

Bioaugmentation of soils by increasing microbial richness: missing links

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Summary

It is generally assumed that increased microbial diversity corresponds to increased catabolic potential and, hence, to better removal of metabolites and pollutants. Yet, microbial diversity, more specifically richness of species in environmental samples and sites, is difficult to assess. It is proposed to interpret this diversity more in the framework of Pareto's law, i.e. 20% of the species govern 80% of the energy flux of the ecosystem. Ecological studies should attempt to delineate the main energy fluxes and that group of species playing quantitative key roles in the system. Consequently, bioaugmentation should aim at the rearrangement of the group of organisms dominantly involved in the overall energy flux, so that specific catabolic traits necessary for the clean up of pollutants are part of that active group. For soil ecosystems, the capacity of plant roots as creators of physical and chemical discontinuity should be used more strategically to bring about such rearrangements. Overall, this paper identifies a number of ecological concepts, such as the Pareto law, the Gompertz model and plant community-induced microbial competence, which may, given careful underpinning, open new perspectives for microbial ecology and biodegradation.

Introduction

At present, several studies focus on improving the bioremoval capacity of soil by inoculating specific strains or consortia; the latter is commonly labelled as bioaugmentation (Alexander, 1994). This approach corresponds to increasing the metabolic capabilities of the microbiota present in the soil. Such an increase is actually the result of an enlargement of the genetic capacity present at that site. In that respect, bioaugmentation corresponds to an increase in the gene pool and, thus, the genetic diversity

of that site. In principle, this genetic diversity could be increased by augmenting the microbial diversity. Diversity is a function of two components: (i) the total number of species present, known as species richness or species abundance; and (ii) the distribution of individuals among those species, known as species evenness or equitability. In microbial ecology, in view of the difficulty of estimating the different biomass levels of each species, diversity often relates to species richness. Yet, in order to increase the degradative potential of a microbial community, one does not necessarily need a diversity of microbial species or genes, but the right competence for certain actions under the given conditions. That competence refers to the capability of important genes to be activated and, thus, to be participating in the energy flux of the system. Thus far, the aspects of what amount of energy is driving the community and what metabolic traits are required to trap this energy are rarely or never taken into consideration when dealing with soil bioaugmentation studies.

In this article, different approaches to measure and to increase the microbial richness of soil will be discussed in relation to the hypothesis that only a minor part of the organisms consumes a major part of the energy flow.

The current emphasis of biodiversity of soil systems

Over the last century, the richness component of diversity has been estimated by a variety of methods such as plating, plain and fluorescence microscopy and, recently, DNA and RNA analysis (Fig. 1). However, opinions diverge. Torsvik *et al.* (1990) reported ≈ 4000 different genome equivalents g^{-1} soil, which suggests that there are perhaps 1000 or even more species g^{-1} soil; Truper (1992) suggested that 2–3 million bacterial species might exist in the biosphere.

For years, plating (cfu) on different media was the technique for investigating microbial diversity. However, the relative proportion of bacteria growing on agar plates to those counted by fluorescence microscopy varies from 0.1–1% in pristine forest soils to 10% in environments such as arable soil. This implies that investigations based on bacterial isolates may include only a minor part of the total bacterial diversity (Amann *et al.*, 1995). In the last 10 years, molecular techniques have allowed the investigation of bacterial communities without culturing, which gives a more reliable view, particularly of the richness

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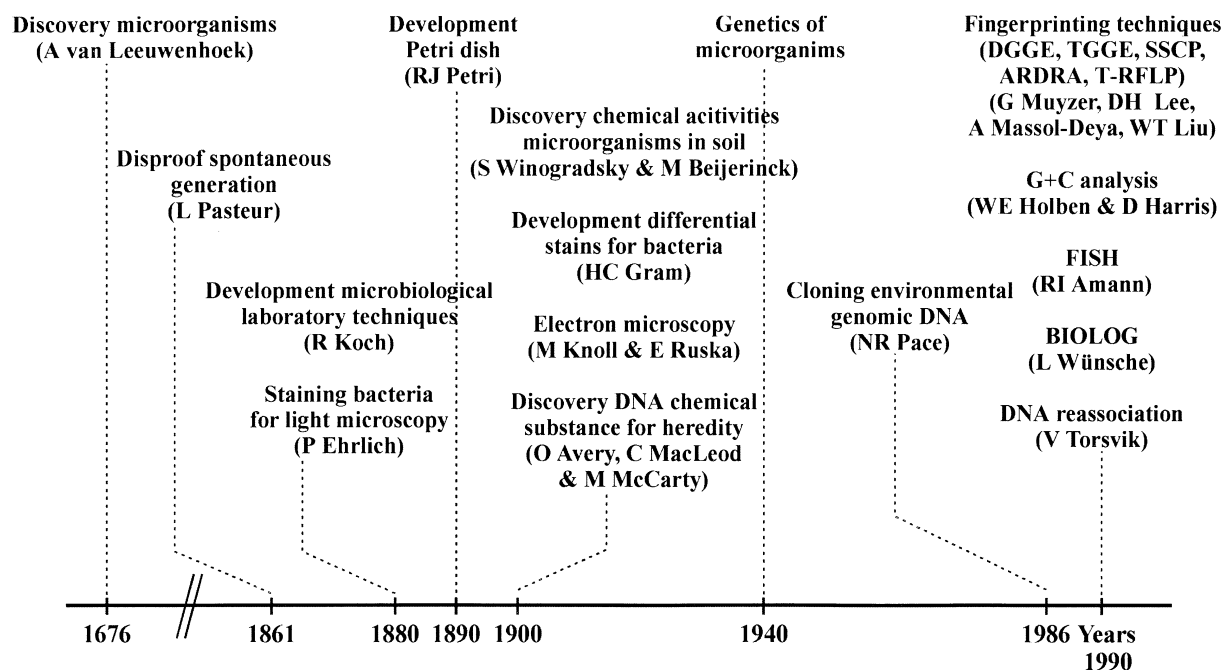


Fig. 1. Timescale of the most important events in microbiology (Pelczar *et al.*, 1993).

component of diversity (Pickup, 1991; Stackebrandt *et al.*, 1993; Amann *et al.*, 1995; Holben and Harris, 1995). A first attempt to study the culturable but also unculturable species of ecosystems was made by cloning random fragments of environmental genomic DNA and then sequencing clones containing rRNA genes (Pace *et al.*, 1986). Because this technique is very laborious, several researchers started to use polymerase chain reaction (PCR) to amplify rRNA genes selectively from total microbial community DNA. This technique uses different primer sets to amplify the ribosomal genes of all types of organisms (Archaea, Bacteria or Eukarya) present in an environmental sample. The PCR fragments obtained can then be cloned and sequenced (Amann *et al.*, 1995; Hugenholtz and Pace, 1996), or separated and visualized by fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993). In DGGE and TGGE (thermal gradient gel electrophoresis), double-stranded DNA fragments with the same length but different basepair sequence, obtained after PCR of rRNA genes, are separated in a denaturant or thermal gradient polyacrylamide gel respectively (Muyzer *et al.*, 1993). Very familiar also is the single-strand conformation polymorphism (SSCP) method, in which single strands of PCR-amplified rRNA genes with the same length but different conformational structure are separated in a polyacrylamide gel (Lee *et al.*, 1996). Another more recently developed molecular technique for rapid analysis of microbial community diversity is called terminal restriction fragment length polymorphism (T-RFLP). It uses PCR

of 16S rRNA genes, in which one of the two primers used is fluorescently labelled. The PCR product is subsequently digested with restriction enzymes, and the fluorescently labelled terminal restriction fragment is measured precisely using an automated DNA sequencer (Liu *et al.*, 1997). With these techniques, a pattern of bands is obtained in which, in theory, each band corresponds to one particular species or 'ribotype'. In this way, an idea of the microbial richness of an ecosystem is obtained in addition to information about changes in the gross community structure. Another DNA fingerprinting technique that is also based on PCR amplification of rRNA genes and in combination with restriction of the amplified fragments is amplified ribosomal DNA restriction analysis (ARDRA) (Massol-Deya *et al.*, 1995). This technique seems to give too many bands per species to provide reliable and robust genotypic characterization at the community level, but is feasible for monitoring specific populations in microbial communities and for assessing the diversity of bacterial isolates and cloned genes (Torsvik *et al.*, 1998). As the microbial diversity of soil is so large, it may be better to focus on specific subgroups of microorganisms rather than on the total rRNA gene pool. Examples of subgroups that have been analysed in this way are methylotrophic members of the γ - and α -Proteobacteria (Henckel *et al.*, 1999), α - and β -Proteobacteria (Gomes *et al.*, 2001), actinomycetes (Heuer *et al.*, 1997), ammonia-oxidizing bacteria (Kowalchuk *et al.*, 1998), *Acidobacterium* (Barns *et al.*, 1999), Archaea (Øvreas *et al.*, 1997) and fungi

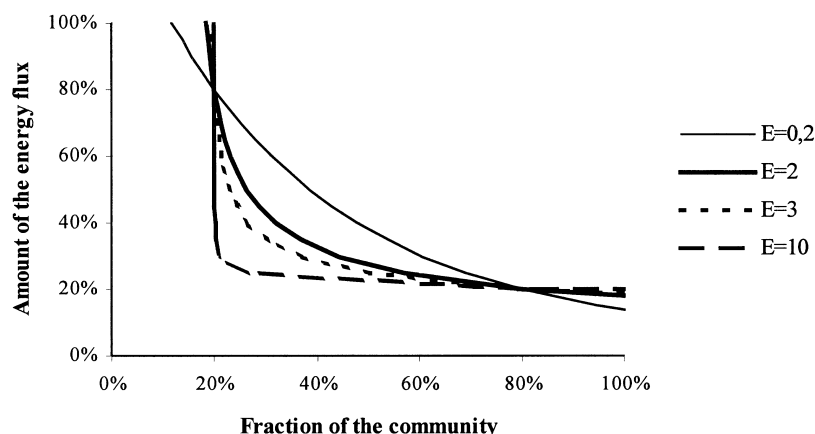


Fig. 2. Representation of different Pareto curves [energy flux = $1/(\text{fraction of the community})^E$] (Pareto, 1897). According to Pareto, about 80% of all energy flux is governed by about 20% of the species in a community. By changing environmental conditions, one could try to decrease E (see $E = 0.2$), so that the energy flux is more evenly distributed over the community. In this way, suppressed bacteria could become active and fulfil an important role in the community.

(Smit *et al.*, 1999). Extraction of RNA instead of DNA, followed by reverse transcription-PCR (RT-PCR), gives a picture of the metabolically active microorganisms in the system (Weller and Ward, 1989; Felske and Akkermans, 1998; Nogales *et al.*, 1999). In the same way, fluorescent *in situ* hybridization (FISH), in which fluorescent oligonucleotides target rRNA sequences (Amann *et al.*, 1990), community phospholipid fatty acid analyses (PLFA) (Zelles *et al.*, 1994) and *in situ* PCR (Hodson *et al.*, 1995) are also used to study the active microbiological component of a soil. Community-level physiological profiles that are based on response patterns of environmental samples inoculated into each of the 96 wells of a Biolog plate (Garland and Mills, 1991; Wünsche *et al.*, 1995; El Fantroussi *et al.*, 1999a) and the measurement of short-term respiration responses of microbial communities to the addition of simple substrates (= *in situ* catabolic potential) (Degens, 1998) can give an estimation of the functional diversity of a soil. To get an idea of the total genetic diversity of bacterial communities, base composition profiles expressed as mol percentage guanine + cytosine (%G+C) (Holben and Harris, 1995; Torsvik *et al.*, 1996; Nüsslein and Tiedje, 1998) and DNA reassociation and the so-called 'C₀t curves' are used (Britten and Kohne, 1968; Torsvik *et al.*, 1998). In this last technique, the reassociation time is a measure of the genetic diversity in a sample, as the more different genomes there are in a sample, the slower the DNA reassociation occurs after initial denaturation.

Yet, all these methods do not necessarily reveal the organisms largely involved in the mainstream energy flux of the ecosystem. To achieve this, the molecular ecology studies should be complemented with metabolic mass balance studies. As a matter of fact, systemic microbial ecologists focusing on overall soil processes have suggested that only a few organisms (10–20) are directly significant at a particular site (Greenwood, 1968; Hattori, 1973). For example, in the rhizoplane and rhizosphere of

established tea bushes, two *Bacillus* species, i.e. *Bacillus subtilis* and *Bacillus mycoides*, comprised the major part of the bacterial community during favourable and unfavourable conditions (Pandey and Palni, 1997). Clearly, this aspect about how many species are controlling the major part of the energy flux at a site needs to be assessed by current molecular methods on the one hand and by detailed studies of metabolic conversions on the other hand. At this point, one should take the so-called Pareto law into consideration (Pareto, 1897; Buchanan, 2000). Pareto's law is a model used in economics, which states that the number of individuals with wealth W is proportional to $1/W^E$. The exponent is always between 2 and 3, so that 20% of the people own 80% of the wealth. Pareto's law is also valuable in biological sciences. Overall population growth in a periodic environment follows the Pareto distribution (Dimitrov *et al.*, 1998). In bioinformatics, where protein and DNA data are processed, analogues to Pareto's law are used (Jain and Ramakumar, 1999). Even in toxicological tests, the exponential and the Pareto models are considered to be the true underlying models to determine distribution for the tolerance concentrations (LD₅₀) of a population (Moermans and Nelis, 1994). It is meaningful, in the context of this paper, to look at that part of the microbial community that controls 80% of the metabolic flux. The bottom line is that one should try to designate the main metabolic processes and, concomitantly, try to see whether a species detected in a particular ecosystem belongs to the determinative 20% or to the subsisting 80% of the community. A short list of methods suited to quantify temporal variation of those genomes controlling 80% of the energy flux should be developed. Particularly promising in this respect are the mRNA-based techniques (Fleming *et al.*, 1998) and proteomics (Hecker and Engelmann, 2000), as both methods focus on the expression of catabolic genes in active organisms. Pelz *et al.* (1999) observed through immunocapture and

isotopic ratio mass spectrometry that, in a four-member 4-chlorosalicylate-degrading consortium, a *Pseudomonas* sp. strain can degrade the primary substrate and takes 80% of the carbon, whereas the other three strains take the remaining 20%. This *Pseudomonas* sp. strain also makes up 80% of the community, resulting in low biodiversity. It thus appears worthwhile to explore methods of modifying the composition of the ruling fraction so that other species might participate in the major conversion processes (Fig. 2). The latter could alter the overall degradation capacity of the soil ecosystem. To delineate the energy-yielding processes in soil systems and link them to a diverse array of genomes that governs these processes is a challenge for the next decade of microbial ecology.

Improved biodegradation by augmenting species richness

Diversity can be enhanced by adding a strain or a mixture of strains to the soil. According to the concept of the carrying capacity of microbial communities, the added populations stabilize at 10^3 cfu g⁻¹ soil (Gompertz, 1825; Corman *et al.*, 1987; Crozat *et al.*, 1987; Postma *et al.*, 1990; Vandenhove *et al.*, 1991; Vandepitte *et al.*, 1995). This number appears to be independent of the ecosystem used (Fig. 3). This concept needs to be examined for more cases before it can be generalized. Nevertheless, according to estimates based on the Gompertz growth kinetic (Gompertz, 1825), the addition of any species capable of living under the given environmental conditions will give rise to increased biodiversity of the soil for days to months. In addition, several tests with the seeding of strains have had a positive effect on the degradation of certain compounds in soil (Table 1). Hence, inoculation is a valuable approach to broadening the biodegradation potential of soil.

A variant is not to focus on the inoculant but on genetic information that can be transferred from an introduced donor strain to the well-established and competitive indigenous bacterial populations of soil (Table 2). An advantage of this approach is its independence of the long-term survival of the introduced donor strain, which is often the major bottleneck in bioaugmentation processes (Akkermans, 1994).

A third approach (often applied but undeservedly not highly regarded) is the introduction of an unspecified group of bacteria such as those present in soil, sludge, manure or compost. Barbeau *et al.* (1997) used a pentachlorophenol (PCP)-acclimated activated soil biomass produced in a mixed soil slurry bioreactor and a flocculent biomass obtained from an air-lift immobilized soil bioreactor to bioaugment a PCP-impacted sandy soil successfully. Under anaerobic conditions, Mikesell and

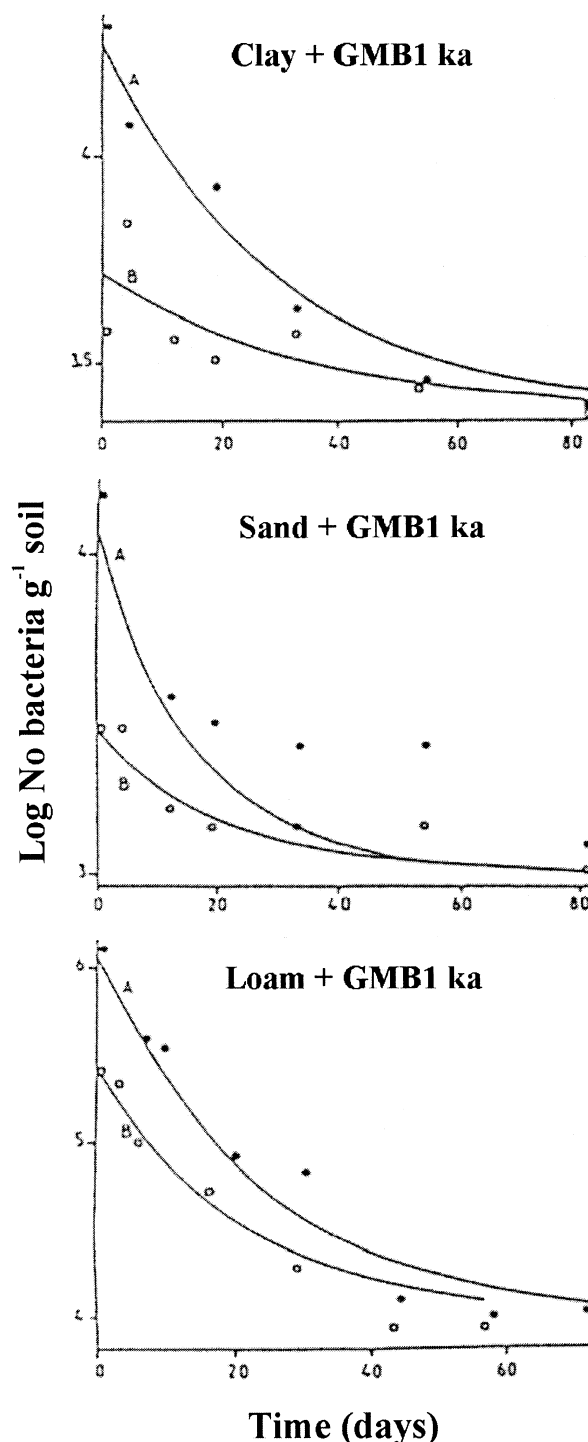


Fig. 3. Survival kinetics (experimental data and fitted curves) of *Bradyrhizobium japonicum* GMB1 ka in three soils (clay, sand and loam) according to the Gompertz model (Gompertz, 1825). For each soil, two inoculation levels are shown (A and B). The introduced bacteria attain a carrying capacity of 10^3 – 10^4 cfu g⁻¹ soil (Corman *et al.*, 1987).

Table 1. Cases of successful use of inoculants for the bioaugmentation of polluted soils.

Inoculant	Degraded compound	Reference
<i>Arthrobacter</i> sp.	Isopropyl <i>N</i> -phenylcarbamate (IPC) and isopropyl <i>N</i> -3-chlorophenylcarbamate (CIPC)	Clark and Wright (1970)
<i>Pseudomonas stutzeri</i> and <i>Pseudomonas aeruginosa</i>	Parathion	Barles <i>et al.</i> (1979)
<i>Pseudomonas cepacia</i>	2,4,5-Trichlorophenoxyacetic acid	Kilbane <i>et al.</i> (1983)
<i>Flavobacterium</i> sp.	Pentachlorophenol	Crawford and Mohn (1985)
<i>Pseudomonas aeruginosa</i>	Tetramethylthiuram disulphide	Shirkot and Gupta (1985)
Active anaerobic sewage sludge	Pentachlorophenol	Mikesell and Boyd (1988)
<i>Pseudomonas cepacia</i> BRI6001	2,4-Dichlorophenoxyacetic acid	Comeau <i>et al.</i> (1993)
<i>Pseudomonas fluorescens</i>	2,5-Dichlorobenzoate	Crowley <i>et al.</i> (1996)
Consortium	Atrazine	Alvey and Crowley (1996)
Consortium	Atrazine	Grigg <i>et al.</i> (1997)
<i>Pseudomonas</i> sp. strain ADP	Atrazine	Shapir and Mandelbaum (1997)
Activated soil	Pentachlorophenol	Barbeau <i>et al.</i> (1997)
<i>Alcaligenes eutrophus</i> TCP	2,4,6-Trichlorophenol	Andreoni <i>et al.</i> (1998)
<i>Desulfotobacterium frappieri</i> PCP-1	Pentachlorophenol	Beaudet <i>et al.</i> (1998)
<i>Agrobacterium radiobacter</i> J14a	Atrazine	Struthers <i>et al.</i> (1998)
Consortium	Atrazine	Newcombe and Crowley (1999)
<i>Ralstonia eutropha</i> JMP134	2,4-Dichlorophenoxyacetic acid	Daane and Häggblom (1999)
<i>Pseudomonas</i> sp. strain P51	1,2,4-Trichlorobenzene	Tchelet <i>et al.</i> (1999)
<i>Pseudomonas pseudoalcaligenes</i> POB310 (pPOB)	3-Phenoxybenzoic acid	Halden <i>et al.</i> (1999)
<i>Sphingomonas paucimobilis</i> EPA505	Polycyclic aromatic hydrocarbons	Straube <i>et al.</i> (1999)
<i>Desulfomonile tiedjei</i>	3-Chlorobenzoate	El Fantroussi <i>et al.</i> (1999b)
<i>Escherichia coli</i> (pAtzA)	Atrazine	Strong <i>et al.</i> (2000)
<i>Arthrobacter</i> sp. strain B1B and <i>Ralstonia eutrophus</i> H850	Polychlorinated biphenyl	Singer <i>et al.</i> (2000)
<i>Arthrobacter</i> RP17	Phenanthrene	Schwartz <i>et al.</i> (2000)
<i>Ralstonia basilensis</i> RK1	2,6-Dichlorophenol	Steinle <i>et al.</i> (2000)
<i>Ralstonia eutropha</i> JMP134	2,4-Dichlorophenoxyacetic acid	Roane <i>et al.</i> (2001)

Boyd (1988) enhanced the degradation of pentachlorophenol in soil using biologically active anaerobic sewage sludge, previously shown to dechlorinate chlorophenols. The results clearly demonstrated that the dechlorinating activity present in sludge could be transferred to soil through bioaugmentation. These microbial associations may also be used as such, without adaptation to the pollutant. Indeed, these ecosystems normally contain such a high diversity of microorganisms that the species necessary to destroy the pollutant may be present in these inocula or an interaction (at the catabolic and genetic level) between different microorganisms may result in improved removal of the contaminant. Moreover, in such samples, the co-operating species might be spatially in optimal configuration relative to one another (see below). An interesting inoculum for this strategy is

the soil or sludge from river banks. In the stream water, different and time-varying pollutants are present (Guimaraes *et al.*, 2000). These microbial habitats come into contact with different pollutants, potentially increasing and varying with time the catabolic potentials of the bacteria that are present (Admiraal *et al.*, 2000). By this approach, the difficult task of isolating and characterizing a certain bacterium able to degrade a specific compound or the time needed to adapt the bacterial community to a specific chemical can be diminished in the bioaugmentation process.

Overall, it appears that there are different strategies for increasing the (catabolic) diversity of soil, ranging from adding specific strains, specific mobile genetic elements, to adapted microbial associations and highly diverse microbial communities generated by nature. The key

Table 2. Cases of successful use of gene transfer for the bioaugmentation of polluted soils.

Gene or plasmid	Degraded compound	Reference
pFL40	2,2-Dichloropropionate	Brokamp and Schmidt (1991)
RP4::Tn4371	Biphenyl	De Rore <i>et al.</i> (1994)
pJP4	2,4-Dichlorophenoxyacetic acid	DiGiovanni <i>et al.</i> (1996)
pEMT1 and pEMT3	2,4-Dichlorophenoxyacetic acid	Top <i>et al.</i> (1998; 1999)
pJP4	2,4-Dichlorophenoxyacetic acid	Newby <i>et al.</i> (2000a,b)
pJP4 and pEMT1	2,4-Dichlorophenoxyacetic acid	Dejonghe <i>et al.</i> (2000)

question is how to add these strains or microbial associations in a way that achieves a positive effect. In other words, the genomes introduced should be competent to participate in the main energy flux processes. In studies published so far, little attention is given to the aspects of how these allochthonous organisms are positioned in relation to the autochthonous microbiota, particularly in capturing an important part of the overall energy influx. An important approach might be to evaluate the population levels relative to the dynamics proposed by the Gompertz model (Gompertz, 1825), for which molecular methods are very enabling tools. Populations with prolonged biomass levels above the postulated 10^3 cfu g⁻¹ subsistence value could signal a participation in the mainstream of the overall energy flux. This certainly needs further validation.

Soil heterogeneity and microbial competence

Soil can be divided into an assemblage of sites offering different conditions of growth, leading to structural heterogeneity. Species diversity can be maintained if a site is capable of supporting similar numbers of individuals for each of the different species present in the community. If this is not possible, then the type that is best adapted to the most productive niche (task) will become dominant in the community, and diversity will be low. In a recent study, Kassen *et al.* (2000) found a strong unimodal relationship for *Pseudomonas fluorescens* species between diversity and productivity in heterogeneous, but not in homogeneous, environments.

The spatial heterogeneity of the soil can be increased by the root system of plants (Angers and Caron, 1998) and the digging of invertebrates (Lavelle *et al.*, 1997). Owing to the movement of roots and mesofauna through the soil, new niches are created enhancing the chance that certain bacteria can establish in the soil. In addition, the burrows of earthworms contribute to macroporosity and so influence water infiltration and aeration (Lee and Foster, 1991; Devliegher and Verstraete, 1997). Water infiltration can facilitate the movement of bacteria through the soil, whereas aeration can stimulate the growth of certain bacteria, resulting in disturbance of the microbial balance in soil. It can be appropriate to use mixed instead of monoplant cultures to promote microbial diversity (Nüsslein and Tiedje, 1999). For example, different plants have roots with different structures or that move to different depths in the soil, disturbing the soil system in a different way (Angers and Caron, 1998). Different plants also excrete different metabolites and deposit different residues and thus constitute food sources for different groups of bacteria (Westover *et al.*, 1997; Jones, 1998; Griffiths *et al.*, 1999; Yang and Crowley, 2000). Thus, growing a diverse community of plants in a soil brings

about spatial heterogeneity, which may correspond with multiple bioaugmentation phenomena 'from inside'. Indeed, the different plants will generate different microbial populations actively involved in the metabolism of their respective inputs to the soil. Clearly, a key question is whether the proposed line of multiplant rhizoremediation is more effective than the current, often applied practice of monoculture rhizoremediation (Macek *et al.*, 2000).

Need for research

Molecular methods have shown that soil microbial communities have a very high species richness. Yet, it has not been established whether Pareto's law is also applicable in these systems. This aspect warrants in-depth studies because microbial ecology needs to be able to differentiate the populations governing the energy fluxes in the soil system.

The introduction of allochthonous organisms or genes is an effective way of increasing species richness and has also been shown to be effective in terms of soil depollution. To make such additions more effective, it is necessary to examine whether the species exceed or just attain the subsistence population levels, as represented by the Gompertz model (Gompertz, 1825). A better understanding of these population levels and kinetics is needed to judge the competence of an added catabolic trait.

Finally, the concept of using higher plants and biota as generators of soil heterogeneity, and thus as providers of variable (micro)sites and concomitantly more niches for soil microorganisms, deserves to be explored effectively. The fact that an increase in structural heterogeneity of a soil would result in increased species richness, coupled to an augmented purification capacity of the soil, needs to be corroborated.

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