Vitenskapskomiteen for mattrygghet
Norwegian Scientific Committee for Food Safety

VKM Report 2016: 42

Health and environmental risk evaluation of microorganisms used in bioremediation

Opinion of the Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety

Report from the Norwegian Scientific Committee for Food Safety (VKM) 2016: 42
Risk assessment on Health and environmental risk evaluation of microorganisms used in bioremediation

Opinion of the Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety
16.08.2016

ISBN: 978-82-8259-232-1
Norwegian Scientific Committee for Food Safety (VKM)
Po 4404 Nydalen
N – 0403 Oslo
Norway

Phone: +47 21 62 28 00
Email: vkm@vkm.no

www.vkm.no
www.english.vkm.no

Suggested citation: VKM. (2016) Health and environmental risk evaluation of microorganisms used in bioremediation. Scientific Opinion of the Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety, ISBN: 978-82-8259-232-1, Oslo, Norway.

Health and environmental risk evaluation of microorganisms used in bioremediation

Authors preparing the draft opinion

Ida Skaar (chair), Nana Asare (VKM staff), Jörn Klein, Arinze Okoli, and Anders Ruus

(Authors in alphabetical order after chair of the working group)

Assessed and approved

The opinion has been assessed and approved by the Panel on Microbial Ecology. Members of the Panel are: Ida Skaar (chair), Tor Gjøen, Jacques Godfroid, Anders Jelmert, Jörn Klein, Arinze Okoli, Arne Tronsmo and Bjørnar Ytrehus.

(Panel members in alphabetical order after chair of the Panel)

Acknowledgement

The Norwegian Scientific Committee for Food Safety (Vitenskapskomiteen for mattrygghet, VKM) has appointed a working group consisting of both VKM members and external experts to answer the request from the Norwegian Food Safety Authority/Norwegian Environment Agency. Project leader from the VKM secretariat has been Nana Asare. The members of the working group Ida Skaar, Arinze Okoli, Jörn Klein (Panel on Microbial Ecology), Anders Ruus and Christopher Miles (Panel on Contaminants) are acknowledged for their valuable work on this opinion. The Panel on Microbial Ecology is acknowledged for comments and views on this opinion.

Competence of VKM experts

Persons working for VKM, either as appointed members of the Committee or as external experts, do this by virtue of their scientific expertise, not as representatives for their employers or third party interests. The Civil Services Act instructions on legal competence apply for all work prepared by VKM.

Table of Contents

Summary

In 2015, The Norwegian Environment Agency requested the Norwegian Scientific Committee for Food Safety (VKM) to collate an overview of bioremediation of polluted ground based on bioaugmentation described in literature for the degradation of various types of pollutants, (including hydrocarbons, heavy metals, chlorinated compounds, explosives etc.).The assessment of genetically modified microorganisms (GMO), phytoremediation, bioremediation based on natural attenuation, bio-stimulation or biodegradation, including composting, are not included in this report.

For each chemical type, the Norwegian Environment Agency requested a brief description of the most common classes of microorganisms with respect to the properties that are relevant to health and environmental risk assessments. Furthermore, VKM was asked to evaluate whether the current information requirements in the declaration form, which importers, distributors or manufactures of microbiological products in Norway are required to submit, (cf. the Regulation on microbiological products), provide sufficient basis to assess the health and environmental risks involved in the use of bioremediation for the clean-up of polluted ground in Norway.

VKM appointed a working group consisting of members of the Panel on Microbial Ecology. The Panel on Microbial Ecology has reviewed and revised the draft prepared by the working group, and the assessment has been adopted.

Bioaugmentation, the focus of this report, involves supplementation of pre-grown microbial cultures to existing microbial population to biodegrade toxic substances to non-toxic substances by metabolic conversion. This technological approach is apparently cost-effective, more convenient and causes less environmental stress compared to conventional remediation approaches. However, the introduction of exogenous microbial species, strains or consortia to the endogenous microbial community comes with an inherent risk of adversely affecting the functionality of the native community and the ecosystem at large.

The first part of this report provides an updated scientific overview of existing current knowledge on bioremediation with focus on bioaugmentation as a technology in decontaminating primarily hydrocarbons, heavy metals, halogenated compounds, and explosives as pollutants. The overview briefly describes the hazardous implications of environmental contaminants and methods for mitigating them by bioaugmentation (*in situ* and *ex situ*). The rationale for the use of microorganisms in remediating polluted environments and some microbial features utilized in bioremediation (anaerobic and aerobic biodegradation) are presented. Finally, possible health and environmental implications of the microorganisms involved are discussed.

Based on scientific assessment of the information requirements laid down in the declaration form of the Regulation on microbial products, the VKM Panel concludes that the information

requirements in their current form are not sufficient to conduct a health and environmental risk assessment of added microorganisms for bioremediation of polluted ground in Norway. The second part of this report include specific recommendations from the working group concerning information needed to update the regulation on microbiological products, as further specified in the guidelines to the regulation for the use of microorganisms in bioremediation, based on initial findings.

There seems to be a general lack of accuracy when it comes to specification of the microbial content and concentrations that are included in the product. Without proper taxonomic classification, no meaningful risk assessment is feasible. The taxonomic affiliation of the organisms present in the product should be specified to at least species, preferably strain level.

The declaration should in our opinion not necessarily rely on specific methods, as long as the methods described are scientifically adequate. However, the identification should be based on new molecular methods, for e.g. the potential role of the microorganism in the product acting as a pathogen, toxigen or an allergen, its association to intestinal dysbiosis or genes coding for antibiotic resistance can be identified. Rather than specifying a list of specific antibiotics employ generic classes of antibiotics as stipulated in the Nordic Ecolabelling guidelines. The Panel recommends a multiphasic approach to future assessments as this allows for the implementation of current and most effective methods as they are developed and verified.

There seems to be lack of emphasis on environmental impacts, especially on the potential for persistence and spread in the environment (terrestrial or aquatic), the potential for pathogenic effects on domestic or wild vertebrates, arthropods or plants. Furthermore, there is little emphasis on the effects with increased use and accumulation, persistence and spread in terrestrial and aquatic environments and on long-term effects on the microbial community.

The form (vegetative, viable spores (bacteria and fungi) or cysts (protozoans)) of the microorganism present should be specified. If the product contains organisms that form endospores, spores or cysts, procedures for activating the spores or cysts and for further cultivation should be described.

The declaration should provide information about the procedures and quality controls securing a product without contaminations, pathogens, or known relevant virulence or resistance factors that may increase health or environmental risks. The safety reassurances provided by producers of bioremediation products should also cover properties related to allergenicity, sensitization, plant pathogenicity and environmental impacts. How the microbes in the product and their pathogenic properties develop with time through and after shelf life should also be described.

In our opinion, a declaration should include information about intended use and instructions for use, if specific precautions (personal protection, waste, containers etc.) need to be taken. Furthermore, information relating to user groups should be provided; for example if the

product is suitable for use in certain settings and environments such as areas in close proximity of facilities for vulnerable people (immunocompromised, infants, elderly, pregnant women etc.) or production animals.

The term “Environmental Damage” is not sufficiently defined. What kinds of shift in the microbial community and local community can be expected in the receiving environment, especially if exposure is chronic and frequent? The document focuses only on the introduction of foreign genes into the ecosystem. The environment can also be permanently altered (or damaged) if the introduction of the new organisms results in the extinction of the naturally existing closely related species. In addition, metabolic products that might affect resident microbial communities could be valuable information.

A re-evaluation of current national and international regulatory and policy frameworks may be necessary. This can include an evaluation of the most appropriate instruments (e.g. product declaration forms, regulations, standards, codes of practice, etc.) to use for strengthening these frameworks to mitigate risks to human health and the environment.

Key words: VKM, (benefit and) risk assessment, Norwegian Scientific Committee for Food Safety, Norwegian Environment Agency, bioremediation, bioaugmentation, microorganisms

Sammendrag

Miljødirektoratet ga i 2015 Vitenskapskomiteen for mattrygghet (VKM) i oppdrag å utarbeide en litteraturoversikt over bioremediering basert på bioaugmentering for nedbryting av ulike typer forurensere (inkludert hydrokarboner, tungmetaller, klorinerte forbindelser, eksplosiver etc.). Bioremediering er en metode som brukes for å rense forurenset jord og vann ved hjelp av primært mikroorganismer. Bioaugmentering innebærer å tilsette pre-kultiverte mikrobielle kulturer til eksisterende mikrobielle populasjoner for å fremskynde nedbrytning av toksiske forbindelser til ikke-toksiske alternativer i forurenset jord og vann. Vurdering av genmodifiserte mikroorganismer (GMO), fytoremediering, bioremediering basert på naturlig attenuering, biostimulering eller biologisk nedbrytning, inkludert kompostering, er ikke inkludert i denne rapporten.

For hver kjemikalietype ønsket Miljødirektoratet en kort beskrivelse av de vanligste klassene av mikroorganismer med hensyn til egenskaper som er relevante for helse- og miljørisikovurderinger. Videre ble VKM spurt om å vurdere om dagens krav til informasjon i deklarasjonsskjemaet, som den som importerer, produserer eller omsetter mikrobiologiske produkter i Norge er pålagt å levere, (jf. Forskrift om mikrobiologiske produkter), gir tilstrekkelig grunnlag for å vurdere helse- og miljørisiko ved bruk av bioremediering for rensing av forurenset grunn i Norge.

VKM utpekte en arbeidsgruppe med medlemmer fra Faggruppen for mikrobiell økologi. Faggruppen for mikrobiell økologi har gått gjennom og revidert arbeidsgruppens utkast og godkjent risikovurderingen.

Rapportens første del inneholder en oppdatert litteraturoversikt over dagens kunnskap om bioremediering basert på bioaugmentering. Oversikten beskriver kort konsekvenser av miljøforurensinger og metoder for å redusere dem ved bioaugmentering (*in situ* og *ex situ*). I denne delen presenteres også begrunnelsen for å bruke mikroorganismer i remediering av forurensede miljøer, og enkelte mikrobielle egenskaper som anvendes i bioremediering (anaerob og aerob biologisk nedbryting). Mulige helse- og miljøeffekter av mikroorganismene som benyttes er også diskutert.

Med bakgrunn i tilgjengelig kunnskap, mener VKM at bioaugmentering ser ut til å være kostnadseffektiv, enklere å bruke og forårsake mindre belastning på miljøet enn konvensjonelle remedieringsmetoder. Imidlertid vil fremmede mikrobielle arter, stammer eller sammensetninger som tilsettes eksisterende mikrobielle populasjoner, kunne innebære risiko for at funksjonaliteten blir påvirket negativt. Det gjelder både det eksisterende mikrobielle samfunnet og økosystemet som helhet.

Basert på en vitenskapelig vurdering av dagens krav til informasjon i deklarasjonsskjemaet, konkluderte VKMs arbeidsgruppen at kravene ikke gir tilstrekkelig grunnlag for å gjennomføre en vurdering av helse- og miljørisiko knyttet til mikroorganismer brukt for bioremediering av forurenset grunn i Norge.

I rapportens andre del gir VKM spesifikke anbefalinger om hva slags informasjon som er nødvendig for å oppdatere forskriften.

I dagens forskrift synes det å være en generell mangel på presisjon ved spesifisering av det mikrobielle innholdet og konsentrasjoner i produktet. Uten en grundig klassifisering av mikroorganismene, er det ikke mulig å gjennomføre risikovurdering av produktet. Tilhørigheten til mikroorganismen i produktet bør spesifiseres til minst artsnivå, helst også stammenivå.

Etter VKMs vurdering bør ikke deklarasjonen av mikrobielle produkter avhenge av spesifikke metoder så lenge metodene som benyttes er vitenskapelig adekvate. Identifiseringen bør baseres på nye molekylære metoder, f.eks. den aktuelle mikrobens potensiale som patogen, allergen eller toksigen, eventuelle assosiasjoner med mikrobiell ubalanse i kroppen eller gener som koder for antibiotikaresistens kan identifiseres. I stedet for en liste med spesifikke antibiotika bør det angis generiske klasser av antibiotika, på samme måte som i retningslinjer for Svanemerket. Det bør tilstrebes en bred metodisk tilnærming, slik at nye og mer effektive analyser eller evalueringsmetoder kan tas i bruk så snart de er publisert og validert.

Dagens forskrift ser også ut til å legge liten vekt på miljøpåvirkninger av fremmede mikroorganismer. Det gjelder spesielt potensialet for vedvarende påvirkning og spredning av mikroorganismene i jord og i vann, og potensialet for å påføre ville eller tamme virveldyr, leddyr eller planter sykdom. Videre er det lagt liten vekt på effektene ved økt bruk og akkumulering, persistens og spredning både innendørs og i jord og vann, og langtidseffekter på det mikrobielle miljøet.

Den aktuelle mikrobens form (vegetative, levedyktige sporer (bakterier og sopp) eller cyster (protozoer)), bør spesifiseres. Dersom produktet inneholder organismer som danner endosporer, sporer eller cyster, bør prosedyrer for aktivering og videre kultivering beskrives.

Deklarasjonen bør også gi informasjon om prosedyrer og kvalitetskontroller som sikrer et produkt uten kontaminanter, patogener eller kjente relevante virulens- eller resistensfaktorer som kan øke risiko for helse og miljø. Sikkerhet som er angitt av produsenter av mikroorganismer til bioremediering, bør også dekke egenskaper relatert til allergenisitet, følsomhetsreaksjoner, plantepatogenitet og miljøpåvirkning. Hvordan mikrobene i produktet og deres sykdomsframkallende egenskaper utvikles over tid, under og etter lagring, bør også beskrives.

Etter VKMs vurdering bør en deklarasjon inneholde informasjon om bruksområder og instruksjoner for bruk når det er behov for spesielle forholdsregler (beskyttelsesutstyr, avfall, emballasje osv.).

Videre bør deklarasjonen inneholde informasjon relatert til brukergrupper. Det kan for eksempel være om produktet kan brukes i spesielle sammenhenger og miljøer, som arealer tilknyttet fasiliteter for sårbare grupper (personer med nedsatt immunforsvar, barn, eldre, gravide osv.), eller lokaler for produksjonsdyr.

Begrepet “miljøskade” er ikke tilstrekkelig definert i dagens forskrift. Hva slags endring av mikrobielle samfunn og miljøet lokalt kan for eksempel forventes i det eksponerte miljøet dersom eksponeringen er kronisk og frekvent? Dokumentet fokuserer kun på introduksjonen av fremmede gener i økosystemet. Miljøet kan også bli permanent påvirket eller skadet dersom introduksjon av en ny organisme resulterer i utryddelse av nært beslektede arter som finnes naturlig i miljøet. I tillegg kan det være verdifullt å få informasjon om metabolske produkter som kan påvirke eksisterende mikrobielle samfunn.

En revurdering av gjeldende nasjonale og internasjonale regulatoriske og politiske rammeverk kan være nødvendig. Dette kan omfatte en evaluering av de mest hensiktsmessige virkemidlene for å styrke disse rammeverkene og redusere risiko for human helse og miljøet. Eksempler på virkemidler kan være produkterklæringer, forskrifter, standarder, og regler for god praksis.

Abbreviations and glossary

1.1 Abbreviations

AB	Antibiotic
ARD	Aromatic ring dioxygenases
BAP	Benzo[a]pyrene
CFU	Colony-forming units
CYP	Cytochrome P450-dependent monooxygenases
DBP	Dibenzo[a,l]pyrene
DCE	Dichloroethane
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
DNA-SIP	DNA stable isotope probing
EC	European Commission
EEC	European Economic Community

EPA	Environmental Protection Agency
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HG	Mercury
HR	Hazardous rating
IARC	International Agency for Research on Cancer
ISO	International Organization for Standardization
GMO	Genetically modified organism
MEHG	Methylmercury
$NAHCO_3$	Sodium bicarbonate / sodium hydrogen carbonate
NIH	National Institutes of Health
NO	Nitric oxide
OECD	Organization for Economic Co-operation and Development
PAHS	Polycyclic aromatic hydrocarbons
PCBS	Polychlorinated biphenyls
PCE	Perchloroethene / Perchloroethylene

PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonate
TCE	Trichloroethylene
UV	Ultraviolet light
VC	Vinyl chloride
YOPI	Young, old, pregnant, immune compromised

1.2 Glossary

Bioaugmentation - The supplementation of pre-grown microbial cultures to existing microbial population to enhance degradation of toxic and xenobiotic compounds in contaminated soil and water.

Bioremediation - The use of organisms to degrade toxic substances to less toxic alternatives.

Biostimulation - The injection of nutrients and other supplementary components to native microbial populations to enhance the rate of propagation and thereby stimulate the degradation of pollutants/unwanted compounds.

Background as provided by the Norwegian Environment Agency

Introduction

Bioremediation is a method to remove chemical substances from contaminated soil or water. Bioremediation is defined by Cookson Jr (1995) as: «the application of biological treatment to the cleanup of hazardous chemicals by metabolic conversion into non-toxic substances". According to OECD (2015) bioremediation is a cost-effective and gentle method for the treatment of polluted masses, compared to conventional treatment, such as excavation and incineration of masses or burying in specialized landfills. There are numerous examples in published literature documenting efforts to clean up different types of hazardous chemicals such as heavy metals, explosives, chlorinated compounds etc by bioremediaton. Applications of bioremediation vary from the household level to large-scale industrial projects. In Norway, field trials with bioremediation have amongst others been performed on soils contaminated with hydrocarbons and explosives.

Three strategies of bioremediation are mentioned in the literature today; natural attenuation, biostimulation and bioaugmentation. This assignment is concerned with the use of bioaugmentation; the addition of specialized microorganisms, in the bioremediation of contaminated soils.

Regulatory background

Plans to utilize bioremediation as a treatment procedure of polluted masses are assessed pursuant to the Act on Protection against pollution and waste (Pollution Control Act of 13 March 1981) and the Regulation on the limitation of pollution (Pollution regulations of 1 June 2006 no. 931). If the bioremediation measure includes the addition of specialized organisms in a product, the products will be assessed as any other product under the Product Control Act of 6 November 1976, in addition to separate regulations of 22 January 1998 no. 93 relating to the Declaration and labeling of microbiological products involving release to the environment (Regulation on microbiological products). The purpose of the Regulation on microbiological products is to prevent microorganisms in microbiological products from causing damage to health or adverse environmental effects such as disruption of ecosystems, pollution, or waste. According to the Regulation any person that manufactures or imports microbiological products or places them on the market in Norway has a duty to declare any information necessary for an assessment of the risk the product poses to damage of human health or detrimental environmental effects. The information is to be given in a declaration form (cf. appendix of the regulation) and amongst others include a description of the product and its composition, area of application, mode of use, and degradation products, antibiotic resistance and any pathogenic properties of the microorganisms. The guidelines to the regulations provide detailed description of the type of

information and what documentation is required in to satisfactorily declare the product. The information provided shall give the authorities a basis to assess the health and environmental risks associated with the use of the products.

The Norwegian Environment Agency considers that there is a need to update according to current knowledge, the information requested and the methods by which this information has been obtained, to sufficiently evaluate the health and environmental risks of added microorganisms in bioremediation. The Norwegian Environment Agency also sees the need for an updated scientific assessment of the current knowledge regarding bioremediation of contaminated soil to support the current risk assessment of microorganisms in the bioremediation of polluted masses.

Terms of reference as provided by the Norwegian Environment Agency

The Norwegian Environment Agency asks VKM to:

1) Collate an overview of bioremediation of polluted ground based on bioaugmentation described in literature for the degradation of various types of pollutants including hydrocarbons (including diesel, gasoline, kerosene, PAHs etc.), heavy metals, chlorinated compounds (PCBs), explosives etc. For each type of chemical briefly describe the most common microorganism classes with regards to properties relevant for environmental and health risk assessment (spore formation, survival, proliferation, pathogenicity, and more).
2) On the basis of initial assignment and the Regulation on microbiological products, assess if the information requirements in the declaration form of the Regulation of Microbial products (and as further specified in the guidelines to the regulation) provide sufficient grounds to conduct a health and environmental risk assessment of added microorganisms for bioremediation of polluted ground in Norway.
3) If no in section 2) assess which information requirements must / should be set in order to adequately risk assess added microorganisms in bioremediation of polluted ground focusing on (a) general requirements (b) specific requirements for classes of microorganisms for example if related to the degradation of specific contaminants / pollutants

It must be considered whether the proposed information requirements may be obtained using internationally recognized methods available today. The assessment must include an evaluation of whether there is sufficient knowledge to carry out risk assessments of added microorganisms in bioremediation in Norway. Critical knowledge gaps for risk assessment of microorganisms must be included in the assessment.

The following is not included:

- Assessment of genetically modified microorganisms (GMMO) are not included in the assignment as they are regulated under a different set of legislation and related procedures
- Phytoremediation is not included as it concerns the use of plants
- Bioremediation based on natural attenuation and bio-stimulation is not included
- Biodegradation, including composting, that fall under other legislation, is not included

Assessment

1 Literature

1.1 Background literature provided by the Norwegian Environment Agency

Regulations of 22 January 1998 no. 93 relating to the Declaration and labeling of microbiological products: https://lovdata.no/dokument/SF/forskrift/1998-01-22-93 https://www.regjeringen.no/en/dokumenter/declaration-and-labelling-of-microbiolog/id440456/

Cookson, JT (1995) Bioremediation Engineering: Design and Application, McGraw Hill, New York

OECD (2015) Biosafety and the Environmental Uses of Micro-Organisms: Conference Proceedings, OECD Publishing

1.2 Literature searches

The following search terms and combinations thereof were employed: Bioremediation, remediation biotechnology, in-situ/ex-situ bioremediation, hydrocarbons, heavy metals, metal pollution, petroleum pollution, bioremediation & halogen, bioremediation and halo-organic substances, nitrogen, nitro-aromatic compounds, phosphorus, bacteria, fungi, aerobic/anaerobic biodegradation, bioremediation & antibiotic resistance, soil bacteria & resistome, health and environmental implications.

Sources: Pubmed, oria.no, Google scholar, ISI web of knowledge, and Scopus.

Search results were analysed for those that were of relevance. Each working group member performed relevance screening independently. The reference lists in selected citations were further assessed to identify additional articles that were not identified by the initial searches.

2 Introduction

Abandoned polluted areas, but also advancements in the chemical industry combined with increased sophistications in various technologies comes with the release of a more complex mixture of pollutants from production processes to end-product use and disposal into the environment. Consequently, remediation technologies have had to adapt to meet the growing demand and concern from the public and regulatory authorities towards more sustainable approaches concerning disposal of such toxic waste effluents. Thus, exploring environmental biotechnology could make significant contributions in decontaminating polluted environments in a sustainable manner. One such approach is bioremediation that employs microorganisms to biodegrade toxic substances to non-toxic substances by metabolic conversion. Bioremediation competes effectively with conventional approaches such as incineration; destruction of pollutant by combustion, solidification; encapsulation into cement, and thermal desorption. Presently, the petroleum, creosote, solvent and to a lesser extent, the pesticide industries actively employ bioremediation in their clean-up processes (OECD, 2015; Tyagi et al., 2011).

Since bioremediation harnesses natural processes and low energy input is required, this technological approach is apparently cost-effective, more convenient and causes less environmental stress compared to conventional remediation approaches (OECD, 2015). However, there are some potential risks with the use of bioremediation in cases where the process may fail because of low bioavailability of the substance, or extreme toxicity in the microenvironment, and the partial breakdown of compounds to intermediates that are more toxic than the parent compound, to mention a few (OECD, 2015; Tyagi et al., 2011). With bioaugmentation, the focus of this report, the introduction of exogenous microbial consortia to the endogenous microbial community comes with an inherent risk of adversely affecting the functionality of the native community and the ecosystem at large (Tyagi et al., 2011). Most countries have thus developed guidelines or protocols for the risk assessment of the release of microorganisms into the environment.

In Norway, the use of microorganisms in bioremediation as a treatment procedure of polluted masses are assessed pursuant to the Act on Protection against pollution and waste (Pollution Control Act of 13 March 1981) and the Regulation on the limitation of pollution (Pollution regulations of 1 June 2006 no. 931). If the bioremediation measure includes the addition of specialized organisms in a product, the products will be assessed as any other product under the Product Control Act of 6 November 1976, in addition to separate regulations of 22 January 1998 no. 93 relating to the Declaration and labelling of microbiological products involving release to the environment (Regulation on microbiological products). In view of these regulations, the Norwegian Environment Agency sees the need for updating the information requested and the methods by which such information is obtained, based on current knowledge in order to evaluate the health and environmental risks related to the use of microorganisms in bioremediation efficiently.

This report provides an updated scientific overview of existing current knowledge on bioremediation with focus on bioaugmentation as a technology in decontaminating primarily hydrocarbons, heavy metals, halogenated compounds, and explosives as pollutants. In addition, the report will include specific recommendations from the working committee concerning information needed to update the regulation on microbiological products, as further specified in the guidelines to the regulation for the use of microorganisms in bioremediation, based on initial findings. The following are not within the mandate of this report:

- Assessment of genetically modified microorganisms (these are regulated under a different set of legislation and related procedures)
- Phytoremediation is not included as it relates to the use of plants
- Bioremediation based on natural attenuation and bio-stimulation
- Biodegradation regarding composting (falls under other legislation)

3 Hazardous implications of environmental contaminants

3.1 Aromatic Hydrocarbons

The deleterious impact of polycyclic aromatic hydrocarbons (PAHs) on human health and the surrounding ecosystem is due to their persistence in the environment and toxicity to the living component of the ecosystem. Persistence in the environment and toxicity to living things are linked directly to the constituent aromatic rings – number of rings and molecular topology or pattern of ring linkage. Persistence of PAHs, especially the high molecular weight PAHs (compounds with four or more aromatic rings), is generally due to size and angularity of the molecule (Heitkamp and 1987; Heitkamp et al., 1987; Kanaly and Harayama, 2000); increased size and angularity results in a concomitant increase in hydrophobicity and electrochemical stability (Zander, 1983; Zhang et al., 2000). Toxicity to living organisms is also associated with the size of PAHs. For example, PAH genotoxicity increases with molecular size up to at least four or five fused benzene rings (Cerniglia, 1992).

Exposure to PAHs in the trophic web exerts acute toxicity and/or possess mutagenic, teratogenic, or carcinogenic properties (Kanaly and Harayama, 2000; Luch, 2009; Menzie, 1992). For example benzo[a]pyrene (BaP) and dibenzo[a,l]pyrene (DBP), are respectively classified as carcinogenic (IARC Group 1) or probable (IARC Group 2) carcinogenic to human [http://www.iarc.fr/]. In the EU, BaP has been flagged as an environmental pollutant of high concern [http://echa.europa.eu/addressing-chemicals-of-concern], and has a reported toxicity value range of 0.22 ug/l to 1.5 ug/l in the aquatic environment [http://echa.europa.eu/documents/10162/13638/svhc_supdoc_pitch_publication_3296_en.pdf]. In the U.S. both BaP and DBP are considered priority pollutants (Louvado et al., 2015), and BP is included as 1 of 12 target compounds or groups defined in the Environmental Protection Agency's strategy for controlling persistent, bioaccumulative and toxic pollutants (Louvado et al., 2015). In humans and animals, metabolic activation of PAHs such as BaP or DBP is mainly catalysed by cytochrome P450-dependent monooxygenases (CYPs) (Luch, 2005). Studies of various carcinogenic PAHs revealed that CYP-mediated metabolism mainly produces the anti-diol-epoxides (Luch, 2005), which are the main DNA-binding metabolites that mediate the biological effects of their parent PAH compounds (Xue and Warshawsky, 2005).

In microorganisms, a study conducted on 16 well-known PAHs namely, napthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, pyrene, fluoranthene, chrysene, benz[a]anthracene, benzo[k]fluoranthene, benzo[b]fluoranthene, benzo[a]pyrene, benzo[ghi]perylene, dibenz[a,h]anthracene, indeno[1,2,3-cd]pyrene, revealed that harmful effects of the substances are largely determined by the extent to which a substance is partitioned between water, soil and sediment (Verbruggen, 2012). The

study assumes that certain effects of all 16 PAHs occur at the same concentrations in organisms that live in water, soil and sediment, and that they cause effects in the same way. It was also found that the internal effective concentration of PAHs does not differ between organisms in soil, water and sediment; however, large differences between the effective concentrations of the substances outside the organisms were observed. The harmful effects of the substances are thus, largely determined by the extent to which they partition between water, soil and sediment, and is taken up from water.

Natural sources such as fossil fuels and anthropogenic sources in combination with global transport result in contamination of the environment with PAHs. Petroleum refining and transport activities are major contributors to localized loadings of PAHs into the environment, such as marine environments (Coelho, 2010; Fang et al., 2008; Guitart et al., 2007; Guitart, 2010). However, PAHs released into the environment, such as terrestrial environments, may also have their sources from, for example, gasoline and diesel fuel combustion (Notar et al., 2001). From these sources PAHs find their ways in air, soil and sediment, surface water, groundwater, road runoff, vegetation and food chain (Edwards, 1983; Sims, 1983).

3.2 Heavy metals

Heavy metals, some of which are toxic, generally refer to any relatively dense metal, and a few examples are given below. Metals are natural elements and constituents of the environment, however anthropogenic activities have altered their geochemical cycles rendering some of them pollutants in many cases, thus posing a threat to the environment and human health. Metals have been linked to birth defects, cancer, neural damage, and liver and kidney damage among others (WHO-IPCS, 1990; WHO-IPCS, 1992; WHO-IPCS, 1995). For instance, lead may cause developmental neurotoxicity and impaired neurobehavioural functioning in children (WHO-IPCS, 1995). Furthermore, long term exposure to lead may cause adverse cardiovascular, effects (increased systolic blood pressure, associated with increased risk of cardiovascular mortality) in adults (WHO-IPCS, 1995). Chronic kidney disease (reduction in glomerulus filtration rate) is another toxic effect of lead (WHO-IPCS, 1995). Regarding cadmium, well characterised chronic toxicities resulting from exposure are effects on kidneys and bones (WHO-IPCS, 1992). Cadmium may induce bone effects both by a direct effect on the bone tissue or indirectly via cadmium induced renal damage. Cancer is yet another chronic effect associated with cadmium exposure and The International Agency for Research on Cancer (IARC) has classified cadmium as a human carcinogen (Group 1; on the basis of occupational studies). Regarding mercury, different species exist (organic and inorganic). Humans may be exposed to methylmercury (MeHg) e.g. through the consumption of sea food, MeHg affects the development of the fetal brain and may cause neurologic damage in adults (Dietz et al., 2013). In the natural environment, toothed wales (Odontoceti) appear to be a particularly vulnerable group, accumulating high concentrations of Hg in the central nervous system, leading to neurochemical effects (Dietz et al., 2013). Other adverse effects of MeHg include cardiovascular and reproductive effects, as well as impaired immune function (Wolfe et al., 1998). Neurotoxic effects could also be found in of neurotoxic effects of mercury in beluga

whales (Delphinapterus leucas), ringed seals (Pusa hispida), and polar bears (Ursus maritimus) (Krey et al., 2015).

3.3 Halo-organic substances

Halogenated organic compounds constitute one of the largest groups of chemicals that can potentially pollute the environment. They have inter alia been used as

- pesticide compounds, such as in the controversial fluorine-containing pesticide “1080” (Blakey) or dichlorodiphenyltrichloroethane (DDT) and its metabolites (dichlorodiphenyldichloroethane (DDD) and dichlorodiphenyldichloroethylene (DDE) (Garrison et al., 2014)
- components of dielectric fluids, such as the forbidden polychlorinated biphenyl (PCB) formerly used in transformers and capacitors
- and flame retardants in textiles (Horrocks, 2011)

This broad use of halo-organic substances has resulted in widespread dissemination in the environment (Häggblom and Bossert, 2003).

Furthermore, are halo-organic by-products such as Tetrachlorodibenzodioxin (Thuan et al., 2013), perfluorooctanoic acid (PFOA), surfactants, fire foams and other halogen substituents able to increase the hydrophobicity of organic compounds, subsequently increasing thereby the tendency to bioaccumulate in food chains and to sorb to soil (Mohn, 2004). Known examples of bioaccumulating compounds are dichlorodiphenyltrichloroethane (DDT) (Lukyanova et al., 2016) perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) (Baduel et al., 2014; Franklin, 2016; Perez et al., 2014; Surma et al., 2015; Wen et al., 2015; Zhang et al., 2015).

3.4 Nitro-aromatic compounds

Nitro-aromatic compounds are regarded as stable, persistent and are generally considered as poisonous when exposed by oral, subcutaneous, intraperitoneal or intramuscular routes. They exhibit human mutagenic and carcinogenic potential, and are potent uncouplers of oxidative- and photo-phosphorylation. Toxic fumes of NOx may be emitted during decomposition. All these properties render them hazardous to the environment. Therefore, all nitro-aromatic compounds are given hazardous rating (HR) 3, where 3 denotes the worst level of hazardness/toxicity (Sax and Lewis, 1999).

The electron-withdrawing character of nitro-aromatics makes oxidative attack by oxygenase from aerobic bacteria difficult. The susceptibility to electrophilic attack resulting in reduction of nitro groups is increasing with increasing number of nitro-groups and electron withdrawing substituents. The result is that nitro-aromatic compounds are readily reduced to more reactive carcinogenic derivatives when introduced into mammalian systems. Epidemiological data indicate that nitro-aromatic compounds (including nitrobenzene,

dinitrotoluenes, mono- and di-nitrophenols) are powerful carcinogens, and they are consequently referred to as priority pollutants (EPA, 2003).

Consequently, waste generated from mono-, di-, poly-nitro-aromatics are regulated by the US Environmental Protection Agency as toxic wastes (Nishino et al., 2000).

4 Methods for mitigating pollutants

4.1 In situ bioremediation

In-situ bioremediation is the use of microorganisms to degrade pollutants in place to less toxic products. One method of application is monitored natural attenuation, also referred to as intrinsic bioremediation that employs indigenous microorganisms without human intervention but requires site-characterization and long-term monitoring for implementation (OECD, 2015).

Although emerging technologies are on the rise, bioaugmentation as well as biostimulation have become pivotal in in-situ bioremediation due to their complementary nature (Tyagi et al., 2011). Bioaugmentation, the focus of this report, involves supplementation of pre-grown microbial cultures to existing microbial population to enhance degradation of toxic and xenobiotic compounds in contaminated soil and water. Due to the complexity of most polluted sites, customized strategies are often required to meet specific environmental conditions. These include addition of pre-adapted pure strain or consortium, introduction of genetically engineered bacteria or relevant genes packaged into a vector among others (Tyagi et al., 2011). The selection of competent microbes based on microbial communities inhabiting target sites and use of a consortium as opposed to pure cultures have been found to be most effective as it provides the metabolic diversity as well as robustness needed (Nyer et al., 2003; Rahman et al., 2002; Tyagi et al., 2011). Additionally, the target metabolism of the bioremediation system depends on the contaminant of concern since some pollutants such as petroleum hydrocarbons are degraded aerobically whereas others take the anaerobic pathway and yet a third group that are biodegraded under either conditions (OECD, 2015; Tyagi et al., 2011). Concurrent bioaugmentation with anaerobic halorespiring and aerobic degrading bacteria in the remediation of polychlorinated biphenyls in contaminated sites is a highly efficient process achieved by the corresponding sequential halorespiratory activity that converts higher chlorinated congeners to lower congeners susceptible to aerobic degradation (Payne et al., 2013). There are, however, some drawbacks concerning the process as microbial inoculants are produced under controlled conditions in bioreactors and as such do not always survive the biotic and abiotic stresses (fluctuations and extremes in temperature, pH, nutrient, competition between introduced and indigenous biomass among others), when exposed to the complex natural habitat. The use of microbial cell encapsulation or immobilization provides support by controlling the flow of nutrients, lowering toxicity in the microenvironment and offers improved survival rates by shielding cells and enabling efficient biodegradation (Tyagi et al., 2011).

Nutrient supply is essential in microbial degradation under all conditions (Horel and Schiewer, 2009; Tyagi et al., 2011). Injection of nutrients and other supplementary components such as oxygen to the native microbial population enhances the rate of propagation and thereby stimulates the degradation of unwanted compounds, and is

referred to as biostimulation. In the case of petroleum hydrocarbon spills where the carbon source is readily available to the existing microorganisms, addition of nitrogen and phosphorus, the rate-limiting nutrients substantially improves the degradation process and thus the decontamination rate. In some contamination sites, there has been need for the concurrent application of biostimulation as well as bioaugmentation to produce synergistic effects (Tyagi et al., 2011). These two techniques are thus complementary rather than competing.

The primary function of fungi in ecosystems is that of decomposition, by nature. Mycoremediation refers to the deliberate use of fungi to degrade contaminants. Bioventing, biosparging and bioslurping are various in situ remediation technologies that have evolved to meet specific environmental conditions related to groundwater and employ additives like oxygen in order to facilitate the degradation process.

It is worth noting that phytoremediation (excluded from this assessment) involves the use of plants to remediate based on their ability to remove, transfer, concentrate or metabolize elements by various processes influenced by their roots, is particularly useful in mitigating heavy metal pollutants ((EPA, 2006), see section 4.4 for details).

4.2 Ex situ bioremediation

As its name implies, ex situ bioremediation usually involves removal by excavation or extraction and transportation of contaminants or waste to process site for treatment and can simply be classified by the physical state of the contaminated material for which the application is required, namely, solid-phase, liquid-phase and slurry-phase bioremediation (EPA, 2006).

Solid-phase bioremediation utilizes an aboveground treatment center. The most common treatments for solids are biopiles, land farming and composting (EPA, 2006). Land farming is a simple process in which the excavated soil is spread over a characteristic pad with the capacity to collect any residual fluid from the soil, which is then turned regularly to allow transport of oxygen and thereby enhance the degradation process of endogenous microbes (Rittmann et al., 2001). Soil biopiles involves a collection area equipped with an aeration system/vacuum pump that allows oxygen to readily reach the existing microbial community and potentially suck up volatile contaminants. These are typically 2-3 m high and to stimulate degradation, environmental conditions such as moisture, pH, temperature, nutrients are controlled (EPA, 2006). In composting the soil is mixed with organic wastes and bulking agents such as straw or corncobs to enhance porosity and thereby degradation.

Treatment of solid-liquid mixtures such as sludge wastes and slurries employs bioreactors among others. Constructed wetlands that involve the biological assimilation, breakdown and transformation of pollutants are commonly used for liquids (EPA, 2006). Concurrent applications of bioaugmentation or phytoremediation are usually employed.

Taken together, in-situ bioremediation is considered simpler and convenient but long-term whereas ex-situ bioremediations are short-term but inconvenient due to the need for excavation or extraction, and transportation in some cases. Generally, these processes, with their inherent benefits as well as drawbacks are adapted for specific situations.

4.3 Polycyclic Aromatic Hydrocarbons

Apart from the use of microorganisms, PAHs can also be removed from polluted environments by chemical methods, thermal methods and UV light irradiation. UV light technology has been used in PAH removal from solid matrices such as soil and sludge (Dong et al., 2010),(Zhang et al., 2008). UV rays cause photodegradation of PAHs by breaking the benzene ring in PAH's structure in the presence of radicals (Zhang et al., 2008),(Guieysse et al., 2004). Titanium oxide, methyl orange and ammonium chloride are examples of catalysts that can aid photodegradation of PAHs by UV light (Zhang et al., 2008).

In the chemical method, the two most commonly applied techniques to clean up PAH contaminated sites are soil washing by water and solvent (polar) extraction. The main factor which governs the efficiency of this process is the solubility of PAHs in the extraction solvent. These two approaches are however inefficient, toxic and expensive, especially with respect to removal of high molecular PAHs. Various alternative extraction agents are being studied, including surfactants, biosurfactants, microemulsions, natural surfactants, cyclodextrins, vegetable oil and solution with solid phase particles. These extraction agents have been found to remove PAHs from soil at percentages ranging from 47 to 100% for various PAHs (Lau et al., 2014), but information on the toxicity of these approaches is lacking.

Thermal treatment (indirect heating) is also used in the removal of PAHs, especially from petroleum sludge, which contains high level of high molecular weight hydrocarbons. In most thermal treatments, degradation of PAHs is achieved at temperature range of 250ºC – 650ºC using $Ca(OH)_2$ + $NaHCO_3$ as an additive (Pakpahan et al., 2013). However, high temperatures used for this treatment cause the modification of PAH structure, such as intramolecular rearrangement and molecular weight growth by acetylene addition. These contribute to the presence of residual PAH in the resultant biochars, sometimes in concentrations above the recommended safe levels (Pakpahan et al., 2013).

4.4 Heavy metals

Conventional methods for removal of metals from soil or water include for instance land filling (excavation, transport and deposition of contaminated soil), fixation (chemical processing of soil to immobilize the metals in soil, often followed by measures to prevent penetration by water), leaching (applying acid solutions to desorb and leach metals from soil), precipitation or flocculation (in water) followed by sedimentation and disposal of sludge, ion exchange (in water), reverse osmosis (in water), microfiltration (of water), electrodialysis (of water), and evaporation (Wise et al., 2000).

Bioremediation measures against metal pollution may include different mechanisms. Microbes may be used to covert metals into non-toxic forms. The process may involve oxidation, binding, immobilization, volatilization, or transformation. It must be pointed out that the metals are elements and thus cannot be degraded; however, remedial processes transform their oxidation state or organic complex (Wise et al., 2000).

Phytoremediation (not assessed here) is another bioremediation measure and refers to the use of plants and associated microorganisms to remove specific contaminants from soil, sludge, sediments or water. Phytoremediation may also include different processes, including phytoextraction (uptake of contaminants from soil or water by plant roots and translocation into biomass), phytofiltration (adsorption to e.g. roots to minimize movement in water), phytostabilization (reduction of mobility and bioavailability to prevent migration to water or the food chain) and phytovolatilization (e.g. absorption of metals by plants, followed by conversion into volatile forms that are released to the atmosphere).

4.5 Halo-organics

Halo-organic compounds are not easy degradable by nature. Technically possible, but often non-economic, in situ remediation methods for halo-organic contaminants include i.a. and often in combination:

- Thermal remediation, such as the use of microwave remediation of hexachlorobenzene treated soil (Calvert and Suib, 2007)
- Chemical remediation, such as the use of zero-valent iron against chlorinated hydrocarbon compounds via reductive dehalogenation (Gillham and O'Hannesin, 1994)
- Physical remediation, such as by soil vapour extraction (Albergaria et al., 2012)

Phytoremediation is also for halo-organic compounds increasingly used. Plants remediate halo-organic compounds by phytotransformation (metabolic conversion), phytostabilisation or by supporting microbial bioremediation. Examples for phytoremediation include the use of poplar trees in remediation of trichloroethylene (TCE) (Gordon et al., 1998) or *Echinacea purpurea* against polycyclic aromatic hydrocarbons (PAHs) (Liu et al., 2015).

Microbial bioremediation of halo-organics in a stand-alone approach is difficult, as the potential for bioremediation requires that a halo-organic compound can be biodegraded, partly or completely destroyed by metabolism.

4.6 Nitro-aromatics

The reduction of nitro-groups to nitroso derivatives, hydroxylamines or amines is catalyzed by nitroreductases. Anaerobic transformation of nitro-aromatics by reduction of nitro groups to aromatic amines is receiving increased attention (Razo-Flores et al., 1997). It has been shown that most of the poly-nitro-aromatics are susceptible to degradation only under anaerobic conditions (Nishino and Spain, 2002; Zhang and Bennett, 2005). However, single

species of anaerobes are rarely capable of complete conversion of nitro-aromatic substrate to CO_2 or methane (Razo-Flores et al., 1997). Thus, synergistic effort of a consortium of microbes is required for complete as well as partial degradation of several compounds, as e.g. dinitrotoluene, 3,5-dinitrobenzoic acid, 2-, 3- and 4-nitrophenol or TNT (Alexander, 1999; Hess et al., 1990).

The rate of reduction of nitro compounds is determined by the chemical properties of the entire molecule, i.e. number of rings, number and types of substituents. Several different pathways are postulated for degradation of a wide range of nitro-aromatics, involving complex systems of bacterial and fungal extra-cellular non-specific enzymes.

Anaerobic bacteria reported to perform biodegenerating effects on common nitro-aromatics are shown in Table 1 (Appendix I).

5 Rationale for the use of microorganisms in remediating polluted environments

Hazardous pollutants are frequently introduced and thereby pollute the environment despite attempts by various countries to prohibit or reduce this through regulations. Being recalcitrant, the pollutants can persist and accumulate in the environment if not removed. Therefore, efficient ways to remove or convert them into forms that are harmless or utilizable in the ecosystem are required and being steadily developed. Microorganisms are found everywhere, can adapt to most environmental conditions and possess inherent abilities to metabolize most compounds and utilize them as energy sources or bio-convert them into forms that can easily be absorbed and/or utilized by other life forms in the ecosystem. This ubiquity, versatility, malleability and adaptability makes microorganisms and lower plant forms efficient agents of bioremediation. For example, bioremediation can be applied to areas that are not accessible without excavation, e.g. in the remediation of groundwater contamination, which is cost effective. Moreover, advances in microbial culture techniques, genome sequencing, genetic engineering and 'omics' are providing unprecedented insights into different biochemical pathways, regulatory networks to carbon flux in particular environments and particular compounds, and molecular adaptation strategies to changing environmental conditions. These have enabled the identification and engineering of organisms, which can be utilized in the remediation of contaminated extreme environments. Thus, identified bioremediation-useful genes of some extremophiles can be engineered into other microorganisms (or vice versa) (Brim et al., 2000); the microorganisms are subsequently 'trained' and used to remediate contaminated sites.

Justification for the use of microorganisms to remove polluting hazardous substances, similar to other biotechnological fields employing microorganisms, is the safety and efficiency of the process. Unlike chemical remediation of environmental pollutants whose products in themselves can constitute environmental hazards, and can be injurious to resident indigenous microorganisms, the final products of bioremediation are the environmentally benign compounds of fatty acids, CO_2, NH^{4+} or H_2O, while the intermediate by-products are usually energy sources for other organisms. However, some intermediate metabolic products can be toxic (Podar et al., 2015). Vinyl chloride, a partially dehalogenated analog of tetrachloroethane, is a known carcinogen (Delgado et al., 2014; Holmes et al., 2006; McCue et al., 2003; Nielsen and Keasling, 1999). Nonetheless, it is not impossible to discover or engineer microbial life forms that can metabolize and thus remediate such toxic intermediates (Brim et al., 2000).

Application of microorganisms as bioremediators is not without some risks given that some microorganisms are agents of diseases. This has informed the use of non-pathogenic strains

in bioremediations. In addition, potential pathogenic genes can be identified and removed from the genomes of bioremediators with high promise through genetic engineering techniques. For specially engineered or where non-indigenous microorganisms are applied to particular sites, once the contaminants are completely metabolized or removed, their food chain is cut-off and the microbes should theoretically die and become food sources themselves for aquatic and terrestrial organisms. However, there are exceptions where the microorganisms continue to live.

6 Microbial features utilized in Bioremediation

The diversity of microorganisms that can potentially be used for remediation purposes is huge. Some important microbial features utilized in bioremediation are described below. However, properties relevant for environmental and health risk assessment (spore formation, survival, proliferation, pathogenicity etc.) for such a vast variety of microorganisms would be too extensive to describe in this report.

6.1 Bacteria

6.1.1 Polycyclic Aromatic Hydrocarbon Bioremediation

There is a substantial body of literature highlighting the great diversity in microbes (bacteria and fungi; fungi is described in a later section) that are capable of degrading PAHs. For example at the time of writing this report, a PubMed search with the string 'Bioremediation, Aromatic hydrocarbon, Bacteria' returned 6218 hits. These microbes harbour different and often interconnected pathways required to achieve the bioremediation of sites contaminated with PAHs and other co-occurring contaminants. Several review articles have summarized bacteria involved in PAH biodegradation, for example (Bressler and Fedorak, 2000; Cerniglia, 1984; Cerniglia, 1997; Diaz and Prieto, 2000; Dua et al., 2002; Fernandez-Luqueno et al., 2011; Giessing and Johnsen, 2005; Johnsen and Karlson, 2005; Johnsen et al., 2005; Kanaly et al., 2000; Meckenstock et al., 2004; Samanta et al., 2002; Watanabe, 2001; Zylstra et al., 1997). The bacteria described in these articles were isolated based on the traditional isolation methods and pure culture studies of their PAH degrading abilities. The most recent reviews (Aranda, 2016; Castillo-Carvajal et al., 2014; El Amrani et al., 2015; Fuentes et al., 2014; Jeon and Madsen, 2013; Louvado et al., 2015; Vila et al., 2015) also included more modern identification methods some of which do not require isolation and culture of the bacterial species. Although bacterial activities in PAH degradation are known to be distinct in both terrestrial and aquatic environments, this report focused on bacteria associated with PAH degradation in terrestrial environments. A historical approach in chronicling common PAH degrading bacteria genera has been adopted. This highlights different technological advancements that enabled the bacterial identification in PAH degradation.

In published articles leading up to the year 2000 (see also review by Kanaly and Harayama (2000)), the usual bacterial culprits in soil PAH biodegradation include species in the genera *Pseudomonas* (Barnsley, 1975), *Sphingomonas* (Khan et al., 1996), *Alcaligenes* (Weissenfels, 1991), *Mycobacterium* (Kelley, 1995), *Rhodococcus* (Walter, 1991), *Gordona* (Kästner, 1994), *Flavobacterium* (Trzesicka-Mlynarz and Ward, 1995), *Cycloclasticus* (Geiselbrecht et al., 1998), *Burkholderia* (Juhasz, 1997). Studies in the identification of bacteria in PAH degradation were mainly by isolation, pure culture and single substrate incubation.

The years 2000 to 2010 witnessed multiple substrate incubation, mixed bacteria culture and the use of molecular techniques such as PCR and pyrosequencing in studies related to bacteria PAH degradation. During this time frame, additional new species were described in association with PAH degradation. These belong, predominantly, to the genera *Achromobacter* (Wang, 2008), *Acinetobacter* (Nwanna, 2006), *Aeromonas* (Saagua, 2002), *Aquamicrobium* (Andreoni et al., 2004), *Arthrobacter* (Lors et al., 2004), *Bacillus* (Hori, 2000), *Brevibacterium* (Farahat, 2008), *Comamonas* (Widada et al., 2002), *Corynebacterium* (Lors, Tiffreau et al. 2004) (Lors et al., 2004), *Methylobacterium* (Andreoni et al., 2004), *Micrococcus* (Hori, 2000), *Moraxella* (Samanta et al., 2001), *Rhizobium* (Andreoni et al., 2004), *Stenotrophomonas* (Andreoni et al., 2004), and *Staphylococcus* (Mallick et al., 2007).

Using a combination of substrate enrichment, co-incubation, and the advanced techniques of 'omics', DNA stable isotope probing (DNA-SIP) technology, deep sequencing and pyrosequencing between 2010 and 2016, additional genera were added to the list of PAH degrading bacteria. For example, *Cupriavidus* and *Luteimonas* were recently identified in degradation of PAH in PAH-polluted soil (Jones et al., 2014). Another genus which has not previously been associated with PAH degradation, namely *Hydrogenophaga*, was the most abundant components of a pyrene-degrading consortium that were identified from soil contaminated with high molecular weight polycyclic aromatic hydrocarbons (Sun et al., 2010). Further, several new bacteria genera, namely, *Acidovorax, Rhodoferax, Pigmentiphaga* and *Hydrogenophaga*, were added to the list of PAH degrading bacteria from studies that employed the DNA-SIP technology in early response to PAH degradation (Cebron et al., 2011; Jones et al., 2011; Martin et al., 2012; Singleton et al., 2006). Also, some yet-to-be cultured members of *Proteobacteria* were revealed to be relevant in the biodegradation of anthracene and pyrene (Jones et al., 2011; Singleton et al., 2006), although their abundance was simulated by barcoded pyrosequencing (Singleton et al., 2013; Singleton et al., 2011).

6.1.2 Halo-organic substances

Microorganisms have evolved a variety of metabolic strategies for cleaving the carbon-halogen bond. Chloroorganic aliphatic and aromatic compounds serve for those bacteria as carbon and energy sources, or as terminal electron acceptors (Smidt and de Vos, 2004; Zanaroli et al., 2015). The range of biologically-mediated dehalogenation reactions and processes are summarized in a number of reviews (El Fantroussi et al., 1998; Erable et al., 2005; Ewald et al., 2007; Fetzner, 1998; Haggblom, 1992; Hardman, 1991; Janssen et al., 1994; Kurihara and Esaki, 2008; Li et al., 2010; Maltseva et al., 1999; Olaniran et al., 2004; Reineke and Knackmuss, 1988; Smidt and de Vos, 2004; Zanaroli et al., 2015). Häggblom and Bossert (Häggblom and Bossert, 2003) provide a brief overview of aerobic and anaerobic microorganisms, and their abilities to metabolize halogenated organic compounds.

So far, the following bacterial genera are mainly used for bioremediation of Halo-organic substances:

- *Desulfitobacterium* (Renpenning et al., 2015; Smidt and de Vos, 2004; Wiegel et al., 1999; Zanaroli et al., 2015)
- *Clostridium* (Li et al., 2015; Lütke-Eversloh, 2014)
- *Spirochaeta* (Kaufhold et al., 2013; Vandermeeren et al., 2014)
- *Sedimentibacter* (Jugder et al., 2015; Justicia-Leon, 2012; Maphosa et al., 2012)
- *Pseudomonas* (Hamid et al., 2013; Kaur and Parihar, 2014; Maphosa, 2010)
- *Burkholderia* (Dobslaw and Engesser, 2015; Löffler et al., 2013; Su et al., 2013)

It is important to stress that the diversity of bacteria able for biologically-mediated dehalogenation is not yet fully described, and the above mentioned list of genera is not exhaustive.

6.2 Fungi

Even if the literature on mycoremediation is less comprehensive than for bacteria, there still is a substantial body of literature highlighting the great diversity. The search string 'Soil, Bioremediation, Fungi' returned 457 hits from the last 5 years in PubMed. Bacteria are more commonly used in bioremediation, but fungal mycelia exhibit the robustness of adapting to highly restrictive environmental conditions often experienced in the presence of persistent pollutants, which makes them more useful compared to other microbes (Chanda et al., 2016). Fungi hold in general a broad spectrum of biodegrading enzymes, and both mushrooms, (toxigenic) filamentous fungi and yeasts have been shown to have effect when used in bioremediation of various pollutants as eg. PAHs, nitro-aromatic compounds, chlorinated substances, and heavy metals (Singh, 2006). As an example are white-rot fungi considered effective in degrading a wide range of organic molecules due to their release of extra-cellular lignin-modifying enzymes with a low substrate-specificity. They can therefore act upon various molecules that are broadly similar to lignin. The enzymes present in the system employed for degrading lignin include lignin-peroxidase (LiP), manganese peroxidase (MnP), various H_2O_2 producing enzymes and laccase (Singh, 2006).

7 Anaerobic biodegradation

7.1 Aromatic Hydrocarbons

In contrast to aerobic biodegradation of PAH (discussed in Section 8.1), information on anaerobic PAH degradation is limited. Aerobic PAH degradation in contaminated sites will lead to oxygen depletion resulting in an anaerobic or microaerobic environment. In such environments with decreased redox potential, denitrifying, sulphate-reducing and methanogenic bacteria become the dominant players in PAH degradation (Riser-Roberts, 1998), (Christensen et al., 2004) (al-Bashir et al., 1990), (Meckenstock, 1999), (Rockne et al., 2000). Bacteria anaerobic biodegradation of PAH is slow, however its ecological significance in terms of the impacts of intermediate breakdown products is relevant.

7.2 Heavy metals

With regards to metals, all forms of degradation (hereby anaerobic) are not applicable/relevant since metals as elements are not broken down per se.

7.3 Halo-organic substances

Anaerobic processes for halo-organics compounds has been applied for products like

- Chlorinated solvents such as PCE, TCE, TCA, DCA, CCl4, chloroform and methylene chloride (Backhus et al., 1997; Doong and Chang, 2000)
- Chlorobenzenes including di- and tri-chlorobenzene (Adrian and Görisch, 2002)
- Most pesticides including DDT, DDE, dieldrin, 2,4-D and 2,4,5-T (Baczynski et al., 2010)

7.4 Nitro-aromatic compounds

The reduction of nitro-groups to nitroso derivatives, hydroxylamines or amines is catalyzed by nitroreductases. Anaerobic transformation of nitro-aromatics by reduction of nitro groups to aromatic amines is receiving increased attention (Razo-Flores et al., 1997). It has been shown that most of the poly-nitro-aromatics are susceptible to degradation only under anaerobic conditions (Nishino and Spain, 2002; Zhang and Bennett, 2005). However, single species of anaerobes are rarely capable of complete conversion of nitro-aromatic substrate to CO_2 or methane (Razo-Flores et al., 1997). Thus, synergistic effort of a consortium of microbes is required for complete as well as partial degradation of several compounds, as e.g. dinitrotoluene, 3,5-dinitrobenzoic acid, 2-, 3- and 4-nitrophenol, TNT (Alexander, 1999; Hess et al., 1990).

The rate of reduction of nitro compounds is determined by the chemical properties of the entire molecule, i.e. number of rings, number and types of substituents. Several different pathways are postulated for degradation of a wide range of nitro-aromatics, involving complex systems of bacterial and fungal extra-cellular non-specific enzymes.

Anaerobic bacteria reported to perform biodegenerating effects on common nitro-aromatics are shown in Table 1 (Appendix I).

8 Aerobic biodegradation

8.1 Aromatic Hydrocarbons

The initial step in the aerobic catabolism of a PAH molecule is the oxidation of the PAH to a metabolite with one or two –OH radicals; this is catalysed by the aromatic ring dioxygenases (ARD) and di- or monooxygenases (Demaneche et al., 2004; Kanaly and Harayama, 2000; Seo, 2009). In soil, the diversity of genes encoding ARD among PAH-degrading bacteria has been clustered into two groups associated with Gram-positive and Gram-negative bacteria (Cebron et al., 2008). Following oxygenation, the dihydrodiol intermediate ring structure is cleaved to protocatechute and catechol catalysed by intradiol- or extradiol oxygenases (Finette et al., 1984; Schocken and Gibson, 1984). The protocatechute and catechol are further converted to tricarboxylic acid cycle intermediates (Jiménez, 2004; Nzila, 2013; Seo, 2009).

8.2 Heavy metals

With regards to metals, all forms of degradation (in this instance aerobic) are not applicable /relevant since metals as elements are not broken down per se.

8.3 Halo-organic substances

Aerobic bioremediation typically proceeds through oxidative processes to render the contaminant either partially oxidized to less toxic by-products or to fully oxidize the contaminants. Aerobic culture techniques are relatively simple compared with anaerobic culture methods. Furthermore, are aerobic processes considered the most efficient and generally applicable.

Literature about aerobic bioremediation for halo-organic-substances is not extensive. One of the few published examples is the bioremediation of 1,2 dichloroethane (1,2 DCA) and vinyl chloride (VC) (Davis et al., 2009).

8.4 Nitro-aromatic compounds

Degradation of nitro-aromatic compounds by aerobic bacteria and fungi involves mainly mono- and di-nitro-aromatics as a source of carbon and/or nitrogen and energy by complete mineralization. During the past few decades has various microbes which degrade/mineralize nitro-aromatics aerobically been identified (Table 1, see appendix I), and the catabolic pathways and enzymes involved in aerobic degradation processes have been elucidated.

The main degeneration reactions are:

1. Monooxygenase catalyzed reactions

Monooxygenase adds a single oxygen atom and causes elimination of nitro groups from mono-nitrophenols.

2. Dioxygenase catalyzed reactions

Dioxygenase introduces two hydroxyl groups with the removal of a nitro group as nitrite from the aromatic ring.

3. Meisenheimer complex formation

The partial reduction of the aromatic ring of di- or tri- nitro-compounds through addition of hydride ions leads to formation of a hydride-Meisenheimer complex (Spain, 1995).

4. Partial reduction of aromatic ring

The nitro group is partially reduced to corresponding hydroxylamine and upon hydrolysis yields ammonia.

9 Possible health and environmental implications of the microorganisms involved

9.1 Possible unwanted/unintended effects of bioremediation

The products or by-products of microbial bioremediation may potentially be even more persistent or toxic than the original contaminant, in addition is the real environment that contains contaminants mixed, unevenly distributed, and in different phases (solid, liquid, gas), which may lead to unexpected outcomes. Long-term studies regarding the effects of bioremediation are not known to the authors. Possible risks include spread of pathogens, transmission of antimicrobial-resistance genes and production of biological toxins (see section 10 for details on the recommendations to the regulation on microbial products and its guidelines).

The emergence and spread of antibiotic-resistant bacteria has accelerated the recent years, leading to problems in the treatment of infectious disease caused by bacteria (Rossolini et al., 2014; Ventola, 2015). The development of antibiotic resistance is a natural phenomenon, based on Darwinian principles. The genes for antibacterial resistance can be passed from one generation of bacteria to the next (vertically) and between bacteria of the same generation and bacteria of different species (horizontal gene transfer) (Furuya and Lowy, 2006).

9.1.1 Bacteria

9.1.1.1 Hydrocarbons

The intermediate aerobic PAH degradation products and enzymes are similar to those of other metabolic pathways and are widespread among bacteria (de Lorenzo, 2008). Thus, metabolic intermediates of bacterial PAH degradation have not been associated to any toxicity, but transient toxicity of the early intermediates during product breakdown, (in soil the estimated half-lives of 3-ring phenanthrene and 5-ring benzo[a]pyrene range from 16 to 126 days and 229 to 1400 days respectively (Peng, 2008; Shuttleworth and Cerniglia, 1995), cannot entirely be ruled out. In anaerobic and microaerobic environments, such as deep sea sediments however, many intermediates currently lack analytical confirmation, and may harbour unknown metabolic pathways. For example, the main metabolite in deep sea sediments, cyclopenta(def)phenanthrone, has not yet been detected in pyrene degradation pathways in the available literature (Louvado et al., 2015).

There is limited information in relation to the health implications of the inherent properties of bacteria commonly used in PAH degradation, but pathogenic bacteria species, in general, are not employed in PAH degradation. Nonetheless, acquisition and/or transfer of potential pathogenic genes, such as antibiotic resistant genes, between degrading and resident bacteria cannot be excluded.

9.1.1.2 Heavy metals

Mercury (Hg) is a potentially toxic element that enters the biosphere from natural and anthropogenic sources. Anoxic conditions favour the bacterial transformation of inorganic Hg to methylmercury (MeHg). Methylmercury is the most toxic form of Hg and has a greater potential for bioaccumulation than inorganic Hg. With the exception of a few syntrophic and fermentative firmicutes, Hg methylators are heterotrophic bacteria that use sulphate iron and CO_2 as terminal electron acceptors. The capability of Hg methylation can be predicted by the presence of the hgcAB genes (Podar et al., 2015). *Desulfovibrio* sp. and *Geobacter* are examples of known mercury methylating bacteria.

Podar et al. (2015) found that microbial systems engineered for degradation of chlorinated compounds often contained abundant hgcAB, and pointed out that the MeHg formation potential of dechlorinating systems is consistent with the dechlorinating capacity of some methylating *Deltaproteobacteria* and *Clostridia*, but may also be linked to the presence of hgcAC in some *Dehalococcoides*.

This suggests that there may be a potential for rendering mercury more toxic if contaminated sites are treated with certain microbes to facilitate degradation of organohalides.

Analogue issues may theoretically apply for other metals. For instance, arsenite, As(III) is more mobile and toxic than Aresenate As(V), and a wide varity of bacteria known as As(V)-resistant microbes can reduce As(V) (listed by Yamamura and Amachi (2014).

It is also known that hexavalent chromium, Cr(VI), is toxic, carcinogenic and mobile, while trivalent chromium, Cr(III), is less toxic and less mobile. Murray et al. (2005) showed for instance that microorganisms may indirectly mediate Cr(III) oxidation, as *Pseudomonas putida* strain GB-1 can rapidly oxidize Cr(III) in the presence of Mn(II). The diverse metabolism of wild-type strains of *P. putida* may potentially be used for bioremediation, as it for example has been shown in the laboratory to function as a soil inoculant to remedy naphthalene-contaminated soils (Gomes et al., 2005).

9.1.1.3 Halo-organic substances

Some partially biodegraded/dehalogenated metabolites can be more harmful than the initial compound, both with regard to intrinsic toxicity as well as mobility in the environment. For instance, vinyl chloride (VC), a volatile, toxic metabolic product and a known carcinogen, is a partially dehalogenated analog of tetrachloroethene PCE and TCE (Delgado et al., 2014;

Holmes et al., 2006; McCue et al., 2003; Nielsen and Keasling, 1999). Another example is DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene], a major environmental transformation product of DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl) ethane], which is a more potent androgen receptor antagonist than its parent compound (Kelce et al., 1995). Moreover, products of microbial reductive dechlorination of polychlorinated biphenyls (PCBs) are more effective than parent PCB mixtures at stimulating uterine contractions *in vitro* (Tsuneta et al., 2008).

A publication from 2005 (Ganey and Boyd, 2005) notices that issues of bioavailability are not considered when performing in vitro toxicity assays. Furthermore not only should the toxicity of the parent contaminants be tested, their stable transformation products produced during remediation should be tested as well. This includes bioavailability from an environmental engineering point of view (e.g., how much of the contaminant is not bound to soil constituents) and from the perspective of toxicology (e.g., how much of the exposure dose interacts with target tissue). According to the authors can those issues best be addressed using whole-organism studies (Ganey and Boyd, 2005). In addition, biologically based toxicokinetic and toxicodynamic modelling could be used to address issues of extrapolation to human risk.

9.1.2 Fungi

Filamentous fungi that produce mycotoxins also demonstrate the ability to degrade a wide variety of naturally occurring and anthropogenically generated hazardous wastes. Hence, these are emerging as excellent candidates for bioremediation. However, it appears that several regulatory factors that govern mycotoxin synthesis in these toxigenic strains also regulate their bioremediation abilities. More research is needed before the full potential of mycoremediation can be exploited simultaneously with minimized mycotoxin contamination.

10 Evaluation and recommendations to improve the guidelines for the regulation on microbiological products

This chapter has been jointly written together with the working group on “Health and environmental risk assessment of microbial cleaning products” and subsequently specified for bioremediation.

The guidelines to the regulation of 22 January 1998 no. 93 on the declaration and labeling of microbiological products consists of 9 main parts with a total of 28 specific questions, outlined accordingly as separate sections below [https://www.regjeringen.no/en/dokumenter/declaration-and-labelling-of-microbiolog/id440456/].

Although the guidelines require extensive documentation of bioremediation products, the Panel still considers that there is need for further information and above all a general modernization in the methodological approach. This is discussed in more detail below.

10.1 General information

Part 1 of the declaration (questions 1-9) and the guidelines is generally satisfactory in the current form. However, the contact details (e.g. web address) should be updated and the data sheet following the product (or another documentation stating its trade name) should be provided.

10.2 Composition of the product

Purpose of Part 2: To identify all components of the product, both microorganisms and chemical components.

This part needs to be re-evaluated in order to provide more accurate requirements for specification of the microbial and chemical composition of the product.

Question 10. Specify which microorganisms are present in the product.
An accurate identification of the microorganisms present in the product is crucial for a proper risk assessment. Allowing a specification to genus-level, will likely both increase the risk for allowing the incorporation of unknown pathogenic microorganisms in the product, and will

likely increase the risk for eventual horizontal transfer of AB resistance genes from resistant conspecifics. The taxonomic affiliation of the organisms present in the product should consequently be specified to at least species, preferably strain level. The identification could be e.g. 16 S ribosomal DNA or similar specific methods. A list of suitable methods is provided in Table 1, (p. 927) in (Emerson et al., 2008).

Concern for antibiotic (AB) resistance: Rather than specifying that the microorganism shall not be resistant to a specific list of antibiotics, we recommend that the regulation specifies the classes of antibiotics to which the organisms shall not be resistant to. AB resistance is partly due to mutations, and partly due to horizontal gene transfer. Some of the resistance mechanisms are generic, and highly transferable, and focus on the classes of AB will address this issue better.

This area also would benefit from the new molecular genetical methods, where genes coding for AB resistance can be identified (McArthur et al., 2013).

An overarching improvement in quality assurance of the data would be to specify that the laboratories providing the necessary data, are accredited to the relevant ISO-standard.

Question 11. Specify the concentration(s) of the microorganism(s) present in the product itself.

The concentration of the microorganism(s) in the undiluted product and the anticipated end-user dilution must be provided. CFU enumeration is one option, but may miss strains growing poorly on standard agar counting plates. Currently more powerful microscopical and flow cytometer based methods are developed for a number of applications. Some of these allows specification of metabolically active vs passive cells. “Best practice” for documentation of concentration must be used, and documentation of the choice of method must be given.

Question 12. Specify the chemical substances present: CAS no., chemical name, EC number, classification, content as percentage by weight.

The Panel on Microbial Ecology consider that these issues are fairly well covered by existing regulations. The Panel has no further comments to this question.

10.3 Information on any pathogenic properties of the microorganisms

Purpose of Part 3: to obtain information on any pathogenic properties of the microorganism(s)

Part three of the declaration should be extended in order to give stronger emphasis also to pathogens of animal and plants, as this is significant in environmental risk assessment. These issues are especially impotant to consider for *in situ* bioremediation purposes.

Generally, the regulation on declaration and labelling of microbiological products, the declaration form and "Guidelines for completing the declaration required according to the regulations relating to the declaration and labelling of microbiological products for applications that may involve their release to the outdoor environment" focuses on human pathogens without giving adequate emphasis to pathogens of animals and plants. The latter is significant within ecological context in environmental risk assessment.

General comments:

- There seem to be a general lack of accuracy when it comes to specification of what microorganisms that are included in the product. Without proper taxonomic classification, no meaningful risk assessment is feasible, and the applicant should be obliged to name the species and strain(s) in question as specific and accurate as possible.

- There seem to be a lack of emphasis on environmental side effects, especially on

    - the potential for persistence and spread in the environment

    - the potential for pathogenic effects on different wild species (vertebrates, arthropods, plants)

    - the potential for pathogenic effects on agricultural plants

    - the marine environment (which should be very important in Norway)

- There is little emphasis on the effects with increased use and accumulation, persistence and spread, both , in terrestrial and aquatic environments. The questionnaire should among other things ask about survival ability of the microorganisms in question in different types of environment.

There seem to be a lack of thinking about the potential for disturbance of the ecological environment and resulting long-term effects on microbial community.

Question 13. Has it been reported that the microorganism(s) or other strains of the same species have caused disease/injury in humans, animals or plants (name of the disease, host organism, disease mechanism)

Instead of relying on its own, enclosed list of pathogenic organism, the classification of the microorganisms in question should be based on well-known public lists (i.e. EU directive 93/88/EEC, October 1993; NIH Guidelines on Recombinant DNA (April 2002); Canadian Laboratory Biosafety Guidelines (2nd ed. 1996); CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (4th Edition 1999) or Forskrift om tiltaksverdier og grenseverdier for fysiske og kjemiske faktorer i arbeidsmiljøet samt smitterisikogrupper for biologiske faktorer (forskrift om tiltaks- og grenseverdier); Vedlegg 2: Liste over klassifiserte biologiske faktorer (smitterisikogrupper) https://lovdata.no/dokument/SF/forskrift/2011-12-06-

1358#KAPITTEL_9.) However, none of these classifications are based on the environmental risk of the microorganisms they deal with, and the reference should preferably be one that in addition to assessing risk for human health, also take into account the potential hazard for the environment.

If a microorganism is not listed in any of the recommended sources, or as an alternative to relying on such classification lists, other reliable sources may be used. This may be based on a literature review of the peer-reviewed scientific literature. If there is any uncertainty concerning the risk group the higher group is to be chosen until it has been made clear that the risk justifies placement in a lower risk group.

Not only the pathogenic properties of the actual strain(s) and different strains of the same species should be declared, but also species in the same genus and closely related genera, should be evaluated.

If the microorganism is related to any pathogens, it should be declared how the organism in the product/product organism differs from any closely related disease causing strains with respect to safety:

- What changes have been made to the organism to expect that it will not cause similar diseases like the related strains;
- How has the product organism been characterized to ensure it is not a pathogenic strain.

Even for non-pathogens, their ability as opportunistic organisms should be described, e.g. against immunocompromised individuals; this can be derived from the published literature. Concerning this, if the organism has been shown to cause diseases in immunocompromised individuals, a survey of the immune status of individuals in and around the environment of application should be conducted to help devise means of mitigating the risk of their infection.

Knowledge about genetic exchange between the strain in question and other strains and species should be asked for (see point 21).

It should be declared where and in which amounts the microorganism(s) normally are found. If it is ubiquitous in the environment, this may indicate lower risk of disease and other adverse effects in the environment (see section 13.6).

The declaration should include documentation of knowledge on

  - acute infectious disease in humans
  - effects of chronic exposure in humans (allergies, sensitization etc.)
- infectious disease in terrestrial animals

- infectious disease in aquatic (including marine) animals
- effect on insects and other arthropods, especially pollinating insects
- pathogenic effects on plants
- effects on microbiological ecology in environments during long-term use and/or use of large amounts
- persistence and accumulation in the environment, both , soil and aquatic

The declaration should also state to which degree there is sufficient knowledge on occurrence of virulence and resistance factors of the strain that may

  - be exchanged to pathogenic microorganisms
  - cause the current strain to act as an opportunistic pathogen

It should be stated if there is any knowledge on the bioremediation of complex mixtures.

The agent should be grouped in a Risk Group (1-4) and the underlying Biosafety Level Definition has to be stated, according to e.g. European Economic Community (DIRECTIVE 93/88/EEC, Oct. 1993), NIH Guidelines on Recombinant DNA (April 2002), Canadian Laboratory Biosafety Guidelines (2nd ed. 1996) or CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (4th Edition 1999) (see Appendix II).

Risk Group Databases can be found here:

1. Forskrift om tiltaksverdier og grenseverdier for fysiske og kjemiske faktorer i arbeidsmiljøet samt smitterisikogrupper for biologiske faktorer (forskrift om tiltaks- og grenseverdier); Vedlegg 2: Liste over klassifiserte biologiske faktorer (smitterisikogrupper) https://lovdata.no/dokument/SF/forskrift/2011-12-06-1358#KAPITTEL_9

2. American Biological Safety Association https://my.absa.org/tiki-index.php?page=Riskgroups

3. Public Health Agency of Canada http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/index-eng.php

If a microorganism is not listed in any of the recommended sources, other reliable sources may be used. There may be a publication in the peer-reviewed scientific literature that describes the agent. If there is any uncertainty concerning the risk group the higher group is to be chosen until it has been made clear that the risk justifies placement in a lower risk group

Question 14. Specify the tests that have been made to ensure that the product is not contaminated with unwanted microorganisms, particularly pathogenic microorganisms.

Also here the Panel recommends a revision of the guidelines, especially with emphasis on the methodological approach.

In the guidelines it is stated that "For products consisting solely of identified microorganisms, it will be sufficient to test for general contamination". 'General contamination' should be clearly defined. Is there contamination by human, animal and plant pathogens? A molecular approach should be combined with the culture method given that culture method may be unable to detect pathogenic bacteria forms which when they get into the body can proliferative and cause diseases. This is especially so for viruses which may be difficult to culture without adequate tissue culture and laboratory facilities. A suggestion is to use high-sensitivity molecular detection to first screen for presence of pathogens, potential pathogens, toxin-producing microorganisms and AB resistance genes. Pathogens which cannot be easily detected by standard culture methods will therefore be considered further. Also, this approach first provides an overview of the pathogens/potential pathogens present, which will then guide the culture approach and makes the culture approach more targeted.

The declaration should provide information about

- how the quality control and assessment is performed.
- which procedures that are in place for securing absence of pathogens
- any testing for known relevant virulence or resistance factors that may increase health or environmental risk
- how the microbial content within the products develops with time through and after shelf life. May contamination or other changes occurring during use increase virulence?

The application in our opinion should not necessarily rely on specific methods, as long as the methods described are scientifically adequate. A multiphasic approach should be emphasized.

The product needs to be pure before being dispersed to the environment. A pure product will ensure adequate characterization of the microorganisms and the eventual breakdown product during processing (i.e. before release to the environment).

Question 15. Specify recommended precautions to be taken in connection with use of the product (respiratory equipment, personal protective equipment, hygienic measures, etc)

This section need to be elaborated. In our opinion a declaration should include information on:

- the intended use and instructions for use

- special precautions if there are nursing homes, kindergarten or similar in close proximity to the bioremediation site.
- if the product can be used in environments with food production or animal production
- specific precautions need to be taken with waste, containers etc.
- specific precautions for use, personal protection
- storage in relation to how storage influences the microbiological composition and pathogenic properties

10.4 Information on inactivation of microorganisms

Purpose of Part 4: to determine whether or not the product contains live microorganisms. Unless the presence of live microorganisms is important for the performance of the product, they should be inactivated.

The Panel considers the guidelines to part 4 to be generally satisfactory in the current form, as products for bioremediation must contain live microorganisms.

Question 16. Does the product contain live microorganisms, including viable spores (bacteria and fungi), or cysts (protozoans)?

If the product contains organisms that form endospores, spores or cysts, procedures for activating the spores or cysts and for further cultivation should be described.

10.5 Information on where and how the product is to be used

Purpose of Part 5: to obtain information that gives an indication of the risk of unwanted establishment and dispersion of the microorganism in the environment in which the product is intended for use.

The Panel recommends that the guidelines are extended for part 5. The Panel especially suggests more emphasis concerning possible hazards for particular risk groups and clarification on the where and how the products should be used or should not be used. Possible short or long term impacts for the environment, and methods for inactivation or for sanitary quarantine of the contaminated area when used unintentionally should be discussed.

Question 17. Where is the product intended to be used, and is this environment appreciably different from the environment from which the microorganisms have been isolated?

Since the main focus of part 5 is to obtain information on unintended effects, it would be necessary to state likely environments (different from the environment of exposure) which can also inadvertently exposed to the product and in which the microorganisms can thrive. It will be necessary to describe similarities between the intended and these likely-to-be exposed unintended environments; measures on how the product(s) will be excluded from these environments should be clearly stated.

The risks linked to the use of strains which belong to species known to include opportunistic pathogens and possible hazards for particular risk groups (YOPI – young, old, pregnant, immune compromised) should be clarified; this is linked to possible restrictions in areas in close proximity to e.g. hospitals, retirement homes, and child care.

Question 18. How are the microorganisms released to the environment?

Even if notifiers provide information supporting the exposure assessment for the microorganism, models for predicting environmental fate or expression are lacking.

It should be discussed what kinds of shift in the microbial community and local community can be expected in the receiving environment, especially if exposure is chronic and frequent.

It seems it has been taken for granted that the form of the organism to be released (spores/endospores/cysts/live vegetative cells) will also inform the type of method to be used in the release. Given that companies provide only requested information ignoring information not explicitly requested, it might be necessary to also state that the form of the organism to be released and the method of spread that is adopted.

Question 19. With respect to the microorganisms, what are the typical concentrations, quantities (quantity per unit of volume, weight or area) and frequencies of application, and the total number of applications?

Data on the persistence of vegetative forms and spores in the environment should be provided, and conditions for germination of the latter should be described.

Prescriptions related to further application of the product need to be provided. Particular attention should be given in case the microorganism establishes itself in the environment (sporulation).

To help predicting how the existing ecosystem will be affected by introduction of the product information on the colonization ability (including competitive ability in relation to closely related species) in the new environment should be provided.

10.6 Description of the microorganism

Purpose of Part 6: to obtain information on any traits of the microorganisms that have a strong bearing on the risk of injury to health or environmental damage.

The Panel suggest that this part is revised. Some specific considerations are discussed below.

"Environmental Damage" is not sufficiently/explicitly defined. The declaration form focuses only on the introduction of foreign gene into the ecosystem. The environment can also be permanently altered (or damaged) if the introduction of the new organisms results in the extinction of the naturally existing closely related species (see also comment on Question

19). How the metabolic by-product arising from the bioremediation alters, for example, pH, alkalinity/acidity, etc., and how they affect resident microbial communities could be a necessary information too.

Question 20. Have the microorganisms been deliberately altered since their isolation, and if so how?

The effect of the alteration on the genetic makeup and physiology of the organisms should also be determined; e.g. a comparison with the isogenic parental wildtype at genomic and physiological (proteomic and metabolomics levels)

Although this document emphasizes deliberate alteration, non-deliberate (unexpected alteration) should also be determined, for example, after the isolate would have been passaged several times. In such cases the strains current environment (laboratory or Culture center) will be quite different from the original environment of isolation. Information such as passage number and duration of storage in the Culture Collection Centre will be useful. Therefore, information on how the Master Seed Culture and Working Cultures are maintained is necessary.

Question 21. For products containing bacteria: what pattern of resistance do they show to antibiotics (including synthetic antibacterial agents)?

Rather than specifying a list of specific antibiotics employ generic classes of antibiotics. The latter seems more adaptable to new emergent antibiotics and are well suited to (if necessary) rank the risk for antibiotic resistance based on resistance patterns / mechanisms (some of which may be transferable). It could e.g. be specified that the microorganisms cannot show antibiotic resistance to aminoglycosides, macrolides, Beta-lactam, tetracyclines, fluoroquinolones or other quinolones according to EUCAST or Nordic AST or other equivalent method. This is a disk diffusion method (Nordic AST refers to EUCAST). For comparison, the Norwegian legislation asks for resistance against amoxicillin/clavulanic acid, ampicillin, cephalothin, chloramphenicol, erythromycin, fucidic acid, lincomycin, methicillin, norfloxacin, oxytetracycline, penicillin, trimethoprime/sulfamethoxazole and vancomycin.

Question 22. Do the microorganisms have special survival mechanisms, for example the formation of spores in bacteria?

Some microorganisms form survival and dispersal structures. These are called endospores in bacteria, spores and conidia in fungi and cysts in protozoans (though the term spores is often used for protozoans as well). Adverse environmental conditions often trigger the formation of spores and spore like structures.

10.7 Ecological effects related to degradation processes

Purpose of Part 7: to obtain information on any environmental effects of the product, with special emphasis on the formation of harmful intermediate products during the degradation process.

Question 23. Give a brief description of metabolic pathways for the degradation of the pollutants in question or, if appropriate, of similar compounds

We consider that these issues are well covered by existing regulations. We have no further comments to this question.

Question 24. Does the degradation process involve the formation of stable intermediate products with different and/or environmentally more harmful properties than the original compounds (toxicity, biodegradability, bioaccumulation potential, solubility in water, carcinogenicity)? Specify the chemical identity of any environmentally harmful intermediate products.

A comprehensive list of suggested tests is provided in the guidelines. However, in general such lists are in danger of being obsolete and consequently must be updated on a regular basis. In order to avoid this, we suggest a more general formulation, as e.g.: If a literature review does not provide adequate documentation, standard tests generally accepted as the currently best tests for the purpose (e.g. OECD/ISO tests) are recommended as a starting point for answering question 23 and 24. Even if such tests are carried out under conditions which may be significantly different from natural environmental conditions, they will give a good indication of whether harmful intermediate compounds are formed. Appropriate tests should be selected on the basis of whether the product is to be used in a fresh-water, marine or terrestrial (soil) environment. The rationale for the use of the selected test should be given.

Documentation may also be obtained by means of simulated environmental studies. However, a satisfactory simulation study can constitute an entire research project in itself, and often requires extensive resources."

Given that the pollutants could be complex mixtures, as noted in the guidelines for question 23 it will be difficult for applicants to specify the chemical identity of any environmentally harmful intermediate products. Another alternative will also be to change "any environmentally harmful intermediate products to "main environmentally harmful intermediate products".

Question 25. Can the product have undesirable effects on important natural microbial processes in the environment, for example nitrogen/phosphorus cycles and carbon mineralization, or by altering pH or oxygen concentration?

Biological functions usually are extremely specific Bioremediation could consequently disturb the ecosystem. In order for the correct microbes to be present, the appropriate

environmental conditions, levels of nutrients, and contaminants need to be met. As an example very low (<3) and very high (>9) pH values or sudden changes in pH can significantly inhibit microbial growth, gas solubility in soil water, nutrients availability and solubility of heavy metals (Mohee and Mudhoo, 2012). The availability and/or toxicity of metals necessary (often in trace amounts) for plants and microbes is usually dependent on pH and metals are generally becoming more mobile/available at higher pH values. Information on possible physiological and biochemical ecological changes and the prediction of the development of natural microbial communities (resistant to change, resilient or development of new but functionally similar communites) is consequently valuable (Allison and Martiny, 2008). However, given the complexity, is it difficult to provide a complete overview of possible undesirable effects on important natural microbial processes in the environment. Consequently, we consider that these issues are well covered by existing regulations and have no further comments to this question.

10.8 Other relevant information

Question 26. Give any other information in the form of empirical or test data that is relevant to the hazard to health or the environment posed by use of the microbiological product, and to which the importer / manufacturer has or should have access.

We consider that these issues are well covered by existing regulations and have no further comments to this question.

10.9 Overall assessment of risk to human health and the environment

Question 27 Give an overall assessment of the risk to human health and the environment posed by use of the product.

The guidelines here are very open and general. Specific guidelines should be provided on how this should be done, as for instance demand simple risk assessment diagrams.

The question should be more specified. A risk assessment should be given for:

1) Health hazards caused by microorganisms in the product
   a) Intended use of the product
   b) Wrong (unintended) use of the product
2) Environmental hazards caused by microorganisms in the product
   a) Intended use of the product
   b) Wrong (unintended) use of the product
3) Health hazards caused by chemicals in the product
   a) Intended use of the product
   b) Wrong (unintended) use of the product
4) Environmental hazards caused by chemicals in the product

a) Intended use of the product
b) Wrong (unintended) use of the product

11 Uncertainties

For the purpose of clarity and transparency in risk assessment processes, it is recommended that assessments identify areas of uncertainties and state clearly their subsequent impact on the overall assessment outcome since this is critical in subsequent selection of risk management options (EFSA draft opinion).

Most of the work done on bioremediation of most compounds are lab experiments or *in situ* pilot experiments. There is therefore limited data from full field-scale bioremediation systems for specified sources of uncertainty to be iterated.

The interactions between even a well-described microorganism and the biotic and abiotic environment they are released into are complex. Taking also into consideration the inherent lability of biological systems, there will always be considerable uncertainty about the long-term impact on health and environment regarding the use of microorganisms in remediation.

The long-term effects of bioremediation is uncertain due to the risk of evolution of pathogenicity in microbes that are continuously exposed to the environment.

Generally, the degree of uncertainty regarding the effects of increased use of bioremediation might have on health and environment, inherently has to be high. This uncertainty is partly related to the dearth of scientific research on adverse effects of the application of bioremediation in the Norwegian environment, but even if these knowledge gaps (see section 13) were filled before microorganisms are introduced into a complex ecosystem (including the wide variety of human behaviour into the concept of “ecosystem”), uncertainty of potential impacts on health and environment may still prevail.

Notably, decisions will have to be made ahead of conclusive scientific evidence accordingly. However, the resulting ambiguity of a mixture of scientific knowledge and non-objective assumptions may be acceptable to the public if the processes and decisions are made open and the uncertainty well communicated (Van Der Sluijs, 2005).

12 Conclusions (with answers to the terms of reference)

This report is divided into two main parts according to the assignment given by The Norwegian Environment Agency.

The first part is an overview of bioremediation of polluted ground based on bioaugmentation described in literature for the degradation of various types of pollutants including hydrocarbons, heavy metals, chlorinated compounds, and explosives. The most common microbial classes with regards to properties relevant for environmental and health risk assessment (e.g. spore formation, survival, proliferation, and pathogenicity) are described.

Based on scientific assessment of the information requirements laid down in the declaration form of the Regulation of Microbial products, VKM concludes that the information requirements in their current form are not sufficient to conduct a health and environmental risk assessment of added microorganisms for bioremediation of polluted ground in Norway.

Consequently, VKM has suggested updated information requirements that producers and importers must / should meet that can be used as a basis for the risk assessment of health and environmental risks associated with added microorganisms for bioremediation of polluted ground. Both general requirements and more specific requirements are suggested. The proposed information requirements can be obtained using internationally recognized methods available today.

On the subject matter regarding the evaluation of whether there is sufficient knowledge to carry out risk assessments of added microorganisms in bioremediation in Norway, the VKM Panel is of the opinion that critical knowledge gaps exists. Specific knowledge on the microorganisms intended for usage for remediation purposes in Norway (including gene characteristics) and their performance and interactions in the environment is needed to perform risk assessments.

Assessment of genetically modified microorganisms (GMO) is not included as these are regulated under other legislation and procedures. In compliance with the assignment are phytoremediation, bioremediation based on natural attenuation and bio-stimulation or biodegradation, including composting, not included in this report.

13 Data gaps

Notably, the working group did not evaluate declaration forms regarding specific products on the Norwegian market and the extent of usage. Iterated below are data gaps identified in literature:

- There is no information on the ecological impact of the transient biodegradation intermediates of PAH degradation.
- The intermediate products of the slow anaerobic PAH biodegradation will persist long in the environment. Information on the impact of these on the environment is lacking.
- Most of the work done on bioremediation of nitro-aromatic compounds are lab experiments or in situ pilot experiments. Data are lacking from full field-scale bioremediation systems.
- Work done on aerobic bioremediation of halo-organics is scarce. Similar to nitro-aromatics, most of the published work has been performed in small-scale environments.
- The long- term effects of microbial bioremediation on halo-organic mixtures is unknown.

14 References

Adrian L., Görisch H. (2002) Microbial transformation of chlorinated benzenes under anaerobic conditions. Research in Microbiology 153:131-137.

al-Bashir B., Cseh T., Leduc R., Samson R. (1990) Effect of soil/contaminant interactions on the biodegradation of naphthalene in flooded soil under denitrifying conditions. Appl Microbiol Biotechnol 34:414-9.

Albergaria J.T., Maria da Conceição M., Delerue-Matos C. (2012) Remediation of sandy soils contaminated with hydrocarbons and halogenated hydrocarbons by soil vapour extraction. Journal of environmental management 104:195-201.

Alexander M. (1999) Biodegradation and Bioremediation. Academic Press San Diego CA, USA.

Allison S.D., Martiny J.B. (2008) Colloquium paper: resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci U S A 105 Suppl 1:11512-9. DOI: 10.1073/pnas.0801925105.

Andreoni V., Cavalca L., Rao M.A., Nocerino G., Bernasconi S., Dell'Amico E., Colombo M., Gianfreda L. (2004) Bacterial communities and enzyme activities of PAHs polluted soils. Chemosphere 57:401-12. DOI: 10.1016/j.chemosphere.2004.06.013.

Aranda E. (2016) Promising approaches towards biotransformation of polycyclic aromatic hydrocarbons with Ascomycota fungi. Curr Opin Biotechnol 38:1-8.

Backhus D.A., Picardal F.W., Johnson S., Knowles T., Collins R., Radue A., Kim S. (1997) Soil-and surfactant-enhanced reductive dechlorination of carbon tetrachloride in the presence of Shewanella putrefaciens 200. Journal of contaminant hydrology 28:337-361.

Baczynski T.P., Pleissner D., Grotenhuis T. (2010) Anaerobic biodegradation of organochlorine pesticides in contaminated soil–significance of temperature and availability. Chemosphere 78:22-28.

Baduel C., Lai F.Y., Townsend K., Mueller J.F. (2014) Size and age-concentration relationships for perfluoroalkyl substances in stingray livers from eastern Australia. Sci Total Environ 496:523-30. DOI: 10.1016/j.scitotenv.2014.07.010.

Barnsley E.A. (1975) The bacterial degradation of fluoranthene and benzo[alpyrene. Can J Microbiol 21:1004-8.

Blakey C. Assessment of 1080 Use in New Zealand. Hohonu:94.

Bressler D.C., Fedorak P.M. (2000) Bacterial metabolism of fluorene, dibenzofuran, dibenzothiophene, and carbazole. Can J Microbiol 46:397-409.

Brim H., McFarlan S.C., Fredrickson J.K., Minton K.W., Zhai M., Wackett L.P., Daly M.J. (2000) Engineering Deinococcus radiodurans for metal remediation in radioactive mixed waste environments. Nat Biotechnol 18:85-90. DOI: 10.1038/71986.

Calvert C.A., Suib S.L. (2007) An initial study into the use of microwave remediation of hexachlorobenzene treated soil using selected oxidants and coated graphite rods. Journal of Soils and Sediments 7:147-152.

Castillo-Carvajal L.C., Sanz-Martin J.L., Barragan-Huerta B.E. (2014) Biodegradation of organic pollutants in saline wastewater by halophilic microorganisms: a review. Environ Sci Pollut Res Int 21:9578-88. DOI: 10.1007/s11356-014-3036-z.

Cebron A., Louvel B., Faure P., France-Lanord C., Chen Y., Murrell J.C., Leyval C. (2011) Root exudates modify bacterial diversity of phenanthrene degraders in PAH-polluted soil but not phenanthrene degradation rates. Environ Microbiol 13:722-36. DOI: 10.1111/j.1462-2920.2010.02376.x.

Cebron A., Norini M.P., Beguiristain T., Leyval C. (2008) Real-Time PCR quantification of PAH-ring hydroxylating dioxygenase (PAH-RHDalpha) genes from Gram positive and Gram negative bacteria in soil and sediment samples. J Microbiol Methods 73:148-59. DOI: 10.1016/j.mimet.2008.01.009.

Cerniglia C.E. (1984) Microbial metabolism of polycyclic aromatic hydrocarbons. Adv Appl Microbiol 30:31-71.

Cerniglia C.E. (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351-368.

Cerniglia C.E. (1997) Fungal metabolism of polycyclic aromatic hydrocarbons: past, present and future applications in bioremediation. J Ind Microbiol Biotechnol 19:324-33.

Chanda A., Gummadidala P.M., Gomaa O.M. (2016) Mycoremediation with mycotoxin producers: a critical perspective. Appl Microbiol Biotechnol 100:17-29. DOI: 10.1007/s00253-015-7032-0.

Christensen N., Batstone D.J., He Z., Angelidaki I., Schmidt J.E. (2004) Removal of polycyclic aromatic hydrocarbons (PAHs) from sewage sludge by anaerobic degradation. Water Sci Technol 50:237-44.

Coelho F., Sousa, S., Santos, L., Santos, A.L., Almeida, A., Gomes, Newton, C.M., Cunha, A. (2010) PAH degrading bacteria in an estuarine system. In:Hamamura, N., Suzuki, S., Mendo, S., Barroso, C.M., Iwata, H., Tanabe, S. (Eds.), Interdisciplinary Studies on Environmental Chemistry. Biological Responses to Contaminants. Terrapub Vol. 1:77-87.

Cookson Jr J.T. (1995) Bioremediation engineering: design and application McGraw-Hill, Inc.

Davis G.B., Patterson B.M., Johnston C.D. (2009) Aerobic bioremediation of 1, 2 dichloroethane and vinyl chloride at field scale. Journal of contaminant hydrology 107:91-100.

de Lorenzo V. (2008) Systems biology approaches to bioremediation. Curr Opin Biotechnol 19:579-89. DOI: 10.1016/j.copbio.2008.10.004.

Delgado A.G., Kang D.W., Nelson K.G., Fajardo-Williams D., Miceli J.F., 3rd, Done H.Y., Popat S.C., Krajmalnik-Brown R. (2014) Selective enrichment yields robust ethene-producing dechlorinating cultures from microcosms stalled at cis-dichloroethene. PLoS One 9:e100654. DOI: 10.1371/journal.pone.0100654.

Demaneche S., Meyer C., Micoud J., Louwagie M., Willison J.C., Jouanneau Y. (2004) Identification and functional analysis of two aromatic-ring-hydroxylating dioxygenases from a sphingomonas strain that degrades various polycyclic aromatic hydrocarbons. Appl Environ Microbiol 70:6714-25. DOI: 10.1128/AEM.70.11.6714-6725.2004.

Diaz E., Prieto M.A. (2000) Bacterial promoters triggering biodegradation of aromatic pollutants. Curr Opin Biotechnol 11:467-75.

Dietz R., Sonne C., Basu N., Braune B., O'Hara T., Letcher R.J., Scheuhammer T., Andersen M., Andreasen C., Andriashek D., Asmund G., Aubail A., Baagoe H., Born E.W., Chan H.M., Derocher A.E., Grandjean P., Knott K., Kirkegaard M., Krey A., Lunn N., Messier F., Obbard M., Olsen M.T., Ostertag S., Peacock E., Renzoni A., Riget F.F., Skaare J.U., Stern G., Stirling I., Taylor M., Wiig O., Wilson S., Aars J. (2013) What are the toxicological effects of mercury in Arctic biota? Sci Total Environ 443:775-90. DOI: 10.1016/j.scitotenv.2012.11.046.

Dobslaw D., Engesser K.H. (2015) Degradation of toluene by ortho cleavage enzymes in Burkholderia fungorum FLU100. Microbial biotechnology 8:143-154.

Dong D., Li P., Li X., Xu C., Gong D., Zhang Y., Zhao Q., Li P. (2010) Photocatalytic degradation of phenanthrene and pyrene on soil surfaces in the presence of nanometer rutile TiO2 under UV-irradiation. Chemical Engineering Journal 158:378-383. DOI: http://dx.doi.org/10.1016/j.cej.2009.12.046.

Doong R.-a., Chang S.-m. (2000) Relationship between electron donor and microorganism on the dechlorination of carbon tetrachloride by an anaerobic enrichment culture. Chemosphere 40:1427-1433.

Dua M., Singh A., Sethunathan N., Johri A.K. (2002) Biotechnology and bioremediation: successes and limitations. Appl Microbiol Biotechnol 59:143-52. DOI: 10.1007/s00253-002-1024-6.

Edwards N.T. (1983) Polycyclic Aromatic Hydrocarbons (PAR's) in the Terrestrial Environment-A Review. J Environ Qual 12:427–441.

EFSA draft opinion. Guidance on Uncertainty in EFSA Scientific Assessment. http://www.efsa.europa.eu/sites/default/files/160321DraftGDUncertaintyInScientificAssessment.pdf.

El Amrani A., Dumas A.S., Wick L.Y., Yergeau E., Berthome R. (2015) "Omics" Insights into PAH Degradation toward Improved Green Remediation Biotechnologies. Environ Sci Technol 49:11281-91. DOI: 10.1021/acs.est.5b01740.

El Fantroussi S., Naveau H., Agathos S.N. (1998) Anaerobic dechlorinating bacteria. Biotechnol Prog 14:167-88. DOI: 10.1021/bp980011k.

Emerson D., Agulto L., Liu H., Liu L. (2008) Identifying and characterizing bacteria in an era of genomics and proteomics. Bioscience 58:925-936.

EPA. (2003) U.S. Environmental Protection Agency. EPA's Report on the Environment (2003 Draft). U.S. Environmental Protection Agency, Washington, DC, 2008.

EPA. (2006) In Situ and Ex Situ Biodegradation Technologies for Remediation of Contaminated Sites. Engineering Issue.

Erable B., Maugard T., Goubet I., Lamare S., Legoy M.D. (2005) Biotransformation of halogenated compounds by lyophilized cells of Rhodococcus erythropolis in a continuous solid-gas biofilter. Process Biochemistry 40:45-51. DOI: 10.1016/j.procbio.2003.11.031.

Ewald E.M., Wagner A., Nijenhuis I., Richnow H.H., Lechner U. (2007) Microbial dehalogenation of trichlorinated dibenzo-p-dioxins by a Dehalococcoides-containing mixed culture is coupled to carbon isotope fractionation. Environ Sci Technol 41:7744-51.

Fang M.D., Ko F.C., Baker J.E., Lee C.L. (2008) Seasonality of diffusive exchange of polychlorinated biphenyls and hexachlorobenzene across the air-sea interface of Kaohsiung Harbor, Taiwan. Sci Total Environ 407:548-65. DOI: 10.1016/j.scitotenv.2008.09.021.

Farahat L.A., El-Gendy, N.S. . (2008) Biodegradation of Baleym Mix crude oil in soil microcosm by some locally isolated Egyptian bacterial strains. Soil Sediment Contam 17:150–162.

Fernandez-Luqueno F., Valenzuela-Encinas C., Marsch R., Martinez-Suarez C., Vazquez-Nunez E., Dendooven L. (2011) Microbial communities to mitigate contamination of PAHs in soil--possibilities and challenges: a review. Environ Sci Pollut Res Int 18:12-30. DOI: 10.1007/s11356-010-0371-6.

Fetzner S. (1998) Bacterial dehalogenation. Appl Microbiol Biotechnol 50:633-57.

Finette B.A., Subramanian V., Gibson D.T. (1984) Isolation and characterization of Pseudomonas putida PpF1 mutants defective in the toluene dioxygenase enzyme system. J Bacteriol 160:1003-9.

Franklin J. (2016) How reliable are field-derived biomagnification factors and trophic magnification factors as indicators of bioaccumulation potential? Conclusions from a case study on per- and polyfluoroalkyl substances. Integr Environ Assess Manag 12:6-20. DOI: 10.1002/ieam.1642.

Fuentes S., Mendez V., Aguila P., Seeger M. (2014) Bioremediation of petroleum hydrocarbons: catabolic genes, microbial communities, and applications. Appl Microbiol Biotechnol 98:4781-94. DOI: 10.1007/s00253-014-5684-9.

Furuya E.Y., Lowy F.D. (2006) Antimicrobial-resistant bacteria in the community setting. Nat Rev Microbiol 4:36-45. DOI: 10.1038/nrmicro1325.

Ganey P.E., Boyd S.A. (2005) An approach to evaluation of the effect of bioremediation on biological activity of environmental contaminants: dechlorination of polychlorinated biphenyls. Environ Health Perspect 113:180-5.

Garrison A.W., Cyterski M., Roberts K.D., Burdette D., Williamson J., Avants J.K. (2014) Occurrences and fate of DDT principal isomers/metabolites, DDA, and o,p'-DDD enantiomers in fish, sediment and water at a DDT-impacted Superfund site. Environ Pollut 194:224-34. DOI: 10.1016/j.envpol.2014.07.025.

Geiselbrecht A.D., Hedlund B.P., Tichi M.A., Staley J.T. (1998) Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading Cycloclasticus strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of puget sound Cycloclasticus strains. Appl Environ Microbiol 64:4703-10.

Giessing A.M., Johnsen A.R. (2005) Limited microbial degradation of pyrene metabolites from the estuarine polychaete Nereis diversicolor. Chemosphere 61:1281-7. DOI: 10.1016/j.chemosphere.2005.03.093.

Gillham R.W., O'Hannesin S.F. (1994) Enhanced degradation of halogenated aliphatics by zero-valent iron. Ground water 32:958-967.

Gomes N., Kosheleva I.A., Abraham W.R., Smalla K. (2005) Effects of the inoculant strain Pseudomonas putida KT2442 (pNF142) and of naphthalene contamination on the soil bacterial community. FEMS microbiology ecology 54:21-33.

Gordon M., Choe N., Duffy J., Ekuan G., Heilman P., Muiznieks I., Ruszaj M., Shurtleff B.B., Strand S., Wilmoth J. (1998) Phytoremediation of trichloroethylene with hybrid poplars. Environmental Health Perspectives 106:1001.

Guieysse B., Viklund G., Toes A.-C., Mattiasson B. (2004) Combined UV-biological degradation of PAHs. Chemosphere 55:1493-1499. DOI: http://dx.doi.org/10.1016/j.chemosphere.2004.01.021.

Guitart C., Garcia-Flor N., Bayona J.M., Albaiges J. (2007) Occurrence and fate of polycyclic aromatic hydrocarbons in the coastal surface microlayer. Mar Pollut Bull 54:186-94. DOI: 10.1016/j.marpolbul.2006.10.008.

Guitart C., Garcia-Flor, N., Miquel, J.C., Fowler, S.W.; Albaiges, J. (2010) Effect of the accumulation of polycyclic aromatic hydrocarbons in the sea surface microlayer on their coastal air-sea exchanges. J Mar Syst 79:210-217.

Haggblom M.M. (1992) Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol Rev 9:29-71.

Hamid T.A., Hamid A., Zulkifly A., Hamdan S., Ariffin S.H.Z., Huyop F. (2013) Purification and properties of a new dehalogenase enzyme from Pseudomonas sp. B6P grow in 3-chloropropionate (3CP). African Journal of Biotechnology 10:610-614.

Hardman D.J. (1991) Biotransformation of Halogenated Compounds. Critical Reviews in Biotechnology 11:1-40. DOI: Doi 10.3109/07388559109069182.

Heitkamp M.A., Cerniglia, C. E., . (1987) The effects of chemical structure and exposure on the microbial degradation of polycyclic aromatic hydrocarbons in freshwater and estuarine ecosystems. Environ. Toxicol. Chem. 6:535–546.

Heitkamp M.A., Freeman J.P., Cerniglia C.E. (1987) Naphthalene biodegradation in environmental microcosms: estimates of degradation rates and characterization of metabolites. Appl Environ Microbiol 53:129-36.

Hess T., Schmidt S., Silverstein J., Howe B. (1990) Supplemental substrate enhancement of 2, 4-dinitrophenol mineralization by a bacterial consortium. Applied and Environmental Microbiology 56:1551-1558.

Holmes V.F., He J., Lee P.K., Alvarez-Cohen L. (2006) Discrimination of multiple Dehalococcoides strains in a trichloroethene enrichment by quantification of their reductive dehalogenase genes. Appl Environ Microbiol 72:5877-83. DOI: 10.1128/AEM.00516-06.

Horel A., Schiewer S. (2009) Investigation of the physical and chemical parameters affecting biodegradation of diesel and synthetic diesel fuel contaminating Alaskan soils. Cold Regions Science and Technology 58:113-119.

Hori M.O.N., Amund, D.I. . (2000) Degradation of anthracene by bacteria isolated from oil polluted tropical soils. Naturforsch C55:890–897.

Horrocks A.R. (2011) Flame retardant challenges for textiles and fibres: new chemistry versus innovatory solutions. Polymer Degradation and Stability 96:377-392.

Häggblom M.M., Bossert I.D. (2003) Dehalogenation : microbial processes and environmental applications Kluwer Academic Pub., Boston.

Janssen D.B., Pries F., van der Ploeg J.R. (1994) Genetics and biochemistry of dehalogenating enzymes. Annu Rev Microbiol 48:163-91. DOI: 10.1146/annurev.mi.48.100194.001115.

Jeon C.O., Madsen E.L. (2013) In situ microbial metabolism of aromatic-hydrocarbon environmental pollutants. Curr Opin Biotechnol 24:474-81. DOI: 10.1016/j.copbio.2012.09.001.

Jiménez J., Miñambres, B., García, J., Díaz, E. (2004) Genomic Insights in the Metabolism of Aromatic Compounds in Pseudomonas. .In: Ramos, J.-L. (Ed.), Pseudomonas. Springer, US:425–462 DOI: http://dx.doi.org/10.1007/978-1-4419-9088-4_15.

Johnsen A.R., Karlson U. (2005) PAH degradation capacity of soil microbial communities--does it depend on PAH exposure? Microb Ecol 50:488-95. DOI: 10.1007/s00248-005-0022-5.

Johnsen A.R., Wick L.Y., Harms H. (2005) Principles of microbial PAH-degradation in soil. Environ Pollut 133:71-84. DOI: 10.1016/j.envpol.2004.04.015.

Jones M.D., Crandell D.W., Singleton D.R., Aitken M.D. (2011) Stable-isotope probing of the polycyclic aromatic hydrocarbon-degrading bacterial guild in a contaminated soil. Environ Microbiol 13:2623-32. DOI: 10.1111/j.1462-2920.2011.02501.x.

Jones M.D., Rodgers-Vieira E.A., Hu J., Aitken M.D. (2014) Association of Growth Substrates and Bacterial Genera with Benzo[]pyrene Mineralization in Contaminated Soil. Environ Eng Sci 31:689-697. DOI: 10.1089/ees.2014.0275.

Jugder B.-E., Ertan H., Lee M., Manefield M., Marquis C.P. (2015) Reductive Dehalogenases Come of Age in Biological Destruction of Organohalides. Trends in biotechnology 33:595-610.

Juhasz A.L., Britz, M.L., Stanley, G.A. (1997) Degradation of fluoranthene, pyrene, benz[a]anthracene and dibenz[a,h]anthracene by Burkholderia cepacia. J Appl Microbiol 83:189–198.

Justicia-Leon S.D. (2012) Microbes and monitoring tools for anaerobic chlorinated methane bioremediation.

Kanaly R.A., Bartha R., Watanabe K., Harayama S. (2000) Rapid mineralization of benzo[a]pyrene by a microbial consortium growing on diesel fuel. Appl Environ Microbiol 66:4205-11.

Kanaly R.A., Harayama S. (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J Bacteriol 182:2059-67.

Kaufhold T., Schmidt M., Cichocka D., Nikolausz M., Nijenhuis I. (2013) Dehalogenation of diverse halogenated substrates by a highly enriched Dehalococcoides-containing culture derived from the contaminated mega-site in Bitterfeld. FEMS microbiology ecology 83:176-188.

Kaur P., Parihar L. (2014) Bioremediation: Step towards Improving Human Welfare. Annual Research & Review in Biology 4:3150.

Kelce W.R., Stone C.R., Laws S.C., Gray L.E., Kemppainen J.A., Wilson E.M. (1995) Persistent DDT metabolite p,p'-DDE is a potent androgen receptor antagonist. Nature 375:581-5. DOI: 10.1038/375581a0.

Kelley I., Cerniglia, C. E. (1995) Degradation of a mixture of high-molecular-weight polycyclic aromatic hydrocarbons by a Mycobacterium strain PYR-1. . J Soil Contam 4:77–91.

Khan A.A., Wang R.F., Cao W.W., Franklin W., Cerniglia C.E. (1996) Reclassification of a polycyclic aromatic hydrocarbon-metabolizing bacterium, Beijerinckia sp. strain B1, as Sphingomonas yanoikuyae by fatty acid analysis, protein pattern analysis, DNA-DNA hybridization, and 16S ribosomal DNA sequencing. Int J Syst Bacteriol 46:466-9. DOI: 10.1099/00207713-46-2-466.

Krey A., Ostertag S.K., Chan H.M. (2015) Assessment of neurotoxic effects of mercury in beluga whales (Delphinapterus leucas), ringed seals (Pusa hispida), and polar bears (Ursus maritimus) from the Canadian Arctic. Sci Total Environ 509-510:237-47. DOI: 10.1016/j.scitotenv.2014.05.134.

Kulkarni M., Chaudhari A. (2007) Microbial remediation of nitro-aromatic compounds: an overview. J Environ Manage 85:496-512. DOI: 10.1016/j.jenvman.2007.06.009.

Kurihara T., Esaki N. (2008) Bacterial hydrolytic dehalogenases and related enzymes: occurrences, reaction mechanisms, and applications. Chem Rec 8:67-74. DOI: 10.1002/tcr.20141.

Kästner M., Breuer-Jammali, M., Mahro, B. (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH) Appl Microbiol Biotechnol 41:267–273.

Lau E.V., Gan S., Ng H.K., Poh P.E. (2014) Extraction agents for the removal of polycyclic aromatic hydrocarbons (PAHs) from soil in soil washing technologies. Environ Pollut 184:640-9. DOI: 10.1016/j.envpol.2013.09.010.

Li F.B., Li X.M., Zhou S.G., Zhuang L., Cao F., Huang D.Y., Xu W., Liu T.X., Feng C.H. (2010) Enhanced reductive dechlorination of DDT in an anaerobic system of dissimilatory iron-reducing bacteria and iron oxide. Environ Pollut 158:1733-40. DOI: 10.1016/j.envpol.2009.11.020.

Li Z., Yoshida N., Wang A., Nan J., Liang B., Zhang C., Zhang D., Suzuki D., Zhou X., Xiao Z. (2015) Anaerobic mineralization of 2, 4, 6-tribromophenol to CO 2 by a synthetic microbial community comprising Clostridium, Dehalobacter, and Desulfatiglans. Bioresource technology 176:225-232.

Liu R., Dai Y., Sun L. (2015) Effect of Rhizosphere Enzymes on Phytoremediation in PAH-Contaminated Soil Using Five Plant Species. PloS one 10:e0120369.

Lors C., Tiffreau C., Laboudigue A. (2004) Effects of bacterial activities on the release of heavy metals from contaminated dredged sediments. Chemosphere 56:619-30. DOI: 10.1016/j.chemosphere.2004.04.009.

Louvado A., Gomes N.C., Simoes M.M., Almeida A., Cleary D.F., Cunha A. (2015) Polycyclic aromatic hydrocarbons in deep sea sediments: Microbe-pollutant interactions in a remote environment. Sci Total Environ 526:312-28. DOI: 10.1016/j.scitotenv.2015.04.048.

Luch A. (2009) On the impact of the molecule structure in chemical carcinogenesis. EXS 99:151-79.

Luch A., Baird, W.M. (2005) Metabolic activation of polycylic aromatic hydrocarbons. In: A Luch (ed.): The Carcinogenic Effects of Polycyclic Aromatic Hydrocarbons. Imperial College Press, London:19–96.

Lukyanova O.N., Tsygankov V.Y., Boyarova M.D., Khristoforova N.K. (2016) Bioaccumulation of HCHs and DDTs in organs of Pacific salmon (genus Oncorhynchus) from the Sea of Okhotsk and the Bering Sea. Chemosphere 157:174-180. DOI: 10.1016/j.chemosphere.2016.05.039.

Lütke-Eversloh T. (2014) Application of new metabolic engineering tools for Clostridium acetobutylicum. Applied microbiology and biotechnology 98:5823-5837.

Löffler F.E., Yan J., Ritalahti K.M., Adrian L., Edwards E.A., Konstantinidis K.T., Müller J.A., Fullerton H., Zinder S.H., Spormann A.M. (2013) Dehalococcoides mccartyi gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and family Dehalococcoidaceae fam. nov., within the phylum Chloroflexi. International Journal of Systematic and Evolutionary Microbiology 63:625-635.

Mallick S., Chatterjee S., Dutta T.K. (2007) A novel degradation pathway in the assimilation of phenanthrene by Staphylococcus sp. strain PN/Y via meta-cleavage of 2-hydroxy-1-naphthoic acid: formation of trans-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid. Microbiology 153:2104-15. DOI: 10.1099/mic.0.2006/004218-0.

Maltseva O.V., Tsoi T.V., Quensen J.F., 3rd, Fukuda M., Tiedje J.M. (1999) Degradation of anaerobic reductive dechlorination products of Aroclor 1242 by four aerobic bacteria. Biodegradation 10:363-71.

Maphosa F. (2010) Chasing organohalide respirers: ecogenomics approaches to assess the bioremediation capacity of soils Wageningen Universiteit (Wageningen University).

Maphosa F., Passel M.W., Vos W.M., Smidt H. (2012) Metagenome analysis reveals yet unexplored reductive dechlorinating potential of Dehalobacter sp. E1 growing in co-culture with Sedimentibacter sp. Environmental microbiology reports 4:604-616.

Martin F., Torelli S., Le Paslier D., Barbance A., Martin-Laurent F., Bru D., Geremia R., Blake G., Jouanneau Y. (2012) Betaproteobacteria dominance and diversity shifts in the bacterial community of a PAH-contaminated soil exposed to phenanthrene. Environ Pollut 162:345-53. DOI: 10.1016/j.envpol.2011.11.032.

McArthur A.G., Waglechner N., Nizam F., Yan A., Azad M.A., Baylay A.J., Bhullar K., Canova M.J., De Pascale G., Ejim L., Kalan L., King A.M., Koteva K., Morar M., Mulvey M.R., O'Brien J.S., Pawlowski A.C., Piddock L.J., Spanogiannopoulos P., Sutherland A.D., Tang I., Taylor P.L., Thaker M., Wang W., Yan M., Yu T., Wright G.D. (2013) The comprehensive antibiotic resistance database. Antimicrob Agents Chemother 57:3348-57. DOI: 10.1128/aac.00419-13.

McCue T., Hoxworth S., Randall A.A. (2003) Degradation of halogenated aliphatic compounds utilizing sequential anaerobic/aerobic treatments. Water Sci Technol 47:79-84.

Meckenstock R.U. (1999) Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol Lett 177:67-73.

Meckenstock R.U., Safinowski M., Griebler C. (2004) Anaerobic degradation of polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol 49:27-36. DOI: 10.1016/j.femsec.2004.02.019.

Menzie C.A., Potocki, B.B., Santodonato, J. (1992) Exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. 26:1278–1284. DOI: http://dx.doi.org/10.1021/es00031a002.

Mohee R., Mudhoo A. (2012) Bioremediation and sustainability: research and applications John Wiley & Sons.

Mohn W.W. (2004) Biodegradation and bioremediation of halogenated organic compounds, Biodegradation and bioremediation, Springer. pp. 125-148.

Murray K.J., Mozafarzadeh M.L., Tebo B.M. (2005) Cr (III) oxidation and Cr toxicity in cultures of the manganese (II)-oxidizing Pseudomonas putida strain GB-1. Geomicrobiology Journal 22:151-159.

Nielsen R.B., Keasling J.D. (1999) Reductive dechlorination of chlorinated ethene DNAPLs by a culture enriched from contaminated groundwater. Biotechnol Bioeng 62:160-5.

Nishino S., Spain J. (2002) Biodegradation, transformation and bioremediation of nitroaromatic compounds. Hurst, CJ, Crawford, RL, Knudsen, GR, McInerney, MJ, Stetzenbach, LD Manual of Environmental Microbiology 2nd. Edition. Editorial American Society for Microbiology, Washington DC:987-996.

Nishino S.F., Spain J.C., He Z. (2000) Strategies for aerobic degradation of nitroaromatic compounds by bacteria: process discovery to field application. In: Spain, J.C., Hughes, J.B., Knackmuss, H.J. (Eds.), Biodegradation of Nitroaromatic Compounds and Explosives. Lewis Publ., New York,. pp. 7-62.

Notar M., Leskovsek H., Faganeli J. (2001) Composition, distribution and sources of polycyclic aromatic hydrocarbons in sediments of the Gulf of Trieste, Northern Adriatic Sea. Mar Pollut Bull 42:36-44.

Nwanna I.E.M., George, G.O., Olusoji, I.M. . (2006) Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria. Afr J Biotechnol 5:823–828.

Nyer E.K., Payne F., Sutherson S. (2003) Discussion of environment vs. bacteria or let's play,'name that bacteria'. Groundwater Monitoring & Remediation 23:32-44.

Nzila A. (2013) Update on the cometabolism of organic pollutants by bacteria. Environ Pollut 178:474-82. DOI: 10.1016/j.envpol.2013.03.042.

OECD. (2015) Biosafety and the Environmental Uses of Micro-Organisms OECD Publishing.

Olaniran A.O., Pillay D., Pillay B. (2004) Haloalkane and haloacid dehalogenases from aerobic bacterial isolates indigenous to contaminated sites in Africa demonstrate diverse substrate specificities. Chemosphere 55:27-33. DOI: 10.1016/j.chemosphere.2003.10.067.

Pakpahan E.N., Isa M.H., Kutty S.R.M., Chantara S., Wiriya W. (2013) Polycyclic aromatic hydrocarbon removal from petroleum sludge cake using thermal treatment with additives. Environmental Technology 34:407-416. DOI: 10.1080/09593330.2012.698648.

Payne R.B., Fagervold S.K., May H.D., Sowers K.R. (2013) Remediation of polychlorinated biphenyl impacted sediment by concurrent bioaugmentation with anaerobic halorespiring and aerobic degrading bacteria. Environ Sci Technol 47:3807-15. DOI: 10.1021/es304372t.

Peng R.-H., Xiong, A.-S., Xue, Y., Fu, X.-Y., Gao, F., Zhao, W., Tian, Y.-S., Yao, Q.-H. (2008) Microbial biodegradation of polyaromatic hydrocarbons. FEMS Microbiol Ecol Rev 32:927–955 DOI: http://dx.doi.org/10.1111/j.1574-6976.2008.00127.x.

Perez F., Llorca M., Kock-Schulmeyer M., Skrbic B., Oliveira L.S., da Boit Martinello K., Al-Dhabi N.A., Antic I., Farre M., Barcelo D. (2014) Assessment of perfluoroalkyl substances in food items at global scale. Environ Res 135:181-9. DOI: 10.1016/j.envres.2014.08.004.

Podar M., Gilmour C.C., Brandt C.C., Soren A., Brown S.D., Crable B.R., Palumbo A.V., Somenahally A.C., Elias D.A. (2015) Global prevalence and distribution of genes and microorganisms involved in mercury methylation. Sci Adv 1:e1500675. DOI: 10.1126/sciadv.1500675.

Rahman K.S., Thahira-Rahman J., Lakshmanaperumalsamy P., Banat I.M. (2002) Towards efficient crude oil degradation by a mixed bacterial consortium. Bioresour Technol 85:257-61.

Razo-Flores E., Donlon B., Lettinga G., Field J.A. (1997) Biotransformation and biodegradation of N-substituted aromatics in methanogenic granular sludge. FEMS microbiology reviews 20:525-538.

Reineke W., Knackmuss H.J. (1988) Microbial degradation of haloaromatics. Annu Rev Microbiol 42:263-87. DOI: 10.1146/annurev.mi.42.100188.001403.

Renpenning J., Rapp I., Nijenhuis I. (2015) Substrate hydrophobicity and cell composition influence the extent of rate limitation and masking of isotope fractionation during microbial reductive dehalogenation of chlorinated ethenes. Environ Sci Technol 49:4293-301. DOI: 10.1021/es506108j.

Riser-Roberts E. (1998) Remediation of Petroleum-Contaminated Soils: Biological, Physical, and Chemical Processes. . Lewis Publishers, Boca Raton, FL. :542.

Rittmann B.E., Tularak P., Lee K.C., Federle T.W., Itrich N.R., Kaiser S.K., Shi J., McAvoy D.C. (2001) How adaptation and mass transfer control the biodegradation of linear alkylbenzene sulfonate by activated sludge. Biodegradation 12:31-7.

Rockne K.J., Chee-Sanford J.C., Sanford R.A., Hedlund B.P., Staley J.T., Strand S.E. (2000) Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl Environ Microbiol 66:1595-601.

Rossolini G.M., Arena F., Pecile P., Pollini S. (2014) Update on the antibiotic resistance crisis. Curr Opin Pharmacol 18:56-60. DOI: 10.1016/j.coph.2014.09.006.

Saagua M.C., Baeta-Hall, L., Anselmo, A.M. (2002) Microbiological characterization of a coke oven contaminated site and evaluation of its potential for bioremediation. World J Microbiol Biotechnol 18:841–845.

Samanta S.K., Bhushan B., Jain R.K. (2001) Efficiency of naphthalene and salicylate degradation by a recombinant Pseudomonas putida mutant strain defective in glucose metabolism. Appl Microbiol Biotechnol 55:627-31.

Samanta S.K., Singh O.V., Jain R.K. (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol 20:243-8.

Sax I., Lewis R. (1999) Nitro-compounds of aromatic hydrocarbons. Dangerous properties of industrial material 2:2534-2536.

Schocken M.J., Gibson D.T. (1984) Bacterial oxidation of the polycyclic aromatic hydrocarbons acenaphthene and acenaphthylene. Appl Environ Microbiol 48:10-6.

Seo J.-S., Keum, Y.-S., Li, Q.X. (2009) Bacterial degradation of aromatic hydrocarbons. Int. Int J Environ Res Public Health 6:278-309.

Shuttleworth K.L., Cerniglia C.E. (1995) Environmental aspects of PAH biodegradation. Appl Biochem Biotechnol 54:291-302.

Sims R.C., Overcash, M.R. (1983) Polynuclear aromatic compounds (PNAs) in soil-plant systems Residue Rev 88:1–68.

Singh H. (2006) Mycoremediation: fungal bioremediation John Wiley & Sons.

Singleton D.R., Jones M.D., Richardson S.D., Aitken M.D. (2013) Pyrosequence analyses of bacterial communities during simulated in situ bioremediation of polycyclic aromatic hydrocarbon-contaminated soil. Appl Microbiol Biotechnol 97:8381-91. DOI: 10.1007/s00253-012-4531-0.

Singleton D.R., Richardson S.D., Aitken M.D. (2011) Pyrosequence analysis of bacterial communities in aerobic bioreactors treating polycyclic aromatic hydrocarbon-contaminated soil. Biodegradation 22:1061-73. DOI: 10.1007/s10532-011-9463-3.

Singleton D.R., Sangaiah R., Gold A., Ball L.M., Aitken M.D. (2006) Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. Environ Microbiol 8:1736-45. DOI: 10.1111/j.1462-2920.2006.01112.x.

Smidt H., de Vos W.M. (2004) Anaerobic microbial dehalogenation. Annu Rev Microbiol 58:43-73. DOI: 10.1146/annurev.micro.58.030603.123600.

Spain J.C. (1995) Biodegradation of nitroaromatic compounds. Annu Rev Microbiol 49:523-55. DOI: 10.1146/annurev.mi.49.100195.002515.

Su X., Deng L., Kong K.F., Tsang J.S. (2013) Enhanced degradation of haloacid by heterologous expression in related Burkholderia species. Biotechnology and bioengineering 110:2687-2696.

Sun R., Jin J., Sun G., Liu Y., Liu Z. (2010) Screening and degrading characteristics and community structure of a high molecular weight polycyclic aromatic hydrocarbon-degrading bacterial consortium from contaminated soil. J Environ Sci (China) 22:1576-85.

Surma M., Gizejewski Z., Zielinski H. (2015) Determination of perfluorinated sulfonate and perfluorinated acids in tissues of free-living European beaver (castor fiber L.) by d-SPE/ micro-UHPLC-MS/MS. Ecotoxicol Environ Saf 120:436-44. DOI: 10.1016/j.ecoenv.2015.06.029.

Thuan N.T., Dien N.T., Chang M.B. (2013) PCDD/PCDF behavior in low-temperature pyrolysis of PCP-contaminated sandy soil. Sci Total Environ 443:590-6. DOI: 10.1016/j.scitotenv.2012.11.014.

Trzesicka-Mlynarz D., Ward O.P. (1995) Degradation of polycyclic aromatic hydrocarbons (PAHs) by a mixed culture and its component pure cultures, obtained from PAH-contaminated soil. Can J Microbiol 41:470-6.

Tsuneta T., Loch-Caruso R., Quensen J.F., 3rd, Boyd S.A., Hanna M., Grindatti C. (2008) Stimulatory effects of a microbially dechlorinated polychlorinated biphenyl (PCB) mixture on rat uterine contraction in vitro. Environ Res 107:185-93. DOI: 10.1016/j.envres.2008.01.011.

Tyagi M., da Fonseca M.M., de Carvalho C.C. (2011) Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. Biodegradation 22:231-41. DOI: 10.1007/s10532-010-9394-4.

Van Der Sluijs J. (2005) Uncertainty as a monster in the science-policy interface: four coping strategies. Water Science & Technology 52:87-92.

Vandermeeren P., Herrmann S., Cichocka D., Busschaert P., Lievens B., Richnow H.-H., Springael D. (2014) Diversity of dechlorination pathways and organohalide respiring bacteria in chlorobenzene dechlorinating enrichment cultures originating from river sludge. Biodegradation 25:757-776.

Ventola C.L. (2015) The antibiotic resistance crisis: part 1: causes and threats. P t 40:277-83.

Verbruggen E. (2012) Derivation of environmental risk limits for polycyclic aromatic hydrocarbons (PAHs) based on internal residues. http://echa.europa.eu/documents/10162/13639/poster_verbruggen_29_en.pdf.

Vila J., Tauler M., Grifoll M. (2015) Bacterial PAH degradation in marine and terrestrial habitats. Curr Opin Biotechnol 33:95-102. DOI: 10.1016/j.copbio.2015.01.006.

Walter U., Beyer, M., Klein, J., Rehm, H-J. (1991) Degradation of pyrene by Rhodococcus sp. UW1. . Appl Microbiol Biotechnol. 34:671–676.

Wang B.J., Lai, Q.L., Cui, Z.S., Tan, T.F., Shao, Z.Z. . (2008) A pyrene-degrading consortium from deep-sea sediment of the West Pacific and its key member Cycloclasticus sp P1. Environ Microbiol 10:1948-1963.

Watanabe K. (2001) Microorganisms relevant to bioremediation. Curr Opin Biotechnol 12:237-41.

Weissenfels W.D., Beyer, M., Klein, J., Rehm, H. J. (1991) Microbial metabolism of fluoranthene: isolation and identification of ring fission products. Appl Microbiol Biotechnol. 34:528–535.

Wen B., Zhang H., Li L., Hu X., Liu Y., Shan X.Q., Zhang S. (2015) Bioavailability of perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) in biosolids-amended soils to earthworms (Eisenia fetida). Chemosphere 118:361-6. DOI: 10.1016/j.chemosphere.2014.08.009.

WHO-IPCS. (1990) Methylmercury, Environmental Health Criteria 101. World Health Organization, Geneva, Switzerland. 1990.

WHO-IPCS. (1992) Cadmium, Environmental Health Criteria 134. World Health Organization, Geneva, Switzerland. 1992.

WHO-IPCS. (1995) Inorganic Lead, Environmental Health Criteria 165. World Health Organization, Geneva, Switzerland. 1995.

Widada J., Nojiri H., Kasuga K., Yoshida T., Habe H., Omori T. (2002) Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Appl Microbiol Biotechnol 58:202-9.

Wiegel J., Zhang X., Wu Q. (1999) Anaerobic dehalogenation of hydroxylated polychlorinated biphenyls by Desulfitobacterium dehalogenans. Applied and environmental microbiology 65:2217-2221.

Wise D.L., Trantolo D.J., Cichon E.J., Inyang H.I., Stottmeister U. (2000) Remediation engineering of contaminated soils. Marcel Dekker Inc. New York.:1000

Wolfe M.F., Schwarzbach S., Sulaiman R.A. (1998) Effects of mercury on wildlife: a comprehensive review. Environmental toxicology and chemistry 17:146-160.

Xue W., Warshawsky D. (2005) Metabolic activation of polycyclic and heterocyclic aromatic hydrocarbons and DNA damage: a review. Toxicol Appl Pharmacol 206:73-93. DOI: 10.1016/j.taap.2004.11.006.

Yamamura S., Amachi S. (2014) Microbiology of inorganic arsenic: From metabolism to bioremediation. J Biosci Bioeng 118:1-9. DOI: 10.1016/j.jbiosc.2013.12.011.

Zanaroli G., Negroni A., Haggblom M.M., Fava F. (2015) Microbial dehalogenation of organohalides in marine and estuarine environments. Curr Opin Biotechnol 33:287-95. DOI: 10.1016/j.copbio.2015.03.013.

Zander M. (1983) Physical and chemical properties of polycyclic aromatic hydrocarbons. . In Handbook of polycyclic aromatic hydrocarbons. Ed Bjørseth A. (Marcel Dekker, Inc. New York, N.Y): pp 1–26.

Zhang C., Bennett G.N. (2005) Biodegradation of xenobiotics by anaerobic bacteria. Appl Microbiol Biotechnol 67:600-18. DOI: 10.1007/s00253-004-1864-3.

Zhang F., Wei J., Li Q., Jiang R., Yu N., Qin J., Chen L. (2015) Effects of perfluorooctane sulfonate on the immune responses and expression of immune-related genes in Chinese mitten-handed crab Eriocheir sinensis. Comp Biochem Physiol C Toxicol Pharmacol 172-173:13-8. DOI: 10.1016/j.cbpc.2015.04.002.

Zhang F.J., Cortez C., Harvey R.G. (2000) New synthetic approaches to polycyclic aromatic hydrocarbons and their carcinogenic oxidized metabolites: derivatives of benzo[s]picene, benzo[rst]pentaphene, and dibenzo[b,def]chrysene. J Org Chem 65:3952-60.

Zhang L., Li P., Gong Z., Li X. (2008) Photocatalytic degradation of polycyclic aromatic hydrocarbons on soil surfaces using TiO2 under UV light. Journal of Hazardous Materials 158:478-484. DOI: http://dx.doi.org/10.1016/j.jhazmat.2008.01.119.

Zylstra G.J., Kim E., Goyal A.K. (1997) Comparative molecular analysis of genes for polycyclic aromatic hydrocarbon degradation. Genet Eng (N Y) 19:257-69.

15 Appendix I

Table 1. Microbes reported to perform biodegenerating effects on common nitro-aromatics (modified from Kulkarni and Chaudhari (2007)).

		Nitro-benzene	Nitro-benzoate	Mono-nitro-phenol	Dinitro-phenol	DNT	TNT
Aerobic bacteria	*Acidovorax*	x		x			
	Acinetobacter			x			x
	Actinomycete			x			
	Alcaligenes				x		
	Arthrobacter		x	x			
	Bacillus			x			
	Burkholderia		x			x	
	Comamonas	x	x				
	Enterobacter						x
	Hydrogenophaga					x	
	Klebsiella			x			x
	Moraxella			x			
	Mycobacterium						
	Nocardiodes			x			x
	Pseudomonas	x	x	x		x	x
	Ralstonia	x	x	x	x		
	Rhizobium						x
	Rhodococcus			x			x
	Sphingomonas			x	x		x
Anaerobic bacteria	*Clostridium*						x
	Desulfovibrio	x				x	x
	Desulphatomaculem			x			
	Haloanaerobicum	x		x	x		
	Methanobacterium			x			
	Methylobacterium						x
	Sporohalobacter	x		x	x		
Fungi	*Candida*	x					
	Heterobasidium						x
	Hypholoma						x
	Nematoloma						x
	Phanerochaete			x		x	x
	Phlebia						x
	Pleurotus						x

16 Appendix II

European Economic Community (DIRECTIVE 93/88/EEC, Oct. 1993), NIH Guidelines on Recombinant DNA (April 2002), Canadian Laboratory Biosafety Guidelines (2nd ed. 1996) and CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (4th Edition 1999)

European Economic Community (DIRECTIVE 93/88/EEC, Oct. 1993)

(1) Group 1 biological agent means one that is unlikely to cause human disease;

(2) Group 2 biological agent means one that can cause human disease and might be a hazard to workers; it is unlikely to spread to the community; there is usually effective prophylaxis or treatment available;

(3) Group 3 biological agent means one that can cause severe human disease and present a serious hazard to workers; it may present a risk of spreading to the community, but there is usually effective prophylaxis or treatment available;

(4) Group 4 biological agent means one that causes severe human disease and is a serious hazard to workers; it may present a high risk of spreading to the community; there is usually no effective prophylaxis or treatment available.

NIH Guidelines on Recombinant DNA (April 2002)

(1) Risk Group 1 (RG1) agents are not associated with disease in healthy adult humans.

(2) Risk Group 2 (RG2) agents are associated with human disease which is rarely serious and for which preventive or therapeutic interventions are often available.

(3) Risk Group 3 (RG3) agents are associated with serious or lethal human disease for which preventive or therapeutic interventions may be available.

(4) Risk Group 4 (RG4) agents are likely to cause serious or lethal human disease for which preventive or therapeutic interventions are not usually available.

Canadian Laboratory Biosafety Guidelines (2nd ed. 1996)

(1) Risk Group 1 (low individual and community risk) This group includes those microorganisms, bacteria, fungi, viruses and parasites, which are unlikely to cause disease in healthy workers or animals

(2) Risk Group 2 (moderate individual risk, limited community risk) A pathogen that can cause human or animal disease but under normal circumstances, is unlikely to be a serious hazard to healthy laboratory workers, the community, livestock, or the environment. Laboratory exposures rarely cause infection leading to serious disease; effective treatment and preventive measures are available and the risk of spread is limited.

(3) Risk Group 3 (high individual risk, low community risk) A pathogen that usually causes serious human or animal disease, or which can result in serious economic consequences but does not ordinarily spread by casual contact from one individual to another, or that can be treated by antimicrobial or anti-parasitic agents.

(4) Risk Group 4 (high individual risk, high community risk) A pathogen that usually produces very serious human animal disease, often untreatable, and may be readily transmitted from one individual to another, or from animal to human or vice-versa directly or indirectly, or casual contact.

CDC/NIH Biosafety in Microbiological and Biomedical Laboratories (4th Edition 1999)

(1) BIOSAFETY 1 is suitable for work involving well-characterized agents not known to cause disease in healthy adult humans, and of minimal potential hazard to laboratory personnel and the environment.

(2) BIOSAFETY LEVEL 2 is similar to Level 1 and is suitable for work involving agents of moderate potential hazard to personnel and the environment.

(3) BIOSAFETY LEVEL 3 is applicable to clinical, diagnostic, teaching, research, or production facilities in which work is done with indigenous or exotic agents which may cause serious or potentially lethal disease as a result of exposure by the inhalation route.

(4) BIOSAFETY LEVEL 4 is required for work with dangerous and exotic agents which pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease.