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Improvement of bioreporter bacteria-based test systems for the analysis of arsenic in drinking water and the rhizosphere

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Improvement of bioreporter bacteria-based test systems

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To my dear husband and my dear son

for supporting and distracting me

Abstract

Contamination of drinking water with arsenic can be measured in laboratories with atom absorption spectrometry (AAS), mass spectrometry with inductive coupled plasma (ICP-MS) or atom fluorescence spectrometry (AFS) at the relevant concentrations below 50 µg/L. Field test kits which easily and reliably measure arsenic concentrations are not yet available. Test systems on the basis of bioreporter bacteria offer an alternative. Based on the natural resistance mechanism of bacteria against arsenic compounds toxic for humans, bioreporter bacteria can be constructed that display arsenic concentrations with light emission (luminescence or fluorescence) or colour reactions. This is achieved by coupling the gene for the ArsR-protein and arsenic regulated promoters with suitable reporter genes. The resulting bioreporter bacteria report bioavailable arsenic in a dose dependent manner at the toxicologically relevant level of 2 to 80 µg/L and are therewith suitable both for the guideline levels of the WHO of 10 µg/L and for the national standards in South East Asia of 50 µg/L. This alternative method has the advantage of being independent from sophisticated apparatus as by eye detection is feasible and offers the possibility of measuring directly the bioavailable fraction. Bioreporter bacteria are also suitable for *in situ* research. Yet, in order to apply such bioreporter bacteria as a low-cost analytical tool in a regular manner, open questions exist regarding the preservation of the specific activity, the vitality of bioreporter bacteria and the improvement of bioreporter test systems for layman.

The aim of this thesis hence was to optimize and improve bioreporter based test systems to allow easy conservation, storage and transport, and also an application without the need of a sophisticated infrastructure. For that purpose it was intended (i) to develop and validate a method that allows arsenic detection without external calibration (chapter 2) and (ii) to improve the vitality and specific activity of preserved bioreporter bacteria by using different conservation methods and culture conditions (chapter 3). A further goal was (iii) to use arsenic bioreporter bacteria for the detection of arsenic in the rhizosphere of maize plants in

order to investigate transport processes and understand resistance mechanisms of plants (chapter 4). To achieve this, different strains of the bioreporter bacteria *Escherichia coli* DH5α were used in well defined experiments to receive reproducible results. *Zea mays* L. was used as a model plant in easily accessible rhizoboxes.

The main outcome of this thesis is: (i) A multiple cell line approach was developed that permits detection of arsenic without external calibration (chapter 2). For this purpose two different kind of reporter constructions were developed and modified that resulted in strains which differ in their sensitivity against arsenic. Because of this each bioreporter strain starts the visible reaction at a different concentration and in combination the strains display the arsenic concentration at relevant levels. (ii) A preservation method based on vacuum drying with addition of 34% trehalose and 1.5% polyvinylpyrrolidone could be identified that preserved the specific activity and vitality of the bioreporter bacteria over a time range of 12 weeks and that allows their application for over night measurements (chapter 3). (iii) A carrier system for bioreporter bacteria was developed which facilitates the investigation of arsenic distribution in the rhizosphere. It could be shown that the distribution along roots is dependent on the root age and structure (chapter 4). Mainly at laterals and on roots tips higher concentrations of arsenic were detected. In addition a split root experiment indicated that arsenic is translocated within the roots and released also by intact roots.

This thesis shows that bioreporter based test systems can be improved to achieve broad application in low-cost laboratory monitoring as well as in *in situ* experiments thus proving that they are a valuable tool for environmental research and a promising alternative to existing methods.

Zusammenfassung

Arsen ist in einigen Ländern eine der wichtigsten anorganischen Trinkwasserverunreinigungen. Arsenkontaminationen können zwar im für lokale Grenzwerte relevanten Konzentrationsbereich unter 50 µg/L mittels Atomabsorptionsspektrometrie (AAS), Massenspektrometrie mit induktiv gekoppeltem Plasma (ICP-MS) oder Atomfluoreszenzspektrometrie (AFS) im Labor detektiert werden. Eine verlässliche, einfache und regelmäßig anwendbare Feldanalysemethode existiert derzeit aber noch nicht. Testsysteme, die auf Bioreporterbakterien basieren, stellen hierfür eine interessante Alternative dar. Basierend auf den natürlichen Resistenzmechanismen von Bakterien gegenüber Arsenverbindungen können Bioreporterbakterien konstruiert werden, welche durch Lichtemission (Lumineszenz oder Fluoreszenz) oder Farbreaktionen die Konzentration von Arsen in Wasserproben anzeigen. Dafür werden das Gen für das ArsR-Protein und arsenregulierte Promotoren mit geeigneten Reportergenen gekoppelt, die als Signalgeber dienen. Die resultierenden Bioreporterbakterien zeigen bioverfügbares Arsen im toxikologisch relevanten Bereich von 2 bis 80 µg/L dosisabhängig an und erfassen damit sowohl den Grenzwert der WHO von 10 µg/L als auch lokale Grenzwerte einiger Länder von 50 µg/L. Diese Alternativmethode beruht gegenüber gängigen Verfahren der Arsenanalyse auf einem geringen apparativen Aufwand (einfache visuelle Interpretation erreichbar) und bietet die Möglichkeit der direkten Erfassung des bioverfügbaren Anteils. Darüber hinaus können Bioreporterbakterien für die *in situ* Untersuchung von Arsengradienten herangezogen werden. Um die Bioreporterbakterien routinemäßig als kostengünstige Analysemethode einsetzen zu können, müssen jedoch noch Fragen hinsichtlich der Konservierung der lebenden Bioreporterbakterien und der Vereinfachung von Testsystemen geklärt werden.

Das Ziel dieser Promotionsarbeit bestand zum einen darin, Testsysteme, die auf Bioreporterbakterien basieren, für Transport, Lagerung sowie eine einfache Anwendung im Feld oder in Laboratorien mit begrenzter Ausstattung zu verbessern. Dazu sollte (i) eine

Methode entwickelt und validiert werden, die eine einfache Arsenbestimmungen ohne externe Kalibrierung erlauben (Kapitel 2) und (ii) die Haltbarkeit der lebenden Bioreporterbakterien durch verschiedene Konservierungsmethoden verlängert werden (Kapitel 3). Zum anderen sollten (iii) Bioreporterbakterien für die Analyse von Transportprozessen von Arsen in der Rhizosphäre von Maispflanzen herangezogen werden und damit Fragestellungen bezüglich der Arsenumwandlung und -abgabe in und von Pflanzenwurzeln beantwortet werden (Kapitel 4). Um diese Ziele zu erreichen, wurden verschiedene Stämme des Bioreporterbakteriums *Escherichia coli* DH5α in definierten Experimenten eingesetzt, um reproduzierbare Ergebnisse zu erhalten. Einerseits wurden Experimente mit Flüssigkulturen durchgeführt (Kapitel 2 und 3) und andererseits ein Trägersystem eingesetzt, um komplexe Matrizes wie die Rhizosphäre zu untersuchen (Kapitel 4). Dafür wurde zusätzlich *Zea mays* L. als Modellpflanze in leicht zugänglichen Rhizoboxen verwendet.

Die wichtigsten Ergebnisse dieser Arbeit sind: (i) Durch die Entwicklung eines Ampelsystems wurde die Arsenmessung ohne externe Kalibrierung ermöglicht (Kapitel 2). Dazu wurden zwei verschiedene Arten von Reporterkonstruktionen entwickelt, deren Stämme sich jeweils in der Sensitivität der Arsenreaktion unterscheiden. Dadurch erfolgt für jeden Stamm erst bei einer bestimmten Arsenkonzentration die Farbreaktion, und die Stämme erlauben in Kombination das Ableiten der Arsenkonzentration im relevanten Konzentrationsbereich. (ii) Es wurde ein Konservierungsverfahren basierend auf Vakuumtrocknung und unter Zusatz von 34% Trehalose und 1,5% Polyvinylpyrrolidone gefunden, dass die spezifische Aktivität und Lebensfähigkeit der Bioreporterbakterien in einem Zeitraum von 12 Wochen weitestgehend stabil hält und dadurch den Einsatz in einer Übernachtmessung ermöglicht (Kapitel 3). (iii) Es konnte ein Verfahren bzw. ein Trägersystem für die Bioreporterbakterien entwickelt und mit dessen Hilfe die Arsenverteilung in der Rhizosphäre ohne Störung des Systems untersucht werden (Kapitel 4). Dabei wurde gezeigt, dass die Arsenanreicherung an Pflanzenwurzeln vom Alter und Struktur

der Wurzel bestimmt wird. Hauptsächlich im Bereich lateraler Wurzeln und an der Wurzelspitze traten erhöhte Konzentrationen auf. Außerdem verdeutlichte ein Experiment mit geteiltem Wurzelbereich, dass Arsen innerhalb der Wurzel transportiert und auch von intakten Wurzeln wieder in den Boden abgegeben wird.

Die vorliegende Arbeit zeigt, dass bioreporterbasierte Testsysteme soweit verfeinert werden können, dass sie für eine breite Anwendung in der Umweltanalyse eingesetzt werden können. Zusätzlich konnte im Bereich der *in situ* Analyse die Wirksamkeit der Bioreporterbakterien gezeigt und dadurch neue Erkenntnisse über das Schicksal von Arsen in der Rhizosphäre gewonnen werden. Damit konnte ein Beitrag für die Implementierung dieses alternativen Analyseansatzes sowohl für routinemäßige Laboruntersuchungen wie auch für eine erweiterte *in situ* Anwendung geleistet werden.

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Chapter 1

1 General Introduction

General Introduction

Analytical methods for the biological effects of chemical compounds exist in a great variety due to our interest in the environment and concern about products of daily use. Two different analytical approaches have been continuously developed over the past years. One is the chemical analysis, which uses a wide spectrum of analytical instrumentation. This allows highly accurate and sensitive determination of sample composition, but for an extensive analysis of various detectable targets in environmental samples the necessary analytical procedures are often expensive, time-consuming, very complex and require trained personnel. The other approach is based on the use of living cells or organisms as sensing systems and biological tools. The principle is indeed very old, for example mine canaries were used to sense carbon monoxide in Roman times, but only during the last twenty years living cell-based sensing systems have developed into a scientific and technological field of its own.

One class of biological tools are toxicity bioassays that quantify a sample's total negative impact on the test organisms rather than detecting single sample constituents. The end result is an average of all synergistic and antagonistic effects (Belkin 2003). Fish or planktonic crustaceans (Daphnia etc.) are the most widely used test organisms, but numerous other test systems have been standardized, e.g. the bacterial *Vibrio fischeri* bioluminescence test in which the decrease in light emission indicates the toxicity level (Bulich and Isenberg 1981). A different class of bacterial bioassays aims at the detection of bioavailable concentrations of certain organic compounds or metals. The construction principle of such bioreporter bacteria is based on a natural regulatory circuit composed of transcription regulator and promoter or operator which is combined with a promoterless gene that encodes for an easily measurable protein. The activation of the promoter by the transcription regulator caused by interaction with a chemical compound leads then to the expression of the reporter gene. The resulting output signal can be detected, calibrated and interpreted.

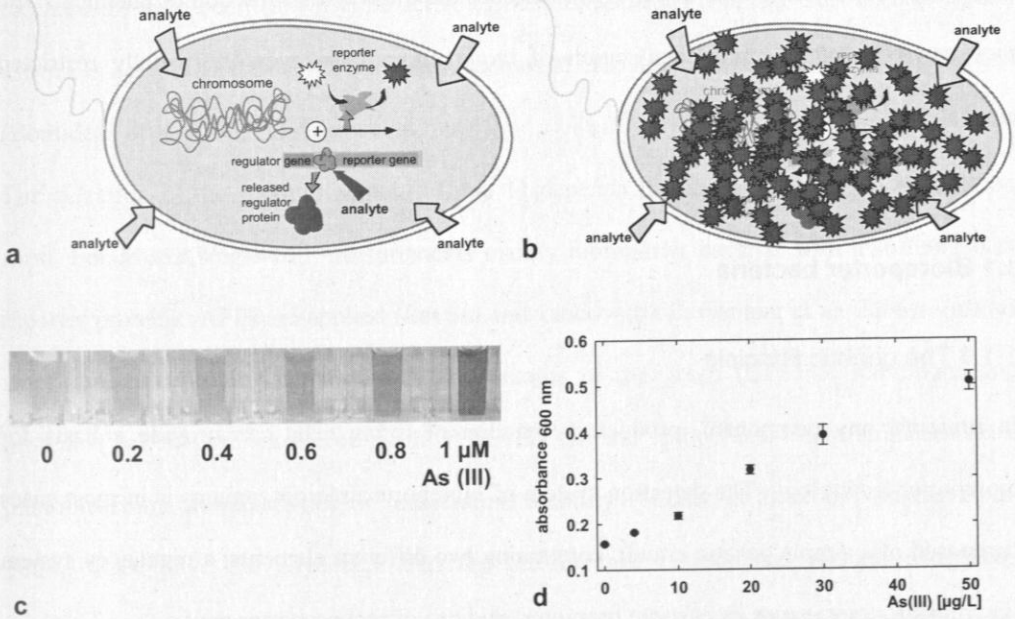

Figure 1. Principle of bioreporter bacteria and calibration curve of signals. a) The analyte leads to a conformation change of the regulator protein and its release from the operator region, the downstream encoded reporter gene is transcribed and subsequently the reporter enzyme expressed (Figure modified from Harms *et al.* (2006)). b) Bioreporter bacteria after contact with analyte. The cell contains the reporter enzyme, which can be detected either itself (e.g. gfp as reporter protein) or its reaction product (e.g. β-galactosidase as reporter protein). c) Colour product of a *lacZ* arsenite bioreporter bacterium at various arsenite concentrations that allows by eye detection. d) Calibration curve of colour signals of a *lacZ* arsenite bioreporter bacterium measured with absorbance spectrometry.

The advantage of sensing techniques based on bioreporter bacteria in comparison to analytical chemistry lies in the cheapness of cultivation and the easiness of genetic engineering and measurement protocols. Bioreporter bacteria consist of large and homogenous populations and offer short generation times and rapid responses. Bioreporter technology can be used as an alternative in laboratories with smaller budgets, for niche application or for rapid multi-

target analysis. Besides this, bioreporters measure the bioavailable fraction of chemicals and they can be used for microlocalization of target compounds even in spatially restricted environments.

1.1 Bioreporter bacteria

1.1.1 The genetic principle

In principle any component, product or reaction of living cells can provide a basis for bioreporter technology. The detection system of bioreporter microorganisms is in most cases composed of a simple genetic circuit, comprising two different elements: a regulatory system (i.e. regulatory gene plus its cognate promoter) and one or more reporter genes.

Two distinct naturally occurring regulatory systems have been widely exploited. For organic compounds the relevant promoter in the metabolic pathway of the bacteria which are able to degrade the target compound is used. For instance to detect linear alkanes Sticher and colleagues (1997) used the *alkBAC* operon of *Pseudomonas oleovorans* encoding the genes involved in hydroxylation of alkanes and dehydrogenation of resulting alkanols. Benzene and its derivatives were sensed with the TOL plasmid from *Pseudomonas putida* mt-2 using the regulatory genes *xylR* and *xylS* (Ikariyama et al. 1997; Willardson et al. 1998), while for naphthalene bioreporters the *nah* operon (Heitzer et al. 1994) was applied. For construction of metal-specific bioreporter bacteria promoters and transcription regulators of genes responsible for microbial metal transformation and resistance mechanism are highly suitable due to their specificity. The resistance genes are mostly organized in operons and are usually plasmid borne (Ramanathan et al. 1997; Bruins et al. 2000). Their expression is strictly regulated and induced by the presence of specific metals in the cell (Ramanathan et al. 1997). Bacterial resistance may be based on extracellular precipitation, sequestration at the cell envelope, intracellular precipitation, redox transformation and active export (Bruins et al. 2000). Such

mechanisms have been shown to be active against toxic metals (Pb, Hg, Cd, As, Sb, Ag, Tl) as well as against dangerously high concentrations of essential metals (Zn, Fe, Ni, Cu, Co, Cr) (Bontidean et al. 2000; Bontidean et al. 2004).

The selection of the reporter protein (Table 1) depends mainly on the desired application form. For *in situ* single-cell measurements mainly bioreporter bacteria with autofluorescent reporter proteins (AFP) are applied (Leveau and Lindow 2002; Gantner et al. 2006). Various autofluorescent proteins mostly based on mutants of the green (GFP) or red fluorescent protein (DsRed) are available with different spectral properties, maturation kinetics, photobleaching characteristics or temperature stability (Shaner et al. 2005; Shaner et al. 2008). They have the advantage that the fluorescence is produced without exogenous substrates, cofactors or ATP (van der Meer et al. 2004). Unfortunately GFP based bioreporters are less sensitive, probably due to autofluorescent background from which low signals are hardly differentiated. For bulk measurements several different types of reporter proteins have been used (Daunert et al. 2000), of which bacterial and eukaryotic luciferases have been particularly popular (van der Meer et al. 2004; Magrisso et al. 2008). Mostly because of their relatively high quantum yields, luciferases have been chosen for highly sensitive applications. For colorimetric or electrochemical detection other reporter proteins like β-galactosidase (*lacZ*) can be used (Daunert et al. 2000). The β-galactosidase is currently probably the most versatile, because various substrates are available for different detection purposes. A very simple method that allows detection by eye is based on the use of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), resulting in a blue colour development (Stocker et al. 2003; Wackwitz et al. 2008). The search for a simple colour detection method has led to the application of novel reporter proteins e.g. cytochrome-c-peroxidase (Wackwitz et al. 2008) and carotinoides from photosynthetic bacteria (Fujimoto et al. 2006).

Table 1. Most common reporter proteins in bioreporter construction

Reporter protein	Reporter gene	Detection method	Comments
Bacterial luciferase	*lux*	Luminescence	Sensitive detection, no natural background in soil, requires aldehyde as substrate and oxygen
Firefly luciferase	*luc*	Luminescence	High quantum yield, sensitive detection, no natural background in soil, requires luciferin as substrate, oxygen and ATP
Fluorescent proteins	*gfp* and variants, DsRed	Fluorescence	No substrate required, highly stable, autofluorescence of natural matrices, requires oxygen
β-Galactosidase	*lacZ*	Colorimetric fluorescence electrochemical chemiluminescence	Different detection methods depending on substrate, requires substrate

1.1.2 The application in environmental research

Bioreporter bacteria have made an excellent career in research institutes in the last years. Starting from biosensors containing living cells as cheaper suppliers of enzymes development went on to bioreporter bacteria, which itself produced detectable signals and could be applied to various media. The progress started with several studies as proof-of-principle that validated the functioning of new constructs. Only recently some studies went beyond the addition of environmental samples in the calibration procedure and started to enter the real environmental application phase (Harms 2007).

First publications dealing with bioreporter bacteria appeared in 1990 (King et al. 1990), which targeted naphthalene. Reporters for other organic compounds like toluene (Burlage et al. 1994), benzene (Tan et al. 1994), aromatic hydrocarbons (Selifonova and Eaton 1996) and middle chain alkenes (Sticher et al. 1997) followed. Only shortly later first metal ions were the target of bioreporter bacteria, starting with aluminium (Guzzo et al. 1992), mercury

(Selifonova et al. 1993), arsenic and cadmium (Corbisier et al. 1993). For the bioreporter targets a shift occurred from environmental contaminants to compounds being part of the environment and even whole organisms. Today, bioreporter bacteria exist that detect water (Axtell and Beattie 2002), plant nutrients like nitrate (DeAngelis et al. 2005), root exudates (Darwent et al. 2003), pathogenic bacteria (Brigati et al. 2007), viruses (Kintzios et al. 2004) and even the communication between bacteria (DeAngelis et al. 2007). The analysed media have changed over the years as well. In the beginning research stretched on *ex situ* analysis of water samples (Selifonova et al. 1993; Tan et al. 1994; Sticher et al. 1997) and soil or sediment extracts (Burlage et al. 1994; Selifonova and Eaton 1996). Nowadays *in situ* research has come into focus e.g. for phyllosphere (Joyner and Lindow 2000; Leveau and Lindow 2001), rhizosphere (DeAngelis et al. 2005) and soil (Hojberg et al. 1999).

Although the application of bioreporter bacteria is mostly restricted to laboratories probably because bioreporter based assays have so far not been certified by environmental regulation authorities (Harms 2007), bioreporter bacteria are often validated using environmental samples and the bioreporter approach is applied for environmental analysis to some extent. Especially for the determination of bioavailability bioreporter bacteria are very useful and they were successfully employed in many cases. For detection of bioavailable iron in freshwater and seawater a *Synechococcus* Fe bioreporter was used and was able to detect trace levels of iron and report varying iron bioavailability in contrast to similar total iron concentrations (Durham et al. 2002; Mckay et al. 2005; Boyanapalli et al. 2007). In another study differences between concentrations of water-extractable and bioavailable fractions of heavy metals in soil were shown by bioreporter bacteria (Ivask et al. 2002; Ivask et al. 2004). These examples indicate that using a living system such as a bioreporter organism will assist in gaining a better understanding of bioavailability from the perspective of a living cell (Boyanapalli et al. 2007). Additionally bioreporter bacteria have been used to provide *in situ* information on rhizosphere conditions at very high spatial resolution (Cardon and Gage

2006). Application ranged from reporting root exudation in relation to shoot nitrate concentration (Darwent et al. 2003), the availability of nitrate in the rhizosphere (DeAngelis et al. 2005) as well as the communication via N-acylhomoserine by rhizobacteria during colonization on plant roots (Gantner et al. 2006).

1.1.3 Comparison with analytical chemistry

Comparing chemical analysis with bioreporter assays gives the impression that the first might have a better point in terms of sensitivity, quantification and identification of unknown molecules, and that it has only the disadvantage of being expensive, time-consuming and requiring trained personnel. But considering the development history of both techniques indicates that the bioreporter approach, which has been in focus of research for nearly twenty years now, has considerably room for improvement. Until today mostly the genetic construction rather than the optimization of detection strategies has been in the centre of research. Only few studies dealt so far for instance with the improvement of detection limits, e.g. by construction of sensitized bioreporter bacteria for heavy metals based on knock-out mutants of metal transporters (Leedjärv et al. 2007) or the simplification of the calibration modus (Wackwitz et al. 2008). Technically both techniques have to face the same problems as sample interferences exist for all analytical techniques. Under certain circumstances bioreporter approaches can even compete with chemical analysis. This was shown for example in South East Asia where massive arsenic contamination occurs. Expensive and complicate even though accurate chemical analysis like atom absorption spectrometry (AAS), mass spectrometry with inductive coupled plasma (ICP-MS) or atom fluorescence spectrometry (AFS) is no option because of the special situation in the affected countries. The fact that there are about 10 millions of probably arsenic contaminated tube wells and even more dependent people and that seasonal and spatial variation in arsenic concentrations occurs taken together with the local social and economic conditions demand cheap testing.

Nearly 200 samples from groundwater wells of the Red River and Mekong River deltas in Vietnam were analysed using luminescent arsenite/arsenate bioreporter bacteria (Stocker et al. 2003) and resulted in more than 90% correct measurements with an overall average of 8.0% false negative and 2.4% false positive results at the WHO guideline of 10 μg/L (Trang et al. 2005). Colorimetric field test kits based on the mercuric bromide stain method or Gutzeit method are reported to be uncertain for concentration ranges below 70 µg/L. A study investigating the reliability showed that false negatives occurred with 68% and false positives with up to 35% at the local guideline level of 50 µg/L (Rahman et al. 2002). A recent study reported a higher accuracy of a Merck kit, yet, the authors recommended improvement of several issues such as training of laymen or simplified and reliable colour signal discrimination as a prerequisite for mass scale screening with such field kits (Jakariya et al. 2007).

In fact both methods can complement each other. Bioreporter bacteria give the biological answer; they detect the bioavailable fraction instead of the total fraction. Moreover due to their small size, bioreporters can be applied to spatial restricted environments, which can only be difficultly accessed by chemical analysis. Using gfp-tagged bioreporter bacteria Bahl and colleagues were able to investigate the concentrations of tetracycline in the intestine of rats (Bahl et al. 2004). Additionally bioreporter bacteria can be used at gradients to show spatial resolution, which presents an enormous advantage for environmental research. Microenvironments with gradients of interest exist for example in biofilms or rhizosphere. Gantner and colleagues used single cell information to investigate AHL-mediated communication by rhizobacteria. They could show that most communicating bacteria were between 4 and 5 µm apart but that even distances of 37 µm in the root tip zone and 78 µm in the root hair zone were bridged by AHL-mediated communication (Gantner et al. 2006). Leveau and Lindow could show the availability of fructose and glucose on bean leaves with a spatial resolution of 10 µm (Leveau and Lindow 2001). In theory the spatial resolution can be

as high as the size of the bioreporter bacteria as long as a non destructive *in situ* measurement of the bioreporter signals is possible. Another advantage of the small size of bioreporter bacteria is the possibility of miniaturization. One bacterial host strain with different genetic reporter circuits can be used in multi-target arrays. Because only small sample volumes are necessary a single sample can be tested with a variety of bioreporter bacteria with different target specificities (Tecon and van der Meer 2008).

1.2 Arsenic

1.2.1 Occurrence in the environment

For many centuries arsenic (As) has almost exclusively been connected with deliberate poisoning, but today its contribution to environmental pollution through anthropogenic use of arsenic containing pesticides, through mining and burning of coal as well as its natural release has come into focus (Leonard 1991). Arsenic has been detected in groundwater of several countries worldwide, like Bangladesh, India, Vietnam, Cambodia, Australia, USA, Canada, Mexico, Argentina and Germany, with concentration levels higher than the WHO drinking water guideline value of 10 µg/L as well as the national regulatory standards (e.g. 50 µg/L in India and Bangladesh (Ahmed et al. 2004; Mukherjee et al. 2006)). Arsenic contamination of groundwater is often based on geological sources and its ingestion leads to chronic health disorders in most of the affected regions of the world (BGS 2001; Smedley and Kinniburgh 2002). In Asia arsenic in groundwater has emerged as the largest environmental health disaster threatening at least 100 million people in the Bengal Basin of Bangladesh and West Bengal, India with cancer and other As-related diseases (Mukherjee and Bhattacharya 2001 ; Bhattacharya et al. 2004).

Arsenic as the 52nd out of 92 elements with respect to its abundance in the earth's crust, with a concentration of 1.8 parts per million, occurs naturally in a wide range of minerals, like

arsenopyrite (iron arsenic sulfide), realgar (arsenic sulfide) and tennantite (copper arsenic sulfide). In soils, the most often found arsenic forms (Figure 2) are inorganic As^{III} (arsenite) and As^{V} (arsenate) (Masscheleyn et al. 1991; Smith et al. 1998). Methylated species, monomethylarsonic acid (MMAA), dimethylarsinic acid (DMAA) and trimethylarsine oxide (TMAO) dominate in biomass, but have also been encountered in soils (Leonard 1991). In addition, As^{V} and As^{III} can be volatilized upon conversion to arsine, MMAA to monomethylarsine (Rahman et al. 2002), DMAA to dimethylarsine (DMA), and TMAO to trimethylarsine (TMA) (Cullen and Reimer 1989). In soils, the bioavailability and thus toxicity of arsenic depends on various soil parameters, like pH, redox potential, other elements, soil and site hydrology as well as plant and microbial components. These parameters influence the adsorption capacity and behaviour of soil colloids, and thus alter the solubility and bioavailability of arsenic (Livesey and Huang 1981; Roy et al. 1986; Goldberg and Glaubig 1988).

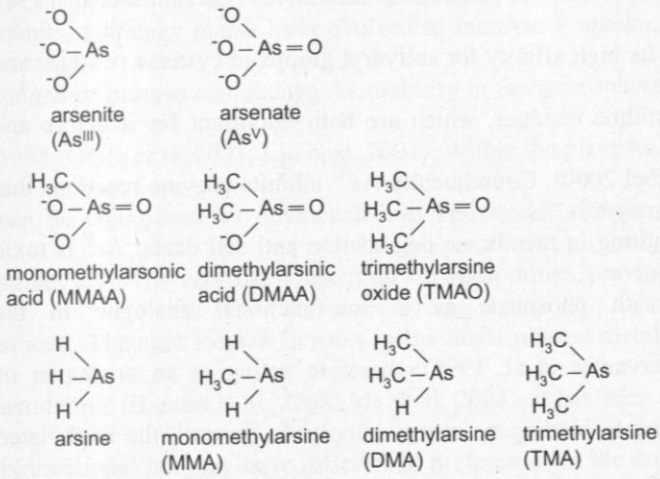

Figure 2. Chemical structure of the most abundant arsenic forms in soil and biomass.

In natural ecosystems arsenic mobilization is primarily driven by microbial mediated biogeochemical interactions. Microbial reduction of As^{V} to the more toxic and mobile As^{III}

species occurs via detoxification (Cervantes et al. 1994) or respiration processes (Ahmann et al. 1994). The As^V-reductase genes involved in As^V reduction have been identified in a number of bacteria (Santini and Stolz 2004). In contrast only a handful of microorganisms capable of respiring As^V have been isolated (Oremland and Stolz 2003). The As^V-respiring microorganisms can use different electron donors (e.g. acetate, hydrogen), and range from mesophiles to extremophiles (Oremland and Stolz 2003). Laboratory studies indicate that microbial processes involved in As^V reduction and mobilization are many times faster than inorganic chemical transformations (Ahmann et al. 1997; Jones et al. 2000) and that these microorganisms may play an important role in As cycling in the sub-surface (Ahmann et al. 1997; Jones et al. 2000; Islam et al. 2004).

1.2.2 Arsenic metabolism in bacteria and plants

The impact of arsenic on biological systems is determined by its concentration and the affected organism. In general, arsenic toxicity depends on its oxidation state: trivalent arsenic forms are about 100 times more toxic than the pentavalent derivatives (Cervantes et al. 1994). The toxicity of As^{III} is based on its high affinity for sulfydryl groups in cysteine residues and for imidazolium nitrogen of histidine residues, which are both important for structure and function of various proteins (Gebel 2000). Consequently As^{III} inhibits enzyme reactions that need free sulfhydryl groups, resulting in membrane degradation and cell death. As^V is toxic because of its competition with phosphate as a non-functional analogue in the phosphorylating metabolism (Cervantes et al. 1994), therefore acting as an uncoupler of oxidative phosphorylation, leading to inadequate energy supply. In general, the methylated species MMAA and DMAA are less toxic forms than oxyanions As^V and As^{III} due to their low solubility and reduced affinity to tissues (Leonard 1991).

The oxyanions of arsenic enter cells via transporters for other compounds. In bacteria, As^V is taken up by phosphate transport systems, such as the ATP-coupled Pst pump (Rensing et al.

1999). One route of entry for As^{III} is over the GlpF polyol transporters (Rensing et al. 1999). Bacterial detoxification of arsenic is often based on inducible ion efflux systems that lower the intracellular concentration by active export (Nies and Silver 1995; Rensing et al. 1999). Because As^{V} can not be transported with this system, the enzyme arsenate reductase catalyzes the reduction of As^{V} to As^{III}, the substrate of the efflux system (Nies and Silver 1995; Rensing et al. 1999). The genes that encode the proteins involved in As resistance are either plasmid or chromosomally borne, and have been best studied in *Escherichia coli*. Plasmid R773 is composed of five genes *arsRDABC* organized in one operon (Chen et al. 1986). The *arsC* gene encodes the As^{V}-reductase (Gladysheva et al. 1994; Oden et al. 1994), *arsA* and *arsB* act as the ATP-dependent As^{III} efflux pumps (Mobley and Rosen 1982) and *arsR* and *arsD* encode two inducer-dependent *trans*-acting repressors that regulate the basal and upper levels of ars operon expression, respectively (Wu and Rosen 1991; Wu and Rosen 1993a; Wu and Rosen 1993b; Chen and Rosen 1997).

In plants As^{V} is taken up through the high affinity P transporters in root membranes. Any adaption strategy plants have evolved to increase P uptake, like the release of organic acid anions or protons can change As mobility in the rhizosphere and As uptake (Geelhoed et al. 1998; Grafe et al. 2001; Liu et al. 2001). Within the plant As^{V} is instantly reduced to As^{III} that can be complexed by phytochelatines and stored afterwards in vacuoles as detoxification strategy. As^{III} is very likely taken up via aquaporines, previously described as transporters for silicon. They are located in roots in the distal plasma membrane domain of endodermis and exodermis (Bienert et al. 2008; Ma et al. 2008). It has been shown that these transporters are bidirectional but may have differential preferences in the direction of transport (Bienert et al. 2008). Recent observation of Xu and colleagues (2007) showed massive As^{III} efflux from tomato roots grown in hydroponic systems.

1.2.3 Arsenic as target for bioreporter bacteria

For detection of arsenic concentrations below the guideline levels of the WHO (10 µg/L) as well as the national standards in South East Asia (50 µg/L) chemical analysis by atom absorption spectrometry (AAS), mass spectrometry with inductive coupled plasma (ICP-MS) or atom fluorescence spectrometry (AFS) is one option. However, to use these techniques trained persons and well equipped laboratories are necessary. In the case of the arsenic contamination in South East Asia repeated testing of many of the 10 million tube wells is crucial due to seasonal variations. Therefore cheaper and simpler methods are required. Most of the now available field methods use a colorimetric approach based on mercuric bromide. In that test As^{V} and As^{III} are reduced with powdered zinc to produce arsine gas (AsH_3), which reacts with mercuric bromide to form arsenic/mercury halogenides that lead to discolouration ranging from yellow to brown depending on the arsenic concentrations. Reports show that this method works imprecisely for concentration ranges below 70 µg/L (Rahman et al. 2002; Jakariya et al. 2007). Beyond that two toxic metals are applied in the analytical procedure (Zn, Hg).

Bioreporter assays offer an alternative. Based on the previously described resistance mechanism of *E. coli* on plasmid R773 bioreporter bacteria for the detection of arsenic have been developed with various reporter proteins over the past years (Cai and DuBow 1996; Ramanathan et al. 1997; Tauriainen et al. 1997; Ramanathan et al. 1998; Tauriainen et al. 1999; Petanen et al. 2001). A set of bioreporter bacteria was produced by Stocker and colleagues (2003) for different detection strategies. In this study, a prototype of a simple colorimetric paper strip was presented based on a *lacZ* strain which was dried on the paper and reacted after rehydration. Furthermore a sensitive and robust lux strain was constructed that has been already used for analysis of groundwater (Trang et al. 2005) and rice (Baumann and van der Meer 2007).

1.3 Outline of the thesis

This thesis has two interrelated foci. The first one addressed the bioreporter itself and its preservation in order to improve bioreporter bacteria-based test systems. Bioassays with bioreporter bacteria are usually calibrated with analyte solutions of known concentrations, as bioreporter output does not only depend on target concentration, but also on incubation time and physiological activity of the cells in the assay. Comparing the bioreporter output with standardized colour tables in the frame of field applications seems rather difficult and error-prone. A new approach controlling assay variations and improving application ease is therefore highly desirable. Long-term maintenance of specific activity and viability of bioreporter bacteria are essential for the functioning of cell-based detection devices, particularly when field application is intended.

The second goal was the application of the bioreporter bacteria in an *in situ* environment to gain insight into the arsenic transport and release along and from plant roots, because the classical concept of arsenic transfer in plants is questioned by recent identification of bidirectional transporters for arsenite and their potential role in plant As status regulation. Established soil based studies that rely on chemical analysis of soil or soil solution require root mat formation which denies investigations along individual roots differing in age and function and show no spatial resolution.

To achieve these goals different strains of the bioreporter bacteria *E. coli* DH5α were used in well-defined experiments to obtain reproducible results. *Zea maize* was used as a model plant in rhizoboxes allowing easy access of the roots. The knowledge obtained is expected to improve the handling of bioreporter bacteria based test systems and the understanding of rhizosphere processes involved in arsenic metabolism.

In addition to the general introduction (chapter 1), the summary and concluding remarks (chapter 5), this thesis comprises three chapters in the form of publications. A multiple cell line approach is presented that allows bioassays without external calibration (chapter 2). Two

distinct types of reporter constructions were developed and tested if they would result in different levels of reaction sensitivity to arsenite. It was further investigated if combinations of such reporter cell lines with variable signal outputs would be suitable to infer arsenite concentration ranges without external calibration and at the required drinking water standards. Chapter 3 concentrates on the long-term preservation of specific activity and viability of the bioreporter bacteria depending on different culture conditions, preservation methods and protection media. In the 4th chapter the application of the bioreporter bacteria in an *in situ* environment is described. It includes (i) the investigation of effects of the soil solution on the bioreporter activity in LB agar and the development of an application strategy for utilisation in rhizobox systems; (ii) the visualisation of As distribution in soil-root system for plants grown on soil contaminated with As and relation of As concentrations to position and development of the roots; (iii) the visualisation of As release in a rhizobox system by a split root experiment and (iv) the determination whether As is released from intact or from injured roots.

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Chapter 2

Internal arsenite bioassay calibration using multiple bioreporter cell lines

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Internal arsenite bioassay calibration using multiple bioreporter cell lines

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Summary

Bioassays with bioreporter bacteria are usually calibrated with analyte solutions of known concentrations that are analysed along with the samples of interest. This is done as bioreporter output (the intensity of light, fluorescence or colour) does not only depend on the target concentration, but also on the incubation time and physiological activity of the cells in the assay. Comparing the bioreporter output with standardized colour tables in the field seems rather difficult and error-prone. A new approach to control assay variations and improve application ease could be an internal calibration based on the use of multiple bioreporter cell lines with drastically different reporter protein outputs at a given analyte concentration. To test this concept, different *Escherichia coli*-based bioreporter strains expressing either cytochrome *c* peroxidase (CCP, or CCP mutants) or β-galactosidase upon induction with arsenite were constructed. The reporter strains differed either in the catalytic activity of the reporter protein (for CCP) or in the rates of reporter protein synthesis (for β-galactosidase), which, indeed, resulted in output signals with different intensities at the same arsenite concentration. Hence, it was possible to use combinations of these cell lines to define arsenite concentration ranges at which none, one or more cell lines gave qualitative (yes/no) visible signals that were relatively independent of incubation time or bioreporter activity. The discriminated concentration ranges would fit very well with the current permissive (e.g. World Health Organization) levels of arsenite in drinking water (10 μg l^{-1}).

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Introduction

In the recent years, new tools for environmental monitoring have been developed on the basis of so-called bioreporter organisms. Bioreporters are often microbial strains which are genetically modified to produce easily detectable output in response to the presence of specific analytes. Several reviews have been dedicated to this rapidly developing field, exposing details of genetic constructions, type of reporters or analyte detection (Daunert *et al.*, 2000; Leveau and Lindow, 2002; Belkin, 2003; van der Meer *et al.*, 2004; Harms *et al.*, 2006). The detection system of bioreporter microorganisms is in most cases composed of a simple genetic circuitry, comprising a regulatory system (i.e. regulatory gene plus its cognate promoter) and one or more reporter genes. Commonly used reporter proteins are the chromogenic β-galactosidase (*lacZ*) or β-glucuronidase (*gusA*), luminescent luciferases (*luc* or *luxAB*) or autofluorescent proteins (GFP and variants) (Ivask *et al.*, 2002; Paitan *et al.*, 2004; Werlen *et al.*, 2004; DeAngelis *et al.*, 2005).

For quantitative measurements, bioreporter assays need to be calibrated with analyte solutions of known concentrations, which are typically analysed along with the samples of interest (Hakkila *et al.*, 2004; Harms *et al.*, 2005). The reason for this is that the bioreporter output (colour, luminescence or fluorescence) does not only depend on analyte concentration, but also on assay incubation time and the numbers and physiological activities of the cells in the assay (Jansson, 2003; Stocker *et al.*, 2003; van der Meer *et al.*, 2004; Wells *et al.*, 2005). The latter two variables are very difficult to control, in particular when dried or immobilized cells are used (Bjerketorp *et al.*, 2006). Simple applications like bioreporter field test kits or paper test strips are hampered by the difficulty to compare the resulting colour signals with standardized colour tables. Therefore, a robust and reliable system for internal calibration would be highly desirable.

The problem of variable incubation time and uncertain cellular activity could be overcome by the simultaneous use of multiple bioreporter cell constructs, each giving qualitative information about the presence or absence of analyte in a certain concentration range. Bioreporters

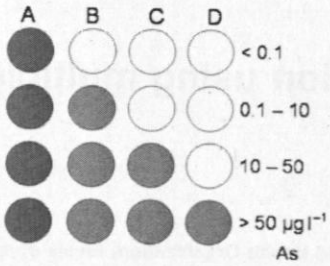

Fig. 1. Principle of the internal calibration approach. (A)–(D) represent bioreporter cell lines differing in their sensitivity to arsenite.

could be designed and combined in such a way that not the actual individual output but the combination of reacting cell lines indicates the analyte concentration range in unknown samples without the need to measure exact rates or endpoints of colour formation by individual bioreporters (Fig. 1). Although this method would not report exact concentrations, the identification of concentration ranges should be sufficient for many applications, where, for instance, only a distinction of concentrations below and above a predefined permissible level is desired. If in combination with a chromogenic reporter protein this would allow signal interpretation by eye without instrumentation. In the case of arsenic in potable water, a distinction between concentrations below and above the World Health Organization (WHO) drinking water standard of 10 µg l^{-1} or the local standard of 50 µg l^{-1} in the most arsenic-threatened countries of South-East Asia would be most desirable.

To test this concept, we used two reporter genes to redesign bioreporter bacteria that had previously been developed for the detection of arsenite in drinking water, exploiting the ArsR sensor transcriptional repressor of the natural arsenic resistance of *Escherichia coli* (Stocker *et al.*, 2003). In the presence of arsenite, ArsR loses affinity for its binding site on the DNA the result of which is depression of the *arsR* promoter. Equally effective in derepressing the *arsR* promoter via ArsR is antimonite. In cells that carry the arsenate reductase ArsC, arsenate (at 25% efficiency) and trimethylarsine oxide (at 10% efficiency) will also lead to derepression (Baumann and van der Meer, 2007). In one series of cell lines, the *arsR* promoter was combined with *lacZ* for β-galactosidase. β-Galactosidase has many advantages and various chromo- and fluorogenic substrates exist to assay LacZ activity (Daunert *et al.*, 2000; Lei *et al.*, 2006). In a second series of cell lines we applied the *ccp* gene. Its product, the cytochrome *c* peroxidase (CCP), has not frequently been utilized in whole-cell bioreporter bacteria so far, but a large variety of *ccp* mutants have been developed which display different catalytic activity for one of its substrates guaiacol (Iffland *et al.*, 2000). The signal outputs of both reporters were thus varied in different ways: for LacZ bioreporters we modified the intervening sequence between *arsR* and *lacZ* in order to modulate *lacZ* expression. For *ccp* we maintained the same reporter synthesis rate, but hypothesized that because of different catalytic activity of the reporters we would get variable output of the cells at the same arsenite concentration. Our research questions therefore were (i) if both types of reporter constructions would result in different levels of reaction sensitivity to arsenite and (ii) if combinations of such reporter cell lines with variable signal outputs would be suitable to infer arsenite concentration ranges without external calibration and at the required drinking water standards.

Results

β-Galactosidase-based bioreporter assay

Bioassays were performed in 96-well plates to allow spectrophotometrical measurements (Fig. 2) as well as visual signal detection by digital imaging (Fig. 3). For each bioreporter strain the bioassay was performed in triplicate with 15 different arsenite concentrations ranging from 0.2 to 100 µg l^{-1}. Strain 2245 was very sensitive and produced visible colour from the lowest arsenite concentration (0.2 µg l^{-1}) with a linear signal increase up to 6 µg l^{-1} and a plateau above 10 µg l^{-1} (Figs 2 and 3). Colour development by strain 1595 began at approximately 10 µg l^{-1}

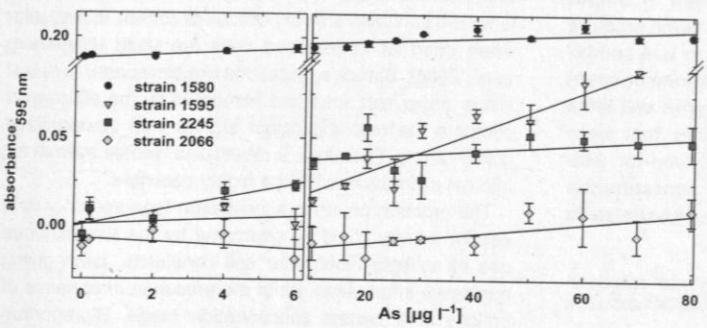

Fig. 2. Colour development after 480 min by four bioreporter strains expressing LacZ measured as absorbance at 595 nm. Data points represent the average of three replicates.

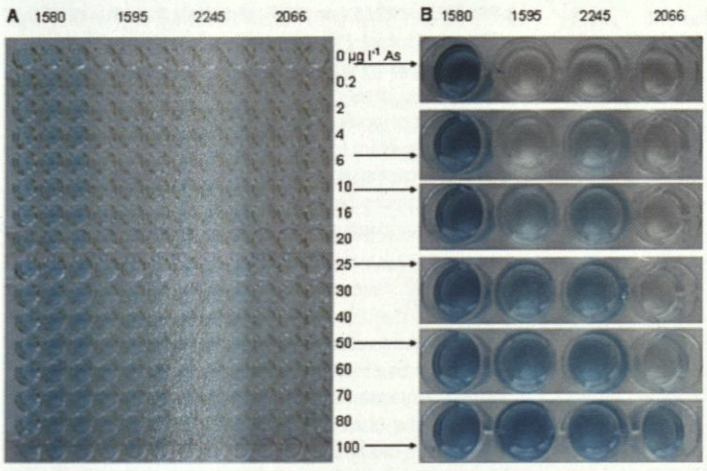

Fig. 3. Colour development from X-gal by four LacZ-based bioreporter strains as response to different arsenite concentrations.
A. Wide concentration range overview of assay carried out in 96-well plates. The digital image was taken after 8 h, whereas colour development proceeded for 22 h.
B. Colour detail in assays performed in 24-well plates. Image taken after 4 h incubation time at 30°C.

arsenite and linearly increased at higher concentrations. Both strains had a relatively low background activity in the absence of arsenite. In contrast, strain 1580 was characterized by a high background and little further signal increase towards higher concentrations. Strain 2066 was the least sensitive and gave visible signals only at very high concentrations (around 50 µg l^{-1}). Visual signal differentiation for neighbouring concentrations was difficult with all strains. The combination of qualitative (yes/no) signals of all four strains appeared suitable for the definition of three arsenite concentration ranges: < 6 µg l^{-1}, 10–50 µg l^{-1} and > 50 µg l^{-1} (Table 1A). The quality of the responses (visible colour or not) was independent of incubation time in a window between 4 and 8 h after induction. During that time frame the colours gained intensity but the pattern of the signals stayed the same. Signal distribution was also independent of cell density for final cell culture turbidity in the assays of OD_{600} of between 0.1 and 1.0 (data not shown).

CCP-based bioreporter assay

In this case, the assays were carried out with colonies exposed to arsenite on agar plates and transferred to paper in order to obtain optimal visual signal interpretation. For each strain the bioassay was performed in four replicates with 10 different arsenite concentrations ranging from 1 µg l^{-1} to 5 mg l^{-1}. The bioreporter strains 1971, 1981 and 1982 displayed relatively similar responses and produced clearly visible, concentration-dependent signals between 4 and 30 µg l^{-1} followed by a plateau (Fig. 4). For the strains 1971 and 1982 a further signal increase could be detected after incubation with 1000 µg l^{-1}. The background activity in the absence of arsenite was high enough to result in visible spots. Strain 2031 showed no background activity without arsenite, while colour production was clearly visible at 4 µg l^{-1} and increased strongly until 20 µg l^{-1}. Strains 2332 and 2340 did not respond to low arsenite concentrations (1–10 µg l^{-1}) and produced slightly increasing signals between 20 µg l^{-1} and 5 mg l^{-1} arsenite. The combination of the qualitative signals of these strains appeared to enable the distinction of arsenite-free controls from the concentrations ranges 1–10 µg l^{-1} and > 20 µg l^{-1} (Table 1B). Similar results were obtained with either twice as many cells per spot or shorter (2 h) and longer incubation (16 h) (data not shown).

Table 1. Arsenite concentration ranges distinguished with combinations of LacZ strains (A) and CCP strains (B).

A	Concentration range (µg l^{-1})	1580	1595	2245	2066		
	< 6	x	x				
	10–50	x	x	x			
	> 50	x	x	x	x		
B	**Concentration range (µg l^{-1})**	**1971**	**1981**	**1982**	**2031**	**2332**	**2340**
	0	x	x	x			
	1–10	x	x	x	x		
	> 20	x	x	x	x	x	x

Strains that give a signal in the respective concentration range are marked with x.

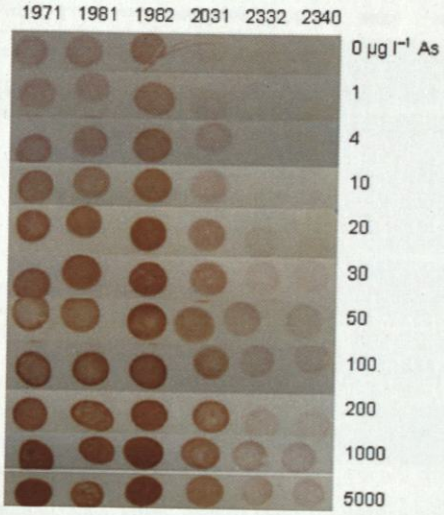

Fig. 4. Colour development in the six bioreporter strains expressing CCP as a function of arsenite concentration in the assay. Colonies were spotted on paper after 4 h incubation with arsenite on LB agar plates at 37°C. Digital image was taken 10 min after adding guaiacol and hydrogen peroxide to develop colour.

mRNA content

Reporter mRNA contents of cells exposed to three arsenite concentrations were quantified in order to determine whether differences in signal intensities for the reporters at identical arsenite concentrations were the result of differential transcription or of enzyme synthesis and enzyme activity. Levels of the *ccp* mRNA in the different CCP bioreporter strains were similar after incubation with the same arsenite concentration, but increased as a function of arsenite concentration (Fig. 5A). An exception was strain 1982, which for unknown reasons contained significantly ($P < 0.05$) less *ccp* mRNA at the highest arsenite concentration. The level of *lacZ* mRNA in the different reporter strains generally increased as a function of arsenite concentration, but varied between all strains. At 100 µg As l^{-1} the levels of *lacZ* mRNA differed significantly ($P < 0.05$) between all strains, while at 10 µg As l^{-1} the difference was only significant between strain 1580 and the other strains ($P < 0.05$). Although strains 1595, 2245 and 2066 had similar levels of mRNA at 10 µg As l^{-1} the colour formation was already visible for the strains 1595 and 2245 but not for 2066. The most likely reason for this difference is the improved *lacZ* ribosome binding site in strain 2245 and 1595 compared with strain 2066, which catalyses a faster translation rate from the same amount of mRNA (de Smit and van Duin, 1994). Altogether the change of the intergenic region between *arsR* and *lacZ* in the different constructs, and the use of two different plasmid vectors for cloning (pMV for 1580 and 1595; pPROBE for 2245 and 2066) did affect the amount of *lacZ* mRNA produced. Notably, *lacZ* expression in strain 1580 without and at 10 µg As l^{-1} did not differ, which is the result of the leaky *arsR* promoter and read-through (Fig. 5B). Basal level of *arsR* expression is required for the natural resistance system to function because of the nature of the *arsR* transcriptional organization. As ArsR is a transcriptional repressor which binds within its own promoter DNA, no *arsR* transcription would take place without leakiness.

Discussion

The aim of this study was to simplify the application of bioreporter bacteria by developing an internal calibration method. The idea was to infer analyte concentrations from combinations of qualitative (yes/no) signals provided by a series of cell lines with different sensitivities for the analyte. The quantitative response of a single cell line used so far in bioreporter assays is replaced by a series of complementary qualitative responses. The advantage of such bioreporter arrays is their relative independence of incubation times and the physiological activity of the cells in the assay. We used two different ways to produce such variable reporter cell lines: (i) by influencing the rate of

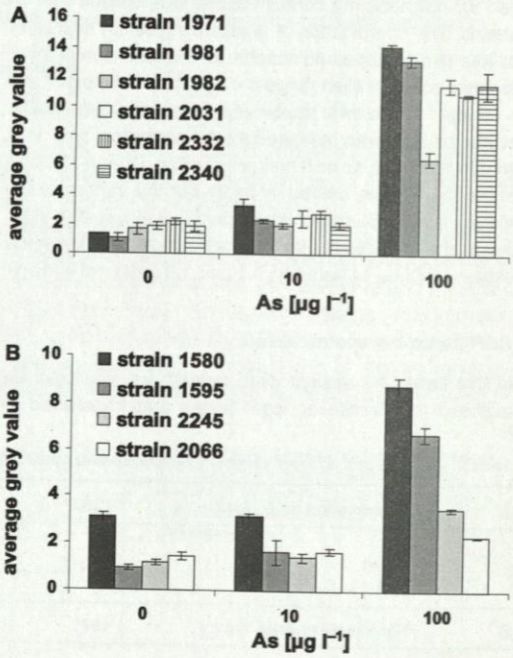

Fig. 5. Comparison of signal intensities after dot blot hybridization of mRNA in the six bioreporter strains expressing CCP (A) and in four bioreporter strains expressing LacZ (B) induced with three different arsenite concentrations for 4 h.

reporter enzyme synthesis (LacZ) or (ii) by influencing the reporter enzyme specific activity (for CCP). Various other ways of obtaining similar effects can be imagined, for instance, by using different stabilities of reporter proteins (Andersen *et al.*, 1998). We focused on an arsenite-detecting bioreporter, for which a simple qualitative assay could be useful in field campaigns notably in South-East Asia (Smith *et al.*, 2000; British Geological Survey, 2001). Several studies have shown that bioreporter strains based on ArsR and the *arsR* promoter react equally well to antimonite and arsenite (Ramanathan *et al.*, 1997; Scott *et al.*, 1997; Tauriainen *et al.*, 1999). Even wolframite will be able to derepress the system although at a 1000-fold higher concentration than arsenite. However, we believe that cross-reaction with antimonite is rather unlikely in groundwater samples, because most arsenite-contaminated samples contain only negligible concentrations of antimonite (Trang *et al.*, 2005). On the other hand, the bioreporter may be of equal use for the detection of antimonite where it prevails as antimonite is a suspected carcinogen. The presence of phosphate and nitrate in the analyte has not significantly affected the assays as reported by Ramanathan and colleagues (1997), Scott and colleagues (1997) and Harms and colleagues (2005).

LacZ bioreporters

Arsenite measurements have been performed before with LacZ bioreporters under control of the ArsR promoter using classical spectrophotometrical, chemiluminescence and electrochemical detection (Scott *et al.*, 1997; Ramanathan *et al.*, 1998), or by chromogenic visualization using X-gal (Stocker *et al.*, 2003). We previously showed that leaky expression from the *arsR* promoter must be reduced to obtain gradually intensifying blue colour formation from X-gal as a function of arsenite concentration. This was accomplished by placing a second ArsR DNA binding site downstream of *arsR* (Stocker *et al.*, 2003). Upon long incubation times, however, cells with this bioreporter construct (i.e. strain 1595) will intensify their blue colour at all arsenite concentrations, reducing the visibility of a good colour scale. We therefore produced one variant of strain 1595 in which the background is further reduced (strain 2066) by altering the ribosome binding site in front of *lacZ* (Fig. S1). Indeed, production of LacZ activity in strain 2066 is very low compared with the others, which was useful for the concept of the multi-strain assay, because the 2066 bioreporter only produces visible blue colour from X-gal at arsenite concentrations above 50 $\mu g\ l^{-1}$. At the engineered unique restriction sites in front of *lacZ* we then replaced fragments of various length originating from the wild-type *lacZ* promoter in *E. coli*, one of which (construct in strain 2245, Fig. S1) appeared to be suitable for the arsenite concentration range below 5 $\mu g\ l^{-1}$. Interestingly, strain 2245 reacted with visibly different blue colour formation from X-gal already at 0.2 $\mu g\ l^{-1}$ arsenite, compared with the negative control. Thus, the responses of strains 2245 and 1595 complement each other very well in the low (0.2–5 $\mu g\ l^{-1}$) and medium (5–50 $\mu g\ l^{-1}$) concentration ranges. Although strain 1580 is not directly useful in showing colour differences at varying arsenite concentrations, it can be used as positive control for the quality of the test.

By probing *lacZ* mRNA levels in the four strains, we could provide evidence that the different LacZ activities in the reporter strains as a function of arsenite is due to both differential transcription efficiency or mRNA stability and translational effects. Two transcriptional effects were clearly seen: first, the inclusion of the secondary ArsR binding site in all constructs except 1580, which reduced mRNA formation in the absence of arsenite, and second, the lower *lacZ* mRNA abundance from the constructs based on plasmid pPROBE (i.e. 2066 and 2245), which might be due to variations in plasmid copy number. However, the strong LacZ activity difference between 2066 and 2245 was not apparent from mRNA levels (Fig. 5B), but the result from the absence (2066) and presence (2245) of a good ribosome binding site. One can conclude that our intention to control the bioreporter sensitivity on the level of reporter enzyme synthesis was successful in that it led to a range of bioreporter strains of quite complementary sensitivities including a nearly constitutive control strain.

CCP bioreporters

The use of CCP as reporter gene resulted in a useful colouration as well, although the assay was a bit less robust than that for β-galactosidase. The main advantage of *ccp* was the availability of a well-characterized set of mutants with variable activity against the classical peroxidase substrate guaiacol (Iffland *et al.*, 2000; 2001). In this case mRNA probing showed that all mutant *ccp* genes were transcribed with similar efficiencies, and as a function of arsenite concentration (except one; Fig. 5A). However, the colouration resulted in very different intensities at the same arsenite concentration for the various reporters. Those strains containing CCP with improved specific activity (1971, 1981, 1982) compared with the wild type (2031) gave high signals at very low arsenite concentrations, but with a relatively high background in the absence of arsenite. Their increased activity confirms previous findings of Wilming *et al.*, who observed turnover numbers of 102 and 26 s^{-1} of strain 1971 and 1982, respectively, compared with 0.35 s^{-1} for the wild-type enzyme (Wilming *et al.*, 2002). The *ccp* gene in strain 1981 contains multiple mutations (Y39H–H60R–N184D–D217G–D224Y) and although the substrate turnover was

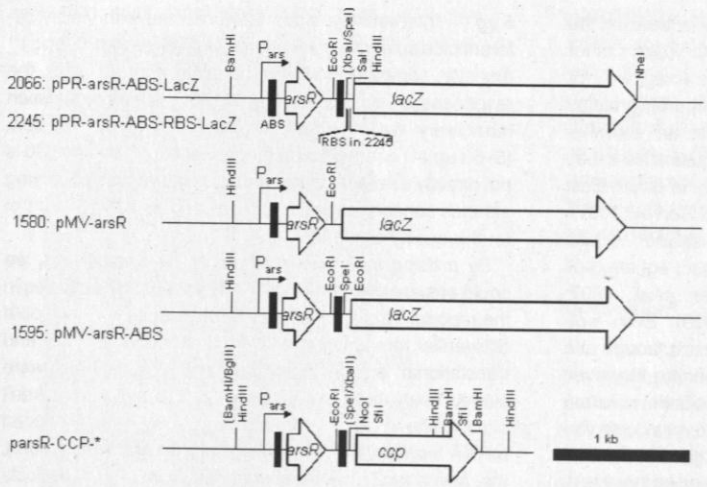

Fig. 6. Schematic picture of the plasmids developed in this study. Genes are represented as arrows, drawn to proportion. Relevant restriction sites for cloning are shown in their usual abbreviations. The black vertical bars symbolize the ArsR binding sites (ABS).

reported to be only slightly higher (0.8 s^{-1}; Iffland *et al.*, 2001) than that of the wild type, a colouration similar to that of 1971 and 1982 for arsenite concentrations up to 100 $\mu g\ l^{-1}$ was observed. The CCP of the bioreporter strains with the lowest observed activity and sensitivity in our tests (2332, 2340) had higher reported turnover numbers (13 s^{-1} and 5 s^{-1} respectively) than the wild-type enzyme (Wilming *et al.*, 2002). The reasons for these discrepancies between reported enzyme activities and signals obtained in our experiments with whole cells are unknown. Measurements of enzyme activities in cell extracts were in accordance with the reported substrate turnover (Fig. S1), which might be an indication for different enzyme activity in extracts and whole cells, or differences in substrate accessibility or transport in whole cells. Our results, however, show that it is possible to receive a set of bioreporter bacteria with varying sensitivity by modifying the catalytic activity of the reporter protein and to use combination of them for internal calibration.

Conclusions

We demonstrated that bioreporter bacteria of various sensitivities can be tailored by modifying different steps of the detection-signalling chain, i.e. the rate of reporter enzyme synthesis and the catalytic activity of the reporter protein. It appears thus possible to construct sets of bioreporter bacteria that, when combined, cover ranges of target concentrations that could not be covered by a single bioreporter organism due to restrictions of, for example, the induction factor, limitations of spectroscopic signal detection or the insensitivity of visual interpretation. Furthermore, our results demonstrate the potential of reporter protein engineering as opposed to the former focus on analyte sensing and gene regulation for bioreporter improvement (van der Meer *et al.*, 2004).

The accomplished modifications altogether resulted in two sets of bioreporter bacteria that can be used to infer analyte concentration ranges without external calibration. This quantitative information originates from combinations of robust qualitative signals of the individual bioreporter strains. The hypothesized working principle was thus shown to function. A further important result is that permissive concentrations of arsenic could be identified with both bioreporter sets: (i) below the WHO limit only two of the four LacZ bioreporter bacteria produced a visible signal, above 10 $\mu g\ l^{-1}$ three or at concentrations above local arsenic standards (50 $\mu g\ l^{-1}$) all four strains were responding with clear signals, (ii) the CCP bioreporter bacteria indicate a concentration below the WHO limit when four out of six strains show signals. This set could also be reduced to four strains, as three strains gave redundant information.

Our approach promises to simplify bioreporter application by layman as it eliminates time-consuming and technically demanding calibration. Especially for simple field detection devices such as paper strips (Stocker *et al.*, 2003) our method would be of great value. Besides its higher robustness it has the advantage of the much easier interpretation of sets of qualitative subsignals than of gradual colour changes.

Experimental procedures

Construction of ArsR plasmids

The following reporter plasmids were constructed (Fig. 6) in which ArsR controls expression of either the *lacZ* gene or the *ccp* gene [for CCP from *Saccharomyces cerevisiae* (Iffland

et al., 2000)]. In plasmid pMV-arsR [strain number 1580 (Stocker *et al.*, 2003)] the *arsR* gene is placed directly upstream of *lacZ* and in the same direction of transcription, so that expression of *arsR* and *lacZ* are under control of the ArsR-regulated promoter in front of *arsR* itself (P_{ars}, Fig. 6). To reduce background *lacZ* expression from the leaky P_{ars} promoter, a second ArsR DNA binding site (ABS) was introduced downstream of *arsR* in plasmid pMV-arsR-ABS [strain number 1595, Fig. 6 (Stocker *et al.*, 2003)]. To change the sequence and ribosome binding site upstream of *lacZ*, we amplified the entire *lacZ* gene from the *E. coli* K12 chromosome while introducing HindIII and NheI restriction sites at the gene extremities. This *lacZ* DNA was then used to replace the *gfp* gene in the broad host range vector pPROBE' (Miller *et al.*, 2000). The *arsR* gene and the downstream secondary ArsR binding site were recovered on a BamHI–SpeI fragment and inserted into pPROBE'–*lacZ* in front of *lacZ* to make pPR-ArsR-ABS-LacZ (strain 2066, Fig. 6). The ribosome binding site (RBS) of the original *lacZ* gene of *E. coli* was amplified together with the entire *lacZ* gene by PCR by using primer LacZ Rev NheI (5'-gctagcttatttttgacaccagaccaactgg-3') and primer LacZ-RBS-For (5'-gcttgtgagcggataacaatttc acacagg-3') and introduced in pPR-ArsR-ABS-LacZ digested by HindIII and NheI (plasmid pPR-arsR-ABS-RBS-LacZ, strain 2245, Fig. 6). Sequences of the *arsR–lacZ* intergenic regions are presented in Fig. S2.

The *ccp* reporter plasmids were all constructed by inserting a BamHI–SpeI fragment containing *arsR* and the second ArsR binding site from pPR-arsR-ABS (Stocker *et al.*, 2003) in a pET15-based plasmid containing the *ccp* gene or its variants (Iffland *et al.*, 2000; 2001), pre-digested with BglII and XbaI (during which the T7 promoter is removed). The *arsR* gene is thus expressed from P_{ars} and on its turn regulates *ccp* expression (Fig. 6). In this manner we produced *E. coli* strains with plasmids pArsR-CCP-WT (strain number 2031, wild-type *ccp*), pArsR-CCP-R48I (strain 2332, R48I mutation in CCP), pArsR-CCP-R48T (strain 2340), pArsR-CCP-R48Q (strain 1971), pArsR-CCP-K2.4 (strain 1981, Y39H-H60R-N184D-D217G-D224Y mutations) and pArsR-CCP-PT1 (strain 1982, R48H mutation). Arsenite bioreporters were constructed in *E. coli* DH5α.

Cell pre-culturing

Arsenite in aqueous solution was measured with cell suspensions of *E. coli* strains (Table 2). Bioreporter strains were plated from –80°C stock cultures on fresh Luria–Broth (LB) plates with appropriate antibiotic selection (ampicillin 100 μg ml^{-1} or kanamycin 50 μg ml^{-1}) and grown overnight at 37°C. Five millilitres of liquid LB medium plus antibiotic was inoculated with a single colony and incubated for 16 h at 37°C. Cultures were diluted in fresh pre-heated LB medium without antibiotics at a 1:50 ratio and incubated until an optical density at 600 nm of 0.5 was reached and then used for triplicate bioassays as described below.

Arsenite assays with E. coli *β-galactosidase (LacZ) bioreporters*

Assay mixtures containing 100 μl of cell suspension (OD_{600} of 0.5, 1:5 diluted in water), 90 μl of arsenite stock solution and 10 μl of X-gal substrate solution (5-bromo-4-chloro-3-indolyl-β-D-galactosidase, Serva, 0.2 mg ml^{-1} in dimethylformamid) were prepared directly in 96-well plates. Cells were exposed to different final arsenite concentrations (0, 0.05, 0.2, 0.5, 2, 4, 6, 10, 20, 25, 30, 40, 50, 60, 70, 80 and 100 μg l^{-1}). Arsenite solutions were prepared by dilution from a 50 mM (3.9 g l^{-1}) sodium arsenite solution commercial stock (Merck) in tap water. The 96-well plates with the assay mixtures were covered with a lid and incubated at 30°C in a rotary shaker (700 r.p.m.) for 22 h. In order to detect the blue colour formed from X-gal conversion, the optical density was measured in a microplate reader (Spectramax 250, Molecular Devices) at 595 nm after 0, 1, 2, 3, 4, 5, 6, 7, 8 and 22 h. The turbidity resulting from the cell suspension itself was subtracted from the measured signal by measuring triplicate assay mixtures without X-gal.

Table 2. Identification and characteristics of *E. coli* DH5α bioreporter constructs.

Strain	Plasmid
LacZ	
1580	pMV-arsR
1595	pMV-arsR-ABS
2245	pPR-arsR-ABS-RBS-lacZ
2066	pPR-arsR-ABS-lacZ
CCP	
1971	pArsR-ABS-CCP-R48Q
1981	pArsR-ABS-CCP-K2.4
1982	pArsR-ABS-CCP-PT1
2031	pArsR-ABS-CCP-WT
2332	pArsR-ABS-CCP-R48I
2340	pArsR-ABS-CCP-R48T

ABS, ArsR binding site; RBS, ribosome binding site.

Arsenite measurements with E. coli *CCP bioreporters*

The CCP bioreporters were cultured as above for the LacZ reporters in small pre-cultures for 16 h at 37°C after which cells were diluted 50-fold and again grown until a culture turbidity of ~0.5 was reached. Cells were then concentrated by centrifugation to achieve a turbidity at 600 nm of ~6. Ten microlitres of these cell suspensions were then spotted on agar plates containing different concentrations of arsenite (0, 1, 4, 10, 20, 30, 50, 100, 200, 1000 and 5000 μg l^{-1}) and briefly dried. After an incubation of 4 h at 37°C the cell spots were transferred to paper by gently pressing a paper sheet (3M chromatography paper, Whatman) of the right diameter directly on the agar surface. Peroxidase activity of the cells was detected by incubating the paper sheets on filter paper which was wetted with 50 mM potassium phosphate buffer (pH 6.0) containing freshly added guaiacol (final concentration 105 mM, Sigma) and hydrogen peroxide (final concentration 170 μM, Merck) (Iffland *et al.*, 2000). Colour development was allowed to proceed until clearly visible, after which the paper sheets were digitally photographed.

Determination of mRNA contents

Reporter strain cultures grown similarly were exposed to 0, 10 or 100 μg l^{-1} arsenite for 4 h at 37°C, after which total RNA

Table 3. Primers for the synthesis of probes for dot blot hybridization.

Primer name	Target	Sequence
sfi_for	*ccp*	5′-GCC ATG GCC AGC ACG GCC ACA CC-3′
R48Trev	*ccp*	5′-GTG CCA AGC AAG AGT GAC TAA TAC GGG-3′
LacZfor	*lacZ*	5′-TCG GTT ACG GCC AGG ACA GT-3′
LacZrev	*lacZ*	5′-CAT CAT TAA AGC GAG TGG CAA CAT-3′

was isolated from 5 ml of culture using a Macherey Nagel Nucleo Spin Kit. Twenty microlitres from 120 µl of total RNA extract from each sample was blotted on a positively charged nylon membrane (Roche) using a Milliblot system (Millipore). The total RNA on the membrane was fixed with UV (1 min at 120 mJ cm^{-2}) and stored at room temperature until hybridization. DIG-labelled DNA probes were synthesized using random primed labelling (DIG DNA labelling kit, Roche) from PCR products generated with specific primers (Table 3) for *lacZ* and *ccp* respectively. Hybridization was performed for 16 h at 57°C for the *ccp* probe and 53°C for the *lacZ* probe using DIG Easy Hyb solution (Roche) and 100 ng μl^{-1} probe. Subsequent washing was performed according to the manufacturer's protocol (Roche). DIG–DNA–RNA hybrids were detected with the NBT-CSIB (Roche) approach as described by the manufacturer. Dot blots were scanned and quantitatively analysed using Image J, in which the signal intensities (i.e. the grey intensities of the dots) are expressed as average grey values.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Specific activity of CCP in cell extracts from CCP bioreporter bacteria incubated for 4 h with 5 different arsenite concentrations.

Fig. S2. Intervening sequences in the arsR-lacZ constructions between the end of arsR (taa, underlined) and the beginning of lacZ (atg, underlined). Relevant restriction sites used for cloning are indicated. The ArsR binding site motif is shaded in grey background. Rbs, ribosome binding site. Plasmid pBGD23 is the original construct used by Scott *et al.*, 1997.

This material is available as part of the online article from http://www.blackwell-synergy.com

Chapter 3

Optimization of preservation conditions of As (III) bioreporter bacteria

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METHODS

Optimization of preservation conditions of As (III) bioreporter bacteria

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Abstract Long-term preservation of bioreporter bacteria is essential for the functioning of cell-based detection devices, particularly when field application, e.g., in developing countries, is intended. We varied the culture conditions (i.e., the NaCl content of the medium), storage protection media, and preservation methods (vacuum drying vs. encapsulation gels remaining hydrated) in order to achieve optimal preservation of the activity of As (III) bioreporter bacteria during up to 12 weeks of storage at 4°C. The presence of 2% sodium chloride during the cultivation improved the response intensity of some bioreporters upon reconstitution, particularly of those that had been dried and stored in the presence of sucrose or trehalose and 10% gelatin. The most satisfying, stable response to arsenite after 12 weeks storage was obtained with cells that had been dried in the presence of 34% trehalose and 1.5% polyvinylpyrrolidone. Amendments of peptone, meat extract, sodium ascorbate, and sodium glutamate preserved the bioreporter activity only for the first 2 weeks, but not during long-term storage. Only short-term stability was also achieved when bioreporter bacteria were encapsulated in gels remaining hydrated during storage.

Keywords Bioreceptor · Arsenic · Preservation · Storage

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Introduction

The ability of bacteria to sense chemicals has been widely employed to determine the bioavailability and concentration of environmental pollutants (King et al. 1990; Sticher et al. 1997; Daunert et al. 2000; Jaspers et al. 2001; Belkin 2003; Leveau and Lindow 2002; Harms et al. 2006). The so-called bioreporter organisms are often microbial strains that are genetically modified to indicate the presence of specific analytes. The detection system of bioreporter microorganisms is in most cases composed of a simple genetic circuitry, comprising a regulatory system (i.e., a regulatory gene plus its cognate target promoter) and one or more reporter genes, which produce a detectable output (van der Meer et al. 2004). Commonly used reporter proteins are the chromogenic β-galactosidase (*lacZ*) or β-glucuronidase (*gusA*), luciferases (*luc* or *luxAB*) or autofluorescent proteins (GFP and variants; Ivask et al. 2002; Paitan et al. 2004; Werlen et al. 2004; DeAngelis et al. 2005).

Arsenic is a worldwide occurring contaminant causing serious health problems particularly in Southeast Asian countries. Bioreporter bacteria for the detection of As (III) and As (V) in potable water have been described and their usefulness for field measurements has been demonstrated (Tauriainen et al. 2000; Stocker et al. 2003; Trang et al. 2005; Fujimoto et al. 2006; Date et al. 2007; Wackwitz et al. 2008), but only few reports address one of the main practical challenges, which is the long-term preservation and maintenance of the bioreporter activity under simple conditions. An original idea was recently presented by Date et al. (2007), who based their As bioreporter on *Bacillus subtilis* and used spores for long-term preservation at room temperature. Unfortunately, the germination of spores to vegetative cells that can be used for the bioassay requires a day or so, which is a drawback for immediate use of the

B. subtilis bioreporter. To our point of view, a ready-to-use bioreporter kit should be easy to handle and inducible in the shortest possible time, while producing a reproducible read-out after a constant induction time (i.e., the contact time with the sample). Ideally, therefore, the bioreporter cells should remain stable and quickly inducible even after long storage (i.e., months) at temperatures that can be maintained at low cost (e.g., 4°C–ambient).

The current standard in the preservation of commercially available bioreporter kits is freeze-drying. Stable activity after storage at −20°C from 10 days up to several months has been reported (Gu et al. 2001; Durand et al. 2003; Shin et al. 2005), yet some tests required renewed cultivation of the bioreporter cells (Tauriainen et al. 2000), since the proportion of active bioreporter cells easily drops below 1% (this study). For convenient field application of bioreporter kits, preservation has to be optimized further to allow shipping and storage at refrigerating to ambient temperatures with only minimal loss of bioreporter activity. One possible technique for achieving this would be to use vacuum drying at ambient temperature rather than freeze-drying. Pedahzur et al. (2004) observed significantly more loss of activity and viability of freeze-dried bioreporter bacteria than of vacuum-dried bacteria stored at 37°C. However, the low survival of less than 5% of the bioreporter bacteria in the Pedahzur report after 35 days storage at 37°C points at the need for better protocols for long-term storage. One of the suggestions for improvement of survival made by those authors was to grow the bioreporter strains under osmotic stress to induce stress response genes and to accumulate compatible solutes (Pedahzur et al. 2004). Several reports have indeed found positive effects of high trehalose concentrations both inside and outside the bacterial cell during the drying process (de Castro et al. 2000; Tunnacliffe et al. 2001; Manzanera et al. 2002). Also, encapsulation of bacteria in organic or inorganic gels has been proposed for increased survival (Cassidy et al. 1995; Premkumar et al. 2002; Polyak et al. 2004).

The objective of this work was to study the effects of different drying conditions and formulations on the survival and response to As (III) of an *Escherichia coli*-based bioreporter. We show that ambient temperature drying in the presence of high trehalose concentrations and polyvinylpyrrolidone (PVP) results in satisfactory survival and activation potential.

Materials and methods

Bioreporter cultivation *E. coli* DH5α strain 1595 (pMV-arsR-ABS) carrying *lacZ* as the reporter gene under transcriptional control of the ArsR protein (Stocker et al. 2003) was used throughout this study. Strain 1595 responds to As (III) and As (V) (Stocker et al. 2003), but for the sake of brevity and since only As (III) was used here, we refer to it as As (III) bioreporter. It was plated from a −80°C glycerol stock on fresh Luria broth (LB) agar plates and grown overnight at 37°C. Bioreporter cultivation then consisted in inoculating a single colony in either 5 ml LB medium or in 5 ml 1:10 diluted LB medium containing 2% NaCl (for preconditioning under osmotic stress). Both variants were incubated at 37°C on a rotary shaker until after 16 h the stationary phase was reached. The cells were harvested by centrifugation (6 min at 3,800×*g*). Preliminary tests had indicated better survival resistance of stationary phase cells compared to cells from the exponential growth phase. We hypothesized that growth in the presence of 2% NaCl could enhance the intrinsic stress response of bioreporter bacteria, perhaps leading to increased drought resistance. All growth media contained 100 μg/ml ampicillin to promote the maintenance of the reporter plasmid.

Bioreporter preservation by vacuum drying Stationary phase bioreporter bacteria (salt-preconditioned or not) were resuspended in different drying protection media to achieve culture optical density at 600 nm (OD_{600}) of 10. Table 1 summarizes the compositions of the drying protection media. Trehalose, sucrose, sodium ascorbate, and meat extract are common ingredients of protection media. Glutamate and raffinose were chosen because they had positive effects on bacterial survival during liquid drying (Malik 1990). Either 10% gelatin or 1.5% PVP served as viscosity enhancers. Ten microliters of the cell suspensions in protection media was applied to an Eppendorf tube and subsequently vacuum-dried at 20°C according to the following protocol: 4 mbar for 2 h, 0.4 mbar for 2 h, and 0.04 mbar for 2 h. Thereafter, the Eppendorf tubes were capped under air and stored at 4°C.

Bioreporter preservation by encapsulation in gels As an alternative to storage in dehydrated form, cells were also encapsulated in hydro- and sol-gels. Therefore, freshly harvested and washed cells were concentrated to $OD_{600}=20$ and mixed with the same volume of either 2% sodium alginate (alg), 1.5% carragenan (car), or 1.5% tetramethylorthosilan (TMOS) or LB agar. The TMOS mixture was prepared according to the protocol of Premkumar et al. (2002). This comprised mixing 4 ml of TMOS (Aldrich) with 2 ml aqua dest and 0.5 ml of 0.1 M HCl. The mixture was then sonicated for 10 min and left to age at 4°C for 1 day. Ten microliters of each mixture was filled into an Eppendorf tube and stored at 4°C.

Recovery of dried and encapsulated cells Cells prepared with all protocols were recovered immediately after preservation and after 2, 4, 6, 9, and 12 weeks storage at 4°C. One

Table 1 Composition of drying media in grams per liter

Variants	GR1	GR2	GR3	GR4	G1	G2	G3	GS15/GS15-NaCl[a]	GS20/GS20-NaCl	GT15/GT15-NaCl	GT20/GT20-NaCl	GT34/GT34-NaCl	PT34/PT34-NaCl
Gelatin	100	100	100	100	100	100	100	100	100	100	100	100	
PVP													15
Sodium ascorbate	10	10	25	10	10	25	25						
Sodium glutamate	50	50	50	50	50	50	75						
Meat extract	3												
Peptone	5												
Raffinose	50	50	50	100									
Sucrose								150	200				
Trehalose										150	200	340	340

Capital letters in the names indicate major components: *G* gelatin, *P* polyvinylpyrrolidone (PVP), *R* raffinose, *S* sucrose, *T* trehalose

[a] The label NaCl refers to bioreporters cultivated in the presence of 2% NaCl

milliliter of sterile tap water was added to a stored batch of cells, upon which the tubes were incubated for 30 min in a thermomixer at 37°C. Afterwards, cell suspensions were used to analyze inducibility with As (III) and viability.

Bioreporter assay for inducibility with As (III) The inducibility of bioreporters with As (III) was measured in 96-well plates. Hundred microliters of cell suspension obtained by re-hydration or resuspension of stored batches was mixed with 100 µl of a test solution containing 1:5 LB medium/water and 0.02% X-gal, without or with 1 µM (78 µg) As (III) per liter. The development of the absorption at 600 nm (A_{600}) was measured in a plate reader for 24 h at 30°C. Every 11 min, data were recorded and the plate was agitated in between. Controls without X-gal added were incubated simultaneously to subtract the culture turbidity from the total absorption in order to derive the absorption resulting from X-gal cleavage by the induced beta-galactosidase reporter protein in the assays. For comparison, the same procedure was performed every time with fresh cells from the stationary phase of an overnight culture. Bioreporter activities at 1 µM As (III) were characterized by the time until the color development started (referred to as the *response time* (RT)), the maximum corrected A_{600} achieved (the *response intensity* (RI)), and the ratio of the RI of induced cell suspensions to the RI of uninduced controls (the *induction coefficient* (IC)). In addition, the duration before the onset of growth (the *lag time* (LT)) was determined for every assay. LT and RT were determined from the intersection of two regression lines through absorption data before and after the observed onset of growth or color development, respectively.

Colony-forming units and determination of total cell counts For determination of colony-forming units (CFU), dilution series of the reactivated cell suspension were prepared and 25 µl of appropriate dilutions plated on LB agar plates containing 100 µg/l ampicillin. The plates were incubated overnight at 37°C before counting the number of colonies. Total bacterial cell numbers were measured with a Multisizer 3 Coulter Counter (Beckmann Coulter, Krefeld, Germany) using a 20-µm capillary.

Whole cell hybridization and total cell counts To estimate the activity state of the cells, quantitative whole cell hybridization with the ribosomal RNA-targeting, CY3-fluorescently labeled probe EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′; Amann et al. 1990) was performed. Cellular levels of ribosomes have been suggested as indicator for viability and potential general activity with single-cell resolution (Amann et al. 1995). Resuspended cell suspensions were fixed in 2% paraformaldehyde for 1 h and washed once with phosphate-buffered saline (PBS) (20 min centrifugation at 3,800×*g*) and twice with 50% ethanol in PBS. Cells were resuspended in 20 µl of 100% ethanol and stored at −20°C until further treatment. For the hybridization, the cell suspension was mixed with 60 µl 0.1% pyrophosphate (pH 8) and briefly vortexed, after which 20 µl of the mixture was spotted into a well of a slide that was coated with 0.1% gelatin. After drying to the air, 20 µl of hybridization buffer (900 mM NaCl, 20 mM Tris/HCl, 10% formamide, 0.01% sodium dodecyl sulfate) together with 5 ng/µl probe was added and the slides were incubated at 46°C for 2 h in humid chambers. The slides were then washed in preheated washing buffer (450 mM NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% sodium dodecyl sulfate) at 48°C for 20 min. Afterwards, the slides were rinsed with distilled water and stained for 3 min with 4′,6-diamidino-2-phenylindole (DAPI, 40 ng/well) for total cell counting. DAPI- and CY3-fluorescing cells were counted using a Zeiss epifluorescence microscope in 15 randomly chosen complete microscopic fields.

Results

Effect of preservation conditions on bioreporter response intensity, response time, and induction coefficient after storage To identify the preservation conditions leading to optimal maintenance of bioreporter activity, we used 1 μM As (III) to induce the beta-galactosidase of bioreporter cells that had been preserved in different ways and stored for different times. Fresh cells harvested in the stationary phase on each sampling day served as controls. Figure 1 shows the maximum response intensities of all variants, which were typically reached within 1 to 4 h after the first appearance of a measurable color formation. For vacuum-dried cells with gelatin as viscosity enhancer (variant abbreviations with a capital G), there is a general trend of declining response intensity with storage time (Fig. 1), sometimes after some increase within the first 2 weeks. Regarding the effects of different amendments (summarized in Table 1), there are some general trends visible. The addition of raffinose with or without meat extract and peptone (GR1–GR4) resulted in relatively fast decline of bioreporter activity, whereas cells dried without raffinose (G1–G3) and/or in the presence of trehalose or sucrose (abbreviations with GS or GT) maintained their initial response intensity for longer times. The combination of NaCl preconditioning and either trehalose or sucrose led to the best results of all cells dried in gelatin. Even better maintenance of a high response intensity was only obtained for PVP-embedded, vacuum-dried cells (PT34 and PT34-NaCl). It is interesting to note that fresh, stationary cells had lower response intensities than many of the dried and reactivated cells.

There was a general trend that storage of bioreporter cells encapsulated in either alginate, carragenan, LB agar, or TMOS resulted in the lowest response intensities, although with TMOS, relative good preservation of the response intensity up to 9 weeks was obtained.

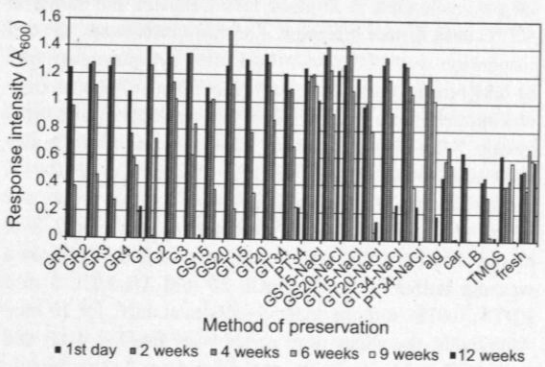

Fig. 1 Response intensities of differently preserved bioreporter bacteria with 1 μM As (III) after various durations of storage compared with responses of stationary phase cells freshly grown on the days of the measurements. Designations of treatments are given in Tables 1 and 2 and in the text

A second quality criterion for bioreporter preservation is the response time, the time needed for bioreporter suspensions to start producing visible color change upon contact with As (III). Selected response times obtained after prolonged storage are listed in Table 2. Most treatments resulted in an immediate increase of the response time (RT initial), which further increased during storage (RT final). Media containing high levels of raffinose (GR4) or sodium ascorbate and sodium glutamate (G3) initially preserved a relatively fast bioreporter response. After 12 weeks, however, the responses of GR4 and G3 were 10 and 13 times slower, respectively, than those of fresh cells. With some other preservation media, bioreporter responses were only detectable until 28 (GR1 and GR3) or 42 (GR2, G1, G2) days (Table 2). Storage of cells in organic (alginate, carragenan, LB agar) and inorganic (TMOS) gels did not result in faster response times than vacuum-drying.

The best maintenance of fast response during prolonged storage was obtained with bioreporter bacteria dried in a combination of trehalose and PVP (PT34, PT34-S). Although cells reactivated on day 1 responded four- to fivefold slower than fresh cells, RT remained stable thereafter for 84 days (data not shown). Preconditioning with sodium chloride did not further improve the response time after storage in this medium (Table 2).

A third quality criterion was the induction coefficient, i. e., the signal intensity with 1 μM As (III) relative to the background signal without arsenite. Fresh cells exhibited induction coefficients between 5 and 6 (Fig. 2). Induction coefficients of preserved cells measured after 6 weeks of storage ranged between 4 and 12 and typically declined thereafter along with the declining response intensity. Bacteria encapsulated in alginate (not shown) and TMOS preserved induction coefficients comparable to those of fresh cells only for the first 6 or 9 weeks, respectively, which then declined to very low values. Preconditioning of cells with sodium chloride generally resulted in higher induction coefficient, which, however, also decreased with time. Only cells preserved in trehalose and PVP (PT34) kept high induction coefficients throughout the entire storage period.

Integration of quality criteria for bioreporter preservation The combination of short response time, high response intensity, and high induction coefficient defines the quality of a bioreporter test. Figure 3 integrates these criteria for the eight best variants after 6 and 12 weeks storage and compares them with predefined limits for acceptable bioreporter response (i.e., quality goals). Regarding the response time, a stricter limit of 6 h (for water testing for consumption on the same day; left rectangle; Fig. 3) and a more relaxed delimiter

Table 2 Effect of storage on response times (RT), colony forming units (CFU), total cell counts, and lag times (LT) of bioreporter bacteria

Variant	Maximum storage [days][a]	RT [h] initial[b]	RT [h] final[a]	RT final/RT fresh cells	CFU initial	CFU final[a]	Total cell counts	LT [h] initial	LT final[a] [h]
Fresh cells	–	1.7	–	1	2×10^7	–	$4\times10^7\pm5\times10^6$	0.9	
GR1	28	5.3	15.5	9.1	8×10^5	3×10^4	$4\times10^7\pm4\times10^6$	2.9	15.0
GR2	42	6.7	18.7	11.0	2×10^5	5×10^2	$3\times10^7\pm8\times10^6$	2.3	21.5
GR3	28	7.3	18.1	10.6	7×10^5	6×10^2	$2\times10^7\pm2\times10^6$	2.1	20.6
GR4	84	7.0	17.7	10.4	3×10^5	2×10^3	$2\times10^7\pm4\times10^6$	2.2	15.9
G1	42	6.3	15.4	9.1	2×10^5	9×10^2	$3\times10^7\pm7\times10^6$	4.1	17.3
G2	42	6.0	14.9	8.8	2×10^5	1×10^3	$3\times10^7\pm2\times10^6$	4.1	16.0
G3	84	6.7	22.1	13.0	3×10^5	2×10^1	$3\times10^7\pm2\times10^6$	4.8	21.0
Alginate	84	3.0	24.3	14.3	2×10^6	2×10^2	$4\times10^7\pm9\times10^6$	1.3	19.7
Carrageenan[c]	14	2.4	4.1	2.4	6×10^6	8×10^5	$4\times10^7\pm2\times10^6$	1.0	2.4
LB agar	84	7.9	21.5	12.6	5×10^4	4×10^2	$1\times10^6\pm1\times10^5$	7.9	19.7
TMOS	84	1.9	23.7	13.9	1×10^7	4×10^1	$3\times10^7\pm5\times10^6$	0.8	14.6
GS15	42	6.5	20.3	11.9	1×10^5	2×10^1	$1\times10^7\pm7\times10^6$	4.2	23.2
GS20	42	5.2	19.5	11.5	5×10^4	2×10^2	$1\times10^7\pm6\times10^6$	3.7	19.9
GT15	42	4.2	17.1	10.1	6×10^4	8×10^1	$1\times10^7\pm7\times10^6$	2.9	15.6
GT20	63	4.4	21.1	12.4	1×10^5	1×10^2	$2\times10^7\pm6\times10^6$	2.6	20.4
GT34	84	4.4	18.9	11.1	8×10^4	5×10^2	$1\times10^7\pm5\times10^6$	2.5	16.8
PT34	84	7.1	7.3	4.3	5×10^5	8×10^3	$4\times10^7\pm5\times10^6$	6.4	7.0
GS15-NaCl	84	5.8	13.7	8.1	3×10^5	4×10^2	$2\times10^7\pm5\times10^6$	4.2	13.5
GS20-NaCl	84	4.9	11.1	6.5	5×10^5	4×10^2	$1\times10^7\pm5\times10^6$	3.1	10.1
GT15-NaCl	84	6.0	21.5	12.6	2×10^5	2×10^3	$1\times10^7\pm3\times10^6$	4.0	19.8
GT20-NaCl	84	4.3	19.8	11.6	4×10^5	7×10^3	$2\times10^7\pm5\times10^6$	2.5	14.0
GT34-NaCl	84	4.1	15.6	9.2	5×10^5	9×10^2	$1\times10^7\pm4\times10^6$	2.1	13.9
PT34-NaCl	84	7.3	10.4	6.1	7×10^5	2×10^4	$3\times10^7\pm5\times10^6$	6.0	8.9

Bioreporter bacteria were vacuum-dried with different drying media and in some cases preconditioned with sodium chloride (marked with *S*) or encapsulated in organic and inorganic gels. The suffix "initial" refers to the value immediately after beginning of treatment, "final" refers to the last day of the indicated maximum shelf life. Total cells counts were determined for each time point and are listed as mean with standard deviation over all time points

[a] Maximum storage refers to the longest storage time allowing measurable reporter response upon reconstitution. Values designated final refer to this day of measurement

[b] Immediately after drying or encapsulation

[c] Stored only for 2 weeks

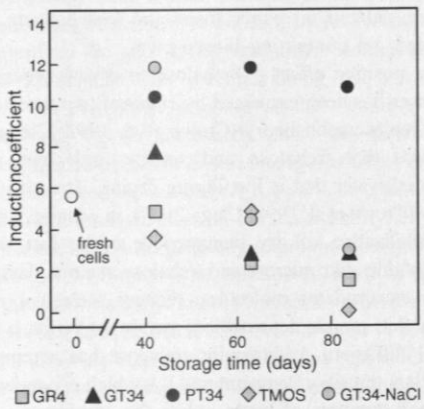

Fig. 2 Induction coefficients with 1 μM As (III) of differently preserved bioreporter bacteria after various durations of storage compared with responses of stationary phase cells freshly grown on the days of the measurements. Designations of treatments are given in Tables 1 and 2 and in the text

of 12 h (overnight testing; right rectangle; Fig. 3) were defined. Response intensities A_{600} above 0.4 (to assure visibility) and induction coefficients above 5 (to allow naked eye interpretation; pluses in symbols) were regarded as acceptable. Variants matching the wide or narrow windows were regarded as potentially suitable for field use. Only fresh cells, but none of the preserved cells matched the 6 h limit. Four variants, three of them using trehalose as an osmoprotectant, appeared suitable for overnight water testing. Of these, only variant PT34 fulfilled the predefined criteria even after 12 weeks of storage and showed good stability of the response characteristics between 6 and 12 weeks. It should be noted that PT34 was also the variant with the shortest response time. The other four variants and all variants not included in Fig. 3 missed the entire set of quality criteria.

Influence of storage on bioreporter viability To study the reasons for changing bioreporter response, bioreporter viability directly after preservation by vacuum drying or

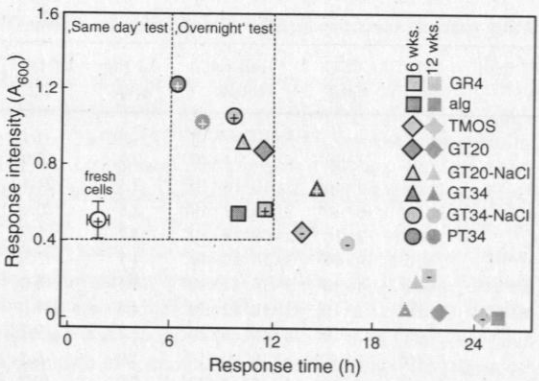

Fig. 3 Quality of selected bioreporter preservations expressed as a combination of response time, response intensity, and induction coefficient after storage for 6 or 12 weeks. *Plus and minus symbols* indicate induction coefficients above and below 5, respectively. *Dashed rectangles* delimit predefined response requirements (quality goals) with regard to visibility (A_{600} above 0.4) and quickness for water testing (response time below 6 or 12 h)

encapsulation and after prolonged storage was analyzed. Hereto, the numbers of colony-forming units (CFU) and total cells in resuspended cultures were determined. Numbers of CFU generally decreased from the initial value of 2×10^7 to 10^7–10^4 at day 1 and during prolonged storage further to 10^4–10^1 per batch of stored cells (Table 2). In contrast, the total number of cells in the batches as derived from Coulter counting typically remained around 10^7. In general, no systematic differences in CFU after storage were measured between vacuum-dried or gel-immobilized cells. An exception was the LB agar variant, which resulted in an order of magnitude lesser CFU. General activity of bioreporters after storage was analyzed by comparing the proportions of cells with high ribosome contents via fluorescence in-situ hybridization using a 16S ribosomal RNA (rRNA) targeting probe relative to total cell counts with DAPI. Interestingly, for preservations involving vacuum storage, the proportion of positively FISH-hybridizing cells remained at 75–85% of all DAPI-stained cells as compared to 80% for cells in a fresh culture. The relative amounts of hybridized gel-encapsulated cells remained stable (70–85%) except for a drop to 50% for cells in the hydro-gel alginate and LB agar after 9–12 weeks of storage (data not shown). Irrespective of the large decline in the ability of stored cells to form colonies, they remained stainable with DAPI and 70–85% of those by rRNA FISH.

As a further characteristic of cells after storage, we determined the lag time before onset of growth (Table 2). Lag times directly after immobilization in alginate, TMOS, and carrageenan were much alike those in fresh cells whereas directly after vacuum drying, lag times were prolonged up to sixfold. Upon prolonged storage, however, the lag times of vacuum-dried cells increased much less than those of the gel-stored cells, with, e.g., PT34 showing only a twofold further decrease in lag phase. Comparison of these viability and activity indicators with the bioreporter response revealed an inverse correlation between CFU counts and bioreporter response times ($y=2\times10^6e^{-0.41x}$, $R^2=0.71$) and a positive linear correlation between lag time before growth and bioreporter response time ($y=0.90x+3.01$, $R^2=0.73$).

Discussion

The aim of this study was to test different media and procedures for their effectiveness to preserve the activity of bioreporter bacteria during long-term storage at 4°C. This is crucial when the development of user-friendly, reliable bioreporter-based arsenite test is intended. Our quality criteria thus included the quickness (response time), robustness (response stability during storage), and ease of naked-eye interpretation (response intensity and induction coefficient). The guiding scenario was that the bioreporters would form the analytical component of As (III) paper test strips like those presented before (Stocker et al. 2003), which, however, did not account for any long-term stability. Although response intensities were quantified in this study, for field use by non-specialists, we had a naked-eye interpretation of qualitative results (on/off) in mind. The best robustness and bioreporter performance was achieved with cells that had been vacuum-dried in the presence of 34% trehalose and PVP as viscosity enhancer. From a practical perspective, it is advantageous that a variant involving vacuum drying gave the best results. It delivers a solid bioreporter matrix which is easier to be immobilized on paper strips and less delicate than a hydrated gel containing bioreporters.

The positive effect of trehalose in drying processes as seen here has been explained by either of two theories. The water replacement theory (Crowe et al. 1992; Clegg 2001) proposes that trehalose and similar molecules replace structural water that is lost during drying. The vitrification theory (Crowe et al. 1998; Clegg 2001), in contrast, attributes the stabilization of dry biomaterials to the fact that dry disaccharides like sucrose and trehalose attain a glassy state, which encapsulates molecules, reduces molecular motion, and inhibits protein denaturation, membrane fusion, and free-radical diffusion. Additionally, trehalose has a remarkably high glass transition temperature (T_g), which is not depressed by small amounts of water unlike for other sugars. Thus, trehalose has exceptional properties for non-ideal storage conditions characterized by high temperature and humidity.

It is obvious from the results that for our purpose, PVP is better suited as a drying matrix than gelatin. Although we

cannot explain this difference mechanistically, earlier studies point at the general importance of the increase of the glass transition temperature of the drying protecting mixture for the stabilization of biological membranes (Bronshtein 2003). We were surprised by the strongly favorable effect of PVP, which we had included in only two preservation variants. Fortunately, those were the ones (high trehalose concentration, with and without salt preconditioning) that gave also the best results with gelatin-embedded cells, so that the PT34 may well be a near-optimal combination within the set of ingredient used in this study. It appears nevertheless worthwhile to further optimize vacuum-drying with PVP in combination with other concentrations of trehalose, a larger range of other ingredients, and modified drying protocols. The latter may have an influence as survival rates were found to strongly depend on remaining water contents (Bullifent et al. 2000; de Castro et al. 2000). Experiments with luminescent arsenic bioreporters (Stocker et al. 2003) in our group showed an increase of activity of these bacterial sensors for about 50% when the drying time was extended by a factor of three (Hellmich R., unpublished data). The strong improvement with PVP as compared to gelatin suggests testing a broader range of synthetic polymers in the future. Hydroxyethyl starch, polyethylene glycol, and polyacrylamide or polysaccharides such as Ficoll and Dextran, and proteins would be good candidates for further testing (Crowe et al. 1997). Optimization of initial cell numbers may also be worthwhile, as cell density effects on cell preservation have been reported (Gu et al. 2001). Cell numbers were not varied in the present study. Preconditioning of the cells with NaCl improved the viability measured as CFU and reduced the lag times of all variants. Improved survival up to 62% after 6 weeks of storage following preconditioning with sodium chloride is comparable with reported data (Manzanera et al. 2002). NaCl conditioning also improved the activity of bioreporter bacteria dried in gelatin. In contrast, salt preconditioning did not further improve the bioreporter response of the "winning" PT34 variant. Since we do not exactly know the physiological changes induced by salt in *E. coli* bioreporters, we prefer not to speculate about the reasons for the variable effect of salt.

Long-term maintenance of the cultivability of bioreporter bacteria was also generally improved with increasing concentrations of sucrose or trehalose. Trehalose at 34% gave the best viability after 12 weeks. Whereas others reported sucrose to be better than trehalose (Gu et al. 2001), our data allow no clear ranking. Reduced cultivability was neither reflected by total cell counts nor by numbers of cells containing ribosomes to an extent needed for positive whole-cell fluorescent hybridization. These two latter properties were little affected by storage and, consequently, of much lower value for the prediction of bioreporter performance than cultivability, which strongly correlated with the bioreporter response time. It is quite obvious that most individual *E. coli* bioreporter bacteria entered a viable but non-cultivable (VNC) state during the drying process, a phenomenon observed for starving enteric bacteria (Xu et al. 1982). Plate counts may thus have underestimated numbers of viable cells, as VNC cells cannot be effectively detected by classical plate count methods (Braux et al. 1997).

It can be concluded that for preservation of bioreporter inducibility the cultivability of the cells is a better marker than their number of ribosomes or intactness of the cell as, e.g., characterized by rRNA FISH.

A striking result remains unexplained at present, i.e., the observation that maximum absorbance values achieved with most of the stored cells were higher than those of fresh cells. It indicates that reactivation from a more inactive state (vacuum-dried or embedded in gels) can result in higher bioreporter activity than reactivation of a fresh, although stationary, culture. Here, it should be noted that exponentially growing cells are much more rapidly induced than stationary phase and therefore, the cells in preserved mixtures probably began to grow before becoming induced. This was a compromise that had to be made because of the poor preservation of exponentially growing cells. Future studies should be focused on further improving reactivation time, perhaps by inducing preservation pathways in exponentially growing cells without compromising inducibility of the *arsR* promoter. Until then, we have identified a procedure for bioreporter preservation which matches realistic requirements of qualitative As (III) testing in drinking water.

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Chapter 4

Visualisation of gradients in arsenic concentrations around individual roots of *Zea mays* L. using agar-immobilized bioreporter bacteria

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Abstract

The classical concept of arsenic transfer into plants through arsenate uptake via phosphate transporters, reduction to arsenite, complexation and compartmentation within vacuoles is challenged by recent identification of bidirectional transporters for arsenite and their potential role in plant As status regulation. Soil-based studies with chemical analysis of soil solution require root mat formation amplifying root effects on their surroundings and additionally denying investigations along individual roots differing in age and function. We tried to overcome these shortcomings by using bioreporter bacteria to visualise the spatial distribution of inorganic arsenic along roots and to characterize inorganic arsenic gradients in the rhizosphere concurrent with root age and branching. Therefore we developed an agar-based carrier element ensuring intimate contact between bioreporters and root-soil system and enabling fast and easy reporter output analysis.

We show that inorganic arsenic distribution is related to root development with the highest bioreporter signal induction around lateral roots, which are known to show the highest expression of transporters responsible for bidirectional arsenite flux. Since there is so far no evidence for an arsenate efflux mechanism this is a strong indicator that we observed rather arsenite than arsenate efflux. No signal was detected along the distal region of young adventitious roots, i.e. the region of extension growth and root hair formation. The novel bioreporter assay may thus complement conventional measurements by providing information on the spatial distribution of inorganic arsenic on mm to cm-scale.

key words: arsenic, bioreporter bacteria, rhizobox, rhizosphere, *Zea mays*

Introduction

Arsenic is a worldwide occurring contaminant causing serious health and environmental problems. Geogenic arsenic (As) is naturally released from weathering rocks (Smedley and Kinniburgh 2002). In addition, human activities like mining and ore processing (Morin and Calas 2006) or the application of As-based pesticides and fertilizers (Welch et al. 2000) have resulted in extensive pollution of groundwater and soils throughout the world (Nordstrom 2002). There is increasing concern that arsenic is transferred into the food chain via the irrigation of food crops. The mobility and thus the availability of As to plants depend on several factors, including the redox dynamics, which affect As speciation, soil mineralogy, i.e. sorption capacity, or presence of competing anions, in particular phosphate and pH (Livesey and Huang 1981; Roy et al. 1986; Goldberg and Glaubig 1988).

Arsenate (As^{V}) is the predominant As form in soils under oxic conditions, whereas arsenite (As^{III}) occurs predominantly in reduced environments (Masscheleyn et al. 1991; Smith et al. 1998). As^{V} is taken up by plants through high affinity P transporters in root membranes. Any adaptation strategy plants have evolved to increase P uptake, like release of organic acid anions and protons, or a shift in number and ratio of high and low affinity P transporters can alter As^{V} mobility and uptake in the rhizosphere (Geelhoed et al. 1998; Grafe et al. 2001; Liu et al. 2001). Inside the plant As^{V} is immediately reduced to As^{III} which can be complexed by phytochelatines and stored in the vacuoles as detoxification strategy (Zhao et al. 2009).

If As^{III} is present in soil solution uptake likely occurs via aquaporines which were previously described as transporters for silicon and are localised in roots in the distal plasma membrane domain of endodermis and exodermis (Bienert et al. 2008; Zhao et al. 2009). It has been shown that these transporters are bidirectional (Bienert et al. 2008). This is in line with the recent observation of Xu et al. (2007), who reported massive As^{III} efflux from tomato roots grown in hydroponic systems. Within only one day nearly all As^{V} applied initially was converted to As^{III}. Xu et al. proved that this change in speciation was due to As^{V} reduction by

the plant and subsequent As^{III} release by the roots and not a result of soil microbial activity or changes in redox potential.

The As speciation in the rhizosphere solution of soil-plant systems is largely unknown. Using a compartment system, in which the bulk soil is separated from the root compartment by a nylon mesh, Vetterlein et al. (2007) found for a quartz substrate supplemented with As^{V}, that As^{III} accumulated in the rhizosphere solution of *Zea mays* L.. However, As^{III} concentrations remained small compared to those of As^{V} and could only be detected close to the root mat. The apparent difference between the soil-based experiment and the hydroponic experiments of Xu et al. (2007) is probably related to the fact that in hydroponic experiments no gradients are built up around roots whereas the slow effective diffusivity in soil retains As near the root. This retention may also have a feed-back on diffusion driven efflux processes. Compartment system experiments are soil based and enable the investigation of gradients between bulk soil and a root mat, which represents the root surface, by measuring the As concentration in the soil solution, however, they are limited in resolution (Fitz et al. 2003; Bravin et al. 2008). Alternative approaches to characterize As gradients around single roots in soil-based systems require high spatial resolution combined with sufficient sensitivity for the prevailing concentrations.

In the recent years, new tools for environmental monitoring of chemicals have been developed on the basis of so-called bioreporter organisms. Bioreporters are often microbial strains which are genetically modified to produce easily detectable output in response to the presence of bioavailable fractions of specific analytes. Several reviews have been dedicated to this rapidly developing field, exposing details of genetic constructions, type of reporters or mechanisms of analyte detection (Daunert et al. 2000; Leveau and Lindow 2002; Belkin 2003; van der Meer et al. 2004; Harms et al. 2006). The detection system of bioreporter microorganisms is in most cases composed of a simple genetic circuitry, comprising a regulatory system (i.e. regulatory gene plus its cognate promoter) and one or more reporter

genes. Commonly used reporter proteins are the chromogenic β-galactosidase (*lacZ*) or β-glucuronidase (*gusA*), luminescent luciferases (*luc* or *luxAB*) or autofluorescent proteins (GFP and variants) (Ivask et al. 2002; Paitan et al. 2004; Werlen et al. 2004; DeAngelis et al. 2005). Bioreporters have been used for fifteen years as an excellent tool to provide *„in-situ"* information on rhizosphere conditions at very high spatial resolution (Cardon and Gage 2006). Application ranged from biosensors reporting root exudation in relation to shoot nitrate concentration (Darwent et al. 2003), via the detection of available nitrate in the rhizosphere (DeAngelis et al. 2005) to the visualization of communication via N-acylhomoserine by plant root-colonizing rhizobacteria (Gantner et al. 2006). For the detection of inorganic arsenic (As^{III}, As^{V}), a variety of bioreporter bacteria have been developed (Ramanathan et al. 1997; Stocker et al. 2003; Wells et al. 2005; Wackwitz et al. 2008) but not yet used to detect arsenic anions in the rhizosphere. The green fluorescent protein (gfp) tagged arsenic bioreporter strain *Escherichia coli* DH5α 1598 developed by Stocker et al. (2003) is a promising candidate for the visualisation of arsenic anions. Although the regulator *arsR* used for construction of bioreporter bacteria recognizes only arsenite, most arsenic bioreporter strains respond also to arsenate after reduction to arsenite in the cell by the arsenate reductase (*arsC*) as part of the whole resistance mechanism. Thus, the applied bioreporter bacteria cannot be used to distinguish between As^{III} and As^{V}. Up to now most arsenic bioreporter strains have been optimized to ensure fast and reliable monitoring under standardized laboratory conditions (Trang et al. 2005). Given the fact that *E. coli* is not adapted to propagation/survival in soil or the rhizosphere, it has to be administered in a way that ensures signal production during an *„in-situ"* deployment to such complex matrices.

It was thus the objective of this work to extend the application of bioreporter bacteria originally designed for use in liquid assays to a heterogenous environment, i.e. the rhizosphere. Although the fluorescent reporter protein would principally enable *„in-situ"* detection on a µm to mm scale by epifluorescence microscopy, we concentrated on

characterizing undisturbed gradients of inorganic arsenic on a mm to cm-scale in the rhizosphere concurrent with root age and branching. Therefore we developed an agar-based carrier element, in analogy to a classical approach for detection of rhizosphere pH by Römheld et al. (1984), which ensured intimate contact between the bioreporter and the root-soil system while enabling fast and easy analysis of reporter outputs. We hypothesized that (i) As^{V} and As^{III} distribution in the rhizosphere is related to root development and that (ii) steep gradients in inorganic arsenic concentrations around individual roots exist as a result of As efflux. To this end, we performed experiments with *Z. mays* plants grown in As^{V} contaminated soil in a rhizobox system that also allowed for split root experiments.

Materials and Methods

Cell pre-culturing

Concentrations of arsenic anions in the rhizosphere were measured with cell suspensions of the green fluorescent protein (gfp) tagged bioreporter strain *E. coli* DH5α 1598 (pPR-*arsR*-ABS) (Stocker et al. 2003). This strain responds to arsenite as well as to arsenate after it has been reduced to arsenite in the cell by the arsenate reductase (*arsC*). The bioreporter strain was plated from -80°C stock culture on fresh Luria-Broth (LB) plates with appropriate antibiotic selection (kanamycin 50 μg mL^{-1}) and grown overnight at 37°C. Liquid LB medium (500 mL) plus antibiotic was inoculated with a single colony and incubated for 16 h at 37°C.

Pre-culturing of *Z. mays* plants

Z. mays seeds were germinated on filter papers soaked with 2% $CaSO_4$ and incubated for 2 days in the dark at 30°C. Seedlings were transferred either to soil compartment systems (see below) or to filter paper soaked with 2% $CaSO_4$ and 50 μM arsenate.

Experimental design

Z. mays (3 plants per compartment system) was grown under controlled conditions (23°C, 75% rel. humidity day and night; 12 h photoperiod with 480 μmol m^2 s^{-1} photosynthetically active radiation) in rhizoboxes (200 × 350 × 20 mm) in which a 20 mm thick layer of soil was covered by a hydrophilic nylon mesh (30 μm) (Fig. 1). The transparent front cover consisted of a metal grid (grid size 6 × 8 mm) overlaid with foil to enable observation of root growth. *Z. mays* seedlings were placed in the soil with their seminal root passing through a cut in the nylon mesh between the nylon mesh and the foil. Thus, roots supplied by the soil with the required nutrients and water but without soil particles adhering to them were obtained, which allowed an undisturbed exposure to the bioreporter-agar-system (see below). The soil used in the rhizoboxes was a mixture of 85% quartz sand (WF 33; Quarzwerke Weferlingen), 10%

quartz silt (Mikrosil SP12; Euroquarz), 5% quartz clay (VP960–943; Quarzwerke Frechen) and different amounts of goethite (G-0 0, G-1 0.1%, and G-4 0.4%) sieved to 1 mm. The substrate was initially supplied with 100 mg N kg^{-1} (NH_4NO_3), 80 mg P kg^{-1} ($CaHPO_4$), 100 mg K kg^{-1} (K_2SO_4), 100 mg Ca kg^{-1} ($CaSO_4 \times 2H_2O$), 50 mg Mg kg^{-1} ($MgCl_2$), a micronutrient solution (3.25 mg Mn kg^{-1} [$MnSO_4 \times 2H_2O$]), 0.79 mg Zn kg^{-1} ($Zn[NO_3]_2 \times 4H_2O$), 0.5 mg Cu kg^{-1} ($CuSO_4 \times 5\ H_2O$ and 0.17 mg B kg^{-1} [H_3BO_3]), and 3.25 mg Fe kg^{-1} (Fe-EDTA). The soil was spiked with different amounts of As^V ($Na_2HAsO_4 \times 7H_2O$); details are given below. The chemical composition of the substrate and establishment of the three treatments was identical to the compartment system experiment described in Vetterlein et al. (2007). It was shown by As species analysis that at the start of the experiment, before plants started to grow, only As^V is present. Neither methylated arsenic species (monomethylarsinate, dimethylarsinate) nor unknown As species as described for the same quartz substrate (Ackermann et al. 2008) and for a floodplain soil (Ackermann et al. EJSS-315-08 under revision) after passing through a redox cycle were initially present.

Rhizobox experiments

Two experiments were performed in the rhizoboxes to detect arsenic anions along roots depending on root age and to show possible release of arsenic anions. For experiment I the whole substrate of one rhizobox was spiked with 2.5 mg As^V per kg ($Na_2HAsO_4 \times 7H_2O$), for experiment II only one half of the rhizobox was spiked with 5 mg As^V per kg, a level sufficient to induce growth depression in *Z. mays* without occurrence of visual symptoms and severe stress. After adding N, K, Mg, and micronutrient solutions, the substrate was allowed to dry before it was thoroughly mixed and sieved. Thereafter, P and Ca were mixed in as powder, and As^V was mixed in as solution. The substrate was again air dried. For details about substrate composition and selection of goethite as an additive see Vetterlein et al. (2007).

In experiment I rhizoboxes were packed homogeneously with the spiked substrate to a bulk density of 1.6 g per cm^3. In experiment II one half of the rhizobox was filled with As^V spiked substrate, the other one with the same substrate without As^V. The two halves were separated by a vertical 2 mm thick bar (Fig. 1). Roots developing between nylon mesh and foil could grow unrestricted only above both halves (with and without arsenate). For the detection of released arsenic anions we focused on roots originating from the As^V-spiked compartment which then crossed the border and grew into the As^V-free compartment. Roots of plants growing only in the As^V-free compartment were used as a negative control. The packed rhizoboxes were inclined at an angle of 50° for root growth along the nylon mesh and watered with deionised water by capillary rise. A water table of 1 cm was maintained at the bottom of the rhizoboxes throughout the duration of the experiment. In both experiments three *Z. mays* seedlings were planted in each rhizobox and plants were grown for 3 weeks. For visualisation of inorganic arsenic in the root zone, roots from both experiments were covered with a thin layer of agar-embedded bioreporter bacteria as described below.

Experiments with seedlings

To verify that the detectable arsenic anions were released by intact roots, an experiment was performed with *Z. mays* seedlings grown on filter paper. The *Z. mays* seedlings were transferred to filter paper soaked with 2% $CaSO_4$ and 50 µM arsenate and grown for 2 days at room temperature. Subsequently the roots of the seedlings were rinsed with a desorption solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$ and 5 mM MES to remove adsorbed As^V and, for analysis of the release of arsenic anions, covered with a thin layer of liquid bioreporter-containing agar as described below.

Preparation of agar-embedded bioreporters

For preparation of agar-embedded bioreporters the overnight culture was adjusted by centrifugation to 4×10^{11}cells mL^{-1}. Preheated LB agar (37°C) was mixed 1:1 with the cell suspension. Subsequently, 1.5 mL of the mixture was poured over provided LB agar plates with 5 cm diameter resulting in layer of about 1 mm depth in the altogether 3 mm thick discs, which could be used for exposure to plant roots. The influence of the model-soil-solution containing a mixture of the below listed ions on the immobilized bioreporter bacteria was investigated in a different set up. To minimize the spreading of the solution, small wells (0.6 cm in diameter) were generated by pressing a form into the bioreporter-agar-system. Into each well 7.5 µL of different arsenite and arsenate concentrations (0.5, 1, 2.5, 5, 10, 25, and 50 µM As,) either in Aqua bidest. or in model-soil-solutions of pH 7 or 3 were applied. The concentrations of the ions in the model soil-solution were established according to previous experiments (Vetterlein et al. 2007; Szegedi et al. 2008) and were as follows: 50 mg L^{-1} K^{+}, 300 mg L^{-1} Cl^{-}, 150 mg L^{-1} SO_4^{2-}, 15 mg L^{-1} PO_4^{3-}, 230 mg L^{-1} Ca^{2+}, 40 mg L^{-1} NH_4^{+}, 40 mg L^{-1} NO_3^{-}, and 7 mg L^{-1} Fe^{2+}. The agar plates were subsequently incubated at 37°C for 2 hours and afterwards scanned to measure the fluorescence at 473 nm using the FLA-3000 (Fuji Film Co.) with filter Y520. For a quantitative digital image analysis Image J was used (Abramoff et al. 2004). Signal intensities (i.e. the grey intensities of the dots) were expressed as average grey values.

Bioreporter-agar and application on root surfaces

For application on the root surface, agar discs with the immobilized bioreporter bacteria were removed from the petri-dish and laid upside down on the roots. Roots were completely covered from the upper end to the root tip to follow differences in inorganic arsenic mobilization or release along the roots. After 2 hours of incubation the agar discs were retrieved and further incubated at 37°C for additional 16 h. Fluorescence measurement was

performed by scanning as described above. For analysis of inorganic arsenic release of seedling roots, the seedlings were placed in petri-dishs and the agar-bacteria-suspension was poured directly onto the roots. The whole system was subsequently incubated for 2 hours at 37°C. Agar discs retrieved after root exposure were analysed for fluorescence on a transilluminator (BST-30G-8R, biostep) and photographed.

Results

Influence of anions and pH on the activity of agar-embedded bioreporters

Agar-embedded bioreporters responded to arsenate and arsenite in a dose-dependent manner (Fig. 2A). A tendency towards signal saturation was observed between 10 and 50 µM As^{V} and As^{III}. Arsenite and arsenate gave similar signal intensities. Similar signal intensities were also obtained with agar-embedded bioreporters exposed to arsenate and arsenite in Aqua bidest. or in model soil solutions of pH 3 or 7 (Fig. 2B). Agar-embedded bioreporter bacteria were thus shown to function without any loss of activity when exposed to typical soil anions and low pH and could be applied for *„in-situ"* experiments in rhizoboxes.

Bioreporter detection of released As anions along *Z. mays* roots

In order to localize zones along the roots with increased arsenate and arsenite concentration as a result of either mobilization or release of inorganic As a complete *Z. mays* root was covered and incubated with agar-embedded bioreporter bacteria (rhizobox experiment I). We could distinguish zones with different fluorescence intensities as a result of bioreporter induction indicating differences in concentrations of bioavailable inorganic arsenic (Fig. 3). In particular in the distal root regions and along laterals strong bioreporter signals were observed (Fig. 3C and 3D, marked with arrow 1). We obtained signals also around dead roots (Fig. 3A and B, brownish discolouration, marked with arrow 2) but not next to older main roots (Fig. 3A and B, marked with arrow 3). No signal was obtained from the main axis of young adventitious roots, corresponding to the extension zone and the zone of root hair formation (Fig. 3C, marked with arrow 4). For the three different goethite treatments the signal intensity was compared based on average grey value analysis. The results show that for increasing goethite amounts the average grey value decreased (Table 1), which is in line with measured soil solution concentrations for these treatments (Vetterlein et al. 2007).

To distinguish between inorganic arsenic released from the roots or mobilized and transported to the root surface, rhizobox experiment II was carried out, in which a compartment with arsenate was separated from a compartment without arsenate. The roots of individual plants were allowed to spread in both compartments. It was clearly visible that bioreporter bacteria in the arsenate-free compartment gave strong signals, when they were exposed to roots, which grew from the arsenate-amended compartment to the arsenate-free one. This indicated that partly arsenate-exposed roots translocated and released inorganic arsenic (Fig. 4).

Induction of bioreporter activity by intact and dead roots

A seedling experiment was performed to demonstrate that the inorganic arsenic detected by bioreporters was released by intact roots. We used *Z. mays* seedlings grown on filter paper to ensure the integrity of the roots and allow easier handling. The roots of the seedlings were either left intact before incubation with the bioreporter bacteria or cut. Roots of seedlings that were grown with arsenate induced bioreporter signals (Fig. 5A and 5B) with no visible difference between intact (Fig. 5A) and cut roots (Fig. 5B). In control experiments with seedlings grown without arsenate no induction of bioreporter activity was detected (Fig. 5C).

Discussion

When exposed to increasing concentrations of either arsenite or arsenate, LB-agar-embedded bioreporter bacteria were capable of sensing, visualizing and spatially resolving the distribution of this common soil contaminant. This kind of bioreporting was apparently quite robust as there was no obvious influence of typical soil ions or pH between 3 and 7. This was in seeming contrast with the findings of Harms et al. (2005), who reported that iron interfered with arsenic bioreporter bacteria. As preliminary liquid culture assays (see online resource) also showed some interference of ions other than arsenic anions and adverse effects of low pH, it appears that the LB-agar buffered the pH and kept interfering ions away from the bioreporter bacteria. However, arsenic anion-dependent signals were obtained and could be calibrated against concentrations of arsenic anions. Whereas in a liquid culture based assay of Stocker et al. (2003), a twofold lower response to As^{V} in comparison to As^{III} was reported for *E. coli* (pJAMA-arsR), we found the same sensitivity for As^{V} and As^{III}. The reason for this discrepancy is unclear; however, equal signals from both species potentially allow for an exact quantification of inorganic arsenic without knowledge of the actual speciation.

Bioreporters embedded in agar versus exposure as suspensions

Earlier investigations of rhizosphere processes using bioreporters typically relied on bacterial species indigenous to soil, which upon genetic engineering and introduction into soil systems were observed for longer time periods (for a review see Cardon & Gage (2006)). In such applications it is crucial that the bioreporter bacteria can cope with soil conditions, which may require competing with other soil bacteria, to grow in the rhizosphere, and to avoid being grazed by predators. Moreover, the prevailing, and possibly fluctuating, conditions should have no negative impact on bioreporter fitness as this would affect the spatial response pattern.

In contrast to these investigations, the guiding idea of the present study was to utilize the bioreporter in analogy to chemical indicators such as agar layers containing bromocresol purple that had been placed on roots for pH measurement in the rhizosphere (Römheld et al. 1984). As the agar-embedded indicator elements, i.e. molecules or bioreporter bacteria, respectively, are fixed in space, such systems are capable of freezing the visual effects of an analyte's two-dimensional distribution. Our bioreporters are applied for a short time (2 hours) in a predefined, homogeneous arrangement (1.5×10^{10} cells per cm^2 agar). They do thus neither need to grow in the system, i.e. the signal output does not depend on parameters like energy or nutrient supply from soil, nor do they seem to be affected by soil chemical conditions. Finally, immobilisation allows easy and non-destructive bioreporter retrieval from the system for signal interpretation. These features taken together result in a spatial signal resolution on a mm to cm scale across entire root systems. The only comparable approach to the one chosen here was described by Darwent et al. (2003) and Paterson et al. (2006) for the detection of exudation intensity in relation to nitrate nutrition. In their study, lux-marked bioreporters were applied as a solution to selected zones of the root and CCD imaging applied to quantify bioluminescence directly on the root system.

Gradients in concentrations of arsenic anions with increasing distance from the root surface

The formation of gradients near the root surface is a classical feature of the rhizosphere (Hinsinger et al. 2009). Element concentrations at the root surface can be increased as a result of nutrient transport to the root surface (e.g. assisted by exudate-mediated mobilization) exceeding the uptake capacity of the plant. Conversely, uptake exceeding transport to the root surface or precipitation of minerals leads to reduced concentrations. Compartment system experiments with root mats indicated neither depletion nor accumulation of As^V, whereas the competing anion phosphate was mobilized (Vetterlein et al. 2007). The simulation of these

experiments by the model RhizoMath, which couples chemical speciation, transport and root activity, predicted As^V accumulation in the rhizosphere only if competition of As^V and P^V for membrane transporters was included (Szegedi et al. unpublished data). The imaging technique in the present experiments clearly showed for some roots an accumulation of inorganic arsenic in the rhizosphere. Whether these increased concentrations reflect As^V or As^{III} is discussed below in more detail.

As^V and As^{III} distribution along different parts of the root system

Increased concentrations of arsenic anions close to root surfaces were predominantly observed along laterals. Fifteen to 21 days old roots and the distal region of young adventitious roots gave no signals. It is well known that element uptake by roots does not occur uniformly along the whole root axis (Hinsinger et al. 2009). Uptake of Mg and Ca is particularly pronounced in the distal (younger) regions, since the suberised endodermis of older regions restricts the radial flow of these nutrients (Clarkson 1991). Phosphate uptake primarily occurs in the zone with active root hairs, and declines in the more proximal (older) root regions (Ernst et al. 1989; Shin et al. 2004). For Si transporters, which constitute the pathway for As^{III} transport (Bienert et al. 2008; Ma et al. 2008), it is known that they are mainly expressed in laterals but not in root hairs (Ma et al. 2001). The fact that we observed the strongest gradients in signal induction around laterals can be taken as a hint that we visualized the result of As^{III} uptake and release. We never observed such strong gradients around young adventitious roots where root hairs were visible with the naked eye. However, this point needs further investigation, either by comparing wild type roots with hairless mutants as they are available for rice (Ma et al. 2001) or by adapting our agar system to use in conjunction with confocal fluorescence microscopy to obtain a higher spatial resolution.

As^{V} accumulation versus As^{III} release

An alternative and promising approach to a distinction between As^{V} accumulation around roots and As^{III} release by roots is the split root experiment (experiment II). The detectable inorganic As along the roots in the compartment not containing any As^{V} has to be a result of translocation within the root followed by its release. This is in agreement with the As^{III} root efflux observed by Xu et al. (2007) in hydroponic experiments and the accumulation of As^{III} close to the root mat in compartment system experiments (Vetterlein et al. 2007). While there is mechanistic evidence for bi-directional diffusion-driven transport of As^{III} via aquaporins (Bienert et al. 2008; Ma et al. 2008; Mitani et al. 2009) similar evidence is missing for an active As^{V} efflux. An efflux of As^{V} similar to the one described for P^{V} (Mimura 1999) is unlikely as As^{V} is reduced to As^{III} in plants immediately after being taken up (Delnomdedieu et al. 1994; Bleeker et al. 2006).

Translocation of As in *Z. mays* plants from the roots to the shoots and leaves has been shown (Gulz et al. 2005; Raab et al. 2007) and a compilation of As-speciation data from the xylem reveals that As^{III} is by far the dominant species in the xylem (Zhao et al. 2009). No data for translocation within the root system itself are available, but it is known that xylem flow within the roots can be reversed in relation to changes in water potential gradients (Burgess and Bleby 2006). As^{III} could thus have been translocated with a water stream to where it was detected by the bioreporters.

In hydroponic as well as in soil-based systems, As^{III} accumulation was related to As concentrations in root tissue (Vetterlein et al. 2007; Xu et al. 2007). While complete transformation of As^{V} to As^{III} has been observed in hydroponic systems within one to three days as a result of As^{III} root efflux, in soil-based systems As^{III} is confined to the immediate vicinity of the roots and is restricted to a minor fraction of the initial As^{V}. If we assume a solely diffusion-driven efflux, differences between hydroponic and soil-based systems can be

explained by retarded As^{III} diffusion in soil. The steep gradients visualized with our bioreporter method around individual roots are in line with this assumption.

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Table 1: Average grey value of reporter-agar-systems after incubation on *Z. mays* roots in quartz sand with three different amounts of goethite.

Treatment	Number of reporter slices	Mean ± standard deviation
G-0	9	175.4 ± 25.1
G-1	7	146.3 ± 27.2
G-4	9	121.8 ± 35.5

Figure captions

Figure 1: Experimental design. A) Rhizobox, B) schematic in side view showing the nylon mesh (dashed line) separating some of the roots from the soil (left side) and in front view showing the bar separating the rhizobox into an arsenic-amended (As^V+) and an arsenic free (As^V-) compartment. Note that some roots cross the bar. C) Close up to show soil particle free roots on the right side of the nylon mesh.

Figure 2: Signal production by bioreporter bacteria immobilized in LB agar upon exposure to different concentrations of arsenite and arsenate. As^V and As^{III} was applied either in Aqua bidest. or in model-soil-solutions with pH 3 or 7. A) Image of the agar plate system signal intensities after exposure to As^V and As^{III} supplied in the model soil solution of pH 3. A similar image was obtained with pH 7 (not shown). B) Signal intensities displayed as average grey values for As^{III} (filled symbols) and As^V (empty symbols) supplied either in Aqua bidest. at pH 7 (diamonds), or in the model soil solution at pH 7 (triangles) and pH 3 (squares).

Figure 3: Root covered with agar-embedded bioreporters (A) and corresponding fluorescent bioreporter response (lighter) reflecting arsenic-mediated induction (B). Blow-up of details to illustrate signals adjacent to side roots (C) and the root tip region (D). Arrows indicate certain root zones: 1) distal root and laterals, 2) dead roots, 3) older main roots, 4) main axis of young adventitious roots.

Figure 4: Root system after 3 weeks growth in a system composed of an arsenate-amended (A, right) and an arsenate-free compartment (A, left). Blow-up of a section of a root (arrow) that grew from the compartment with arsenate across the border to the compartment without arsenate (B, left), corresponding signals of agar-embedded bioreporters that had been exposed to the same area (C).

Figure 5: Bioreporter signals induced by roots of seedlings grown on filter paper with arsenate (50 μM) (A: intact root, B: cut root) and without arsenate (C). The white arrow indicates the cut in the root.

Figure 1

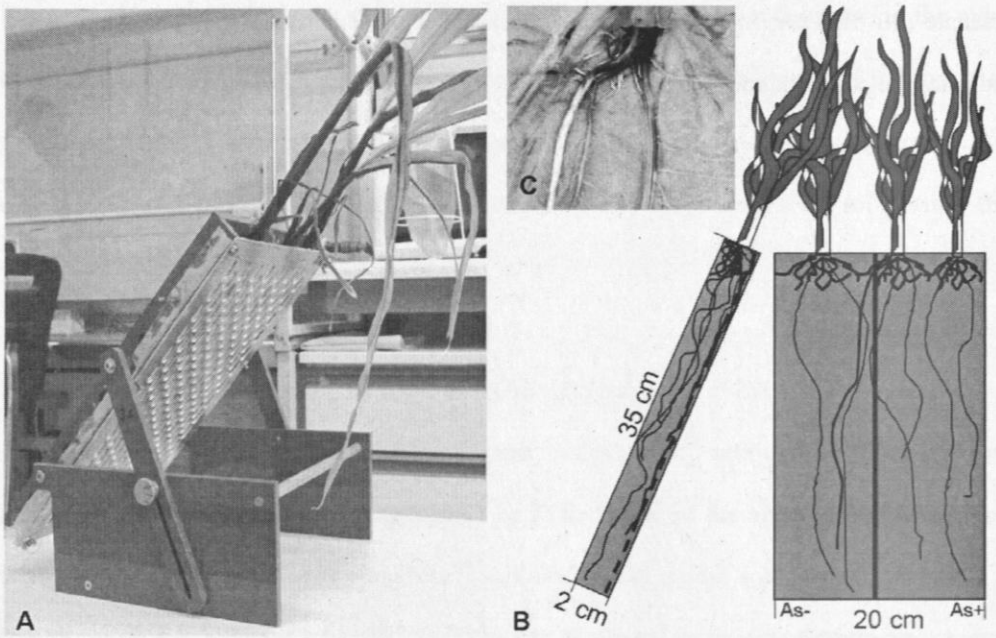


Figure 2

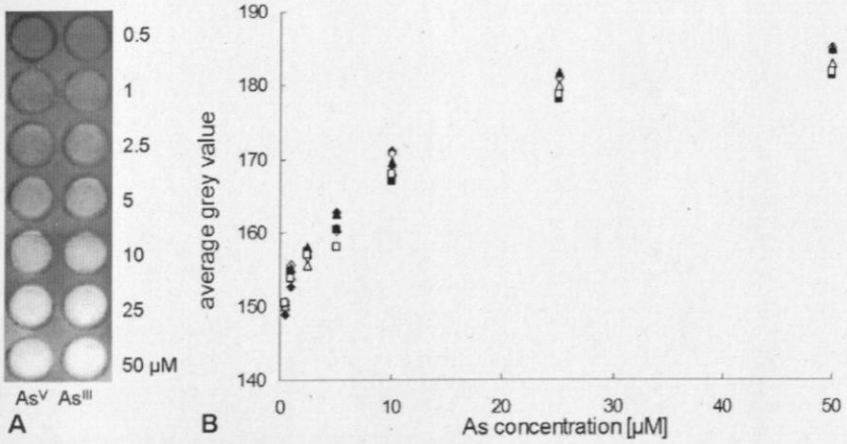

0.5
1
2.5
5
10
25
50 µM
As^V As^III
A
B
average grey value
190
180
170
160
150
140
0
10
20
30
40
50
As concentration [µM]

Figure 3

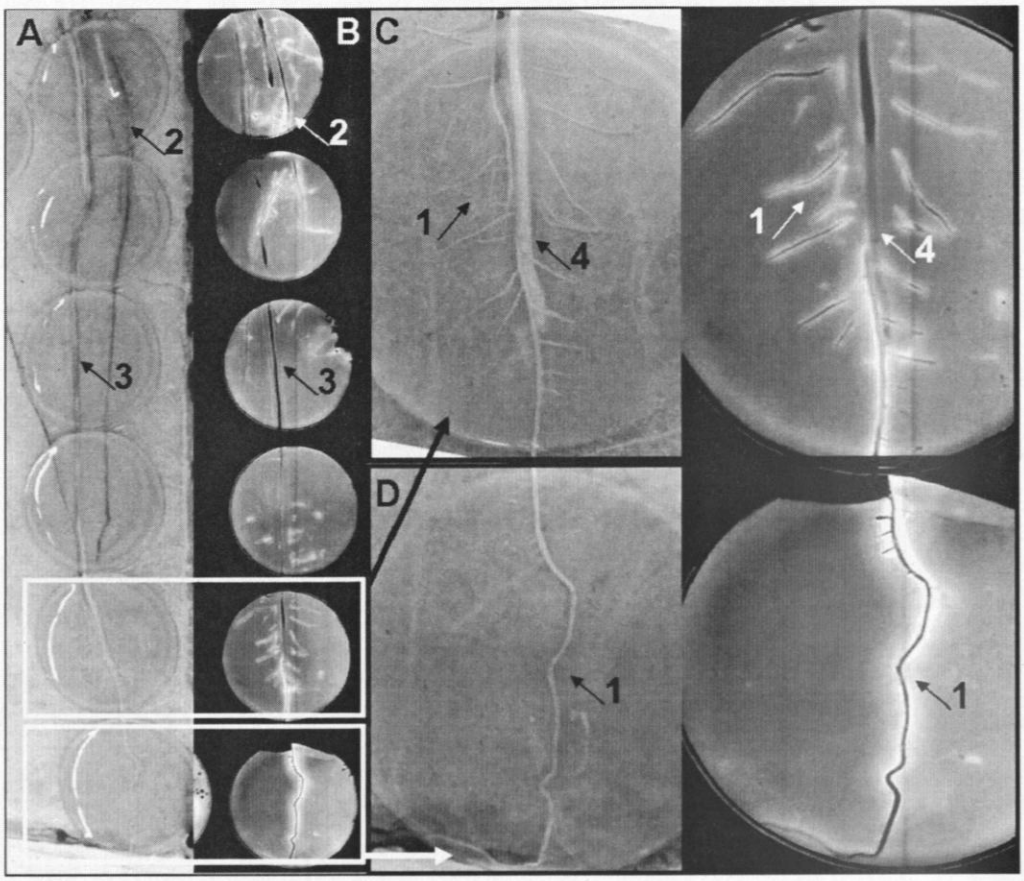


Figure 4

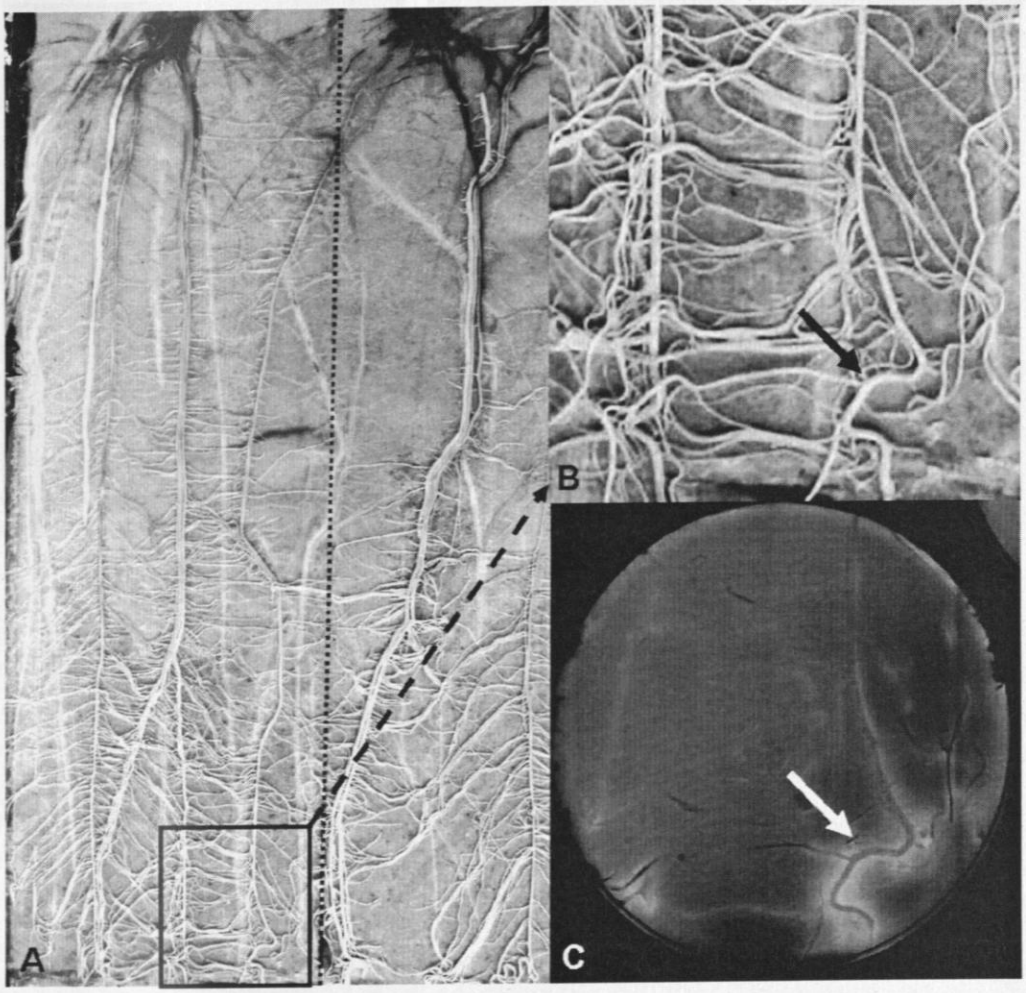
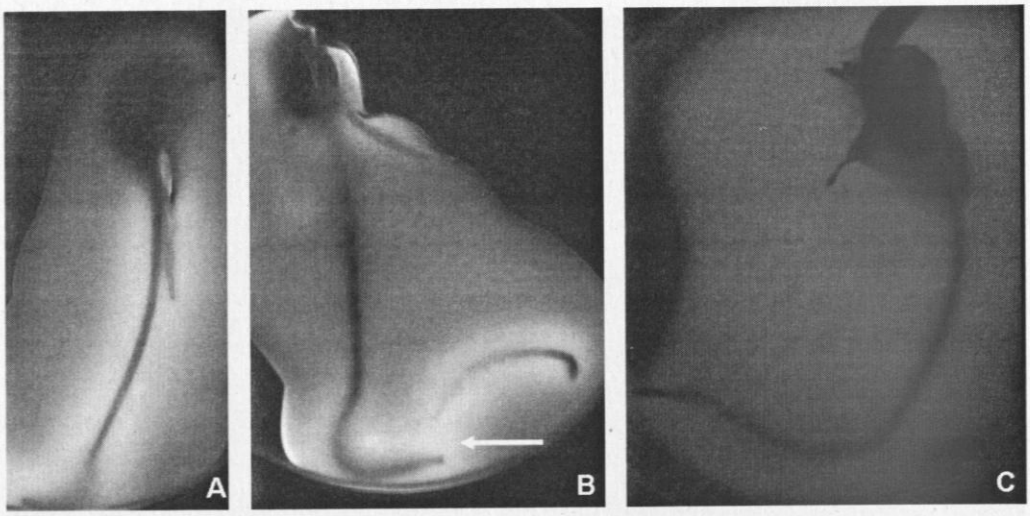


Figure 5



Supplementary Material

Influence of soil solution ions and pH on bioreporter activity in liquid culture assays

Material and Methods. For the application of the bioreporter bacteria in liquid culture assays, overnight cultures of the bioreporter bacteria were diluted 1:50 in LB and then grown under the same conditions to an optical density at 600 nm (OD_{600}) of 0.6, harvested by centrifugation, and resuspended in the same volume of modified MM9 medium (100 mL MM9 salts solution, 2 mL 1 M $MgSO_4$, 0.1 mL 1 M $CaCl_2$ solution, and 10 mL 20% w/w glucose solution per litre; MM9 salts solution contains 5 g NaCl, 10 g NH_4Cl, 54.8 g MOPS (3-(*N*-morpholino)propanesulfonic acid), 51.0 g MOPS sodium salt, 0.59 g Na_2HPO_4 * $2H_2O$, and 0.45 g KH_2PO_4 in 1 L of water, final solution adjusted to pH 7). Arsenite solutions were prepared by dilution from a 50 mM (3.9 g L^{-1}) sodium arsenite stock solution (Merck) in Aqua bidest. In order to determine the influence of the main ions occurring in the soil solution on the bioreporter activity, 96-well plate assays with 1 µM arsenite were spiked with the following ions: K^+ (25, 50 and 75 mg L^{-1}), Cl^- (150, 300 and 450 mg L^{-1}), SO_4^{2-} (75, 150 and 225 mg L^{-1}), PO_4^{3-} (7.5, 15 and 22.5 mg L^{-1}), Ca^{2+} (115 and 230 mg L^{-1}), NH_4^+ (20, 40 and 60 mg L^{-1}), NO_3^- (20, 40 and 60 mg L^{-1}), and Fe^{2+} (3.5, 7 and 12.5 mg L^{-1}). Additionally the influence of a model soil-solution containing a mixture of all listed ions as well as the influence of lower pH values (3.5, 4, 4.5, 5) was analysed. The concentrations of the ions in the model soil-solution were established in previous experiments (Vetterlein et al. 2007; Szegedi et al. 2008). The 96-well plates with 200 µl of the assay mixtures in triplicates were covered with a lid and incubated at 37°C in a rotary shaker (700 rpm) for 4 hours. Green fluorescence of the gfp produced by the bioreporters as a response to As was measured in a microplate spectrofluorometer with excitation at 480 nm and emission at 520 nm (Spectramax Gemini XS, Molecular Devices) and the optical density was measured in a microplate reader (Spectramax 250, Molecular Devices).

Results. The response of the bioreporter bacteria to different concentrations of relevant soil ions was investigated with the liquid culture bioreporter assay. A significant signal reduction was measured for most ions like chloride, sulphate, calcium and nitrate at various concentrations (Fig. ESM1). The lowest activity was observed with Fe^{2+} independent of the concentration used. Application of the model-soil-solution in the assay resulted in a signal reduction of about 75%. No signals were detectable after exposure to pH values below 5.

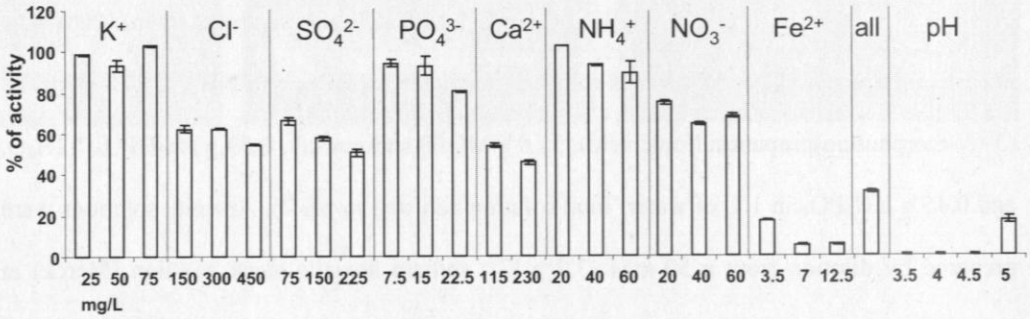


Figure ESM1: Activity of bioreporter bacteria after exposure to ions at various concentrations and 1 µM As measured as green fluorescence. Bioreporter activity of cells incubated only in medium and As served as a control. Column "all" refers to the model-soil-solution containing all ions as described in the text.

Chapter 5

5 Summary and concluding remarks

Summary and concluding remarks

Using bioreporter bacteria for detection of contaminants offers an alternative to the known chemical analytical methods. Due to their easy production in terms of genetic engineering, cheap cultivation, as they reproduce by themselves with short generation times, and easy handling they are an interesting alternative especially for applications requiring high throughput or that are limited by low available budgets. Another advantage lies in their large improvement and optimization potential with respect to specificity and selectivity of the sensing and reporting proteins. Because of the small size of bioreporter bacteria they can also be used for *in situ* analysis at interfaces or gradients. But up to now, bioreporter bacteria have also their shortcoming such as limitations in sensitivity or unsatisfying shelf life. The goal of this study was thus to improve arsenic bioreporter bacteria with respect to their shelf life and easiness of handling for drinking water analysis and to show their usefulness for *in situ* studies.

5.1 Bioreporter bacteria in drinking water analysis

Up to now only few applications of bioreporter bacteria for the analysis of arsenic in drinking water in large-scale environmental monitoring campaigns have been reported (Trang et al. 2005). One reason might be lack of acceptance by regulatory authorities, as the bioreporter bacteria are genetically modified organisms and their application outside laboratories is strictly regulated. Another aspect might be the not yet satisfying shelf life of preserved bioreporter and the consequent requirement of cultivation even for field application. The microbial bioreporter response, operational stability and long-term use depend on the preservation or immobilization strategy used and still need further optimization. Although several authors have shown that freeze-dried cells preserve their viability during years (Miyamoto-Shinohara et al. 2000; Miyamoto-Shinohara et al. 2008), this has not been

reported in the case of bioreporters. In order to preserve activity of arsenite bioreporter bacteria during storage at 4°C we tested different culture conditions, preservation methods and protection media. The most satisfying, stable response to arsenite after 3 months storage followed by rehydration of the bioreporters was obtained with cells that had been dried in the presence of 34% trehalose and 1.5% polyvinylpyrrolidone. Still these bioreporter cells did not match all criteria of a test that could be used to verify water safety within 6 h (water testing for consumption on the same day). For overnight testing (reaction time 12 h) however the bioreporter bacteria dried in trehalose appeared suitable. This variant fulfilled the criteria of reaction time within 12 hours, response intensity and induction coefficient above a predefined threshold even after 12 weeks of storage and showed good stability of the response characteristics between 6 and 12 weeks. Although results could still be better and need further improvement, we have identified a procedure for bioreporter preservation which matches realistic requirements of qualitative arsenic testing in drinking water. It appears nevertheless worthwhile to further optimize vacuum-drying with polyvinylpyrrolidone in combination with other concentrations of trehalose, a larger range of other ingredients like synthetic polymers (hydroxyethyl starch, polyethylene glycol, polyacrylamide or polysaccharides such as Ficoll and Dextran) and modified drying protocols.

Another aspect in applying bioreporter bacteria for field measurements is the ease of application. We developed an approach that promises to simplify bioreporter application by layman since it eliminates time consuming and technically demanding calibration. Besides its higher robustness it has the advantage of the much easier interpretation of sets of qualitative sub-signals than of gradual colour changes. Therefore bioreporter bacteria were tailored to various sensitivities by modifying different steps of the detection-signalling chain, i.e. the rate of reporter enzyme synthesis in LacZ bioreporter bacteria and the catalytic activity of the reporter protein in CCP bioreporter bacteria. The accomplished modifications altogether resulted in two sets of bioreporter bacteria that can be used to infer analyte concentration

ranges without external calibration and both sets could identify permissive concentrations of arsenic: (i) below the WHO limit only two of the four LacZ bioreporter bacteria produced a visible signal, above 10 µg/L three or at concentrations above local arsenic standards (50 µg/L) all four strains responded with clear signals; (ii) the CCP bioreporter bacteria indicated a concentration below the WHO limit when four out of six strains show signals. Especially for simple field detection devices such as paper strips (Stocker et al. 2003) this new method would be of great value.

5.2 Bioreporter bacteria for arsenic *in situ* detection in the rhizosphere

Because of the increasing concern about arsenic uptake into the food chain rhizosphere processes such as the uptake of inorganic arsenic species, arsenic efflux into the rhizosphere and arsenic detoxification by plants require further investigation. We contributed to this research field by applying LB-agar-embedded bioreporter bacteria to the root-soil system of *Zea mays*. As the agar-embedded indicator elements, i.e. bioreporter bacteria, are fixed in space, the system is capable of freezing the visual effects of an analyte's two-dimensional distribution. The imaging technique showed that As accumulation in the rhizosphere is related to root development. Increased As concentrations close to root surfaces were predominantly observed along laterals. Fifteen to 21 days old roots and the distal region of young adventitious roots gave no signals. We never detected strong gradients around young adventitious roots where root hairs were visible with the naked eye. The fact that we observed the strongest gradients in signal induction around laterals can be taken as a hint that we visualized the result of As^{III} uptake and release. For a distinction between As^{V} accumulation around roots and As^{III} release by roots we performed a split root experiment showing that the detectable As along the roots in the compartment not containing any As^{V} has to be a result of translocation within the root followed by its release.

The novel bioreporter assay has the advantage that the bioreporters are applied for a short time (2 h) in a predefined, homogeneous arrangement. They do thus neither need to grow in the system, i.e. the signal output does not depend on parameters like energy or nutrient supply from soil, nor do they seem to be affected by soil chemical conditions. Finally, immobilisation allows easy and non-destructive bioreporter retrieval from the system for signal interpretation. These features taken together result in a spatial signal resolution on a mm to cm scale across entire root systems and the bioreporter assay complements very well conventional measurements that are based on soil solution sampling.

5.3 Concluding remarks about bioreporter bacteria, their limits and perspectives

Chemical analysis has certainly a better starting point in terms of sensitivity, quantification and identification of unknown molecules. But considering the relatively short development history of the bioreporter approach reveals that there is plenty room for improvement and high potential of bioreporter bacteria especially due to the wealth of tailoring possibilities (van der Meer et al. 2004). My work contributed to the improvement of bioreporter based assays and showed at the same time one of its major lacks. I believe that the limited and hardly predictable shelf life is probably the main reason for the fact that easy and reliable bioreporter based test kits are rare. The shelf life of bioreporter bacteria is not only important for the durability of the test itself but also for the reliability as only the complete population of the test gives the correct response. This is even more important if only one strain and its gradual colour change are used for quantification. Next to improving the general procedure by excluding external calibration the multiple cell line approach presented in this thesis minimizes this source of malfunction. Because each cell line has a wider range (OD 0.1 - 1.0) regarding the cell density for the correct qualitative response the whole approach becomes

more robust. The assay relies on four or more qualitative answers which in sum give the quantitative answer. This is an important step towards implementation of bioreporter assays. The fact that the strains with difference in sensitivities resulting in the described calibration could be produced confirms the above mentioned statement that bioreporter bacteria are not yet at the end of improvement. Especially the ongoing genome studies revealing the great natural genetic diversity and the ever growing databases for available genetic parts (e.g. http://partsregistry.org/) that could be used for bioreporter construction promise high potential for future applications of bioreporter based assays. One future application might lie in the field of nanoparticles. They are increasingly used in consumer products and due to the increased surface area and reactivity the properties differ from "normal" materials and could lead to increased bioavailability and toxicity as well, but data on potential environmental effects are rare. For metallic nanoparticles bioreporter bacteria have already been used. A combination of a general toxicity test based on *Vibrio fischeri* and metal-specific recombinant bioreporter bacteria allowed to clearly differentiate the toxic effects of metal oxides *per se* and solubilized metal ions (Heinlaan et al. 2008). In general bioreporter bacteria that respond to environmental stress seem to be a promising tool for detecting different kind of ecotoxical nanoparticles (Mortimer et al. 2008). Another future application could also be arsenic contaminations in food. There are already studies that investigated arsenic concentration in rice with bioreporter bacteria (Baumann and van der Meer 2007) as arsenic enters the food chain after irrigation with arsenic contaminated water.

The application of bioreporter bacteria for *in situ* research has been performed in several different ways and showed the high advantage of bioreporter bacteria for this purpose (Darwent *et al.*, 2003; DeAngelis *et al.*, 2005; Gantner *et al.*, 2006; Paterson *et al.*, 2006). The study performed here delivered nice visualisation of the root zones and their contribution to available arsenic. But unfortunately quantification was not achieved. For this goal a differentiation between arsenite and arsenate would be of great value. Up to now most arsenic

bioreporter bacteria are based on the resistance mechanism of plasmid R773 and do therefore not distinguish between arsenite and arsenate. Specific arsenite detectors have been constructed in photosynthetic bacterium *Rhodovulum sulfidophilum* with a carotenoid as the reporter protein. Fujimoto and colleagues used only the promoter region of the ars operon and the *arsR* gene and not the entire operon or *arsC* gene (Fujimoto et al. 2006). Other construction strategies would be based on deletion of the arsenate reductase in the bioreporter bacteria. Tests in the 80's showed a loss of arsenate resistance after deletion of *arsC* (Chen et al. 1986). Furthermore the uptake systems for arsenate or arsenite could be knocked out, respectively and these bioreporter bacteria would then sense only one kind of the ions and would allow differential quantification.

One point which is always mentioned when bioreporter are praised as a new technology is their importance for resolving gradients at a high spatial resolution. Some questions which I find important at that point are listed and partly answered below. Do such steep gradients exist? In aqueous environments for example low molecular mass molecules diffuse over a distance of 36 µm in only 0.3 s (van der Meer et al. 2004). For such applications the velocity of reaction would be much too slow compared with the diffusion processes. Up to now reaction times between 5 min (Axtell and Beattie 2002) and several hours have been reported, but the speed of response could probably be further improved (van der Meer et al. 2004). Another point is the signal intensity, which has to be high enough for single cell analysis. The autofluorescent proteins need in general high levels of expression, which makes them not suitable for constructs with weak promoter or single chromosomal copy (Larrainzar et al. 2005). Additionally the application modus of the bioreporter bacteria can influence the gradients as they are mostly applied in a solution (Darwent et al. 2003; Paterson et al. 2006). Here our approach offers an interesting alternative. The bioreporter bacteria are fixed in a gel matrix, e.g. LB-agar. Hence, the system is capable of freezing the visual effects of an analyte's two-dimensional distribution. The access to the investigated system e.g. rhizosphere

forms another problem. How can one enter the system *in situ* or detect the signals? Solving this problem seems to be too complicated for some questions; therefore small subsamples (like rhizosphere and bulk soil) are retrieved and investigated in an assay with bioreporter bacteria. Maybe spatial resolution is overestimated and distinction between different rhizosphere zones and bulk soil sufficient for many objectives like concentration differences of quorum sensing molecules (DeAngelis et al. 2007), oxygen distribution (Hojberg et al. 1999) and nitrate availability (DeAngelis et al. 2005). On the other hand model systems like rhizoboxes allow for undisturbed access to the area of interest but they probably do not reflect the real condition in the environment. After all the spatial root growth is restricted and the development of a root mat promoted. Another aspect is that bioreporter bacteria are per definition genetically modified before they are used, thus they are not native bacteria for example of a biofilm and need to be introduced into the system. In addition, the expression of the reporter gene may alter the physiological behaviour in contrast to the wild-type cell (Lenz et al. 2008). One may thus conclude that bioreporter bacteria are a valuable tool for *in situ* analysis of gradients or transport processes as long as one is aware of their limitations.

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Publications

1. **Kuppardt A.**, Vetterlein D., Harms H., Chatzinotas A.: Visualisation of gradients in arsenic concentrations around individual roots of *Zea mays* L. using agar-immobilized bioreporter bacteria. Plant and Soil (accepted) DOI: 10.1007/s11104-009-0154-3

2. **Kuppardt A.**, Chatzinotas A., Breuer U., van der Meer J., Harms H. (2009): Optimization of preservation conditions of As (III) bioreporter bacteria. Applied Microbiology and Biotechnology 82, 785-792

3. **Wackwitz A.**, Harms H., Chatzinotas A., Breuer U., Vogne C., van der Meer J., (2008): Internal arsenite bioassay calibration using multiple bioreporter cell lines. Microbial Biotechnology 1, 149-157

4. Busam S., McNabb M., **Wackwitz A.**, Senevirathna W., Beggah S., van der Meer J., Wells M., Breuer U., Harms H. (2007): Artificial neural network study of whole-cell bacterial bioreporter response determined using fluorescence flow cytometry. Analytical Chemistry 79, 9107-9114

Oral presentations

1. **Wackwitz A.**, Breuer U., Chatzinotas, A., Harms H. (2007): Development of a bioreporter-based arsenic test system. Arsen-Workshop. Leipzig

2. **Wackwitz A.**, Breuer U., Chatzinotas, A., Harms H. (2006): Development of a biosensor-based arsenic test strip. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie. Jena

Posters

1. Hellmich R., Chatzinotas A., **Kuppardt A.**, van der Meer J., Harms H. (2009): Entwicklung eines anwendungsbereiten Arsenbiosensors auf Basis von Bioreporter-Bakterien. 6. Deutsches BioSensor Symposium. Freiburg

2. **Wackwitz A.**, Harms H., Breuer U., Chatzinotas A., van der Meer J. (2007): Internal bioassay calibration via the use of multiple bioreporter cell lines. Jahrestagung der Vereinigung für Allgemeine und Angewandte Mikrobiologie. Osnabrück

3. **Wackwitz A.**, Breuer U., Chatzinotas, A., van der Meer, J.R., Harms H. (2006): Development of a biosensor-based arsenic test strip. International Symposium on Environmental Biotechnology. Leipzig (first scientific award poster prize from Trends in Biotechnology, Elsevier)

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Leipzig, 27.August 2009 Anke Kuppardt