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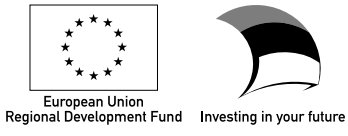

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers that will be referred to by their Roman numerals in the text.

I **Nõlvak, H.**, Sildvee, T., Kriipsalu, M., Truu, J. **2012**. Application of microbial community profiling and functional gene detection for assessment of natural attenuation of petroleum hydrocarbons in boreal subsurface. Boreal Environment Research, 17, 113–127.

II **Nõlvak, H.**, Truu, J., Limane, B., Truu, M., Cepurnieks, G., Bartkevics, V., Juhanson, J., Muter, O. **2012**. Microbial community changes in TNT spiked soil bioremediation trial using biostimulation, phytoremediation and bioaugmentation. Journal of Environmental Engineering and Landscape Management, *accepted*.

III **Nõlvak, H.**, Truu, M., Truu, J. **2012**. Evaluation of quantitative real-time PCR workflow modifications on 16S rRNA and *tetA* gene quantification in environmental samples. Science of the Total Environment, 426, 351–358.

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Author's contribution

Paper I: The author participated in sampling, is responsible for the microbiological analysis (about 80%), the data analysis (about 85%) and writing the manuscript (about 90%).

Paper II: The author is responsible for the microbiological analysis (about 30%), the data analysis (about 50%) and writing the manuscript (about 85%).

Paper III: The author is responsible for planning the experiment (about 80%), for the microbiological analysis (100%), the data analysis (about 90%) and writing the manuscript (about 90%).

ABBREVIATIONS

BrdU	5-bromo-2`-deoxyuridine
BTEX	Benzene, toluene, ethylbenzene, xylene
CPT	Cycling probe technology
C_t	Threshold cycle
DCE	Dichloroethene/Dichloroethylene
DGGE	Denaturing gradient gel electrophoresis
E	Polymerase chain reaction amplification efficiency
ETBE	Ethyl *tert*-butyl ether
FAM	6-carboxy fluorescein
FRET	Fluorescence resonance energy transfer/Förster resonance energy transfer
HMX	High melting explosive/High-velocity military explosive
HPLC	High pressure liquid chromatography/High performance liquid chromatography
IAC	Internal amplification control
KOD	Kinetic outlier detection
LH-PCR	Length heterogeneity polymerase chain reaction
LNA	Locked nucleic acid probe
LUX	Light upon extension primer
MB	Molecular beacon
MGB	Minor groove binding probe
MNA	Monitored natural attenuation
NTC	No template control
PAH	Polyaromatic hydrocarbon
PCA	Principal component analysis
PCB	Polychlorinated biphenyl
PCE	Tetrachloroethene/Tetrachloroethylene
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RDX	Research department explosive
RISA	Ribosomal intergenic spacer analysis
RT-PCR	Reverse transcriptase polymerase chain reaction
SIP	Stable isotope probing
SOD	Shape based kinetic outlier detection
SSCP	Single strand conformation polymorphism
TCE	Trichloroethene/Trichloroethylene
TGGE	Temperature gradient gel electrophoresis
TNT	2,4,6-trinitrotoluene
TPH	Total petroleum hydrocarbons
T-RFLP	Terminal restriction fragment length polymorphism

1. INTRODUCTION

The increasingly industrialized global economy that has emerged over the last century has led to dramatically elevated releases of anthropogenic chemicals into the environment, resulting in contamination of many areas. Contamination can be a result of improper chemical production (i.e. oil spills from drilling, explosives from manufacturing), transport (i.e. oil spills from tankers or pipelines), storage (i.e. chemicals from leaking storage tanks), usage (i.e. pesticides and fertilizers from agriculture, explosives from munitions firing) or disposal processes (i.e. explosives from demilitarization facilities). Organic chemicals released into the environment may impact whole ecosystems (i.e. the Gulf of Mexico oil spill and Arctic oil spills have caused loss of species richness), drinking water supplies or directly influence human health (Farhadian et al., 2008; Gerhardt et al., 2009; Yang et al., 2009; Mrozik and Piotrowska-Seget, 2010).

Concurrently with increasing pollution levels, avid interest in the development of strategies for the remediation of environmental contaminants using physical, chemical and biological processes has emerged. As classic "suck and truck" strategies followed by off-site treatments are expensive, *in situ* bioremediation processes such as monitored natural attenuation (MNA), biostimulation, bioaugmentation and rhizoremediation have become attractive methods to rehabilitate contaminated sites (Ayoub et al., 2010). The aforementioned bioremediation techniques rely extensively on the presence of an active microbial degrader population able to transform the bioavailable contaminants into harmless or less dangerous compounds. The bioremediation processes are, however, complex in contaminated environments, and their effectiveness must be demonstrated by continuous monitoring through chemical, biological and environmental indicators (Andreoni and Gianfreda, 2007).

One method that is increasingly used in the monitoring of bioremediation efficiency is quantitative polymerase chain reaction (qPCR), which enables quantification of the abundance and expression of taxonomic (i.e. rRNA) and functional gene markers within the environment from the domain level down to the quantification of individual species or phylotypes (Smith and Osborn, 2009). The quantitative data generated can be used to relate variation in gene abundances with variation in abiotic and biotic factors and process rates (Sharma et al., 2007) making this method especially suitable for bioremediation monitoring. However, target gene quantification results from environmental samples depend on a number of factors, such as the method and quality of DNA extraction, the subsequent presence of inhibitory substances in the extracted microbial community DNA, the qPCR chemistry used, the amplification efficiency achieved and the overall quality of the resultant datasets (Sharma et al., 2007; Smith and Osborn, 2009). Despite the increasing use of qPCR in environmental monitoring, the reports often fall short of considering the aforementioned factors influencing the outcome of target gene quantification. Nevertheless, the best possible quality of qPCR reactions and target gene quantifications should be ensured in order to adequately support overall decision-making regarding the implementation of bioremediation.

2. THE AIM OF THE STUDY

The main aim of this thesis was to evaluate the scope of different aspects affecting gene enumerations from environmental samples by quantitative polymerase chain reaction (qPCR) used for the estimation of bioremediation potential.

The specific aims were:

- to study the effect of qPCR workflow modifications (the variation in microbial community DNA extraction methods, qPCR chemistry type, qPCR kits from different manufacturers, the determination of inhibition rate) on target gene quantification results from environmental samples;
- to improve the quality of qPCR quantification data by employing reaction outlier removal based on the developed amplification data quality control procedure;
- to assess the effect of absolute and relative quantification data improvement of target genes on the evaluation of bioremediation potential in residual oil and TNT-contaminated environmental matrices.

3. LITERATURE REVIEW

3.1. Bioremediation in contaminant removal from polluted environments

All of the major elements found in biological organisms, as well as some of the minor and trace elements, are cycled between biotic and abiotic forms in predictable and definable ways. The biogeochemical cycles are mainly driven by ubiquitous microbial activities. The diverse degradative capabilities of microbes that have evolved for natural organic and mineral compounds also form the basis for degradation pathways that are applicable in environmental technology for the bioremediation of contaminants (i.e. petroleum hydrocarbons, pesticides, explosives) spilled into the environment (Travis et al., 2008). Bioremediation is defined as a managed or spontaneous process in which biological, especially microbial, degradation acts on pollutant compounds, thereby remedying or eliminating environmental contamination (Madsen, 1991). Many contaminants (i.e. pesticides, polyaromatic hydrocarbons, explosives) can be seen as mainly carbon and to a lesser extent other nutrient reservoirs (depending on the type of pollutants present) for microbes able to metabolize the compound befitting the goal of bioremediation to fully degrade the contaminants or at least render the pollutants harmless (Diplock et al., 2009). Microbial activities can be harnessed for contamination prevention by removing or at least reaching the acceptable levels of possible pollutants and excess nutrients in treated waters (i.e. wastewater, surface runoff from agricultural areas, landfill leachate) before these are directed into the environment. In addition to preventative capacity, bioremediation is applied even more extensively for the treatment of already polluted environmental matrices (i.e. soil, groundwater), both on site (*in situ*) and in specialized treatment facilities off-site (*ex situ*). As off-site treatments tend to be expensive, the *in situ* bioremediation processes, such as monitored natural attenuation (MNA), biostimulation, bioaugmentation and rhizoremediation, have increasingly become an attractive way to rehabilitate contaminated sites, especially those polluted by organic contaminants (Ayoub et al., 2010; Table 1).

The competent microbial community and the whole bioremediation process of xenobiotics is influenced by a multitude of environmental parameters such as temperature, the availability of oxygen (or an alternative electron acceptor), the type and concentration of nutrients, salinity, pressure, water activity, pH and process-inhibiting co-contaminants on the site as well as the chemical composition, physical state, concentration, availability and toxicity of the target contaminant. If any of these factors is suboptimal or absent at the field site, the success rate of applied bioremediation may decrease. In well-aerated environments the low levels of nutrients are often the most rate-limiting factors (Nikolopoulou and Kalogerakis, 2009; Santos et al., 2011); however, in sediments, wetlands, salt marshes and the subsurface layer of beaches oxygen tends to become the limiting factor instead of nutrients (Venosa and Zhu, 2003).

Table 1. Prevalent organic contaminants in the environment.

Contaminant	**Main sources**	**Risks/effects**	**Contamination and subsequent bioremediation cases**
Aliphatic hydrocarbons i.e. alkanes, alkenes	Crude oil and oil products	Toxic, some compound isomers (i.e. hexane) neurotoxic.	Tanker Prestige oil spill; biostimulation (Jiménez et al., 2006) Former industrial site; bioaugmentation (Alisi et al., 2009) Industrial dump site, MNA (Salminen et al., 2004).
Monoaromatic hydrocarbons i.e. BTEX (benzene, toluene, ethylbenzene, xylene)	Oil products	Toxic, carcinogenic. Reduce biodiversity.	Tanker Prestige oil spill; biostimulation (Gallego et al., 2006) Underground fuel storage tank leakage at gas station, MNA (Takahata et al., 2006). Underground gasoline storage tank leakage, MNA (Baldwin et al., 2008)
PAHs (polyaromatic hydrocarbons)	Oil products, coal conversion facilities, wood processing, incineration of waste.	Toxic, carcinogenic, mutagenic (depending on compound). Accumulate in food chains, reduce biodiversity.	Aged creosote-contaminated soil; phytoremediation (Robinson et al., 2003) Military testing site; phytoremediation (Siciliano et al., 2003) Oily sludge-contaminated soil; biostimulation (Liu et al., 2010)
Halogenated compounds i.e. TCE (trichloroethene), PCE (tetrachloroethene), DCE (dichloroethene) PCB (polychlorinated biphenyl)	Industrial solvents, wood preservatives.	Toxic, potentially carcinogenic. Accumulate in environment.	Kelly Air Force Base, bioaugmentation (Major et al., 2002). PCE plume derived from dry-cleaning operations (Lendvay et al., 2003). TCE contaminated groundwater, biostimulation (Dugat-Bony et al., 2012)

Table 1. Continuation.

Contaminant	**Main sources**	**Risks/effects**	**Contamination and subsequent bioremediation cases**
Nitroaromatics i.e. TNT (trinitrotoluene), RDX (research department explosive)	Munitions	Toxic, carcinogenic.	Surroundings of former ammunition plant; biostimulation (Gerth and Hebner, 2007) Training range, natural attenuation (Clausen et al., 2004) Demilitarization plant; MNA, bioaugmentation, rhizoremediation (van Dillewijn et al., 2007)
Pesticides i.e. malathion, heptachlor, aldrin, atrazine	Agriculture	Toxic, potentially carcinogenic. Accumulate in food webs, reduce biodiversity.	Atrazine in agricultural soil; biostimulation, bioaugmentation (Silva et al., 2004). Lindane at production site, rhizoremediation (Abhilash et al., 2011)

Many xenobiotics can also be degraded via anaerobic pathways; however, aerobic degradation is often preferable, as the most rapid degradation of some prevalent contaminants (i.e. oil products) occurs when oxygen is utilized as an electron acceptor for microbial metabolism (Nikolopoulou and Kalogerakis, 2009; Santos et al., 2011). Excess moisture also reduces soil aeration, whereas low water content levels can lead to decreased microbial activity (Mashreghi and Prosser, 2006). The activity and abundance of the microbial community is also affected by the temperature of the environment – the rate of biodegradation can decrease significantly with low temperatures in boreal and cold climate sites (Venosa and Zhu, 2003). Temperature can also influence the properties and availability of some contaminants (i.e. oil) (Mercer and Trevors, 2011; Tyagi et al., 2011). Changes in pH can affect the microbial community directly, as the extreme values of pH inhibit microbes' degradative ability, or indirectly by affecting the solubility of nutrients (Radwan, 2008). In addition to abiotic factors, a few biotic factors such as competition for resources within the microbial community and predation by protozoans affect bioremediation.

The downside of bioremediation approaches is the fact that the circumstances prevailing at the field site significantly influence the choice of technique to be applied, the success of which usually requires the customization of the chosen technique for specific field site conditions. Bioremediation is generally a slow process and successful application in the laboratory under controlled conditions does not imply similar success or transformation rates in an uncontrolled environment at a field site (Diplock et al., 2009). Furthermore, the potential for bioremediation cannot be efficiently monitored through the measurement of a single parameter. Nevertheless, the eco-friendly, cost-effective and low-maintenance nature of bioremediation approaches over chemical or physical treatments for environmental clean-up of hazardous contaminants has proved advantageous in many cases of contamination treatment (Yang et al., 2009). Furthermore, bioremediation can be applied over vast areas as an additional clean-up strategy when the physical and chemical strategies have run their course but have not achieved complete cleanup (Nikolopoulou and Kalogerakis, 2009). When the bioremediation process is implemented, its effectiveness has to be demonstrated by continuous monitoring through chemical, biological, and environmental indicators (Andreoni and Gianfreda, 2007).

3.1.1. Monitored natural attenuation

Natural attenuation is defined as the reduction in toxicity, mass and/or mobility of a contaminant without human intervention, owing to naturally occurring physical (i.e. sorption, volatilization, dispersion) and biological (biodegradation) processes. Of these, microbial processes are often the dominant reactions driving the natural attenuation of contaminants. In order to verify whether natural attenuation is ongoing and sustainable, the associated processes are monitored over time (Röling and van Verseveld, 2002). Although no action is required to initiate or continue the process, natural

recovery is considered the result of a deliberate, thoughtful decision following detailed site assessment and characterisation (Perelo, 2010). In several countries where monitored natural attenuation (MNA) is routinely applied (i.e. the USA, Great Britain, Germany), three lines of evidence are necessary to demonstrate the efficiency of the process: 1) an observed decrease of the contaminant at the field site; 2) laboratory assays indicating that micro-organisms from the site have potential to transform contaminants from the site; 3) evidence that biodegradation potential is realized in the field (Smets and Pritchard, 2003; Rügner et al., 2006). MNA (also referred to as intrinsic bioremediation, bioattenuation or passive remediation) is considered to be most effective for low-risk sites with low-level or diffuse contamination, where human health and ecological risks are not immediate or substantial (Magar and Wenning, 2006).

In order to demonstrate the conformity of MNA to the lines of evidence required and also to verify that there is no risk to the environment or to human health, intrinsic remediation processes are monitored. The initial site characterisation verifies whether the mechanisms of natural attenuation are sufficient to meet remedial goals in an acceptable time frame. For those systems in which MNA is proved to be viable, the loss of contaminants, the presence and distribution of geochemical and biochemical indicators as well as direct microbial evidence of natural attenuation at the field site need to be demonstrated routinely (USEPA, 2007). For years, chemical analyses demonstrating the decay of the target compounds, the appearance of metabolites or end products and changes in terminal electron acceptor concentrations were prevalent standalone methods in natural attenuation monitoring (van Stempvoort and Biggar, 2008), which have been used to monitor the natural attenuation of BTEX-contaminated aquifers (Reusser et al., 2002; Roychoudhury and Merrett, 2006) and soil contaminated by TNT (van Dillewijn et al., 2007) and aliphatic hydrocarbons (Serrano et al., 2008), among other applications. Since microbial degradation has been recognized as the key process in bioremediation, the role of biological and molecular analyses characterizing the composition and activity of the microbial population has, in combination with chemical analysis, been steadily increasing at contaminated sites, especially in the last decade. Methods targeting microbial community composition and structure, abundance and activity, have been used to assess natural attenuation in soils and groundwater contaminated with petroleum (Bento et al., 2005), BTEX compounds (Takahata et al., 2006) or gasoline (Baldwin et al., 2008), among other pollutants. No standardized protocols exist for natural attenuation monitoring, and the design of the monitoring procedure applied for routine estimations of ongoing natural attenuation is largely dependent on the characteristics of the field site; the numerous monitoring techniques available enable versatile design options (Smets and Pritchard, 2003; Rügner et al., 2006). Regardless of design details, the monitoring process needs to be reliable, easy to maintain and of reasonable cost.

Heavy dependency on field site conditions while relying on intrinsic processes can be a severe disadvantage of MNA processes: pollutant susceptibility to degradation may change drastically depending on variations in environmental factors (Farhadian et al., 2008). Biodegradation of the contaminant by the intrinsic microbial community is often limited either by the concentration of an appropriate electron acceptor or a nutrient required during the biodegradation. The MNA approach requires thorough site characterization before any decisions about bioremediation implementation can be made, and due to possibly changing conditions at field sites, extensive long-term monitoring (i.e. several months to decades). Despite its several disadvantages, MNA has its niche in bioremediation approaches by virtue of its minimized cost compared to engineered options, as well as avoidance of land disruption and human exposure (Andreoni and Gianfreda, 2007). As MNA is most effective at low contaminant concentrations, it is regarded as a good follow-up to active remediation measures that have already been implemented and become unfeasible (Takahata et al., 2006; USEPA, 2007). MNA is also the solution for sites where other bioremediation techniques cannot be applied due to economic or logistic limitations (i.e. far-off cold climate sites) (van Stempvoort and Biggar, 2008).

3.1.2. Biostimulation

The microbial processes of intrinsic bioremediation are often constrained by unfavourable conditions such as low levels or nutrients and electron donors or the low bioavailability of pollutants at contaminated field sites. These limitations can be overcome with the addition of determined growth-limiting nutrients (i.e. nitrogen, phosphate, potassium), electron acceptors/donors (i.e. oxygen) or surfactants (i.e. rhamnolipids) to the contaminated environment to promote the catabolic potential of the indigenous microbial community and accelerate pollutant degradation on biostimulation approach.

Nutrients (i.e. nitrogen, phosphorus) often become limiting factors, especially when the contaminant functions as a C source (i.e. petroleum products) (Röling and van Verseveld, 2002). The addition of nutrients in the form of inorganic and organic fertilizers is the most frequent biostimulation application. Numerous types of amendments such as inorganic fertilizers (Garcia-Blanco et al., 2007; Delille et al., 2009), wastewater sludge (Fernández-Luqueño et al., 2008), sewage sludge compost (Hamdi et al., 2007), vermicompost (Contreras-Ramos et al., 2008), municipal solid waste compost (Sayara et al., 2011), manure (Liu et al., 2010) and biosolids (Sarkar et al., 2005) have been utilized to enhance the degradation of petroleum products in the subsurface of contaminated sites. Water environments, especially marine oil spills, are somewhat more difficult to manage with biostimulation, as added nutrients are diluted and may be washed out by wave action. To combat these effects, slow release and oleophilic fertilizers have been developed (Nikolopoulou and Kalogerakis, 2009). However, it must be ensured that nutrients are maintained in the

treated matrices; microbes are unable to utilize dissolved nutrients that are washed out quickly (Lee et al., 1999; Tyagi et al., 2011). On the other hand, excessively high nitrogen levels can be toxic and can inhibit microbial activity; in an aquatic environment, excess nutrients can also cause algal blooms (Nikolopoulou and Kalogerakis, 2009). Therefore nutrient injections must be thoroughly optimized according to the conditions of each treated site.

The majority of organic contaminants can be degraded both aerobically and anaerobically; the type and dominant degradation pathway depends on the availability of terminal electron acceptors in given conditions. Available electron acceptors are often utilized in a sequence related to their energy yields per unit of oxidized organic carbon in the following order: aerobic respiration, denitrification, Mn(IV) and Fe(III) reduction, sulfate reduction and methanogenesis (Bouwer and Zehnder, 1993). Aerobic conditions have been found to be preferable in bioremediation applications, as aerobic biodegradation processes tend to occur considerably more rapidly due to the greater potential energy yield compared to other terminal electron acceptors. However, in contaminated environments oxygen diffusion may be limited, and available oxygen is consumed faster than it can be replaced. In such situations favourable conditions for biodegradation are maintained by air, oxygen or hydrogen peroxide injections to contaminated matrices. Among other applications, this approach has also been used on the mesocosm scale to enhance PAH degradation in groundwater (Richardson et al., 2012), DCE degradation in soil and groundwater (Olaniran et al., 2006) and in field scale to enhance vinyl chloride degradation in groundwater (Begley et al., 2012). Although the rate of aerobic biodegradation is higher than that of anaerobic biodegradation, anaerobic processes are more dominant in several field conditions and may be the only possible solution for pollutant removal, as it is often difficult to inject oxygen into underground waters or deep subsurface layers. The contaminant-acclimatized microbial community can then be supported by injections of respective electron acceptors such as sulfate (Sublette et al., 2006) or chelated-ferric iron (Da Silva et al., 2005) to ensure the sustainability of the contaminant degradation process.

Besides other factors, the limited bioavailability of a pollutant can severely decrease the biodegradation efficiency at contaminated sites. The bioavailability of a pollutant and therefore the efficiency of biodegradation can be improved by the addition of biosurfactants to the contaminated matrices. Biosurfactants (i.e. rhamnolipids, surfactin) are small biodegradable detergent-like molecules produced by microbes, which can enhance the solubilization of a contaminant (i.e. PAHs), disperse oil into smaller droplets or disrupt pollutant-soil bonds (Nikolopoulou and Kalogerakis, 2009; Fernández-Luqueño et al., 2011). The use of biosurfactants to enhance the biodegradation rate is well studied (Rahman et al., 2002; Bordoloi and Konwar, 2009) and used widely in biostimulation applications, mostly for the treatment of petroleum and PAH contamination (Cui et al., 2008; Das et al., 2008; McKew et al., 2007; Sanscartier et al., 2009).

Like other in situ bioremediation techniques, biostimulation requires rigorous site characterization before any decision about technology implementation can be made, and continuous monitoring of nutrient availability to ensure the proper interval of treatments. However, biostimulation enables naturally occurring microbes to adapt better and faster to the spill environment, resulting in a shorter lag phase and faster contaminant degradation (Nikolopoulou and Kalogerakis, 2009). In addition to the aforementioned prevalent amendment variants environmental conditions can also be improved in order to obtain optimal values of pH, electron donors, moisture content and temperature, making biostimulation a versatile bioremediation technique. Different biostimulation amendments can be used in unison, such as the simultaneous addition of nutrients and biosurfactants (McKew et al., 2007). Furthermore, biostimulation can provide suitable nutrients and conditions to both indigenous and exogenous microbes, often making combinations with bioaugmentation more efficient than the two techniques applied separately (Olaniran et al., 2006; Baek et al., 2007; Hamdi et al., 2007).

3.1.3. Bioaugmentation

Many pollutants are complex compounds or a mixture of different contaminants that are degradable only by a specific set of microorganisms and pathways. Even when the appropriate catabolic microbes are present in the intrinsic microbial community at the contaminated site, the abundance and activity of the microorganisms may be too low for successful bioremediation. In such cases bioaugmentation of highly concentrated and specialized populations (single strains or consortia) able to degrade the xenobiotic compounds of interest is used to enhance the degradative capacity of the microbial community and the transformation rate of the pollutants severalfold. The most commonly-used options for bioaugmentation covering the catabolic degradation route of the contaminant are: addition of a pre-adapted pure bacterial strain; addition of a pre-adapted consortium; introduction of genetically engineered bacteria; addition of biodegradation-relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms (El Fantroussi and Agathos, 2005).

Successful application of bioaugmentation is dependent on the identification, isolation and characterization of appropriate microbial strains, and their subsequent survival and catabolic activity once released into the target habitat (Thompson et al., 2005). No microorganisms or their groups are universally applicable to bioaugmentation, but many microbes are metabolically versatile and capable of degrading a wide spectrum of substrates. Gram-negative bacteria have been the prevalent inoculums either in consortiums or individually in bioaugmentation trials. *Pseudomonads* have been used to degrade aliphatic (Ueno et al., 2006), aromatic (Heinaru et al., 2005; Yu et al., 2005) and halogenated compounds (Niu et al., 2009), as well as pesticides like atrazine (Shapir and Mandelbaum, 1997) and explosives (van Dillewijn et al., 2007),

among other xenobiotics. *Sphingomonads* and *Acinetobacter* strains have mainly been used for the degradation of various aromatic compounds (Ruberto et al., 2003; van Herwijnen et al., 2006; Coppotelli et al., 2008); representatives of numerous other gram-negative genera have also been successfully, albeit less frequently, used to degrade wide variety of contaminants (Mrozik and Piotrowska-Seget, 2010; Tyagi et al., 2011). Of gram-positive bacteria, members of *Dehalococcus* genus are used extensively for the degradation of halogenated compounds (Major et al., 2002; Mao et al., 2012; Popat et al., 2012), while *Rhodococcus* strains have been applied for the degradation of fuel additive ethyl *tert*-butyl ether – ETBE (Fayolle-Guichard et al., 2012), aromatic compounds (Gentili et al., 2006) and halogenated compounds (Semprini et al., 2009). Other gram-positive bacteria such as *Mycobacterium* and *Bacillus* harnessed for PAH degradation (Yu et al., 2005; Jacques et al., 2008; Silva et al., 2009b) have been used to a lesser extent in bioaugmentation applications (Mrozik and Pietrowska-Seget, 2010). In addition to bacteria, fungi like *Achremonium*, *Aspergillus*, *Verticillium* and *Penicillium* can be used as inoculums for the degradation of various aromatic compounds (Mancera-López et al., 2008; dos Santos et al., 2008; Silva et al., 2009a;b).

Several studies have observed that the improvement of the bioremediation activity might be temporary, and the number of inoculated microorganisms decreases shortly after the addition of the biomass to the site, consequently nullifying the effect of the accelerated removal rate of the pollutant (Blumenroth and Wagner-Döbler, 1998; Bouchez et al., 2000). The relationship of the inoculated microorganisms with its new biotic and abiotic environments, in terms of survival, activity and migration, can be decisive in the outcome of any bioaugmentation strategy (El Fantroussi and Agathos, 2005; Pandey et al., 2009). Therefore, a comprehensive assessment of both abiotic and biotic environmental factors and their impacts on the bioaugmentation process are significant to confer the optimal efficiency to the process at the field site (Mrozik and Piotrowska-Seget, 2009; Pandey et al., 2009). It has been suggested that the best way to increase the survival of the inoculum is to base the selection of competent microbes on prior knowledge of the microbial communities inhabiting the target site (Thompson et al., 2005; Hosokawa et al., 2009); if this is not possible, candidate microbes should be chosen from the same ecological niche as the polluted area (El Fantroussi and Agathos, 2005). Apparently, indigenous microbes (pre-selected for bioaugmentation) are more likely to persist and propagate when reintroduced into the site, as compared to transient or alien strains to such habitat (Thompson et al., 2005). This also explains the reported poor performance of highly adapted commercial microbial cultures (Venosa et al., 1992; Simon et al., 2004). From an applied perspective, using a microbial consortium rather than a pure culture for bioremediation is more advantageous, as it provides the metabolic diversity and robustness needed for field applications (Heinaru et al., 2005; Jacques et al., 2008). The effects of predation, competition and low availability of nutrients are sometimes combated by encapsulation of the selected microbes

into the carrier materials (i.e. agar, alginate, gellan gum, gelatin gel, κ-carrageenan, activated carbon etc.) generating protective barriers around microorganisms and providing temporary nutrition, resulting in a better survival rate of the bacterial strains upon inoculation (Moslemy et al., 2002; Parameswarappa et al., 2008; Liang et al., 2009). The bioavailability of some contaminants (i.e. some PAHs, biphenyls) can be enhanced by using surfactant-producing strains as inoculum (Gentry et al., 2004).

Alternatively is argued that more important than the survival of introduced microbes is the survival of catabolic traits. Genetic information encoding the degradation of xenobiotic compounds is often found on plasmids or other mobile elements and can also potentially be transferred to the local microbial community from dead inoculum (Top et al., 2002). Therefore this approach has the advantage of being independent of the survival and the propagation of the donor strains and may be useful in unfavourable conditions for inoculum survival (Dejonghe et al., 2001). Despite several successful plasmid-mediated bioaugmentation trials at lab scale (Top et al., 1999; Bathe et al., 2005; Mohan et al., 2009), this approach has rarely been used at field scale, a few examples concerning the pesticide atrazine (Strong et al., 2000) and oil compounds degradation (Jussila et al., 2007) can be found. As in the case of genetically engineered microbes exhibiting enhanced degradative capabilities that have been tested extensively at lab scale for bioaugmentation purposes (Rodrigues et al., 2001; Monti et al., 2005; Massa et al., 2009), plasmid-mediated bioaugmentation at field scale is hindered by unforeseen risks (i.e. horizontal gene transfer to the native microbial community) associated with their release into the environment (Pandey et al., 2009) as well as limited public acceptance.

Likewise to several other bioremediation techniques (i.e. biostimulation, rhizoremediation), bioaugmentation is well characterized at lab scale under controlled conditions. Reports of successful field-scale trials are still fewer due to the aforementioned difficulties, and it has been suggested that combinations with other techniques (i.e. biostimulation) might prove beneficial in speeding up bioremediation (Silva et al., 2004; Hamdi et al., 2007). The fate of the bioaugmentation process depends heavily on the characteristics of each field site and therefore the monitoring of degradation processes and inoculum survival and its abundance is of high priority to enable any meaningful predictions of the process results (El Fantroussi and Agathos, 2005).

3.1.4. Rhizoremediation

Harnessing plants capable of metabolizing organic contaminants directly or in unison with the microbial community in soil is another option for in situ bioremediation. Phytoremediation is defined as the use of green plants to degrade, stabilize and/or remove environmental contaminants (Gerhardt et al., 2009). Phytoremediation is further divided into phytostabilisation, phytoextraction, phytovolatilization and rhizodegradation, owing to the predomi-

nant contaminant-affecting process (Wenzel, 2009), the latter of the four being mainly dependent on microbial processes in the plant root zone.

Rhizoremediation (also rhizodegradation, microbe-assisted phytoremediation) utilizes the complex interactions involving roots, root exudates, rhizosphere soil and microbes that result in the degradation of contaminants to non-toxic/less-toxic compounds. Plant roots stimulate rhizosphere microbial communities by aerating the soil and releasing nutrients through root exudates as well as providing niches to protect bacteria against desiccation and other abiotic and biotic stresses (Kuiper et al., 2004). Rhizospheric microorganisms in turn promote plant growth by nitrogen fixation, nutrient (i.e. phosphorus) mobilization, production of plant growth regulators, decreasing plant stress hormone levels, providing protection against plant pathogens and degradation of pollutants before they negatively impact the plant (Chaudhry et al., 2005; Segura et al., 2009). Consequently these mutual interactions, also known as the rhizosphere effect, result in an elevated number, diversity and metabolic activity of microbes able to degrade contaminants or support plant growth in the close vicinity of roots compared to bulk soil (Ramos et al., 2000; Kent and Triplett, 2002).

In addition to substantial amounts of root-exuded sugars, amino acids and organic acids (as much as 40% of plants photosynthate can be deposited in soil (Kumar et al., 2006)) usable for microbes as carbon and energy sources, plants produce and depose through the roots secondary metabolites such as isoprenoids, alkaloids and flavonoids. The structure of many secondary plant metabolites resembles those of contaminants (i.e. PCBs, PAHs), and they can induce catabolic genes in microbes that also can degrade the xenobiotic analogue (Singer et al., 2003). For instance, the growth of PCB-degrading bacteria and PCB degradation is enhanced by flavonoids apigenin and naringin (Fletcher and Hegde, 1995). Easily degradable root-exuded compounds can also serve as co-metabolites in processes in which contaminants cannot be used as a sole carbon source (i.e. aerobic degradation of trichloroethylene (Hyman et al., 1995)) due to the negative energy balance (Reichenauer and Germida, 2008). Plant roots, along with some rhizospheric bacteria, may also excrete biosurfactants, thus increasing the bioavailability and uptake of pollutants (Schwitzguébel et al., 2002; Kuiper et al., 2004). This aspect can be especially beneficial in aged soils with low contaminant bioavailability that generally appear to be much less responsive to rhizodegradation than freshly spiked soil (Dams et al., 2007; Gunderson et al., 2007).

Naturally occurring rhizoremediation may be suppressed by the toxicity of the contaminant or several environmental factors such as low nutrient levels. One possible solution that has been proposed is the use of endophytic bacteria that colonize the internal tissues of the plant without causing a negative effect since there is less competition for nutrients in the roots and bacteria are physically protected from adverse changes in the environment (Reinhold-Hurek and Hurek, 1998). However, successful remediation by endophytic bacteria requires the transport of the pollutant to the plants' internal tissues, and the success of this process depends on soil, contaminant and plant properties (Sung

et al., 2001). Despite evidence that endophytic bacteria can enhance the in situ phytoremediation of TCE and BTEX compounds in field experiments (Weyens at al., 2009a; b), this phytoremediation option is still rarely used. As the composition of root exudates depends on plant species (Segura et al., 2009), and this exerts selective pressure on the rhizospheric microbial community, rhizoremediation applications are optimized by the selection of suitable plant-microbe pairs and support for their growth. For this purpose, combinations with bioaugmentation and biostimulation to facilitate plant and microbe growth are used (White et al., 2006; van Dillewijn et al., 2007).

Even enhanced rhizoremediation may be considerably slower than ex situ treatments due to environmental restrictors at field sites such as competition by weed species which are better adapted to the site (Nedunuri et al., 2000), limited plant growth in heavily and unevenly contaminated soil and the presence of plant pathogens and other biotic and abiotic stressors (Gerhardt et al., 2009). Furthermore, rhizoremediation is only effective in the rooting zone and is unsuitable for usage in deeper subsurface layers. Some toxic contaminant metabolites can also bioaccumulate in plants, making strict regulations of plant material treatment necessary. However, despite the aforementioned shortcomings rhizoremediation is emerging as one of the most effective means by which plants can affect the remediation of organic contaminants, particularly large recalcitrant compounds (Gerhardt et al., 2009). In addition to its relatively low maintenance costs, no size restrictions for the area and environmentally friendly nature, the quality and texture of the soil is also improved by the addition of organic materials, nutrients and oxygen via plant and microbial metabolic processes. Despite the challenge of introducing phytoremediation from the lab and greenhouse scale to the field, rhizoremediation has been used to treat field sites contaminated with petroleum hydrocarbons (Nedunuri et al., 2000; Siciliano et al., 2003), PAHs (Robinson et al., 2003; White et al., 2006), TNT (van Dillewijn et al., 2007), BTEX (Barac et al., 2009) and TCE (Weyens et al., 2009a;b). Like the other bioremediation techniques, detailed monitoring is essential for process efficiency and environmental safety considerations and in order to avoid undesired effects.

3.2. Monitoring microbial processes of bioremediation

The analysis of microbial populations in the soil and groundwater of contaminated sites undergoing bioremediation has become the cornerstone of bioremediation monitoring and subsequent decision-making. Numerous methods, both traditional culture-based approaches and rapidly developing molecular methods, are available for environmental monitoring; the choice of method used depends on the question investigated as well as the availability of knowhow and technical means.

In culture-based methods such as live-dead staining, plate-counting, bioluminescence monitoring, data are obtained by analyzing material derived

from microbial growth. Culture-based environmental monitoring methods are limited, as only a small percentage of microorganisms (approximately 1%) in the soil and other environmental matrices are culturable. Furthermore, even for culturable bacteria, there is no guarantee that activity measured in the lab is relevant to that which occurs under the range of conditions that exist in soil (Hirsch et al., 2010). Hence the culture-based methods, while beneficial for investigating specific problems, have been dwarfed by the numerous molecular bioremediation monitoring approaches over the last decade (Desai et al., 2010; Hirsch et al., 2010; van Elsas and Boersma, 2011).

Molecular methods circumvent the need for cultivation by targeting the DNA or RNA extracted directly from environmental matrices, enabling access to most of the community in the addressed habitat. DNA extracted from environmental samples represents the total metagenome, including components that are not active or are no longer viable, and can be used to evaluate bioremediation potential. RNA is synthesized only by actively-growing cells and can be used to identify the functioning members of the targeted microbial communities. Depending on the research question, either taxonomic (i.e. 16S or 18S rRNA) or functional genes are targeted (Cébron et al., 2008; Kao et al., 2010). As microbial community DNA or RNA is used as the starting material for most molecular analyses, it must be ensured that the extraction methods used guarantee the high yield and purity of the template. Low-quality template material extractions will strongly affect the results of the microbial community analysis and can lead to erroneous decision-making in bioremediation applications.

The diversity of the microbial community can routinely be evaluated using various fingerprinting methods such as denaturing/temperature gradient gel electrophoresis (DGGE/TGGE), length-heterogeneity polymerase chain reaction (LH-PCR), terminal restriction fragment length polymorphism (T-RFLP), ribosomal intergenic spacer analyses (RISA) and single-strand conformation polymorphism (SSCP). Even more comprehensive coverage of community diversity and composition can be achieved using microarrays or new-generation high-throughput sequencing; the active proportion of the community can be estimated when RNA is targeted. Quantitative polymerase chain reaction (qPCR) and reverse transcriptase PCR (RT-PCR) enable measurement of the targeted community or active microbial group abundance based on DNA and RNA templates respectively. Another possibility to estimate the active part of the community is to use stable isotope probing (SIP) or 5-bromo-2`-deoxyuridine (BrdU) staining. Each of these aforementioned methods used for microbial bioremediation monitoring has their distinctive advantages but also limitations (Table 2).

Even though some methods (i.e. qPCR) are more popular than others for environmental monitoring, none of these methods, including traditional culture based approaches, can be overlooked or dismissed, as they may prove useful in answering specific research questions. In various cases a combination of several monitoring methods is useful or even unavoidable for investigation of testable hypothesis (van Elsas and Boersma, 2011).

Table 2. Molecular methods used in bioremediation monitoring.

Method	**Interpretation of results**	**Advantages**	**Disadvantages**	**Examples of bioremediation cases**
Fingerprinting	Provides snapshot views of microbial diversity and community make-up by separating out PCR products derived from universal primers. High reproducibility.	Allows easy and rapid comparisons between samples. Methods with different resolution and reproducibility available.	Only a dominant part (≥0.1 %) of the target community is accessed. Limited resolution. Change in the fingerprint can reflect change in the number of populations above the detection threshold instead of change in diversity. Possible coincide reads caused by different amplicon co-migration. Susceptible to 16S rRNA gene microheterogenity.	Phenolic compounds; bioaugmentation (DGGE; Juhanson et al., 2009). Acid mine drainage; bioreactors (TGGE; Martins et al., 2011). Diesel oil; biostimulation, bioaugmentation (T-RFLP; Vázques et al., 2009). Phenolic compounds; biostimulation (SSCP; Lin et al., 2007). PCB; biostimulation, bioaugmentation (RISA; Petríc et al., 2011). TCE; biostimulation (LH-PCR; Connon et al., 2005).
Clone libraries	PCR-generated amplicons cloned into vectors, introduced into the host strains, isolated by plasmid extraction and sequenced. Provides information about dominant sequence types in the community. Medium reproducibility.	Easy census of target genes in community. Allows diversity estimates and direct data on unique sequences.	Laborious preparation of sample. Cloning bias affects the interpretation of true microbial diversity. Limited overview of target gene/organism diversity. Microarray analysis and high-throughput sequencing are rapidly replacing this technique.	RDX, HMX (high melting explosive), herbicides; bioreactors (Perchet et al., 2008).

Table 2. Continuation.

Method	Interpretation of results	Advantages	Disadvantages	Examples of bioremediation cases
qPCR/RT-qPCR	DNA/cDNA sequences are amplified and quantified. Provides information on the presence and abundance of targeted genes in microbial community. High reproducibility.	Robust and high-throughput technique. High sensitivity.	Several PCR biases and artifacts (see section 3.3). Only species over 0.1–1% abundance are visible.	PAH; natural attenuation (Kao et al., 2010). Aromatic compounds of gasoline; natural attenuation (Baldwin et al., 2008).
SIP and BrdU	A label (i.e. ^{13}C, BrdU) is incorporated into the nucleic acids or phospholipid fatty acids (PLFA) of actively-growing cells. Provides information on the presence, diversity and activity of the community. High reproducibility.	The relation between structure and function can be elucidated. Detection is sensitive to genus, species or functional group level. In situ activity can be assessed.	Presence of opportunists blurring the data. Activity of microbes can be very low.	PAH; bioreactors (Singleton et al., 2005). Toluene; natural attenuation (Winderl et al., 2010). Benzene; natural attenuation (Herrmann et al., 2010).
Microarrays	Fluorescently labelled target (DNA/RNA) is simultaneously hybridized to a large set of oligonucleotide probes on a chip. Provides information on community diversity and make-up, the relative abundance of different taxa and individual sequences. Medium reproducibility.	Very high throughput. Monitoring is rapid and sensitive.	Only known and chipped genes are targeted. Possible cross-hybridizations with low-homology sequences. Complicated chip design.	Diesel-oil; bioaugmentation, biostimulation (Lin et al., 2010). TCE; natural attenuation (Nemir et al., 2010). TCE; biostimulation (Dugat-Bony et al., 2012).

Table 2. Continuation.

Method	**Interpretation of results**	**Advantages**	**Disadvantages**	**Examples of bioremediation cases**
High-throughput (massive parallel) sequencing	Large numbers of sequences are processed simultaneously to gain information on total and active members of the community. Medium reproducibility.	All-in-one analysis of microbial community sequences. Very high throughput. High potential for comparative studies.	PCR amplification prior to sequencing creates bias. Caution with data interpretation needed due to occurring artifacts/errors. Need for specific capability and knowhow in order to analyse immense amount of data obtained.	Toluene; natural attenuation (Fowler et al., 2012). PAH; natural attenuation (Berdugo-Clavijo et al., 2012).

3.3. The principle and potential of quantitative PCR in bioremediation monitoring

The basic goal of quantitative PCR is to distinguish and measure precisely specific nucleic acid sequences in a sample, even if there is only a very small quantity. The conventional PCR technology has gone through several development steps to fulfil this goal.

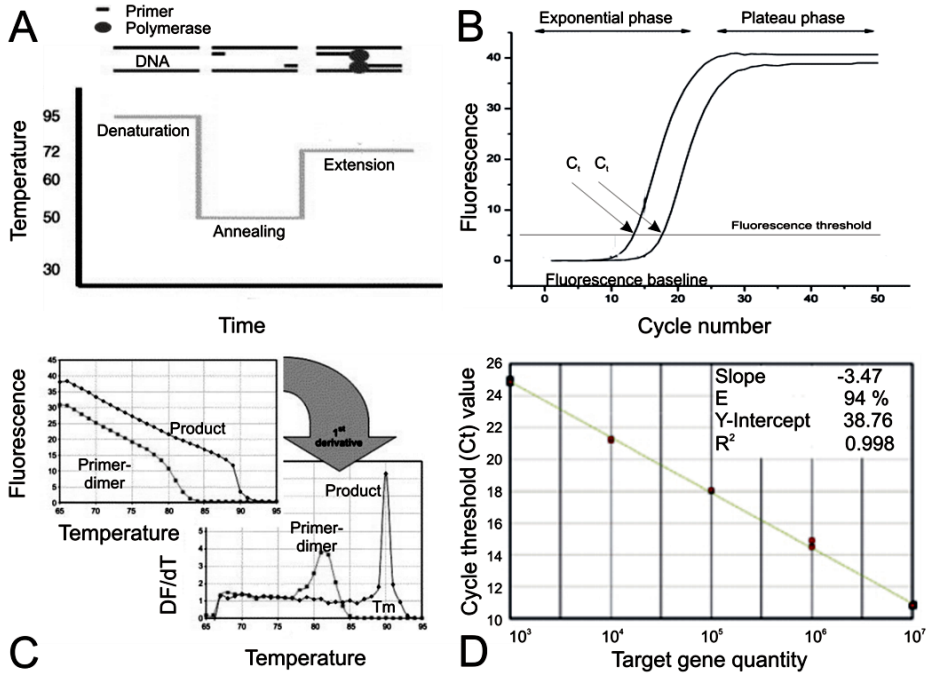

Figure 1. Essential aspects of quantitative PCR (modified from Kubista et al., 2006; Smith and Osborn, 2009). **A** – **The PCR temperature cycle:** the temperature is raised to about 95 °C to melt the double-stranded DNA; the temperature is lowered to let primers anneal; the temperature is set to 72 °C to let the polymerase extend the primers. **B** – **Quantitative PCR amplification curves:** a fluorescence threshold level is set sufficiently above the fluorescence baseline and the number of cycles required to reach threshold, C_t, are registered. **C – Melting curve analysis:** dye fluorescence drops rapidly when the DNA melts. The melting point is defined as the inflection point of the melting curve, which is most easily determined as the maximum in the negative 1st derivative of the melting curve. The amplicon produced from the target product is typically longer and melts at higher temperature than the primer-dimers. **D** – **Standard curve:** the dilution series of known concentrations of template DNA are amplified upon qPCR and plotted as the linear regression of the C_t values of the amplification curves versus the log of the initial gene copy number. QPCR descriptors are shown (box). Quantification of the unknown target template is determined by comparison of the C_t values of the target template against the standard curve.

The polymerase chain reaction (PCR) was developed in the mid 1980s by Mullis and his colleagues (Saiki et al., 1985), and enables amplification of essentially any nucleic acid sequence present in the complex sample in a cyclical process in order to generate a large number of identical copies that can be readily analysed. The targeted DNA template is identified with two short synthetic and sequence-specific oligonucleotides (primers) that also act as the initiation points for the synthesis, which is carried out by polymerase using nucleotide triphosphates (dNTP) as building blocks (Fig. 1A). The reaction gives rise to essentially the same amount of product independently of the initial amount of DNA template molecules present in the reaction mixture, making the target quantification at the end of the reaction exceedingly difficult and questionable.

Quantitative PCR (also referred to as real-time PCR or quantitative real-time PCR), enabling target gene enumerations was subsequently developed in 1992 (Higuchi et al., 1992). In qPCR the template amplification is recorded during the course of the reaction via an increase in fluorescence signal in every cycle, which is directly proportional to the amplified DNA (Fig. 1B; Fig. 2).

Basic equation of PCR kinetics:

Eq. 1 $N_c = N_0 * E^C$

Eq. 2 $E = N_{C+1} / N_C$

Estimation of starting concentration:

Eq. 3 $N_0 = N_t / E^{Ct}$

Estimation of fold difference:

$$\text{If } C_{t,A} = C_{t,B} \qquad \text{If } E_A = E_B$$

Eq. 4 $= N_{0,A} / N_{0,B} = (N_{t,A} / E_A^{Ct,A}) / (N_{t,B} / E_B^{Ct,B}) = E_B^{Ct,B} / E_A^{Ct,A} = E^{Ct,B-Ct,A}$

Figure 2. Equations used in the analysis of quantitative PCR data (modified from Ruijter et al., 2009). **Eq. 1:** The basic equation for PCR kinetics states that the amount of amplicon after *c* cycles (N_c) is the starting concentration of the amplicon (N_0) times the amplification efficiency (E) to the power *c*. The PCR efficiency in this equation is a number between 1 and 2 (2 indicates 100% efficiency). **Eq. 2:** The PCR efficiency can be defined as the increase in amplicon per cycle. During the exponential phase of the PCR reaction efficiency is constant. **Eq. 3:** Equation 1 can be inverted to calculate the starting concentration (N_0) from the user-defined fluorescence threshold (N_t), the efficiency and the fractional number of cycles needed to reach the threshold (C_t). **Eq. 4:** The starting concentration of amplicon A ($N_{0,A}$) can be expressed relative to that of amplicon B ($N_{0,B}$) by direct division of these starting concentrations. When the fluorescence thresholds for both amplicons are equal, the expression ratio can be "simplified". Further reduction of the number of parameters requires that the efficiencies of both amplicons (E_A and E_B) are equal.

During the initial cycles the fluorescence signal is weak and cannot be distinguished from the background. Quantification of the starting template of the target gene in the sample is achieved by determining the threshold cycle (C_t) at the exponential phase of amplification when the amount of target amplified is proportional to the starting template from a range of standards constructed from known amounts of the target gene in question. At the latter part of the reaction the fluorescence signal levels off and saturates due to the reaction running out of some critical components. In order to understand and make use of the qPCR reaction, the following concepts are essential (Dorak, 2012; LinRegPCR (11.0) manual):

Amplicon – qPCR-amplified target sequence.

Amplicon group – A set of samples in which the same pair of primers is used to amplify the DNA-of-interest.

Exponential (log-linear) phase – The section of the qPCR amplification curve which best represents the exponential phase of the qPCR reaction, when the levels of generated fluorescence exceed baseline fluorescence, but reagents have not yet begun to be limiting. In this phase the amplification efficiency is similar across samples regardless of the starting concentration.

Plateau phase – The endpoint phase of the qPCR reaction in which there is significant depletion of one or more reaction components. In the plateau phase the amplification curves of the quantitative PCR are no longer exponential and the PCR efficiency drops to zero.

Fluorescence threshold – The threshold is set either automatically or manually at a fixed amount of fluorescence in the region associated with an exponential growth of PCR product above the highest fluorescence baseline signal level.

C_t – Reflects the number of cycles needed for fluorescence generated within the reaction to reach the fluorescence threshold. This is inversely correlated to the logarithm of the copy number of the initial target gene, and is used to calculate the target gene's starting concentration per sample comparing its C_t value to a standard curve or a reference sample. Samples with higher starting concentrations will reach this threshold earlier and will have a low C_t value.

Fluorescence background – The fluorescence of the reference fluorochrome (ROX or Fluoresceine) used to correct for variations in pipetting and/or fluorescence outside the reaction wells. The background is handled by the PCR system.

Fluorescence baseline – Measured fluorescence when no amplification-specific fluorescence can yet be determined. This includes fluorescence from cDNA, primers and unbound reporters.

PCR efficiency (E) – Efficiency is calculated from the slope of the amplification curve in the exponential phase. Ideally the PCR efficiency is 100%, meaning that in each cycle the amount of amplicon doubles.

Tm – Melting temperature, at which the double-stranded amplicon separates in the melting curve analysis.

Some qPCR chemistries (i.e. SYBR green), where the fluorophore is incorporated into the final PCR product, enable the melting (dissociation) curve analysis after the completion of the PCR cycling to confirm that the fluorescence signal was generated only from the target templates and not from nonspecific PCR products. In the melting curve analysis the temperature is gradually increased and the fluorescence is continuously measured as function of temperature (Fig. 1C). Initially the fluorescence decreases gradually with increasing temperature; however, when the temperature is reached at which the double-stranded DNA separates, the fluorescent reporter dissociates and fluorescence drops abruptly. This temperature is referred to as melting temperature and is determined by the length and sequence of the amplified product. Possible primer-dimers and nonspecific products have different melting temperatures than the target amplicon and can be readily distinguished through such analysis (Kubista et al., 2006; Valasek and Repa, 2005).

Quantification of the initial target sequences of an unknown concentration can be described either in relative or in absolute terms. In relative quantification changes in the unknown target are expressed relative to a co-amplified steady-state reference gene (Bustin, 2010). It is difficult to apply such approach to the study of prokaryotes, due to the absence of valid steady-state reference genes (Smith and Osborn, 2009). In absolute quantification protocols, the numbers of a target gene are determined from a standard curve generated from the amplification of the target gene present at a range of known template concentrations, and the C_t values of each known concentration. A simple linear regression of these C_t values is plotted against the log of the initial copy number (Fig. 1D). Quantification of the unknown target template is determined by comparison of the C_t values of the target template against the standard curve.

Quantitative PCR has been established as a powerful tool in many fields of research and among others is widely applied in microbial ecology to quantify the abundance and expression of taxonomic and functional gene markers within the environment (Smith and Osborn, 2009). The value of QPCR as a rapid, automated, high-throughput, sensitive and reproducible monitoring tool has also been recognized in bioremediation studies (Baldwin et al., 2008; Kao et al., 2010). However, qPCR workflow is a multistep process, in which the variability and uncertainty of biological and technical nature as well as inappropriate experimental design is often encountered, leading to inconsistent or even misleading results (Love et al., 2006; Bustin, 2010). QPCR studies are subjected to experimental variability; therefore the comparison of absolute gene copy numbers generated in different qPCR assays is not reliable, while relative quantities can be compared across multiple qPCR experiments (Smith et al., 2006). PCR detects nucleic acids rather than living cells, so there is a risk of "free" nucleic acids from dead cells causing overestimations of gene abundances. Furthermore, silent and pseudogenes giving false-positive results also exist (Wolffs et al., 2005). Finally, the quantification of genes present at low copy number will be less accurate, as it will include a higher proportion of

non-template derived signals compared to genes present in high copy number (Smith et al., 2006). Therefore the different aspects of qPCR workflow should be considered cautiously and reported with sufficient experimental detail to enable the evaluation of the quality of the results by the reader (Bustin et al., 2009).

3.3.1. Choice of qPCR chemistry

The selection of fluorescence chemistry type to be used in qPCR is one of the first steps of a planned study. Since the first studies in which classical intercalator ethidium bromide was used as the fluorescent reporter (Higuchi et al., 1992), a wide range of different fluorescence chemistries offered by different manufacturers with distinctive pros and cons have been developed. The most suitable, practical and cost-efficient fluorescence chemistry for the purposes of the planned study should be chosen.

Based on Buh Gašparič et al., (2010) the numerous different fluorescent reporters can broadly be categorized as:

1) Sequence-unspecific DNA-labeling dyes (i.e. SYBR green, Evagreen etc.);

2) Techniques involving double-labeled probes

a) Hybridization probes (i.e. molecular beacons – MB)

b) Hydrolysis probes (i.e. TaqMan, cycling probe technology probes (CPT), locked nucleid acid probes (LNA), minor groove binding probes (MGB), light-up probes etc.);

3) primer-based technologies (i.e. AmpliFluor, Plexor, light upon extension (LUX), scorpion, sunrise primers).

For bioremediation monitoring or the evaluation of other aspects of environmental risk in biotechnological processes, SYBR green and TaqMan reporter systems are commonly used, the use of other qPCR chemistries (i.e. molecular beacons and LUX-primers) could seldom be found in the literature (Table 3).

The fluorescence signal generation mechanisms of different fluorescent reporters vary extensively (Fig. 3). Like any other intercalating dye, the fluorescence of SYBR green increases significantly when it binds to double-stranded DNA. In the qPCR, the intensity of fluorescence increases proportionally to amplicon concentration. In probe-based technologies (i.e. TaqMan hydrolysis probes), each probe targeting an additional conserved region within the target amplicon sequence has a reporter fluorophore (i.e. 6-carboxy fluorescein (FAM)) covalently attached to one end and a quencher (the molecule that absorbs the emission of fluorescent reporter when in close, 6 to 10 nucleotides, vicinity) attached to the other. As long as both dyes remain in close proximity, the signal is quenched due to fluorescence resonance energy transfer (FRET), and it is released only when dyes become physically separated on the template extension by the 5` exonuclease activity of the polymerase (Holland et al., 1991). Hybridization probes known as molecular beacons consist of a sequence-specific loop region flanked by two

Table 3. Comparison of qPCR chemistries and examples of their application in environmental technology studies.

QPCR chemistry	**Advantages**	**Disadvantages**	**Examples of use in monitoring**
SYBR green (intercalating dye)	Enables melting curve analysis. Low cost to establish new assay; cost-effective to use.	Binds to any double-stranded DNA (target amplicon, non-specific products, primer-dimers). May bind preferentially to specific DNA sequences (Giglio et al., 2003). Does not enable multiplex gene quantifications. Requires extensive assay optimization. Lower repeatability between runs (Buh Gašparič et al., 2010).	Bioremediation of aliphatic hydrocarbons (Powell et al., 2006; McKew et al., 2007). Bioremediation of aromatic hydrocarbons (Baldwin et al., 2008; Cébron et al., 2008; Kao et al., 2010). Biodegradation of halogenated compounds (Ahn et al., 2007). Bioremediation of herbicides (Gonod et al., 2006; Monard et al., 2008).
TaqMan (hydrolysis probe)	Binding is target-sequence specific. It enables multiplex co-amplification and the co-quantification of several genes by using probes with different fluorophores in one reaction mix.	Needs an additional conserved site (for probe annealing) within a short amplicon sequence. Melting curve analysis is not possible. Needs relatively long probes. Requires two-step PCR, which is not optimal for the polymerase, to function properly. Expensive.	Bioremediation of aliphatic hydrocarbons (Cyplik et al., 2011). Bioremediation of aromatic hydrocarbons (Beller et al., 2002; Winderl et al., 2008; Kazy et al., 2010). Bioremediation of halogenated compounds (Himmelheber et al., 2007; Lee et al., 2008).
Molecular beacon (hybridization probe)	Binding is target sequence specific. Probes are shorter and have higher hybridization specificity than TaqMan. Enables multiplex co-amplification and co-quantification of several genes by using probes with different fluorophores in one reaction mix. Enables melting curve analysis.	Needs an additional conserved site (for probe annealing) within a short amplicon sequence. Can be very sensitive to changes in assay conditions (Andersen et al., 2006). Low repeatability between repeated runs (Buh Gašparič et al., 2010). Complicated probe design. Expensive: higher cost than other probes.	Bioremediation of aromatic hydrocarbons (Ye et al., 2009a;b).

Table 3. Continuation.

QPCR chemistry	Advantages	Disadvantages	Examples of use in monitoring
LUX (light upon extension primers)	Binding is target sequence specific. Enables multiplex co-amplification and co-quantification of several genes by using primers with different fluorophores in one reaction mix. Enables melting curve analysis. Hairpin primers prevent primer-dimer formations and mispriming. Less expensive than probe-based technologies.	Complicated primer and assay design. Assay setup can be more time consuming compared to other chemistries (Buh Gašparič et al., 2010).	Antibiotic resistance determination in wastewater (Börjesson et al., 2009a; b).

inverted repeats. Reporter and quencher dyes are attached to each end of the molecule, and the fluorescence is quenched by the formation of a hairpin structure. Upon binding to a complementary target sequence the beacon unfolds, leading to separation of the fluorophore from the quencher and an increase in fluorescence (Tyagi and Kramer, 1996). The LUX technology includes a self-quenched fluorogenic primer and a corresponding unlabeled primer. The labelled primer has a short sequence tail of 4–7 nucleotides on the 5` end that is complementary to the 3` end of the primer, to which the fluorophore is attached. The resulting hairpin secondary structure provides optimal quenching of the fluorophore; the primer is dequenched upon its integration into a PCR product, and its fluorescence increases up to eight-fold (Nazarenko et al., 2002).

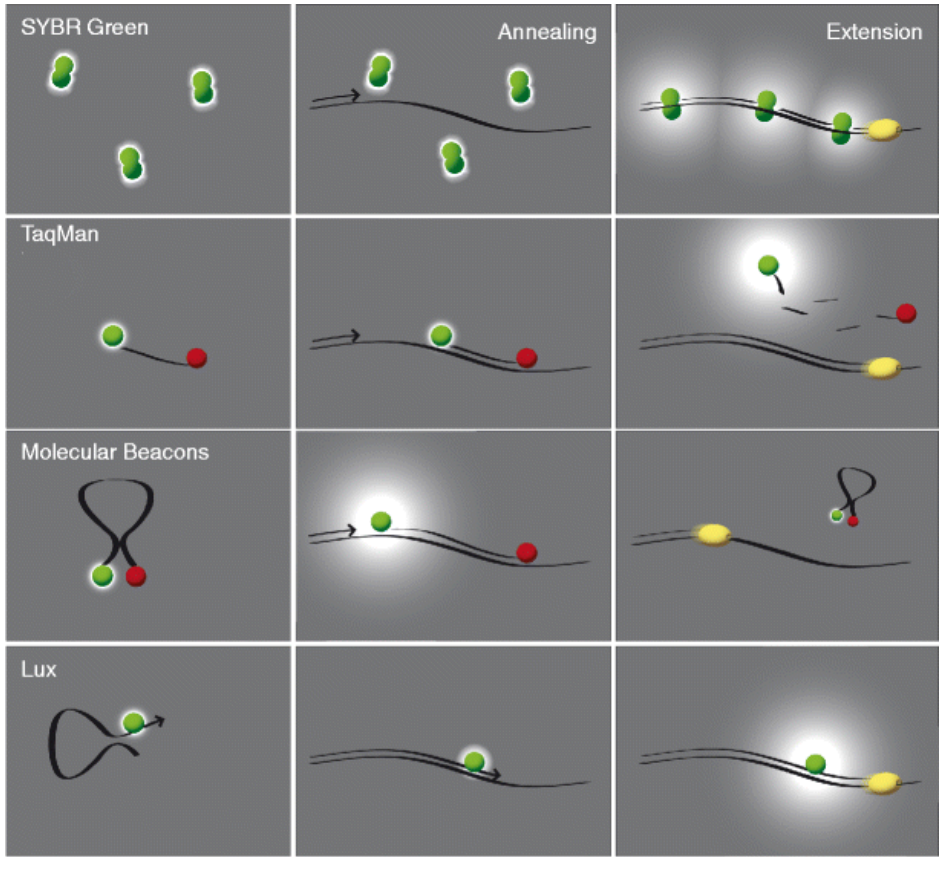

Figure 3. Schematic representation of quantitative PCR with different chemistries. The SYBR green, TaqMan probe, molecular beacon and LUX primer chemistries are presented during the primer annealing step and after the extension, when the new strand of DNA is synthesized by DNA polymerase (in yellow), with a reporter fluorophore shown in green and a quencher shown in red (modified from Buh Gašparič et al., 2010).

3.3.2. Sampling and DNA extraction

Sampling and DNA extraction from collected environmental samples are often excluded from the discussion of qPCR workflow, yet it is recognized that these first steps in the analysis process are critical for the later quantitative interpretation of the obtained data (Sharma et al., 2007).

The details of sampling procedure, such as the number of replicate samples taken, whether sampling is randomized or at regular intervals and whether or not there is bulking and mixing of sub-samples depend on the scientific question of the study, the target biota, the analytical methods to be used and the properties of the sample material (i.e. soil) (Schleuß and Müller, 2001; Hirsch et al., 2010). The overall sampling protocols used to provide a representative sample of any site are well-established and should be followed in order to eliminate possible sampling-derived bias from the experiment (Hirsch et al., 2010).

The choice of method used for nucleic acid extraction is a major determinant of the final target gene quantification, especially from soil samples. Different nucleic acid isolation methods result in target gene number variability (Smith et al., 2006), as each extraction protocol introduces its own biases with respect to extraction efficiency, quality and the quantity of the extracted DNA (Martin-Laurent et al., 2001; İnceoğlu et al., 2010). The key issues causing the variability between different protocols are the efficiency of the release of microbial cells from soil particles, the efficiency of the lysis of bacterial cells and the co-extraction of inhibitory substances (i.e. organic matter, clay particles, humic acids), which all depend on the individual soil sample analysed. The bead beating used for the separation of microbial cells from soil particles may result in enhanced shearing of the DNA of the cells with the most fragile envelopes, and enzymatic lysis may not affect those bacteria that are resistant to excessively soft lysis (İnceoğlu et al., 2010). A protocol optimized to extract genomic DNA from the majority of microbial community will be biased against both tougher and more fragile propagules. The inhibitory substances are either able to bind to nucleic acids inhibiting their purification (Moran et al., 1993; Desai and Madamwar, 2007) or are capable of inhibiting post-extraction analysis (Tebbe and Vahjen, 1993; van Elsas and Boersma, 2011) including qPCR. Of other practicalities, it must be kept in mind that the purification steps following DNA extraction may incur losses of material, and frequent freezing and thawing upon repeated manipulation can impact the integrity of the DNA sample (van Elsas and Boersma, 2011).

It is argued that the numerous available protocols should suit most research needs even if the adopted protocol may depend on the sample type and target community (Hircsh et al., 2010). Yet, for ecological investigations that require an appreciable number of samples to be analysed, it is not possible to optimize protocols for every soil or soil treatment. Moreover, in order to be able to compare copy numbers of target genes, the same DNA extraction-purification

protocol must be maintained throughout the study for all analysed samples (Smith et al., 2006).

3.3.3. Design and optimization of qPCR assay

The qPCR assay design is directly dependent on the aim of the study, the target gene sequence, the qPCR chemistry chosen and whether or not multiplexing (simultaneous analysis of more than one target in the same reaction) will be used. The target specificity of any qPCR assay is determined by the design of two oligonucleotide primers that flank the short (ideally 50–150 bp) DNA sequence to be amplified, and in the case of some qPCR chemistries internal probes (Smith and Osborn, 2009). The requirement for prior sequence data of the specific target gene in question is a major disadvantage of qPCR – only known genes can be quantified (Wolffs et al., 2005). The majority of the high diversity of microbes in the environment is still unknown, and therefore the primers (and probes) designed on known sequences may miss a considerable part of the community (Hong et al., 2009).

All qPCR assays, irrespective of the fluorescence chemistries to be applied, make use of primers that should form stable complexes with the targeted sequences but not with any other sequences or form primer-dimers. Primer-dimers are caused by complementarity between the designed primers (particularly their 3`-ends); their formation interferes with the formation of specific target products on qPCR because the two reactions compete for reagents, leading to erroneous target gene quantification results. Samples containing only a few target molecules are especially vulnerable to that bias (Kubista et al., 2006). When multiplexing is planned, complementarity must be avoided between all the primers used. When the chosen qPCR chemistry allows (i.e. SYBR green, LUX), the primer-dimer formations are routinely checked using melting curve analyses (Fig. 1C). Upon probe-based qPCR chemistries, the primer-dimer formation is either checked on gel-electrophoresis or with the addition of sequence non-specific BOXTO dye, enabling melting curve analysis of the reaction (Lind et al., 2006).

Primers used with SYBR green chemistry can be designed using any primer design program (i.e. Primer3Plus); for other more complicated qPCR chemistries, specific primer and probe design programs are available on the homepages of biotechnology firms (i.e. D-LUX™ (Invitrogen) for LUX, Beacon Designer™ (Premier Biosoft) for TaqMan). Despite the plentiful primer and probe design programs available, knowledge of the following practicalities concerning different chemistries is still useful, as it helps to evaluate the program-proposed solutions. The high content of G and C bases in the primer sequence increases its specificity. The TaqMan probe should be situated as close as possible to the forward primer without overlapping; it should not have a guanine nucleotide (natural quencher) at the 5` end or have more G-bases than C-bases; the melting temperature of the probe should be 8–10 °C above the melting temperature of the primers. LUX-primers are labeled

with a single fluorophore on a G or C base close to the 3` end of the primer; a tail of 5–7 nucleotides complementary to the 3` end of the primer is added to the 5` end of the primer to form a blunt-end hairpin (ΔG from -1.6 to -5.8 kcal/mol) when the primer is not incorporated into double-stranded DNA (Nazarenko et al., 2002). Specific quantification of multiple targets that are amplified within a reaction can be performed using a differentially labelled primer or probes. A good probe, independent of chemistry, should have low background fluorescence, high fluorescence upon target formation (a high signal to noise ratio), and high target specificity (Kubista et al., 2006).

Upon assay optimization the optimal primer annealing temperature, the duration of cycling steps, the concentrations of primers and other reagents as well as the detection limit of the assay is determined empirically. The annealing temperature depends on the primers and is theoretically a few degrees below the melting temperature of the two primers, ensuring target-specific binding (Kubista et al., 2006). SYBR green and LUX assays typically use three-step temperature cycling (Fig. 1A), while in TaqMan assays the annealing and extension steps are often combined and performed at the same temperature (i.e. 60 °C). The use of elevated elongation temperature is probably more important to melt any secondary structures that may form in the template and may block extension (Kubista et al., 2006). It has been suggested that extensive optimization of primer concentrations used in SYBR green qPCR assays may be required to ensure that only the target product is formed (Smith and Osborn, 2009). Even more extensive primer concentration optimization is required in multiplex assays in which the parallel reactions compete for reagents, and limiting amounts of primers are used to minimize this competition (Kubista et al., 2006). The sensitivity of qPCR allows for the quantification of very low copy numbers of target genes. This should, however, be backed up by providing information on the amplification signal detected, if any, within the no template control (NTC) (Smith and Osborn, 2009). The quantification of low numbers of the target gene may be artificially increased by the presence of the amplification signal within the reaction that is equivalent to that quantified within the NTC.

3.3.4. Standard curve properties

Irrespective of the fluorescence chemistry used, absolute quantification of the target gene in an unknown sample is carried out in essentially the same manner in most studies: the numbers of a target gene are determined from a standard curve generated by amplification of the target gene present at a range of known template concentrations, and the C_t values of each known concentration (Fig. 1D). Any quantification result depends on the quality of the calibration standard. In contrast to relative quantification, in which the same steady-state housekeeping gene can be used as a reference in numerous assays, in absolute quantification an independent, reliable and highly reproducible standard is necessary for each gene to be analysed. There are many factors,

both technical (i.e. uncertainties in initial quantification of the standard curve template and serial dilutions of the standard curve template) and empirical (i.e. determination of the fluorescence threshold), that can contribute bias to the construction of a standard curve and therefore to the subsequent quantification of the unknown template (Love et al., 2006).

Plasmids carrying the target gene are typically used for standard curve creation. The amount of DNA in the primary standard is usually estimated via spectrophotometry, introducing the range of measurement uncertainty of the instrument used. The logarithmic (10-fold) dilutions of the primary standard covering up to 6 or 7 orders of magnitude in the expected unknown concentrations range are amplified and the obtained C_t values are plotted against log-transformed concentrations of serial dilutions of the target gene (Fig. 1D). In this step the accuracy of the dilution process as well as the somewhat arbitrary setting of fluorescence threshold can influence the final quantification outcome. It has been shown that lesser dilution series may occasionally be necessary when the amplification efficiencies in log-scale dilutions are too variable for accurate target gene quantifications from environmental samples (Töwe et al., 2010). In microbial ecology it is not, however, always possible to use small-scale standard curves, because environmental samples can cover a broad range of gene copies. In order to obtain reliable quantification results, a few practicalities must also be kept in mind. The repeated freezing and thawing of templates used to construct standard curves should be avoided, as it affects their DNA concentration. It should also be ensured that the C_t value of the most diluted template DNA used to construct the standard curve is at least a log-fold lower (3.3 cycles – equivalent to 10-fold dilution) than the C_t value of the NTC (Smith and Osborn, 2009), in case any amplification in NTC is recorded.

The quality and reliability of the standard curves can be controlled by a few individual standard curve specific descriptors: slope, efficiency (E), R^2 and Y-intercept (Fig. 1D). Ideally, the mathematically-calculated slope of the standard curve should be –3.32, which corresponds to 100% efficiency or two-fold amplification at each cycle. Slope values from -3.1 to -3.6 are deemed to be sufficient for usable standard curves. The efficiency (E) of the reaction is calculated using the following equation: $E= (10^{(-1/slope)}-1)*100\%$. This efficiency value expresses the quality of standard dilutions only and not the efficiency of individual samples tested (Töwe et al., 2010). R^2 is used to assess the fit of the standard curve to the data points plotted. The closer the value to 1, the better the fit; for a good quality qPCR standard, the R^2 value should be $\geq$0.99. The Y-intercept of the standard curve indicates the sensitivity of the reaction, as it corresponds to the C_t of a diluted standard containing only a single target molecule (Kubista et al., 2006; Smith et al., 2006). Y-intercept around 40 indicates a good sensitivity of the reaction. If the qPCR instrumentation used for amplifications requires individual standard curve application in each separate run, the standard curves created need to be highly reproducible and not statistically different (Smith et al., 2006). Verifying the

reproducibility of the standard curve specific descriptors helps to fulfil this notion (Smith et al., 2006; Töwe et al., 2010). Any number of regression lines can have similar R^2 and slope values due to parallel lines having the same slope, the uniqueness of the reaction is determined by the Y-intercept value; variations in Y-intercept value would result in differences in the absolute values of the gene copies obtained.

It has been shown that even highly reproducible standard curves may result in statistically significant differences in target gene copy numbers for the same template (with equivalent C_t values) when target gene numbers are quantified within separate qPCR assays. This is due to the log nature of the curve, whereby minor differences in C_t values and standard curves result in great differences in gene copy numbers. Therefore absolute gene copy numbers determined from standard curves of different qPCR runs should be compared with caution (Smith et al., 2006).

3.3.5. Evaluation of qPCR inhibition

The susceptibility of qPCR assays to inhibitory substances co-extracted alongside template DNA from environmental samples potentially leading to inaccurate target gene quantification or false-negative results has long been known (Stults et al., 2001). Despite constant efforts to improve DNA extraction protocols, the co-extraction of PCR-inhibiting humic (Tebbe and Vahjen, 1993), tannic and fulvic acids (Kreader, 1996), as well as other similar compounds (Watson and Blackwell, 2000), cannot be completely prevented. The need for inhibition downsizing or at least evaluation is evident and over the years different strategies have been proposed (Beller et al., 2002; van Doorn et al, 2009; Schneider et al., 2009). Most contaminant removal strategies successful on PCR (i.e. removing inhibitors by cleanup procedures; scavenging inhibitors by proteins) are not applicable on the qPCR approach as they alter unpredictably the DNA amount analysed (Schneider et al., 2009). Consequently modifications of two types of strategies (sample dilutions and internal amplification controls) are used.

The concentrations at which inhibitors in the template DNA no longer affect the target gene amplification are not known a priori and are determined empirically (Stults et al., 2001). The DNA extracts are mostly diluted several-fold in order to lower the concentration of inhibitors and in some cases to enable any amplification of the target gene at all (Schneider et al., 2009). This is done by either testing the effect of different dilutions on the target gene quantification (Stults et al., 2001; Töwe et al., 2010) or by adding different template DNA dilutions to the known amount of the autonomous reference DNA sample to determine the amount of the template that no longer affects the reference DNA quantification (Volkmann et al., 2007; Schneider et al., 2009). Nevertheless, this approach is not free of bias, as it has been shown that the dilution factors themselves can have an effect on quantification results

(Smith et al., 2006). In addition, serial dilutions may dilute targets in low copy numbers below the detection limit (Volkmann et al., 2007).

The second frequently-used option for inhibition evaluation when performing qPCR has been internal amplification controls (IAC). The classic approach of IAC used in PCR reactions (a non-target DNA sequence that is co-amplified with the target under the same reaction conditions and in the same reaction tube (Hoorfar et al., 2004)) is not applicable to qPCR, as the competition for reaction components can affect target gene quantification (van Doors et al., 2009). Therefore, separate reactions should be used for each target and IAC. The IAC sequence is usually incorporated into plasmid, the known amount of IAC is mixed with environmental DNA and the IAC amplification results are compared to the IAC standard curve. When recovery of IAC is below 100%, the quantification data of the target gene is corrected using the corresponding efficiency factor (Beller et al., 2002; Cébron et al., 2008). To date, most IACs have been applied in a single concentration, yet it is argued that the selection of a single concentration may not ensure the accuracy of inhibition evaluation, especially for samples with weak inhibition (van Doors et al., 2009). As a solution, the usage of a range of concentrations of IACs yielding a calibration curve for each individual environmental DNA sample is proposed. One drawback of this approach is the resulting dramatic increase in the number of reactions that must be performed, which is of particular concern when large-scale screening of samples is required or in cases where only a small amount of template DNA is available. To date, such approach has only been applied when using the high density and low-volume microarrays of the Biotrove OpenArray platform (van Doors et al., 2009).

3.3.6. QPCR amplification efficiency

It has been recognized that even though methods that do not rely on the estimation of the efficiency of PCR amplification may provide reproducible and sensitive data, they do not quantify DNA with precision (Karlen et al., 2007). Therefore, high and comparable amplification efficiency values are the key for the reliable quantification of target genes from environmental samples using qPCR (Töwe et al., 2010).

In theory, the PCR reaction generates copies in an exponential fashion, with a doubling in each cycle, but this is only true if the PCR functions with 100% efficiency. In reality, the PCR is almost never perfect, as the number of experimental variables (i.e. properties of primers, amplicon length and sequence, secondary structures, presence of inhibitors, presence of primer-dimers and other non-functional templates) influence PCR kinetics and consequently amplification efficiency in the exponential phase (Karlen et al., 2007; Ruijter et al., 2009). In order to obtain accurate and reproducible results, reactions should have an efficiency that is as close to 100% as possible, and the efficiency should be similar for both target and reference.

Some papers report that the mean amplification efficiency of the analysed samples can be calculated from the slope of the standard curve (Pfaffl et al., 2001; Nolan et al., 2006; Smith and Osborn, 2009). However, biological samples are complex and may contain inhibitory substances that are not present in standards based on purified templates and this may reduce PCR efficiency (Kubista et al., 2006). It has been recognized for some time that a standard curve-derived efficiency does not represent the true mean efficiency of the samples (Ramakers et al., 2003; Schefe et al., 2006), and only expresses the quality of the standard dilutions (Töwe et al., 2010). Nevertheless, the aforementioned efficiency evaluation is used in a handful of papers that report amplification efficiency when analysing environmental samples (Liu et al., 2010; Dandie et al., 2011; Philippot et al., 2011), leading to potentially erroneous estimations of target gene abundance. Instead, estimations of the PCR efficiencies of each individual PCR reaction should be used. Several models and algorithms have been proposed for this purpose (Ramakers et al., 2003; Zhao and Fernald, 2005; Karlen et al., 2007; Rutledge and Stewart, 2008; Spiess et al., 2008; Ruijter et al., 2009).

Methods to estimate amplification efficiency can be grouped into two approaches: the linear regression algorithms and nonlinear curve-fitting models (logistic or sigmoid curve fit). One option to apply the linear regression method is to generate serial dilutions of every sample in question and to perform multiple PCR reactions on each dilution. The C_t values are then plotted against the log of the dilution and a linear regression is performed from which the mean efficiency can be derived. It is proposed that such a serial dilution based method requires at least a set of 24 diluted samples to function properly (Karlen et al., 2007) which raises questions regarding its robustness and feasibility. Several empirical sigmoidal curve-fit methods have been proposed for PCR efficiency evaluation and template quantification (Liu and Saint, 2002; Rutledge and Stewart, 2008; Spiess et al., 2008), but it has been shown that the sigmoidal models depend on input concentrations (Ruijter et al., 2009) and are generally of poor resolution and precision (Karlen et al., 2007). To date, the most widely used method for determining individual amplification efficiencies is an improved logistic curve-fitting model applied in the LinRegPCR program (Ruijter et al., 2009). The program performs fluorescence baseline-correction on the data and identifies the exponential phase of the reaction by plotting the fluorescence on a log scale and fitting a regression line to a subset of data points in the log-linear phase leading to estimation of the efficiency of each PCR reaction from the slope of the fitted regression line (Ramakers et al., 2003; Ruijter et al., 2009). The possibility of performing automated baseline correction adds weight to that efficiency determination option, as baseline estimation errors are directly reflected in the observed PCR efficiency values (Ruijter et al., 2009). The LinRegPCR method requires much less PCR reactions than the serial dilution method and is considerably faster to implement. Furthermore, it has been shown that the two methods display comparable accuracy in measuring efficiency values but

the reproducibility of the results is higher on LinReg approach (Karlen et al., 2007).

Similarly to other aspects concerning qPCR performance, it is worth keeping in mind a few practicalities concerning the estimation and use of PCR efficiencies. More robust and statistically coherent estimations of the PCR reaction efficiency are obtained by using the mean efficiency of the amplicon group instead of individual efficiency values (Čikoš et al., 2007). This approach also decreases intra- and inter-assay variability. Regardless of whether a target gene will be normalized against a reference gene or plotted against a standard curve, the amplification efficiencies of the target and the reference must be comparable (Bustin et al., 2009; Töwe et al., 2010). It has been reported that sometimes the comparable amplification efficiencies cannot be achieved within log-scale serial dilutions and that standard dilutions and environmental samples only had similar amplification efficiencies in the case of comparable copy numbers (Töwe et al., 2010). In such cases small-scale (1:5, 1:4) dilution series may be the solution.

3.3.7. QPCR data evaluation, quantification strategies and statistical analysis

QPCR data analysis includes an evaluation of raw data quality and reliability, and the generation of reportable results. Quality assurance and the implementation of appropriate statistical methodologies for data handling and processing are essential to obtaining valid biological results (Bustin et al., 2009).

Raw qPCR data is usually first analysed visually in terms of the possible severe aberration of amplification and melting curves from the majority of samples; individual measurement results determined as flawed are excluded from further analysis. In the case of environmental samples, divergences in the GC content of a specific gene present in different organisms may also lead to the formation of a multiple or blunt peaks on melting curve analysis (Sharma et al., 2007). The specificity of such reactions should be controlled on an agarose gel before any omission decision is made. The majority of studies in the field of bioremediation and environmental monitoring do not proceed beyond this quality control step (Cébron et al., 2008; Börjesson et al., 2009a;b; Petrić et al., 2011). However, to obtain a reliable quantification, PCR runs must show amplification curves or efficiencies derived from those which do not significantly differ from each other, as small alterations in amplification efficiencies due to inhibitors and other reaction variables give rise to several-fold differences in final gene copy numbers (Bar et al., 2003). This cannot be estimated visually based on amplification and melting curves, and outlier detection methods pointing out dissimilar samples from the majority have been proposed as a solution. The univariate kinetic outlier detection (KOD) method (Bar et al., 2003) compares the PCR efficiencies of individual test samples with the mean efficiencies of a chosen reference sample set (i.e.

standard curve, whole set of tested environmental samples), and samples with significantly different PCR efficiency are considered to be outliers. KOD identifies outliers that differ by 1.3–1.9-fold in their quantity from normal samples with a P value of 0.05. Recently, methods based on the mathematical analysis of the difference in the shape of amplification curves have also been proposed. Tichopad and co-authors (2010) used fitting of the exponential phase of the amplification curve with a suitable model and calculation of the Z-score statistics with two parameters related to amplification efficiency. Sisti and co-authors (2010), on the other hand, used the non-linear fitting of Richards` equation to parameterize the whole PCR trajectory (SOD – Shape based kinetic Outlier Detection). Methods based on the shape of the amplification curve and KOD methods appear to be equally specific, but the latter is slightly less sensitive than the other outlier detection methods (Sisti et al., 2010; Tichopad et al., 2010).

The choice of reference sample set is critical for further analysis regardless of the technical details of different outlier detection methods. Typically, standard curve samples are used as the reference for test samples. When analyzing environmental samples, however, the use of highly homogeneous samples as a reference set may result in overly sensitive outlier detection that discriminates against minor deviations in C_t. In such cases more robust procedures, such as using the entire set of reactions for calibration, "leave-one-out" classification (sequentially removing one sample and testing it against others) or the repeated exclusion of outliers and redefinitions of the reference, may be of advantage (Tichopad et al., 2010). The precision of outlier removal also depends on the size of the reference set – at least 10 reference measurements should be available for every assay (Tichopad et al., 2010). By excluding aberrant measurements from further analysis, false results can be avoided, the spread of results in a group of replicates can be reduced and the potential of qPCR to detect smaller differences in DNA amount is improved.

Several varying data treatment options have been proposed for the analysis of qPCR results, in all cases the methods used are not fully assumption-free and the final quantification result is somewhat influenced by the subjective decisions made by the analyser. The basic choice in real time PCR data calculations is between absolute quantification employing a standard curve to derive the gene copy number of the input template and relative quantification relying on a comparison of the target gene versus a reference gene in the analysed sample. The "gold standard" for absolute quantification is the cycle-threshold (C_t) method, which relies on the assumption that the quantity of PCR product in the exponential phase is proportional to the initial amount of target DNA and that the reaction efficiency is uniform in tested samples (Guescini et al., 2008). A fluorescence threshold is set either arbitrarily by the researcher or automatically by qPCR instrumentation, with the condition that it lays within the exponential phase of the reaction. Varying recommendations for placing the quantification threshold can be found in the literature (Tuomi

et al., 2009), but in most studies high and very low threshold settings have been avoided due to variation in plateau phase levels and background fluorescence. Other notable absolute quantification methods include the second derivative (C_p) method, the C_{y0} method and sigmoidal curve fitting. The C_p method calculates the cycle at which exponential amplification can no longer be sustained (the second derivative of the qPCR fluorescence intensity reaches its maximum value) and the curve begins to taper into the plateau phase (Luu-The et al., 2005). The upside of this method is the minimal involvement of decision by the user, while the downside is its inferior precision compared to the C_t method in assays with variable baseline and plateau conditions (Durtschi et al., 2007), which is likely to be the case when analyzing environmental samples. The C_{y0} method is based on the fit of Richards` equation to qPCR data (Guescini et al., 2008). It does not require the assumption of uniform reaction efficiency or any choice of threshold level by the user, but is lacking in robustness and ease of use. Sigmoidal curve fitting methods rely on empirically finding the best-fitting sigmoidal model for each amplification curve. Sigmoidal curve fitting does not rely on the standard curve, but it is experimentally cumbersome and the results are purely descriptive, leading to possibly unreliable biological conclusions (Karlen et al., 2007). It has also been shown that the accuracy and precision of this method is markedly impaired when amplification efficiency is reduced (Guescini et al., 2008). It has been shown that the other described methods besides sigmoidal curve fitting are equally precise and accurate in optimal amplification conditions (Guescini et al., 2008), but the C_t method is still preferable, as it is the most stable and straightforward to use (Karlen et al., 2007).

The relative quantification is based on calculating the difference in C_t values (ΔC_t) between the target gene and the reference gene and performing a subsequent comparison of the ΔC_t-s of the different samples (Bustin et al., 2009). Alternatively, in microbial ecology and bioremediation monitoring studies the obtained absolute gene quantification results are used to perform target gene normalizations against reference genes (Cébron et al., 2008). In both cases the amplification efficiencies of both genes need to be similar in order to guarantee valid results. In environmental monitoring it is difficult to find steady-state reference genes and in many studies 16S rRNA genes are used as reference genes in normalizations (Kandeler et al., 2006; Cébron et al., 2008). However, the use of 16S rRNA as a reference gene or marker for quantifying the abundance of the whole bacterial community in complex environmental samples is controversial as the number of 16S rRNA genes per cell varies between one and 15 copies (Klappenbach et al., 2001). 16S rRNA gene could be used as a valid reference gene targeting particular groups of microbes with group-specific primers and taking the 16S rRNA number per cell (i.e. typically 10 copies in bacilli, 7 in enterobacteria, 4 in pseudomonads, 1 in nitrifiers and 1 in the majority of archaea that have been sequenced) into account (Lee et al., 2009). The number of studies targeting functional genes as

references or enumerating specific portions of a community is growing as they are usually found with only one copy per cell (Hirsch et al., 2010).

As a final step of qPCR data handling the appropriate implementation of statistical methodologies is necessary in order to obtain valid and meaningful biological results (Bustin et al., 2009). Standard parametric tests are used most frequently to evaluate quantified gene abundances and gene ratios (i.e. respective to the different bioremediation method applied), even though they depend on assumptions, such as the normality of distributions, whose validity cannot always be expected (Pfaffl, 2004). The analysis of variance is frequently conducted using t-tests (Kandeler et al., 2006; Jung et al., 2011) and one-way and two-way ANOVA (Cébron et al., 2008; Yoshida et al., 2009; García-Lledó et al., 2011). To assess the level of significance between the two analysed groups, paired or unpaired t-tests (Nyysönen et al., 2006; García-Lledó et al., 2011), a Mann-Whitely U-test (Monard et al., 2008) or a Wilcoxon signed-rank test is used. Additionally, the Pearson's correlation analysis can be applied between the two matched groups (Morales et al., 2010). In addition to standard parametric tests, several non-parametric tests, such as the Kruskal-Wallis test (El Azhari et al., 2008; Petrić et al., 2011), are also frequently encountered in the literature concerning environmental monitoring. Permutation and randomization tests that make no distributional assumptions about the data are also deemed to be useful in the analysis of qPCR data (Pfaffl et al., 2004).

4. MATERIAL AND METHODS

The results of three experimental studies – two bioremediation case studies (Papers I–II) and one methodological assessment (Paper III) – are presented in this dissertation. In all of the conducted experiments, quantitative PCR was used to quantify taxonomic and functional target genes in the studied environmental matrices.

4.1. Monitored natural attenuation (MNA) experiment (Paper I)

The MNA experiment was conducted at the Laguja landfill in southern Estonia. Industrial and municipal wastes were deposited in the landfill from the early 1970s until its closure in 2004. At the time of its closure, the landfill covered 1.4 ha and contained about 50 000 tons of waste. A shallow, 1 ha pond with no outlet receiving landfill leachate and surface runoff was located in the lowermost section of the landfill. Fuel tank sediments, bilge water, various kinds of oily waste (fuel oil, lubricating oil etc.) and oil-contaminated water were dumped into the pond from 1974 to 1993.

In the period of 2002–2004, the integrated remediation plan for Laguja landfill was implemented, which included the removal and treatment of oily leachate and sediments from the former oil-pond, capping of the landfill with locally excavated topsoil and the creation of a surface flow constructed wetland for further treatment of landfill leachate. During landfill exploitation, oily wastes were dumped not only in the oil-pond but also into the main body of the landfill, resulting in continuous leaching of oily water from the capped waste storage area to the newly constructed wetland. Despite the aforementioned remediation actions, residual oil contamination was still present at the time of the MNA experiment (2004–2008). Average residual total petroleum hydrocarbon (TPH) contamination in the subsurface around the constructed wetland was 80 mg kg^{-1}, and some hotspots receiving landfill leachate had TPH concentrations of up to 960 mg kg^{-1}; in the water of constructed wetland, TPH were below detection limit. MNA was applied as a technology to complement previous remediation activities.

All experimental details are described in Paper I. In brief, the subsurface soil samples for preliminary site characterisation were taken during the installation of groundwater monitoring wells in November 2006 and September 2007, and groundwater samples were obtained in September 2008. Subsurface soil was used in an enrichment culture experiment, in order to estimate the response of soil microbial community to elevated concentrations of contaminants (crude oil, diesel fuel and hexadecane). The post-incubation enrichment cultures were further incubated in either xylene vapours or liquid culture containing hexadecane to obtain xylene and alkane degrading bacterial isolates. The isolated bacterial strains showing biodegradative capacity were

taxonomically identified using 16S rRNA gene sequencing. The catabolic potential of the indigenous microbial communities as well as isolated bacterial strains was assessed by PCR amplification of 11 different functional genes involved in hydrocarbon degradation pathways and by enumerating functional populations related to phenol and alkane degradation in groundwater using the qPCR approach. The structure of the microbial community was estimated using DGGE-fingerprinting.

4.2. Laboratory-scale trinitrotoluene (TNT) bioremediation experiment (Paper II)

The effect of biostimulation, bioaugmentation, rhizoremediation and combinations of these treatments on TNT removal was studied in a laboratory-scale bioremediation experiment. The substrate used (a mixture of industrial quartz and peat) in the pot experiment mimicked the soil of explosives-contaminated Adazi military camp in Latvia. Adazi polygon is the largest military training area (7746.5 ha) in the Baltic States and has been used for this purpose for over 70 years. The experimental details concerning the setup of the experiment and the treatments applied are described in Paper II.

The fate of TNT and its metabolites was estimated using high pressure liquid chromatography (HPLC) analysis. The metabolic profile of the artificial soil microbial communities indicating their functional diversity was analysed using Biolog EcoPlates and subsequent principal component analysis (PCA). Microbial community structure was assessed using DGGE-fingerprinting and subsequent PCA and one-way permutational multivariate analysis (PERMANOVA). Universal, *Pseudomonas* and *Stenotrophomonas* genus-specific primers targeting 16S rRNA genes were used in the qPCR approach to evaluate the response of the whole bacterial community as well as two specific bacterial groups with known biodegradative abilities to different bioremediation treatments. In order to evaluate the effect of TNT-spiking and subsequent biostimulation/bioaugmentation and plant treatments on the whole bacterial community as well as its functional abilities, the obtained qPCR data was subjected to the Kruskal-Wallis one-way analysis of variance by ranks and Mann-Whitney test. All experimental details are described in Paper II.

4.3. Application and modifications of qPCR

QPCR methodology was applied in all of the conducted experiments to quantify 16S rRNA and functional genes in order to estimate the bioremediation potential of targeted microbial communities.

Standard curves for gene quantifications were created essentially in the same manner in all of the conducted studies. Target gene fragment (16S rRNA, *LmPH*, *alkM* – Paper I; *Pseudomonas* and *Stenotrophomonas* group

specific 16S rRNA – Paper II; *tetA* – Paper III) was PCR amplified from bacterial strains possessing the gene (Papers I–III); *nirS* and *nosZ* gene fragments were PCR amplified from *Pseudomonas fluorescens* PAO1 (unpublished data). As no strains carrying the *nirK* gene were available, the amplicon was obtained from an environmental sample (unpublished data). For IAC, fragment of bacteriophage λ DNA was PCR amplified (Papers I, III). The PCR reaction mixtures contained 1xPCR buffer with $(NH_4)_2SO_4$ (75 mM Tris-HCl, pH 8.8; 20 mM $(NH_4)_2SO_4$; 0.01% Tween 20), 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.0008 mM (each) of forward and reverse primers (Table 4), 0.5 U of Taq DNA polymerase (Fermentas, Lithuania) and a specific DNA template. All PCR amplifications were performed on Eppendorf Mastercycler or Thermal cycler PCR machines. The details of the amplification programs used for each gene amplification are described in respective papers (Papers I–III), and in Table 4A in the case of *nosZ, nirS* and *nirK* gene amplifications. The obtained PCR products were cloned using the InsT/Aclone PCR cloning kit (Fermentas), plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen, CA, USA) and controlled by PCR amplifications and sequencing with BigDye™ chemistry (Applied Biosystems, CA, USA). The number of copies of standard plasmids in the extract was calculated as follows:

$$N = \frac{c}{m} Na \quad (1)$$

where N is the number of target gene copies per liter of solution, c is the concentration of extracted plasmid DNA (g L^{-1}), m is the target gene fragment containing plasmid vector mass (Da) and Na is the Avogadro number. For all of the standards, DNA stock solutions of 10^9 plasmid copies μl^{-1} were prepared. Serial dilutions ranging from 25 to 10^8 (total, *Pseudomonas* and *Stenotrophomonas* group specific 16S rRNA; *LmPH*; *alkM*; *nosZ*; *nirS*; *nirK*; IAC) or from 6 to 10^8 (Paper III – total 16S rRNA; *tetA*; IAC) target gene copies μl^{-1} were used for standard curve creation on qPCR.

Different qPCR chemistries (SYBR Green and LUX™) and kits were used in the studies: Maxima SYBR Green Master Mix (Fermentas; Papers I–III; *nosZ*, *nirS* and *nirK* amplifications), Maxima Probe qPCR Master Mix (Fermentas; Paper III) and Platinum® qPCR Supermix-UDG with ROX (Invitrogen, UK; Paper III). The qPCR assays were performed either on Rotor-Gene®Q (Qiagen; Papers I–II; *nosZ*, *nirS* and *nirK* amplifications) or ABI Prism 7900 (Applied Biosystems; Paper III) qPCR systems. All reactions were performed in the total volume of 10 µl, containing 5 µl of the respective qPCR kit master mix used, 0.0002 mM of forward and reverse primers (Table 4), DNA template and sterile distilled water adding up to final volume. The details of the qPCR programs used in each study are described in Table 4B and respective papers (Papers I–III). Immediately after every qPCR amplification assay, melting curve analysis was also performed. All of the performed qPCR reactions from the analysed samples and standards were run

in triplicate except for *nosZ*, *nirS* and *nirK* amplifications (unpublished data), where the standards were run in four replicates. The qPCR data were analysed using either Rotor-Gene Series software version 2.0.2 (Papers I–II; *nosZ*, *nirS* and *nirK* amplifications) or Sequence Detection Software (SDS), version 2.4.2 (Paper III).

In Papers I and II the amplification and melting curves of the qPCR reactions were inspected visually, the deviating reaction data was omitted from further analysis, and initial target gene copy numbers of analysed samples were deduced from the standard curves. In Paper III, three-step amplification quality control and the outlier removal procedure for the analysis of environmental samples preceding target gene quantification was developed. The quality control system consists of visual inspection of deviating amplification and melting curves, the determination and omission of irregular fluorescence reads preventing the individual amplification efficiency calculations in LinRegPCR program (Ruijter et al., 2009) and kinetic outlier detection (KOD) statistical method (Bar et al., 2003) for the detection of samples with dissimilar efficiencies. In all cases, the amplification data of standard curves and samples analysed were subjected to identical treatment. The described qPCR amplification data quality control system is also applied for the estimation of the quality of the experimental data of *nosZ*, *nirS* and *nirK* amplifications (unpublished data). After the reaction outliers were omitted from further study, the target gene's copy numbers were deduced from standard curves. In order to estimate the possible differences in target gene quantification results arising from the different analytical methods used, the described procedure for the estimation of qPCR data quality was applied in retrospect to the qPCR data of Papers I and II. The differences in pre- and post-quality control target gene quantification data were estimated using a two-tailed paired *t*-test.

The presence of PCR inhibitors was evaluated by mixing known amounts of IAC with environmental samples and estimating its effect on IAC DNA amplification (Papers I, III). The aforementioned amplification quality control system was also applied to the data analysis of the inhibition measurement experiment (for Paper I data in retrospect). When recovery of IAC differed from 100%, the quantification data of the target genes was corrected using the corresponding inhibition factor. In order to estimate the relative abundance of functional communities, the analysed functional genes were also normalised against 16S rRNA genes (Papers I–III). Normalisations were conducted either using the obtained target gene copy numbers (Papers I–III) or by using amplicon-specific amplification efficiencies and C_t values (Paper III) as proposed by Ruijter et al. (2009). The latter was also applied in retrospect to the normalisation of the quantification data from Papers I–II, in order to estimate the possible differences in the results arising from the use of different quality control methodologies for qPCR data.

Table 4. Characteristics of primer sets and amplification programs used, A – for PCR amplifications of *nosZ*, *nirS* and *nirK* genes; B – for all conducted qPCR experiments.

A						
Primer	**Primer sequence 5'-3'**	**Target gene**	**Amplicon size (bp)**	**Primer reference**	**PCR program**	**PCR reference in the current study**
nosZF	CGYTGTTCMTCGACAGCCAG	*nosZ*	453	Kloos et al., 2001	1 cycle: 94°C 2min; 10 cycles: 94°C 30s, 58°C 30s[1], 72°C 1min; 25 cycles: 94°C 30s, 53°C 30s, 72°C 1min; 1 cycle: 72°C 20min	Unpublished data
nosZ1622R	CGSACCTTSTTGCCSTYGCG			Throbäck et al., 2004		
nirSCd3aF	AACGYSAAGGARACSGG	*nirS*	425	Kandeler et al., 2006	1 cycle: 94°C 2min; 7 cycles: 94°C 30s, 63°C 1min[2], 72°C 1min; 28 cycles: 94°C 30s, 57°C 1min, 72°C 1min; 1 cycle: 72°C 10min	Unpublished data
nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA					
FlaCu	ATCATGGTSCTGCCGCG	*nirK*	473	Hallin and Lindgren, 1999	1 cycle: 95°C 5min; 10 cycles: 95°C 30s, 60°C 1min[1], 72°C 1min; 25 cycles: 95°C 30s, 58°C 1min, 72°C 1min; 1 cycle: 72°C 5min	Unpublished data
R3Cu	GCCTCGATCAGRTTGTGGTT					
B						
Primer	**Primer sequence 5'-3'**	**Target gene**	**Amplicon size (bp)**	**Primer reference**	**qPCR program**	**qPCR reference in the current study**
785FL	ggactacGGATTAGATACCCTGGTA G*T*CC[3]	16S rRNA	156	Paper I	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 63°C 30s[4], 72°C 30s[5] Melting curve: 65–90°C	Paper I–III
919R	CTTGTGCGGGTCCCCGTCAAT					

Table 4. Continuation.

Pse-F2	GGTCTTCGGATTGTAAAGCAC	16S rRNA	184	Juhanson et al., 2009	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 58°C 30s, 72°C 30s[5] Melting curve: 65–90°C	Paper II
Pse-R	CCGGGGMTTTCACATCCAAC					
Ste-F	TTGTCCTTAGTTGCCAGCAC	16S rRNA	192	Paper II	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 58°C 30s, 72°C 30s[5] Melting curve: 65–90°C	Paper II
Ste-R	CCGGACTGAGATAGGGTTTC					
Phe00L	gacgcCRATYGACGARCTGCG*T*C[3]	*LmPH*	209	Modified from Heinaru et al., 2005	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 63°C 30s, 72°C 30s[5] Melting curve: 65–90°C	Paper I
Phe212	GTTGGTCAGCACGTACTCGAAG GAGAA			Watanabe et al., 1998		
alkM-F2	TGGGGNATGAGTGCTGCWTT	*alkM*	203	Paper I	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 61°C 30s, 72°C 30s[5] Melting curve: 65–90°C	Paper I
alkM-RL	ccagagNTTATTNTTCCANCTATGC TC*T*GG[3]			Modified from Margesin et al., 2003		
tetA F2L	cagccTCAATTTCCTGACGGGC*T*G[3]	*tetA*	96	Börjesson et al., 2009a	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 40 cycles: 95°C 15s, 62°C 30s[4], 72°C 30s[5] Melting curve: 60–90°C	Paper III
tetA R2	GAAGCGAGCGGGTTGAGAG					
nosZF	CGYTGTTCMTCGACAGCCAG	*nosZ*	453	Kloos et al., 2001	1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 61.5°C 30s, 72°C 30s[5] Melting curve: 75–95°C	Unpublished data
nosZ1622R	CGSACCTTSTTGCCSTYGCG			Throbäck et al., 2004		

Table 4. Continuation.

nirSCd3aF	AACGYSAAGGARACSGG	*nirS*	425	Kandeler et al., 2006	1 cycle: 95°C 10min; 6 cycles: 95°C 15s, 63°C 30s[2], 72°C 30s, 80°C 30s[5]; 40 cycles: 95°C 15s, 58°C 30s, 72°C 30s, 80°C 30s[5]; 1 cycle: 95°C 15s, 60°C 30s, 72°C 10min Melting curve: 60–95°C	Unpublished data
nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA					
FlaCu	ATCATGGTSCTGCCGCG	*nirK*	473	Hallin and Lindgren, 1999	1 cycle: 95°C 10min; 6 cycles: 95°C 15s, 63°C 30s[2], 72°C 30s, 80°C 30s[5]; 40 cycles: 95°C 15s, 61°C 30s, 72°C 30s, 80°C 30s[5]; 1 cycle: 95°C 15s, 60°C 30s, 72°C 10min Melting curve: 60–95°C	Unpublished data
R3Cu	GCCTCGATCAGRTTGTGGTT					
λ7403FL	cacctcGACCGGACATGAAAATGAGG*T*G[3]	Bacteriophage λ DNA	109	Paper I	1 cycle: 50°C 2min; 1 cycle: 95°C 10min; 45 cycles: 95°C 15s, 63°C 30s[4], 72°C 30s[5] Melting curve: 65–90°C	Paper I, III
λ7512R	ATCAGTATGCAGCTTCACCAGTGC					

[1] The annealing temperature drops 0.5 °C after every cycle.
[2] The annealing temperature drops 1 °C after every cycle.
[3] When LUX™ fluorescence chemistry is used, the fluorophore is attached to the underlined T base; the sequences of artificial tails added to the primer sequence and enabling hairpin formation (Nazarenko et al., 2002) are denoted in lower case.
[4] Fluorescence recording point when LUX™ fluorescence chemistry is used.
[5] Fluorescence recording point when SYBR Green I fluorescence chemistry is used.

5. RESULTS AND DISCUSSION

5.1. The effect of qPCR workflow and data analysis procedure modifications on the results of target gene quantification from environmental samples (Paper III)

Quantitative PCR, which is regarded as a precise and sensitive method, has become mainstream methodology in environmental monitoring over the last decade (Smith and Osborn, 2009). Even though qPCR-related aspects such as target amplification efficiency and its comparability to standard curve dilutions as well as the quality of the obtained datasets have pivotal roles in trustable target gene quantifications, these aspects are rarely estimated and taken into account in the aforementioned research field (Sharma et al., 2007; Smith and Osborn, 2009). Hence the variability in 16S rRNA and functional gene quantification from environmental samples in relation to modifications in qPCR workflow and subsequent data evaluation and analysis was examined. The detailed results and conclusions of the study are presented in Paper III.

It was found that the quality of qPCR amplification datasets depended largely on the properties of the target amplicon and the qPCR chemistry used. SYBR green qPCR yielded considerably better quality amplification datasets than LUX^{TM} qPCR (Paper III). Variable sequences between the conserved primer binding positions in the target amplicons (i.e. 16S rRNA) also reduced the quality of the obtained datasets. This was further confirmed by amplifications of denitrification-related genes (*nosZ, nirS, nirK*; Appendix Table 1). The relatively low quality of the *nosZ, nirS* and *nirK* gene amplification datasets is probably also influenced by amplicon length (Table 4), which is significantly longer than the proposed ideal of 50 to 150 bp (Smith and Osborn, 2009). Primer pairs targeting *nosZ* and *nirS* genes had two to five degenerated nucleotide positions in each primer (Table 4), most probably also significantly contributing to the aforementioned effect. Despite the shortcomings, the applied primers targeting denitrification-related genes are regularly used in recent denitrification assessment studies (Bárta et al., 2010; Djigal et al., 2010; Dandie et al., 2011; Rasche et al., 2011). Due to the absence of superior primer sets, the rigorous quality control of the amplification and acknowledgement of the reaction variables is the next best thing that can be done to ensure realistic results.

Reliable target gene quantification from environmental samples also hinges on high and comparable target gene amplification efficiency. Mean amplification efficiency is routinely calculated from the slope of the standard curve in environmental monitoring studies (Liu et al., 2010; Dandie et al., 2011; Philippot et al., 2011) which, in reality, expresses only the quality of standard dilutions (Töwe et al., 2010). The standard curve slope derived and the sample's individual measurement-based mean amplification efficiencies were found to be significantly different; the former being misleadingly high (Paper III Table 3). Therefore, the individual efficiencies of amplified samples

were used in all analyses. Amplification efficiencies were influenced by the same parameters that reduced overall datasets quality (Paper III Fig.1–2; Table 5). It also became apparent that more variable target amplicon sequences (i.e. 16S rRNA, *nirS*) showing reduced amplification data quality are more prone to statistically significant differences in amplification efficiencies compared to standard curves (Paper III; Table 5) which affects the target gene quantification results. A similar bias-creating tendency was noted in other studies (Paragraph 5.2.1 and 5.3.1). As in many cases it might not even be realistic to achieve a comparable amplification efficiency between standard and sample amplifications due to the complexity of environmental samples, the next best option is to report the difference in measured amplification efficiencies as background information and take those into consideration in interpreting the target gene quantification results.

For a decade, internal amplification controls (IAC) for inhibition rate evaluation in DNA extracted from environmental samples have been regularly used to avoid target gene multiplicity underestimations (Beller et al., 2002; Cébron et al., 2008; de Vet et al., 2011). However, IAC amplification efficiencies in several tested experiment variants showed statistically significant differences compared to the respective standard curve (Paper III). This indicates that incorrect estimations of inhibition factors, probably due to the heterogeneity of inhibitory substances in environmental samples, can occur, introducing further bias into target gene quantification instead of reducing it. Consequently, each individual study should consider whether the inhibition rates are high enough and the amplification efficiencies of internal standard dilutions and reference DNA similar enough to avoid the possible addition of further bias into target gene quantification data.

Target gene quantification data indicated that modifications in qPCR workflow steps (i.e. variations in DNA extraction methodology, qPCR chemistry, inhibition measurement, data quality estimations) can significantly influence the gene quantification results from environmental samples (Paper III Supplementary Table 1). For instance, different DNA extraction methods yielded as much as an order of magnitude variation in calculated target gene copy numbers (Paper III Supplementary Table 1) confirmed by the analysis of variance (Paper III Table 4) while the implemented amplification data quality control influenced the target gene multiplicity estimations up to 40%. Therefore, even though the target gene copy numbers obtained with different qPCR workflows are compared between different studies (Cébron et al., 2008; Chon et al., 2011), these estimations are not viable due to the incomparability of such data. If comparisons are necessary, target gene normalizations as a percentage of another gene are recommended (Smith and Osborn, 2009; de Vet et al., 2011). The effect of qPCR workflow modifications on *tetA* gene normalizations against 16S rRNA genes (Paper III Table 5) revealed quite stable results for good quality datasets regardless of the DNA extraction method or qPCR chemistry used.

It was confirmed that modifications in qPCR workflow steps significantly influence the target gene quantification results from environmental samples. For environmental monitoring purposes the most suitable method workflow relating to the characteristics of a particular experiment should be chosen in order to ensure the quality and truthfulness of the results obtained (Sharma et al., 2007).

Table 5. Comparison of mean amplification efficiencies of experimental samples analysed with respective standard curves. All reactions presented were performed with SYBR green qPCR chemistry. Standard deviations are presented in brackets. NS – not significant.

Amplification target	**Study reference**	**E_{mean} of standard curve**	**E_{mean} of samples analysed**	**P**
nosZ	Unpublished data	1.765 (0.099)	1.721 (0.076)	<0.05
nirK		1.764 (0.090)	1.793 (0.079)	NS
nirS		1.414 (0.015)	1.493 (0.102)	<0.01
LmPH	Paper I	1.782 (0.041)	1.776 (0.038)	NS
Total community 16S rRNA		1.756 (0.028)	1.796 (0.030)	<0.001
IAC		1.912 (0.022)	1.898 (0.020)	NS
Total community 16S rRNA	Paper II	1.756 (0.028)	1.723 (0.032)	<0.0001
Pseudomonas-specific 16S rRNA		1.965 (0.017)	1.945 (0.023)	<0.001
Stenotrophomonas-specific 16S rRNA		1.900 (0.026)	1.886 (0.021)	<0.05

5.2. The application of qPCR in the evaluation of residual oil degradation potential (Paper I)

The microbial potential for pollutant degradation in the groundwater of Laguja landfill, which was undergoing natural attenuation, was estimated using target gene (*LmPH* coding large subunit of multicomponent phenol hydroxylase, *alkM* coding alkane hydroxylase, 16S rRNA) quantifications on qPCR and subsequent normalizations among other monitoring methods. The detailed results and conclusions are presented in Paper I.

In brief, it was found that the results of target gene quantification were influenced by qPCR inhibition, which ranged from 0 to 27.8 %; similarly to several earlier studies (Beller et al., 2002; Cébron et al., 2008), the quantification data of the targeted genes was corrected using the corresponding inhibition factor. The *alkM* genes were present (Paper I Table 3) at the field site in non-quantifiable proportions. *LmPH* genes coding the key enzyme for

aerobic phenol metabolism (Watanabe et al., 1998), on the other hand, were quantifiable despite being scarce in groundwater (Paper I Fig. 4a; Appendix Table 2). Generally, the higher *LmPH* gene copy numbers were detected in groundwater monitoring wells with residual oil contamination compared to uncontaminated wells (Paper I Table 1), which corresponds well to previously reported results about contamination boosting the growth of indigenous catabolic microbes (Margesin et al., 2003; Basile and Erijman, 2010). The 16S rRNA gene quantification results providing background information about the total bacterial community at the study site followed a similar trend showing up to two orders of magnitude higher abundance (Paper I Fig. 4A; Appendix Table 2) in monitoring wells with residual oil contamination. A comparison of the relative abundance of functional communities at different field site locations (Paper I Fig. 4b; Table 6) revealed relatively even distribution (despite their scant numbers) indicating stable bioremediation potential towards phenol compounds. Such even distribution of catabolic community can probably be related to the site's long pollution history as well as to the present situation, where only residual oil contamination is present at the site.

5.2.1. The impact of qPCR data quality control implementation on MNA monitoring

In order to estimate the impact of the developed qPCR data quality control system (Paper III) and other modifications in qPCR data analysis on the results of the bioremediation monitoring, the developed methodology was implemented in retrospect to assess the MNA experimental data. All amplification data of groundwater samples yielding quantifiable results (*alkM* gene amplification data was not used) and respective qPCR standards (for 16S rRNA and *LmPH* genes as well as for IAC) were subjected to the analysis.

It has been recognized that reliable target gene quantification from environmental samples hinges to a large degree on the quality of the datasets, as well as on the high and comparable amplification efficiency values of both standard curve dilutions and individual samples tested (Töwe et al., 2010). The removal of deviating amplification data based on a visual inspection of the amplification and melting curves used in the original study (Paper I; Appendix Table 1) can be seen as the first step of the qPCR quality control procedure. Implementation of the entire quality control procedure resulted in the detection of several more deviating amplification reads from all of the analysed datasets (Appendix Table 1). All determined reaction outliers were omitted from further analysis. No statistically significant differences between the standard curve and the mean values of amplification efficiency related to the environmental samples were detected for the *LmPH* gene and IAC. On the other hand, 16S rRNA gene amplifications showed incomparable mean amplification efficiency values for standard curve and environmental sample amplifications (Table 5), which can introduce some bias into the absolute gene

quantification results. This supports the trend towards the occurrence of statistically significant differences in the amplification efficiencies of environmental samples versus respective standard curves for target amplicons with variable sequences which was also noted in other experiments (Paper III; Paragraph 5.1; 5.3.1).

The recalculated IAC recovery rates for groundwater samples ranged from 75.7% to 100%, indicating that inhibition rates were somewhat overestimated in the original study. The pairwise *t*-test confirmed that the slight modifications to the IAC standard due to improved estimation of qPCR data quality resulted in a statistically significant difference in measured inhibition rates ($p<0.01$). The re-quantification of *LmPH* genes, which took into account the newly established inhibition factors, resulted in the detected functional gene range of 9 to 462 copies per ml of groundwater in different sampling locations. Despite significant differences in inhibition rates established with different analysis methods, the functional gene abundances detected with the modified data analysis workflow showed no statistically significant difference. In fact, the differences between the two quantification results ranged only from zero to 3 copies of *LmPH* genes per ml of groundwater for the individual samples tested (Appendix Table 2). The recalculated 16S rRNA gene copy numbers ranged from $2.7*10^5$ to $2.7*10^7$ copies per ml of groundwater, thus varying somewhat from those reported in the original study (Appendix Table 2); the detected difference was not, however, statistically significant. Therefore, despite some alterations in measured target gene (*LmPH*, 16S rRNA) copy numbers in the environmental samples, the detected abundance dynamics of the target gene at the field site (Paper I Fig. 4A) remained unchanged.

The results of absolute target gene quantification depend on the series of applied qPCR workflow steps and are not readily comparable among different studies (Smith and Osborn, 2009). Instead, normalizations of detected functional genes against reference genes (usually 16S rRNA in environmental microbiology) are implemented. In the original study the measured target gene copy numbers were used for normalizations (Paper I Fig. 4B). However, such approach does not take into account the possible varying amplification efficiencies of different amplicons and uses data that has itself already undergone several calculations. In order to avoid such possible bias, the formula taking into account the C_t values and the amplification efficiencies of each individual sample (Ruijter et al., 2009) was used in retrospect. A comparison of the two sets of normalization results (Table 6) reveals that the functional communities present in groundwater at the field site were on average underestimated 11.5 times (7.3 to 18.8 times for individual boreholes) in the original study. Hence the bioremediation potential in MNA experiment was somewhat underestimated in the original publication (Paper I) and is actually more profound.

Table 6. The relative abundance of *LmPH* genes in groundwater and pond water samples from Laguja landfill. Borehole labels are given in Paper I Fig. 1.

Percentage of *LmPH* genes relative to 16S rRNA gene copy number	**Sampling location (borehole number)**					
	1	4	6	P1	P2	Pond
Original study (Paper I)	0.00057	0.0028	0.00052	0.015	0.0058	0.0006
Current study	0.0107	0.0263	0.0055	0.1093	0.0669	0.0068

5.3. The application of qPCR in the evaluation of TNT bioremediation potential (Paper II)

The effect of bioaugmentation, biostimulation, rhizoremediation and their combinations on TNT removal and on the microbial community involved was assessed in a 28-day laboratory pot experiment. QPCR was used to estimate the abundance of the total bacterial community as well as two functionally important phylogenetic groups (*Pseudomonas* and *Stenotrophomonas*) known to possess TNT degradation capacity (Cho et al., 2008; Travis et al., 2008) by targeting 16S rRNA genes with universal, *Pseudomonas* genus-specific and *Stenotrophomonas* genus-specific primers. The detailed results and conclusions are presented in Paper II.

In brief, all of the applied bioremediation treatments resulted in decreased concentrations of TNT in the soil (Paper II Fig. 1), with rye cultivation combined with biostimulation-bioaugmentation treatment having the most profound effect. Contrary to previous findings (Gong et al., 1999), no inhibitory effect of TNT on microbial abundance was recorded (Paper II Table 4). Instead, the survival and elevation of the introduced *Stenotrophomonas* and especially *Pseudomonas* strains was noted in TNT-contaminated samples (Paper II Fig.4 and Table 4), fulfilling an important prerequisite for the successful application of bioaugmentation (Thompson et al., 2005). This phenomenon can most likely be attributed to the selective pressure of TNT promoting the growth of microbes able to utilize the pollutant. The recorded strong impact of bioaugmentation on the functional pattern and phylogenetic structure of the microbial community (Paper II Fig. 2, 3) further supported this finding. Plants enhanced the overall abundance of the microbial community, but in the case of blue fenugreek, cultivation did not significantly affect the proportions of functional microbial communities in soil or the rate of TNT degradation. Rye cultivation, on the other hand, had a positive effect on TNT removal (Paper II Fig. 1). Contrary to previous findings, where rhizoremediation had overshadowed bioaugmentation in TNT removal from soil (van Dillewijn et al., 2007), the simultaneous application of biostimulation and bioaugmentation treatments resulted in more profound effects in this study.

5.3.1. The impact of qPCR data quality control implementation on TNT bioremediation monitoring

The impact of modifications in qPCR amplification quality estimation as well as other alterations in qPCR data analysis on 16S rRNA gene quantifications for TNT bioremediation assessment was estimated through the reanalysis of TNT degradation pot experiment qPCR amplification data.

In the original study (Paper II) the reaction outliers were determined by visual analysis of amplification and melting curves. The implementation of subsequent steps of the quality control procedure resulted in the detection of several more deviating amplification reads from all of the standard curves and environmental amplification related datasets (Appendix Table 1). All determined reaction outliers were omitted from further study. Even though most of the re-analysed qPCR amplification datasets were of relatively good quality (except for the *Pseudomonas*-specific 16S rRNA standard curve), significant differences between the mean amplification efficiency values of the generated standard curves and the analysed experimental samples were recorded for all primer sets used (Table 5). This result corresponds with findings in Paper I (Paragraph 5.2.1) and Paper III (Paper III Fig. 1) highlighting the difficulty of generating standard curves with comparable amplification efficiency to analysed environmental samples, especially when the targeted sequences are quite variable. Despite the notion that comparable amplification efficiency is a requirement for precise target gene quantification (Töwe et al., 2010) this is evidently not easily achievable in practice. If this possibly bias-creating difference cannot be avoided, it should at least be reported and taken into account when interpreting the target gene quantification results.

In order to estimate the impact of qPCR amplification data quality improvement on the result of TNT bioremediation monitoring, the targeted 16S rRNA genes were re-quantified (Appendix Table 3) and compared to the original report (Paper II Table 4) using the paired *t*-test. Quantification results were compared per treatment type as these introduce different compounds into the soil possibly affecting the microbial community, DNA extraction and subsequent qPCR amplification to a varying degree. The results indicated that total community 16S rRNA gene quantification had somewhat overestimated the absolute target gene copy numbers in non-planted as well as in TNT-spiked and biostimulated experiment variants ($P<0.05$) in the original study. While a comparison of *Pseudomonas*-specific 16S rRNA quantifications did not yield any meaningful differences, *Stenotrophomonas*-specific 16S rRNA genes were found to have been somewhat underestimated in the experimental variants that used rye cultivation ($P<0.05$). Despite the fact that in this case the conclusions made based on absolute gene quantifications in the original study (Paper II) remained unchanged, these findings accentuate the impact of qPCR amplification data quality on the recorded target gene quantification and therefore bioremediation monitoring results. The pre-experiments had indicated no inhibition in the qPCR reactions of the samples analysed, and therefore no IAC for inhibition measurement was used in this study.

It has been stated that the comparison of absolute target gene copy numbers between different studies is not valid due to differences in qPCR workflows applied (Smith and Osborn, 2009). Instead, target gene normalizations reducing the impact of qPCR workflow details are preferable. The normalizations in the original study (Paper II Fig. 4) were based on calculated absolute target gene copy numbers – the method generally used in environmental microbiology research (Cébron et al., 2008). However, the clearly different amplification efficiencies of the amplicon groups (Table 5) undermine the credibility of such analysis. Therefore, for target gene normalization re-analysis calculation, a formula based on amplicons' C_t values and amplification efficiencies was used (Ruijter et al., 2009). A comparison of the relative abundance of targeted phylogenetic groups gauged in the original study (Fig. 4A) and in the re-analysis (Fig. 4B) revealed that the proportion of targeted bacterial groups in the TNT bioremediation experiment had been severely underestimated in the original study. The re-normalizations resulted in a 2.08 to 5.97 times (on average 3.62 times) higher relative abundance of *Pseudomonas* group and a 7.21 to 25.98 times (on average 13.43 times) higher relative abundance of the *Stenotrophomonas* group (Fig. 4, Appendix Table 3) in the analysed samples. While the general occurrence patterns of the *Pseudomonas* group in different bioremediation treatments remained unchanged, the occurrence patterns of the *Stenotrophomonas* group in TNT spiked but not amended soil was subjected to changes (Fig. 4). The general results and conclusions, such as the elevation of targeted phylogenetic groups indicating the survival of the introduced microbial consortium and the selective pressure of TNT recorded in the original study (Paper II; Paragraph 5.3), remained unchanged. However, the variation magnitude recorded in the target gene normalization results generated by the different data analysis methodology applied highlights the impact that varying analysis methods can have on bioremediation monitoring and subsequent decision-making.

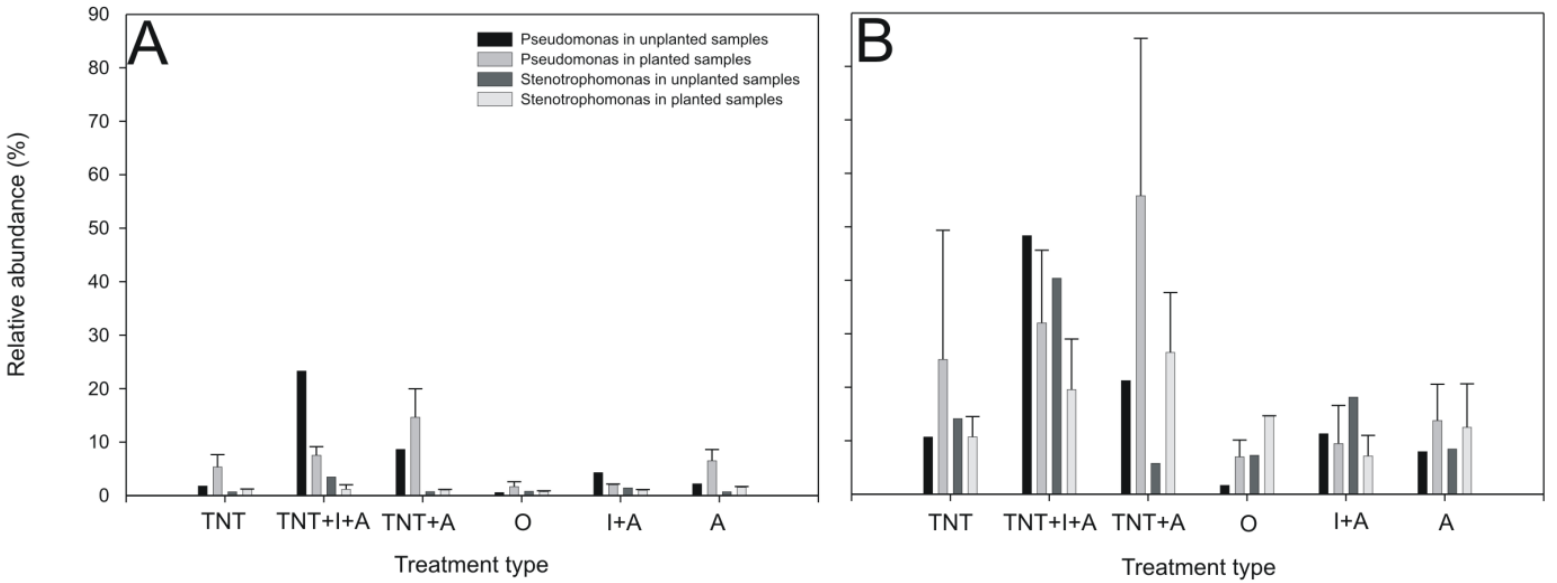

Figure 4. Changes in the relative abundance of *Pseudomonas* and *Stenotrophomonas* groups recorded by 16S rRNA gene quantifications in different bioremediation treatments designated by (A) – normalisations of calculated target gene copy numbers or (B) – normalisations using the amplification efficiency and C_t values of target gene amplifications. Group mean and standard deviation are shown. Treatment type abbreviations: TNT – TNT spiked soil, I – bioaugmentation, A – biostimulation, O – only planted or nonplanted samples, + indicates combination of different treatments.

6. CONCLUSIONS

Over the last decade, quantitative PCR, regarded as a precise and sensitive method, has become mainstream technology for monitoring the state of microbial processes in environmental matrices during bioremediation. Target gene quantification from environmental samples is a multistep process and its results are influenced by several qPCR workflow related variables (i.e. DNA extraction method, amplification efficiency and quality). However, so far these factors are rarely taken into account in environmental monitoring by qPCR. Hence, the effect of qPCR workflow and analysis process modifications on target gene quantification and normalization results and their impact on bioremediation evaluation were assessed in this study. Based on the results, the following conclusions can be made:

- The qPCR amplification efficiency as well as the overall quality of amplification datasets depends largely on the qPCR chemistry and primer pair used as well as on the properties of the target amplicon. The efficiency and quality of SYBR green qPCR amplifications from environmental samples were generally good and stable, while LUX™ qPCR amplification datasets from soil samples exhibited significantly poorer quality as well as low and more fluctuating amplification efficiency. Therefore SYBR green may be the preferable qPCR chemistry for the analysis of complex environmental samples. Target gene amplifications with primer pairs possessing several degenerate base-positions (i.e. primers targeting *nirS* and *nosZ* genes) resulted in reduced mean amplification efficiencies. Target amplicon properties such as its excessive length and great sequence variability also lower the mean amplification efficiency and make it more prone to statistically significant differences in amplification efficiencies compared to standard curves.
- Most DNA extraction methods are insufficient to remove all inhibitory substances affecting PCR amplification; consequently, internal amplification controls (IAC) for inhibition rate evaluation are used. Implementation of qPCR amplification data outlier removal can result in statistically significant differences in recorded inhibition rates compared to no-outlier removed datasets. Nevertheless, this disparity did not significantly affect the target gene quantification results. More problematic is the occasionally recorded statistically significant difference between IAC and the respective standard curve, which may lead to incorrect estimations of inhibition rates and subsequently introduce further bias into bioremediation estimations instead of reducing it. Hence, it should either be confirmed that the bias that may have been introduced is minimal, or IACs that do not rely on standard curves should be used.
- Even though comparisons of target gene copy numbers are routinely made in the literature, such estimations are not valid, as recorded target gene abundance is strongly influenced by qPCR workflow characteristics such as the DNA extraction method used. It was revealed that the application of qPCR amplification data quality control can significantly affect the target gene

quantification results. Even though in this study the dynamics of target gene abundance in bioremediation estimations remained mostly unchanged, this is not guaranteed in other qPCR applications. Therefore, it is essential to ensure high quality of amplification datasets for valid bioremediation monitoring and subsequent decision-making.

- The relative abundance of functional groups determined by target gene normalizations against the reference gene is deemed to be a more appropriate parameter when comparisons between studies using varying qPCR workflow are necessary. For normalization purposes, usually calculated target gene quantification results are used. However, when target and reference gene amplification efficiencies are not comparable such approach is not credible. Instead, normalizations based on amplification efficiency and the C_t values of target gene amplifications should be used.

On the basis of our findings, it can be concluded that modifications in qPCR workflow and analysis procedure steps can significantly influence target gene quantification and normalization results from environmental samples and consequently also bioremediation related decision-making. For environmental monitoring purposes, the most suitable method workflow relating to the characteristics of individual conducted experiments should be chosen to ensure the quality and truthfulness of the results obtained.

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SUMMARY IN ESTONIAN

Kvantitatiivse polümeraasi ahelreaktsiooni modifikatsioonide mõju märklaudgeeni kvantifitseerimisele keskkonnaproovidest bioremediatsiooni hindamisel

Keskkonna reostumine erinevate saasteainetega (nt. naftasaadused, klooorgaanilised ühendid) on muutunud kriitiliseks probleemiks üle maailma kahjustades inimtervist, kahandades puhta joogivee varusid ning mõjutades terveid ökosüsteeme. Et klassikalised „pumpa-ja-töötle" saastuse eemaldamise meetodid on töömahukad ja kallid, on viimastel kümnenditel hakatud aina enam rakendama erinevaid bioremediatsiooni tehnoloogiaid. Bioremediatsioon põhineb saasteainete füüsikaliste, keemiliste ja bioloogiliste transformatsiooniprotsesside rakendamisel, millest enamasti olulisim on saasteainete mikroobne lagundamine. Ehkki bioremediatsiooni meetodid on enamasti aeganõudvad, on nende populaarsuse kasvule aidanud kaasa võrdlemisi lihtne rakendatavus, kohandatavus suurtele aladele ning odavus. Samas mõjutavad muutuvad keskkonnatingimused saasteaineid lagundavat mikroobikooslust ja seeläbi ka bioremediatsiooniprotsessi efektiivsust. Seetõttu kaasneb bioremediatsiooni rakendamisega enamasti ka keemiliste ja mikrobioloogiliste parameetrite pikaajaline seire, et hinnata toimuvate protsesside käiku ja jätkusuutlikkust.

Kvantitatiivne polümeraasi ahelreaktsioon (qPCR) on aina sagedamini kasutust leidev metoodika saasteaineid lagundava mikroobikoosluse esinemise ja arvukuse hindamiseks bioremediatsiooni seirel. QPCR on kiire ja tundlik meetod, mis võimaldab sõltuvalt püstitatud küsimustest ja valitud tehnilistest vahenditest (eelkõige praimeritest) nii taksonoomiliste kui funktsionaalsete märklaudgeenide arvukuse määramist hõimkonna tasemest liigi tasandini. Kasutades reaktsiooni märklauana mikroobikoosluse DNA-d on võimalik hinnata saasteainete lagundamise potentsiaali; mikroobikoosluse mRNA baasil sünteesitud cDNA märklauana kasutamisel on võimalik hinnata ka uuritava mikroobikoosluse aktiivse osa arvukust. Määratud arvukusi muude seireparameetritega (nt. saasteaine kontsentratsioon) kõrvutades on võimalik hinnata bioremediatsiooniprotsesside efektiivsust ja kulgemist uuritavas kohas.

Märklaudgeenide arvukuse määramise edukus keskkonnaproovidest sõltub mitmetest faktoritest, näiteks mikroobikoosluse DNA eraldamise meetodist ja kvaliteedist, inhibiitorite esinemisest eraldatud DNA-s, qPCR reaktsioonikeemia tüübist, märklaudjärjestuse amplifikatsiooni efektiivsusest ja tulemuste analüüsi kvaliteedist. Ehkki qPCR kasutamine bioremediatsiooni seirel on sagenenud, pööratakse eelnimetatud asjaolude arvestamisele praktilistes rakendustes seni veel vähe tähelepanu. Sellest tulenevalt oli antud töö eesmärgiks hinnata erinevate qPCR reaktsiooni- ja analüüsiprotsessi modifikatsioonide mõju keskkonnaproovidest märklaudgeeni absoluutse ja suhtelise arvukuse määramisele, mida omakorda kasutatakse bioremediatsiooni potentsiaali hindamiseks.

Töö käigus saadud tulemused on järgmised:

- QPCR amplifikatsiooni efektiivsus ja kvaliteet sõltuvad suurel määral kasutatavast praimeripaarist, qPCR-i reaktsioonikeemiast ning paljundatava märklaudjärjestuse omadustest. Testitud qPCR-i reaktsioonikeemiatest osutus eelistatumaks SYBR green, mis erinevalt LUX^{TM} reaktsioonikeemiast, tagas enamjaolt hea ja stabiilse kvaliteediga tulemused. Märklaudjärjestuse amplifikatsiooni efektiivsust langetasid nii selle ülemäärane pikkus ja varieeruvus kui kõdupositsioonidega praimerite kasutamine. Lisaks täheldati varieeruvate märklaudjärjestuste puhul sageli statistiliselt olulisi erinevusi keskkonnaproovide ja standardkõverate keskmise amplifikatsiooniefektiivsuse vahel, mis võib mõjutada märklaudgeeni arvukuse määramise ja seeläbi bioremediatsiooni potentsiaali hindamise tulemusi. Seni on kirjanduses enamasti esitatud standardkõvera tõusust tuletatud reaktsiooniefektiivsusi keskkonnaproovidele vastava parameetrina; antud töös aga näidati, et selline lähenemine ülehindab reaktsiooni efektiivsust võrreldes proovide individuaalsete amplifikatsiooniefektiivsuste keskmisega.
- Kasutatavad meetodid mikroobikoosluse DNA eraldamiseks keskkonnaproovidest ei ole enamasti piisavad eemaldamaks kõiki PCR reaktsiooni inhibeerivaid ühendeid. Seetõttu tehakse sageli kindlaks ka iga testitava proovi inhibitsioonimäär, et kindlustada võimalikult realistlik märklaudgeenide arvukuse hinnang uuritavas keskkonnas. Selgus, et märklaudjärjestuste amplifikatsioonitulemuste kvaliteedikontroll ja võõrväärtuste eemaldamine võib viia statistiliselt oluliselt erinevate määratud inhibitsioonikoefitsentideni. Viimane ei mõjutanud siiski oluliselt testitud märklaudgeenide arvukuse hinnanguid. Realistliku bioremediatsiooni potentsiaali hindamise seisukohalt on kriitilisem, et täheldati statistiliselt olulisi erinevusi inhibitsiooni määramiseks kasutatud kontrolljärjestuse ja vastava standardkõvera amplifitseerimise efektiivsustes. Sellistel juhtudel võib inhibitsioonikoefitsendi arvessevõtt ettearvamatult kallutada märklaudgeenide arvukuse määramise ja seeläbi bioremediatsiooni potentsiaali hindamise tulemusi.
- Märklaudgeenide määratud arvukusi võrreldakse sageli kirjanduses omavahel. Antud uuringust selgus, et säärastel võrdlustel pole alust kuna modifikatsioonid qPCR reaktsiooni- ja analüüsiprotsessis mõjutavad tugevalt (kuni kümnekordne vahe määratud geeni arvukuses) märklaudgeenide arvukuse hinnanguid. Lisaks leiti, et märklaudgeeni amplifitseerimise tulemuste kvaliteedi kontrollimine ja võõrväärtuste eemaldamine võib teatud juhtudel oluliselt mõjutada määratud arvukusi. Kuigi antud uuringus ei mõjutanud see varasemaid bioremediatsiooni kulgemise kohta tehtud järeldusi, ei saa eeldada, et see alati nii oleks. Niisiis, kõrge qPCRi kvaliteedi tagamine on äärmiselt oluline realistlikku bioremediatsiooni olukorda peegeldavate tulemuste saavutamiseks.
- Kui võrdlused erinevate uuringute vahel osutuvad vajalikeks, soovitatakse kasutada märklaudgeeni normaliseeringut mõne teise geeni suhtes. Selgus, et mõningatel juhtudel on selleks alust – kui määratud märklaudgeeni arvukus kõikus vastavalt erinevatele DNA eraldamise meetoditele üle 10 korra, siis

normaliseeringute puhul jäid vastavad andmed kõik ühe suurusjärgu piiresse. Enamasti kasutatakse andmete normaliseerimisteks märklaudgeenide määratud absoluutseid arvukusi. Samas näidati, et märklaud- ja referentsgeeni amplifikatsiooni efektiivsused on sageli erinevad, mis omakorda mõjutab mõningal määral vastavate geenide arvukuse hinnanguid. Tagantjärele igast individuaalsest keskkonnaproovist määratud märklaud- ja referentsgeeni amplifikatsiooni efektiivsusel ja C_t-l põhinevat normaliseerimise meetodit kasutades selgus, et bioremediatsiooni potentsiaali oli esialgsetes uuringutes selle näitaja põhjal üle 10 korra alla hinnatud.

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APPENDIX

Table 1. The effect of qPCR amplification data quality control implementation on reaction outlier detection. All reactions presented were performed using SYBR green qPCR chemistry.

Target gene/sequence	**Sample type**	**Reaction outliers detected by visual control**	**Reaction outliers detected by quality control procedure**	**Reference**
LmPH	Standard dilutions	0/27	2/27	Paper I
	Environmental	1/18	1/18	
16S rRNA	Standard dilutions	2/30	6/30	
	Environmental	1/18	3/18	
IAC	Standard dilutions	1/30	3/30	
	IAC in presence of environmental sample	0/30	0/30	
Total community 16S rRNA	Standard dilutions	2/27	4/27	Paper II
	Environmental	0/51	3/51	
Pseudomonas-specific 16S rRNA	Standard dilutions	6/30	10/30	
	Environmental	0/51	4/51	
Stenotrophomonas-specific 16S rRNA	Standard dilutions	3/27	4/27	
	Environmental	1/51	4/51	
nosZ	Standard dilutions	Not applied separately	22/38	Unpublished data
	Environmental		18/99	
nirS	Standard dilutions		11/23	
	Environmental		20/99	
nirK	Standard dilutions		6/24	
	Environmental		17/99	

Table 2. The quantification of 16S rRNA and *LmPH* genes in the groundwater of Laguja landfill. The quantification results are presented as target gene copy numbers per ml of groundwater. Borehole labels are given in Paper I Fig. 1. (A) – quantification results in the original study (Paper I); (B) – quantification results in this study.

Sample	**16S rRNA (A)**	**16S rRNA (B)**	***LmPH* (A)**	***LmPH* (B)**
1	$2.04*10^6$	$2.49*10^6$	11	9
4	$1.65*10^7$	$1.64*10^7$	465	462
6	$2.54*10^7$	$2.65*10^7$	185	187
P1	$2.67*10^5$	$2.71*10^5$	40	42
P2	$6.15*10^5$	$6.77*10^5$	36	38
Pond	$8.07*10^6$	$8.10*10^6$	48	48

Table 3. The quantification of 16S rRNA genes of total and selected functional groups of the microbial community in TNT degradation pot experiment soil samples. Quantification results are presented as the number of gene copies per gram of soil. Sample labels are given in Paper II Table 1. (A) – quantification results in the original study (Paper II); (B) – quantification results in this study.

Sample	**Total 16S rRNA (A)**	**Total 16S rRNA (B)**	***Pseudo-monas* 16S rRNA (A)**	***Pseudo-monas* 16S rRNA (B)**	***Stenotrophomonas* 16S rRNA (A)**	***Stenotrophomonas* 16S rRNA (B)**
1	$7.65*10^8$	$7.12*10^8$	$1.37*10^7$	$2.28*10^7$	$5.29*10^6$	$7.87*10^6$
2	$3.11*10^8$	$2.99*10^8$	$9.42*10^7$	$7.61*10^7$	$1.08*10^7$	$1.38*10^7$
3	$6.54*10^8$	$6.34*10^8$	$5.64*10^7$	$5.88*10^7$	$4.84*10^6$	$5.48*10^6$
4	$5.83*10^8$	$5.64*10^8$	$3.28*10^6$	$3.06*10^6$	$4.62*10^6$	$4.91*10^6$
5	$3.82*10^8$	$3.67*10^8$	$1.64*10^7$	$1.71*10^7$	$5.45*10^6$	$5.93*10^6$
6	$5.24*10^8$	$5.07*10^8$	$1.16*10^7$	$1.11*10^7$	$3.69*10^6$	$4.06*10^6$
1R	$5.56*10^8$	$5.12*10^8$	$1.69*10^7$	$1.66*10^7$	$6.05*10^6$	$6.51*10^6$
2R	$4.24*10^8$	$3.83*10^8$	$3.87*10^7$	$3.67*10^7$	$9.75*10^6$	$1.08*10^7$
3R	$7.95*10^8$	$7.56*10^8$	$7.39*10^7$	$7.76*10^7$	$9.46*10^6$	$1.06*10^7$
4R	$7.11*10^8$	$5.27*10^8$	$5.16*10^6$	$4.86*10^6$	$4.12*10^6$	$4.37*10^6$
5R	$6.37*10^8$	$1.16*10^9$	$1.23*10^7$	$1.18*10^7$	$2.64*10^6$	$3.14*10^6$
6R	$4.52*10^8$	$1.96*10^9$	$3.89*10^7$	$5.10*10^7$	$7.63*10^6$	$7.74*10^6$
1A	$1.91*10^9$	$2.58*10^9$	$1.46*10^8$	$2.47*10^8$	$2.33*10^7$	$1.19*10^7$
2A	$3.44*10^9$	$2.20*10^9$	$2.04*10^8$	$3.29*10^8$	$1.89*10^7$	$2.01*10^7$
3A	$8.58*10^8$	$8.13*10^8$	$1.71*10^8$	$1.91*10^8$	$1.27*10^7$	$1.45*10^7$
4A	$2.12*10^9$	$2.66*10^9$	$5.48*10^7$	$5.64*10^7$	$1.89*10^7$	$2.73*10^7$
5A	$3.24*10^9$	$2.97*10^9$	$6.97*10^7$	$9.06*10^7$	$2.15*10^7$	$1.64*10^7$
6A	$1.66*10^9$	$2.01*10^9$	$7.23*10^7$	$7.55*10^7$	$2.35*10^7$	$1.64*10^7$

Table 4. The relative abundance (%) of *Pseudomonas* and *Stenotrophomonas* groups recorded using 16S rRNA gene amplifications on qPCR in samples with different bioremediation treatments. Treatment type abbreviations are from Figure 4. (A) – normalization results in the original study (Paper II); (B) – normalization results in this study.

Treatment type	***Pseudomonas* in unplanted samples (A)**	***Pseudo-monas* in unplanted samples (B)**	***Pseudo-monas* in planted samples (A)**	***Pseudo-monas* in planted samples (B)**	***Stenotropho-monas* in unplanted samples (A)**	***Stenotropho-monas* in unplanted samples (B)**	***Stenotropho-monas* in planted samples (A)**	***Stenotropho-monas* in planted samples (B)**
TNT	1.79	10.68	5.34	25.19	0.69	14.13	1.16	10.71
TNT+I+A	23.29	48.36	7.53	32.02	3.47	40.39	1.14	19.55
TNT+A	8.63	21.25	14.64	55.81	0.74	5.78	1.02	26.50
O	0.56	1.67	1.66	6.98	0.79	7.27	0.74	14.61
I+A	4.29	11.31	2.05	9.44	1.43	18.14	0.99	7.14
A	2.21	7.97	6.49	13.78	0.7	8.45	1.56	12.48

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Other publications:

Limane, B., Muter, O., Juhanson, J., Truu, M., Truu, J., **Nõlvak, H**. (2011). Characterization of microbial community structure after application of different bioremediation approaches in TNT contaminated soil. In: Environmental Engineering 8th International Conference, 19–20 May 2011, Vilnius, Lithuania; proceedings pp 188–194.

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Publikatsioonid:

Nõlvak, H., Truu, J., Limane, B., Truu, M., Cepurnieks, G., Bartkevics, V., Juhanson, J., Muter, O. (2012). Microbial community changes in TNT spiked soil bioremediation trial using biostimulation, phytoremediation and bioaugmentation. *J. Environ. Eng. Landsc*., *accepted*.

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