The tritrophic trinity: a source of pollutant-degrading enzymes and its implications for phytoremediation

Andrew C Singer*, Ian P Thompson and Mark J Bailey

Barring bioavailability and nutritional limitations, virtually all organic anthropogenic chemicals can be naturally biodegraded. It is to this phenomenon we owe thanks to the long established 'tritrophic trinity' of microbe–plant–insect interactions. Over hundreds of millennia these organisms have coevolved, producing hundreds of thousands of different chemicals that are used to attract, defend, antagonize, monitor and misdirect one another. In comparison, the numbers of truly novel chemicals of anthropogenic origin are negligible. It is only now that we are beginning to appreciate the fortuitous evolution of xenobiotic-degrading enzymes from these interactions. We argue that success in phytoremediation can be hastened through understanding the structure, sources, uses and targets of these secondary metabolites. Owing to recent developments in molecular biology, particularly stable isotope probing, we eagerly anticipate highly significant insights into trophic interactions, particularly in the rhizosphere, providing phytoremediation with a solid mechanistic understanding.

Addresses

Centre for Ecology & Hydrology – Oxford, Mansfield Road, Oxford, OX1 3SR, United Kingdom

*e-mail: acsi@ceh.ac.uk

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Abbreviations

gfp green-fluorescent protein
PAH polycyclic aromatic hydrocarbon
PCB polychlorinated biphenyl
SIP stable isotope probing
SPME secondary plant metabolite

Introduction

How do microbes biodegrade synthetic compounds to which they have had no prior exposure? This question has lingered in environmental microbiology circles for decades. In a recent review [1•], it was argued that the pollutant-degrading abilities found in microorganisms evolved from the continued supply of naturally created pollutant analogues, such as dioxins [2•], biphenyl [3], and volatile organic compounds [4•,5]. Moreover, one may argue that the world was always a polluted place,

with pollutant analogues constantly entering our environment from geothermal and volcanic activity [6], comets [7] and space dust [8], the latter of which can deliver to Earth approximately 100 t of organic dust per day. Hence, life itself, however it began, must have subsequently evolved amongst 'pollutants'. In this light, it is hardly surprising that pollutant-degrading bacteria are found in virtually every gram of soil on earth.

Geologically more recent pollutant analogues are delivered in the form of secondary plant metabolites (SPMEs). SPMEs are considered non-essential chemicals for the basic metabolic processes of the plant — typically derived from isoprenoid, phenylpropanoid, alkaloid or fatty acid/polyketide pathways [9]. Their roles are subject to debate (see Hadacek [10] for a review) and are referred to by a number of terms: allelopathy — chemicals which adversely impact on other (competing) plants [11,12], photosynthate — root exudates consisting of low-molecular weight sugars, amino acids and organic acids [13], phytohormones/phytoalexin — chemicals that regulate the protective responses of plants against both biotic and abiotic stresses [14], phytosiderophores — chemicals used in the acquisition of essential nutrients [13] and phytoanticipin — antimicrobial compounds [15].

In this review, we discuss how identifying the role of SPMEs in the rhizosphere can facilitate our understanding of the mechanics of phytoremediation. Relevant literature is presented as support for our hypothesis, and we also take this opportunity to speculate on exciting, promising avenues for further research in phytoremediation.

The tritrophic trinity

SPMEs provide a tool by which plants [12], microbes [16] and insects [17] can communicate, while developing and maintaining positive and negative facultative and obligate relationships with one another. These relationships are dynamic, as members of this tritrophy 'break the code' of mutualism, or 'make alliances' with alternate partners, resulting in unchecked gains for the rogue or cheater. To retain mutualism, members of this tritrophy must continually adapt — modifying 'the code' — to ensure fitness. This dynamic is arguably one of the driving forces behind the evolution of pollutant-degrading enzymes. This tritrophic source of pollutant-degrading enzymes is arguably the more actively evolving class of enzymes within the global network of suprametabolism, which represents all metabolic enzymes of microbial origin [18]. We argue that pollutant analogues (e.g. SPMEs), within the network of suprametabolism have important

Figure 1

Network of suprametabolism. Chemical compounds derived from plants, insects and microorganisms are represented by circles, the areas of which are proportional to the chemicals' molecular weight and hydrophobicity (i.e. larger circles represent relatively larger and more highly hydrophobic chemicals). Biodegradation steps are represented by arrows, where the widths are proportional to the antiquity of the catalysing enzyme (i.e. narrower arrows represent relatively more recently evolved metabolic enzymes). The average number of biodegradation reactions necessary for a chemical to reach central metabolism is 3.3. Red circles represent common chemical intermediates, whereas blue circles reflect different degrees of novel chemical structures. The more distant a chemical is from a central intermediate, the more inherently recalcitrant the chemical is likely to be — necessitating additional (potentially novel) biochemical steps to reach central metabolism. Adapted from Pazos *et al.* [18].

implications for predicting the fate of pollutants (Figure 1). Polychlorinated biphenyls (PCBs) were among the first pollutants to be definitively linked to enhanced xenobiotic degradation by SPMEs such as limonene, cymene, carvone and pinene [19,20], but only a few studies have since investigated the efficacy of SPMEs in the attenuation of other pollutants [1••].

Examining the meta-rhizosphere

Exciting advances in molecular biology have facilitated novel insight into trophic interactions and signalling mechanisms with immediate applications to phytoremediation. Casavant *et al.* [21••] used a *gfp*-labelled *P. fluorescens* A506 to detect low concentrations of toluene (0.2 μ m) and trichloroethylene in the rhizosphere. The authors demonstrated increased *gfp* expression in the root-colonising biosensor population when the plant rhizosphere was exposed to toluene. Critically, in the context of this review, the authors noted 14% more induced cells in uncontaminated rhizosphere soil than bulk soil, indicating the presence of natural inducers. The sugges-

tion that the *gfp* promoter for toluene was induced by SPMEs, was confirmed when it was shown to be induced by a variety of alkyl-substituted benzene derivatives and branched alkenes, all of which could be produced by barley roots. The authors also demonstrated that the promoter was sensitive to isoprene, a known inducer of recalcitrant pollutants [1••]. This technique could easily be used to screen plants for their potential to phyto-stimulate pollutant degradation. Plants that produce more SPMEs of the kind that induce a toluene biosensor might be highly applicable to phytoremediation of pollutant-contaminated soil.

Narasimhan *et al.* [22••] described a 'rhizosphere metabolomics' approach for phytoremediation similar to the 'field application vector' approach reported by Lajoie *et al.* [23], who engineered a PCB-degrading bacterium with the capacity to grow, *in situ*, on a selective carbon source. Narasimhan *et al.* [22••] engineered a microbial degrader to utilise the predominant root exudates of *Arabidopsis*. This nutritional bias provided the bacterium with a selective advantage in the *Arabidopsis* rhizosphere. Rhizosphere metabolomics was then used to identify root exudates that afforded a nutritional bias for the inoculum. Phenylpropanoids, chosen for their abundance in the rhizosphere, served as a sole carbon source for the inoculum, *Pseudomonas putida* PML2, which was *gfp*-tagged to facilitate its identification, location and cell division on the plant root. The authors also produced an auxotrophic mutant to ensure the isolate was dependent on the root exudates for growth and subsequently demonstrated that SPMEs were exuded in sufficient amounts to bias growth of the inoculum and enhance PCB degradation. This approach — rhizosphere metabolomics — is a highly instructive technique for understanding the role of SPMEs in the rhizosphere and provides a tool for engineering phytoremediation systems.

Kuiper *et al.* [24] provide a similarly instructive study where they use a bacterium isolated from the rhizosphere of a grass grown in polycyclic aromatic hydrocarbon (PAH)-polluted soil. The bacterium degraded the PAHs and, thus, protected the plant from the pollutant. The authors examined the root exudates of the host plant, which were found to be high in the sugars, glucose and fructose, and the organic acids, succinic acid and citric acid [25••]. Using a reporter mutant, they tested the exudates for their influence on the indigenous upper (*dox*) and lower (*nah*) naphthalene-degradation pathway genes. As observed by Narasimhan *et al.* [22••], the plant provided a growth substrate for the inoculum, which in turn exhibited elevated expression of the naphthalene-degrading genes.

The diversity of PAH-degrading genes has always been problematic for assessing the diversity and catabolic potential of a soil. Considerable effort has been invested

in isolating PAH degraders, however, increasingly, these studies find a poor correlation between genetic homology and function in respect to PAH degradation. Widada *et al.* [26] isolated nineteen naphthalene and phenanthrene degrading bacteria, consisting of at least seven genera, of which only 32% hybridised to the *phnAc* probe and none to the *pahAc* probe. These observations demonstrate the diversity of PAH-degrading bacterial enzymes. Moreover, the study indicated the need to investigate additional sources of PAH-degrading bacteria to provide a reliable indication of the diversity in nature. Given the potential for SPMEs to induce PAH-degradation, the rhizosphere is a likely source of this diversity.

Siciliano *et al.* [27], in a ground-breaking study, reported evidence for the ability of plants to selectively enhance the prevalence of pollutant-degrading endophytes. The authors demonstrated that alkane monooxygenases and naphthalene dioxygenase were 2–4 times more prevalent in endophytes than in the surrounding soils. A similar observation was made for three nitroaromatic degrading genes, which were 7–14 times more prevalent in endophytic bacteria. Of particular note, the prevalence of naphthalene dioxygenase-containing endophytes doubled upon spiking of the rhizosphere with petroleum, indicating that the population density of pollutant-degrading endophytes is positively correlated to the presence and concentration of contaminants in the soil. It could be argued that a population of microorganisms or pollutant-degrading pathways are actively maintained in the plant to facilitate the detoxification and removal of potentially harmful and recalcitrant pollutants within the transpiration stream (i.e., akin to the human liver). Owing to the notorious recalcitrance of many environmental bacteria to isolation in the laboratory, molecular methods will continue to prove invaluable in determining their roles and capabilities. Pollutant-degrading endophytes may become particularly relevant to phytoremediation when addressing contaminants such as trichloroethylene [28] and methyl *tert*-butyl ether [29], which can be routinely assimilated in the transpiration pathway of the plant. Genetic engineering of the endophyte to degrade a particularly recalcitrant pollutant may prove fruitful in response to the constant supply of a readily bioavailable selective carbon source (i.e. pollutant) and a low C:N growing medium (i.e. vascular system) [30^{••}]. It will be interesting to see how the role of endophytes in phytoremediation will be borne out in the years to come. We anticipate the avenue of endophyte-assisted phytoremediation will elevate the profile of phytoremediation in the near future as a viable solution to mobile and semi-mobile pollution problems.

Aken *et al.* [31] provided evidence of the biodegradative potential of a phytosymbiotic bacterium. *Methylobacterium* sp. strain BJ001 was isolated from a tissue culture and plantlets (*Populus deltoids* × *nigra* DN34), and its capacity

to transform 2,4,6-trinitrotoluene and mineralise hexahydro-1,3,5-trinitro-1,3,5-triazine and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine to CO₂, possibly via co-metabolism, was observed in pure culture. The authors suggested that the capacity to transform these compounds might be related to the ability of the isolate to metabolise C1 carbon substrates, which are frequently generated from nitramine degradation.

Application of technological advances

The past four years have seen the application of a number of technological advances, such as microarrays, proteomics and bioinformatics, for the study of microbial ecology, trophic interactions and biogeochemical and nutrient cycling. One of the most powerful of these new techniques is stable isotope probing (SIP), which relies on the incorporation of isotopically-labelled substrates such as fatty acids, DNA and RNA into the biomass of the active microorganisms. Following extraction of the labelled biomass, insight into the structure and identity of the active members of a microbial community can then be gained with unprecedented clarity and precision [32,33[•],34].

Butler *et al.* [35[•]] used ¹³C-CO₂ pulse labelling to enrich annual ryegrass (*Lolium multiflorum* Lam. Var. Gulf) with ¹³C-labelled photosynthate. The microbial populations actively involved in cycling rhizodeposition were identified through the analysis of ¹³C-labelled phospholipid fatty acids. However, because many phospholipid fatty acids are ubiquitous to all organisms (e.g. 16:0), the approach was limited to qualifying gross changes in microbial communities. Ostle *et al.* [36], also used the ¹³C-CO₂ pulse labelling technique to measure the incorporation of recently assimilated plant carbon into soil microbial RNA and DNA pools. This study confirmed the rapid transfer of photosynthate carbon into rhizosphere microorganisms. The incorporation of ¹³C-CO₂ into photosynthate by the plant, exudation into the rhizosphere and incorporation into biomass and respiration into ¹³C-CO₂ took as little as 5 h. Johnson *et al.* [37], demonstrated the same efficient conversion of photosynthate into ¹³C-CO₂ by arbuscular mycorrhizal symbionts in a pasture plant where arbuscular mycorrhizae mycelium respired ¹³C-CO₂ within 9 hrs of the pulse-labelling. A novel study by Bruneau *et al.* [38^{••}] incorporated the pulse-labelled ¹³C-CO₂ technique with a laser ablation stable isotope ratio mass spectrometer to determine the fate of photosynthate in both the root and the rhizosphere soil. The authors clearly demonstrated rapid translocation of the photosynthate to the roots, which remained in the rhizosphere for more than four weeks. The authors noted, however, that the contribution of photosynthate to the soil carbon pool was negligible as compared to the size of the initial soil carbon pool, yielding no significant change to the mean value of bulk δ¹³C (‰) values. Therefore, while this approach has outstanding promise, studies will need to address the detection limit problem through, for

example, the use of artificially low carbon soils and earlier timepoints (e.g. hours to a few days). With ever increasing detection limits, honed technique, and creative experimental designs, stable isotope labelling will no doubt provide exciting and intriguing insights into the black box of microbial ecology and trophic interactions.

Conclusions: optimism for phytoremediation

Advances in phytoremediation will be enhanced by a more thorough understanding of the dynamic mechanisms of the tritrophic trinity. This will be facilitated through recent developments and novel applications in molecular biology (e.g. SIP, metagenomics, proteomics, microarray and marked organisms) [39–41], mobile pollutant-degrading functions (e.g. plasmids and integrative- and conjugative-elements) [42] and biodegradation chemical signatures such as isotopic fractionation (^{13}C : ^{12}C) and enantioselective degradation [43,44]. Although beyond the scope of this review, synthetic and phytosurfactants [45,46] warrant further investigation for their ability to modify the soil chemical parameters, thereby increasing bioavailability and degradation [47]. Developments in traditional plant breeding and engineering will soon enable the selection of plants that exude a phyto-

surfactant which is compatible with a soil pollutant, thereby facilitating phytoremediation without the costs and potential hazards of synthetic surfactants. In light of these exciting developments, we are optimistic of the realisable potential of phytoremediation and its development as a discipline (Figure 2).

Update

Recent work by Barac *et al.* [48^{••}], conclusively demonstrates the contribution of an endophyte to the remediation of toluene, particularly that which was contained within the transpiration stream of the plant. The authors tested three bacteria for their capacity to aid in toluene degradation. The first bacteria, *Bacillus cepacia* BU0072, was an engineered derivative of *B. cepacia* L.S.2.4, a natural endophyte of yellow lupine. The second bacteria was a transconjugant of strain BU0072, *B. cepacia* strain VM1330, containing the toluene degrading gene (pTOM). The third bacteria, *B. cepacia* G4 (pTOM, TOI⁺), is a soil bacterium that had also been used as the donor strain for the toluene degradation plasmid found in strain VM1330. After applying the isolates to surface sterilized yellow lupine seeds, the resulting plantlets were assessed for their ability to resist increasing

Figure 2

Comparative research interest in environmental microbiology-related disciplines (1988–2003). An electronic literature search (ISI Web of Science, e.g. search 'Phytoremediation') was carried out within the following four temporal periods: 1988–91, 1992–95, 1996–99, 2000–03, to quantify the publications containing the keyword 'phytoremediation'. Additional keywords were quantified for comparative purposes based on their relevance to modern environmental microbiology and phytoremediation, these were: antimicrobial, antisense, biofilm, bioinformatics, biosensor, bioremediation, microarray, microbial ecology, phytoextraction and proteomics. The total publications within a field are indicative of its research base, it is also symptomatic of its applicability to medicine. Hence, we have normalized the publication trends between disciplines by assessing the % change in total published papers from one temporal period to the next. The resulting series indicates 6 of the 11 disciplines with a >90% increase in publications from the 1996–99 to 2000–03 temporal period: phytoremediation (90%), microbial ecology (122%), bioinformatics (237%), phytoextraction (313%), proteomics (696%), and microarray (2252%)—half of which are technology-based disciplines. We estimated the total publications for the 2004–07 period by linear or binomial regression ($R^2 > 0.9$; Figure 2). The extrapolations indicate a 77–78% increase in publications for phytoremediation and phytoextraction, followed closely by bioinformatics (76%), proteomics (75%), microarray (74%) and microbial ecology (65%). These technology-driven disciplines will provide the fine focus adjustment and higher power objectives enabling research to target and better understand the biological mechanisms underlying phytoremediation.

toluene concentrations and degrade toluene dissolved in the transpiration stream within hydroponic and non-sterile soil systems. The authors found that strain VM1330 was capable of protecting its host against the phytotoxic effects of toluene, while also lowering the emissions of toluene from the transpiration stream. The other two bacteria were significantly less successful on both accounts. The authors anticipate the application of engineered endophytes will become a general strategy for the remediation of water-soluble organic pollutants.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Singer AC, Crowley DE, Thompson IP: **Secondary plant metabolites in phytoremediation and biotransformation.** *Trends Biotechnol* 2003, **21**:123-130.
- Highly relevant synthesis of the literature in which secondary plant metabolites are investigated for their induction of xenobiotic-degrading microorganisms.
2. Meharg AA, Killham K: **A pre-industrial source of dioxins and furans.** *Nature* 2003, **421**:909-910.
- Demonstrates that dioxins and furans, by-products of organochlorine production and inefficient burning of organic carbon in the presence of chlorine, also originate from anthropogenic burning of peat.
3. Liu B, Beuerle T, Klundt T, Beerhues L: **Biphenyl synthase from yeast-extract-treated cell cultures of *Sorbus aucuparia*.** *Planta* 2004, **218**:492-496.
4. Hamilton JTG, McRoberts WC, Keppler F, Kalin RM, Harper DB: **Chloride methylation by plant pectin: an efficient environmentally significant process.** *Science* 2003, **301**:206-209.
- Recent evidence of abiotic conversion of chloride to methylchloride within senescent or dead plant material — a highly relevant environmental pollutant involved in stratospheric ozone depletion.
5. Baraldi R, Rapparini F, Oechel WC, Hastings SJ, Bryant P, Cheng Y, Miglietta F: **Monoterpene emission responses to elevated CO₂ in a Mediterranean-type ecosystem.** *New Phytol* 2004, **161**:17-21.
6. Zolotov MY, Shock E: **A thermodynamic assessment of the potential synthesis of condensed hydrocarbons during cooling and dilution of volcanic gases.** *J Geophys Res* 2000, **105**:539-559.
7. Greenberg J: **Cosmic dust and our origins.** *Surf Sci* 2002, **500**:793-822.
8. Marciano V, Benitez P, Palacios-Pru E: **Acyclic hydrocarbon environments > n-C18 on the early terrestrial planets.** *Planetary and Space Science* 2003, **51**:159-166.
9. Dixon R: **Natural products and plant disease resistance.** *Nature* 2001, **411**:843-847.
10. Hadacek F: **Secondary metabolites as plant traits: current assessment and future perspectives.** *Crit Rev Plant Sci* 2002, **21**:273-322.
11. Bais HP, Vepachedu R, Gilroy S, Callaway RM, Vivanco JM: **Allelopathy and exotic plant invasion: from molecules and genes to species interactions.** *Science* 2003, **301**:1377-1380.
12. Bais HP, Park S-W, Weir TL, Callaway RM, Vivanco JM: **How plants communicate using the underground information superhighway.** *Trends Plant Sci* 2004, **9**:26-32.
13. Gleba D, Borisjuk NV, Borisjuk LG, Kneer R, Poulev A, Skarzhinskaya M, Dushenkov S, Logendra S, Gleba YY, Raskin I: **Use of plant roots for phytoremediation and molecular farming.** *Proc Natl Acad Sci USA* 1999, **96**:5973-5977.
14. Schmelz EA, Engelberth J, Alborn HT, O'Donnell P, Sammons M, Toshima H, Tumlinson JH III: **Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants.** *Proc Natl Acad Sci* 2003, **100**:10552-10557.
15. Wittstock U, Gershenzon J: **Constitutive plant toxins and their role in defence against herbivores and pathogens.** *Curr Opin Plant Biol* 2002, **5**:300-307.
16. Challis GL, Hopwood DA: **Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species.** *Proc Natl Acad Sci* 2003, **100**:14555-14561.
17. Li W, Schuler MA, Berenbaum MR: **Diversification of furanocoumarin-metabolizing cytochrome P450 monooxygenases in two papilionids: specificity and substrate encounter rate.** *Proc Natl Acad Sci* 2003, **100**:14593-14598.
18. Pazos F, Valencia A, Lorenzo VD: **The organization of the microbial biodegradation network from a system-biology perspective.** *EMBO reports* 2003, **4**:1-6.
19. Gilbert ES, Crowley DE: **Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. strain B1B.** *Appl Environ Microbiol* 1997, **63**:1933-1938.
20. Kim BH, Oh ET, So JS, Ahn Y, Koh SC: **Plant terpene-induced expression of multiple aromatic ring hydroxylation oxygenase genes in *Rhodococcus* sp. strain T104.** *J Microbiol* 2003, **41**:349-352.
21. Casavant NC, Thompson D, Beattie GA, Phillips GJ, Halverson LJ: **Use of a site-specific recombination-based biosensor for detecting bioavailable toluene and related compounds on roots.** *Environ Microbiol* 2003, **5**:238-249.
- The authors provide evidence of the utility of a *gfp*-marked microbial biosensor to detect toluene-like secondary plant metabolites, *in situ*.
22. Narasimhan K, Basheer C, Bajic VB, Swarup S: **Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls.** *Plant Physiol* 2003, **132**:146-153.
- A highly relevant study in plant-microbe interactions. Provides conclusive evidence for the role of secondary plant metabolites in stimulating and sustaining microbially-mediated pollutant degradation in the rhizosphere.
23. Lajoie CA, Layton AC, Sayler GS: **Cometabolic oxidation of polychlorinated biphenyls in soil with a surfactant-based field application vector.** *Appl Environ Microbiol* 1994, **60**:2826-2833.
24. Kuiper I, Bloemberg GV, Lugtenberg BJJ: **Selection of a plant-bacterium pair as a novel tool for rhizostimulation of polycyclic aromatic hydrocarbon-degrading bacteria.** *Mol Plant-Microbe Interact* 2001, **14**:1197-1205.
25. Kuiper I, Kravchenko LV, Bloemberg GV, Lugtenberg BJJ: ***Pseudomonas putida* strain PCL 1444, selected for efficient root colonization and naphthalene degradation, effectively utilizes root exudate components.** *Mol Plant-Microbe Interact* 2002, **15**:734-741.
- This study exemplifies an optimised plant-microbe system. The plant provides photosynthate for the growth of a root-colonising microorganism, which in turn is induced to degrade the soil contaminant and protect the plant from the pollutant's phytotoxic effects.
26. Widada J, Nojiri H, Kasuga K, Yoshida T, Habe H, Omori T: **Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites.** *Appl Microbiol Biotechnol* 2002, **58**:202-209.
27. Siciliano S, Fortin N, Mihoc A, Wisse G, Labelle S, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks MK *et al.*: **Selection of specific endophytic bacterial genotypes by plants in response to soil contamination.** *Appl Environ Microbiol* 2001, **67**:2469-2475.
28. Ma X, Burken JG: **TCE diffusion to the atmosphere in phytoremediation applications.** *Environ Sci Technol* 2003, **37**:2534-2539.

29. Hong MS, Farmayan WF, Dortch IJ, Chiang CY, McMillan SK, Schnoor JL: **Phytoremediation of MTBE from a groundwater plume.** *Environ Sci Technol* 2001, **35**:1231-1239.
30. Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mergeay M, van der Lelie D: **Endophytic bacteria and their potential applications.** *Crit Rev Plant Sci* 2002, **21**:583-606.
An excellent review of a fascinating topic suggesting many avenues for its relevance to phytoremediation.
31. Aken BV, Yoon JM, Schnoor JL: **Biodegradation of nitro-substituted explosives 2,4,6-trinitrotoluene, hexahydro-1,3,5-trinitro-1,3,5-triazine, and octahydro-1,3,5,7-tetranitro-1,3,5-tetrazocine by a phytosymbiotic *Methylobacterium* sp. associated with poplar tissues (*Populus deltoides* X *nigra* DN34).** *Appl Environ Microbiol* 2004, **70**:508-517.
32. Jeon CO, Park W, Padmanabhan P, DeRito C, Snape JR, Madsen EL: **Discovery of a bacterium, with distinctive dioxygenase, that is responsible for *in situ* biodegradation in contaminated sediment.** *Proc Natl Acad Sci* 2003, **100**:13591-13596.
33. Manefield M, Whiteley AS, Griffiths RI, Bailey MJ: **RNA stable isotope probing, a novel means of linking microbial community function to phylogeny.** *Appl Environ Microbiol* 2002, **68**:5367-5373.
Primary publication demonstrating the use of RNA-SIP for identifying the dominant phenol degrader in a bioreactor.
34. Radajewski S, Webster G, Reay DS, Morris SA, Ineson P, Nedwell DB, Prosser JL, Murrell JC: **Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing.** *Microbiology* 2002, **148**:2331-2342.
35. Butler JL, Williams MA, Bottomley PJ, Myrold DD: **Microbial community dynamics associated with rhizosphere carbon flow.** *Appl Environ Microbiol* 2003, **69**:6793-6800.
Utilization of the ^{13}C pulse-chase labelling and phospholipid fatty acid analysis for obtaining information on the structure of the microbial communities actively involved in cycling of rhizodeposition.
36. Ostle N, Whiteley AS, Bailey MJ, Sleep D, Ineson P, Manefield M: **Active microbial RNA turnover in a grassland soil estimated using a (CO_2)-C-13 spike.** *Soil Biol Biochem* 2003, **35**:877-885.
37. Johnson D, Leake JR, Ostle N, Ineson P, Read DJ: ***In situ* (CO_2)-C-13 pulse-labelling of upland grassland demonstrates a rapid pathway of carbon flux from arbuscular mycorrhizal mycelia to the soil.** *New Phytol* 2002, **153**:327-334.
The study provides an excellent example of how stable isotope labelling can aid in the qualitative and quantitative understanding of a complex system.
38. Bruneau PMC, Ostle N, Davidson DA, Grieve IC, Fallick AE: **Determination of rhizosphere C-13 pulse signals in soil thin sections by laser ablation isotope ratio mass spectrometry.** *Rapid Commun Mass Spectrom* 2002, **16**:2190-2194.
A combination of soil micromorphology and isotope methodologies is employed to determine the fate of root-derived ^{13}C , originating from a CO_2 pulse-labelled tracer. The first example of laser ablation stable isotope ratio mass spectrometry being applied in such a novel and exciting way — no doubt this technique will shed great insight into the mechanics of the rhizosphere in years to come.
39. Radajewski S, McDonald IR, Murrell JC: **Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms.** *Curr Opin Biotechnol* 2003, **14**:296-302.
40. Cook KL, Saylor GS: **Environmental application of array technology: promise, problems and practicalities.** *Curr Opin Biotechnol* 2003, **14**:311-318.
41. Schloss PD, Handelsman J: **Biotechnological prospects from metagenomics.** *Curr Opin Biotechnol* 2003, **14**:303-310.
42. Meer JR van der, Sentchilo V: **Genomic islands and the evolution of catabolic pathways in bacteria.** *Curr Opin Biotechnol* 2003, **14**:248-254.
43. Barth JA, Slater G, Schuth C, Bill M, Downey A, Larkin M, Kalin RM: **Carbon isotope fractionation during aerobic biodegradation of trichloroethene by *Burkholderia cepacia* G4: a tool to map degradation mechanisms.** *Appl Environ Microbiol* 2002, **68**:1728-1734.
44. Singer AC, Wong CS, Crowley DE: **Differential enantioselective transformation of atropisomeric polychlorinated biphenyls by multiple bacterial strains with different inducing compounds.** *Appl Environ Microbiol* 2002, **68**:5756-5759.
45. Read D, Bengough A, Gregory P, Crawford J, Robinson D, Scrimgeour C, Young I, Zhang K, Zhang X: **Plant roots release phospholipid surfactants that modify the physical and chemical properties of soil.** *New Phytol* 2003, **157**:315-326.
46. Hallett P, Gordon D, Bengough A: **Plant influence on rhizosphere hydraulic properties: direct measurements using a miniaturized infiltrometer.** *New Phytol* 2003, **157**:597-603.
47. Singer AC, Smith D, Jury W, Hathuc K, Crowley DE: **Impact of the plant rhizosphere and augmentation on remediation of polychlorinated biphenyl contaminated soil.** *Environ Toxicol & Chem* 2003, **22**:1998-2004.
48. Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, van der Lelie D: **Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants.** *Nature Biotechnol* 2004, doi:10.1038/nbt960.
This is a ground-breaking study in endophyte-aided phytoremediation, which is certain to revolutionize the field of phytoremediation.