historically polluted soil and the poplar plant, was completely submerged in water from the first appearance of the leaves (13 month after planting) until the sampling time. This treatment is intended

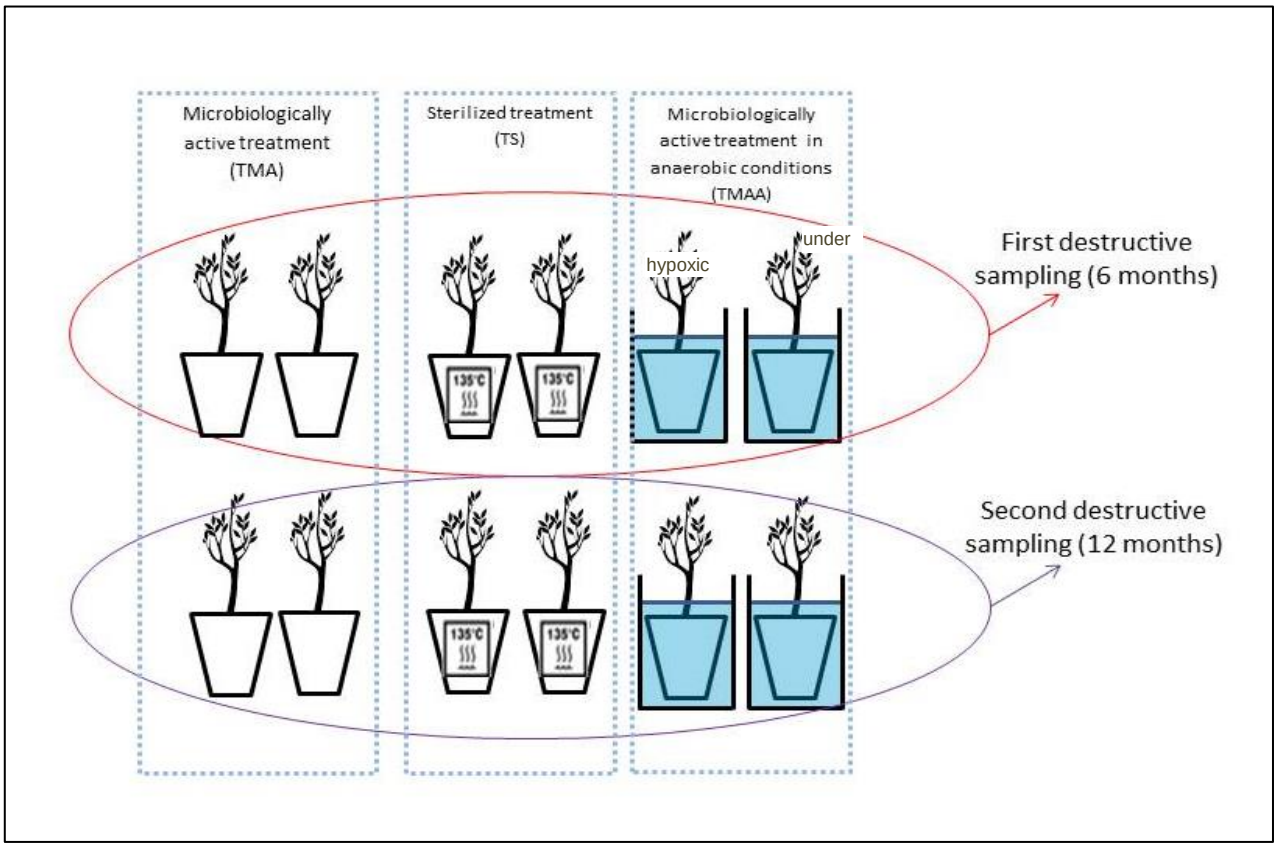

Figure 4 Experimental set-up

Figure 5 The microcosms at the beginning of the trial. From the left: to the right: microbiologically active treatment, sterilized treatment, and microbiologically active treatment under anaerobic conditions

to limit the oxygen concentration in the soil.

Poplars have been planted as unrooted 20 cm long cuttings and were grown in the greenhouse of the Institute Agro-environmental and Forest Biology (IBAF-CNR) of Rome, under natural light and temperature (figure 5). Pots of the treatment TMA and TS were regularly watered in order to maintain a level of humidity equivalent to approximately 65% of the soil water retention capacity. The flooded pots (TMAA) were maintained completely submerged from the first appearance of leaf until the sampling time.

During the trial 2 destructive sampling have been carried out, 6 month and 12 month after planting respectively; each of them affected 2 microcosms for each treatment (figure 4).

3.3 Plant physiology and growth monitoring

The physiology and the growth of the plants in the microcosms were monitored during the entire duration of the year long trial. Every week plants were visually checked in order to observe any retard in the growth or sign of stress, a **photographic reporting** has been compiled.

The physiological status of the poplar growing in the greenhouse was assessed through the leaf chlorophyll content monitoring and the fluorescence measures. A Minolta chlorophyll meter, commonly named SPAD, was used to measure the **leaf chlorophyll content** (Figure 6). It provides is a non-destructive estimation of the total plant chlorophyll, quantifying subtle changes or trends in plant health long before they're visible to the human eye. Clamping the meter over leafy tissue, an index of chlorophyll content is produced by the system. The following equation is then used to convert the SPAD measurement into chlorophyll content.

$$\text{chlorophyll content}\left[\frac{\mu g}{cm^{-2}}\right] = \frac{99*SPAD}{144-SPAD}$$

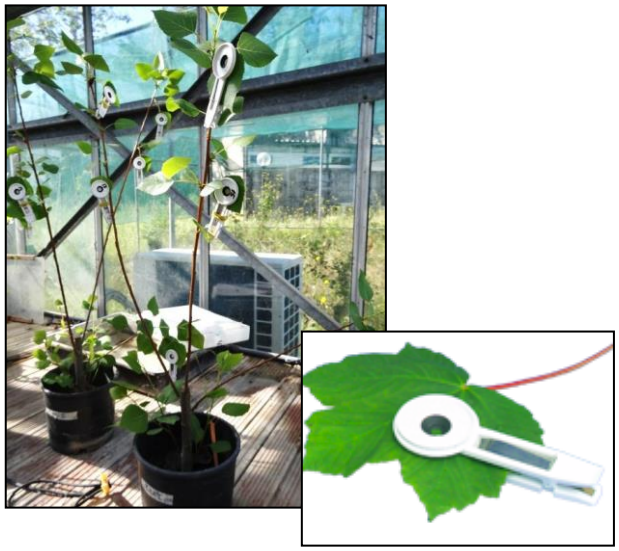

Figure 7 Fluorescence measurement with Hansatech fluorimeter (dark adaptation step)

The **fluorescence of the leaves** was measured with a fluorimeter from Hansatech (figure 7). A small portion of the life area was covered with a lightweight leafclip for approximatly 20 min in order to pull down the photochemistry activity. After the 20 min step of dark adaptation a rapid excitation light was pulsed on the life area, in order to induce a corresponding pulsed fluorescence emission, the latter is expressed as Fv/Fm index.

This parameter is widely used to indicate the maximum quantum efficiency of Photosystem II and it is a sensitive indication of plant photosynthetic performance with healthy samples typically achieving a maximum Fv/Fm value of approx. 0.833. Values lower than 0.800 will be observed if a sample has been exposed to some type of biotic or abiotic stress factor which reduces the capacity for photochemical quenching of energy within PSII.

Chlorophyll content and fluorescence were measured on 5 leaf per poplar, evenly spread over the entire length of the plant; measures were repeated twice, in autumn and in spring, accordingly with the presence of the leaves on the tree.

Two month after planting and prior to sampling, the **plant structure** was examined: the number of branches, their length, and the number of leaves on each plant were recorded. During the sampling, the **biomass** of aboveground organs (leaves and branches free of the original cutting) were weighted and reported on a dry weight basis. In order to estimate the water content of each plant tissue, a portion of leaves and branches from each microcosm were weighted and oven dried at 105 °C and re-weighted after 24h and at 3 hours steps until the weight became stable. The percentage water content has been calculated as follows , where M_d and M_w are dry and wet weight respectively:

Figure 6 Chlorophyll content measurement with SPAD

$$h(\%) = \frac{(M_w - M_d)}{M_w} * 100$$

3.4 Sampling

During the *ex situ* trial two destructive sampling have been carried out, 6 month and 12 month after planting; each of them affected 2 microcosms for each treatment, as previously illustrated in figure 4.

Two weeks before to sampling the pots have been left to dry without being watered, in order to facilitate the separation between soil and roots.

Each microcosm has been split into the following matrices:

-soil

-roots (separating the roots grown inside the pot from the roots grown outside the pot)

-leaves

-branches

-original cutting

Figure 8, Microcosms sampling

Roots were separated from the soil by shaking.

Each sampled matrix has been weighted, identified and wrapped in aluminum foil (figure 8).

The soil have been homogenized in an alu-foil tray and approximately 25 g of each sample has been set aside for microbiological analysis, closed in a plastic bag to avoid the drying of the sample and processed within 48 h.

A portion of leaves, branches and roots (1-2 g) has been set aside for the analysis of antioxidants and stored in aluminum package at -70°C until the analysis.

The remaining portion of branches and leaves have been wrapped in aluminum foil and stored at -70°C.

The remaining roots have been conserved at a temperature of 4 °C for 2 days until the washing-step, except for the samples collected 6 month after planting, which were frozen at -70°C prior to be processed with the other root samples.

The remaining soil portion has been conserved in aluminum package, at ambient temperature for one day, before the sieving and lyophilisation treatments.

The figure 9 describes the sample splitting for each microcosm.

3.5 PCB analysis

All the solvents and reagents employed in this study were of pesticide quality being purchased acetone, hexane, tolouene, Na_2SO_4, silica gel from Chebios S. r. l (Italy) and mass-labelled PCB standards from Wellington Laboratories Inc. (Canada).

The pre-treatment operations have been performed with caution in order to avoid loss of the more volatile analytes. Samples and extracts have been processed carefully, in order to avoid a long contact with plastic materials, potentially realising phthalates that could interfere in analysis, and/or capable of adsorbing lipophilic molecules as PCBs.

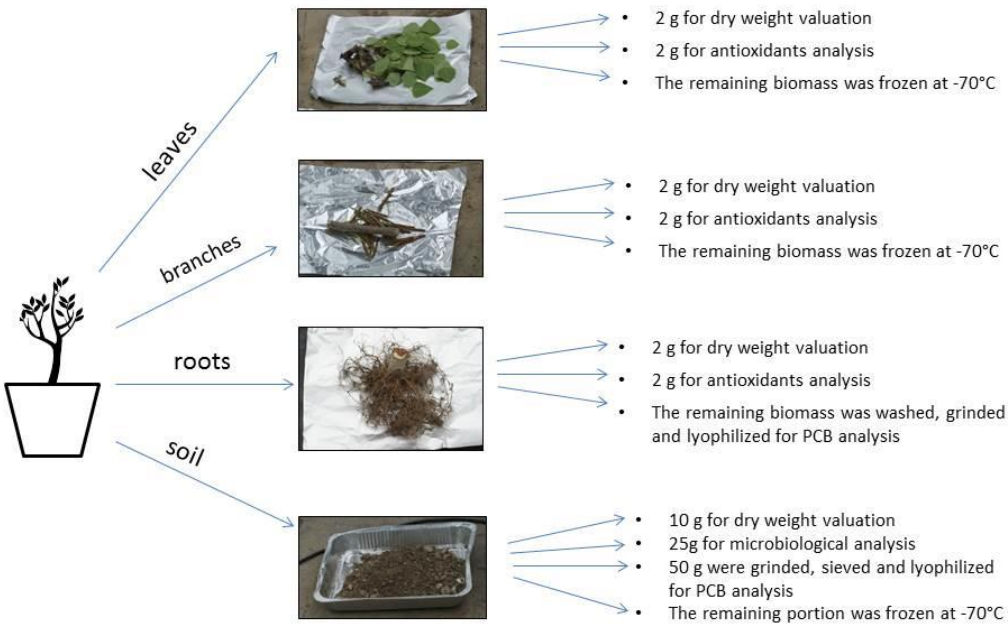

Figure 9, Microcosms splitting during the sampling

3.5.1 Sample pre-treatment

The PCB analysis has been carried out on soil and root matrices; currently leaves and branches have not been analyzed as it is assumed that PCBs are too strongly sorbed to soil particles to be taken up by roots and translocated within the plant to any significant extent (Whitfield Åslund, Zeeb et al. 2007), as a study on poplar tissues of Meggo and Schnoor confirmed (Meggo and Schnoor 2013). PCB content in leaves and branches will be investigated only in case PCBs will be found in root samples at significant concentrations.

Figure 10, Root washing and air drying

For the pre-treatment of the **soil samples**, the procedure was as follows:

Each sample has been grinded in a ceramic mortar and sieved at 2,0 mm. All tools were rinsed with acetone between uses.

In order to prepare the **root samples** for the PCB analysis, procedure was as follows:

During the sampling roots were separated from soil by shaking, the root fragments still dispersed in the soil were recovered after the sieving. In order to remove the small soil particles attached to the root, to be able to analyze only the PCB molecules adsorbed by the plant, excluding the PCBs adsorbed on the external root surface, the root samples were washed accordingly with the method proposed by Barillot et al. (Barillot, Sarde et al. 2013): hand-shacked vigorously for 10 minutes in air and for a further 10 minutes in NaCl solution (0,9%). Then the sample was quickly washed under running water over a

metal sieve. In order to make a clear distinction between adsorbed and absorbed PCBs, a further washing step of the root material with a surfactant (e.g. Tween) would be necessary (Barillot, Sarde et al. 2013). In this study root samples have been frozen before the washing step, it could cause the damage of the cell wall and the potential penetration of the surfactant into the root tissues; as surfactants may yield interferences in PCB analysis, the last washing step has been skipped. Finally the samples were air-dried and grinded into a fine powder in a Moulinex mill (Figure 10). All tools were rinsed with acetone between uses.

In order to avoid PCB volatilization freeze-drying was preferred to oven-drying. Soil and roots where lyophilized for 25 hours in a Virtis Freezmobile 12ES. Samples were stored in glass bottles and kept dry in a vacuum desiccator until the time of analysis.

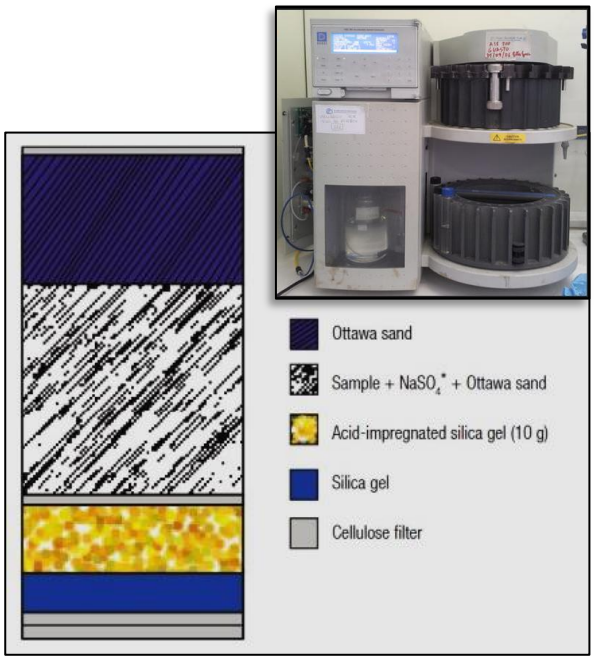

Figure 11, The Accelerate Solvent Extraction system (ASE 200, Dionex) is shown on the top-right side. On the bottom-left side the preparation of the extraction cell for selective extraction has been reported (Dionex Technical Note, 210).

3.5.2 PCB extraction and clean up

The PCB extraction and clean-up have been achieved with the support and the analytical instrumentation of the institute of atmospheric pollution research , IIA-CNR (Rome).

PCBs were extracted from biomass and soil samples using either Soxhlet or selective pressurized liquid extraction (PLE), depending on the instrument availability. The use of both extraction methods was validated by extracting a number of samples by both PLE and Soxhlet.

The **selective pressurized liquid extraction** was performed coupling the accelerated solvent extraction (ASE) with the in-line clean-up, according to the guidelines of the Dionex Technical Note 210. A 33-mL cell was prepared as shown in figure 11, by inserting two cellulose filters at the outlet end of the extraction cell and by adding a layer of activated silica gel (1 g) and then a layer of the acid-impregnated silica gel (10 g) with a cellulose filter on top.

The sample was prepared mixing 2 g of hydromatrix with 5 g of sample and 2 g of $NaNO_4$; any remaining cell volume was filled with Hydromatrix.

The extraction was performed in ASE 200 from Dionex (figure 11), according to the following conditions:

Extraction Solvent: n-Hexane
Pressure: 1500 psi
Temperature: 100 °C
Static Time: 5 min
Static Cycles: 2
Flush: 60%
Purge: 90 s

One analytical duplicate for each sample and one analytical blank (Hydromatrix) for every five samples were also extracted and processed.

The **Soxhlet extraction** was performed according to the EPA guidelines (U.S. EPA, 1996). An aliquot of 10 g dry biomass or soil samples was mixed with 5 g of NaSO4 and 10 g of hydromatrix, and transferred in a Soxhlet thimble.

The extraction was performed for 24 h using toluene as the extraction solvent (approximately 250 ml).

One analytical duplicate for each sample, and one analytical blank (Hydromatrix) for every nine samples were also extracted and processed.

A labelled standard (PCB-LCS-H, from Wellington Laboratories Inc.) was added to each sample as extraction standard.

Extracts from Soxhlet needed a clean-up step in order to eliminate the interfering compounds from the sample. Those molecules can interfere with the PCB congeners of interest in the gas chromatogram or can influence the GC-procedure (i.e. contamination of the chromatographic system). Whereas the selective pressurized liquid extraction achieves very selective extraction, without the need of a subsequent clean-up step as an on-line clean-up is already provided during the extraction step, by the inclusion of sorbents in the extraction cell.

Samples extracted with Soxhlet were cleaned-up in **columns packed with acid-impregnated silica gel**.

Samples were reduced to a volume of 1 mL prior to clean-up.

The glass columns (200 mm x 15 mm i.d.) have been filled (from bottom to top) with:

-2 g of anhydrous sodium sulphate,

-2 g of activated silica gel,

-2 g of $AgNO_3$ (10%),

-0,5 g of activated silica gel,

-10 g of acid-impregnated silica gel (H_2SO_4 44%)

-2 g of activated silica gel

-2 g of anhydrous sodium sulphate.

After adding 100 ml of hexane to the column, extract was loaded onto the surface by means of a Pasteur pipette. Final elution of the extracts occurred with a total volume of 150 ml of hexane.

The elutes from the selective pressurized liquid extraction and from column clean-up were concentrated and transferred to a vial with 50 µl of labelled standard (PCB-ISS-H, Wellington Lab, 20 pg/µl).) as internal standard.

3.7 PCB detection

Extracts were analysed on a Thermo Scientific TRACE Ultra Gas Chromatograph, TSQ Quantum mass spectrometer. The gas chromatograph was equipped with a TG-XLB fused silica capillary column, (60 m x 0,25 mm i.d. x 0,25 µm f.t.) from Thermo Scientific. The carrier gas was helium at a flow-rate of 1 ml/min.

A sample volume of 2,0 µl was injected into a split-splitless injector operating in the splitless mode, at a temperature of 290°C. The temperature of the GC-MS transfer line was 280°C. The oven temperature program started at 140°C for 1min. Subsequently, the

temperature was increased to 200°C at 60°C/min., held for 5 min and to 245°C at 6°C/min., held for 10 min, finally to 325 °C at 6°C/min and held for 7 min (Figure 9).

In order to lower the detection limits , the option MS-MS was used, with electron ionisation, under the following conditions: source temperature 250°C, source electron energy 70 eV, source emission current 50 µA, MS collision gas pressure 1,5 mTorr (Ar), MS collision energy 29 V.

The quantitation of the individual PCB congeners was performed by internal standard multi-point calibration using 6 calibration standard solutions (P48-M, Wellington Laboratories) in three replicates, from 0,1 pg/µl to 5 ng/µl. The compounds are quantified using the ratio of the analyte and internal standard response (peak area). The instrumental limit of quantitation (LOQ, the concentration at which quantitative results can be reported with a high degree of confidence), has been determined with the approach based on parameters from the analytical curve (Ribani, Collins et al. 2007). LOQ values for each indicator PCB congener are reported in figure 5.

The GC-MS determination has been achieved with the support and the analytical instrumentation of the institute of atmospheric pollution research , IIA-CNR (Rome).

PCB congener IUPAC number	LOQ (pg)
28	43,73
52	25,46
101	11,00
153	26,59
138	29,65
180	27,97

Table 5, Limits of quantitation for PCB indicator congeners

3.8 Microbiological analysis

Soil samples from the microcosms were analyzed in order to assess the abundance and the activity of the microbial community, evaluating its evolution over time. All the microbiological analysis have been carried out by the Water Research Institute (IRSA-CNR) of Rome.

Details on the methods and the results are reported in *Annex 3.*

3.9 Antioxidant analysis

Biomass samples (roots, branches and leaves) from the poplars grown in the greenhouse have been analyzed to investigate the activity of the antioxidant enzymes as stress indicator. Analysis have been carried out by the Institute of Agro-Environmental and Forest Biology (IBAF-CNR) of Rome.

As the analysis has not been accomplished by the present researching group, the methodology and the results are reported in *Annex 4.*

3.10 Statistical analysis

SigmaPlot software, version 12.5, was used to perform Anova analysis and Studen's t-test. The significance level of 0,05 was utilized to indicate whether the treatments were significantly different from each other and from the control. A multiple comparison procedure (Dunn's method) was used in

order to isolate the group or groups that differ from the others.

4 Results and discussion

4.1 Plant stress condition and plant growth

The poplars planted in the field experiment showed grate survival and growth rates. One year after planting trees reached an height of about 4 meters (figure12a). The tamarisks, instead, did not survived, probably due to a delay in planting.

With regards to the poplars grown in microcosms the following results have been observed.

Fluorescence analysis showed F_v/F_m indices ranging between 0,76 and 0,82, as averages of the values measured on each leaf. The lowest values were observed 4 months after planting in hypoxic and sterilized treatments. The measurements performed 1 year after planting have never fallen below 0,80 value, without any relevant difference among the treatments. These results reveal the presence of a moderate stress that become more marked at the end of the growing season (in winter, 4 month after planting), when the leaves on the plants were older and had been exposed longer to the testing conditions. In this case, imposed stress factors, especially hypoxia (which lead to a F_v/F_m value of 0,76) caused a reduction of the capacity for photochemical quenching of energy within Photosystem II, limiting the photosynthetic performance of the plant (Maxwell and Johnson 2000).

Figure 12 a. Poplars planted in the contaminated site, 18 months after planting.

These results are consistent with two studies on poplar species, according to which stomatal closure induced by flooding is the cause of photosynthesis decrease (Regehr, Bazzaz et al. 1975, Kreuzwieser, Hauberg et al. 2009). and with the assumption that plants restrict O2 consumption in order to avoid the occurrence of complete anoxia (Geigenberger 2003).

Leaf chlorophyll content, ranged between average values of 27,97 and 32,90 $\mu g/cm^2$. At the end of the first plant growing season (4 month after planting) we measured the lowest values, with a minimum for sterilized and hypoxic treatments. The difference between each of them (TS and TMAA) and the microbiologically active treatment (TMA) was statistically significant ($P \leq 0{,}001$) (Figure 12). One year after, the difference in chlorophyll content between the treatments was not significant. Moreover the average concentrations were higher than those measured previously. Sterilization and flooding conditions may have negatively influenced photosynthetic pigments, inducing problems in the photochemical process; however the stress imposed by the treatments was more evident in old leaves (in winter) than in young leaves (beginning of the summer).

The spectral quality of light reflected from leaves, manifested in leaf colour, has generally been relied upon as an indicator of plant stress. Signs of premature yellowing, and necrosis were observed after 4 month after planting in the poplars grown in hypoxia (TMAA) (figure 13). Any evident stress sign appeared on the other microcosms. Premature leaf senescence in flooded plants has been attributed to the failure of the roots to supply the shoots with inorganic ions (especially nitrogen) (Drew 1983, Kreuzwieser, Hauberg et al. 2009).

The **plant structure development** shown some difference between treatments. The sprouting of all the plants under sterilized treatment was delNeagu, D., Arduini, F., Quintana, J. C., di cori, P., Forni, C., & Moscone, D. (2014). Disposable electrochemical sensor to evaluate the phytoremediation of the aquatic plant Lemna minor L. towards Pb2+ and/or Cd2+. *Environmental Science & Technology*.

ayed of 1 week (*Annex 5*, photo 4B). Poplars under hypoxic conditions do not produced any new branches during the second growing season. In flooded plants the extension of branches and the number of newly formed leaves were also reduced compared to the other treatments.

Some of the above mentioned features are visible on the photographic reporting in *Annex 5.*

Results from **antioxidant measurements** show significant differences between the treatments; more detailed results are reported in *Annex 4.* Phenolics, flavonoids and ascorbate (reduced form) were found in higher concentrations in the tissues of plants grown under hypoxic conditions ($p < 0,050$ for ascorbate and phenolics in woody tissues and for flavonoids in root tissues). This could be an index of stress occurrence; it has been found that there is considerable increase in antioxidants levels following biotic and abiotic stresses.(Gill and Tuteja 2010). The antioxidant defence machinery protects plants against oxidative stress damages; phenols, flavonoids and ascorbate serve as reactive oxygen species (ROS) scavengers by locating and neutralizing radicals before they damage the cell, thus important for plants under adverse environmental conditions (Gill and Tuteja 2010).

It would seem that the plant ecophysiological, structural, and biochemical responses are in agreement; taken together the evidence strongly suggests the presence of a stress under the hypoxic condition.

Figure 13 Necrosis in leaves of hypoxically treated plants.

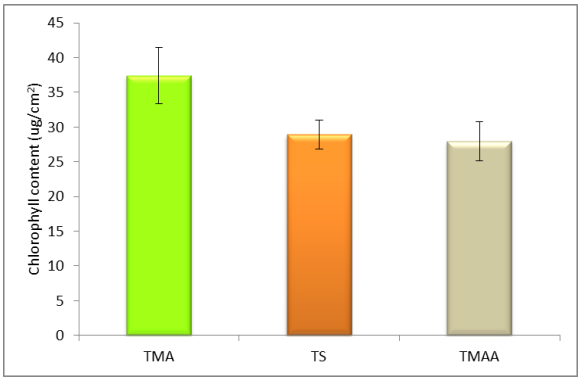

Figure 12 Chlorophyll content in leaves, average values measured for each treatment 4 month after planting.

Figure 14 Root development in poplars grown under hypoxia. The picture has been taken during the sampling and it is not explicative of the trial conditions.

Despite this, by contrast with what one might expect in plant under stress conditions, a similar or even greater aboveground biomass production has been observed in the plants grown in hypoxia as compared with the other treatments. The flooded plants produced also a considerable radical biomass, a part of the root system was concentrated on the top layer of the pot, other roots emerged from the holes in the bottom of the pot and developed in the water surrounding it (figure 14).

Even if the literature generally includes the growth inhibition among the plant flooding responses, however the considerable biomass production of the poplars grown under hypoxia could be explicated with the hydrophilic nature of poplar species and with the particular high flood-tolerance of the specific poplar clone Monviso. These results are consistent with the study of Kreuzwieser *et al*. which have not observed any significant limitation in the shoot growth of grey poplar trees under hypoxic treatment even if during the experiment (1) the roots of the hypoxia-treated trees switched from mitochondrial respiration to alcoholic fermentation with an assumed impaired energy metabolism and (2) biosyntheses were inhibited (Kreuzwieser, Hauberg et al. 2009).

The relevant radical biomass production in flooded poplars, and the plastic roots development in search of more oxygenated areas, are the evidences of the poplar ability to survive hypoxic conditions.

4.3 PCB concentrations in soil

A general decrease in the concentration of each PCB congener is evident 6 month after the experiment set-up in all the plant-treated microcosms (figure 15). This difference is particularly marked ($p<0,05$ for PCB 101, 138 and 152) in aerobic conditions (TMA) compared to the control.

One year after the trial set up, the concentrations of indicator congeners seem

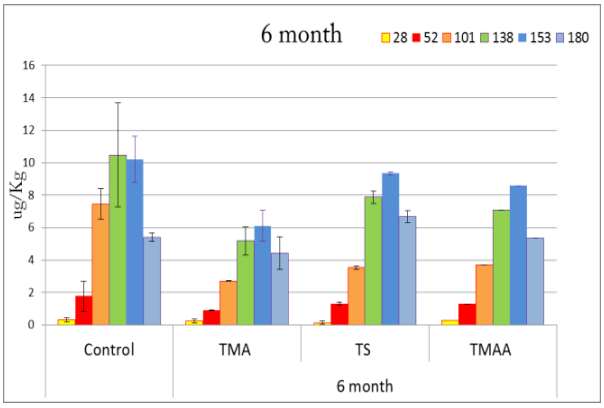

Figure 15 Concentrations of the six PCB indicator congeners 6 months after the trial, in the control, in the microbiologically active treatment (TMA), in the sterilized treatment (TS) and in the hypoxic treatment (TMAA). Each colour represents one of the congeners, identified with corresponding IUPAC numbers in the top right of the picture.

to be more similar to the control compared to the concentrations measured in the previous sampling (figure 16)

If we focus on the trend of low chlorinated congeners, we can observe for PCB 28 (2,4,4'-Trichlorobipheny), 6 month after trial set-up, a decrease of 30% in microbiologically active treatment (TMA) and of 62% in TS. PCB 52 (2,2',5,5'-Tetrachlorobiphenyl) decreased of 48% and 26% in sterilized treatment (TS) (Figure 16). However, one year after the microcosms set-up, an increase of tri- and tetra-chlorinated congeners is found in all experimental

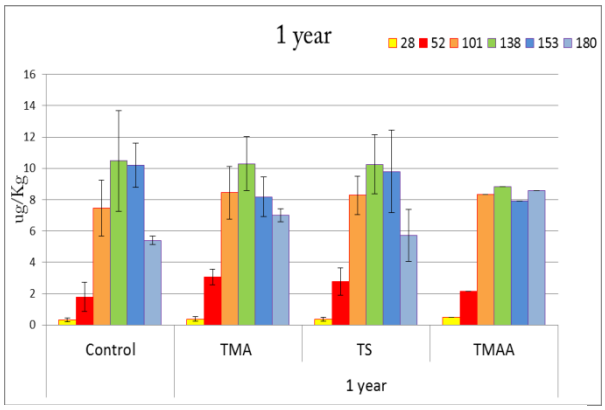

Figure 16 Concentrations of the six PCB indicator congeners 12 months after the trial, in the control, in the microbiologically active treatment (TMA), in the sterilized treatment (TS) and in the hypoxic treatment (TMAA). Each colour represents one of the congeners, identified with corresponding IUPAC numbers in the top right of the picture.

conditions compared to the concentrations measured in the previous sampling. The increase is particularly evident in the aerobic treatments, where we can see a 3 and 2 fold increase, respectively for TMA and TS in tetra-chlorobiphenyl concentrations (PCB52), as shown in figure 16.

The concentrations of PCB101 (2,2',4,5,5'-Pentachlorobiphenyl) follow the same trend

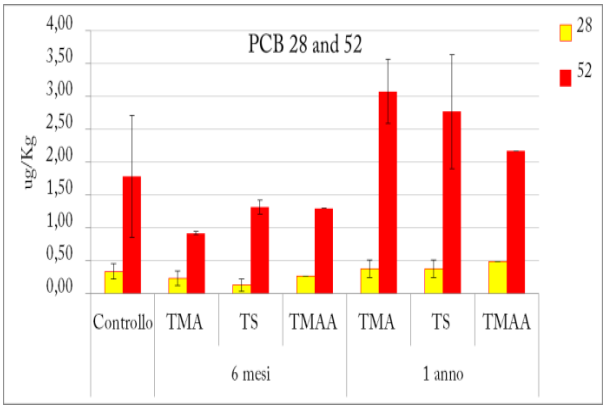

Figure 16 Concentrations of two low chlorinated congeners (PCB 28, 2,4,4'-Trichlorobipheny and PCB 52, 2,2',5,5'-Tetrachlorobiphenyl) 6 and 12 months after the trial set-up, in the control, in the microbiologically active treatment (TMA), in the sterilized treatment (TS) and in the hypoxic treatment (TMAA).

of the lower chlorinated congeners (figure 17).

Differences between the treatments are less evident in high-chlorinated PCBs (figure 18). However, as mentioned before, the results show an evident reduction of esa- and hepta-chlorinated biphenyls in the microbiologically active treatment (TMA) 6 months after planting ($p<0,05$ for esa-chlorinated PCBs). An important shift is observable for PCB 180 (2,2',3,4,4',5,5'-Heptachlorobiphenyl) in hypoxic treatment (TMAA) one year after planting; the concentration increased of 59% compared to the control (Figure 18).

From the results presented above, it seems that during the first 6 months of experiment biodegradation mostly affected the low-chlorinated PCBs, particularly in microbiologically active treatment (TMA). Presumably this is the effect of aerobic degradation by the indigenous microbial community present in the historically contaminated soil used for microcosms preparation. In fact, we did not observe the same decrease of low chlorinated PCBs in the sterilized treatment and in the hypoxic-one (TS and TMAA). This hypothesis is consistent with the findings of a study on biodegradation of long-term PCB-contaminated soil (Mackova, Prouzova et al. 2009). The increase of all PCB congeners during the 2nd half of the experiment could be attributable to the break-down of higher chlorinated PCBs, leading to less chlorinated degradation products. These products are often the same congeners present in the original polluted matrix (Passatore, Rossetti et al., 2014) (Quensen, Tiedje et al. 1988, Tiedje, Quensen III et al. 1993). The PCBs that we analysed are among the most stable congeners, they are therefore the harder to be degraded and they represent the congeners commonly accumulated after a first bio-degradation step (Furukawa, Tomizuka et al. 1979).

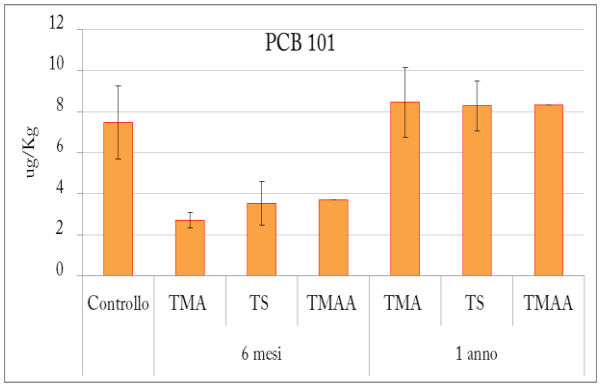

Figure 17 Concentrations of PCB 101 (2,2',4,5,5'-Pentachlorobiphenyl) 6 and 12 months after the trial set-up, in the control, in the microbiologically active treatment (TMA), in the sterilized treatment (TS) and in the hypoxic treatment (TMAA).

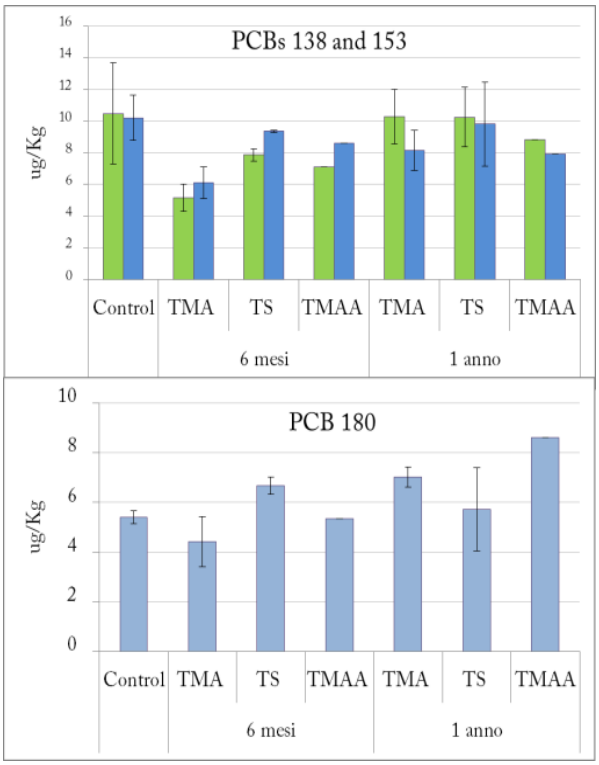

Figure 18 Concentrations of PCB 138 (2,2',3,4,4',5-Esachlorobiphenyl), PCB153 ((2,2',4,4',5,5'-Esachlorobiphenyl) and 180 (2,2',3,4,4',5,5'-Heptachlorobiphenyl) 6 and 12 months after the trial set-up, in the control, in the microbiologically active treatment (TMA), in the sterilized treatment (TS) and in the hypoxic treatment (TMAA).

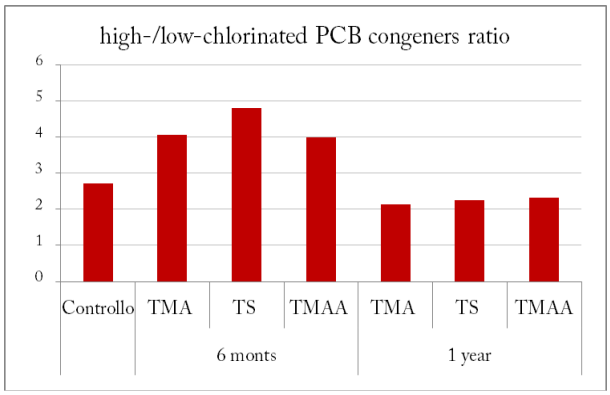

Figure 19 Ratio between PCBs 28, 52 and 101, and PCBs 138, 153 and 180 6 months and 1 year after the microcosm set-up.

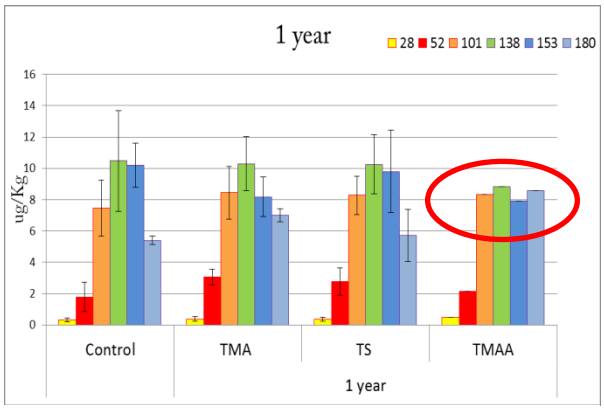

Figure 20 Concentrations of the six PCB indicator congeners 12 months after the trial, the red circle highlights a change in the congeners pattern measured in hypoxic treatment.

It is interesting to highlight a shift in the PCB chlorination degree occurred during the experiment: the ratio between high and low-chlorinated PCBs increased in all treatments 6 month after microcosm set-up; and after 1 year of experiment the ratio begun to decrease, highlighting a degradation of higher chlorinated congeners toward lower chlorinated-ones (figure 19). This trend confirms the assumption of an initial degradation of lower chlorinated and less persistent PCBs, followed by the beginning of a degradation of higher chlorinated and more stable PCBs. Biodegradability, in fact, decreases with increased number of chlorine atoms in the PCB molecule (Furukawa 2000).

With regard to the difference observed between the treatments, the microbiologically active treatment (TMA) stood out for a more accentuate degradation of PCBs during the first 6 months of experiment, effect of aerobic degradation by the indigenous microbial community present in the historically contaminated soil used for microcosms preparation, as discussed above.

PCB concentrations in the sterilized treatment (TS) 6 months after the trial set-up were significantly different ($p<0,05$ for PCB 52 and 180) from the other aerobic treatment (TMA) and almost similar to the control. However, the difference between microbiologically active and sterilized treatment drastically reduced 1 year after planting. This trend means that autoclave sterilisation killed the microbial community present in the soil; however autoclaving created a new environment to colonize, allowing the microorganism survived in resistant spores or the microorganisms present on poplar cuttings to restore, after 1 year, a bacterial community similar to that one of TMA treatment. This assumption is supported by the dehydrogenases analysis results described in *Annex 3*: the higher value measured for dehydrogenases activity corresponds to the sterilized treatment 6 months after planting.

Finally, in the hypoxic treatment (TMAA), we observed a change in congener pattern 1 year after the experiment set-up (figure.20).

This could be the evidence of different degradation pathways taking place in the partially anoxic environment, compared to the degradation occurred in aerobic treatments (TMA and TS) (Field and Sierra-Alvarez 2008, Aken, Correa et al. 2009). It is interesting to note also a sort of anomaly occurred in hypoxic treatment: during the

experiment: one of the container, where the pots were soaked-in, broken and the soil has been subject to an intermittent flooding during the rest of the experiment. Even it is not possible to draw conclusion about this "anomalous treatment", it is interesting to note that the concentration measured in the intermitted flooded-pot were 3-4 folds higher compared to control and to other treatments. This occurrence could be associated with the findings of Meggo et al that reported a dynamic biotransformation activity in marginally aerobic, intermittent flooded rhizosphere (Meggo, Schnoor et al. 2013).

4.4 PCB concentrations in roots

PCBs were unexpectedly found in root tissues at concentrations even higher than in soil, ranging from 0,3 to 89,5 µg/Kg for each PCB congener (figure 21).

Modelling for plant uptake predicts that being hydrophobic molecules such as PCBs (log k_{ow}>3.5) too strongly sorbed to soil particles, the roots are not able to translocate them within plants to any significant extent (Whitfield Åslund, Zeeb et al. 2007); experiments confirm that plants absorb PCBs in very low amounts (Iwata, Gunther et al. 1974, Strek and Weber 1982). For these reasons the presence of PCBs that we observed in root samples could be due to the adsorption to the root exterior surface rather than to an active absorption into root tissues. PCBs in plant tissues follow the general trend of increasing their concentration with decreasing their chlorination; consequently lower chlorinated PCB congeners are taken up much more readily than higher chlorinated ones (Iwata, Gunther et al. 1974, Strek and Weber 1982). In our experiment PCB congener pattern in root samples is similar to that observed in control soil (figure 21). According to the literature above mentioned, this result shows that the presence of PCBs in root tissues is due to the adsorption to the root exterior surface.

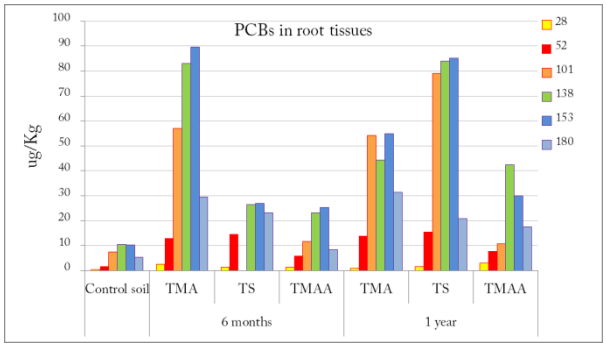

Figure 21 PCB concentration in root tissues, compared to control soil sample

The results show that roots act as bioconcentrator of PCB. This is ascribable to root exudates and to cell turnover, which increase organic carbon content on the root surface, improving consequently the adsorption of PCB molecules on the organic matrix (Haque, Schmedding et al. 1974, Choi, Nfodzo et al. 2012). However, in order to make a clear distinction between adsorbed and absorbed PCBs, a further washing step of the root material with a surfactant (e.g. Tween) would be necessary (Barillot, Sarde et al. 2013).

However, PCB root accumulation has a small influence on soil PCB concentration as the contribution of the roots in the mass balance of a microcosm is minimal (approximately 1% of the total weight).

5 Conclusions

Poplar Monviso was confirmed as an excellent clone for supporting and stimulating PCB biodegradation in a multi-contaminated soil. This plant also showed an unexpected capacity to produce biomass under flooding treatment. Consequently the use of this latter condition in which aerobic and anaerobic microsites can coexist in rooted soil could be a promising strategy. In flooded rhizosphere aerobic and anaerobic processes can simultaneously occur and PCB complete mineralization might be potentially achieved, as found in coupled aerobic/anaerobic bioreactors by Tartakovsky *et al*. (Tartakovsky, Michotte et al. 2001).

In the greenhouse experiment each treatment influenced at different times and in different ways the PCB transformation. The aerobic microbiologically active microcosms (TMA) led to an initial overall decrease in the concentrations of indicator PCBs (28, 52, 101, 138, 153, 180) followed by their subsequent increase during the second half of the experiment. This result shows that PCB biodegradation is a complex process, which follows a not linear trend and in which some congener decrease and some other increase. The evidence of the overall degradation is the change in the ratio between congeners (e.g. high-/low-chlorinated PCBs).

Particularly interesting are the results obtained from the hypoxic microcosms (TMAA) one year after the treatment. In this condition the evident increase in the highest chlorinated indicator PCB (180), is presumably due to the transformation of higher chlorinated congeners, confirming the effectiveness of this treatment. Consequently, flooding can probably trigger anaerobic processes giving hypothetically rise to a metabolic chain where the anaerobic dechlorination products become the substrate for aerobic biodegradation occurring in the oxygenated microsites of the rhizosphere.

Although the complete microbial community analyses are still in progress, the results obtained show that the microbiologically active treatment soil and the sterilized-one (TMA and TS) acted differently on the degradation processes. The aerobic microbial community was initially more active in TMA condition. Six months later the sterilized soil was re-colonized, and although a lower number of bacteria was observed as compared to other conditions, this treatment showed the highest value of dehydrogenase activity. As result of the re-colonization of TS microcosms, 1 year after the treatment the difference in PCB concentration between the TMA and TS conditions was no more observable. In fact the amount of each indicator PCB was quite similar in microbiologically active and sterilized treatment, showing a restoration of the soil degradative potential.

By monitoring the concentrations of the 6 indicator PCBs we could just speculate on the real degradation dynamics which occurred in the soil. In order to follow the time course of the bio-degradation taking place in experimental trials a further analytical analysis of PCBs would be needed, which could lead to the detection of other congeners present in the contaminated matrix.

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Annex 1

Supplementary tables:

-SA: Summary of the main bacterial strains involved in PCB degradation processes.

-SB: Summary of the main fungal strains involved in PCB degradation processes.

-SC: Summary of the main plant species involved in PCB remediation

Table SA Summary of the main bacterial strains involved in PCB degradation processes.

Details about metabolism, the PCB typology utilized as substrate (S), the main metabolic products (P) and the environmental matrix where the strains were isolated are also provided.

Microorganisms	Metabolism and role of PCBs	Substrates (S) and metabolic products (P)	Environmental source	References
Acinetobacter strain P6	Aerobic co-metabolism	**S:** mono- to tetra-CB **P:** ring meta-cleavage products and chlorobenzoic acids	Soil	(Furukawa, Tomizuka et al. 1983)
Achromobacter spp.	Aerobic co-metabolism	**S:** mono-CB (ortho-, meta- and para-substituted) and di-CB (ortho - and para-substituted) **P**: para-chlorobenzoic acids	Sewage effluent	(Ahmed and Focht 1973)
Alcaligenes spp.				
Strain JB1 (formerly *Pseudomonas sp. JB1*)	Aerobic co-metabolism	**S**: mono- to tetra-CB **P:** mono- and di-chlorobenzoates (Parsons, Sijm et al. 1988) and ring-cleaved compounds (Commandeur, May et al.)	Soil (Commandeur, May et al.)	(Parsons, Sijm et al. 1988, Commandeur, May et al. 1996)
Strain Y42	Aerobic co-metabolism	**S:** mono-to tetra-CB **P**: meta-cleavage products and chlorobenzoic acids	Aquatic sediment	(Furukawa, Tomizuka et al. 1979)
Arthrobacter spp.				
Strain B1B	aerobic co-metabolism	**S**: tri- to penta-CB **P:** phenylhexdienoate and chlorobenzoate	Soil	(Gilbert and Crowley 1997)
Strain LPS10B	Aerobic co-metabolism	**S**: 4-chlorobenzoic acid degradation	PCB contaminated freshwater environment	(Pettigrew, Breen et al. 1990)
Burkholderia spp.				

Microorganisms	Metabolism and role of PCBs	Substrates (S) and metabolic products (P)	Environmental source	References
B. xenovorans strain LB400 (formerly *Pseudomonas* sp. and *Burkholderia cepacia)*	Aerobic co-metabolism	**S:** di- to esa-CB (including congeners with adjacent chlorines and double ortho-substituted congeners (Bopp 1986)) ; hydroxylated derivatives of PCBs (2-OH-4-CB, 3-OH-4-CB and 4-OH-4-CB) (Tehrani, Lyv et al. 2012) **P**: hydrodiols, catechols and chlorobenzoate (Haddock, Horton et al. 1995, Seeger, Timmis et al. 1997)	PCB contaminated soil (Bopp 1986)	(Bopp 1986, Haddock, Horton et al. 1995, Seeger, Timmis et al. 1997, Nielsen, Tolker-Nielsen et al. 2000, Tabacchioni, Bevivino et al. 2002, Tehrani, Lyv et al. 2012)
*B. cepacia strain P166 (*Formerly *Pseudomonas cepacia)*	Aerobic Sole carbon and energy sources	**S:** 4-CB, 2-CB and 3-CB **P**: chlorobenzoate and inorganic chloride	Industrial sewage effluent	(Arensdorf and Focht 1995)
Phylum Chloroflexi				
*Dehalococcoides mccartyi strain 195 (*formerly *D. ethenogenes 195)*	Anaerobic metabolism	**S**: tetra- to penta-CB, attacking the double-flanked chlorines **P**: less chlorinated congeners	Anaerobic digester sludge	(Fennell, Nijenhuis et al. 2004, Bedard 2008, Pieper and Seeger 2008, Sowers and May 2013)
Dehalococcoides mccartyi strain CBDB1	Anaerobic metabolism	**S**: tetra- to octa-CB, attacking para and meta chlorines **P**: less chlorinated congeners (Adrian, Dudková et al. 2009)	Riverine sediments	(Adrian, Rahnenführer et al. 2007, Adrian, Dudková et al. 2009)

Microorganisms	Metabolism and role of PCBs	Substrates (S) and metabolic products (P)	Environmental source	References
Strain DEH10	Anaerobic metabolism	**S**: 2,3,4-substituted congeners, attacking also the double-flanked chlorines **P**: unflanked tetra- and tri-CB	Estuarine sediment	(Fagervold, May et al. 2007)
Strain DF-1	Anaerobic metabolism	**S**: PCBs with doubly flanked chlorines (Wu, Milliken et al. 2002); limited to the PCBs chlorinated on a single ring (Bedard 2008)	Estuarine sediments	(Wu, Milliken et al. 2002, Bedard 2008)
Strain o-17	Anaerobic metabolism	**S**: flanked ortho/meta CB (Cutter, Watts et al. 2001); only congeners chlorinated on a single ring , e.g., 2,3,5,6-tetrachlorobiphenyl (Bedard 2008)	Historically polluted estuarine sediments	(Cutter, Watts et al. 2001, Bedard 2008)
Strain SF1	Anaerobic metabolism	**S**: 2345-, 2356-, 236-, and 235-substituted congeners **P**: unflanked tetra- and tri-CBs	Estuarine sediments	(Fagervold, May et al. 2007)
Strain VL-CHL1	Anaerobic metabolism	**S**: penta- through hepta-CBs **P**: tri- and tetra-CBs	Contaminated marine sediment	(Zanaroli, Balloi et al. 2012)
Cellulomonas strain T109	Aerobic co-metabolism	**S**: Aroclor 1242	Uncontaminated soil	(Hernandez , Koh et al. 1997)
Comamonas testosteroni	Aerobic Sole carbon and energy sources	**S:** di-to tetra-CB, chloro-hydroxybiphenyls **P**: benzoic acid derivatives (Francova, Mackova et al. 2004)	Sewage and paper mill sludge (Boyle, Silvin et al. 1992)	(Boyle, Silvin et al. 1992, Francova, Mackova et al. 2004)
Corynebacterium strain MB1	Aerobic co-metabolism	**S:** bi- and tetra-CB (only congeners with open 2,3 sites) **P**: chlorobenzoic acids	Contaminated site	(Bedard, Unterman et al. 1986)
Enterobacter strain SA-2	Aerobic Sole carbon and energy sources	**S**: di- and tri-chlorobenzenes, mono- and di-CB	Landfill containing PCB-contaminated dredge spoils from tropical African ecosystem	(Adebusoye , Picardal et al. 2007)

Microorganisms	Metabolism and role of PCBs	Substrates (S) and metabolic products (P)	Environmental source	References
Hydrogenophaga taeniospiralis strain IA3-A	Aerobic co-metabolism	**S**:all mono-, most di-, and several tri-CB congeners of Aroclor 1248 **P**: chlorobenzoic acid and *ortho*-chlorinated *meta*-cleavage products	PCB-contaminated soil	(Lambo and Patel 2006)
Janibacter strain MS3-02	Aerobic Sole carbon and energy sources	**S**: Aroclor 1242	Soil around of an incinerator plant	(Sierra, Valera et al. 2003)
Paenibacillus strain KBC101	Aerobic co-metabolism	**S**: di- to nona-CB; including some recalcitrant coplanar PCBs	Sediments from a waste water treatment facility	(Sakai, Ezaki et al. 2005)
Pseudomonas spp.				
P. cepacia strain CP9	Aerobic Co-metabolism	**S**: mono- to tetra-CB; limited to the congeners with open 2,3-sites	River sediments	(Fava, Zappoli et al. 1991)
Strain CH07	Aerobic Co-metabolism	**S:** some mono- trough hepta-CB congeners in Clophen A-50, including coplanar congeners. **P**: chlorides and less chlorinated PCBs	Marine sediments	(De, Perkins et al. 2006)
P. pseudoalcaligenes strain KF707	Aerobic Likely co-metabolism	**S**: mono- through tri-CB. **P:** chlorinated benzoic acids	Soil from a biphenyl manufacturing plant	(Oh, KOH et al. 2003)

Microorganisms	Metabolism and role of PCBs	Substrates (S) and metabolic products (P)	Environmental source	References
P. putida strain LB400	Aerobic Co-metabolism	**S**: di- trough esa-CB, including congeners substituted at the 2 and 3 carbons of both rings and congeners with no adjacent unchlorinated carbons (Bedard, Unterman et al. 1986) **P**: HOPDA (2-hydroxy-6-oxo-6- phenylhexa-2,4-dienoic acid), mono-, di-, and trichlorobenzoic acids (Boyle, Silvin et al. 1992)	Sewage sludge (Bedard, Unterman et al. 1986) Plant rhizosphere (3) (Mackova, Prouzova et al. 2009)	(Bedard, Unterman et al. 1986, Boyle, Silvin et al. 1992, Mackova, Prouzova et al. 2009)
Strain SA-6	Aerobic Sole carbon and energy sources	**S**: di- and tri-chlorobenzenes, mono-and di-CBs, chlorinated on both rings	PCB-contaminated dredge spoils	(Adebusoye , Picardal et al. 2007)
P. stutzeri	Aerobic Co-metabolism	**S**: some di- through hexa- CB congeners in Delor 103 (Tandlich, Brežná et al. 2001, Dudášová, Lukáčová et al. 2012)	Plant rhizosphere (Mackova, Prouzova et al. 2009) Long-term contaminated soil (Tandlich, Brežná et al. 2001, Dudášová, Lukáčová et al. 2012)	(Tandlich, Brežná et al. 2001, Mackova, Prouzova et al. 2009, Dudášová, Lukáčová et al. 2012)
P. testosteroni	Aerobic Co-metabolism	**S**: 4-CB and 4,4'-dichlorobiphenyl **P**: 4-chlorobenzoic acid	PCB-contaminated freshwater	(Pettigrew, Breen et al. 1990)
Ralstonia spp.				
*R. eutropha strain H850 (*formerly *Alcaligenes eutrophus)*	Anaerobic facultative Co-metabolism	**S**: tetra-to esa-CB, predominantly ortho-substituted (Bedard, Haberl et al. 1987) **P**: chlorobenzoic acids and chloroacetophenones	PCB-containing dredge spoils	(Bedard, Haberl et al. 1987, Singer, Gilbert et al. 2000)

Microorganisms	Metabolism and role of PCBs	Substrates (S) and metabolic products (P)	Environmental source	References
Strains SA- 3 and SA-4	Aerobic Sole carbon and energy source	**S**: di- to penta-CB **P**: chloride and chlorobenzoate	Landfill containing PCB-contaminated dredge spoils	*(Adebusoye , Ilori et al. 2008)*
Rhodococcus spp.				
R. erythropolis strain TA421	Aerobic Co-metabolism	**S**: 2-CB, 3-CB, 4-CB, 2,3-CB,2,4-CB, 2,5-CB and 4-CB	Intestine of termites	(Chung, Maeda et al. 1994)
R. rhodochrous	Aerobic Co-metabolism	**S**: dichloro-through tetra-CB **P**: HOPDA, benzoylbutanoic acid, dichlorobenzoylpropionic acid, phenylacetic acid, hydroxybenzoic acid, benzoic acid, and mono- and dichlorobenzoic acids	Paper mill sludge	(Boyle, Silvin et al. 1992)
Strain RHA1	Aerobic Co-metabolism	**S**: Kanechlors 200, 300, 400, and 500, which includes mono- to octachlorobiphenyls. **P**: di- and trichlorobenzoic acids (intermediate metabolites)	Soil sample contaminated by g-hexachlorocyclohexane (an insecticide)	(Seto, Kimbara et al. 1995)
Sinorhizobium melitoti	Aerobic Sole carbon and energy source	**S**: 2,4',4-CB **P**: 2-hydroxy-6-oxo-6-phenylhex-2,4-dienoic acid (HOPDA)	Rhizosphere	*(Tu, Teng et al. 2011, Tu, Teng et al. 2011)*
Stenotrophomonas strain JSG1	Aerobic Sole carbon and energy source	**S**: some tri- through hexa-CB congeners in Aroclor 1254 and 4-chlorobenzoic acid **P**: less chlorinated congeners and inorganic chloride ions	Dye industrial effluent	(Ganesh-Kumar, Kalimuthu et al. 2013)

Table SB Summary of the main fungal strains involved in PCB degradation processes.

Details about the PCBs typology utilized as substrate (S), the main metabolic products (P) and the environmental matrix where the strains were isolated are also provided.

Fungal strain	Type of fungus	Substrates (S) and metabolic products (P)	Environmental source	References
Aspergillus niger	Filamentous fungus	**S**: Lower chlorinated PCB congeners	Culture collection	(Dmochewitz and Ballschmiter 1988)
Bjerkandera adusta UAMH 8258 and 7308	White rot fungus	**S**: some di-, tri-, tetra- and hexa-CBs	Culture collection	(Beaudette, Davies et al. 1998)
Ceriporia sp. ZLY-2010	White rot fungus	**S:** high chlorinated biphenyls **P**: benzoic acid	Culture collection	(Hong, Gwak et al. 2012)
Coriolopsis polyzona	White rot fungus	**S**: Delor 106 mixture .	Culture collection	(Novotný, Vyas et al. 1997)
Doratomyces spp.	Filamentous fungi	***S:** high and low chlorinated PCBs*	PCB-contaminated soil	(Mouhamadou, Faure et al. 2013)
Gautieria crispa	Ectomycorrhiza	**S**: di- through tri-CBs	Culture collection	(Donnelly and Fletcher 1995)
Grifola frondosa	Edible mushroom	**S**: di- through hexa-CBs **P**: dichloro-methoxy-phenol		(Seto, Nishibori et al. 1999)
Hirneola nigricans (syn. *Auricularia polytricha)*	White rot fungus	**S**: Delor 106, a commercial PCB-mixture	Cultivated mushroom native of Vietnam	(Šašek, Volfová et al. 1993)
Lentinus tigrinus	White rot fungus	**S**: di- up to penta-CBs in historically Aroclor 1260-contaminated soil	Maize stalk	(Federici, Giubilei et al. 2012)
Myceliophthora thermophile S2-1	Filamentous fungus	***S**:* PCB 28, 52, 101, 118, 138, 153, and 18, regardless of the number of chlorine substituents	PCB-contaminated soil	(Mouhamadou, Faure et al. 2013)
Paecilomyces lilacinus	Filamentous fungus	**S**: Low chlorinated PCBs **P**: mono- and di-hydroxylated CBs, muconic acid derivatives and the corresponding lactones	Wood chip piles	(Sietmann, Gesell et al. 2006)
Penicillium chrysogenum	Filamentous fungus	**S**: 2-CB, 4,4'-CB and 2,2',5,5'-CB	Historically polluted soil (PCBs and heavy metals)	(Tigini, Prigione et al. 2009)

Fungal strain	Type of fungus	Substrates (S) and metabolic products (P)	Environmental source	References
Phanerochaete chrysosporium	White rot fungus	**S**: 4,4'-CB (Dietrich, Hickey et al. 1995) **P**: 4-chlorobenzoic acid, 4-chlorobenzyl alcohol and chloride (Dietrich, Hickey et al. 1995)	Culture collection	(Eaton 1985, Dietrich, Hickey et al. 1995)
Phlebia brevispora TMIC33929	White rot fungus	**S**: tetra-, penta- and hexa-congeners of coplanar PCBs **P**: para-dechlorinated and para- and meta-methoxylated metabolites	Culture collection	(Kamei, Sonoki et al. 2006)
Phoma eupyrena	Filamentous fungus	***S**:* PCB 28, 52, 101, 118, 138, 153, and 18, regardless of the number of chlorine substituents	PCB-contaminated soil	(Mouhamadou, Faure et al. 2013)
Pleurotus ostreatus	White rot fungus	**S**: di- up to hexa-CBs (Kubatova, Erbanova et al. 2001, Čvančarová, Křesinová et al. 2012) **P**: chlorinated derivatives of hydroxy- and methoxy-biphenyls and monoaromatic structures (Čvančarová, Křesinová et al. 2012)	Culture collection	(Kubatova, Erbanova et al. 2001, Čvančarová, Křesinová et al. 2012)
Pycnoporus cinnabarinus	White rot fungus	**S**: mono-CB	Culture collection	(Schultz, Jonas et al. 2001)
Radiigera atrogleba	Ectomycorrhiza	**S**: di- up to tetra-CBs	Culture collection	(Donnelly and Fletcher 1995)
Saccharomyces cerevisiae	Yeast	**S**: 4,4′-CB **P**: hydroxylated metabolites		(Layton, Sanseverino et al. 2002)
Scedosporium apiospermum	Saprophytic filamentous fungus	**S**: 2-CB, 4,4'-CB and 2,2',5,5'-CB	Historically polluted soil (PCBs and heavy metals).	(Tigini, Prigione et al. 2009)
Thermoascus crustaceus	Filamentous fungus	***S**:* PCB 28, 52, 101, 118, 138, 153, and 18, regardless of the number of chlorine substituents	PCB-contaminated soil	(Mouhamadou, Faure et al. 2013)
Trametes versicolor	White-rot fungus	**S**: low and high-chlorinated PCB-congeners	Culture collection	(Ruiz-Aguilar, Fernández-Sánchez et al. 2002)

Fungal strain	Type of fungus	Substrates (S) and metabolic products (P)	Environmental source	References
Trichosporon mucoides	Yeast	**S:** Low chlorinated PCBs **P**: mono- and di-hydroxylated CBs, muconic acid derivatives and the corresponding lactones	Dioxin-contaminated soil.	(Sietmann, Gesell et al. 2006)

Table SC Summary of the main plant species involved in PCB remediation.

Details about the role of the plant in the PCB degradation process, the measured bio-accumulation factor (BAF) and the experimental conditions are also provided.

Species	**Observed plant effects** (BAF, bio-accumulation factor = =(PCBplant)/(PCBsoil)	**Experimental conditions**	**References**
Amoracia rusticana	Support of the bacterial growth	Pot experiments (Ionescu, Beranova et al. 2009, Mackova, Prouzova et al. 2009) Buckets and field plots (Mackova, Prouzova et al. 2009)	(Ionescu, Beranova et al. 2009, Mackova, Prouzova et al. 2009)
Brassica nigra	Increased oxygen diffusion Increased amendment infiltration Microbial enrichment	Mesocosms	(Singer, Smith et al. 2003)
Carex aquatilis and *Carex normalis*	Oxygen diffusion in saturated sediment, allowing at the same time aerobic and anaerobic degradation processes (Smith, Schwab et al. 2007) Uptake and translocation (Shoot BAF=0,45 and 0,29)(Zeeb, Amphlett et al. 2006)	Greenhouse conditions Field experiment (Whitfield Åslund, Zeeb et al. 2007)	(Zeeb, Amphlett et al. 2006, Smith, Schwab et al. 2007, Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008)
Cucurbita pepo e *Cucurbita moschata*	Stimulation of soil microbial community development (Qin, Brookes et al. 2014) Root uptake and translocation (shoot BAF=0,14- 0,20 and 0,29; part of shoot BAF= 2) (White, Parrish et al. 2006, Zeeb, Amphlett et al. 2006, Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008)	Greenhouse conditions (White, Parrish et al. 2006, Zeeb, Amphlett et al. 2006) Pot and field bin trials (White, Parrish et al. 2006) Field experiment (Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008)	(White, Parrish et al. 2006, Zeeb, Amphlett et al. 2006, Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008, Qin, Brookes et al. 2014)

Species	**Observed plant effects** (BAF, bio-accumulation factor = =(PCBplant)/(PCBsoil)	**Experimental conditions**	**References**
Festuca arundinacea	Uptake and translocation (Shoot BAF=0,03 and 0,1) (Zeeb, Amphlett et al. 2006, Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008)	Greenhouse conditions (Zeeb, Amphlett et al. 2006) Field experiment (Whitfield Åslund, Zeeb et al. 2007)	(Zeeb, Amphlett et al. 2006, Whitfield Åslund, Zeeb et al. 2007, Whitfield Åslund, Rutter et al. 2008)
Glycine max	Increased bacterial activity Slight translocation (maximum measured shoot BAF=0,28)	Greenhouse conditions	(Li, Liu et al. 2011)
Medicago sativa	Increased soil bacterial diversity	Field experiment	(Tu, Teng et al. 2011)
Morus spp.	Microbial activity stimulation (release of phenolic compounds and soil aeration improvement)	Plants grown in specially designed portable "rhizotron pots"	(Leigh, Fletcher et al. 2002)
Nicotiana tabacum	Microbial activity stimulation	Experiment in pots, buckets and field plots	(Mackova, Prouzova et al. 2009)
Panicum virgatum	Microbial activity stimulation	Pot experiment in growth chamber	(Chekol, Vough et al. 2004)
Phalaris arundinacea	Microbial activity stimulation	Pot experiment in growth chamber	(Chekol, Vough et al. 2004)
Pinus nigra	Increased abundance of PCB degraders	Naturally vegetated site	(Leigh, Prouzová et al. 2006)
Populus deltoides x nigra DN34	Microbial activity stimulation (Meggo, Schnoor et al. 2013) Enantioselective uptake and biotranformation of PCB 95 (Zhai, Hu et al.)	Soil microcosms (Meggo, Schnoor et al.) Hydroponic culture (Zhai, Hu et al.)	(Zhai, Hu et al. 2011, Meggo, Schnoor et al. 2013)
Salix caprea	Increased abundance of PCB degraders	Pot experiments (Ionescu, Beranova et al. 2009) Naturally vegetated site (Leigh, Prouzová et al. 2006)	(Leigh, Prouzová et al. 2006, Ionescu, Beranova et al. 2009)

Species	**Observed plant effects** (BAF, bio-accumulation factor = =(PCBplant)/(PCBsoil)	**Experimental conditions**	**References**
Salix viminalis and Salix alaxensis	Increased catabolic and phylogenetic bacterial diversity (de Cárcer, Martín et al. 2007, Slater, Gouin et al. 2011) Removal of 4-CBA (intermediate product of aerobic microbial degradation of PCB) (Deavers, Macek et al. 2010)	Hydroponic culture and sterile cell suspension (Deavers, Macek et al. 2010)	(de Cárcer, Martín et al. 2007, Deavers, Macek et al. 2010, Slater, Gouin et al. 2011)
Spartina pectinata	Oxygen diffusion in saturated sediment, allowing at the same time aerobic and anaerobic degradation processes	Greenhouse experiment	(Smith, Schwab et al. 2007)
Vicia cracca	Uptake in shoots (shoot BAF=1,1)	Field experiment	(Ficko, Rutter et al. 2010)
Zea mais	Increased bacterial activity Slight translocation (maximum measured shoot BAF=0,19)	Greenhouse experiment	(Li, Liu et al. 2011)

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Annex 2

- Hybrid poplar genotype Monviso (*P. generosa* X *P. nigra)* fact sheet, from the Phytoremediation plant database, published on IBAF-CNR web-site, author: Laura Passatore

- *Tamarix gallica* fact sheet , from the book "Le piante che depurano l'acqua", author: Laura Passatore

P. generosa x P. nigra

clone Monviso

GENERAL INFORMATION

Fam.: *Salicaceae*
Species*: P.* x *generosa* A. Henry x *P. nigra* L.
Synonims*: P. ×euramericana P. ×robusta,*
Mother*: P.×generosa* 103-86 (*P. deltoides* '583' Iowa, USA x *P. trichocarpa* '196' Oregon, USA)
Father: *P. nigra* '715-86' (*P. nigra* '12' Piemonte, ITA x *P. nigra* '7' Umbria, ITA)
Selector: Alasia Franco - Cavallermaggiore (CN) - Italia
Sex: female

European patent for plant innovation.

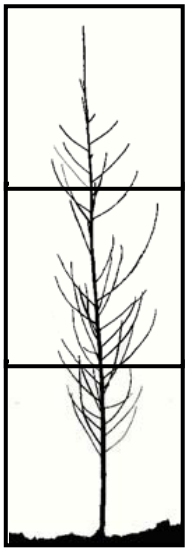
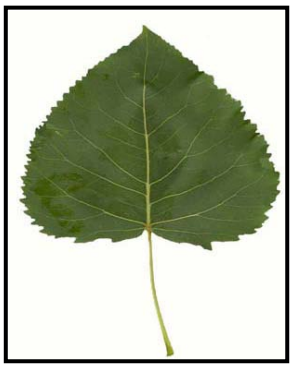


Photo: source –F. Picco, A. Giorcelli, G. Castro (CRA) chiave dicotomica per il riconoscimento in vivaio dei principali cloni di pioppo coltivati nell'unione europea, dicembre 2007.

DESCRIPTION

Foliage: narrow, conical crown
Leaves colour: green
Trunk: straight, densely branched.



Photos: source – Photo: source –F. Picco, A. Giorcelli, G. Castro (CRA) chiave dicotomica per il riconoscimento in vivaio dei principali cloni di pioppo coltivati nell'unione europea, dicembre 2007.

CULTIVATION

Soil tolerances: it adapts to different kinds of soil, even the most difficult. It can easily tolerate the lack of water.
Cultivation: biannual / five years harvest. No need to prune
Trunk: the wood is quite heavy, (basal density 0,31 g/cm^3), suitable for energy biomass, plywood, paper industry, pellets.

RESISTANCE

To spring defoliation: very high
To rusts: very high
To Marssonina brunnea infection: very high
To cortical necrosis: very high
To brown spots: high
To mosaic virus: very high
To woolly aphid Phloeomyzus passerinii: median
To wind: median

USE IN PHYTOREMEDIATION

----------Experiment 1----------

Contaminants of concern	Exachlorocyclohexane (HCH) isomers
Plant species	Poplar clones: I-214, AF2 and Monviso
Interaction of plant and contaminants: Tolerant plant (enhancement of microbial community) / phytoremediation	Phytoremediation
Mechanism involved in phytoremediation: Phytostabilisation/rhizodegradation/phytoaccumulation/phytodegradation/phytovolatilization/ evapotraspiration	Rhizodegradation
Types of microorganisms associated with the plant	Aerobium bacteria *Arthrobacter* strains
Laboratory/field experiment	Field experiment
Initial contaminant concentration	Mean of HCH isomers concentration (±S.E.) in soil samples. Total HCH: 0,125±0,008, α-HCH:0,044±0,031; β-HCH:0,058±0,052; γ-HCH:0,017±0,002; δ-HCH: 0.006±0,001
Length of Experiment	One growing season
Post-experiment contaminant content	The table below provides the means of individual HCH isomers and total HCH concentrations (± S.E.) in soil and rhizosphere samples (ppm) after three treatments.

SAMPLE	α-HCH	β-HCH	γ-HCH
Soil before poplar cuttings planting	0.044 ±0.031 ab	0.058 ±0.052 a	0.017 ±0.002 abc
I-214	0.044 ±0.074 ab	0.046 ±0.015 abcd	0.011 ±0.001 cd
I-214 + bacteria	0.042 ±0.015 ab	0.047 ±0.058 abcd	0.016 ±0.001 abc
I-214 + ORC + compost	0.047 ±0.035 a	0.054 ±0.055 abc	0.020 ±0.003 a

Post-experiment plant condition	Biomass production of the three investigated clones was as good as in the uncontaminated areas.

Soil characteristics

The table below provides some physico-chemical and biological characteristics of soil from experimental site

Sand (%)	75.25
Silt + Clay (%)	24.75
pH (H_2O)	7.72
Total N (g / Kg)	0.16
Organic C (g / Kg)	12.24
Organic matter (g / Kg)	21.11
Electrical conductivity (mS / cm)	462.0
Bacteria (CFU x 10^7 g^{-1})	58.80
Actinomycetes (CFU x 10^7 g^{-1})	13.10
Fungi (CFU x 10^5 g^{-1})	18.00

CFU: colony-forming unit.

Age of plant at 1st exposure
(seed, post-germination, mature)

Cuttings

Requirements for phytoremediation
(specific nutrients, addition of oxygen)

Bacteria inoculation and soil amendment with commercial compost and with oxygen release compound (ORC, IXPER® 75C Calcium Peroxide, Solvay, S.A).

Contaminant storage sites in the plant
(root, shoot, leaves, no storage)

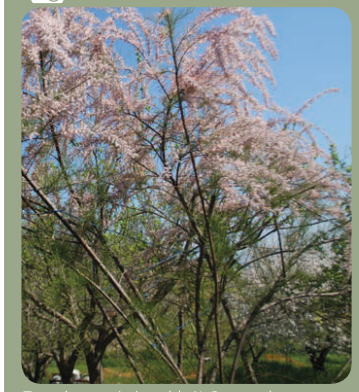
Harvestable plant parts were only slightly contaminated. Only 10% of samples were contaminated by a 0.03 mg Kg^{-1} of the β-HCH isomer.

Reference

D Bianconi, MR De Paolis, AC Agnello, D Lippi, F Pietrini, M Zacchini, C Polcaro, E Donati, P Paris, S Spina, A Massacci, 2010. Field-scale rhyzoremediation of a contaminated soil with hexachlorocyclohexane (HCH) isomers: the potential of poplars for environmental restoration and economical sustainability. In: *Handbook of Phytoremediation* Editors: Ivan A. Golubev ©2010 Nova Science Publishers..

Tamarix spp. *Tamerice*

Fam. Tamaricaceae



Tamarix. spp., insieme (ph: N. Guyennon)

fiore (ph: N. Guyennon)

T. gallica L. (ph: V. Buono)

frutto (ph: L. Passatore)

semi (ph: L. Passatore)

Generalitá

Specie arbustive o piccole arboree, dal portamento asimmetrico, con minutissime foglie, ridotte a squame per ridurre la superficie traspirativa, adattamento agli ambienti aridi. Capaci di sopravvivere su dune marittime e paludi salmastre, hanno sviluppato un sistema di escrezione del sale in eccesso attraverso le foglie, sotto forma di gocce che possono scendere dalla chioma come una pioggia; si tratta di piante freatofite, che per sopravvivere a condizioni di siccità sono provviste di un apparato radicale che può raggiungere diversi metri di profondità alla ricerca di acqua.

Coltivata in parchi e giardini, è apprezzata per la leggerezza della chioma, dalle sfumature grigio-azzurre, e per l'abbondante ed appariscente fioritura primaverile che ricopre i rami di racemi rosa.

È una pianta che viene nominata spesso negli scritti, a cominciare dalle citazioni bibliche fino alle poesie di Virgilio e di Pascoli, che ne riprendono il riferimento, e alle "tamerici salmastre ed arse" di D'Annunzio.

Il nome è riconducibile all'arabo "*tamàr*"- palma, forse per la comunanza di habitat.

Nome inglese: Tamarisk

Descrizione

Tipo ecologico: xerofita

Altezza massima (m): 1-6 m, a seconda della specie

Sistema radicale: particolarmente sviluppato, molto ramificato, include sia radici laterali che verticali. Profondità massima del sistema radicale: 20 m (Zohary, 1961)

Foglie: ridotte a squame sovrapposte, ricoprenti quasi totalmente i rami

Fiori: piccoli, di colore bianco o rosa, disposti in racemi. Ogni fiore ha una corolla con 4-5 petali, a seconda della specie

Frutti: capsule dalla forma allungata, più larga alla base, che a maturazione si aprono liberando semi dotati di peli per la diffusione anemofila

Sviluppo

Ciclo vitale e stagionalità: perenne, la maggior parte delle specie presenti in Italia è decidua

Velocità di crescita: media

Coltivazione

Temperatura (°C): 0/40, a seconda della specie

Esposizione: pieno sole

Profondità dell'acqua (m): 0,00. Possibile la coltivazione in idroponica.

Resistenza/tolleranza: pianta rustica, resistente alla salsedine e ai venti forti, anche salmastri. Bene adattata a terreni sabbiosi, aridi e salini. Tollera poco i ristagni idrici e i terreni molto calcarei. Non teme il freddo ma le gelate possono danneggiare i rami più giovani.

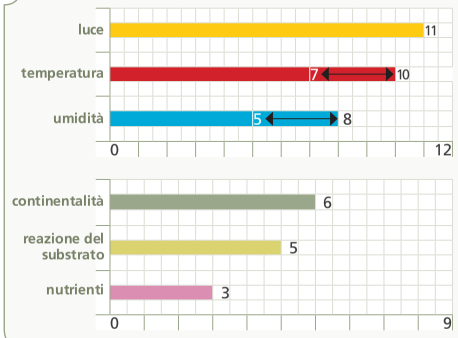
Condizioni di trofia: crescita buona anche su suoli molto poveri di sostanze nutritive.

Densità d'impianto (piante/m²): mantenere una distanza tra le piante di 5-6 m

Riproduzione gamica: produce numerosi semi che vengono dispersi dal vento

Riproduzione agamica: si moltiplica con grande facilità mediante talee; l'attecchimento è molto rapido

Indici di Ellenberg (come modificati da Pignatti)



Provenienza e diffusione

Origine: medio oriente, coste mediterranee e aree desertiche dell'Africa

Distribuzione: Aree mediterranee di Europa e Africa, Sud Africa, Asia, Australia, Stati Uniti, Argentina

Grado di naturalizzazione in Italia: specie autoctone, comuni su tutto il territorio

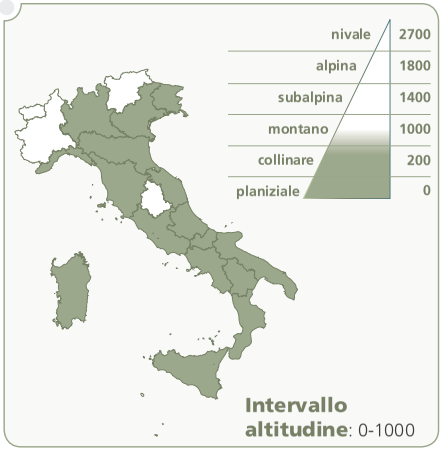
Potenziale invasivo: alto

Passaporto fitosanitario: non richiesto

Altre specie, sottospecie, cultivar e varietá diffuse e utilizzate in italia

Tra le specie di *Tamarix* autoctone, le più diffuse sono *T. africana* e *T. gallica*, molto simili tra loro, distinguibili per dettagli nella morfologia del fiore.

Presenza in Italia



Intervallo altitudine: 0-1000

Applicazioni

Fitorimedio: la tamerice è una pianta resistente alla presenza di *metalli* nel substrato; è stato documentato che può secernere il cadmio assorbito attraverso le foglie, liberandosene insieme alle secrezioni saline. Sembra che all'aumentare della salinità del substrato aumenti anche la capacità di assorbimento del metallo (Manousaki *et al.*, 2008).

Recenti ricerche hanno dimostrato come la tamerice possa agire su suoli alcalini contaminati da idrocarburi policiclici aromatici (IPA), nella sperimentazione condotta *T. aphylla* ha accelerato la rimozione di alcuni IPA e ha limitato il dilavamento di tali componenti a strati più profondi del suolo (Betancur-Galvis, 2012).

Risulta quindi una specie interessante per il fitorimedio di acque salmastre e terreni aridi, poiché oltre ad avere un alto tasso traspirativo e una buona produzione di biomassa, riesce a colonizzare ambienti ove altre piante non potrebbero crescere.

Annex 3

Microbiological analysis of the soil samples

Analysis were carried out by the soil laboratory of IRSA-CNR, Rome.

Soil subsamples from the microcosms were analyzed in order to assess the abundance and the activity of the microbial community, evaluating its evolution over time. The measurements have been conducted at the same time of the chemical analysis.

Materials and methods

Microbial abundances (N bacteria/g soil) were determined by epifluouorescence direct counting, employing DAPI as the fluorescent dye. A gram of soil was placed in a sterile 15 ml test tube with a 9 ml filter-sterilized (0.2 μm) fixing solution (PBS, 4% formaldehyde, 0.5% Tween 20). Then the test tube was shaken for 5 min (400 rpm) to break up any aggregations of bacteria. After shaking, the suspension was left for 24 h so that the larger soil particles could settle out. An aliquot of supernatant (100 μl) was pipetted into a sterile tube containing 2 ml of sterilized physiological solution. Then 200 μl of DAPI (4′,6-diamidino-2-phenylindole) were added. The DAPI was in contact with the supernatant for 20–30 min in the dark at 4 °C. The solution was then filtered through a 0.2 μm black membrane, which was subsequently mounted on a glass microscope, and the bacteria were counted by a Leica epifluorescence microscopy (Barra Caracciolo et al., 2005).

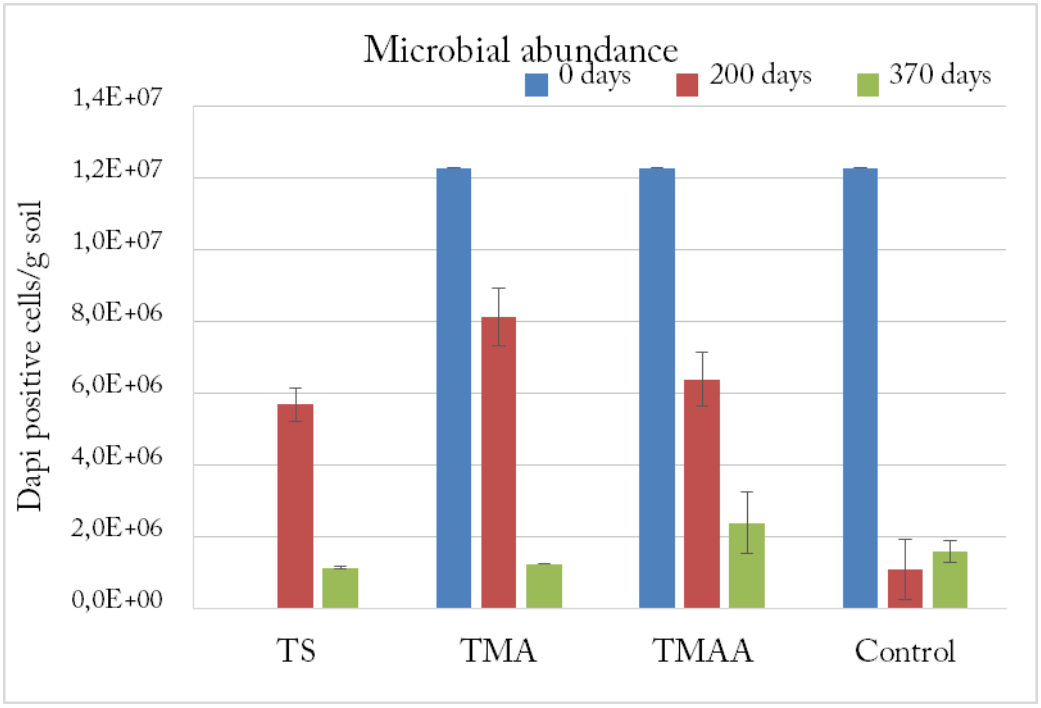
Cell viability was determined using a two-dye fluorescent bacterial viability kit (Molecular Probes® LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit.). The kit stain mixture distinguishes viable bacterial cells from dead ones on the basis of membrane integrity. The kit contains two nucleic acid stains. The green fluorochrome (SYBR green II) is a small molecule that can penetrate intact plasma membranes, while the larger red fluorochrome (propidium iodide) penetrates only compromised membranes. Bacterial suspensions incubated in the presence of both stains simultaneously will fluoresce either green (i.e., viable) or red (i.e., dead), depending on their viability. The excitation and emission maxima for these dyes are about 497 and 520 nm for SYBR green and 490 and 630 nm for propidium iodide, respectively (Alonso et al., 2002).

Dehydrogenase activity: soil dehydrogenase activity was used as microbiological indicator to evaluate the effect of the pollutants on soil functioning. Soil dehydrogenase activity was determined using the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) solution to triphenylformazan (TPF), measured in two replicates using; 6 g of soil were collected and anlyzed as reported by Tabatabai (1994), modified by Bending et al. (2007) and reported in Grenni et al., 2009. Soil dehydrogenase activity was expressed as μg TPFg−1 dry soil.

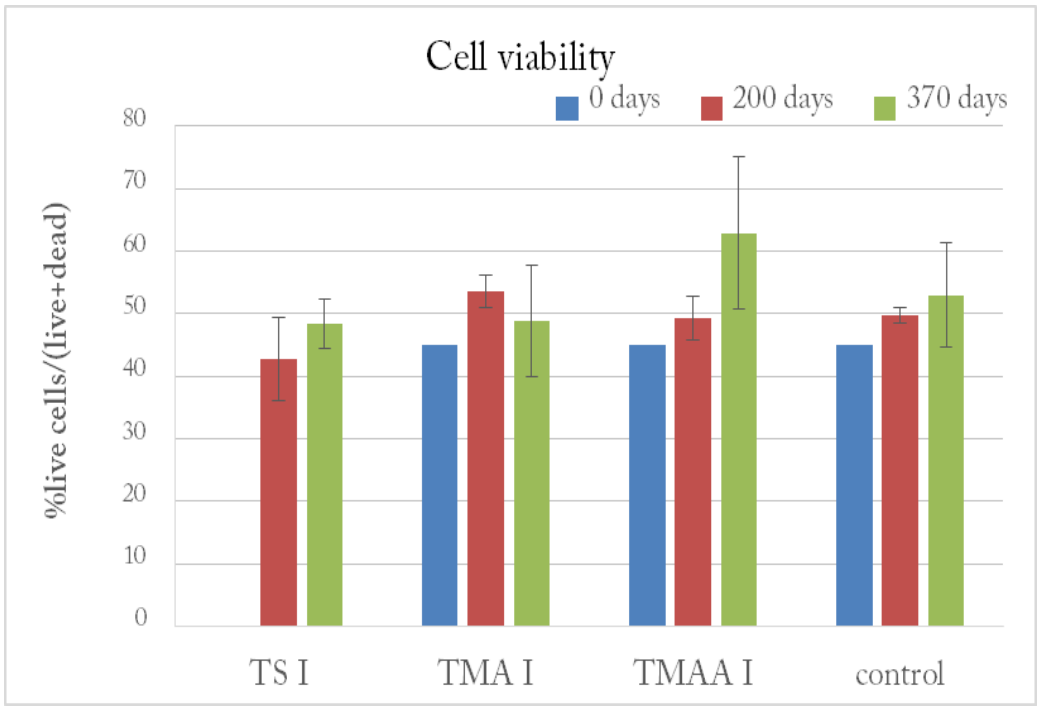
Results

A general decrease in **microbial abundance** (N. cells/g soil) was observed from day 0 to day 370.

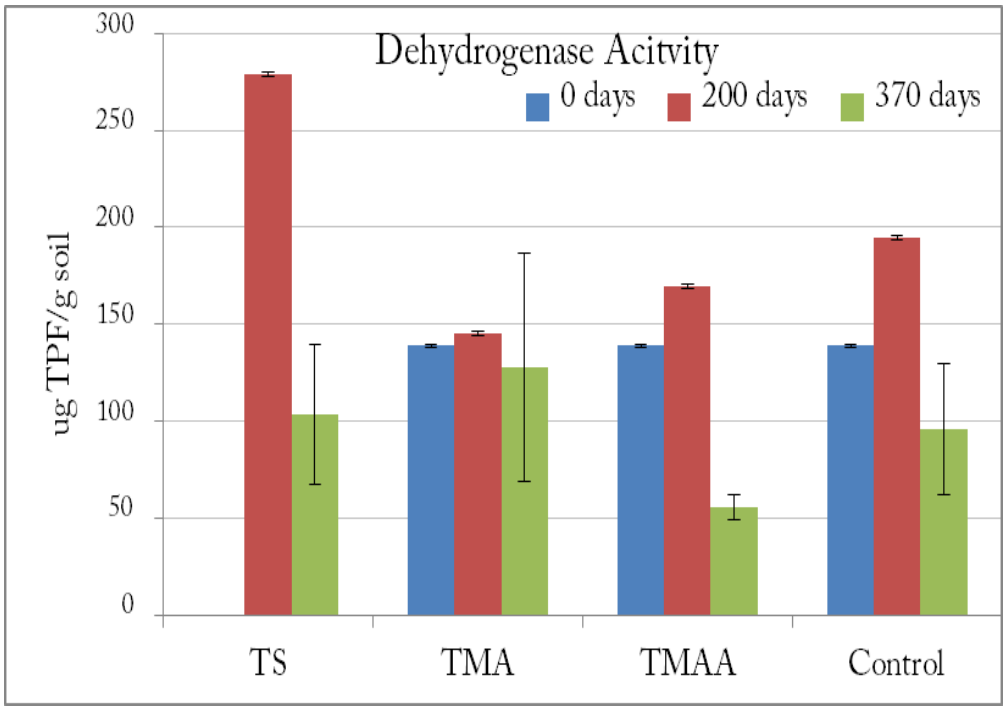
In the sterilized microcosms (TS), 6 month after planting (200 days), the microbial abundance increased significantly compared to day 0, showing a microbial recolonization from the surrounding environment (including from the cuttings) and from the soil cysts and spores survived to the sterilization.



The **cell viability** was not significantly affected neither from the treatment or from the time.



The **dehydrogenase activity** was not different among the treatments except for the sterilized microcosm (TS) in which it was significantly higher compared with the other treatments 6 month after the microcosm set-up (day 200).



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Annex 4

Plant antioxidant analysis

Analysis were carried out by Dott. Isabel Nogués, PhD at IBAF-CNR of Rome

When stress conditions are persistent, adaptation strategies are activated, which include ecophysiological, structural, and biochemical responses. Though each of the various stress conditions to which a plant may be subjected has distinctive characteristics and elicits its own specific responses, they all can lead to an oxidative status that accounts for a substantial part of the damage inflicted to stress organisms. The oxidative stress is the consequence of an unbalance between reactive oxygen species (ROS) formation and the ROS scavenging capacity of the defense system. In normal and in stress conditions, the redox equilibrium and the capacity of ROS scavenging have a key role for the normal development of a plant and for the perception, signaling, and adaptation to stress. To be able to scavenge the continuously produced ROS, plants possess an efficient cooperative system formed by enzymatic and non-enzymatic antioxidants. The latters include phenolics, flavonoids, and metabolites from the ascorbate–glutathione cycle (Nogués et al., 2012). In the present study phenolics, flavonoids and ascorbate were used as indicators of the plant stress eventually caused by the imposed hypoxic conditions (TMAA treatment). Antioxidants were extracted from portion of plant tissues (leaves, roots and branches), previously stored at -70°C.

Materials and methods

Total **phenolics** and **flavonoids** were extracted twice with 80% methanol (1.5 ml) for 3 min in ultrasonic bath.

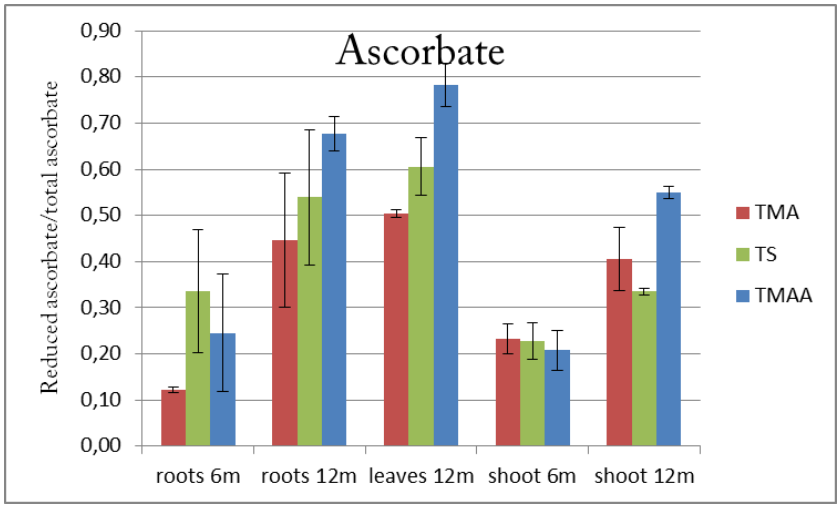
The amount of extracted total phenolics was determined with the Folin–Ciocalteau reagent as described by Singleton and Rossi (1965). Gallic acid was used as standard and the total phenolics were expressed as mg of gallic acid equivalents per g of fresh weight (Nogués et al., 2012).

Total flavonoid content was measured using the aluminium chloride method described by Chang et al (2002).The absorbance was read at wavelength 415 nm. Analysis was done in duplicate for each extracts. Standard solutions of quercetin with concentration 40 -100 μg/mL were used to obtain a standard curve. The total flavonoid content was reported as mg of total quercetin equivalents per g of fresh weight.

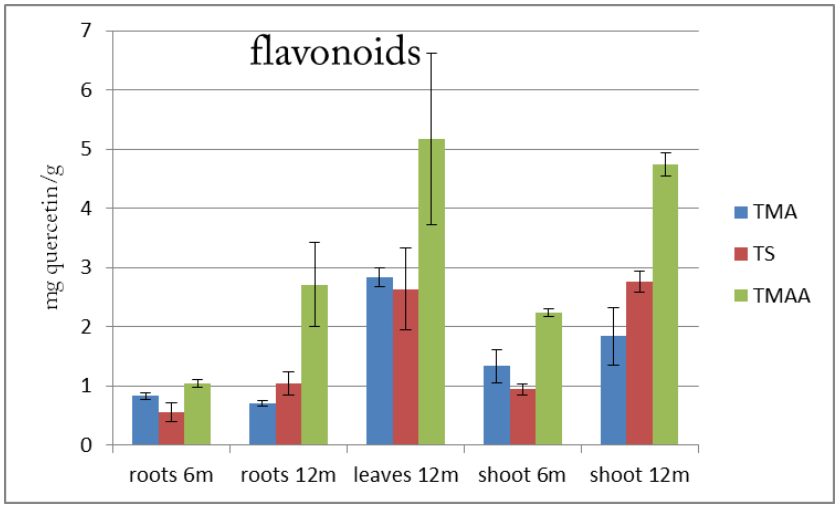
For determination of **ascorbate**, the plant tissue (about 100 mg fresh weight) was dissolved in 1.5 ml 3% perchloric acid, and the mixture was centrifuged (5,000 rpm, for 20 min) at 4°C. The pH was adjusted to 7 by adding 300–400 μ l of a sodium carbonate solution. The reduced (ASC) and oxidized (DHA) ascorbate measurement is based on the reduction of Fe3+ to Fe2+ by ascorbic acid in acidic solution. The Fe2+ forms complexes with bipyridyl, producing a pink colour that absorbs at 525 nm. DHA was reduced to ASC by pre-incubating the sample with dithiothreitol (DTT). The excess DTT was removed with N-ethylmaleimide, and the total ascorbate was determined. The amount of DHA was the difference between total ascorbate and the ASC. The contents were calculated using a standard curve (Hernandez et al., 2010).

Results

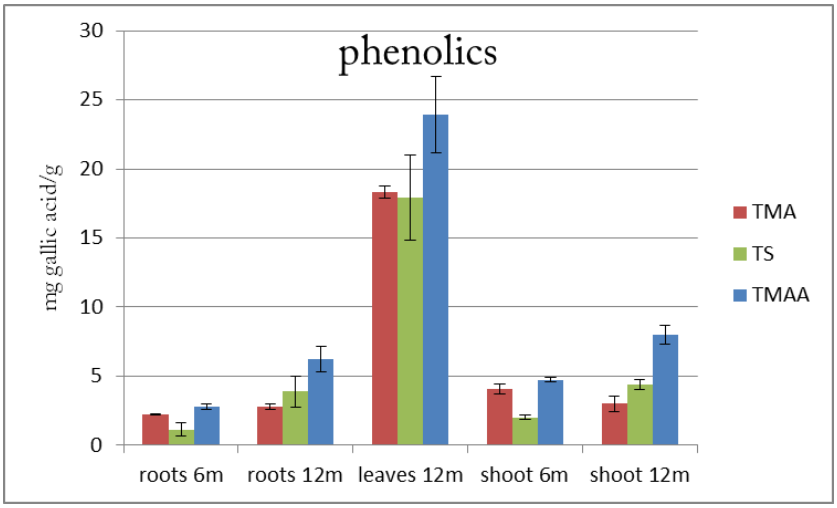
The ratio between the reduced form of ascorbate and the total ascorbate was measured in leaves 12 months after planting, in roots and shoot biomass 6 and 12 months after planting. Results showed values comprised between 0,12 al 0,78, with a general trend of lower ratio values in microbiologically active treatment (TMA) and higher values in the treatment under hypoxia (TMAA) as showed in the histogram below. A significant difference ($p < 0,05$) between the treatment was observed for ascorbate ratio in shoot tissues.



The total flavonoid content ranged between 0,56 and 5,17 mg of quercetin equivalents per g. Higher values were obtained in the hypoxic treatment for all the plant tissues analysed, with significant differences between the treatments observed for root content.



Total phenolic content ranged between 1,14 and 23,92 mg of gallic acid equivalents per g; the trend was the same of the antioxidants described above: higher values for hypoxic treatment (TMAA) and no relevant differences between microbiologically active treatment (TMA) and sterilised treatment (TS). Significance was observed for the difference in phenolics content in shoot tissues of poplar under hypoxia compared to other treatments.



For all the measured antioxidants, the differences between the hypoxic treatment (TMAA) and the other microcosms increase over time, showing the higher differences after 1 year of experiment.

Results are discussed in the PART III, chapter 4.1 of the present manuscript.

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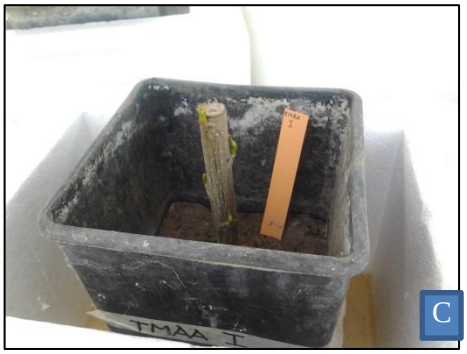
Annex 5

Photograph reporting of the microcosm trial.

A) Microbiologically active treatment; B) Sterilized treatment; C) Treatment in hypoxic conditions

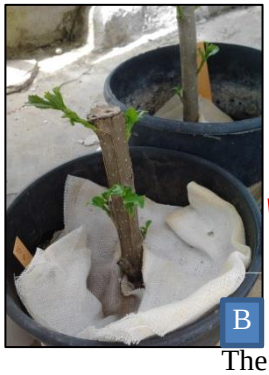
1) T=0 (May 2013)



2) T=6 days (May 2013)

3) T=8 days(May 2013)

4) T=11 days(May 2013)

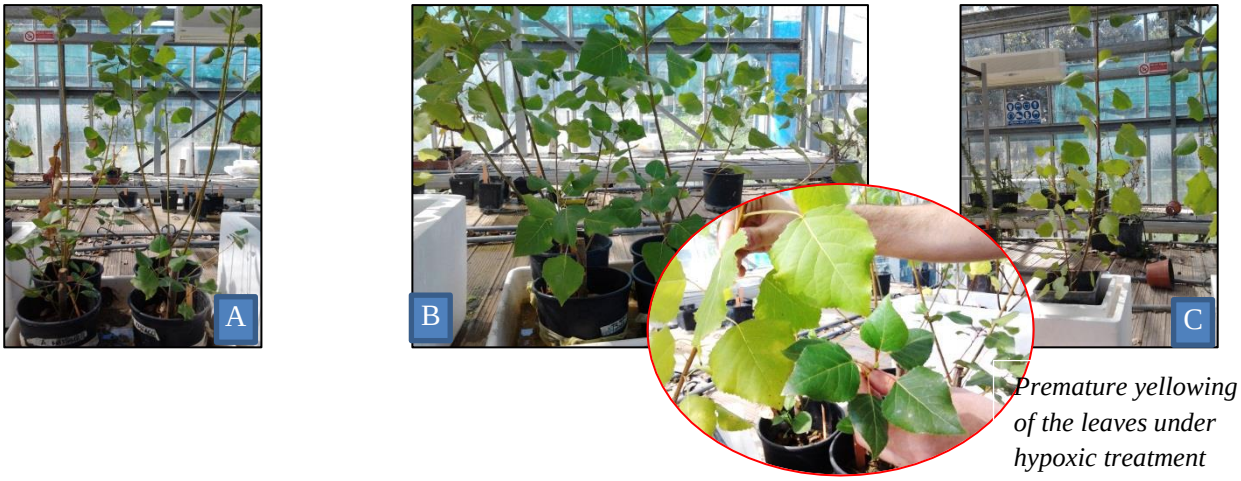


The sprouting delay in the sterilized treatment

5) T=13 days (May 2013)

6) T=20 days(June 2013)

7) T=4 month (September 2013)

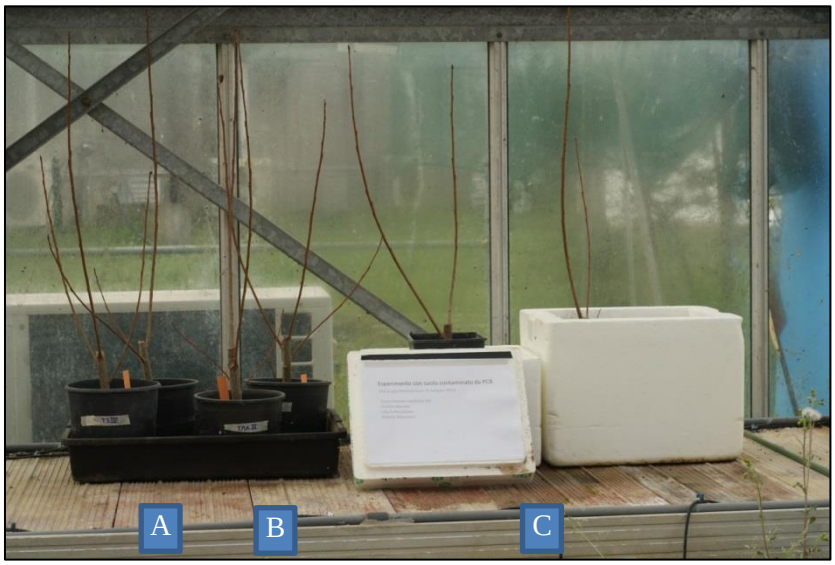


Premature yellowing of the leaves under hypoxic treatment

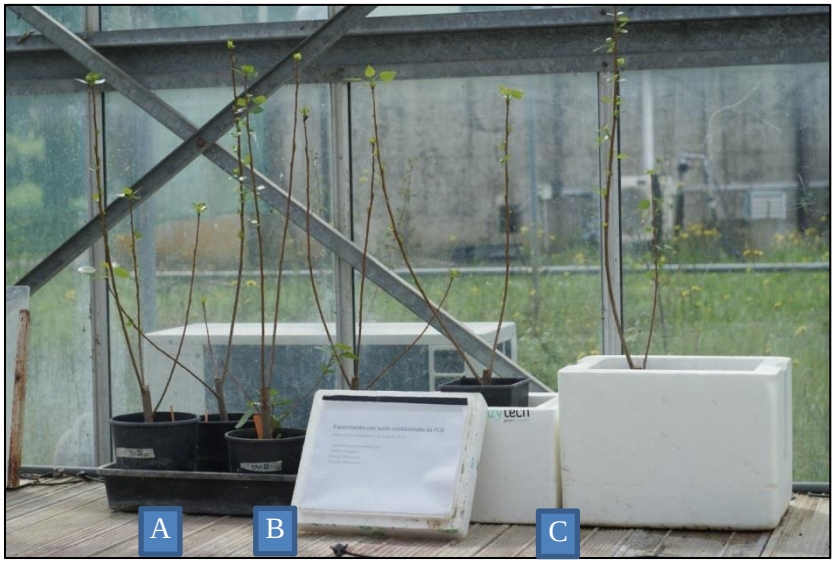
8) T=4,5 month (September 2013)

Necrosis of the leaves under hypoxic treatment

9)T=9 months (February 2014)



10) T= 11 months (April 2014)



11) T= 1 year (May 2014)

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