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Laboratory tests for aerobic bioremediation of the contaminated sites in the Czech Republic

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Abstract: Laboratory-scale testing methods applicable to evaluation of contaminated subsurface microbial communities are discussed in relation to their potential in supporting effective site bioremediation. Both culture-dependent and culture-independent techniques are considered here with special emphasis on their capacity to contribute to bioremediation system design, in optimal cases by providing information on contaminant degradation rates. In this regard, microbial soil respiration tests seem to be the most useful tool since microbial soil respiration is a sensitive and easily measurable parameter for determination of metabolic activity within the sample and is closely related to other microbial parameters such as microbial biomass.

Keywords: culture-dependent and culture-independent techniques; contamination; microorganism; dehydrogenase; molecular method

Remediation of sites with subsurface contamination has become common and widespread within the past decades. The term "subsurface contaminated sites" generally refers to soil, land, and rock formations (possibly also building constructions) affected by inappropriate management of waste products or accidental releases of toxic chemicals. Waste dumps, abandoned industrial places, unprotected depositories of toxic chemicals, former military bases or mining areas are typical examples of subsurface contaminated sites. Data collected for 38 European countries (including 27 member states of EU) reported around 342 000 identified contaminated sites and more than 2.5 million potentially contaminated sites (Panagos et al. 2013). For the Czech Republic the number of identified contaminated sites is currently close to 5 000 while the number of potentially contaminated sites is twice as high (SEKM 2009, Cikánková et al. 2015). The Czech sites mostly belong to old contaminated areas where either the polluter is unknown or polluter does not exist anymore.

Approximately half of the subsurface contaminated sites in Europe are polluted with mineral oil, aromatic and polycyclic aromatic hydrocarbons (Panagos et al. 2013), where aerobic bioremediation is the most common remediation technology. At a relatively high number of contaminated sites in the Czech Republic (13.2 per 10 000 inhabitants compared to average 2.46 for the 38 European countries (Panagos et al. 2013)), the long-term demand for priority evaluation as well as very high costs of site remediation put great emphasis on the contaminated site risk assessment procedure. According to Czech methodologies (MŽP 2011) the risk assessment should not only decide whether risk is acceptable or not but in the latter case also suggest the most suitable options to manage the risk and implement remediation. In practice, this means specifying: (1) remediation limits; (2) the most suitable remediation strategy and technology, and (3) the economic parameters of the remediation – all this in relatively short time and with limited budget.

If aerobic bioremediation is considered for site remediation a lot of information is required before its design can start. A thorough laboratory assessment of the site microbiology is generally required to indicate whether bioremediation is an appropriate treatment technology (US EPA 1991). The laboratory examination should mainly: (1) evaluate the presence of appropriate degrading microorganisms and

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assess their potential to transform contaminants; (2) assess potential toxicity of contaminants to the microorganisms; (3) evaluate nutrient requirements to enhance degradation activity, and (4) evaluate the compatibility of the site geochemistry with the nutrient solution proposed for addition. It is not easy to satisfy all these requirements in current contaminated site management in the Czech Republic.

Thus this paper aims to provide critical assessment of the laboratory testing approaches available today to assess feasibility of the *in-situ* aerobic bioremediation projects. Both culture-dependent and culture-independent laboratory techniques are considered within the assessment.

IN-SITU AEROBIC BIOREMEDIATION IN SUBSURFACE CONTAMINATED SITES

In-situ aerobic bioremediation is based on application and/or support of microorganisms to degrade subsurface contaminants through oxidation processes. Oxygen is considered here as the most common electron acceptor for microbial respiration as well as the agent for aerobic degradation of a wide range of organic compounds. The intensity of the subsurface aerobic biodegradation depends on many other factors apart from oxygen delivery (Malla et al. 2018); these factors include the indigenous microorganism population involved, the physicochemical status of the soil (nutrient availability, pH, temperature, water content), the quality, quantity and bioavailability of contaminants (Margesin et al. 2000), and the spatial contaminant distribution (Jørgensen 2007). The aerobic bioremediation process is usually focused on the unsaturated zone and on the capillary fringe where the low-density hydrocarbons typically stay. Bioremediation is then generally considered as an indispensable, ecofriendly and cost-effective solution for restoring contaminated sites (Desai et al. 2010).

The design of an aerobic bioremediation system should ideally be based on knowledge of the particular microorganisms inhabiting the contaminated site, including their metabolic processes and their reaction to changes in environmental conditions. Microorganisms are able to carry out quite a broad array of processes which all, besides oxidation, may result in lowering the concentration of the original contaminant. Binding, volatilisation, immobilisation or chemical transformation may be mentioned as examples of such processes. Biological reduction of the subsurface contamination is also typically associated with abiotic remediation, so biological and abiotic remediation are often difficult to distinguish (Diplock et al. 2009). The specialised knowledge needed for aerobic bioremediation is in practice unfortunately not easily available, and the use of microbes in bioremediation is experimental rather than knowledge-based.

Compared to purely aquatic samples, the three phase subsurface structures represent a much greater challenge to the development and applications of laboratory scale investigation methods. One tonne of surface soil may harbour more bacteria taxa than all the oceans combined (Nybroe et al. 2006). Soil is structurally complex due to its various contents of sand, silt, clay, organic debris and mucoid and humified material. Humic acids and clay minerals can inhibit enzymatic reactions that are part of molecular detection assays. Bacteria commonly adhere to surfaces of minerals and organic matter. These particles can mask the bacterial cells, making direct, *in situ*, microscopy of the soil environment a difficult task. As bacterial cells are difficult to release from their association with soil particles, even the efficiency of extraction-based methods is compromised. The bulk deep-subsurface soil represents an oligotrophic and highly heterogeneous environment, which makes it difficult to employ assays demonstrating the metabolic activity of target cells, including their cultivability. Within a scale of a few microns, some cells in the soil may be metabolically active or growing, others can be dormant or inactive, and still others may be dead. Therefore it is important to address what type of information a given detection method can provide concerning the activity of the target cells (Nybroe et al. 2006).

Applicability of a specific bioremediation strategy for a specific site may be investigated in the laboratory by measuring the decay of the target compounds or the appearance of metabolites. Such a simple approach does not however differentiate abiotic from biotic processes and also does not provide information on physiological status or performance of the degrader population (Diplock et al. 2009). Without evidence of microbial involvement, there is no way to verify that the contaminant did not simply volatilise, migrate off site, sorb to subsurface solids, or change form *via* abiotic chemical reactions (National Research Council 1993).

Various key parameters have been identified that can be used to predict the likely performance of a bioremediation strategy. These included quantification of the total cultivable degrader populations, an evaluation of hydrocarbon bioavailability (Paton et al. 2003) and soil respiration as an indicator of bioremediation status and potential (Dawson et al.

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2007). In another work, soil respiration, dehydrogenase activity (DHA) and microbial counts were used to quantify microbial activities in the course of bioremediation (Margesin et al. 2000). Some authors quantify not only bacterial degraders and but total heterotrophs as well (Diplock et al. 2009).

General guidance on laboratory-scale simulations of biodegradation processes is also provided through the international technical standards. The highest relevance for the scope of this paper was found for ISO 11266: 1994E which relates to laboratory testing for biodegradation of organic chemicals in soil under aerobic conditions. Determination of physical and chemical parameters is specified here together with the assessment of microbial activity through an appropriate biodegradable reference compound or by determining active biomass. The disappearance of a test compound and the formation of metabolites, carbon dioxide, and other volatile and non-extractable residue are also mentioned as recommended parameters (ISO 11266: 1994E). This standard is, however, mainly applicable to predicting possible future behaviour of a certain organic compound or set of compounds under specific conditions of uncontaminated soil. It is hardly applicable to rapid determination of biodegradation kinetics for real contaminated samples.

Culture-dependent techniques

Culture-dependent techniques involve isolation and characterisation of microorganisms growing in cultivation media. Classical culture-dependent techniques such as colony forming units (CFUs) and most probable number (MPN) are culture-dependent techniques that have been widely used to estimate microbial abundance of a cultivable community. However, the effectiveness of culture-dependent techniques is quite limited, because only 0.1–10% of microorganisms occurring in soil are capable of growing under laboratory conditions (Littlefield-Wyer et al. 2008). Problems associated with noncultivable microorganisms are also frequently found during evaluation of microbial communities in petroleum impacted environments (Bachoon et al. 2001). Many microbes may not have been isolated in the laboratory or may have specific community associations that prevent the isolation of pure cultures for analysis (Whiteley and Bailey 2000). Better simulation of *in situ* growth conditions can be done using natural, low nutrient media and by replacing agar with another solidification agent (Nybroe et al. 2006).

Culture-dependent techniques have often been used for the monitoring of the microbial response in hydrocarbon-contaminated environments. Microorganisms may be examined here by plate counts, by MPN method, by determination of the number of hydrocarbon degrading organisms as determined by counts on selective plates or by selective MPN (Bachoon et al. 2001). Illustrative examples include here the application of cultivation methods to: diesel fuel or lubricant oil contaminated soils through counting of heterotrophs and hydrocarbon degrader bacteria (Margesin et al. 2000, Diplock et al. 2009, Wang et al. 2016); BTEX (benzene, toluene, ethylbenzene and xylene) contamination through counting of heterotrophs (Modrzyński et al. 2016); BTEX and PAHs (polycyclic aromatic hydrocarbons) contaminated soils through heterotrophs and nitrate-reducing bacteria determination (Hollender et al. 2003); and heavy hydrocarbons contaminated soils through aerobic and anaerobic heterotrophs, total gram-negative bacteria, sulphite-reducing bacteria, *Pseudomonas*, moulds, yeast and *Actinomycetes* estimation (Coccia et al. 2009).

The numbers of petroleum degraders and total heterotrophs as well as quantification of the bioavailable fraction of the contaminant allow an estimation of how bioremediation would progress. Soils with very low population densities were associated with the lowest rates of biodegradation (Table 1) (Diplock et al. 2009). In another work, linear correlations were found between total petroleum hydrocarbon (TPH) degradation rate and TPH degrader population and between alkane degradation rate and alkane degrading bacteria population during the first three weeks of incubation. Correlation equations may be useful for predicting TPH and alkane degradation in oil-contaminated soil incubated in the laboratory at room temperature in relation to increases in the abundance of degrading microbial population measured by the MPN procedure (Wu et al. 2017).

It follows from the above, that culture-dependent techniques help us to identify key populations capable of carrying out specific metabolic processes, and subsequently enhance the understanding of the functioning of microbially-mediated processes (Ngom and Liu 2014). They are not, however, enough to characterise the soil microbiota and its significance for the functioning of soils (Insam 2001) and their activity in soil (Frankenberg and Dick 1983). A measurement of microbial biomass in soil gives an indication of soil condition and the potential for metabolic activity (Sandrin et al. 2009). If restricted only to culture-dependent techniques, our understanding of

Table 1. Laboratory scale techniques to assess the feasibility of *in-situ* aerobic bioremediation

Parameter	Culture-dependent techniques	Culture-independent techniques		
		dehydrogenase activity	molecular methods	respiration activity
Cost* (EUROS)	• 10–20 (heterotrophs) • 30–40 (TPH degraders)	• 20–30	• 20–45 (FISH, PCR) • 30–50 (qPCR)	• 10 (titration) • 55 (coulometry)
Time requirements** (days)	• 7–10 (heterotrophs)[1,3] • 7–14 (TPH degraders)[2,3] • 7–124 (fast and slow growing heterotrophs)[2,4]	• 2[5,6,7,8]	• 2 (PCR, FISH)[6]	• 1–100[1,7,10,11,12,13] • till respiration rate declines[14,15]
Assessment of *in-situ* biodegradation rate for TPH	• semiquantitative (*in-situ* bioremediation suitable if degrader number exceeds 5 000 CFU/g)[11]	• semiquantitative	• linear correlation between qPCR method and TPH degradation[17]	• mg CO_2-C/100 g[7] • µg CO_2/g 24 h[6] • mg CO_2/g 24 h (titration method)[5] • µg CO_2/g h[5, 20]
			• relationship between qPCR method and TPH degradation[16,18]	• linear correlation between soil respiration and TPH degradation[6] • exponential correlation between soil respiration and TPH degradation for the concentration of TPH below 10 000 mg/kg[11]
	• linear correlation between MPN test and biodegradation rate[3, 17]	• linear correlation between DHA and biodegradation rate[19,20]		
Differentiation of community composition	possible by definition of cultivation conditions	impossible under ISO Standards conditions	dependent on the method used (e.g., PLFA analysis[5], PCR based methods, microarray[9,16] – species)	impossible to specify

*Range of cost estimated per sample for all the relevant activities, including sample pretreatment, the analysis itself, and interpretation of results. Prices follow from actual market research in the Czech Republic; **Range of time estimated per sample for all the relevant activities including sample pretreatment, the analysis itself and interpretation of results; [1]Hollender et al. (2003); [2]Foght and Aislabie (2005); [3]Wu et al. (2017); [4]Modrzyński et al. (2016); [5]Schinner et al. (1996); [6]Margesin et al. (2000); [7]Riffaldi et al. (2006); [8]ISO 23753-1, 2005; [9]Rastogi and Sani (2011); [10]Paton et al. (2006); [11]Diplock et al. (2009); [12]ISO 16072, 2011; [13]ISO 11266, 1994; [14]Eisentraeger (2005); [15]ISO 17155, 2002; [16]Yergau et al. (2009); [17]Liu et al. (2015); [18]Baek et al. (2009); [19]Galiulin et al. (2012); [20]Polyak et al. (2018); TPH – total petroleum hydrocarbon; CFU – colony forming unit; MPN – most probable number; DHA – dehydrogenase activity; PLFA – phospholipid fatty acid

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the microbial ecology and physiology associated with bioremediation would be incomplete and likely to be biased because environmental factors that influence microbial activity and function, such as resource competition and biotic and abiotic interactions, would not be taken into consideration (Ngom and Liu 2014).

Culture-independent techniques

Microbial biomass and activity are generally closely related because it is through the biomass that the transformations of the important organic elements (C, N, P, and S) occur. Therefore, the ideal parameter for assessing the role of microorganisms in various soil processes would correlate not only with microbial activity but also with microbial biomass (Frankenberg and Dick 1983). The traditional methods for quantifying the activities of soil microorganisms in the presence of hydrocarbon pollutants, such as soil fumigation, carbon respiration measured as CO_2, and soil enzyme activity (Guo et al. 2012) are consistent with this assumption, as are modern molecular techniques used to study microbial population and functionality at contaminated sites.

Dehydrogenase activity. Soil enzymes have been reported to be useful soil quality biological indicators due to their relationship with soil biology, being operationally practical, sensitive, integrative, and easy to measure (Utobo and Tewari 2015), and in exhibiting the fastest responses to external disturbances. Enzyme activity measurements can be an indirect assessment of the activity of a specific group of microorganisms in the soil (Baldrian 2009). They can also indicate changes in the biological status of soil due to hydrocarbon pollution and its bioremediation (Riffaldi et al. 2006). Enzymes suitable for monitoring of TPH removal from soil include catalases, ureases and widely reported dehydrogenases (Polyak et al. 2018).

Dehydrogenases are the main representatives of the oxidoreductases in soil (Kaczyńska et al. 2015). Dehydrogenases, as respiratory chain enzymes, play a major role in energy production by organisms. They transfer two hydrogen ions to coenzymes and thus soil organic matter is oxidised. Through these coenzymes, hydrogens enter the respiratory chain or participate in reducing processes of biosynthesis (Schinner et al. 1996). Therefore, DHA can be used as an indicator of biological redox systems and as a measure of microbial activity in the soil (ISO 23753-1, 2015). In the environment, many specific types of dehydrogenases occur. They differ in terms of, among other things, the coenzyme type (Kaczyńska et al. 2015). Dehydrogenases occur in all living microorganisms (Moeskops et al. 2010). It is considered that they exist as an integral part of intact cells but do not accumulate extracellularly in the soil.

Dehydrogenase activity is measured by a colorimetric method, using 2,3,5-triphenyltetrazolium chloride (TTC) (ISO 23753-1, 2015) or 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-S-phenyl tetrazoliumchloride (INT) (Schinner et al. 1996) as substrate. The substrate is added into a soil sample. The reaction product released after incubation is extracted and then determined by photometry (ISO 23753-1, 2015).

Dehydrogenase levels can indicate the type and significance of pollution in soil (Utobo and Tewari 2015). An evaluation of soil biological activities as a monitoring instrument for the decontamination process of TPH contaminated soil has been done using measurements of various parameters. Illustrative examples of the study include measurement of organic carbon content, soil microbial respiration, soil ATP, and dehydrogenase, β-glucosidase and lipase enzyme activities. Residual hydrocarbon was positively correlated with that of the organic carbon content, microbial respiration and with β-glucosidase activity, while both soil lipase activity and DHA were negatively correlated with the hydrocarbon content. Lipase activity was further found to be the most useful parameter for testing hydrocarbon degradation in soil (Riffaldi et al. 2006). The response of autochthonic microbial community and biological parameters (microbial soil respiration, DHA, catalase activity and total microbial count) on contamination with car fuels (petrol, diesel) and engine oils (new and waste after 10 000 km) has also been evaluated. Addition of petroleum substances led to a decrease of DHA (Wolińska et al. 2016). In another study, it was found that petroleum products affect the soil dehydrogenase in various ways. Biodiesel, diesel oil and fuel oil stimulated these enzymes, while petrol acts as an inhibitor (Kaczyńska et al. 2015). Dehydrogenase further demonstrated the best sensitivity to TPH in the study where urease, catalase and dehydrogenase enzymes were examined and where dehydrogenase activity was concluded to be one of the most useful microbial parameters for testing bioremediation methods (Polyak et al. 2018).

In summary, soil enzyme tests cannot be used to estimate *in situ* matter fluxes as they only provide an estimate of the potential to biodegrade a certain substrate under optimised conditions. To characterise the soil, or to understand differences among soils, additional methods are necessary (Insam 2001).

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Microbial soil respiration. Several petroleum products are known to be toxic, but at the same time these compounds may also serve as a substrate for microbial respiration and growth (Modrzyński et al. 2016). Microbial soil respiration is defined as oxygen uptake or carbon dioxide evolution by bacteria, fungi, algae and protozoans and includes the gas exchange of aerobic and anaerobic metabolism (Schinner et al. 1996). Oxygen consumption is less sensitive than CO_2 evolution as oxygen consumption may arise from biotransformation and not necessarily hydrocarbon mineralisation. Production of CO_2 on the other hand, actually provides data on mineralisation and is very useful for assessing biodegradability in solid media like soil and sediments. Valuable data can be obtained when both O_2 consumption and CO_2 evolution are measured simultaneously (Chikere et al. 2011).

Numerous respiration methods are available such as (*i*) determination of CO_2 release by titration; (*ii*) coulometric determination of total CO_2 evolved; (*iii*) periodic analysis of headspace gas samples for CO_2 by gas chromatography, and (*iv*) continuous monitoring of headspace CO_2 levels with infrared CO_2 analysers. These methods are measures of the overall soil microbial activity and showed correlation or good agreement with target hydrocarbon degradation (ISO 11266 1994, Sanscartier et al. 2009, ČSN EN ISO 16072, 2011). Lowering of target contaminant concentrations can also be monitored in parallel with soil respiration but is often ignored as it entails additional effort and expense (Sanscartier et al. 2009).

Microbial soil respiration as indicated by the amount of evolved CO_2 has been used as an instrument for the monitoring of decontamination process of a TPH contaminated soil (Margesin et al. 2000), for predicting TPH degradation (Wu et al. 2017), or to examine the effect of TPH contamination on respiration activity (Wolińska et al. 2016). The rates of TPH biodegradation have been calculated with a good accuracy from oxygen consumption and CO_2 formation (Hollender et al. 2003). In later studies, the rate of respiration correlated with the total number of heterotrophs, the number of degraders and rate of TPH degradation (Diplock et al. 2009), and with TPH degradation and organic carbon content (Riffaldi et al. 2006).

In a subsequent study, the microbial soil respiration was simulated after the addition of oil derivatives and diesel at a range of different doses (Wolińska et al. 2016). Also, in another study, microbial activity monitored as CO_2 release and ([^{3}H])leucine incorporation was strongly stimulated at low exposure levels of gasoline vapors and strongly inhibited at high exposure levels (Modrzyński et al. 2016).

Summarising, microbial soil respiration measurements are frequently used as a sensitive and easy analysable microbial parameter for the determination of contaminant degradation (Table 1) or inhibition of microbial activity (Hollender et al. 2003). Respiration activity has limitations in being unable to distinguish between CO_2 released from microbial activity and CO_2 released from abiotic processes (ČSN EN ISO 16072, 2011).

Molecular techniques. A more comprehensive assessment of the microbial structure and function of the microbial community at the site of contaminations as whole requires the use of molecular techniques (Bachoon et al. 2001, Gałązka et al. 2018). Microbial molecular techniques rely on the characterisation of cellular constituents such as nucleic acids, fatty acids, proteins and other taxa-specific compounds (Rosselló-Mora and Amann 2001). Several detailed reviews on principles of methods have already been published (Malik et al. 2008, Rastogi and Sani 2011, Loick and Weisener 2014, Li et al. 2017, Dangi et al. 2019) so this part of the review is concerned mainly with their application in bioremediation of TPH contaminated soils.

A wide variety of molecular techniques based on direct isolation and analysis of biomolecules has been developed and applied for describing and characterising the phylogenetic and functional diversity of microorganisms in contaminated environments (Malik et al. 2008, Loick and Weisener 2014). The techniques are divided, for the purposes of this review, into two major categories, partial community analysis and whole community analysis (Rastogi and Sani 2011).

The first method, based on the partial community analysis approach, includes polymerase chain reaction (PCR) tests where total DNA/RNA is used as a template for the characterisation of microorganisms (Rastogi and Sani 2011). Microbial ecology has also been used extensively focusing on amplification of conserved genes such as 16S rRNA and 18S rRNA from an environmental sample (Desai et al. 2010, Rastogi and Sani 2011). One of the reasons for using the latter is that the number of ribosomes in a cell (rRNA) is a definitive factor of activity (Loick and Weisener 2014).

When changes in the amounts of specific groups or species of organisms or the expression of certain genes are of interest, real-time PCR (qPCR) is a method of choice (Loick and Weisener 2014). This technique can simultaneously detect and quantify the amplified product while the reaction is occurring. Several

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environmental studies have used techniques based on PCR for the monitoring of TPH degrading population in TPH contaminated soil (Bachoon et al. 2001, Malik et al. 2008, Baek et al. 2009, Yergeau et al. 2009) and confirmed the correlation between qPCR method and TPH degradation (Table 1, Liu et al. 2015).

Microbial communities were analysed during bioremediation processes by fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TTGE) (Malik et al. 2008, Desai et al. 2010, Loick and Weisener 2014, Wang et al. 2016).

Methods utilising stable isotopes, such as stable isotope probing (SIP), offer more detailed insights into the metabolic activities of microbial communities than determination of linking between community structure and activity of microorganisms can provide. In the SIP method, an active community utilises the labelled substrate and incorporates the isotopes within its biomass. Using molecular techniques, the phylogenetic identity of labelled biomolecules can be established (Rastogi and Sani 2011) as, for example, in a study by Modrzyński et al. (2016).

Characterisation of community structure and indirect detection of microbial biomass can be determined by the method based on analysing phospholipid fatty acids (PLFAs). This method was applied, for example, in the investigation of bioremediation of diesel-contaminated soil (Margesin et al. 2007, Siles and Margesin 2018). The advantage of this method is that PLFAs indicate the presence of living organisms since they rapidly degrade upon cell death (Yao et al. 2015). However, many fatty acids are common to different microorganisms.

The analysis of the suite of proteins produced by bacterial cultures (proteomics) can be used to find variation in the composition and production of proteins and detection of many proteins that are important in the physiological response of microbes in the presence of pollutants, but few laboratories are currently applying it to environmental concerns. Proteomics technology needs to develop further for environmental clean-up at a more reasonable cost (Dangi et al. 2019).

The methods mentioned above have some limitations. Fingerprinting methods can have limitations due to identical mobility of different 16S rRNA sequences. The limitation of methods utilising stable isotopes can be in relation to insufficient substrate incorporation. Therefore, further optimisation of methods is required (Malik et al. 2008).

To obtain a more comprehensive view of the role of molecular techniques in bioremediation and recognising the limitations of particular methods, it is suggested that the use of a combination of molecular methods could be more effective. Combinations of methods such as DGGE, qPCR and culture-based method (Kao et al. 2010), or DGGE, qPCR, PLFA and culture-based method (Pacwa-Płociniczak et al. 2016), or fluorescence *in situ* hybridisation (FISH) and SIP (Loick and Weisener 2014), were used in studies of TPH contaminated soil.

Whole community analysis methods can divide into methods analysing the whole genome and methods known as postgenomic. Whole genome techniques attempt to analyse all the genetic information present in total DNA extracted from an environmental sample or pure culture (Rastogi and Sani 2011). Postgenomic approaches such as metaproteomics (proteins resulting from translation), metatranscriptomics (expressed genes), metabolomics (cellular metabolites produced in microbial cells), and fluxomics (determining rates of metabolic reactions) reveal the link between genetic potential and functionality in microbial communities (Rastogi and Sani 2011, Dangi et al. 2019). Multi-omics approaches provide deeper insights in the cellular function and gene products interacting within the environment (Malla et al. 2018). Both metaproteomics and community metabolomics have been used in the characterisation of microbial populations in one of the largest crude oil polluted areas in the world, the semi-closed Mediterranean sea (Bargiela et al. 2015). The data generated through the application of multi-omics tools provide insight into complex microbial metabolic pathways but the use of the multi-omics approach for bioremediation is so far limited due to high sample processing costs and the requirement for specialised instrumentation (Dangi et al. 2019).

Finally, information obtained by molecular methods depending on the extraction of DNA or RNA from the sample matrix can be biased because of incomplete microbial cell lysis. Additionally, DNA can persist in the environment for prolonged periods, making the differentiation between dead, living and active cells difficult if not impossible (Loick and Weisener 2014). Furthermore, techniques based on DNA analysis do not provide information on the gene expression (functionality) as it occurs under *in situ* conditions (Rastogi and Sani 2011).

No single molecular technique exists that can adequately describe the entire microbial diversity and associated catabolic genes at contaminated sites (Malik et al. 2008). Combination of methods is reco-

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mmended to minimise misinterpretation as well as oversight of less abundant, but important members of the microbial community (Loick and Weisener 2014).

Comprehensive and systematic critical assessment of laboratory-scale bioremediation methods have clearly identified soil respiration testing as the most suitable technique for the identification of contaminant degradation rates required for the design of bioremediation systems in the preliminary phase of site remediation. Microbial soil respiration tests provide a direct reflection of the metabolic activity for a specific sample from a specific contaminated site, which no other culture-dependent and culture-independent technique is able to provide.

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