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Future prospects of the plant microbiome in phytoremediation

Phytoremediation: state-of-the-art and a key role for the plant microbiome in future trends and research prospects

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Phytoremediation is increasingly adopted as a more sustainable approach for soil remediation. However, significant advances in efficiency are still necessary to attain higher levels of environmental and economic sustainability. Current interventions do not always give the expected outcomes in field settings due to an incomplete understanding of the multicomponent biological interactions. New advances in -omics are gradually implemented for studying microbial communities of polluted land *in situ*. This opens new perspectives for the discovery of biodegradative strains and provides us new ways of interfering with microbial communities to enhance bioremediation rates. This review presents retrospectives and future perspectives for plant microbiome studies relevant to phytoremediation, as well as some knowledge gaps in this promising research field. The implementation of phytoremediation in soil clean-up management systems is discussed, and an overview of the promoting factors that determine the growth of the phytoremediation market is given. Continuous growth is expected since elimination of contaminants from the environment is demanded. The evolution of scientific thought from a reductionist view to a more holistic approach will boost phytoremediation as an efficient and reliable phytotechnology. It is anticipated that phytoremediation will prove the most promising for organic contaminant degradation and bioenergy crop production on marginal land.

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1. Introduction and retrospect of the phytoremediation research

Developments in science and technology have increased food production, but also resulted in the use of a wide range of synthetic chemicals, and thus in an exponential increase in their production and consequently the generation of wastes (Panagos *et al.* 2013, Zhao *et al.* 2015). Recognition of the threats posed by contaminants and their impact on natural processes spurred the search for efficient, cost-effective remediation technologies to remove pollutants from the environment. As a less expensive and environmental friendly remediation technology compared to physical (excavation and landfill) and chemical methods, phytoremediation drew increasing attention because it exploits the synergistic interactions between plants and microorganisms to completely eliminate or otherwise mitigate the noxious effects of environmental contaminants *in situ* (Pilon-Smits 2005). Other advantages of phytoremediation include the contribution to atmospheric CO_2-fixation, increasing local biodiversity, mitigating negative effects of erosion, and the fact that plant biomass produced during phytoremediation can be economically valorised in the form of bioenergy (biogas, biofuels, combustion, heating) or industrial feedstock (Weyens *et al.* 2009, Gomes 2012).

Phytoremediation is not a new technology but it has significantly evolved over the past decades due to the evolution of scientific thought, and technology, which leads to new avenues of the science being explored, furthering the understanding behind it (McCutcheon and Rock 2001). In 1940, Miller already showed that plants metabolise xenobiotics analogously to the transformation and conjugation in mammals, and later the feasibility of degrading organics was elucidated in the 'green liver model' (Sandermann 1994). For inorganics, the metal-tolerant plant species observed by Andrea Cesalpino in 1940, were rediscovered by Brooks *et al.* 1977, who proposed the term hyperaccumulator, to describe plants that accumulate nickel in dry matter in concentrations up to 100 times greater than those tolerated by non-accumulator plants (Brooks *et al.* 1977). From 1990 on, rapid advances were made in phytoremediation and approaches including phytodegradation, phytovolatilisation, phytostabilisation, phytoextraction and rhizoremediation reached the market (Salt, Smith and Raskin 1998, Dietz and Schnoor 2001, Suresh and Ravishankar 2004, Pilon-Smits 2005, Leigh 2006, Marmiroli, Marmiroli and Maestri 2006, Pilon-Smits and Freeman 2006, Vangronsveld *et al.* 2009, Wenzel 2009). The range of

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xenobiotic compounds targeted by phytoremediation has grown ever since (Marmiroli, Marmiroli and Maestri 2006, Gao, Ellis and Wackett 2010).

The worldwide market of phytoremediation increased from \$15-18 billion yr^{-1} in 1998 to \$34-54 billion yr^{-1} in 1999, and the US market would reach \$235-400 million by 2005 as was estimated (Glass 1999). However, phytoremediation did not achieve its predicted potential as a commercial technology (Conesa *et al.* 2012). Causing factors are the limitation of clean-up to areas amenable for plant growth, the risk of accumulation of toxic products and contamination of the food chain, the possible volatilisation of organic contaminants, and the often limited ability of plants to intercept, take-up, tolerate and accumulate trace elements. These drawbacks lead to the following properties of phytoremediation: uncertainty, variable effectiveness at the field scale, and in the case of trace elements, long remediation time, which have withhold investments (Schwitzguébel *et al.* 2002, Todd and Raina 2003, Linacre, Whiting and Angle 2005, Mench *et al.* 2010, Olaniran, Balgobind and Pillay 2013).

In the course of time, solutions to many of the limitations were proposed. The attention switched from hyperaccumulators to high biomass-producing, fast-growing and deep-rooting tree species (Vangronsveld, Herzig, Weyens *et al.* 2009). In addition to traditional breeding programs, plant transgenesis was pioneered in the 1990s, to increase the ability of plants to metabolise and detoxify xenobiotic compounds (Abhilash, Jamil and Singh 2009, Maestri and Marmiroli 2011, Kotrba 2013). For example, transgenic tobacco plants (French *et al.* 1999, Hannink *et al.* 2001, Travis *et al.* 2007) and transgenic hybrid aspen (*Populus sp.*) (Van Dillewijn *et al.* 2008) expressing bacterial nitroreductases have enabled effective remediation of explosives at the field scale (Rylott and Bruce 2009, Van Aken 2009, Rylott *et al.* 2011), and similarly, plant transgenics has allowed improved PCB degradation (Sylvestre, Macek and Mackova 2009), and increased plant tolerance for trace elements (Kärenlampi *et al.* 2000, Stearns *et al.* 2005, Kotrba 2013). Many studies have also focused on the use of plant-associated bacteria (Weyens, van der Lelie, Taghavi *et al.* 2009, Glick 2010) and mycorrhizal fungi (Bücking 2011, Colpaert *et al.* 2011, Harms, Schlosser and Wick 2011, Leung *et al.* 2013) to improve phytoremediation. Numerous microbial mediated functions can be addressed in order to

enhance plant health and productivity, and thus phytoremediation (**Box 1**). Due to the importance of the soil habitat to plants, many studies have focused on the rhizosphere: application of microbial inoculants (Weyens, van der Lelie, Taghavi *et al.* 2009, Ma *et al.* 2011) or modification of rhizosphere microbial communities through nutrient addition (Makris, Sarkar and Datta 2010, Muter *et al.* 2012), or rhizoengineering (Narasimhan *et al.* 2003, Dzantor 2007, Aken, Correa and Schnoor 2010). In addition, the use of endophytic microorganisms in phytoremediation received renewed interest (Rylott 2014, Zhu *et al.* 2014, Hardoim *et al.* 2015, Ijaz *et al.* 2015).

For a long time reductionist approaches (one-compound-at-the-time, one microorganism, artificial root exudates, sterile substrate, etc.) have dominated phytoremediation research, though more fundamental research is needed to better understand the interactions between plants and the microbiome as a whole, before scientifically sound interventions can be performed in the field (de Lorenzo 2008). That is because applying techniques developed under controlled laboratory, and greenhouse conditions, to highly complex and heterogeneous field conditions has proven difficult (Khan *et al.* 2013, Sessitsch *et al.* 2013).

Consequently, phytoremediation was revitalised within the concept of "phytotechnology" (Marmiroli, Marmiroli and Maestri 2006). Phytotechnology builds on the practical experience from agriculture, forestry, horticulture (Salt, Smith and Raskin 1998), and the term 'technology' emphasizes the integration of ecology within a multidisciplinary context (Olson, Reardon and Pilon-Smits 2004, Bell *et al.* 2014). A main pillar of a more integrated phytoremediation can be provided by the advances in next-generation sequencing (NGS) technologies launching an era of meta -omics (metagenomics, metatranscriptomics, metaproteomics, metabolomics) (Desai, Pathak and Madamwar 2010, Uhlik *et al.* 2013, Bell, Joly, Pitre *et al.* 2014). These state-of-the-art tools have tremendously increased our understanding of the function of plants, microorganisms, and the interactions between them in a natural context (Bakker *et al.* 2013, Mendes, Garbeva and Raaijmakers 2013, De-la-Peña and Loyola-Vargas 2014). Furthermore, the decreasing costs of high-throughput sequencing technologies allow a further extensive

integration (Ramos *et al.* 2011, Bell, Joly, Pitre *et al.* 2014), offering a wide range of opportunities for optimisation and a better understanding of phytoremediation.

This review zooms in on the complexity of plant-associated microbial communities in the rhizosphere, and we present an overview of the literature showing how integrated data on the plant microbiome using -omics and the biochemistry of contaminant degradation, provide potential new avenues for the design a *new generation of phytotechnologies.* Despite the enormous progress that already has been made, some challenges still exist, which call for the intensification of fundamental research at the microbial community-level and biochemical level. In addition, the implementation of phytoremediation in larger clean-up programs needs to be studied in order to reach maximum applicability, which is also discussed. We conclude with the future trends and research prospects, that will determine the success of phytoremediation on the market.

2.   Microbiome complexity

The interactions between plants and microorganisms are tremendously complex and the multispecies networks resulting from the associations have consequences for plant growth, productivity, resilience against abiotic stresses and thus phytoremediation (Bakker, Berendsen, Doornbos *et al.* 2013, Mendes, Garbeva and Raaijmakers 2013, Rout and Southworth 2013, De-la-Peña and Loyola-Vargas 2014). While huge progress has been made in the understanding of the molecular interactions between plants and model microbial partners (Barret, Morrissey and O'Gara 2011, Knief, Delmotte and Vorholt 2011, Segura and Ramos 2013), there is still a lack of knowledge of the multi-trophic interactions between plants and microorganism at the community level. It is well known that the rhizosphere supports a much higher microbial density than the bulk soil, described as the 'rhizosphere effect' (Smalla *et al.* 2001, Haichar *et al.* 2008, Bulgarelli *et al.* 2013), and that the specific recruitment of particular microbes by the plant can be influenced by contaminant concentration (Siciliano *et al.* 2001, Sipila *et al.* 2008, Andria, Reichenauer and Sessitsch 2009, Kawasaki, Watson and Kertesz 2011, Bell *et al.* 2014, Yergeau *et al.* 2014). In fact, it is argued that biodegradation cannot be attributed to a single taxon, but by a group of microorganisms (Uhlik *et al.* 2009, Bell *et al.* 2011). Moreover, although specific

microorganisms are able to detoxify inorganic and organic contaminants, their effect is highly influenced by the rest of the microbial community. Further, a review of literature shows that (i) contaminant degraders have to be present in sufficiently high numbers to obtain a considerable effect, (ii) plants may favour or select microorganisms that promote plant growth and plant protection when contaminant concentrations are low, and opportunistic microorganisms that do not contribute to phytoremediation may compete effectively for plant-produced compounds (Berendsen, Pieterse and Bakker 2012), (iii) microorganisms can act additively or synergistically on each other for contaminant degradation and other functions (Hibbing *et al.* 2010), and (iv) biodegradation by microorganisms can be independent of their effect on plant growth. Hence, all active (micro)organisms affect each other in one way or another. Although certain functions can be attributed to specific microorganisms, it is clear that the total microbiome and its interaction with the plant determine phytoremediation outcomes. These findings highlight the need for a holistic framework in ecological and biochemical studies in phytoremediation.

3.   Towards a better management of the plant-microbiome

The diversity of microorganisms associated with plants is in the order of 10^{11} microbial cells per gram root, and hundreds to ten thousands prokaryotic species have been reported in the rhizosphere (Mendes, Garbeva and Raaijmakers 2013). In contrast, traditional culture-based techniques typically pick up only a limited fraction of soil microbial communities and thus grossly underestimate microbial diversity (Pham and Kim 2012). The yet to be cultured majority harbors an enormous reservoir of uncharacterised organisms, genes, and enzymatic processes.

To this end, culture-independent techniques are invaluable to estimate microbial diversity and dissecting the function of microbial systems relevant to plant growth, contaminant degradation, and the environment (Desai, Pathak and Madamwar 2010, Orgiazzi *et al.* 2015). These culture-independent techniques encompass the application of an increasingly broad collection of next generation analytical tools such as 16S rRNA gene sequencing (Caporaso *et al.* 2012), stable isotope probing (SIP) in combination with shotgun metagenomics (Chen and Murrell 2010, Verastegui *et al.* 2014), functional gene microarrays (*e.g.* GeoChip, PhyloChip) (Schatz *et al.* 2010, Tu *et al.* 2014), metatranscriptomics (Schenk, Carvalhais and Kazan 2012),

metaproteomics (Siggins, Gunnigle and Abram 2012), and metabolomics (De Cesare *et al.* 2011). Characterisation of enzymes, regulatory pathways, interaction networks, and new microbial species discoveries have been accomplished at unprecedented resolutions and frequencies. In addition to studying whole living systems in their natural context (Li *et al.* 2012), these methods enable the study of single cells in complex communities (Muller and Nebe-von-Caron 2010, Li, Canniffe, Jackson *et al.* 2012).

There are basically two ways to apply –omics. The first, descriptive -omics, collects as large a dataset as possible regarding the molecules or processes of interest, and then tries to make sense of it all, *i.e.* monitoring of biological communities in a passive way. The second way is to devise high-throughput chemical and biological tools to perturb the biological system (*e.g.* by the addition of oxidoreductants, fertilisers, soil physical disturbance, plant harvesting, etc.) and even allow the creation of new ones that do not exist naturally, *i.e.* manipulative -omics.

In the following paragraphs, we show how -omics approaches can be used to garner functional insights into microbial systems for the purpose of applying phytoremediation in the field, and development of improved biostimulants (**Table 1**). Although we focus on specific contaminants, the underlying ideas can be applied in different research fields such as bioenergy and sustainable agriculture.

3.1 Microbial and plant -omics — for aliphatic and aromatic hydrocarbons

Stable isotope probing (SIP), metagenomic and transcriptomic analysis are well suited for characterising the mode of action by which microorganisms degrade hydrocarbon contaminants, activate enzymes and whole metabolic pathways, interact with each other and their environment, and for unraveling novel regulatory pathways. Experiments involving SIP with U-^{13}C labeled naphthalene, phenanthrene, pyrene, fluoranthene or benz[a]anthracene, added to polycyclic aromatic hydrocarbons (PAH) contaminated soil, revealed that a wide range of microbial taxa displayed hydrocarbon metabolism, including some yet to be cultured ones (**Table 1**) (Jones *et al.* 2011). Using a similar approach, Uhlik *et al.* 2012, found that some strains derived carbon

from naphthalene as well as from biphenyl/benzoate in a mixed contaminated soil, pointing out broader biodegradation abilities of some soil microbiota.

Despite the known capability of root exudate compounds to act as microbial growth substrates (Lopez-Guerrero *et al.* 2013) and as inducers or inhibitors of catabolic pathways (Kamath, Schnoor and Alvarez 2004, Rentz, Alvarez and Schnoor 2004, Rentz, Alvarez and Schnoor 2005, Martin *et al.* 2014), questions remain about the function of root exudates *in situ* (Dennis, Miller and Hirsch 2010, Shi *et al.* 2011, Shi *et al.* 2013). This prompted researchers to test whether the addition of a series of root exudates affects the mineralisation rate and diversity of degradative bacteria. In a first study, Cébron *et al.* 2011, spiked microcosms with ^{13}C-labelled phenanthrene and ryegrass exudates, and found that phenanthrene dissipation was not different for the treatments over 12 days, though root exudates favoured a higher diversity of bacteria and increased the abundance of PAH-ring hydroxylating dioxygenase genes, suggesting that increased interspecies competition for root exudates may reduce catabolic gene expression. In a second study, Sun et al., 2010, demonstrated that the removal efficiency of phenanthrene and pyrene was higher in soil treated with only root exudates, than when complete plant roots were added. Thirdly, comparing alfalfa and wildrye root exudates on hydrocarbon mineralization, Philips *et al.* 2012, showed that some root exudate compounds stimulated mineralisation rates and increased gene copy numbers of degradative genes, while others decreased mineralisation rates, which made them conclude that the success of a given phytoremediation experiment is likely influenced by the relative abundance of specific root exudates compounds. Together, these studies demonstrate that the most effective rhizodegradation may not naturally occur in the rhizosphere of plants grown on contaminated soil, and thus appropriate interventions are needed. This may include alternative plant species selection, or exploiting the naturally evolved plant-microbiome communication signals for rhizosphere microbiome engineering (Quiza, St-Arnaud and Yergeau 2015) (**Table 1**).

In a more quantitative study, Bell *et al.* 2013a, sought to examine how shifts in microbial species abundance could affect hydrocarbon degradation rates. They showed that antibiotic treatment (gentamicin and vancomycin) of a hydrocarbon-contaminated Arctic soil, increased

hydrocarbon degradation rates compared to the no-antibiotic control, while bacterial and fungal abundances were reduced. The antibiotic effects were not observed when also nutrients were added. In this case, nutrient addition promoted a larger fungal community which was suggested to account for the differences in degradation. Overall, the study led to the conclusion that a large part of the microbial population is not actively involved in hydrocarbon degradation and may compete with hydrocarbon-degrading microorganisms for nutrients and space. Similar approaches based on *e.g.* functional inhibitors may further our insight in the active bioremediating communities in natural soil environments. This may eventually lead to the rational design of a 'minimal rhizosphere' microbiome, characterised by a minimal set of functional genes necessary to fulfill a specific function (*e.g.* contaminant degradation and plant-growth promotion) (Raaijmakers 2015). However, this is still very challenging and requires an enhanced understanding of the plant microbiome feedbacks, and of the interactions between individual members of the microbiome and with environmental conditions. In this context, lower complexity microbial community studies (*e.g.* in wastewater treatment plants) have provided striking insights in the functioning of microbial communities, and have allowed for targeted community manipulations selecting desirable functions (**Box 2**).

Plant, bacteria and fungi are highly form close associations within the rhizosphere in non-contaminated soil, but the degree of linkage between the groups can be altered in contaminated soils (Bell, El-Din Hassan, Lauron-Moreau *et al.* 2014). In a field study, Bell *et al.* 2014 showed that fungi were more sensitive to hydrocarbon contamination than bacteria, and a closer association was formed between native willow species and fungal communities, than between willow cultivars and bacterial communities. Johnson *et al.*, 2010, found previously that the associations between indigenous arbuscular mycorrhizal fungi and native plant species were more mutualistic than combinations with foreign plant species. Together, the health of native or non-native plant cultivars may be differentially affected by the strength of their associations with indigenous fungi. In this view, a 'back to the roots' framework is suggested to investigate native trees in their natural habitat and interactions with the indigenous microbiota, to identify symbiotic partners that show a synergistic, or at least not antagonistic relationship, with *e.g.* inoculated bacterial strains. If the associations between plants and fungal taxa are specific and

predictable, then future inoculation studies can take advantage of the naturally evolved plant mycorrhizal fungi co-evolution to promote effective bioremediating communities (**Table 1**).

3.2 Plant-microbe insights guiding rhizoengineering — for polychlorinated biphenyls

The very few bacteria capable of mineralising di- and polychlorinated congeners that have been isolated and cultured (Pieper 2005, Leigh *et al.* 2006, Pieper and Seeger 2008, Mackova *et al.* 2009), or were obtained upon genetic manipulation and/or plasmid transfer, was considered a main impediment to highly chlorinated congener biodegradation (Villacieros *et al.* 2005, Rein *et al.* 2007, Hernandez-Sanchez, Lang and Wittich 2013). The value of cultivation-independent analysis to detect PCB-degrading bacteria and functional genes was demonstrated by Leigh *et al.* in 2007. Using SIP with ^{13}C-biphenyl and functional gene analyses, 75 different genera were found that derived carbon from biphenyl, with only 1 strain detected by both cultivation-based approaches and SIP (Leigh *et al.* 2007). In addition, GeoChip detected many genes associated with the catabolism of biphenyl and a variety of aromatic ring hydroxylating dioxygenase (ARHD) subunits, including several ARHD-sequences that were distantly related from all known enzymes. Using similar SIP-based experiments, several other studies identified novel PCB-metabolizing strains that can be stimulated in phytoremediation (**Table 1**).

There is a body of evidence that many plant secondary metabolites (l-carvone, limonene, p-cymene, isoprene, linoleic acid, terpenes, flavonone) may act as signal molecules, triggering the bacterial metabolisation of PCBs during rhizoremediation (Donnelly, Hegde and Fletcher 1994, Gilbert and Crowley 1997, Toussaint *et al.* 2012). However, some root-exudate compounds can also decrease PCB degradation (Vrchotova *et al.* 2013). To further unravel the basis of PCB rhizoremediation, studies have focused on elucidating the signaling interactions between plant species and the whole microbiome, and the influence of specific flavonoids on PCB-degradation by individual members of the microbiome. Slater, Gouin and Leigh 2011, addressed the abilities of two tree species to promote PCB degradation via the release of phytochemicals. The results indicated that willow root crushates enhanced PCB-loss in biphenyl-treated mesocosm with congeners PCB 77, 105, 169, while the root crushates of white spruce and salicylate alone did not. These results suggest that PCB-biostimulation is plant specific, and

involves a shift in the microbial community composition (Slater, Gouin and Leigh 2011). From a screening using quantitative real-time polymerase chain reaction (RT-qPCR) of the *bphA* gene, Pham *et al.* 2015 identified ten flavonoids that did not support bacterial growth, but that acted as inducers of the *Rhodococcus erythropolis* U23A biphenyl pathway (**Table 1**). The level of induction was significantly influenced by the nature and concentration of the flavonoid in the growth medium, as well as by the co-substrate used for growth, suggesting that the optimisation of rhizoremediation requires the adjustment of multiple parameters (Pham *et al.* 2015). Although the research is still in the developmental stages, the overall strategy from identifying the plant-secreted inducers, to manipulation of the plant physiological state via rhizoengineering, is highly promising for developing new degradation strategies in future (Narasimhan, Basheer, Bajic *et al.* 2003, Quiza, St-Arnaud and Yergeau 2015).

In a combined bacteria-mycorrhiza study, cultivation-independent analyses showed that the inoculation of zucchini with arbuscular mycorrhizal fungi (*Acaulospora laevis*, *Glomus caledonium*, *Glomus mosseae*) enhanced the dissipation rates of Aroclor 1242 compared to the non-mycorrhizal control, which was attributed to increased bacterial *bphA* and *bphC* gene abundance in the mycorrhizosphere, and the dominance of specific soil bacterial phyla (Qin *et al.* 2014). These results provide strong evidence that AM-fungi can enhance PCB degradation, and delivers novel ways to enhance their abundance and potency.

3.3 *Advances in the field of endophytes — for water-soluble, volatile organic pollutants*

Phytoremediation is a sustainable solution to treat BTEX and TCE-contaminated sites, if some physical limitations are overcome: the fact that highly water-soluble compounds seem to enter the root xylem faster than the soil and rhizosphere microflora can degrade them, and the problem of volatile compounds and their metabolites being released into the environment by leaf evaporation (Barac *et al.* 2004, Weyens *et al.* 2009). During plant passage the compounds may be transformed by the plant or may be degraded by stimulation of microorganisms residing inside plant tissues. The advantages of using endophytes over rhizospheric bacteria are numerous (Newman and Reynolds 2005, Weyens *et al.* 2009, Gaiero *et al.* 2013, Afzal, Khan and Sessitsch 2014). Perhaps the most important advantage is the longer and more intense contact between

contaminant and microbial catalysts in the plant, which increases the rate and efficiency of phytodegradation (Barac, Taghavi, Borremans *et al.* 2004, Lee, Wood and Chen 2006, Weyens, Van der Lelie, Artois *et al.* 2009, Weyens *et al.* 2010). Another advantage of inoculating plants with endophytes is that at higher abundance, their collective catalytic activity will be higher compared to rhizosphere microbiota (Andria, Reichenauer and Sessitsch 2009, Bulgarelli, Schlaeppi, Spaepen *et al.* 2013). Biostimulation protocols based on the isolation and characterisation of culturable TCE-degrading endophytic bacteria, and the subsequent re-inoculation of selected plant-colonisers engineered with contaminant-degrading genes, have been successfully used to improve the remediation of contamination with water-soluble, volatile organics (Barac, Taghavi, Borremans *et al.* 2004, Mastretta *et al.* 2006, Weyens, Van der Lelie, Artois *et al.* 2009, Weyens *et al.* 2010) and mixes of organic and inorganic contaminations (Weyens, Croes, Dupae *et al.* 2010, Weyens *et al.* 2012, Weyens *et al.* 2015) (**Table 1**).

It was demonstrated that after stable establishment and enrichment of the engineered endophyte *Burkholderia cepacia* L.S.2.4 containing the pTOM-Bu61 plasmid (coding for toluene degradation) in yellow lupine, the plasmid was spread into aboveground plant endophytes by horizontal gene transfer, and this improved the plant tolerance and degradation of toluene that otherwise would have been volatilised (Barac, Taghavi, Borremans *et al.* 2004, Taghavi *et al.* 2005). A subsequent study showed that inoculation of poplar under *in situ* conditions with *P. putida* W619-TCE enhanced TCE-degradation and this was also attributed to horizontal gene transfer of TCE metabolic activity to members of the poplar's leaf endophytic population (Weyens, Van der Lelie, Artois *et al.* 2009). As these studies indicate, plasmids can play an important role in microbial community modulators in phytoremediation. It is to note that maintenance of functionality, rather than a detailed account of 'who does what' is more important. The rhizosphere and endosphere are considered hotspots of genetic exchange between bacteria (Ronchel, Ramos-Diaz and Ramos 2000, Van Elzas, Turner and Bailey 2003, Heuer and Smalla 2012), and in these environments plasmids act as a source of genomic innovators to adapt the bacteria to the prevailing stress conditions (Ochman, Lawrence and Groisman 2000, Sentchilo *et al.* 2013). Metagenomic sequencing has been used to discover the 'mobilome' from rhizosphere soils (Heuer and Smalla 2012, Jorgensen *et al.* 2014, Wang *et al.* 2014, Wei *et al.*

2014), confirming that various genes involved in trace element resistance and recalcitrant organics degradation are plasmid-borne. Metagenomics coupled with (meta)transcriptomics can be used to mine the rhizosphere and endosphere for degradative elements, quantify transformation efficiency within microbial communities, and determine how plants and indigenous microbial communities are affected by plasmid introduction (**Table 1**). In all, combining the use of engineered endophytes with the potential of horizontal gene-transfer is anticipated to be one of the most promising ways to improve the phytoremediation of organics, reflected by the ongoing interest in this topic (Afzal, Khan and Sessitsch 2014, Rylott 2014, Shukla, Upadhyay and Dubey 2014, Zhu, Ni, Liu *et al.* 2014, Ijaz, Imran, Anwar ul Haq *et al.* 2015).

3.4   Microbial and plant -omics —for trace element phytoextraction

While plants adopted for phytoextraction are commonly pre-selected for traits such as high accumulation of trace elements (hyperaccumulators), and/or high biomass and rapid growth, recent studies have shown that the success of phytoextraction depends heavily on the recruitment of specific rhizosphere microbiota from the surrounding environment (**Table 1**). Bell *et al.* 2015, introduced three willow cultivars to a contaminated landfill, and they found that total zinc (Zn) accumulation in willow was better explained by fungal community structure 4 months post-planting than 16 months post-planting as determined by using Ion Torrent sequencing. Certain dominant ectomycorrhizal fungi (*Sphaerosphorella brunnea* and *Inocybe sp.*) were negatively correlated to the accumulation of Zn (Bell *et al.* 2015). The authors suggested that the microbiome has the greatest impact on plant function during the early stages of growth, and the plant-fungus specificity may be critical. Another study evaluated microbial community structure and the presence of functional genes in the rhizosphere of *Pteris vittata* ('Chinese brake') grown on arsenic (As) contaminated soil (Xiong *et al.* 2010). While As dramatically reduced microbial diversity, the rhizosphere soil had a higher metabolic versatility than non-rhizospheric soils, with genes related to As-resistance, sulfur reduction, phosphorus use and denitrification being enriched in *P. vittata* rhizosphere soils (Xiong, Wu, Tu *et al.* 2010). This study employed Biolog metabolic profiling, geochemical analyses and functional gene microarray analysis (GeoChip

3.0) (Xiong, Wu, Tu *et al.* 2010). Yet another study profiled the interplay between the metal-accumulating *Arabidopsis halleri* plant and its rhizosphere microbiome, showing that *A. halleri* accumulated higher levels of cadmium and zinc when grown on natural soil than on gamma-irradiated soil (Muehe *et al.* 2015). Strong alteration of the soil microbial community composition and overall cell numbers were observed across the treatments, and the identification of highly abundant taxa (*Streptomyces, Agromyces, Nitrospira*) were hypothesised to be involved in the microbial effect on plant metal uptake. Together, these studies point out the importance of the (early) recruitment of a host-specific microbial community that may enhance trace element uptake and detoxification. These insights provide new clues about plant-microbial functional linkages that may be coupled directly to phytoextraction practices.

Targeting microbes to enhance trace element bioavailability and increase phytoextraction efficiency is considered a challenging task, because of the intrinsic relationships of these microbes with their host and the potentially thousands of microbial taxa that have varying effects on trace element bioavailability and sequestration. To investigate live plant-microbe interactions in the bioenergy relevant crop maize, a suite of tools based on plant metabolomics and proteomics was utilized, evaluating the effect of metal-resistant bacteria inoculation on copper uptake by maize plants grown in soils contaminated with trace elements (Li *et al.* 2014). The metal-resistant PGPB were applied by means of soil inoculation before sowing and seed coating, yielding higher copper uptake by maize compared to other inoculation methods. Metabolomics provided a picture of the effect of PGPB inoculation on plant physiology (upregulation of photosynthesis, hormone biosynthesis, and tricarboxylic acid cycle metabolites were found), while proteomics provided insight in plant development and stress. A decrease in plant anti-oxidative enzyme activities was observed following inoculation, indicating alleviation of metal phytotoxicity by the PGPB (Li, Pidatala, Shaik *et al.* 2014). This study is a good example of a multi-disciplinary approach to increase our insights into the interactive forces between, in this case, a single inoculated bacterial strain and the host plant. More studies that utilise meta-organism-based approaches (*i.e.* selecting and managing complementary plants and microbiomes) are needed and are sure to provide useful insights. Approaches that can be used to study the interactions between hyperaccumulator plants and their microbial rhizobiome on the

whole-plant level, using -omics analyses and powerful imaging techniques, have been recently reviewed (Visioli, D'Egidio and Sanangelantoni 2014, Jones *et al.* 2015). These efforts will enable a better plant selection and management of the plant microbiome to increase phytoextraction efficiency.

While studies have been undertaken to identify the plant genetic trait loci that are associated with trace element uptake, translocation and tolerance (Ishikawa, Ae and Yano 2005, Payne *et al.* 2007, Zhang *et al.* 2008), so far, no specific breeding programs have been developed that evaluate cultivars for their interaction with the rhizosphere microbiome. Currently, our knowledge of specific plant phenotypic traits and root exudates *in situ* is still too limited to provide specific targets that can be used in plant breeding programs to optimise phytoextraction. To this end, integrated plant-microbiome studies are essential by providing novel gene targets that can be exploited to enhance beneficial associations of plants with microorganisms. This is one of the important information gaps that still remain, and can be addressed using studies that employ reductionist and holistic approaches to phytoremediation.

3.5 New generation of biostimulants

As stated in Kamilova and de Bruyne (2013), the European market for biostimulants reached 500 million Euro in 2013 of which a part consists of microbes, and the area of use in Europe is estimated at 3 million ha. In this, the total area of brownfields has not yet been taken into consideration. Biostimulants that are based on microorganisms are also increasingly applied in phytoremediation. Several successful applications of biodegradative microbial biostimulants have been described (Zhuang *et al.* 2007, Weyens, van der Lelie, Taghavi *et al.* 2009, Segura and Ramos 2013, Sessitsch, Kuffner, Kidd *et al.* 2013, Afzal, Khan and Sessitsch 2014, Arslan *et al.* 2015). However, these biostimulants did not always fulfill the expectations, were less effective than their chemical counterparts, and were often not commercially attractive enough for further development and implementation, calling the need for an intensification of fundamental research in this area (Goldstein, Mallory and Alexander 1985, Thompson *et al.* 2005, van Dillewijn *et al.* 2007). Understanding the responses of specific microbes at the community level will greatly facilitate the rational design of a new generation of biological biostimulants (**Table 2**). Also

timing, frequency, mode of application, and length of the intervals between multiple applications are being tested in field cases (Afzal *et al.* 2013), as well as the influence of biotic and abiotic factors (indigenous microbes, soil type) that determine the success of biostimulant application (Afzal *et al.* 2011, Trabelsi and Mhamdi 2013, Schmidt *et al.* 2014).

4. Implementation in an integrated soil restoration program

In many cases, phytoremediation will be used in integrated soil clean-up programs, in so-called 'treatment trains', which is especially useful in cases where mixed contaminants necessitate the use of more than one technique to effectively remediate sites (**Figure 1**). Efficient functioning of plant-microbial communities is critical to plant health, growth and phytoremediation. In this respect, the proper coupling of phytoremediation with other soil treatments is needed and this implies that many variables and multifactorial interactions need to be considered and studied (**Figure 1**). Site homogenisation by tillage has frequently been associated with decreased microbial biomass, and mixed effects on bacterial diversity (Feng *et al.* 2003, Helgason, Walley and Germida 2010), which could be attributed to a reduction in the number of available niches as soil is homogenised. In addition, the natural role of mycorrhizal fungi has been marginalised through intensive land-use, as microbial communities have been modified due to high inputs of inorganic fertilisers, herbicides and pesticides (Johansson, Paul and Finlay 2004). Overall, the potential effects of soil pre-treatments (*e.g.* soil washing, excavation, soil vapor extraction, thermal treatment, etc.) and co-treatment (*e.g.* addition of oxidoreductants, nutrients, crop rotation, invasive species control, afforestation, etc.) on plant-microbiome functioning has to be thoroughly scrutinised (**Figure 1**).

5. Promoting trends, future perspectives and conclusion

The incentives for soil remediation are numerous and varied, and phytoremediation continues to develop and alter under influence of the macro-environmental climate: governmental policy concerning pollutant control and legislation, environmental programs, research funding, biodiversity topics, demand for clean soils, biomass for bioenergy and climate nexus.

Phytoremediation, energy generation and environmental remediation as a whole are tightly coupled to microbial physiology. As has been demonstrated for humans and their microbiome, plants have evolved a deep connection with microbes for nearly all aspects of life

including detoxification of contaminants and supporting plant growth and fitness. Different soils as well as different environmental stresses (*e.g.* mixed contaminants, nutrient deficiency) each trigger separate plant-species-dependent physiological responses. Moreover, the control of contaminants in and on plant environments most likely require a different subset of microorganisms from the surrounding environment (Uhlik, Jecna, Mackova *et al.* 2009). Hence there is a need for optimisation of the soil-plant microbial system on a site-by-site basis. Thereby it is important to take into account that plant species (cultivar) selection is an important tool to change microbial communities, which can significantly impact phytoremediation outcome, and therefore advances in the knowledge of specific plant-microbe interactions are essential.

The demand for reduced levels of toxic compounds in the environment and crops, the rules for land owners, high costs of physical and chemical soil treatments, and sustainable land use, all favour phytoremediation. While excavation probably remains the method of choice for rapid soil clean-up, phytoremediation is a valuable alternative in situations where time is less urgent, for vast contaminated areas and when pollutant concentrations allow plant growth (Vangronsveld, Herzig, Weyens *et al.* 2009). Scientific and technological innovations, as described in this review, create a flow of new ideas and applications. Already many new insights have been obtained by studies that implement -omics technologies in a goal-driven way (**Table 1**). To summarise, (i) it is clear that plant selection is far more important than previously thought, because of the linkage between plant phylogeny and microbial taxa which can be altered under contamination, (ii) the initial colonisation of plants by soil fungi seems to be critical, and (iii) the degree to which plants exert control over microbiota depends, amongst others, on the contaminant concentration. In the years to come, some of the outstanding questions in phytoremediation (**Box 3)** may be answered. Powerful statistical tools will be indispensable to deal with larger datasets and overcome the inherent technical and bioinformatic difficulties, to reveal the underlying microbiological mechanisms behind efficient operations of natural and engineered processes (Prosser 2015).

As the perception of phytoremediation is crucial to the adoption of the technique, it is also important from an economic view, that companies invest in education of environmental experts. Channel partners in the governmental instances are important for the integration of

phytoremediation, and international research programs (EU-FP7 project GREENLAND, COST Action 859, amongst others) are relevant for the start-up and the long-term maintenance of projects, closing the gap between lab and field (Cichocka *et al.* 2010, Mench, Lepp, Bert *et al.* 2010).

Persistent historic pollutants and new emergent pollutants such as pharmaceutical and personal care products continue to occur in the biosphere, and the lack of economically feasible remediation techniques, offers opportunities for phytoremediation. Examples in Belgium are the vast area of predominantly agricultural land (over 300 km^2) enriched with toxic concentrations of trace elements like cadmium, zinc, and lead originating from emissions from the metal smelting industry, which can be restored using short-rotation coppice of willow and poplar (Janssen *et al.* 2015). Progress has already been made to increase the reliability and efficacy of phytoremediation. As a result, several companies are interested in adopting phytoremediation in soil clean-up programs, indicating that phytoremediation is a serious trend in pollution control today (Stephenson and Black 2014).

The market for phytotechnologies is expected to grow during the coming years. The demand for clean soils, contaminant-residue-free crops, the lower costs, and turning remediation into a profitable outcome (bioenergy, biochar, feedstock, etc.) are the main social and economic incentives for phytoremediation. In particular, a growth in the use of microbial endophytes for the degradation of contaminants with a long residence time *in planta* (*e.g.* BTEX, TCE) and rhizoremediation of hydrocarbon contaminants may be anticipated. During the last few years, we have seen impressive advances and new opportunities in the development of molecular tools to monitor and manipulate microbial communities, increasing the efficacy and predictability in phytoremediation systems. This review intends to inform and inspire research and application of phytotechnologies and to contribute to these exciting endeavors in the years to come.

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Table 1. Contaminants, research goals, and representative plant-microbe -omics-based applications and the main findings to offering translation into phytotechnologies

Contaminant	Main goal	Applications and main findings
Hydrocarbons, mineral oil, diesel, PAH[$]	Identifying 'novel' hydrocarbon degrading strains	SIP-coupled metagenomics applied to contaminated soil to identify hydrocarbon metabolising strains in a culture-independent way (Uhlik *et al.* 2012, Rodgers-Vieira *et al.* 2015). Pigmentiphaga were newly associated with naphthalene and phenanthrene degradation, and yet to be cultured γ-Proteobacteria were associated with pyrene and benz[a]anthracene metabolism (Jones, Crandell, Singleton *et al.* 2011).
	Understanding the effect of plant root exudates on biodegradation	Studies using SIP-labeled substrates performed to elucidate the role of root exudates in hydrocarbon degradation (Sun *et al.* 2010, Cebron *et al.* 2011, Xie *et al.* 2012, Shukla *et al.* 2013, Page, Yergeau and Greer 2015). Ryegrass root exudates did no enhance naphthalene dissipation (Cebron, Louvel, Faure *et al.* 2011). Wildrye root exudates were less repressive on hydrocarbon mineralisation than alfalfa root exudates (Phillips *et al.* 2012).
	Characterising the relation between microbial diversity and diesel degradation	Antibiotics were added to soil microcosms and inhibited subsets of the microbial community, this increased diesel degradation when no additional nutrients were added (Bell *et al.* 2013).
	Effect of nutrient addition on	Hydrocarbon degradation with and without the addition of mono-ammonium phosphate was predictable by initial bacterial diversity, and a high abundance of specific

	microbial response	assemblages of Betaproteobacteria (Bell *et al.* 2013).
	Microbial taxonomy composition to predict hydrocarbon degradation rate?	Establishing predictive relationships between microbial community structure (using NGS) and biotransformation rates facilitates monitoring and follow-up of shifting microbial activities over time, and allows to optimise phytoremediation as contaminant composition changes (Chikere, Okpokwasili and Chikere 2011). Shifts in the chemical composition of diesel constituents and the abundance of specific operational taxonomic units (OTUs), as investigated by pyrosequencing, suggested that natural attenuation has occurred at a diesel-contaminated railway site (Sutton *et al.* 2013)
PCBs[*]	Identifying 'novel' biphenyl degrading strains and functional genes	Using SIP-based approaches to identify biphenyl metabolising strains (Leigh, Pellizari, Uhlik *et al.* 2007, Sul *et al.* 2009, Uhlik, Jecna, Mackova *et al.* 2009), which may be important to stimulate. Planting of horseradish resulted in different bacteria that participate in the metabolism of biphenyl, compared to the initial uncultivated bulk soil (Uhlik, Jecna, Mackova *et al.* 2009).
	Understanding the effect of plant secondary metabolites (flavonoids) on biodegradation	Studies using SIP-labeled substrates to elucidate the role of root exudates in PCB congener degradation (Slater, Gouin and Leigh 2011, Toussaint, Pham, Barriault *et al.* 2012, Uhlik *et al.* 2013, Vrchotova, Lovecka, Drazkova *et al.* 2013, Pham, Pino Rodriguez, Hijri *et al.* 2015), for rhizoengineering applications. From a screening, flavone and isoflavone were among the better inducers of the biphenyl catabolic enzymes in *Rhodococcus erythropolis*

		U23A (Pham, Pino Rodriguez, Hijri *et al.* 2015).
	Insight in *bph*-gene organisation	Bacterial genome sequences are used to enhance insights in the gene organisation, diversity, distribution and evolution of *bph* genes in PCB-degrading bacteria (Denef *et al.* 2006, Parnell *et al.* 2010, Chang *et al.* 2015, Suenaga *et al.* 2015), and can be used for genetic engineering applications.
TCE, BTEX[$$]	Effect of re-introduction of engineered bacterial endophytes	Studies to determine the efficacy of engineered endophytes to phytodegradation. Poplar cuttings inoculated with the engineered root endophyte *P. putida* W619-TCE equipped with nickel (Ni) resistance elements, was found to increase root biomass, enhance Ni uptake and reduce TCE-phytotoxicity and evapotranspiration compared to non-inoculated plants (Weyens, Beckers, Schellingen *et al.* 2015).
	Mining for genes that determine endophytic life	Comparative genomic analyses have identified many genetic factors that determine endophytic colonisation of plants and sustaining bacterial life within the host plant (Mitter *et al.* 2013, Ali *et al.* 2014), as well as the life of obligate endobacteria in AM-fungal mycelia networks (Hoffman and Arnold 2010, Ghignone *et al.* 2012) and their role in biodegradation (Wick, Furuno and Harms 2010). This has implications for inoculant development.
	Mining for the presence of plasmids with catabolic genes	Metagenomic mining of the 'mobilome' (*i.e.* the preferential extraction of plasmid DNA) to access the ongoing horizontal gene transfer (Jorgensen, Kiil, Hansen *et al.* 2014).

Trace elements[**]	Identifying the contribution of the plant microbiome to phytoextraction	Studies using taxonomical and functional metagenomic analyses to untangle specific functions of the plant microbiome in trace element phytoextraction (Mirete, de Figueras and Gonzalez-Pastor 2007, Stefanowicz, Niklinska and Laskowski 2008, Xiong, Wu, Tu *et al.* 2010, Hassan *et al.* 2011, Op De Beeck *et al.* 2014, Bell, Cloutier-Hurteau, Al-Otaibi *et al.* 2015, Muehe, Weigold, Adaktylou *et al.* 2015). Initial recruitment of the rhizosphere microbiome was important for Zn uptake by willow on contaminated soil (Bell, Cloutier-Hurteau, Al-Otaibi *et al.* 2015). Predominance of the AM-fungi *Glomus mossae* in the rhizosphere of *Plantago major* suggests its potential use for phytoremediation of trace elements (Hassan, Boon, St-Arnaud *et al.* 2011).
	Understanding the interaction between inoculant and host plant	Study using multi-facetted approaches to obtain insights in single microbe-plant interactions *in situ*, for enhancing trace element phytoextraction (Li, Pidatala, Shaik *et al.* 2014).

[$]The refining of crude oil, storage, distribution, large-scale use and applications have led in many countries to environmental contamination (Gallego *et al.* 2001, Singh and Jain 2003, Van Hamme, Singh and Ward 2003). About 435.000 underground storage tanks with petroleum hydrocarbons have leaked in the United States (Kansas State University, 2005) and nearly 1.5 million underground storage tanks have been closed since 1984, but a large number of sites are still in need of remediation. In Europe, mineral oil and trace elements are major contaminants, contributing around 60 % to soil contamination (Panagos, Van Liedekerke, Yigini *et al.* 2013). Hydrocarbons occur in various forms, but the most common are the petroleum hydrocarbons which include n-alkanes and other aliphatics, (polycyclic) aromatic compounds and minor

constituents (Zhang *et al.* 2005). Many hydrocarbon compounds are toxic to humans, plants, animals, and microorganisms (DeLaune *et al.* 1990, Tang *et al.* 2011).

[*]Polychlorinated biphenyls represent poorly water-soluble, highly persistent and toxic pollutants in the biosphere (Harris 1993, Carpenter 1998). Mid and highly halogenated PCBs are rather recalcitrant toward bacterial degradation and thus they tend to accumulate in the environment (Mackova, Prouzova, Stursa *et al.* 2009, Passatore *et al.* 2014).

[$$]Highly water-soluble and volatile compounds such as benzene, toluene, ethylene, xylene (BTEX) and trichloroethylene (TCE) compounds are toxic environmental pollutants causing serious environmental problems (Barac, Taghavi, Borremans *et al.* 2004).

[**]Due to past and present activities of the hydrometallurgical industry, mining operations, agricultural practices and other sources, a vast area of land is contaminated with trace elements including cadmium, zinc, lead, copper, nickel, arsenic the most frequently found, posing a significant problem in many countries (Vangronsveld, Van Assche and Clijsters 1995, Van Meirvenne *et al.* 2008, Panagos, Van Liedekerke, Yigini *et al.* 2013, Grison 2015). Trace elements are persistent and not biodegradable and moreover, tend to get increasingly concentrated in living organisms along the food chain. The environmental problem inevitably affects public health when humans are being exposed to trace element pollution (Hogervorst *et al.* 2007).

Table 2. List of critical aspects for the development of microbial biostimulants

Criteria	Assessment of the criteria
Candidate microbes	
PGPR and PGPE	Degradative PGPR and PGPE are attractive candidates for use in biostimulants (Barret, Morrissey and O'Gara 2011, Guttman, McHardy and Schulze-Lefert 2014, Teng *et al.* 2015). PGPR that are able to overcome the selective pressure in the rhizosphere, can act beneficial on plant growth through innate PGP-properties and/or disease control (Lugtenberg and Kamilova 2009).
Ideal biostimulant	
Easy	The formulation should be easy to handle and apply
Persistent	Persistent at the plant/soil interface
Efficient	High efficacy, thereby yielding reproducible results
Safe	No undesired side effects, not causing human health problems, animals, or plants. Not affecting biodiversity of concomitant microbial communities
Low-cost	Economically feasible for the applicant (Kamilova and de Bruyne 2013)
Manufacturing	Processes optimisation to enhance stability of the microbes, *e.g.* the use of physical carriers (alginate beads), physical protection, or using spore-forming or desiccant-tolerant microbes (Vilchez and Manzanera 2011, Malusa, Sas-Paszt and Ciesielska 2012, Berg *et al.* 2013).
Inoculation	
Threshold concentration	Cell-number of 10^6 - 10^8 per plant/seed is recommended, however, this needs to be optimized and tested (Thijs *et al.* 2014).

Technique	Seed coating, soil drench, placement of the inoculant in the seed furrow, rhizosphere, foliar inoculation. Optimization and testing are needed (Afzal, Khan, Iqbal *et al.* 2013).
Environmental factors	Understanding, at least in part, the external factors that influence biodegradation efficiency of the inoculant (Afzal, Yousaf, Reichenauer *et al.* 2011, Trabelsi and Mhamdi 2013).
How to improve efficacy?	
Colonisation	A 'conditio sine qua non' for beneficial interactions is the ability of the microorganisms to competitively colonize the roots, show chemotaxis movement towards roots, and in some cases enter the roots (endophytes) (Segura and Ramos 2013).
Understanding mechanisms	Selecting PGP microbes and understanding, at least in part, the mechanism acting between the PGP and host plant, co-evolution, linkage between plants and microbial taxa (Hoeksema 2010, Loper *et al.* 2012, Bell, El-Din Hassan, Lauron-Moreau *et al.* 2014).
Consortia	The use of consortia of complementary microbes that act synergistically, occupy different niches, co-exist and do no show potential negative interactions, have shown to be more efficient under some conditions (Mikeskova, Novotny and Svobodova 2012, Thijs *et al.* 2014, Teng, Wang, Li *et al.* 2015).
Mycorrhizal helper bacteria	Biodegradative bacteria which promote rapid colonisation by ecto-and endomycorrhizal fungi might be double beneficial (enhancing nutrient mobilization from soil minerals, regulating water house holding, contaminant degradation, protection of plants against root pathogens) (Tarkka and Frey-Klett 2008).

|	
Endophytes	The use of endophytes, which colonise the internal plant tissues, and do not have to compete with the large diversity and abundance of microbes in soil, is very promising (Rylott 2014, Ijaz, Imran, Anwar ul Haq *et al.* 2015).
GMO inoculants and alternatives	The use of GMO PGPB in inocula is refused by the public in Europe, and in many other countries worldwide. Solutions come from the use of natural plasmids and natural gene transfer, microbes that can be 'domesticated' and conditioned to *e.g.* higher chlorinated PCB's (Taghavi, Barac, Greenberg *et al.* 2005, Hernandez-Sanchez, Lang and Wittich 2013).
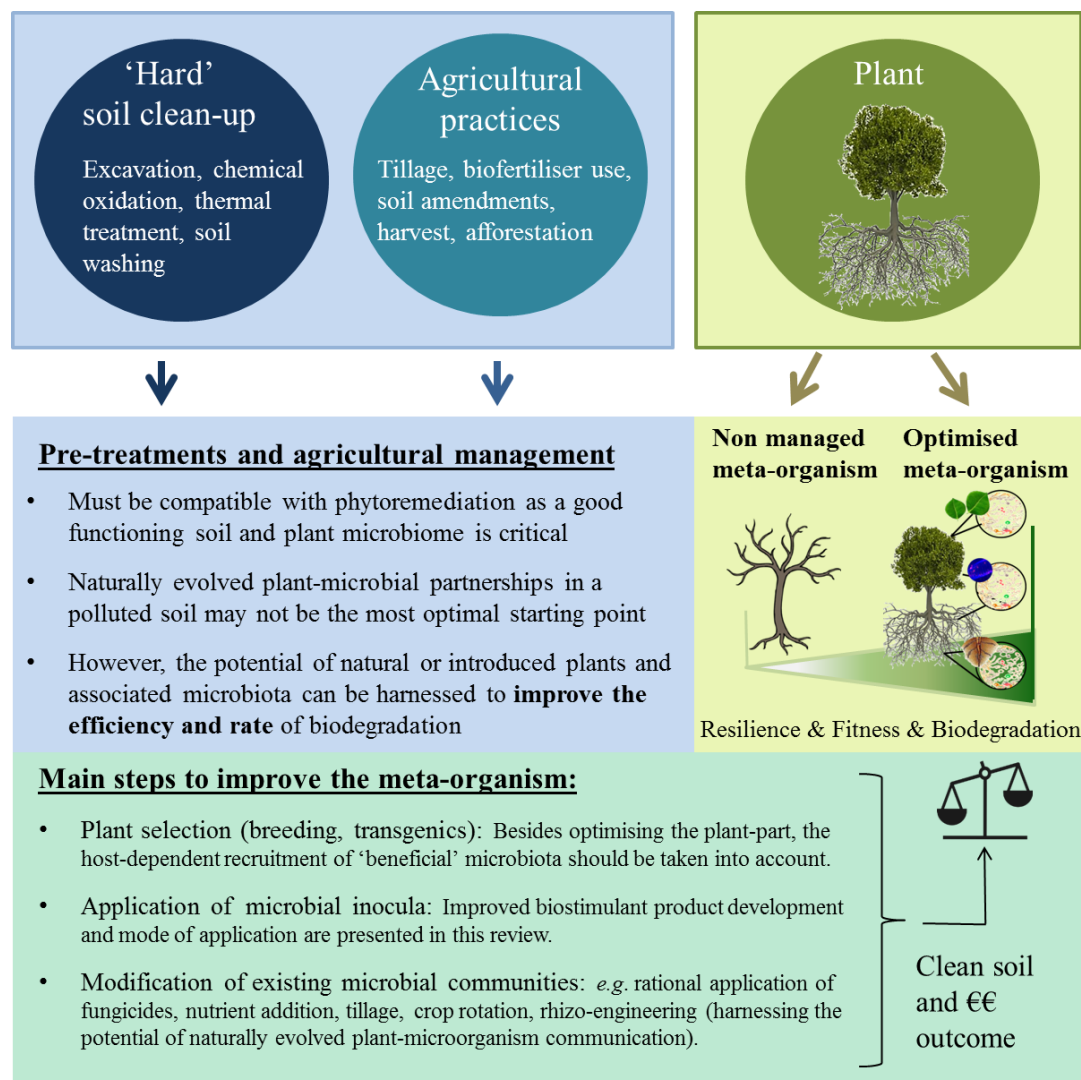
Figure 1. Scheme showing the integration of phytoremediation in soil clean-up treatment trains and optimisation of the plant-microbiome. Identification of the limiting factors to natural attenuation and overview of different approaches (*e.g.* rational plant selection, and microbiome engineering) to turn the plant from a potential low productivity state to a high-productivity, diverse, and resilient state with high phytoremediation activity.

Box 1. Plant and microbial contributions during phytoremediation

Plants contribute to contaminant removal through various ways including pollutant uptake, accumulation, degradation and detoxification, sequestration, stabilisation, volatilisation, and exerting effects on bioavailability of contaminants, soil properties, and microbial activity (Sandermann 1994). Different biotic and abiotic factors determine the uptake of pollutants as has been described in a review by Pilon-Smits *et al.* 2005.
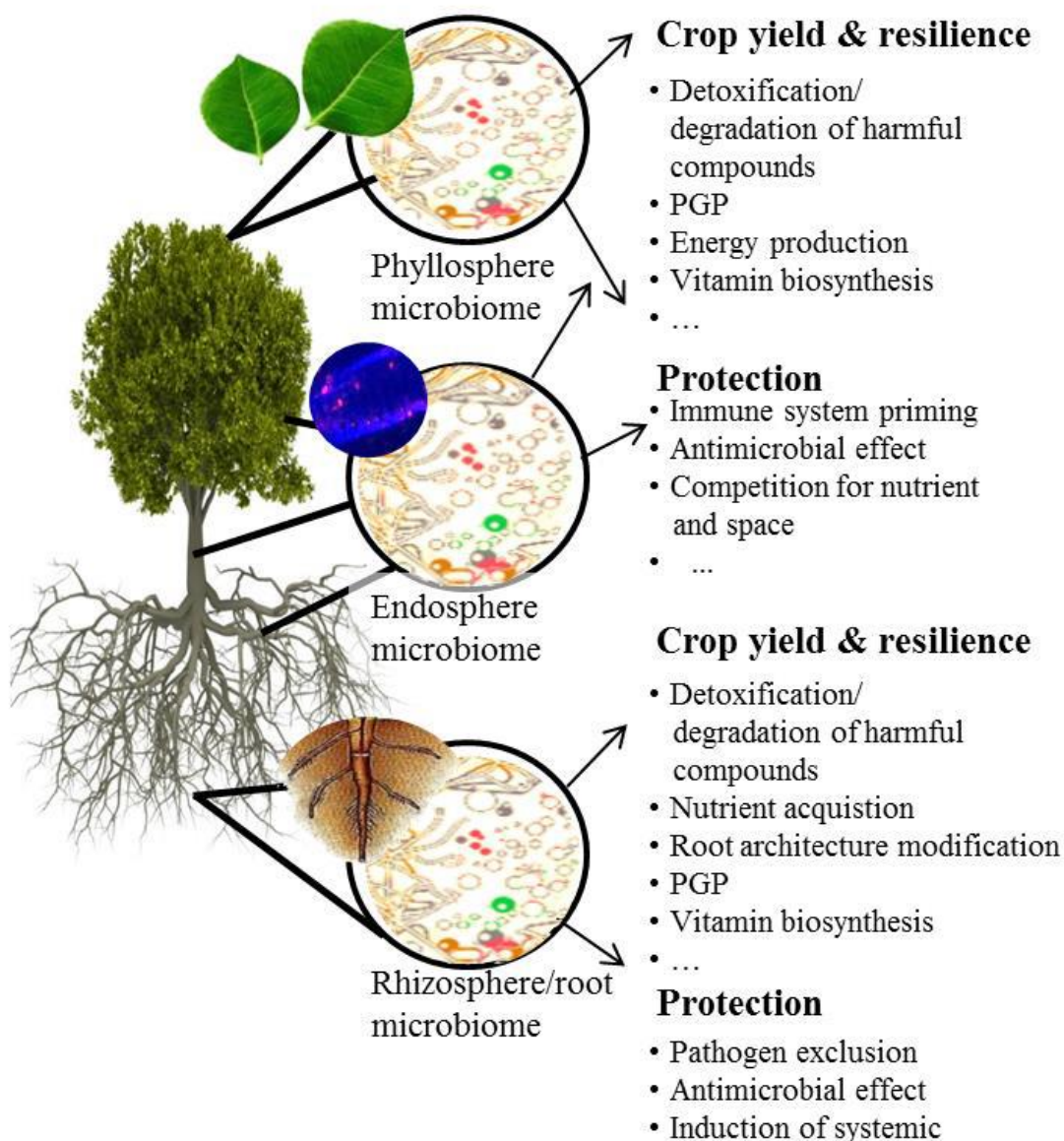
Plants are colonised by microorganisms in cell densities that are far greater than the number of plant cells (Mendes, Garbeva and Raaijmakers 2013). This plant secondary genome, or microbiome is important for numerous physiological functions (Berg 2009, Berendsen, Pieterse and Bakker 2012, Bakker, Berendsen, Doornbos *et al.* 2013, Mitter *et al.* 2013). Some of the microbial mediated functions that enhance beneficial outcome include nutrient cycling, soil organic matter mineralisation, plant-growth promotion (PGP), induction of disease resistance, and response to abiotic stresses.

An essential supportive role of microorganisms involves the degradation and detoxification of xenobiotic compounds in soil (Barea, Azcon and Azcon-Aguilar 2002). This activity may be enhanced in the plant rhizosphere as the plant provides abundant resources (Kuiper *et al.* 2004, Newman and Reynolds 2004, Wenzel 2008, Gerhardt *et al.* 2009). In addition, microorganisms that reside within plant tissue (endophytes) (Porras-Alfaro and Bayman 2011, Gaiero, McCall, Thompson *et al.* 2013), or on aerial plant parts (phyllosphere) (Vorholt 2012) can help to stabilise and/or transform contaminants that have been translocated, which may reduce toxicity and the extent of volatilisation of pollutants to the environment (Weyens, van der Lelie, Taghavi *et al.* 2009, Zhu, Ni, Liu *et al.* 2014). In addition, many plant-associated bacteria (PGPR, PGPE) can improve plant growth and health through their innate PGP-activities such as nutrient solubilisation, metal chelation, nitrogen fixation, production of plant-growth promotion hormones, counteracting stress responses (*e.g.* via 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production), providing a physical and biochemical barrier against pathogens in soil, or by assisting the plant's defense response against pathogenic invasions (Lugtenberg and Kamilova 2009, Glick 2010, Glick 2012).

Box 2. Management of microbial communities in wastewater treatment plants (WWTP)

Advances in network-based modeling (Ruan *et al.* 2006, Finley, Broadbelt and Hatzimanikatis 2009) posed interestingly novel insights into complex ecological and biological associations in WWTP systems (Johnson *et al.* 2014, Ju and Zhang 2014, Ju and Zhang 2015, Ju and Zhang 2015).Using time-series of 16S rRNA data of WWTPs, it was found that biological interactions were the main driver of bacterial community assembly, whereas environmental conditions (*e.g.* sludge retention time and inorganic nitrogen) partly explained phylogenetic and functional variances and indirectly influenced the bacterial community assembly (Ju *et al.* 2014). Taxonomically closely-related taxa tend to co-occur, while negative co-excluding correlations were observed between taxonomically less related species (Ju, Xia, Guo *et al.* 2014), a phenomenon also found in soil bacterial communities (Philippot *et al.* 2010, Goberna *et al.* 2014), which implies a role of competition and habitat filtering in determining bacterial assembly in aquatic and soil environments.

From an engineer's perspective, these observations provide powerful approaches to perturbing biological systems, to allow for selective enrichment of beneficial species and elimination of detrimental microorganisms (Helbling *et al.* 2015), which has applications not only in bioreactors operated for waste and wastewater treatment (Vanwonterghem *et al.* 2014) but also in higher complexity communities in the plant rhizosphere. In addition, network analyses showed great utility in identifying bacteria-bacteria interactions for the biotransformation of novel pharmaceuticals (isoproturon, propachlor, ranitidine, venlafaxine) in wastewater, a significant problem in many countries for which traditional clean-up strategies are not well suited (Helbling, Johnson, Lee *et al.* 2015). Overall, these studies nicely illustrate the merging of screening and design approaches to the generation of more effective and predictable phytotechnologies.

Box 3. Outstanding research questions

(1) The plant-microbiome is considered a wealthy resource of microbial diversity, though, the majority of plant species used in phytoremediation (hyperaccumulators, willow, poplar, grass species) have not yet been studied for their associated microbial communities. Given the diversity of microorganisms in soil and the plant-environment the opportunity to find beneficial plant-microbial partnerships is considerable. Omics coupled with high-throughput isolation (Nichols *et al.* 2010, Stewart 2012) and screening of microbes and their microbial characteristics (nutrition, competition, growth rate), will help in determining the potential activity of microbes and identifying those strains that should be targeted in phytoremediation.

(2) Despite some very definite progress in the description of plant microbiomes, more practical studies are needed to address the process leading from acknowledgement of the occurrence of distinct gene clusters and catalytic activities in single genomes, all the way to comprehending interaction networks, manipulating and management of microbial communities in and on plants. In this context, metagenomic analyses and comparison of plant-associated communities of different cultivars can lead to novel functional insights. An interesting example of this, is the detection of phylotype-linkage between fungal lineages and willow cultivars, grown in hydrocarbon contaminated soil (Bell, El-Din Hassan, Lauron-Moreau *et al.* 2014). Functional analyses will reveal whether plants can benefit from their associated microbiome for plant fitness and tolerance against abiotic stresses.

(3) Although sequencing of phylogenetic markers (*e.g.* 16S rRNA gene fragments) provides valuable insights in the dominant plant colonising strains, too much emphasis on phylogeny may under-evaluate the potential functional variation. Examining function, and the 'mobilome' should be an important component of phytoremediation research.

(4) Although we focused in this review on the bacterial aspect of pollutant degradation in phytoremediation, we opt for a future integration of fungal-bacterial interactions, specifically mycorrhiza.

(5) In the future, the plant microbiome will have a greater importance in plant breeding and engineering strategies in phytoremediation. For now, only plant pathogens were considered

in these approaches. If we know the genetic basis of plants to interact with the microbiome as a whole, then we can use these functional traits as targets in novel plant breeding programs.

(6) Meta-organism approaches should be further adopted in which results from both the plant and microbiota are integrated. Currently, the number of studies exploring interactions via multifaceted -omic techniques is limited. A meta-organism approach is quite challenging due to cost, set-up, training, requirement for labs and machines. Interdisciplinary research and collaboration is necessary between plant physiologists and microbiologists to reach this inspiring goal.

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