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Ochrobactrum tritici immobilized in *Oryza sativa*, sodium polyacrylate and alginate as a novel bioremediation tool

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dedication

Voor alle fantastische mensen die ik ken -met name de steun van familie en vrienden- en zal leren kennen. Dat ik de steun terug kunne geven op het moment des noods!

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abstract

Arsenic pollution is a worldwide problem and although several remediation strategies are present, their costs are high and their effect low. The use of bioremediation as a strategy for cleaning polluted sites has been revealed as a promising approach. One of these strategies is bioaugmentation, i.e. the addition of exogenous bacteria in a polluted site to remove contaminants. In a previous study a strain of the *Ochrobactrum tritici* bacteria was genetically modified by silencing the arsenite efflux pumps of the cell membrane. In this study the arsenic accumulator was immobilized in several supports: an alginate gel, a sodium polyacrylate gel, rice plants, an acrylamide gel, a 2-hydroxyethyl methacrylate gel and a 2-hydroxyethyl acrylate gel. Their performance as a support for arsenic accumulation was tested. Of the gels, the alginate gel and the sodium polyacrylate gel showed the most promising results in terms of water permeability and bacterial survival inside of the gel. The results of the alginate gel indicate an uptake of 29% of the environmental arsenite of 10mM and 2mM and a 19% uptake at arsenite concentrations of 200µM. The inoculated sodium polyacrylate gel took up 6% of the arsenite in a 200 µM arsenite solution. Rice plants without bacteria managed to take up 44% of the arsenic present in a concentration of 20 µM, while rice plants with the mutated bacteria did not significantly take up any arsenite whatsoever. The presence of the bacteria did not increase the arsenic uptake of the plant, but conferred more resistance to arsenic in terms of plant growth. The use of inoculated alginate gels and rice plants for the uptake of arsenite from the environment, suggests experimentation in-situ. The arsenite resistance in rice plants due to the *O.tritici* double mutant in rice plants opens up new research possibilities for the effect of bacteria on the metal metabolism of plants.

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1. introduction

1.1 general

Thousands of people around the world suffer from the adverse health effects of arsenic in their environment (Mandal & Suzuki 2002). They are exposed to either inorganic or organic arsenic, from which inorganic arsenic species pose the biggest threat to human health (Mandal & Suzuki 2002). Arsenic is mainly present in soils, because of human activities and the parent rock of the soil (Mandal & Suzuki). The parent rock contributes much more to the amount of arsenic in soils than the soil type (Mandal & Suzuki 2002). The use of arsenic contaminated drinking water is a major public health concern in countries, such as Bangladesh, China, India, Nepal and Vietnam, and the contamination originates from natural sources in the groundwater (Ng et al. 2003). Arsenic ends up in the food chain and is present for around 65-75% as the inorganic species in many food products, like several dairy and meat products (Mandal & Suzuki 2002). Besides food, humans are exposed to arsenic via the air and water (Mandal & Suzuki 2002). High arsenic pollution is found in Asia, especially in China, Bangladesh and Taiwan (Ravenscroft 2007). In Bangladesh 45 million people were found to use drinking water with levels of arsenic higher than 10µg/L, the maximum concentration recommended by the world health organization (Flanagan 2012). Long term exposure to elevated arsenic levels causes cancers of the skin, bladder, lung and kidneys, skin lesions, pulmonary disease, peripheral vascular disease, arterial hypertension, diabetes mellitus, neuropathy and cardiovascular diseases (Flanagan 2012). These health problems in Bangladesh, caused by chronic arsenic exposure, were found to result in an increase in annual mortality of 43,000 deaths, representing 5.6% of the total deaths in Bangladesh and an economic loss of 10.6 billion euros over the next twenty years (Flanagan 2012).

Faced with the serious problems caused by high concentrations of arsenic in the environment, arsenic removal methodologies are being employed. Different methods are available, such as precipitation of the arsenic with iron oxides, alum, lime and manganese oxides, membrane and adhesion techniques (Kartinen & Martin 1995). Of these classical methods membrane removal scores the best on on-site applicability, removal efficiency and lowest amount of chemicals used (Kartinen & Martin 1995). These latter methods all make use of inorganic chemicals for the remediation. The use of these traditional methods often includes land excavation, close and cover practices and reaction of the contaminants to form non-toxic

compounds. These methods move the contaminants to another place, posing health risk during the transport, leaving the contaminants on spot without removing the problem directly or requiring advanced technological methods (Vidali 2001).

Bioremediation is an option that detoxifies the contaminants, does not pose risks to health and environment and is economically viable (Vidali 2001). Bioremediation is defined as "a managed or spontaneous process in which biological, especially microbial, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination (Madsen 1991). Bioaugmentation is one of the strategies used in bioremediation (Vidali 2001). For bioaugmentation exogenous bacteria are inserted in the soil (Vidali 2001). These inserted bacteria often do not multiply nor stabilize as part of the microbial community of the soil (Vidali 2001). Some draw-backs of bioremediation methods are the long time period required for contaminant removal, the limited number of pollutants and toxics that can be treated and the requirement of expertise and design of the bioremediation strategies (Vidali 2001).

In bioremediation, commonly used organisms are bacteria, plants and fungi (Vidali 2001). Bacteria have a fast metabolism, growth and a large variety of metabolic pathways. The *Ochrobactrum tritici* strain, isolated initially from a wheat rhizoplane, is a highly arsenic resistant microorganism (Sousa et al. 2015). The strain was mutated to bio accumulate intracellular arsenic up to 17 nanogram arsenic per gram of protein, when cells were grown in presence of 1 mM arsenite (Sousa et al. 2015). Two genes were mutated for this *Ochrobactrum tritici* strain, generating the double mutant strain SCII24 (Sousa et al. 2015). The mutated *ArsB* and *Acr3_1* genes encode different membrane proteins that allow the efflux of arsenite from the cytosol (Sousa et al. 2015). In the present work, *O.tritici* double mutant strains were tested in different supports as an arsenic bioremediation tool.

Using modified organisms, it is essential to avoid release of bacteria and one way of immobilizing organisms that are efficient in bioremediation is through the use of hydrogels (Degiorgi et al. 2002; Kaşgöz et al. 2003; Ramachandran et al. 2009; Leung et al. 2000). Hydrogels are polymer networks, which absorb and retain water without dissolving (Degiorgi et al. 2002). These systems can be ionic or neutral hydrogels, with both a different underlying swelling mechanism (Bell & Peppas 1995). The ionic hydrogels are influenced by factors such as concentrations outside of the gel, pH and presence of ions. The neutral polymers are not influenced as much by environmental factors (Bell & Peppas 1995). Hydrogels find yet many different uses in biomedical application as micro encapsulation, matrix entrapment and adhesion (Jen et al. 1995). Their swelling capacity, structure, chemical nature and ease of production make the hydrogels excellent for immobilization of cells.

In metal bioremediation several cases were reported of metal removal from contaminated sites, using alginate, polyacrylamide or acrylate gels (Leung et al. 2000; Kaşgöz et al. 2003; Degiorgi et al. 2002).

Plants are other organisms commonly used in bioremediation and their pollutant removal is categorized as phytoremediation (Vidali 2001). Plants that accumulate metals can be divided in different groups: accumulators, excluders and indicators (Alkorta et al. 2004). Excluders maintain concentrations of the metals in aboveground parts lower than the soil concentrations (Alkorta et al. 2004). Indicators have a similar internal concentration of the metal as outside of the plant (Alkorta et al. 2004). These plants can usually tolerate arsenic concentrations up to 5-20 milligram per kilogram dry weight (Vithanage et al. 2012). The accumulators have higher metal concentrations in the above ground parts and/or in the roots than in the soil and have an excellent use in phytoremediation (Alkorta et al. 2004).

An advantage of phytoremediation over microbial remediation is that plants can extract the metals from the soil to aboveground parts (Alkorta et al. 2004). The inexpensiveness is another advantage of phytoremediation, as well as the nonnecessity of transporting the - to be remediated - soil and its applicability in a wide range of situations (Alkorta et al. 2004). On the other hand, plants used in phytoremediation are vulnerable to natural diseases and the remediation process takes more time than conventional remediation techniques (Alkorta et al. 2004). In addition, the root depth and solubility of the pollutants are influencing the effectiveness and phytoremediation is only applicable for mild pollutant concentrations (Alkorta et al. 2004). Some other plant characteristics, that are important in metal metabolism, are: a profuse root system, high amounts of biomass, rapid growth ex situ, resistance to the metals and accumulation of the metals (Alkorta et al. 2004).

Plants in nature yet act as a support for bacteria. They live in conjugation with bacteria in their rhizosphere but also in the other parts of the plant, having endophytic bacteria (Rajkumar et al. 2009). Most of these bacteria also occur in the soil and can live outside of the plant as well and are therefore called facultative (Rajkumar et al. 2009). Endophytic bacteria are known to solubilize phosphates, fix nitrogen, produce siderophores, plant hormones and organic acids (Rajkumar et al. 2009). The role of endophytic communities in metal resistance of hyperaccumulators is a less explored field of research and ambiguous results have been found about the effects of bacteria on metal resistance (Rajkumar et al. 2009). Metal phosphates and siderophores were found to be produced by the bacteria, increasing the solubility of zinc and the total zinc uptake of the plant (Saravanan et al. 2007).

However, other research found a reduction in nickel and cadmium uptake in tomato plants, when endophytic bacteria, isolated from rice plants, were inoculated on the rhizoplane and this study found a resulting increase in growth of the tomato plant (Madhaiyan et al. 2007). A possible explanation for the increase in plant growth was the immobilization of harmful metals by bacteria (Rajkumar et al. 2009).

Higher contaminant resistance in plants with inoculated bacteria is one of the advantages of plant-bacteria systems for phytoremediation, showing an easier application of genetically modified bacteria and an enhanced contaminant degradation of the inoculated plants compared to non-inoculated plants (Zhuang et al. 2007). Plants can exudate secondary metabolites of the contaminants that allow the growth of specific endophytic bacterial communities that can further metabolize the exudates (Zhuang et al. 2007). The rhizoplane of the plants offers a protected environment for genetically modified bacteria that would normally not reach a stabile population in the microbial communities present in absence of the plant (Zhuang et al. 2007). In *Astragalus sinicus* plant growth-promoting rhizobacteria (PGPR) increased the ability of the plant cells to bind cadmium ions a 9-19 fold and in *Brassica juncea* the PGPR enhanced the plant's ability to accumulate nickel (Zhuang et al. 2007).

1.2 study objectives

The general objective of this work is to develop an active biofilter, combining a support with an arsenic accumulating bacterial strain that contains viable bacterial cells inside, allows diffusion of nutrients and contaminants from the medium to the support, has a good structural stability, minimizes the cell leakage to the environment and is able to take up considerable amounts of arsenic from an arsenic contaminated medium.

The specific goals of the study are analyzing polyacrylamide, polyhydroxyethylacrylate (pHEA), poly(2-hydroxymethacrylate) (pHEMA), alginate and sodium polyacrylate gels on the characteristics mentioned in the general objective (1), investigating the appropriate candidate gels on their arsenic removal (2), analyzing a biofilter with rice plants, *Oryza sativa,* as support for arsenic removal (3) and studying the effect of the inoculated bacteria on the growth parameters of the plant (4).

2. Background

2.1 arsenic, the king of poisons

Arsenic is the 20th most abundant element on earth and is placed on the fourth period and in the fifteenth group of the periodic table. It is mainly known in history for its strong toxicity and was hence called "the king of poisons". Arsenic has one stable isotope, ^{75}As, of the 33 known isotopes and in natural sources this isotope occurs in a hundred percent of the cases (Sun 2010). Arsenic is a metalloid or a semimetal (Helmenstine 2016). Typical characteristics of metalloids are their semiconducting properties and their capability of forming amphoteric oxides (Helmenstine 2016). Arsenic can have -besides a valence state of plus three- a plus five, a zero (elemental arsenic) and a minus three valence state. The main primary arsenic species used in the industry are the amphoteric oxide or As_2O_3 having a valence state of plus three (Greenwood 1984). Arsenic trioxide occurs in natural ores, but the main source is as a byproduct catchment in the mining of other metals. When an ore is heated, the arsenic can be caught volatile in the air as arsenic trioxide (Helmenstine 2016). From AsO_3 different other arsenic compounds can be made, such as sodium *meta*-arsenite:

$AsO_3 + Na_2CO_3 \rightarrow 2NaAsO_2 + CO_2$ (Brauer & Reed 1963).

This sodium *meta*-arsenite is often called sodium arsenite and is an important laboratory reagent. Arsenic trioxide dissolves more readily in bases than it does in acids. An example of a base reaction in the production chain is:

$As_2O_3 + 6NaOH + 2S \rightarrow 2Na_3AsO_3S + 3H_2O$ (Brauer & Reed 1963).

Although arsenic trioxide is less prone to dissolve in acids, it can still dissolve in strong acid, such as hydrochloric acid, resulting in arsenous chloride:

$As_2O_3 + 6HCl \rightarrow 3AsCl_3 + 3H_2O$ (Brauer & Reed 1963).

The variety of reactions that arsenic trioxide can engage in, enables the fabrication of different arsenous products. Besides the acid and base reactions, arsenic trioxide engages in redox reactions. With strong oxidizing reagents arsenite, As(III), can be oxidized to arsenate, As(V).

The oxidation state of arsenate is less stable than metals from its same group in the periodic table (Sun 2010). As a cause of this instability, arsenate "wants" to be reduced and is a strong oxidizer. Arsenite can be reduced to arsenides or elemental arsenic. Arsenides are chemical compounds that contain an arsenic species of minus three. An example of an arsenide that can be formed is Na_3As. Arsenic trioxide can be reduced to the volatile arsine (AsH_3) with the help of nitric acid:

$As_2O_3 + 6Zn^{2+}\ 12HNO_3 \rightarrow 2AsH_3 + 6\ Zn(NO_3)_2 + 6H_2O$ (Brauer & Reed 1963).

Arsine gas is highly toxic due to its reaction with hemoglobin in the body. Arsenides with metals are not toxic as long as the arsenic is bound to the metal (Sun 2010).

In nature, the main inorganic arsenic species in natural waters are arsenate (V) and arsenite (III) (Tsai et al. 2009). In ground water arsenic is usually present as arsenite due to the reducing conditions of these waters (Kartinen & Martin 1995). Whether also significant amounts of arsenate are present in waters, depends on the oxidizing/reduction potential (ORP) of the water and the pH (Kartinen & Martin 1995). In acidic waters the main arsenic species present is arsenate (Kartinen & Martin 1995). Nitrate can oxidize iron to create particles that adsorb arsenic (Tsai et al. 2009). Arsenite is considered as a more mobile and toxic species than arsenate (Tsai et al. 2009). As(III) interferes with the thiol groups of proteins or with the vicinal sulfhydryl groups, damaging basic metabolism of cells (Tsai et al. 2009). Additionally, arsenite can bind to hormone receptors and induce reactive oxygen species (Tsai et al. 2009). Arsenate infers its main toxicity by disturbance in the oxidative phosphorylation by allowing arsenate to be taken up through the same carriers as phosphate (Tsai et al. 2009).

2.2 arsenic in metabolic pathways

2.2.1 organic arsenic species

Arsenic occurs in metabolic pathways in its inorganic form and – after metabolization – in organic forms. Common organic arsenic species are: monomethylarsine, dimethylarsine, trimethylarsine, dimethylarsenate, methylarsonate, arseno lipids and arseno sugars (Bentley & Chasteen 2002). These arsenic species are formed by (trans)methylation and alkylation of mainly inorganic arsenic species, using the metalloid as the acceptor of the added alkyl group (Bentley & Chasteen 2002). The methyl arsines mentioned are volatile species, in contrast to the dimethylarsenate, methylarsonate, arseno lipids and arseno sugars (Bentley & Chasteen). Arseno lipids and arsenosugars contain bigger alkyl groups than methyl (Bentley & Chasteen). However, most of the organic arsenic species contain a methyl group and the other organic species are usually formed by alkylation with an ethyl group (Bentley & Chasteen). The metabolisme of arsenic is different for microorganisms and plants.

2.2.2 arsenic metabolism in microorganisms

Microorganisms have three main detoxification mechanisms for arsenic: intracellular chelation, active efflux and transformation to less toxic organic forms (Tsai et al. 2009). These mechanisms are different for prokaryotes and eukaryotes (Tsai et al. 2009).

In prokaryotes, arsenite is mainly taken up by glycerol transporters and arsenate is taken up via the phosphate transport system; there is no specific uptake system for arsenic due to its toxicity (Tsai et al. 2009). Arsenate is reduced to arsenite by different reductases, depending on the prokaryote, after which the complex is transported to the cell membrane (Tsai et al. 2009). The arsenite pumps, such as ArsB, remove the arsenite from the cell with the help of the ArsA protein, an ATPase, that provides the necessary energy via ATP generation (Tsai et al. 2009). Arsenite can be oxidized to arsenate in some microorganisms, using arsenite oxidases, either in order to use the energy of this oxidation reaction or to convert the more toxic arsenite in the less toxic and less mobile arsenate species (Tsai et al. 2009). Not much research is done on the methylation pathways of arsenic and future studies may reveal more of the uses of these organic arsenic species (Tsai et al. 2009). The general pathway is the reduction of As(V) to As(III), followed by an oxidative addition of a methyl group (Tsai et al. 2009).

These methylated groups are not necessarily less toxic than the non-methylated species, but they are volatile and very mobile (Tsai et al. 2009). These methylated organic arsenics can be used as a carbon source by some bacteria (Tsai et al. 2009). Bacteria are often used in bioremediation and in this study a bacterial strain was used for an arsenic removal biofilter.

Bacterial arsenic resistance can be conferred via the chromosomes or the plasmid (Cervantes et al. 1994). Chromosal arsenic resistance is influenced by the synthesis of arsenate uptake pumps, Pit and Pst systems in *Escherichia coli*, and the synthesis of arsenite oxidizing enzymes (Cervantes et al. 1994). One of the systems, the Pit arsenate pump in *E.coli,* takes up phosphate and arsenate in comparable rates, while the Pst system takes up arsenate poorly (Cervantes et al. 1994). Mutant defectives of the Pit arsenate pump systems are usually arsenate resitant (Cervantes et al. 1994). Besides the transportation of arsenate in the cells, arsenite may be oxidized to arsenate, using enzymes in the cell membrane (Cervantes et al. 1994). This mechanism is considered as arsenic detoxification, since pentavalent arsenic compounds are at least a hundred times less toxic than trivalent arsenic compounds (Cervantes et al. 1994). Plasmid arsenic resistance is conferred by the synthesis of arsenic efflux pumps, reducing the intracellular arsenic concentration (Cervantes et al. 1994). The ArsB membrane transport protein allows an efflux of arsenite from the cells, using energy from an ATPase dependent system in *E.coli* and energy from the membrane potential in *Staphylococcus aureus,*(Cervantes et al. 1994; figure 1). The ArsB protein ATPase subunit forms a complex with the ArsA protein that is activated by

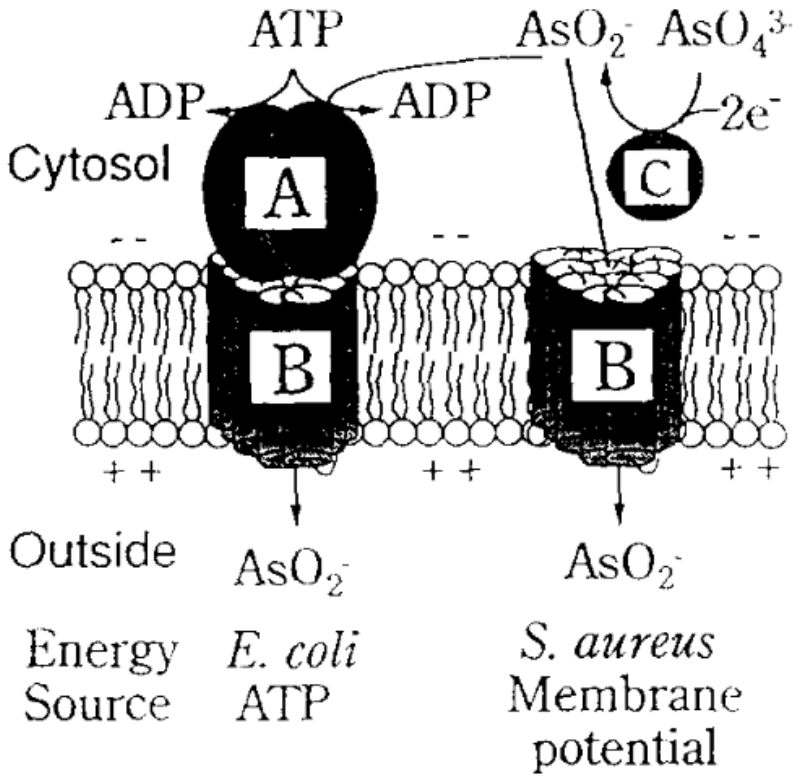

Figure 1: efflux systems of arsenite in *E.coli* and *S. aureus* (Cervantes et al. 1994)

arsenite. Arsenate does not activate the ArsA protein directly, but is first reduced to arsenite by ArsC arsenate reductase (figure 1). Of the membrane transport proteins, beside the ArsB family, another family of membrane transport proteins exist: Acr3p (Sousa et al. 2015). Like the ArsB family these transporters allow the efflux of arsenite from bacterial cell (Sousa et al. 2015). The Acr3p pump is encoded by the *Acr3_1* and *Acr3_2* genes (Sousa et al. 2015). However, the *Acr3_2* gene is not necessary for full arsenic resistance (Sousa et al. 2015). In this study a previously described bacterial strain mutated in the Acr3p and ArsB pump by genetically modifying the *ArsB* and *Acr3_1* genes, was used for the biofilter development (Sousa et al. 2015).

In eukaryotes the arsenic metabolism is again different for groups. In yeasts, arsenite uptake can follow an additional route via sugar metabolism, in the hexose transporters (Tsai et al. 2009). The reduction of the arsenate to arsenite involves a variety of metal complexes produced in the intracellular space (Tsai et al. 2009). Arsenite is a more reactive species than arsenate and can be readily bound to chelators, such as themetallothioneins that have a strong arsenite affinity (Tsai et al. 2009). The chelator-arsenic complexes can be transported to the outside of the cell or inside vacuoles in an ATP dependent way (Tsai et al. 2009).

2.2.3 arsenic metabolism in plants

The main metabolic pathways of arsenic in plants are vacuolar sequestration and volatilization (figure 2). As(V) is taken up by the plant via phosphate transporters, whereas arsenite is taken up by the nodulin 26-like intrinsic protein transporters (NIPs) (Briat 2010). Arsenate is less mobile inside of the plant tissue and is reduced by arsenate reductase to arsenite (Briat 2010). The NIPs family contains aquaporins and other transporters, such as silicon transporters (Briat 2010). These silicon transporters are important especially in rice plants, because they are responsible for loading arsenite into the xylem, transport methylated arsenite and excrete arsenite outside of the root cells (Briat 2010).

Moreover, arsenite can be bound to a phytochelatin and stored in a plant vacuole (Briat 2010). The enzymatic reactions of the sequestration of arsenite are shown in figure 2. Three proteins form a complex (glutamate, cysteine and glycine) after a process of enzymatic reactions and the complex is synthesized into a phytochelatin by phytochelatin synthase (Briat 2010). These phytochelatins form a complex with the arsenite and are transported by members of the ABC transporter subclass, the ATP binding cassette C

transporters (Briat 2010). In humans glutathione (GLH) arsenite complexes can be transported in this manner and in plants several types of plants showed an arsenite transport dependency on glutathione (Briat 2010). Like microorganisms, plants are able to methylate the arsenic. Methylated arsenite can be removed from the plant by volatilization (Briat 2010).

Rice plants take up arsenic mostly as arsenite, arsenate and methylated versions of arsenite, such as monomethylarsonic acid (MMAA) and in limited amounts of dimethylarsinic acid (DMAA) (Abedin 2002). The arsenite and arsenate are taken up by a low-affinity system and a high-affinity system, where both systems act respectively at higher and lower concentrations of arsenic following saturation kinetics (figure 3).

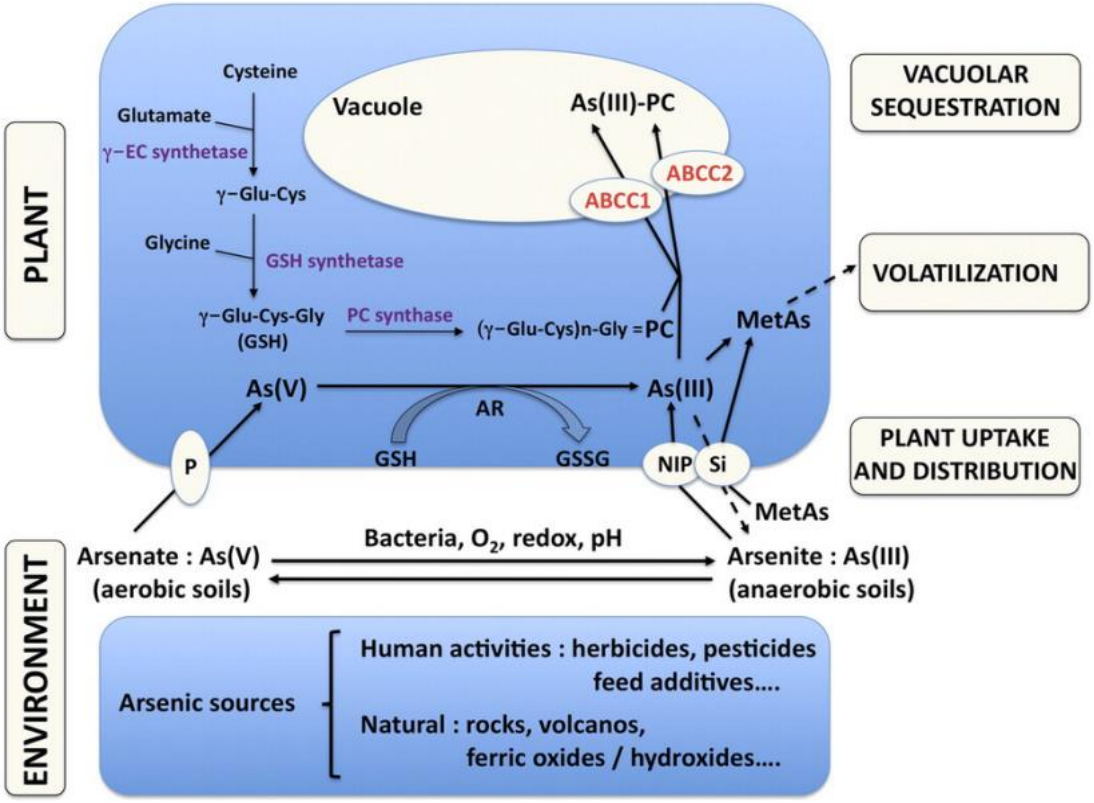

Figure 2: the arsenic cycle in plants and environment (Briat 2010)

Saturation kinetics dictates a limited amount of arsenic uptake that reaches a maximum, after a certain concentration of arsenic. The uptake for arsenite, arsenate, MMAA and DMAA for 0.053 mM are respectively 147, 126, 12.7 and 5.7 mmol per gram of fresh weight of plant per hour (Abedin 2002). Therefore, the rice plants take up arsenite the most, followed by arsenate, MMAA and DMAA.

Because of the chemical similarity between phosphate and arsenate molecules, the addition of phosphate to the medium strongly inhibits the uptake of arsenate from the medium and – since arsenate is reduced to arsenite in the cells – the amount of arsenite in the cells (Abedin 2002).

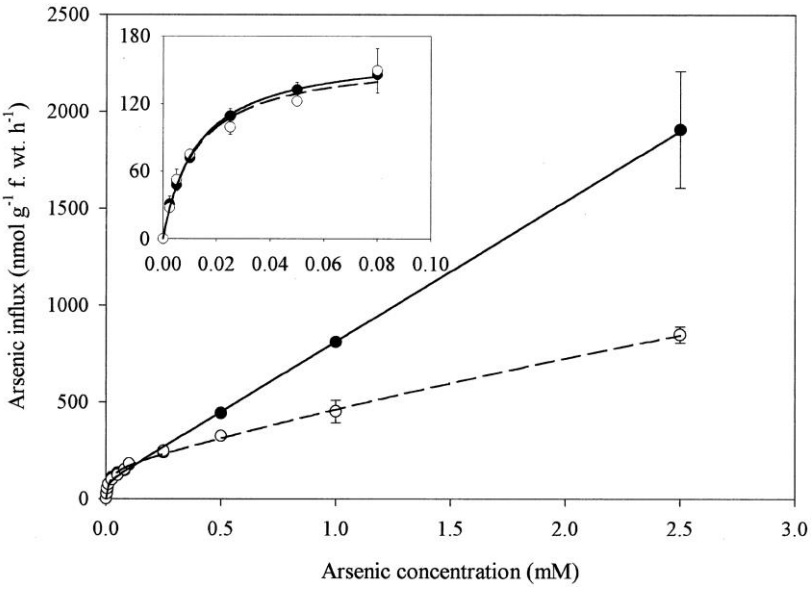

Figure 3: low affinity (curve in small quadrant) and high affinity uptake of arsenite (line) and arsenate (dotted line) in rice plants (Abedin 2002)

2.3 bioremediation

Bioremediation effectively uses microorganisms that can enzymatically convert contaminants to less harmful compounds and was previously defined as "a managed or spontaneous process in which biological, especially microbial, catalysis acts on pollutant compounds, thereby remedying or eliminating environmental contamination" (Madsen 1991). The survival of the organisms used for the remediation is dependent on the environment and key to the effectiveness of the bioremediation is the manipulation of the environmental factors that increase cell survival and/or degradation (Vidali 2001). Different bioremediation strategies can manipulate these environmental factors in favor of the contaminant detoxification. Bioremediation can be classified in in-situ bioremediation and ex-situ bioremediation (Vidali 2001). For the in-situ bioremediation methods, available options are biosparging, bioventing, bioaugmentation and in-situ biodegradation (Vidali 2001). Bioaugmentation can be defined as "the

technique for improvement of the capacity of a contaminated matrix (soil or other biotope) to remove pollution by the introduction of specific competent strains or consortia of microorganisms" (El Fantroussi & Agathos 2005). Biosparging injects high pressure air under the water table of the soil to increase oxygen levels for aerobic contaminant degradation (Vidali 2001). Bioventing is the process of blowing air through the soil with nutrients and oxygen to increase biodegradation in the soil (Vidali 2001). In-situ biodegradation is based on the same principle as bioventing, only that it uses liquid with nutrients to increase biodegradation (Vidali 2001). Ex-situ bioremediation makes use of bioreactors, biopiles, landfarming and composting (Vidali 2001). The function of landfarming is the removal of contaminated soil to a designated area, where the soil is tilled until the contaminant is detoxified (Vidali 2001). In the composting technique soil amendments are added to the -to be contaminated- in order to create a richer microbial community that detoxifies the contaminant more readily (Vidali 2001). The contaminated soil can be more controlled in constructions that enhance the aeration and the leaching of the contaminant to the environment and this technique is called "biopiling" (Vidali 2001). The contaminated soil or water can be directed through a closed engineered system in the "bioreactors" technique (Vidali 2001).

The bioaugmentation method introduces exogenous organisms to the contaminated area. The addition of genetically engineered bacterial strains to contaminated soil or water is mostly done in controlled conditions, such as in the bioreactor technique, because the ecological barriers, present in a natural ecosystem, are hard to overcome (El Fantroussi & Agathos 2005). Introduced modified bacterial strains need to adapt to the new ecosystem in terms of cell survival, migration and activity influenced by the new biotic and abiotic factors (El Fantroussi & Agathos 2005). Succesful bioaugmentation cases were achieved for taking into account the different niches in the ecosystem, modifying microorganisms or consortia of microorganisms yet ubiquitously present in the ecosystem (El Fantroussi & Agathos 2005). For a successful bioaugmentation remediation the population of the genetically modified strain should be monitored, as well as the gene transfer between cells (El Fantroussi & Agathos 2005). Secondly, a way of overcoming the ecological barriers of bioaugmentation, is the use of cell incapsulation (El Fantroussi & Agathos 2005).

Besides the bacteria used in this study, other groups of organisms may yet find and have found their use in bioremediation (Bentley & Chasteen 2002). Different arsenic species, such as areno lipids and arseno sugars are produced by other microorganisms. For example, a marine diatom was found to synthesize arseno lipids (Bentley & Chasteen 2002). Marine algae have been found to accumulate amounts of arsenic higher than 100 milligram of arsenic per kilogram of algae dry weight (Pengprecha et al. 2005). Arsenic species taken up by the plants are converted into different organic species, from which arseno sugars

were found to be produced by marine algae (Pengprecha et al. 2005). These arseno sugars are relatively non-toxic to humans and animals compared with the inorganic species (Pengprecha et al. 2005). Beside the formation of these organic species, fungi are known to volatize inorganic arsenic species to toxic volatized organic forms, from which some have a typical “garlic odor” and these arsenic gases are for a large part unknown (Bentley & Chasteen 2002). A promising example is the fungi strain *Aspergillus oryzae* as biosorbent, that was compared with activated carbon, removing 35 milligram of cadmium(II) per gram of fungi dry weight per eight washing cycles , while activated carbon removed 14 milligram of cadmium(II) per gram weigh in five cycles (Kapoor & Viraraghavan 1995).

2.4 hydrogels

2.4.1 neutral hydrogels, ionic hydrogels and sol-gels

The definition of hydrogels is not constant over time and can be different in many studies. However, in general, hydrogels are hydrophilic polymer networks which may absorb from 10 – 20% (an arbitrary lower limit) up to thousands of times their dry weight in water (Hoffman 2012). The gels have a great swelling capacity and do not dissolve in the liquid (Jen et al. 1996). Hydrogels are hydrophilic polymer networks, that can contain large amounts of water, yet the crystalline regions, crosslinks or entanglements do not allow the gel to dissolve (Bell & Peppas 1995). Hydrogels can find their function in the use of water permeable membranes with filtering pores or as supports for drug delivery or close-contact human body implants, because of their biocompatibility (Bell & Peppas 1995). The mesh size of the pores can be influenced by the amount of crosslinks (Bell & Peppas 1995). The hydrophilicity of the polymer networks is caused by the presence of functional groups at the side of the polymer backbone, being sulfonic acid (R-SO_3H), secondary amide linkage (R-CONH), primary amide linkage (R-CONH2), carboxylic acid (R-COOH) and/or hydroxyl groups (R-OH) (Bell & Peppas 1995). The hydrogels can be divided in two groups: neutral and ionic hydrogels.

Neutral hydrogels find their coherence in covalent bindings between the molecules and are therefore more stable than ionic hydrogels that are kept together by electrostatic forces and the stronger ionic bonds (Bell & Peppas 1995). An important predictor for the hydrophilicity of a neutral hydrogel with covalent bindings is the molecular weight of polymer chains between junctions, being physical entanglements, polymer complexes, physical crosslinks or crystalline regions (Bell & Peppas 1995).

Crystalline regions are polymer chains arranged in a more ordered structure (Bell & Peppas 1995). The molecular weight between crosslinks is related with the hydrophilicity and with the size of the polymer network (Bell & Peppas 1995). The relation between the molecular weight between crosslinks and the other variables offers options for increasing the mesh size of the polymer. From the correlations stated in the reported study (Bell & Peppas 1995) the mesh size of the polymer network can be increased by increasing the molecular weight of the polymer, choosing a polymer with a higher specific volume, a higher fraction of volume polymer and a lower value of the volume of the swelling agent.

The neutral hydrogels have a different swelling behavior than the ionic hydrogels. The ionic hydrogels often have a magnitude higher swelling capacity than neutral hydrogels and are influenced by the environment (Bell & Peppas 1995). Ionic hydrogels are formed by charged groups in the polymer chains that force counter ions from the solution to move into the gel. This double layer of ions causes a higher amount of counter ions inside of the gel than in the swelling agent, resulting in an electric charge between the gel and the swelling agent and forcing water molecules to move in the gel (Bell & Peppas 1995). The swelling capacity is influenced by polar forces, hydrogen bonds and electrostatic forces, as well as the charge repulsion and attraction between molecules in the gel and between the molecules inside and outside of the gel (Bell & Peppas 1995). The pH can influence the ionization of the charged functional groups in the polymer chain, resulting in a change of repulsion forces within the gel (Bell & Peppas 1995). For example, a polymer chain with anionic functional groups will be ionized for a higher pH than its pK_a, resulting in higher ionic forces within the gel at higher pH. The ionic forces within the gel are translated to a higher hydrophilicity, leading to a higher swelling capacity of the ionic hydrogel. Other factors that increase the swelling of the gel are: higher ionic content, lower concentration of the swelling agent and a higher charge of counter ions (Bell & Peppas 1995).

Lastly, there are gels that are not defined as hydrogels. The sol-gel or “solution gelation” method is the method of settling nanoparticles on an existing substrate forming a gel by partial evaporation or the addition of an initiator (Mendez et al. 2009). Sol-gels are mesoporous gels that have a pore size range between 2nm and 50 nm (Owens et al. 2016). The majority of sol-gels nowadays are produced with tetraethyl ortosilicate, which can form varieties of three-dimensional structures depending on the reaction parameters, such as temperature, additives and pH (Owens et al. 2016). Sol-gels are typically formed by metal oxides, usually metal alkoxides, that react in a condensation reaction with each other to form polymers (Mendez et al. 2009). The polymerization can occur in mild conditions, making the gel applicable for uses in microbiology (Owens et al. 2016).

cel-gel immobilization techniques

Hydrogels can find their use in the immobilization of mammalian cells by matrix entrapment, adhesion and micro encapsulation (Jen et al. 1996). These three methods are distinguishable by the goals they aim to achieve, having different gels that are more appropriate to the fulfillments of the gel. The matrix entrapment method focuses on stabilizing the cells in a matrix of polymers, while the adhesion method allows cells to migrate on a surface and proliferate (Jen et al. 1996). The matrix entrapment method is useful for the immobilization of cells for wastewater treatment, since the cells are stabilized inside of the matrix.

Membranes can be categorized as having macropores (pore width > 500 Å), mesopores (between 20 and 500 Å) and micropores (less than 20 Å) (Sing et al. 1985). Pores can also be formed by non-porous membranes through polymer chains (Bell & Peppas 1995).

Micro encapsulation provides a thin microporous membrane (layer width of 10 µm to 100 µm) around a number of cells, protecting – in animal bodies – the cells from immune system responses, such as immunoglobulin proteins, with at the same time still enabling the transport of nutrients, oxygen and waste material through the membrane (Jen et al. 1996). In practice the membranes are tailored in such a way to have a molecular weight cut-off between 50,000 and 100,000 Da (Jen et al. 1996). For the encapsulation of bacteria used for wastewater treatment applications, the protection against immune-response proteins is irrelevant, but genetically modified bacteria cells should be stopped from escaping to the environment. The gels with micro encapsulation can therefore offer options in the stabilization and prevention of cell leakage from genetically modified bacteria to the environment.

The effective diffusion of solutes (D_{eff}) through membranes between 0.1 and 1 µm pore size is positively affected by the diffusion of the solute in pure solvent (D_{iw}), the porosity of the membrane (ε), a partition coefficient that describes the arrangement of the solute through the membrane (K_p) and negatively affected by an irregular structure of the membrane, tortuosity (τ) (equation 1).

$$D_{eff} = D_{iw}\frac{\varepsilon K_p}{\tau}$$

Equation 1: diffusion of solutes through membranes between 0.1 and 1 µm (Peppas & Nicholas 1987)

Secondly, the diffusion (D) in the membranes between 5 to 20 nm divided by the diffusivity in bulk solution (D_∞) is positively correlated with the solute radius divided by the pore radius (λ) (equation 2).

Therefore, the diffusion is positively correlated with a bigger pore size and negatively correlated with a bigger solute size. Logically, the diffusion cannot happen if the λ<0, having a bigger solute radius than the pore radius.

$$\frac{\boldsymbol{D}}{\boldsymbol{D_\infty}} = \mathbf{1} - (\mathbf{1.125})\boldsymbol{\lambda ln\lambda^{-1}} - \mathbf{1.539}\boldsymbol{\lambda} + \boldsymbol{O}(\boldsymbol{\lambda})$$

Equation 2: diffusion of solutes through membranes between 5 to 20 nm (Peppas & Nicholas 1987)

In polymer networks the diffusion over the membrane (D_p) divided by the diffusion in pure solvent (D_s) is positively related to the free volume of solvent in the membrane ($V_{f.1}$), the degree of hydration of the membrane in gram solvent per gram of swollen polymer (H) and negatively affected by the cross-sectional area of the diffusing molecules (q_s), (equation 3). A decrease in the diffusing molecule's size, a higher hydration of the membrane and a higher volume of solvent in the membrane will lead to a higher diffusion across the membrane.

$$\frac{D_p}{D_s} = \exp(-B(q_s/V_{f.1})(1/H - 1)$$

Equation 3: diffusion of solutes through a polymer network (Yasuda et al. 1969)

2.4.2 polyacrylamide gel

chemical properties

Polyacrylamide gels are part of the neutral gels, having covalent bonds between the acrylamide monomers and the cross linker bisacrylamide monomers, forming a dense polymer network (Grattoni et al. 2001). The gel is hydrophilic because of the ionic organic groups at the side of the polymer chain (Grattoni et al. 2001). The mesh formed by the polymer is of a very small size and entangles the water molecules (Grattoni et al. 2001). Polyacrylamide is neurotoxic, mainly in the long nerves of the peripheral nervous system, for many mammals (Johnson et al. 1986). Polyacrylamide is used in wastewater treatment for the polymer chains increase the viscosity of the water, capturing small particles in its chains (Johnson et al. 1986). Besides wastewater treatment, the gel, in different concentration, is used for different electrophoresis gels (Grattoni et al. 2001).

polymerization

A polyacrylamide gel is formed as a polymerization of acrylamide monomers with N,N′-methylenebisacrylamide as cross linker (figure 4). The polymerization follows a vinyl addition polymerization by free radicals (Menter 2000). The vinyl group, CH_2=CH- , is the main part that reacts as an addition reaction to form a polymer (Menter 2000). In a free-radical polymerization an initiator reacts with a monomer and these two molecules start sharing an electron, following the propagation phase in figure 4. The extra electron of the monomer is expressed at the other end of the monomer chain. This free electron at the other end of the chain can react with another monomer to form a dimer (Roberts & Caserio 1977). Again an electron is free at the other end of the dimer and another monomer can be added until a polymer is formed (Roberts & Caserio 1977). This phase of the polymerization is called the propagation and in theory stops when two propagating molecules react, having an initiator at both ends (Roberts & Caserio 1977). In the case of an acrylamide and bisacrylamide gel polymerization, ammonium persulfate, $(NH_4)_2S_2O_8$, acts as the initiator. The initiator of the reaction, the free radical, is the persulfate ion, that is formed after the ammonium persulfate is added to water. Hence, to form the acrylamide polymer, the acrylamide monomer is needed, as well as the ammonium persulfate.

To increase the rate of conversion from monomere to polymere, TEMED, N, N, N', N'-tetramethylethylenediamine, is added to increase the amount of free radicals created by the ammonium persulfate. A mixture with TEMED, but without APS, would not result in a free-radical reaction (Rempp & Merrill 1991).

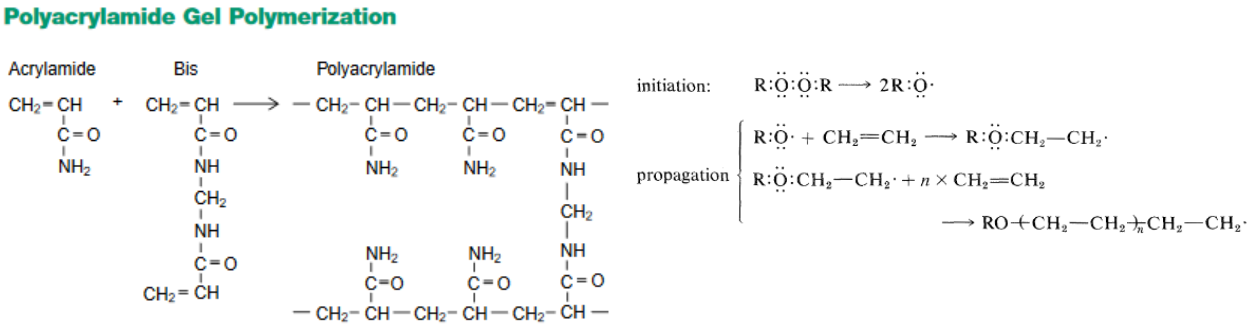

Figure 4: the polymerization of acrylamide with bisacrylamide as cross-linker on the left (Menter 2000) with on the right the basics of a free radical polymerization, represented by the polymerization of an ethene monomer (Roberts & Caserlo 1977)

TEMED forms a redox couple with ammonium persulfate and the reaction between the chemicals creates more free radicals than ammonium persulfate alone (figure 5).

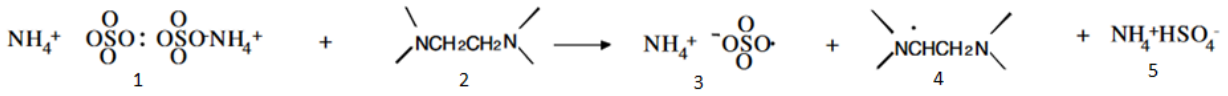

Figure 5: mechanisms of the free radical generating redox reaction with ammonium persulfate (1) and TEMED (2), reacting to ammonium bisulfate (5) and free radicals (3&4) (Rempp & Merrill 1991)

This redox reaction with ammonium persulfate and TEMED generates two free radicals (figure 4). During the polymerization heat is produced, because the reaction from monomer to polymer is exothermic (Rempp & Merrill 1991). The conversion of monomer to polymer is dependent on the initiator concentration, environmental factors such as temperature and a constant rate (Rempp & Merrill 1991). These influencing factors can be summarized with the group constant, G, and the conversion rate over time (t) can be predicted (equation 4).

$$Conversion: \frac{([M_0] - [M])}{[M_0]} = 1 - e^{-Gt}$$

Equation 4: conversion of monomer to polymer over time, with the monomer concentration at time step zero [M_0], the monomer concentration at a specific time [M], the group constant (G) and the time (t) (Yasuda et al. 1969)

In average lab conditions, the polymerization of polyacrylamide is fast, around thirty minutes, and the conversion is more than 95% (Rempp & Merrill 1991, Menter 2000). Polyacrylamide gels are commonly used for different lab techniques like gel electrophoresis, because of their fast polymerization (Rempp & Merrill 1991). The optimal temperature for the polymerization is between 23 and 25 degrees, for at zero and four degrees, the gel is more porous, rigid, inelastic and less transparent (Menter 2000). The role of bisacrylamide, N,N′-Methylenebisacrylamide, monomers in the polymerization of gels, is crosslinking the acrylamide polymer chains. These bisacrylamide monomers have a bigger molecular weight and have two vinyl groups that can enter the addition reaction to form an interconnected network by copolymerization (figure 3). The amount of bisacrylamide in proportion to the acrylamide monomer influences the pore size of the gel and the bisacrylamide is soluble in small proportions in water (National Diagnostics 2016).

Some slight varieties of bisacrylamide that are more strongly water soluble can be used to create a water soluble hydrogel (National Diagnostics 2016). Two of these water soluble cross linkers are N,N'-bisacrylylcystamine (BAC), and N,N'-diallyltartardiamide (DATD) (National Diagnostics 2016).

2.4.3 acrylate gels

poly(2-hydroxymethacrylate gel)

Poly(2-hydroxymethacrylate gel) (pHEMA) gels are neutral hydrogels that can be formed by a free radical generated addition reaction of the monomers to form a polymer (figure 6). The gel is hydrophilic with its many hydroxyl groups within the chain and between different chains (figure 6). Its soft consistency allows small molecules to pass through the gel and it is is often used in living tissues (Achilias & Siafaka 2017). The free radical polymerization is exothermic and releases heat (Achilias & Siafaka 2017). As cross linker normally dimethacrylates are used, such as ethylene glycol dimethacrylate (EGDMA) and diethylene glycol dimethacrylate (DEGDMA) (Achilias & Siafaka 2017). The gel is known to absorb lead and copper ions in its structure and it is adhesive to glass (Achilias & Siafaka 2017).

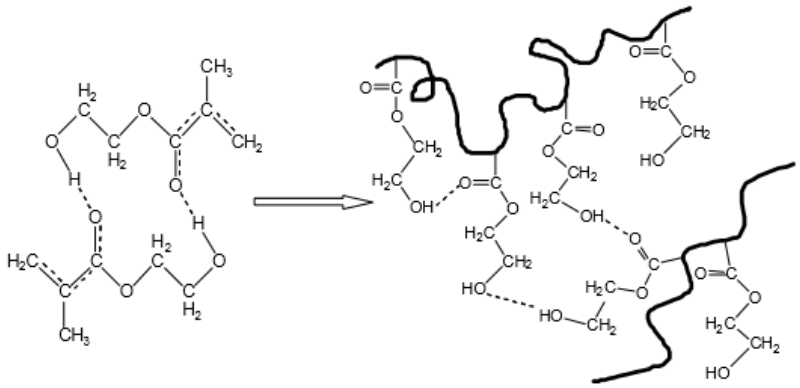

Figure 6: the representation of the polymerization from pHEMA to pHEMA, showing the hydrogen bonds that are present between the C=O group and the –OH group and the hydrogen bonds between the hydroxyl groups. The black line represent chains of the polymerized monomers (Achilias & Siafaka 2017).

polyhydroxyethylacrylate

The polyhydroxyethylacrylate (pHEA) gels are part of the acrylate group, are neutral hydrogels and are slightly different from the methacrylate gels, having a stronger exothermic reaction during the polyermization (Vargün 2003).

The gels react in a free radical polymerization, opening up the C=C double bonds of the monomer, resulting in an addition reaction (Vargün 2003). The reaction is slightly inhibited by oxygen and the reaction shows autoaccelaration, meaning that after the start of the reaction, the reaction rate increases (Vargün 2003). The pHEA gels are hydrophilic because of anionic groups in the side chains. The gel has a poor mechanical strength, but the consistency can be improved by crosslinking (Vargün 2003).

sodium polyacrylate

Sodium polyacrylate consists of a polymer chain of sodium acrylate monomers (Hua & Qian 2001). Sodium molecules are bound to the carboxyl group in the chains (Ewecharoen et al. 2009). The polymer swells because of the osmotic pressure that is present due to the sodium in the network. The swelling of water can be up to 200 times and up to 1000 times its own weight, if the gel is self-polymerized with irradiation (Hua & Qian 2001). Calcium chloride and other salts release cells from the gel because they reduce the osmotic pressure in the gel (Hu et al. 2004). Sodium polyacrylate is able to adsorb nickel to its structure, showing a 44.1mg nickel uptake per gram of sodium polyacrylate in a concentration of 145 gram per liter of contaminated water (Ewecharoen et al. 2009). In an experiment with copper and iron solutions, the sodium polyacrylate gels showed a complexion of iron to its matrix (Leth et al. 2002).

2.4.4 alginate gel

polymerization

Unlike the previous gels, the alginate gel is formed by ionic bonds and not covalent bonds and is an ionic hydrogel. The monomers come from algae and they are composed of mannuronic acid (M) and guluronic acid (G), forming a chain with counterions in its “gaps” (figure 7).

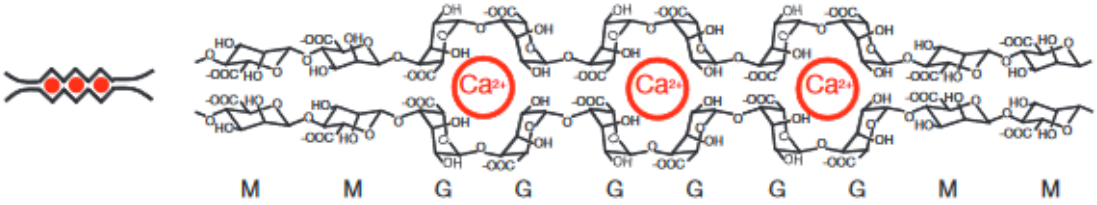

Figure 7: the chemical structure of alginate chains, composed of mannuronic acid (M), guluronic acid (G) and calcium counterions (Ca^{2+}) (Sun et al. 2012)

These "gaps", where the calcium (Ca^{2+}) counterions are located, come to be through the angle of covalent bindings between guluronic acid and mannuronic acid (Sun et al. 2012). Anionic functional groups are present along the alginate chain, having two ethanol groups in the counterion binding site that can form an ionic bond (figure 7). The alginate forms a network, because the Ca^{2+} are distributed throughout the solution (Sun et al. 2012).

production of alginate

Alginate is a product from seaweed and undergoes a series of processes before it can be used in the laboratory (figure 1 appendix). The seaweed has to be granulated into small-sized pieces in order to allow the chemicals to have a bigger reaction surface on the granules, after which the alginate has to undergo several steps to remove the unwanted residues (McHugh 1987). In the seaweed the alginate is present as natural polymer, polymerized with positive ions, mainly calcium ions (McHugh 1987). Via a reaction with sodium ions the alginate forms a salt with these sodium ions and undergoes acidification (McHugh 1987). With the use of formaldehyde some unwanted phenolic compounds form insoluble products that can be removed (McHugh 1987). The alginic acid that follows, is processed by an alkaline treatment with temperatures ranging from 50 until 95 degrees in order to break some of the bonds between the alginate's uronic acids to improve the solubility (McHugh 1987). The non-soluble products can be removed by flotation of these products by a flocculant such as polyacrylamide, after which these floccules can be removed by filtration (McHugh 1987). Too concentrated amounts of alginate will lead to the formation of a gel, used in this research, but useless in the purification step. Using an acid, the alginate can be transformed into a sodium salt (McHugh 1987). In this manner the alginate polymers in seaweed are produced, using chemicals, such as acids, formaldehyde and polyacrylamide.

2.4.5 gel modifications

polyethyleneglycol

Polyethyleneglycols are polymers of ethylene units with a hydroxyl functional group ($-CH_2-CH_2-OH$) on one side. However, when the monomers are polymerized in the presence of water the reactivity of the hydroxyl group becomes very small, due to the size of the polyethers. For poly(*N*-vinyl pyrrolidone) (PVP) polymer hydrogels, polyethyleneglycols (PEGs) can increase the water mobility inside of the gel (Thomas et al. 2014). Poly(*N*-vinyl pyrrolidone) is a commonly used water soluble polymer (Thomas et al. 2014). The PEG chains that can be implemented in different polymers can form hydrogen bonds with the carboxyl groups of the PVP and this process is thought to occur directly via water molecules (Thomas et al. 2014). The PVP macromolecules are cross linked by the PEG via the hydrogen bonds, which forms big spaces in between where water can pass through (Thomas et al. 2014). In this way the water mobility of the gel is improved.

PEG can also be copolymerized with several acrylamides by a free radical polymerisation (Meldal 1992). In this research the combination of the PEG and acrylamide monomer polymerization by free radicals was performed in an attempt to increase the water mobility.

glutaraldehyde

Protein-protein interactions can be established by crosslinking agents. An important and commonly used crosslinking agent is glutaraldehyde (Kapoor 1996). Glutaraldehyde is a homo-bifunctional reagent, reacting with the primary amide groups of proteins and hence forming covalent bonds between them(Kapoor 1996). Furthermore, homo-bifunctional reagents are characterized by their solubility in water and the covalent binding between molecular subunits and within subunits (Kapoor 1996).

sodium silicate

Treating alginate beads with tetramethoxy-ortho-silicate resulted in an encapsulation of enzymes (Heichal-Segal & Earnshaw 1995). Silicate coatings are most commonly produced by tetramethoxy-ortho-silicate, but can also be synthesized by using sodium silicate **(**Ramachandran et al. 2010). The silicate polymers are not hydrogels, but sol-gels. These gels form mesoporous sized membranes that can be used for cell leakage reduction. In a research that used alginate beads with a silicate coating, the beads were left in 30mM sodium silicate for two hours and tested for cell escape and amount of beads fractured

(Ramachandran et al. 2010). The amount of colonies that had escaped in the alginate gel with silicate coating, was very low (figure 8).

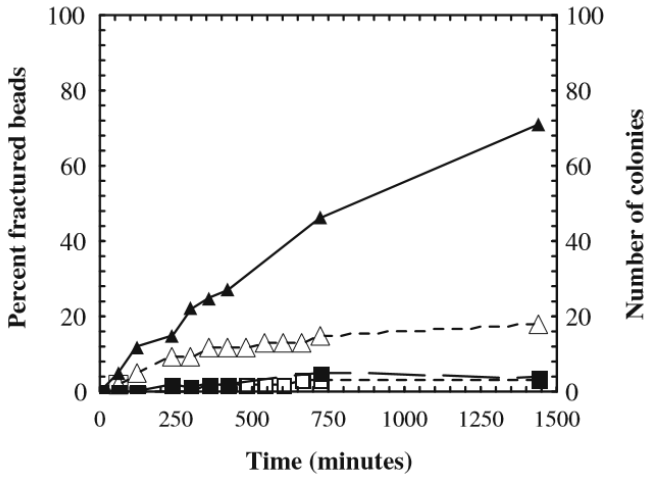

Figure 8: the percentage of fractured beads (open symbols) and number of cell colonies (filled symbols) over time for sole 1.5% alginate beads (triangle) and 1.5% alginate beads with a silicate coating (square) (Ramachandran et al. 2010)

3. gel development

3.1 view of the work plan

An overview of the work that had been done was summarized in figure 9. First of all, the mutated SCII24 bacterium strain of *O.tritici* was tested in different gel supports: polyacrylamide, polyhydroxyethylmethacrylate (pHEMA) and polyhydroxyethylacrylate (pHEA) gel. These polymers are indicated as the first generation gels. The generation I gels were tested for their ability to allow cell survival, cell leakage and water permeability. In the meantime, an arsenic accumulation experiment with rice plants was set up and the work involved in growing the plants was combined in parallel with a science project in a secondary school: the Phytofix project. The students prepared a set of plant samples for the assays of the arsenic uptake by *Oryza sativa* and presented the project in a science fair. The generation II gels were gels that were able to let through water in contrast to the generation I gels. These gels were further developed and tested in a final experiment to see their potential as a bioremediation tool. The aim of these series of gel experiments was to create a gel support that had the following characteristics:

- viable cells inside of the gel

- water permeability
- diffusion of nutrients from the medium to the gel
- good structural stability of the gel
- entrapping the cells and evasion of cell leakage

During the development of the second generation gels, a plant experiment was set up to see effects of the inoculation of *O.tritici* double mutant in *O.sativa* on the bioremediation potential of the plants.

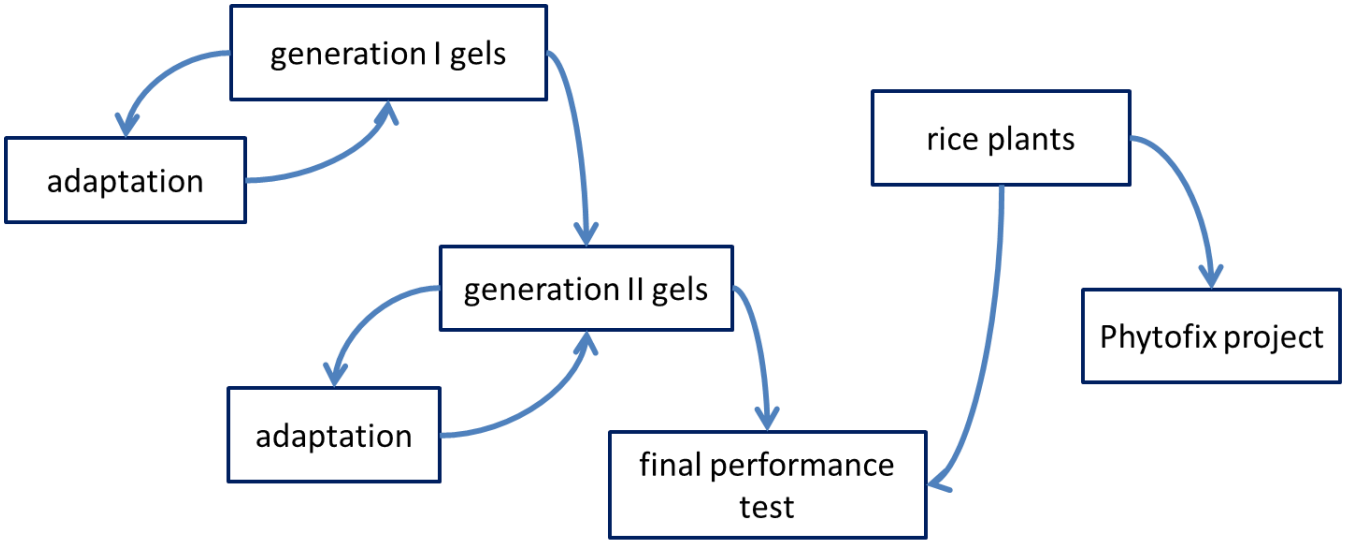

Figure 9: the process of the gel development and the final experiment

3.2 experiments for parameters determination

3.2.1 effect of the monomer and initiator concentration on cell survival in polyacrylamide gels

introduction

From the generation I gels, the bis- and acrylamide gels were the first gels tested.

Acrylamide gel is commonly used for gel electrophoresis and now and then it is employed for immobilization of bacteria (Cassidy et al. 1996). In this experiment the effect of monomer amount and free radical generator (ammonium persulfate) on the amount of bacterial cells was tested.

materials and methods

The bacterium was grown in Luria Broth (LB) medium with a gentamycin concentration of 0.1% and a hygromycin concentration of 0.2%. The suspension was left to grow overnight in a shaker between 30 ˚C and 37 ˚C at 100 rpm to 150 rpm (Agitorb 200, aralab, Portugal). The bacterial growth was determined measuring in the optical density (OD) at 600 nm (Model Evolution 260, ThermoFisher Scientific, Madison, USA). The next day the bacterial suspension was centrifuged for fifteen minutes at 4000 rpm (Centrifuge 5810 R, eppendorf, Hamburg, Germany). The growth medium was discarded and the cells were resuspended in twenty milliliters of 10X diluted LB medium. For the first experiment, four milliliter polyacrylamide gels were made following protocol A in the appendix. In order to test the effect of monomer quantity, another two gels of four milliliters were made following protocol A, except for having 7% bis-/acrylamide solution (6.77% acrylamide and 0. 231% bisacrylamide) instead of the standard 10% bis-/acrylamide solution.

For the second experiment, testing the effect of ammonium persulfate, three gels were made following protocol A in the appendix, but the amount of ammonium persulfate differed: 0.7%, 1.5% and 3%. For both experiments a control was added with no bacteria, 10% bis-/acrylamide solution and 1.5% Ammonium persulfate. Additionally, two bacterial starting concentrations were used of 0.1 and 1 OD. The gels were prepared in a 10 milliliter syringe with the small side burned off, after which they were transferred to 50 mL tube with thirty milliliters of 10X diluted LB medium. The tubes were incubated at 37 ˚C. At the beginning and after seven days the amounts of cells were estimated using the spread plating technique, described in protocol B in the appendix. Of the 7% and 10% polyacrylamide gels microscopic photos were made by scanning electron microscopy following protocol D in the appendix.

Results

The controls did not show any cell growth after seven days. The 10% polyacrylamide gel with 1 OD and the acrylamide 7% with 0.1 OD had their first time interval an uncountable number of cells and their numbers were taken for 10^3 CFU/ml to 10^5 CFU/ml in the solution. Results suggested a negative response in cell growth to a higher concentration of APS used for the polymerization and a slightly lower cell growth response towards higher concentrations of the polymer and cross linker (figure 10 and figure 11). The

amount of colonies inside and outside of the gels was similar, during the start of the monomer testing experiment (figure 11). The absolute amount of colonies was higher outside of the gel than inside of the gel after a week (figure 11). The bacterial strain was inoculated in the gel and the bacteria were present in the medium immediately after dispersion of the gel, meaning that the gels allowed cells to escape. In addition, for the 10% polyacrylamide gel with an optical density of 0.1 OD, the 7% polyacrylamide gel with an optical density of 1 OD and the 10% polyacrylamide gel with an optical density of 1 OD, the amount of colonies was higher outside of the gels than inside of the gels, indicating either a higher growth in the liquid or a migration of cells from the inside of the gel towards the outside. A smaller amount of acrylamide monomer and cross linker indicated a slightly positive effect on the amount of bacteria inside of the gel, having a higher average of colonies in the 7% acrylamide than in the 10% acrylamide gels (figure 11). After two weeks' time, the gel showed in general micro colonies instead of macro colonies (table 1). Furthermore, gels starting with two different initial optical densities, did not show big differences in terms of bacterial cells inside the gel (figure 11).

Table 1: the topologies of the colonies after fourteen days in a 7% bis-/acrylamide gel and a 10% bis-/acrylamide gel at densities of *O.tritici* double mutant of 0.1 OD and 1 OD. Some colonies were shown to form microcolonies (Mic) and others normal sized colonies (Mac).

gel and medium (t=14)	7% monomer/crosslinker				10% monomer/crosslinker			
starting bacterial concentration	0.1 OD		1 OD		0.1 OD		1 OD	
phase	gel	liquid	gel	liquid	gel	liquid	gel	liquid
colony characteristics	no cells	mic & mac	mic	mac	mic	mac	mic	mic & mac

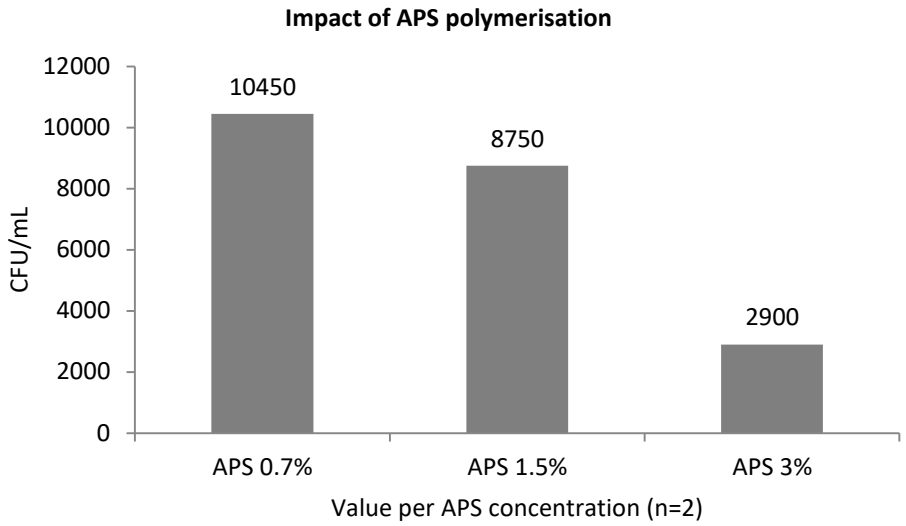

Figure 10: the amount of cells of *O.tritici* double mutant for different concentrations of ammonium persulfate (APS) used for the gel polymerization.

Besides plating, the presence of cells and organic material was confirmed outside and inside of the gels by SEM (figure 12).

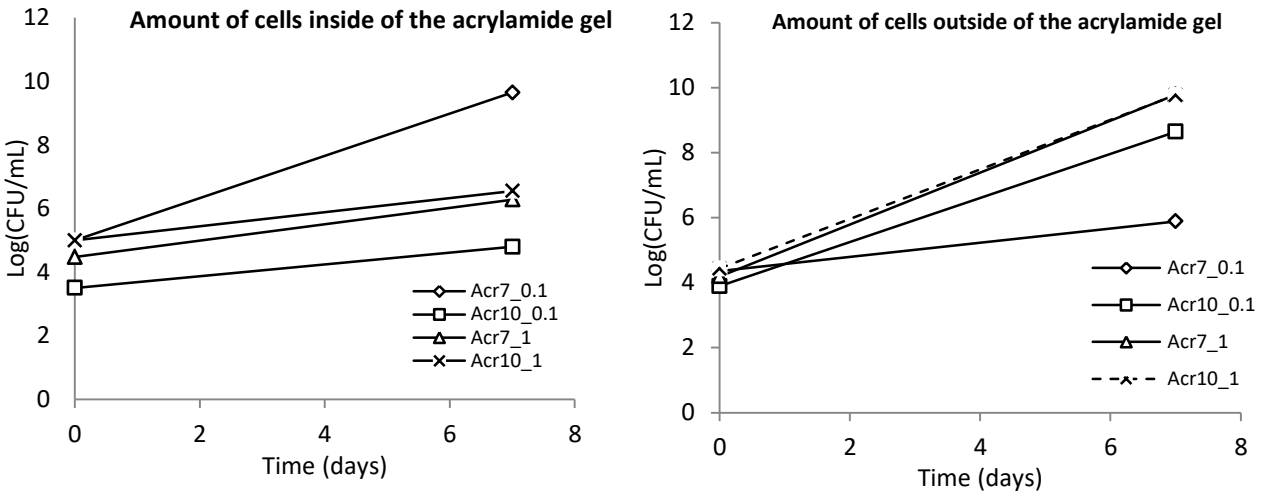

Figure 11: left is the amount of cells of *O.tritici double mutant* over time inside of the polyacrylamide gel, while on the right the amount of cells in the medium outside of the gel is displayed. The cells were put in a 7% acrylamide gel at concentrations of 0.1 OD and 1 OD (Acr7_0.1 and Acr7_1) and in a 10% acrylamide gel with the two different densities (Acr10_0.1 and Acr10_1).

The difference in amount of bacterial cells for different monomer and initiator concentrations could not be tested for significance due to a small sample size, but especially the difference in colony numbers for the different APS concentrations was high, having a difference of 72% between the gel polymerized with 3% APS and the gel polymerized with 0.7%. However, A higher number of cells was expected inside of the polyacrylamide gels compared to the liquid medium outside of the gels, because the cells will sink to the bottom in the liquid, whereas the cells in the gel would be kept in place. The tubes were not shaken and there was no expected higher amount of cells in the gels.

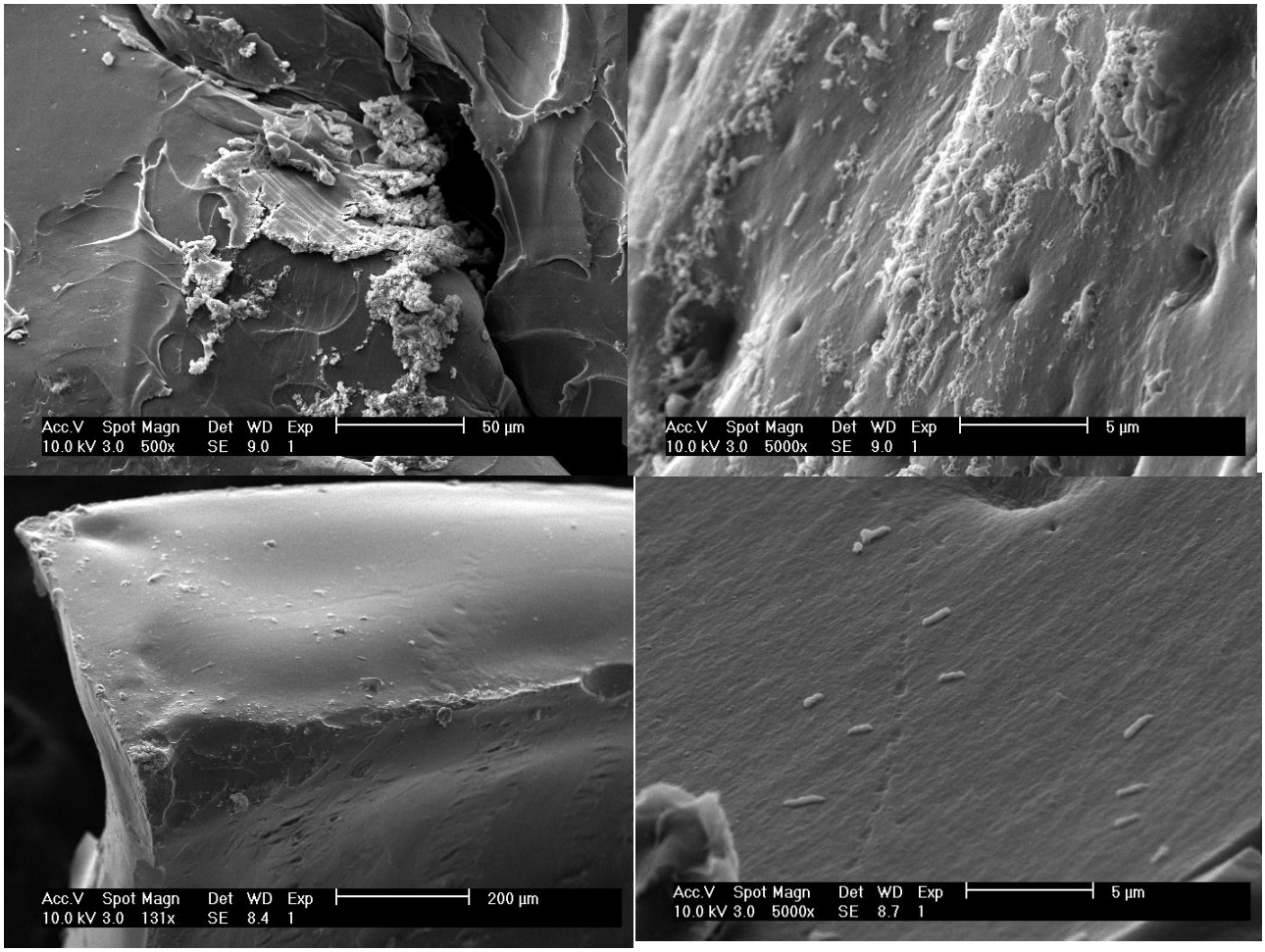

Figure 12: scanning electron microscopy (SEM) micrographs of the exterior of 10% polyacrylamide gel (left up), the interior of 10% polyacrylamide gel (left down), the interior of a 7% polyacrylamide gel (right down) and the exterior of a 7% polyacrylamide gel (right up).

3.2.2 determination of cell survival in pHEA and pHEMA gels

introduction

Other polymer hydrogels that were tested, were poly-2-hydroxyethyl methacrylate (pHEMA) and polyhydroxyethylacrylate (pHEA). These gels were chosen based on reported works for cell encapsulation, in order to confirm if *O.tritici* double mutant would be able to proliferate in the pHEMA and pHEA gels (Degiorgi et al. 2002).

materials and methods

A bacterial suspension was prepared as described previously. The pHEMA and pHEA gels were made following protocol A in the appendix with a bacterial starting concentration of 0.1 and 1 OD. The gels were prepared in a syringe with two open ends, transferred to 50 mL tubes, and suspended in 30 mL of 10X diluted LB medium. The tubes were incubated at 37 ˚C. The gels and medium were plated at time 0, 7 and 20 days following the protocols D and E in the appendix.

results

For the uncountable numbers of cells the data points were left out and at three initial points of the HEMA 0.1, HEA 0.1 gels inside of the gels and the HEMA 0.1 gel in the medium the starting bacterial concentration was taken at 99 cells for zero cells at a dilution of 10^{-2}.The amount of cells inside and outside of the gel increased considerably (figure 13). As expected, inside of the gel the bacterial inoculation of 0.1 OD had a lower start value than the 1 OD gels. Both the pHEA gel with 1 OD and the pHEMA gel with 0.1 OD showed a rapid expansion of cells over the two weeks' time, inside as well as outside of the gel. After two weeks the colonies looked smaller. Similar to the acrylamide gel, the initial optical density did not seem to affect the amount of cells present after the start of the experiment. The pHEMA and pHEA gels were not able to keep the bacteria inside. The colonies measured after twenty days showed many small colonies in both gels.

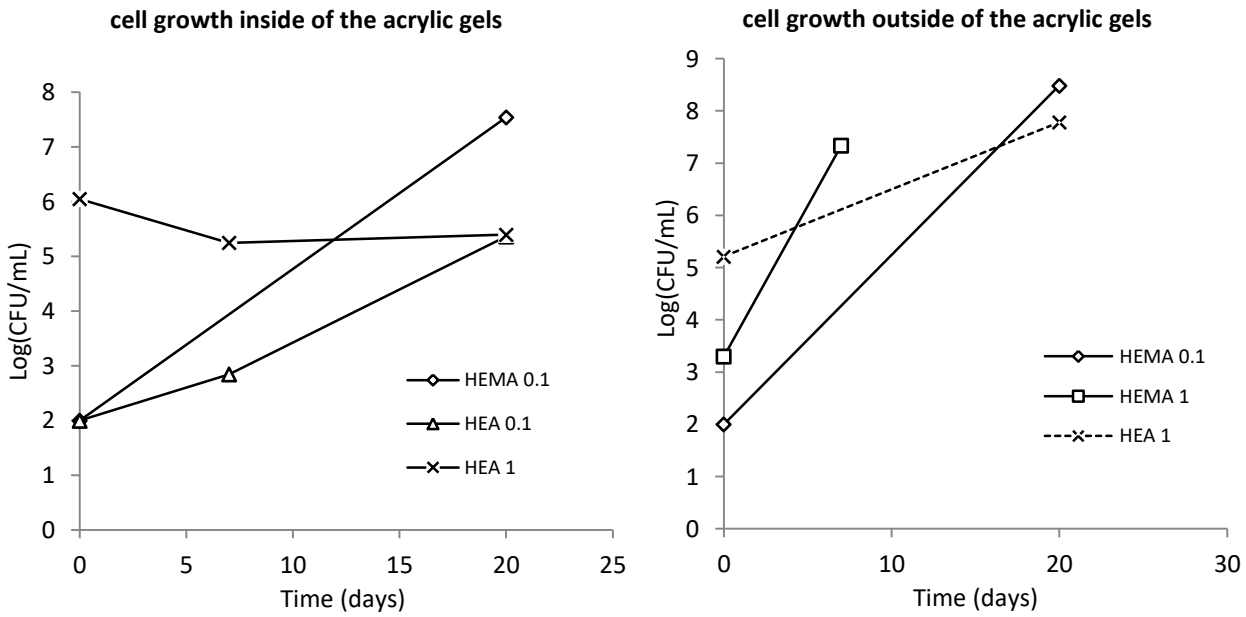

Figure 13: the cell growth was examined inside and outside of the HEMA and HEA gels of *O.tritici* double mutant over time with a starting concentration of 0.1 and 1 OD. After seven days the HEA 1 gel showed no cells for the used dilution of 10^{-2} (dotted line).

3.2.3 preparation of the alginate gels

introduction

Alginate gel was tested as a support for cel immobilization. Some factors that could affect the cell survival and the efficiency of the process were tested, such as the mode of preparation of the beads (using syringe needles or a pipette) and the presence of another gel around the beads (i.e. polyacrylamide gel).

materials and methods

O.tritici double mutant was grown and 4mL of bead mixture was prepared for two 2.5% alginate gels and 2mL for the acrylamide support gel. The beads had 1 OD bacterial suspension and the mixture of Protocol E in the appendix was used. The mixture was either pipetted several times with a pipette of 200 µL or with a syringe with a 0.8 mm needle. The mixture was pushed out in 15 mL 10% $CaCl_2$ solution with a stir magnet. After leaving the beads for fifteen minutes, the beads were washed with PBS and left in 1% glutaraldehyde for one hour. Consequently, the beads were washed with MilliQ ultra-pure water. One set

of beads was added to a 2mL 10% polyacrylamide gel, prepared following procedure C in the appendix. All the gels were dispersed in 15mL of 10X diluted LB medium and incubated at 37 ˚C. After four days, the medium and the gels were plated following protocols D and E in the appendix.

results

There were cells present in all of the gels and for the pipetted gel and the beads in polyacrylamide, less cells were present in the medium. More cells were present in the gels that were prepared by either a needle or a pipette (table 2). The bacteria survived in the small needle of 0.8 mm. The preparation with a pipette and the needle technique bore similar number of cells in the gel. The amount of cells in the medium of the needle prepared beads was higher than in the pipetting technique. The higher amount of cells in the medium could have resulted from the fact that smaller beads have a larger total surface and henceforth enable more cell migration to the liquid medium. The polyacrylamide gel was not able to prevent the cells from escaping to the medium and the amount of cells inside of the beads was not higher.

Table 2: the amount of *O.tritici* double mutant cells present at t=0 for different preparation methods of alginate. For the pipetting a volume of 200 µL was used. The last method embedded the beads in 10% bis-/acrylamide gel.

gel:	phase:	CFU/mL
- needle of 0.8 µm	gel	$1.21x10^4$
	liquid	$1.26x10^4$
- pipette	gel	$1.24x10^4$
	liquid	$8.1x10^3$
- needle of 0.8 µm - beads in acrylamide gel	gel	$1.03x10^4$
	liquid	$8.2x10^3$

3.2.4 waterflow testing

introduction

The polyacrylamide, pHEA and pHEMA gels were tested for their capability of water permeability and diffusion of nutrients from the medium into the gel. Polyacrylamide gels form fine networks of areas with high cross linker densities and less dense cross linker densities (Grattoni et al. 2001). Small percentages of

polyacrylamide are able to pass through water due to bigger less dense cross linker areas, in contrast to the mesoporous structure of the gels with higher amounts of polymer (Grattoni et al. 2001). It was expected, however, that lower percentage of monomer of pHEMA and pHEA gels would allow water to pass through.

materials and methods

Four different gels were made in a non-sterile environment. Their preparation was done inside of a syringe with two open ends. The following gels were made: a 10% bis-/acrylamide gel, a 7% bis-/acrylamide, a 4% bis-/acrylamide, a 2% bis-/acrylamide, a 7% HEA, a 4% HEA and a 12.5% HEMA gel. The gels were made following procedure C in the appendix, only varying the amount of monomer and cross linker. For the pHEMA gel the amount of HEMA and EGMA used was higher than the other gels (12.5%), because the consistency of lower amounts of monomer would result in a fragile structure. The gels were polymerized in syringes with two open ends and were integrated in a peristaltic pump system (Peristaltic pump - Minipuls® Gilson Inc., Villiers-le-Bel, France). At both ends of the syringe, four layers of Parafilm were attached and perforated with little holes. One side of the syringe was connected to the peristaltic pump system with Parafilm, while the other side of the syringe was secured to an Erlenmeyer with multiple layers of Parafilm. The flow rate was set at 0.1 rpm, resulting in a flow of around 0.038 mL/min. After two hours and the next day the amount of liquid in the Erlenmeyer was measured.

results

The 10% polyacrylamide, 7% polyacrylamide, 4% polyacrylamide, 7% pHEA and 4% pHEA gels did not let any water through the gel, even after increasing the flow pressure of the liquid (table 3). The 12.5% pHEMA gel let the water pass, but via the sides of the gel and not through the gel. The 2% polyacrylamide gel did not polymerize well enough to allow a water-flow through test (table 3).

Table 3: observations of the facilitation of water flow through seven different types of gels.

Gel	Polymerization	Water flow
Acrylamide 10%	After the polymerization the gel is sturdy and a light white color.	No water flow is visible through the gel after some hours, not after a day and even not when the water pressure in high above the gel, when the water is accumulating above the gel.
Acrylamide 7%	After the polymerization the gel seems similar to the 10% gel: sturdy and with a slightly white color.	In parallel with the 10% acrylamide gel, the gel does not let any water through, even not after a day time and with a higher water pressure above the gel.
Acrylamide 4%	The gel polymerizes into a sturdy gel, similar looking to the 7% acrylamide gel.	No water is passed through the gel.
Acrylamide 2%	The polymerized links are not visible and the mixture doesn't stay together.	Consistency very watery, not suitable for a flow-through system
7% 2-hydroxyethyl acrylate	The polymerization results in a gel, that is quite elastic and sticks to the sides of the syringe. It seems very transparent.	The water does not pass through the gel after one day's time and with accumulating water above the gel.
4% 2-hydroxyethyl acrylate	The gel is similar to the 7% monomer version, although less resistant and more sticky.	The gel does not let the water pass.
12.5% 2-hydroxyethyl methacrylate	The mixture polymerizes into a sturdy gel, that is white and not transparent. The gel does not stick very well to the sides of the syringe and shrinks a bit.	Water passes next to gel, along the side of the syringe. The gel is not covering the tube well.

3.2.5 diffusion of nutrients in polyacrylamide and alginate gels

introduction

In addition to the water permeability, the diffusion of nutrients and cells inside of the gels was tested for the acrylamide gels and a new gel, an alginate gel. Acrylamide gels with 4%, 7% or 10% of polyacrylamide did not manage to let the water pass, because of a polymer structure that was too dense. The interaction forces with the polymer network that hinder the water molecules partly to go through, might still enable nutrients to pass through. It was expected that via the pores, nutrients can enter the gel, since in gel electrophoresis tiny particles pass through the gel, may it be with electrical charge. In spite of the presence of electrical forces in gel electrophoresis, polyacrylamide gel might facilitate nutrients to pass through.

materials and methods

A strain was grown of *O.tritici,* strain pCHRGFP2, that was genetically modified and had a *gfp* gene in the plasmid (Branco et al. 2013). Three sterilized Erlenmeyer's were prepared with 10mL of 7% acrylamide, 4% acrylamide and 2.5% alginate gel, following protocols C and G in the appendix. An optical density of 1.0 OD was used for the bacteria. For the polyacrylamide gel a 2.5mL "sandwich layer" of polyacrylamide gel was added and for the alginate gel a 2.5 mL "sandwich gel" of 2.5% alginate gel, following protocols A and E in the appendix, except for replacing the bacterial suspension with 10X diluted LB medium. After the preparation of the gels, 100 mL of 10x diluted medium was added to all the Erlenmeyer's and they were incubated at 30 ˚C at 140 rpm. The Erlenmeyer's were examined after ten days and eighteen days under the UV light lamp and the transilluminator (SVL, VILBER, Marne la vallée, France; transilluminator).

results

After ten days a gradient was visible in the alginate gel of green fluorescent protein produced with bacteria, with the bare eye and under the UV light microscope (figure 14). The acrylamide gels had a light green glow, but no gradient was visible, indicating a bacterial presence, but not a bacterial. The medium of the alginate gels contained bacteria, visible as a green glow, while the acrylamide's mediums did not show any green glow. After eighteen days the same trend was visible, showing again a gradient of bacteria in the alginate gel, except for that the medium of the alginate gel did not have a green glow anymore (figure 14). The 4% and 7% polyacrylamide gels are likely to not have any diffusion inside of the gel from the medium, because no gradient was visible after ten and eighteen days. On the other hand, the alginate gel did show a gradient after these time steps and nutrients are likely to diffuse through the gel. The acrylamide "sandwich" gels seemed to successfully stop the bacterial cells from migrating into the medium, while the pores of the alginate "sandwich" gel allowed bacteria to migrate through.

Figure 14: a gradient of green fluorescent protein produced by the *O.tritici* strain under the UV light microscope

3.2.6 construction of gels with ethylene glycol copolymerization

introduction

The alginate gel showed diffusion, in contrast to the first generation gels, therefore it was needed to introduce some modifications to these initial gels. In this adaption phase of the first generation gels, polyethylene glycol (PEG) was integrated in the polyacrylamide, pHEA and pHEMA gels. Hydrophilic groups in the ethylene glycol chains are expected to allow water with nutrients to pass through the gels, because these ethylene glycol chains are copolymerized in the original chains of monomers and cross linkers (Thomas et al. 2014).

materials and methods

The following gels were prepared following protocol A in the appendix, only varying the monomer/cross linker amount and adding ethylene glycol (PEG). The gels were prepared in a syringe with two open ends in a volume of 5mL and in a fifteen milliliter tube for the 2mL gels. The gels consisted of 10% polyacrylamide, 10% pHEA or 12% pHEMA gels. Before the APS and the TEMED were added, a volume of PEG was added to mixtures, in such a way that the total PEG concentration was 2.5%. The mixtures in the tubes had their polymerize initiators added (APS and TEMED), after which the gels were quickly poured over a lamina until the laminas were completely covered by the mixture. After polymerization the syringe gels and the lamina gels were left in a 100 mL bath of deionized water overnight at 37 ˚C. The next day the gels were removed from the water and used in a peristaltic pump system to test their water permeability (Peristaltic pump - Minipuls® Gilson Inc., Villiers-le-Bel, France). The syringe gels were placed back in the syringe and attached to the machine, while the lamina gels were put on top of a tube, adding water on top to test the water permeability.

results

The lamina gels started floating in the deionized water and were broken in the process of water flow testing and their removal from the water bath. Hence, these gels were not suitable for acting as a water facilitating gel due to their weak structure. The syringe gels did not let water pass in the flow-through system.

3.2.7 cell survival in alginate gels

introduction

In a second phase (second generation gels), alginate gels were tested with and without the addition of sodium polyacrylate powder. Sodium polyacrylate was added to give more swelling capacity to the alginate gel and would henceforth allow more cell colonies inside of the gel.

materials and methods

A 0.1 OD suspension of *O.tritici* double mutant was prepared and 4mL of 2.5% alginate gels were prepared in a syringe with two open ends, following protocol E in the appendix. A total of five gels were made: three gels containing alginate and two gels containing alginate and 0.67% sodium polyacrylate. The gels were transferred to fifteen milliliter tubes and 10 mL of 10X diluted medium was added. The gels were

plated at the start of the experiment, after four days, after seven days and after sixteen days, following protocol C in the appendix.

results

The *O.tritici* double mutant strain proliferated in in the pure alginate gels, as well as in the alginate with sodium polyacrylate combination (figure 15). The alginate gels and the mixture of alginate and sodium

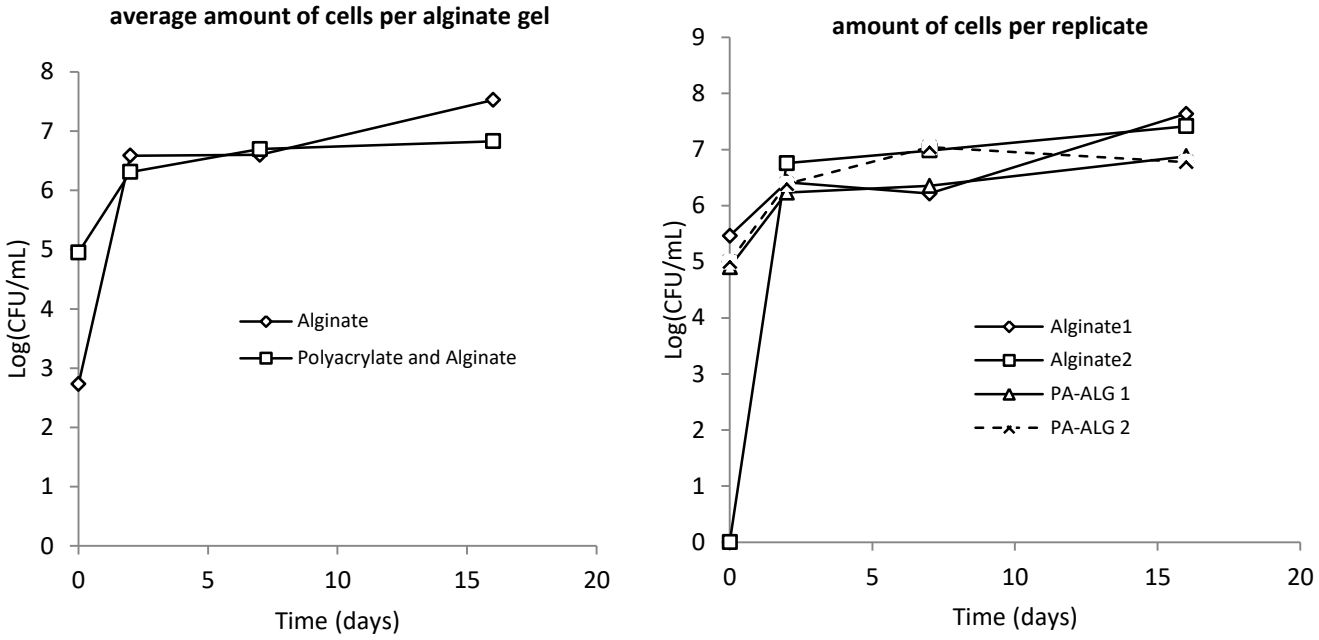

Figure 15: the amount of *O.tritici double mutant* cells over time on average (left) and per replicate (right). The gels used were 2.5% alginate gels and 2.5% alginate gels with 0.67% sodium polyacrylate powder.

polyacrylate gels showed a similar trend in the growth curve as the pure alginate gels, although in the first time interval the amount of cells seemed higher in the alginate with sodium polyacrylate combination and higher in the pure alginate gel after sixteen days (table 4). The initial bacterial growth was higher in the first period and in all the gels the amount of bacterial colonies seemed to stabilize after around four days (table 4).

Table 4: the amount of *O.tritici double mutant* cells inside of the 2.5% alginate gel and the 2.5% alginate gel with 0.67% sodium polyacrylate gel over time.

	Time (days)			
Gel	0	4	7	16
Alginate 2.5% (n=2)	$5.40x10^{2}$	$3.9x10^{6}$	$4.00x10^{6}$	$3.40x10^{7}$
PA-ALG (n=2)	$8.90x10^{4}$	$2.00x10^{6}$	$5.00x10^{6}$	$6.80x10^{6}$

3.2.8 cell growth in sodium polyacrylate gels

introduction

Another type of gel used in the second generation gels was the sodium polyacrylate gel. This gel was tested, based on its high swelling capacity. It was expected that the sodium polyacrylate gel had the capability of supporting bacterial cells, since it was used for cell immobilization (Leth et al. 2002).

materials and methods

A strain of *O. tritici* double mutant was grown and five sodium polyacrylate gels of 15 mL with a bacterial density of 0.1 OD were made in a fifteen milliliter tube. Three gels were made with 0.67% of sterilized sodium polyacrylate powder, from which one control without bacteria. The other two gels had a 1.37% of sodium polyacrylate. The gels were incubated at 37 ˚C and the number of cells was estimated by a plating method following protocol C in the appendix.

results

Cells were capable of growing in the sodium polyacrylate gels .The amount of bacterial cells in the sodium polyacrylate gel strongly fluctuated over time (figure 16). The control gel did not show any cells at the different time intervals. The 0.67% sodium polyacrylate gel showed around 150 million of cells to zero cells inside of the gel; the 1.37% gel fluctuated over around an area of 20 million to 120 million cells.

Table 5: comparing the amount of *O.tritici double mutant* cells over time between alginate (ALG), sodium polyacrylate (PA) and a combination of the two gels (PA-ALG) with for PA 0.67% a too low amount of cells to count in the dilution

	Time (days)			
Gel	0	3;4	7	14;16
PA 0.67% (n=2)	$2.00x10^{7}$	$1.40Ex10^{8}$	-	$1.5x10^{8}$
PA 1.37% (n=2)	$2.60x10^{6}$	$7.10x10^{7}$	$1.20x10^{8}$	$3.20x10^{7}$
Alginate 2.5% (n=2)	$5.40x10^{2}$	$3.90x10^{6}$	$4.00x10^{6}$	$3.40x10^{7}$
PA-ALG (n=2)	$8.90x10^{4}$	$2.00x10^{6}$	$5.00x10^{6}$	$6.80x10^{6}$

The amount of cells in the 0.67% gel did probably not reach zero cells, but low amounts of cells would not have been visible in the dilutions used (10^{-5} and 10^{-6}). When the amount of cells over time was compared between the sodium polyacrylate support and the alginate support, the amount of cells was

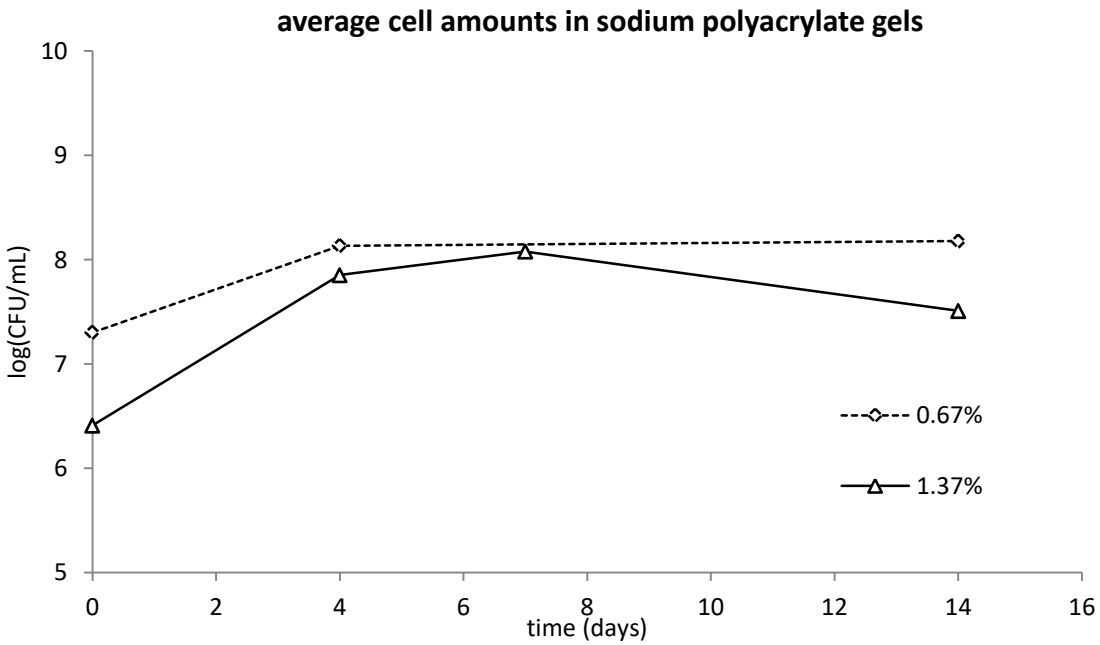

Figure 16 the amount of cells of a *O.tritici* double mutant strain in a 0.67% sodium polyacrylate (n=2) and a 1.37% sodium polyacrylate gel (n=2) with a dotted line indicating an amount of zero cells in the dilution used (10^{-5})after seven days

much higher in the initial phase for the sodium polyacrylate gels (table 5). The number of cells in the alginate gels without sodium polyacrylate, were around a 10x-100x lower than in the 0.67% sodium polyacrylate gels.

3.2.9 cell leakage of alginate, bisacrylamide gels and silicate coating

introduction

Based on results obtained from the alginate and sodium polyacrylate gels, the gels were selected to be coated with sodium silicate in order to prevent cell leakage from the gels. This study was performed in the adaption phase of the second generation gels. Coating alginate beads with sodium silicate solutions creates a homogenous layer of 1µm around the alginate, resulting in a pore size of 10 nm (Ramachandran et al. 2009). The coating of the alginate beads with silicate managed to reduce the amount of bacterial cell that were escaping to the medium (Ramachandran et al. 2009). A study that worked with metal sensing bacteria immobilized in polyacrylamide gels, used gels with only 4% bisacrylamide that showed water permeability (Tantimongcolwat et al. 2014) in contrast to the gels that used polyacrylamide mixtures in this thesis.

materials and methods

Two different experiments were set up. The *O.tritici double mutant* bacterial suspension was prepared with a final optical density of 0.1 OD. The first experiment consisted of attaching gels to the peristaltic pump system (Peristaltic pump - Minipuls® Gilson Inc., Villiers-le-Bel, France) as previously described with a flow rate of around 0.038 mL/min. To test the water permeability of alginate gels and bisacrylamide gels four different gels with a total volume of 4mL were prepared, following protocol A and the aforementioned article (Tantimongcolwat et al. 2014). The bisacrylamide solution was prepared by solving bisacrylamide powder in deionized water at 50 ˚C, leaving the bisacrylamide to solute overnight. Two gels with 4% bisacrylamide concentration were prepared, leaving out the monomer in the protocol A mixture, one 2.5% alginate gel with a silicate coating using a 30 mM sodium silicate bath for two hours and one 2.5% alginate gel with a silicate coating using a 60 mM sodium silicate bath for two hours . The concentration of sodium silicate corresponded to the procedure used in the earlier mentioned research (Ramachandran et al. 2009).

The second experiment used fifteen milliliter tubes with five different gels: one 4% bisacrylamide gel, one 2.5% alginate gel with silicate coating using a 30 mM sodium silicate bath during two hours, one 2.5% alginate gel with silicate coating using a 60 mM sodium silicate bath during two hours and one 2.5% alginate gel combined with 0.67% sodium polyacrylate with a silicate coating using a 30 mM sodium silicate bath during two hours. The gels were prepared according to protocol E in the appendix and the aforementioned article (Tantimongcolwat et al. 2014). The tubes were stored at 37 ˚C.

After one day of incubation, the liquid in the Erlenmeyer's and above the gels in the second experiments were plated following protocol B in the appendix.

results

The bisacrylamide gels did not show any colonies in the medium nor the gel after being subjected to a flow of the 10X diluted LB medium by the peristaltic pump (table 6).The alginate gel with the silicate membrane, using 30 mM sodium silicate, only showed bacteria inside of the gel, while in the medium no bacteria were present. The other silicate coated alginate gel showed bacteria inside- and outside of the gel. The second experiment found bacterial colonies in all the gels and the medium for both time intervals, except for the bisacrylamide gel. The latter gel did not show any colonies in the gel phase and liquid medium after one day. For all the other gels the amount of cells was too high to count them. Because of the absence of cells in the 4% bisacrylamide gels and medium, the medium and gel were plated again after five days, showing high numbers of bacteria.

Table 6: the amount of *O.tritici double mutant* cells in the gel and outside of the gel after one day in the peristaltic pump system experiment

Gel	Phase	CFU/mL
4% bisacrylamide 1	Gel	0
	Medium	0
4% bisacrylamide 2	Gel	0
	Medium	0
2.5% alginate with 30 mM sodium silicate	Gel	$5x10^5$
	Medium	0
2.5% alginate with 60 mM sodium silicate	Gel	$1.2x10^8$
	Medium	uncountable

The silicate coatings did not result in a prevention of cell leakage from the gel, except in the peristaltic pump experiment, where the gel -with a silicate coating, formed with 30 mM sodium silicate - did not show any cell leakage. On the other hand, for the tube experiment the silicate coated gels showed cell

leakage. The 4% bisacrylamide gel did not show any cells after one day in the 15 mL tube experiment and in the flow experiment. However, high amounts of cells were found by plating the liquid medium and the gel after five days.

4. Biofilter development

4.1 materials and methods

4.1.1 the alginate and sodium polyacrylate biofilter

The second generation gels, alginate and sodium polyacrylate gels, were tested for their arsenic removal potential. A bacterial suspension of *O.tritici* double mutant was grown in LB medium with 0.1% gentamycin and 0.2% hygromycin at 37 ˚C and 130 rpm (Agitorb 200, aralab, Portugal). Thirteen PVC tubes of 36 mm length and a volume of 5 mL were washed with 70% ethanol. Every tube was used for one gel of the different groups shown in table 7. A polycarbonate filter with a 0.2 µm pore size was added to one side of the tubes with a glue gun. With 10X diluted LB medium, 4% alginate and the bacterial suspension, a mixture of 5 mL with 1.0 OD bacterial optical density and 3% alginate was prepared. For the sodium polyacrylate gels, four tubes were filled with 10X diluted LB medium, bacterial suspension and sterile sodium polyacrylate powder in a concentration of 0.67%. For the groups that had a control - alginate gels in 200 µM, alginate gels in 2 mM and sodium polyacrylate gels in 200 µM- instead of the bacterial solution, 10X diluted LB medium was added. On the other side of the tubes another polycarbonate filter with 0.2 µm pore size was glued on with a glue gun. The alginate gels were left in 10% $CaCl_2$ for one hour, after which they were immersed in 70% ethanol for one minute and in PBS for 5 minutes. Afterwards, the gels were washed with deionized water. The gels were put in sterile Erlenmeyer's containing 100 mL of 10X diluted LB medium and the appropriate arsenic concentration, described in table 7. The Erlenmeyer's were incubated in a shaker at 30 RPM and 32˚C. One milliliter of the medium was removed at different time intervals (time = 0, 1, 3 and 5 days) and centrifuged at 13300

rpm (Sorvall Legend Micro 17 Centrifuge, Thermo Fisher Scientific). The supernatant was stored and a cell pellets were discarded. The gels were kept at 4 ˚C. The medium samples were plated on solid LB medium plates with 0.1% gentamycin, to test the amount of bacteria present outside of the gel. At the different time intervals the turbidity of the medium was judged qualitatively. After three and five days, the optical density of the bacterial suspension was measured. After five days the inside of the gel was plated on solid LB medium with 0.1% gentamycin. The presence of *O.tritici* double mutant cells in the alginate gel was confirmed by plating and Scanning Electron Microscopy (SEM) for concentrations of 200 µM, 2 mM and 10 mM of sodium arsenite, following protocol D in the appendix. The arsenic inside the medium samples of the different time intervals was analysed with an inductively coupled plasma mass spectrometer (ICP-MS; iCAP Q ICP-MS Thermo Fisher Scientific Inc., Bremen, Germany).

Table 7: the experimental set-up of the arsenic removal by a *O.tritici* double mutant strain in different gel supports.

group:	sodium *meta*-arsenite in medium:	gel type:	amount of gels:	control (n=1):
1	200 µM	alginate 3%	4	yes
2	2 mM	alginate 3%	4	yes
3	10 mM	alginate 3%	1	no
4	200 µM	sodium polyacrylate 0.67%	4	yes

4.1.2 the rice plant biofilter

Over 240 seeds of *Oryza sativa* from the floodplains of the Mondego river were uncapped, grown on wet cotton and distributed over 44 Petri dishes. Consequently, the seeds were washed in 70% ethanol for one minute, in 2.5% hypochlorite solution for 15 minutes, in 2.3:1 sodium hypochlorite with disinfestation solution for 15 minutes and in 1:1 sodium hypochlorite with disinfestation solution during 15 minutes. The disinfestation solution consisted of 1 g/L of Na_3CO_3, 30 g/L of NaCl and 1.5 g/L of NaOH, as an adapted mixture from Hurek et al. 1994. Of the 240 seeds, 200 seeds were grown on forty Petri dishes with 8 g/L agar and a 500 mg/L of yeast extract. After 5 days most of the seeds had germinated and 180 similar developed plants were transferred to long glass tubes. The medium used for the tubes was 15 or 20 mL of Hoagland medium. The medium was an adaptation of earlier used medium, adding 2 mM of ammonium

nitrate (Hoagland & Arnon 1950. The *Ochrobactrum tritici* double mutant strain was grown for 24 hours on LB medium with 0.1% gentamycin at 37 ˚C and 130 rpm (Agitorb 200, aralab, Portugal). After six days of growth, 110 of the 180 the plants, divided by group in table 8, were inoculated with the *Ochrobactrum tritici* double mutant strain, adding a volume of 2.3 mL and 2.0 mL from the bacterial suspension, resulting in a total optical density 0.1 OD. The presence of the bacteria was confirmed by PCR (My cycler©, Biorad, Hercules, USA; transilluminator, Biorad, Hercules, USA). The plants were removed from the original medium, washed with milliQ water and added to a new tube. One hundred and twenty five plants were added to 15 or 20 mL of Hoagland medium with 0, 20 mM or 33 mM of sodium arsenite. After 7 days the plants were removed from the medium. A sample of 1 mL of the medium was taken and frozen at -20 ˚C. The plants were shortly placed on a dry paper to remove the outside liquid, after which the fresh weight, stem length, length of longest root and the number of roots, were measured. In addition to the weight, the length of the longest root, the number of roots and the longest shoot length were measured. The plants were left to dry in an open tube, covered with a sheet of paper for two weeks at 50 ˚C and three days at 70 ˚C. When the plant tissue had completely dried, its weight was measured. The plant material was treated with 5% nitric acid in a total volume of one milliliter, macerated with a pestle inserted in a drill. The nitric acid mixture with plant material was left at 70 ˚C during 4 days. A 1 mL volume of 5% nitric acid was added and the plant tissue was left to digest for a week at 70 ˚C. The 2 mL of digested plant material was stored at 4 ˚C. For the ICP analysis 0.66 mL was mixed with ultra-pure water and nitric acid to have a volume of 10 mL with a concentration of 2% nitric acid. The ICP analysis was performed with an inductively coupled plasma mass spectrometer (ICP-MS; iCAP Q ICP-MS Thermo Fisher Scientific Inc., Bremen, Germany). The medium samples were analysed with x-ray fluorescence (XRF; High Sensitivity Fluorescent X-ray Analyzer SEA6000VX © Hitachi High-Tech Science Corporation). Data was analysed by a Bartlett's test and Shapiro-wilk test to see the homogeneity of variance and the normality of the data. Afterwards, a Wilcoxon test and/or a Kruzkal-Wallis was performed to calculate the significance of differences in data.

Table 8: the set-up of the different plant groups

group	presence of bacteria	sodium arsenite concentration (µM)
B-/As-	no	0
B-/As+	no	20
B+/As-	*O.tritici* double mutant	0
B+/As+	*O.tritici* double mutant	20
B+/As+	*O.tritici* double mutant	33

4.2 results

4.2.1 the alginate and sodium polyacrylate biofilter

The presence of the *O.tritici* double mutant bacterium in the gels was confirmed, the bacterium seemed clustered and more bacteria were present in the alginate gel with 200 µM of sodium arsenite than with the other concentrations (figure 17).

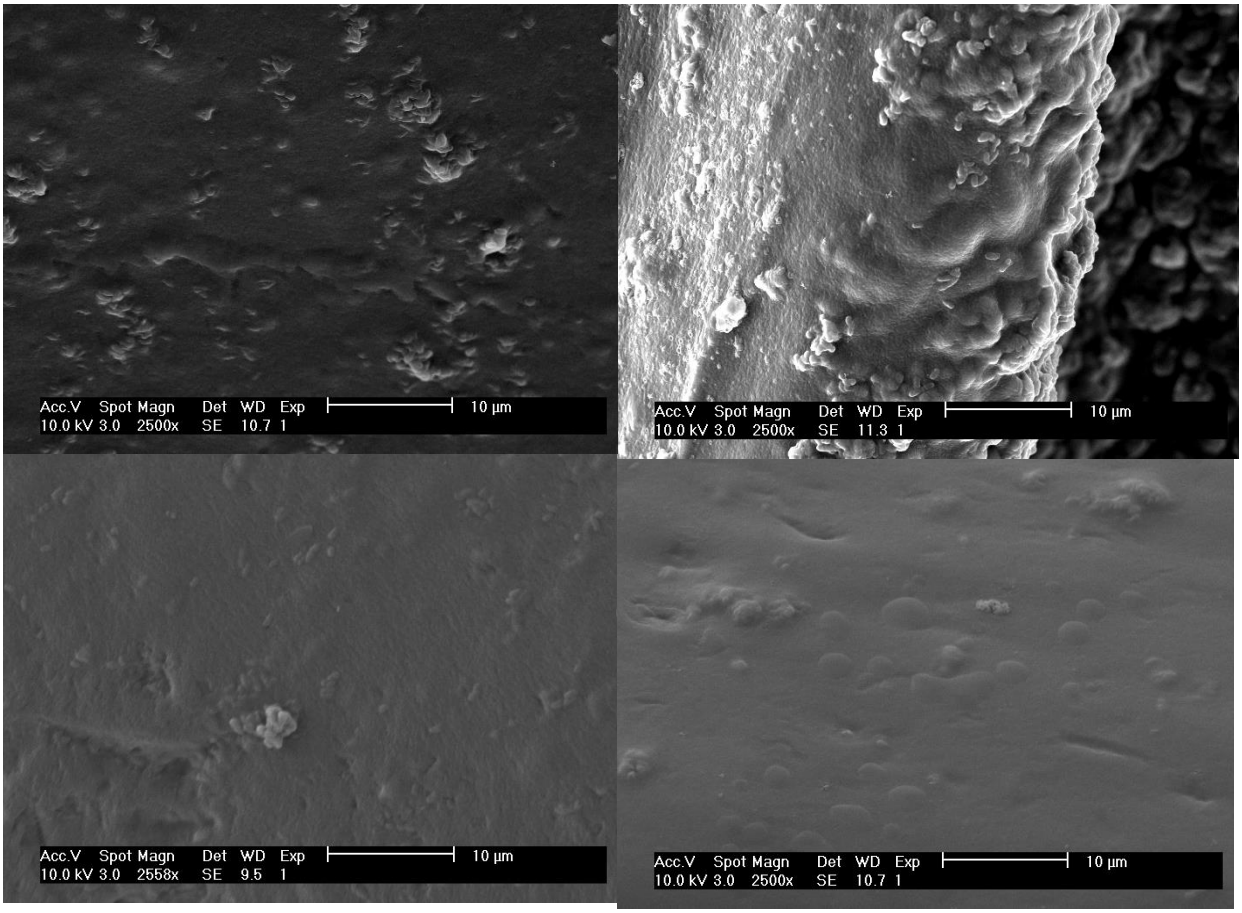

Figure 17: Scanning Electron Microscopy (SEM) pictures of the alginate gel in 200 µM (left and right up), 2 mM (right down) and in 10 mM of sodium arsenite (left down).

The amount of arsenic in the medium decreased drastically initially and gradually increased to a certain amount after time (figure 18). At the start of the experiment, the arsenic concentration was the lowest for all the gels. All the gels except for the controls showed large numbers of cells in the gel after seven days (>

10^7 CFU/mL), confirming the presence of the bacteria. The arsenic concentration inside of the medium for

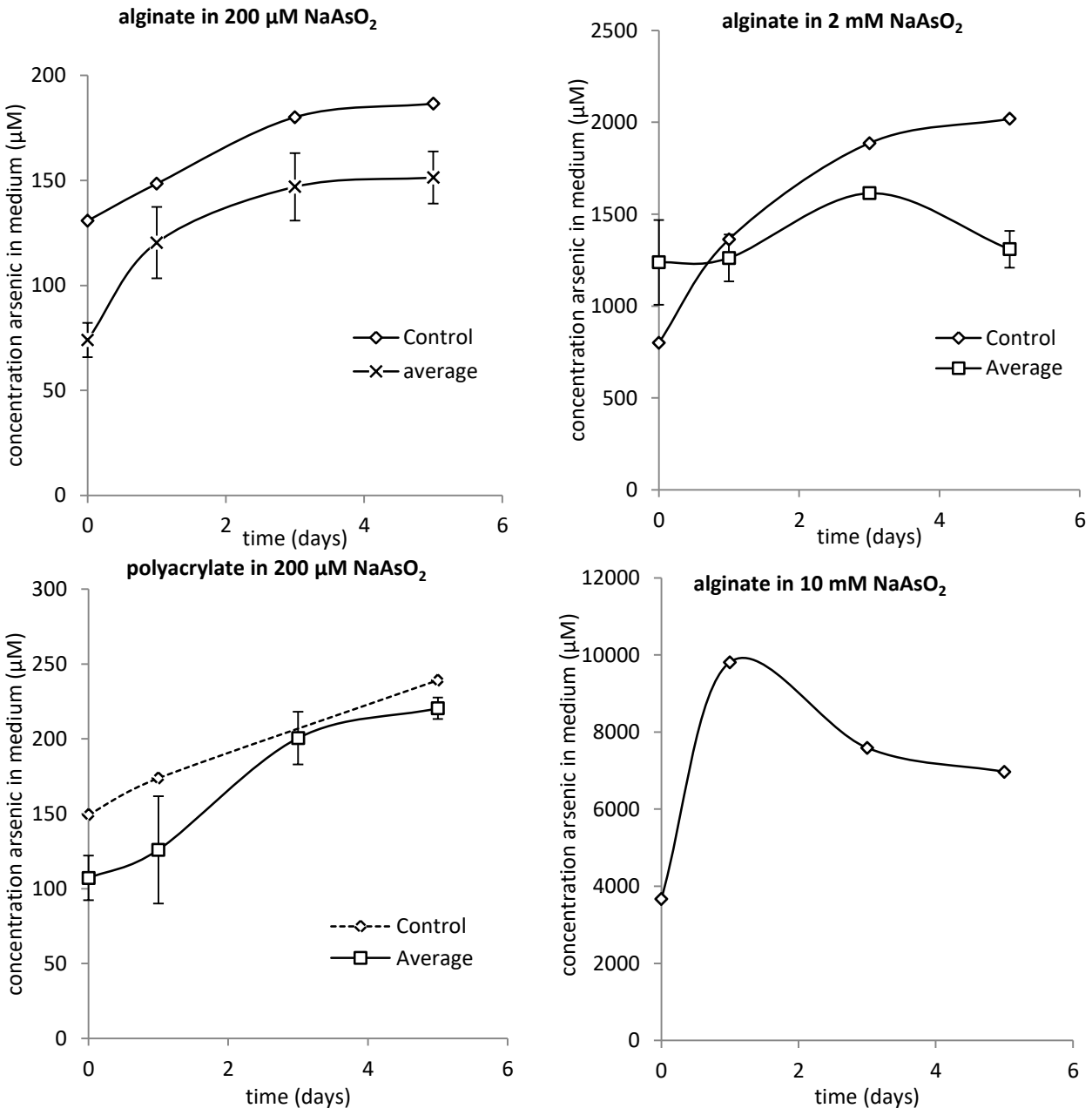

Figure 18: the sodium arsenite concentration in the medium for the *O.tritici* double mutant strains immobilized alginate gels with 200 µM, 2 mM (n=3) and 10 mM (n=1) arsenite and the sodium polyacrylate gel with 200 µM of arsenite (n=3). The error bars represent the standard error and the dotted line for the control in the sodium polyacrylate gel means that an outlier with concentrations much higher than the initial arsenic concentration was left out after three days , The concentration of 10 mM of sodium arsenite did not have a control.

the different gels was more similar to the control, when the average of the replicate gels was taken. The similarity in shape was more evident in the alginate gels in 200 µM sodium arsenite, than the other

alginate gels and the sodium polyacrylate gel. Contrarily to a similar shape in the arsenic removal of the curve, the absolute amounts of arsenic in the medium were lower in the gels with bacteria than in the gels without bacteria, the controls. After five days the concentration of sodium arsenite was almost the initial concentration in the controls going up to 200 µM, 2 mM and 10 mM of sodium arsenite; the concentration of sodium arsenite seemed to stabilize after five days (figure 18). For the gels with bacteria, the end concentration of arsenic was lower than the gels without bacteria.

After five days the average amount of sodium arsenite taken up from the medium was 36.61 mg, 7.65 mg, 452.39 µg and 184.40 µg respectively for the alginate gel with 10 mM of sodium arsenite, the alginate gel with 2 mM of sodium arsenite, the alginate gel with 200 µM of sodium arsenite and the sodium polyacrylate gel with 200 µM of sodium arsenite**.** These values correspond to 28.99%, 29.42%, 18.84% and 6.10% percentage of the arsenic from the medium taken up (figure 19).

The percentages were calculated by dividing the absolute amount of sodium arsenite taken up by the maximum amount of sodium arsenite shown in one of the gels. The theoretical maximum amounts of arsenic in the different gels would be respectively 2600 µg, 26 mg and 130 mg, for the sodium arsenite concentrations of 200 µM, 2 mM and 10 mM. In practice, the controls did not reach this maximum level completely for reasons of gel and medium adhesion and electrostatic forces, so the following maxima were chosen, for the calculation of the percentages. The alginate gel of 10 mM sodium arsenite had a considered maximum value of 126.28 mg, which was the highest concentration observed over time for this gel; the alginate gel of 2 mM had a maximum of 25.99 mg, the highest value of the control; the alginate gel of 200 µM had a maximum of 2400.89 µg, which was also the highest value of the control; the sodium polyacrylate gel of 200 µM had a maximum of 3020.87 µg, also being the highest value of the control. The latter value was higher than the expected amount of arsenic in the medium, while the other values were lower. One of the points of the sodium polyacrylate control, after three days, showed a much higher arsenic concentration than the maximum expected value. This value was 371.36 µM and is 171.36 µM more than expected. This first value was considered as an outlier. The other points at time=5 days of the sodium polyacrylate showed slightly higher concentrations than 200 µM.

The percentage of arsenic taken up from the medium was similar for the alginate gel with 10 mM and 2 mM of arsenic, even though the concentration was five times higher in the 10 mM solution.

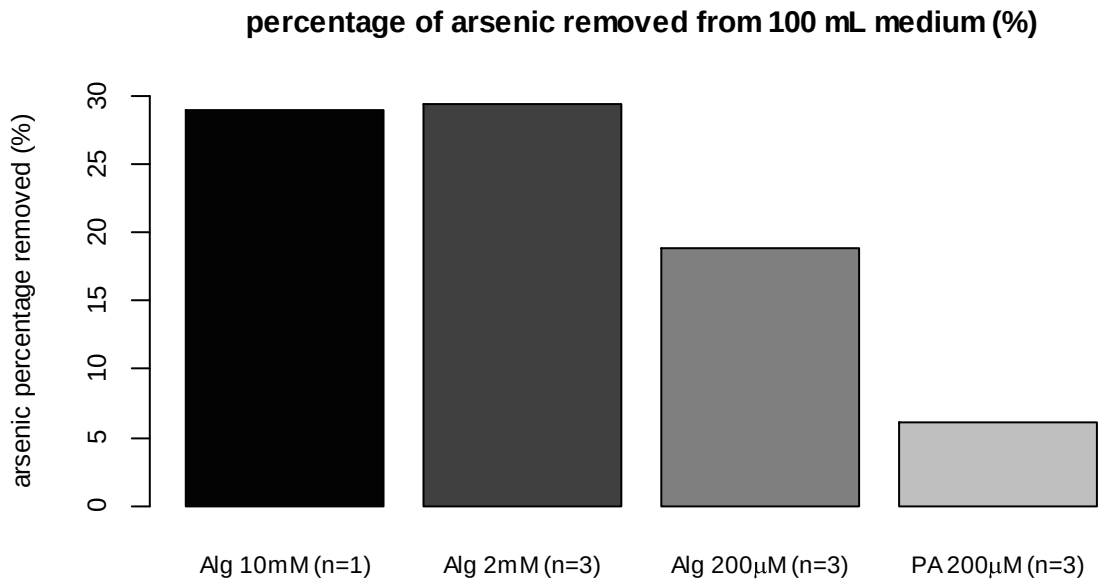

Figure 19: percentage of total sodium arsenite taken up from the medium by the *O.tritici* double mutant strain, embedded in a 2.5% alginate gel (Alg) or a 0.67% sodium polyacrylate gel (PA) in sodium arsenite concentrations of 200 µM, 2 mM and 10 mM.

4.2.2 the rice plant biofilter

Besides the gels, rice plants were analyzed for their arsenic removal, when inoculated with the *O.tritici* double mutant bacteria. The plants that were grown in the absence of arsenic all significantly had less arsenic than the plants that were grown in the presence of arsenic (table 9). The amount of arsenic taken up by the plants that had not been inoculated with the bacterium, was 25.25 µg of the total arsenic taken up, and was significantly higher than the other plant groups with arsenic in the medium (table 9). The plants inoculated with bacteria in the presence of 20 µM and 33 µM sodium arsenite took respectively around 6.61 µg and 9.60 µg arsenic from the medium, not differing significantly (figure 20). The plants without bacteria and 20 µM sodium arsenite, the plants with bacteria and 20 µM sodium arsenite and the plants with bacteria and 33 µM, respectively took up 39.24%, 10.27% and 11.19% of the total amount of arsenic from the medium.

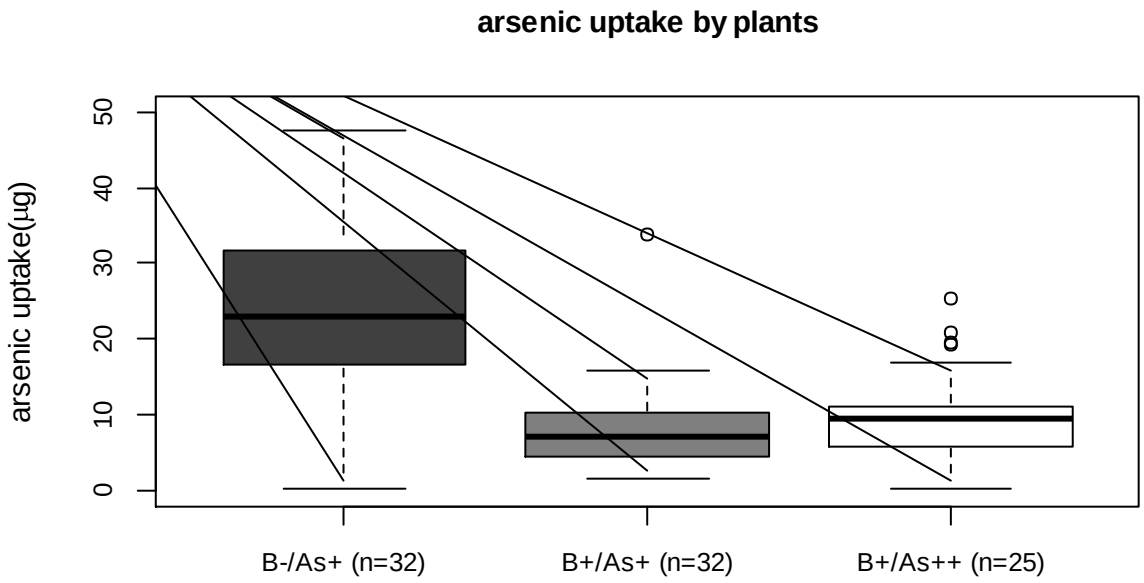

Figure 20: amount of arsenic taken up from the medium by a plant group of no inoculated bacteria and 20 µM of sodium arsenite, a group with inoculated plants in 20 µM of arsenite and a group of inoculated plants in 33 µM of arsenite.

The differences between the plant groups were less evident, when the absolute amount of sodium arsenite per fresh weight and dry weight was considered (figure 2 and 3 in the appendix). Only the plants with no bacteria inoculated and a presence of 20 µM of arsenite were significantly different from the other groups.

Table 9: difference in arsenic uptake by five different groups: plants with no bacteria and arsenite (B-/As-), plants with no bacteria and 20 µM of arsenite (B-/As+), plants with inoculated *O.tritici* double mutant without any arsenite (B+/As-), plants with inoculated bacteria and 20 µM of arsenite (B+/As+) and plants with inoculated bacteria (B+/As+) and 33 µM of arsenite (B+/As++). significance: p<0.05*,p<0.01**,p<0.001***

difference in arsenic uptake (µg)					
group	mean (µg)	B-/As+	B+/As-	B+/As+	B+/As++
B-/As-	0	25.25*	0	6.61*	9.60*
B-/As+	25.25		25.25*	18.63***	15.65***
B+/As-	0			6.61*	9.60*
B+/As+	6.61				2.99
B+/As++	9.60				

The difference in growth variables was significant between the plants with no bacteria and 20 µM of arsenite and all the other plants. The p-values of a Kruzkal-Wallis test within plant groups were respectively 0.008137, 0.4073, 3.403e-05, 0.2381 and 0.02359 for the variables fresh weight, dry weight, stem length, root length and number of roots. The fresh weight, stem length and number of roots were different between all groups of plants. The root length was only different between the plants with no bacteria and arsenite (B-/As-) and the plants with no bacteria and 20 µM of arsenite (B-/As+) and B-/As- and the inoculated plants with no arsenite (B+/As-). The dry weight did not significantly differ between any of the two groups. The latter group was therefore left out of the analysis, because it did not indicate a difference between groups. The stem length, root length and fresh weight showed the highest value for the group without arsenic and bacteria. The number of roots was higher for the group with arsenite and no bacteria compared to the group without both (figure 21).

The values of the stem length and fresh weight were significantly lower for the plants with arsenic and bacteria than for the plants with arsenic and no bacteria. The length of the longest root was also lower in

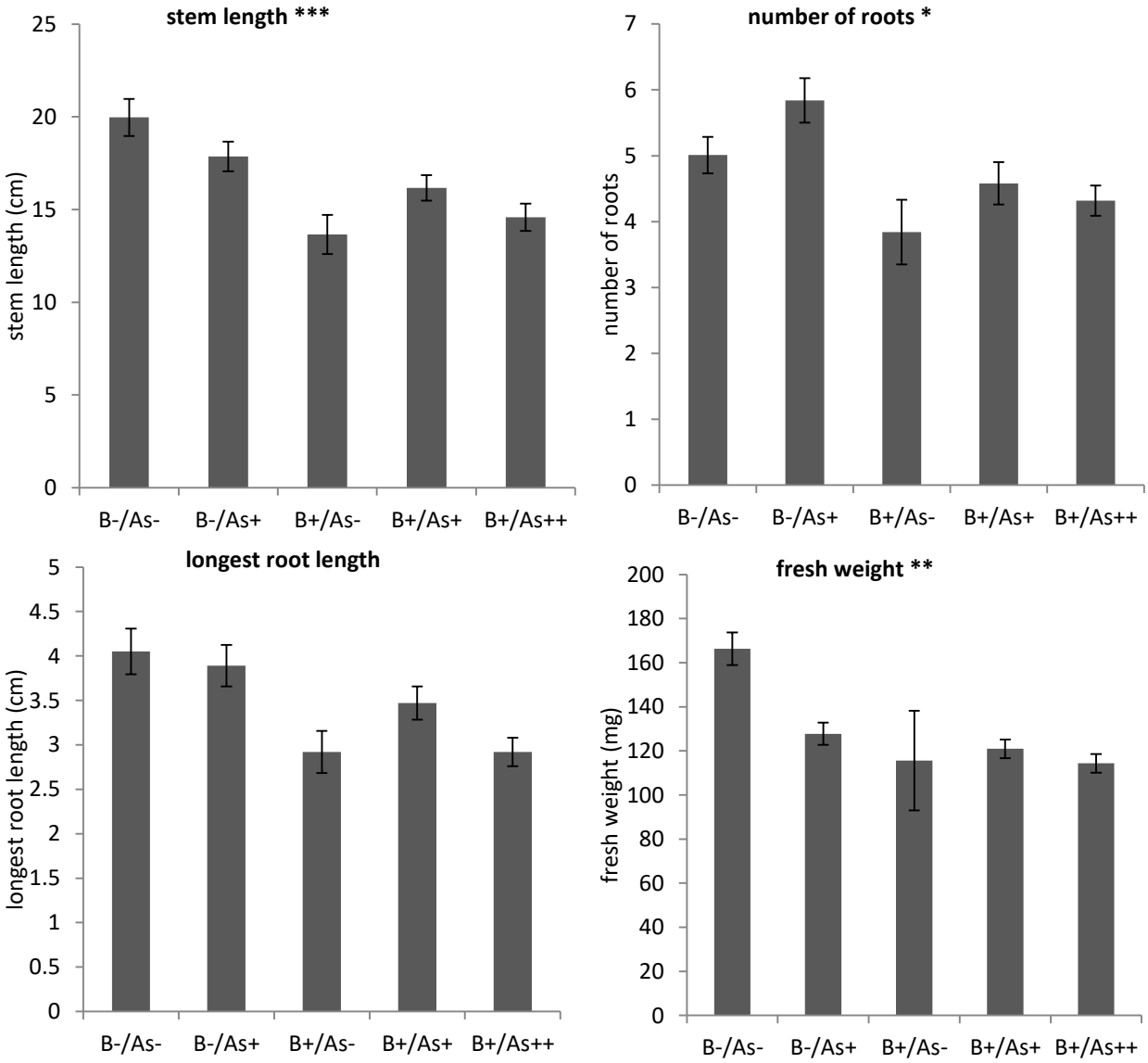

Figure 21: the different growth parameters for the plants without bacteria and no arsenite (B-/As-; n=32), without bacteria and 20 µM of arsenite (B-/As+; n=32), with bacteria and no arsenite (B+/As-; n=32), with bacteria and 20 µM of arsenite (B+/As+; n=32) and with bacteria and 33 µM of arsenite (B+/As++; n=25), having as error bars the standard error and as significance of differences within groups: p<0.05 (*), p<0.01 (**) and p<0.001 (***)

this case, but not significantly. The plant group with no arsenic and the presence of bacteria, B+/As-, had the lowest values for all growth variables.

The presence of 20mM sodium arsenite in the medium increased the stem length by 2.52 cm, longest root length by 0.55 cm and number of roots by 0.74 for the plants inoculated with the *O.tritici* double mutant bacteria. In table 3 in the appendix the exact values of the growth parameters are shown.

5. general discussion

5.1 hydrogel performance

All the preliminary tested gels contained living cells and the cells were able to escape from the gel (table 10). Only the alginate and sodium polyacrylate gels were found to be possible candidates for a bioremediation filter, because of their water permeability and diffusion of nutrients through the gel (table 10). Different results, useful for further research, were found along the path of gel development.

cell viability

Higher amounts of APS in the polyacrylamide gel resulted in lower cell amounts inside of the gel. A possible explanation for the lower amount of cells in presence of a higher APS concentration could be induced by damage to the cell caused by free radicals of the APS, exerting its toxicity in such a way that metals can be toxic due to free radical generation (Lemire et al. 2013). For the polyacrylamide gel, pHEA and pHEMA gels the presence of micro colonies after at least two weeks suggested a lack of nutrients after at least two weeks. Adding sodium polyacrylate powder to alginate gels did not result in a higher amount of cells inside of the gel.

water permeability

The scanning electron microscopy pictures, showed earlier, depicted a gel that had a thick polymer wall for the 7% and 10% monomer/crosslinker gels, from the exterior as well as the interior (figure 12). The surface of the gel did not show a relief-like structure and the bacteria were stuck to a thick polymer wall. Adding a 2.5% concentration of PEG to the alginate gels, the pHEA and pHEMA gel did not lead to an enhancement in the water permeability. Perhaps the pores formed by the PEG did not reach deep enough to let the water pass through. The small membranes of the three different gels were too fragile to act as a membrane to let the water pass.

cell leakage

The results of the two experiments were not strong enough to assume a prevention of cell leakage from either a silicate coated alginate gel or a 4% bisacrylamide gel. The bisacrylamide gels did not contain cells, perhaps as a result of the high amounts of ammonium persulfate in the mixture (10%) as was stated in the referred article (Tantimongcolwat et al. 2014). In the peristaltic pump experiment the -by 30 mM sodium silicate coated- alginate gel did not show cell leakage. However, the -by 60 mM sodium silicate coated- alginate gel did show cell leakage and so did the silicate coated alginate gels in the tube experiment.

Table 10: different hydrogels were tested for their features: vivid cells inside of the gel (1) allowance of water passage through the gel (2) diffusion of nutrients from the medium to the inside of the gel (3) gel structure (4) the leakage of cells from the gel to the medium (5). Some gels were not tested for the diffusion of nutrients (nt).

gel	cell viability (1)	water permeability (2)	diffusion of nutrients (3)	gel structure (4)	cell leakage (5)
polyacrylamide	yes	no	no	strong	yes
pHEA	yes	no	nt	acceptable	yes
pHEMA	yes	no	nt	bad	yes
alginate	yes	yes	yes	acceptable	yes
sodium polyacrylate	yes	yes	nt	acceptable	yes

5.2 removal of arsenic by hydrogel systems

The amount of sodium arsenite over time had a typical trend for the different gels, showing a quick decrease in sodium arsenite at the start of the experiment with the sodium arsenite concentration going back to the original concentration after five days in the controls. An explanation for this trend may be the fact that both the alginate gels and the sodium polyacrylate gels are ionic hydrogels. Based on the features of an ionic hydrogel (Bell & Peppas 1995), the cations in the alginate gels, Ca^{2+}- ions and the cations in the sodium polyacrylate gel, Na^{2+}-ions, may have caused a higher osmotic concentration inside of the gel. The water would have flown inside of the gel, increasing the concentration of arsenic in the medium. Metabolic activity inside of the gel by the bacteria could have caused a fluctuation in the arsenic amounts over time. Additionally, some of the arsenic may have initially been chelated by organic material and would have later on dissociated from the organic matter.

The arsenic final removal at five days of the sodium polyacrylate gels –including the control- was higher than 225 µM, the initial concentration added. The control showed a data point at t=3 with an amount of 371µM that was treated as an outlier. This excess of concentration could be explained by a possible heterogenous distribution of the arsenic in the medium due to charge differences caused by the sodium ions in the gel, contaminated material or a pipetting error. However the chances of a pipetting error or contaminated material are small, since all the replicates showed elevated concentrations of arsenic. Electrostatic forces could be able to repulse the negatively charged arsenite oxyanions, leading to a heterogenous distribution of the ions. More information could be gained by measuring the surface charge of the sodium polyacrylate gel and the exact mechanisms between the negatively charged arsenite oxyanions, the anionic groups of the sodium polyacrylate polymers and the sodium ions should be further investigated.

In earlier research it was found that the *O.tritici* double mutant could not grow in concentrations higher than 1 mM and apparently the strain could survive in a concentration ten times higher (10 mM) inside of the alginate gel (Sousa et al. 2015). It should be tested whether the cells were also able to proliferate in this concentration. This difference in survival by cell immobilization might justify the use of the genetically modified bacteria in elevated concentrations of arsenic in wastewater. Inside of the gel the bacteria would survive, but would not be able to spread to the water due to toxicity of the arsenic. The test with the sodium silicate coating of the alginate did not show an inhibition of cell escape in a water-flow through system nor a closed tube system, as was observed in the reported study (Ramachandran et al. 2010). A possible explanation could be the application of polyallylamine hydrochloride prior to the sodium silicate treatment or the bead shapes in the referred study (Ramachandran et al. 2010) instead of the cylindrical shapes in the present research.

The maximum arsenic removal of 30% by the alginate gels with bacterial cells, was lower than the total amount of mercury removal reported in a study on mercury bioaccumulation by a bacterial strain immobilized in alginate gel (Sinha & Khare 2012). The mercury concentration was much lower than the concentrations used in the alginate gel removal assays. Additionally, the chemical properties and metabolism of mercury are different from arsenic. A study of the cadmium removal from cyanobacteria in alginate gels in concentrations of 0.26 mM of Cadmium showed a removal of nearly 100% after an hour (Rangsayatorn et al. 2004).

It was shown that the biofilters had a pH optimum for the metal removal of 6 and the effect of the pH could infer important consequences as well for the *O.tritici* double mutant strain immobilized in an alginate gel (Rangsayatorn et al. 2004). Temperature did not affect the metal sorption (Rangsayatorn et al. 2004).

The concentrations of metal used in this research were high compared to other research, but another reported study gave some insight in the proportion of metals taken up by the gels in proportion to the bacteria (Degiorgi et al. 2002). Lead and cadmium ions were taken up by adhesion to a 30%pHEA-*co*-20%glycerol gel matrix (6.7% for lead and 6.0% for cadmium ions present) and a high removal of these elements by *E.coli* cells growing in the gel (91.5% for lead ions and 71.3% for cadmium ions) (Degiorgi et al. 2002). For these ions in this neutral hydrogel the majority of the metal ions were removed by the bacteria and not by gel adhesion. However, the amount of contaminated medium used was very low in the referred study, twenty grams of medium, and the concentration of the metal ions was lower, having a 942µM cadmium concentration and a 302 µM lead concentration. Another bacteria was used for the analysis of the uptake of copper and chromium(III) and the concentrations taken up were much lower, being 8.7% and 18.8% of the total amount of ions respectively by the gel and the inoculated bacteria (Degiorgi et al. 2002).

5.3 plants as supports

The rice plant, *O. sativa,* as a support for the arsenic accumulating *O.tritici* double mutant bacterium, did not show a higher uptake than the rice plant without bacteria, in contrast to what was expected. The amount of roots of the rice plant was higher in the presence of arsenic, which could possibly be a phenotypic adaption to the arsenic concentration.

The rice plant showed the highest arsenite uptake and an increase in growth in the absence of the *O.tritici* double mutant bacterium, while in the presence of the metal the length of shoot, length of root and number of roots were bigger, when the bacteria was present. The plant had a higher number of roots in arsenic contaminated medium. These results are in line with results obtained from the earlier referred study, showing a higher metal resistance in plants that were inoculated with –rice isolated- endophytic bacteria (Madhaiyan et al. 2007).

These bacteria were shown to produce ethylene reducing enzymes, lowering plant hormone stress, and reduced metal stress of nickel and cadmium due to possible accumulation in the rhizoplane of the plant (Madhaiyan et al. 2007). Ethylene is a stress hormone produced by plants and is present in higher levels with high metal concentrations, reducing the plant growth (Madhaiyan et al. 2007). An experiment done with tomato plants, canola and Indian mustard plants, showed a relieve of the growth inhibition caused by zinc, lead and nickel in plants inoculated with metal resistant bacteria (Burd et al. 2000). A siderophore overproducing bacterial mutant relieved the growth inhibition even more than just the wild type bacteria, suggesting a relation of the growth stress relieve to the provisioning of iron (Burd et al. 2000). For arsenic metal resistance, bacteria isolated from hyper accumulators were found to have increased metal resistance, growing up to 10 mM of arsenic, to oxidize As(III) to As(V) and to store the arsenic in the biomass (Zhu et al. 2014).

Other endophytes were found to increase the growth and metal accumulation of plants in zinc and lead contaminated environments (Rajkumar et al. 2009). The mechanisms of this increased uptake of metals inside of the plant were thought to be related with the metal solubilization by organic acids, acidification, production of metal phosphates and siderophores, produced by the endophytic bacteria (Rajkumar et al. 2009). This solubilization of non-soluble metals in the rhizoplane would result in a higher uptake of the metals in the plant roots. In our study arsenic was already in soluble state and no immobilized arsenic was present. It should be further investigated if the rice plant in presence of non-solubilized arsenite would show a higher arsenite uptake due to the arsenic accumulating bacterium present on the roots.

5.4 ecological perspective

An effective use of bioaugmentation demands adaptions to overcome the ecological barriers present in the -to be contaminated- sites (El Fantroussi & Agathos 2005). Immobilization of cells and the use of ecological niches for the choice of the bacterial strain that is to be mutated, are important for the improvement of the technique (El Fantroussi & Agathos 2005).

The use of cell encapsulation for bioremediation purposes has several advantages and disadvantages. On one hand the use of gel encapsulation, allows metal accumulators to have adapted environmental conditions compared to outside of the gel.

The supports, being gels or plants, are more controlled than just the environment alone, allowing a possible improvement in efficiency of the traditional remediation strategy (Cassidy et al. 1996). It was found, for example, that the metabolism of encapsulated cells was higher than not encapsulated cells (Cassidy et al. 1996). The possibility of cells escaping was lower for these methods and gels in the shape of beads can be readily stored for a long time (Cassidy et al. 1996). Beads produced, such as alginate beads, are broken down readily in time, are not toxic and have less off-site drifting than classical methods (Cassidy et al. 1996). In medical applications, the chemical properties of the cell encapsulation were adapted in such a way to stop large immunoglobulins from entering inside of the gel (Jen et al. 1996). These findings suggest that there are possibilities of stopping immobilized bacterial cells from leaking to the environment. In the light of the findings that the *O.tritici* double mutant strain could grow inside, but not outside of the gel in elevated concentrations of arsenic, possibilities can be thought of for implications in the environment. The mutated bacterium used in this research, *O.tritici* double mutant, is a genetically modified organism and therefore not allowed to escape to the environment. This work points out that the toxicity of elevated concentrations of arsenic, may prevent the bacterial cells to proliferate outside of the gel.

In this study the main focus was on the supports that were used as a biofilter in conjugation with the *O.tritici* double mutant strain and other organisms could be investigated for their suitability in an arsenic biofilter, either as support or bioaugmentation organism. The high accumulation of arsenic by marine algae and fungi has a potential to be integrated in the gel. Additionally, the algae could be tested for their response to the arsenic accumulating bacteria. Another type of microbial strains that could be integrated in the alginate gel are the cyanobacteria from which one strain was integrated in an alginate gel in the previously described work (Rangsayatorn et al. 2004).

5.5 economic perspective

The amount of maximum contaminant level (MCL) in the United States was decreased from 0.05 mg/L in 1975 to 0.01 mg/L in 2001 (Wang et al. 2011). In order to make it easier to comply with these new standards, a comparison of money cost for small-scale arsenic water treatment was made (Wang et al. 2011). All these methods were conventional remediation techniques without the focus on using organisms for bioremediation. The cost of the chemicals per treatment ranged up to 0.61 $ per 1000 gallons of water treated (Wang et al. 2011).

If the amount of arsenic removed was assumed to be from the previous arsenic MCL to the MCL in 2001 in the US and if the arsenic was present as sodium arsenite, the price per gram of arsenic removed ranged up to 3.46 euro per gram of arsenic removed. In this research the sodium arsenite removed from the 2 mM contaminated medium was 29.4%, similar to the percentage taken up by the gel in 10 mM. For these two data values, a higher absolute arsenic removal was shown for the higher concentration of sodium arsenite. The cost per gram of sodium arsenite can be approached with:

cost = (volume/0.1)*price **(1)**

cost_as = cost / (conc * mol * geleff) **(2)**

cost_as = 0.005756/ conc **(3)**

In these formulae the "volume" was the total volume of the site to be decontaminated in liters and "cost" and "cost_as" were respectively the price of the number of gels used and the price per gram of arsenic removed from the water. The "price" parameter was the price of the 3% alginate and 10 mL of 10% $CaCl_2$ needed for one gel of five milliliter, buying the chemicals in powder form per 5 kg (W201502-5KG) and 500 g (F.W. 147.02) respectively (Sigma-Aldrich 26-07-2017, Flinn Scientific 26-07-2017). An assumption in this model was that the number of gels in the volume was one per 100 mL, according to our set-up, so that the gel efficiency was similar to our research. The latter gel efficiency was taken as "geleff". It was assumed that the arsenic uptake of the gels was proportionate between 2 mM and 10 mM of sodium arsenite. The gel efficiency was the proportion of 0.294 of sodium arsenite removed by the alginate gels in 2 mM and the "mol" variable was the molarity of sodium arsenite. The "conc" variable represented the concentration of sodium arsenite in the -to be decontaminated- volume in mol/liter. The price per gram of arsenic removed reduced with a higher concentration of arsenic contamination (figure 22). The price per alginate removed was lower than the value of most expensive chemicals for the adsorptive media method (Wang et al. 2011) and was in the range of costs of the average adsorptive media method (0.45 euro per gram arsenic) and the maximum prices of ion exchange systems (2.77 euro per gram arsenic; Wang et al. 2011). It was promising that the price of the alginate gels indicated a value in the price range of other small-scale arsenic removal chemicals, although the small-scale arsenic removal methods were set-up as a different system, a flow system, with lower concentrations of arsenic used (Wang et al. 2011).

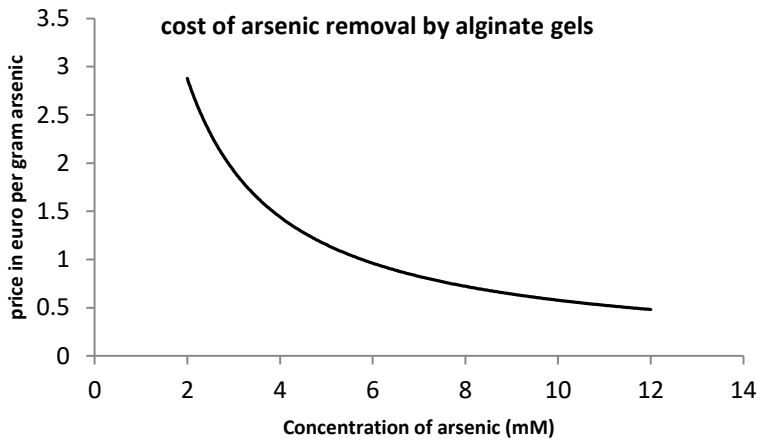

Figure 22: an indication of the cost of removing one gram of arsenic from contaminated wastewater with alginate gels

5.6 prospects for gel-cell immobilization and plant-bacteria interaction

The ionic hydrogels, the second generation gels, showed the passage of water through the gels in contrast to the neutral hydrogels, the first generation gels. The ionic hydrogels have as both their advantage and disadvantage that they are influenced by the environment in their swelling capacity (Bell & Peppas 1995). The alginate gel foundanother function in a probable uptake of arsenite oxyanions in its structure. The swelling capacity and amount of counterions taken up could be further increased by increasing the pH higher than the pKa of the gel, resulting in an ionization of the anionic functional groups in the alginate gel. The gels used performed well in terms of matrix entrapment, but the characteristic of micro encapsulation was missing. A new gel, already used in biomedical applications for its biocompatibility and protection from immunoglobulins, could be used for countering the cell leakage of the inserted bacteria. Two gels that protected mammalian cells from immune system attacks were alginate-polylysine beads and 2-hydroxyethyl methacrylate-*co*-methylmethacrylate (pHEMA-*co*-MMA) (Jen et al. 1995). Both of these gels were adaptions of gels studied in this research, the first gel being an adaption on the ionic alginate hydrogel and the second gel being an adaption on the neutral pHEMA hydrogel.

The bisacrylamide gel had promising features. As explained in previous sections the molecular weight between conjuctions in the neutral hydrogels, such as bisacrylamide gel is influenced by molecular weight of the polymer before crosslinking. Following this characteristic, the amount of monomer can be increased in such a way to find a balance between water passage and prevention of cell leakage. For the polyacrylamide gel it was found that low concentrations of monomer resulted in an incomplete gel formation and too high amounts of polyacrylamide resulted in a prevention of water flow-through. An adaption of a sole bisacrylamide gel was tested and showed promising results in terms of gel formation. Future perspectives of the latter gel could be increasing the monomer to increase a mesh size, still able to pass through the water, but preventing the cells from passing through.

https://drive.google.com/file/d/0B_EB-kc5N136eEh0QmxhX0hMWG8/view?usp=sharing

Thirdly, new innovations may be found in the application of sol-gels. These gels form mesoporous homogeneous small membranes, that could act as a filtering membrane (Owens et al. 2016). Although the silicate coating using 30 mM of sodium silicate did not prevent cell leakage in these experiments, further changes in the production should be tried to find its use as an optimized microbe filter.

Lastly, a combination of plants with sodium polyacrylate gel and bacteria could be an interesting application for bioremediation strategies, since sodium polyacrylate has already found its use in the application to soils for phytostabilization, increasing heavy metal uptake (De Varennes et al. 2009).

The several mechanisms of action of the bacterial metal metabolism that affect the metal uptake in plants, should be explored in future research. The speciation of arsenic might be an influencing factor on the mobilization of arsenic. It should be investigated if arsenic is solubilized in the medium by the bacteria and if the arsenic uptake in the plant is higher, when non-accumulating endophytic communities or natural communities are introduced.

5.7 conclusion

This work showed that the supports that passed the main objectives for a great bioremediation tool, i.e. immobilization of viable cells, allowance of water flow through, nutrients diffusion and a texture that is resistant to environmental conditions, were the 3% alginate gel, the sodium polyacrylate gel and the plain rice plant, without bacteria. The polyacrylamide, pHEMA and pHEA gels did not allow water to pass through the gel –even with the addition of PEG- and were therefore not suitable for bioremediation purposes.

The rice plant support showed the highest arsenic uptake if no bacteria was inoculated, taking up 39.24% of the arsenic. The inoculation of *O.tritici* double mutant cells decreased important growth variables: stem length, longest root length, number of roots and fresh weight. In the presence of the bacterium the plant growth was higher in 20 mM arsenite contaminated medium than in the medium without arsenite, having an average increase in stem length of 2.52 cm, longest root length by 0.55 cm and number of roots by almost one (0.74). The second best arsenic removal system was the alginate-cell gel that showed amounts of total arsenic removed of 28.99%, 29.42% and 18.84% with 2 mM, 10 mM and 200 µM sodium arsenite, respectively. The sodium polyacrylate gel showed relatively the highest concentration of sodium arsenic present in the medium after five days, removing just 6.10% of the total arsenic. Besides the fact that the arsenic removal by the hydrogels was not completely efficient, this work points out an important finding: the possibility of larger removal amounts of arsenic from the medium and a higher survival of the *O. tritici* double mutant bacteria entrapped in alginate gel than previously reported in absence of a gel (Sousa et al. 2015).

The high uptake percentages of the alginate gel with the *O.tritici* double mutant bacteria in elevated concentrations of arsenite, lead to a potential of the gels being used in the bioaugmentation technique. The use of gel immobilization is a good option for the protection of the bacterial strain by possible competitors, creating a more controlled micro bioreactor in the shape of a gel. However, the gels need to be further developed to stop bacterial cells from escaping. Besides the gel, rice plants could be good candidates for the removal of arsenic from contaminated sites with low levels of arsenic. The *O.tritici* double mutant bacteria could be inoculated in the plant to increase the growth parameters of the plant in the presence of arsenic. Nevertheless, the role of endophytic communities on the rice plants' arsenic resistance should be investigated.

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APPENDIX

A.1 Protocols

Protocol A : standardized gel
A mixture was made of consequently 0.1 OD or 1 OD of bacterial suspension, 10X diluted LB medium, 9.67% monomer, 0.33% cross linker, 1.5% of Ammonium persulfate and 0.5 % of TEMED. For the polyacrylamide gel acrylamide was used as a monomer and N,N′-Methylenebisacrylamide as the cross linker. For the pHEMA gel the monomer used was 2-hydroxyethyl methacrylate (HEMA) and the cross linker ethylene glycol dimethacrylate (EGMA). For the pHEA gel the monomer and cross linker used were 2-hydroxyethyl acrylate (HEA) and EGMA. The gel was left to polymerise for fifteen minutes and was washed with PBS and deionized water. For all the gels the proportion of monomer to cross linker was kept constant.

Protocol B: plating of the medium
A volume of 100 µL of medium or diluted was plated on LB solid medium. These solutions were plated using the spread plate technique on solid LB medium, having 0.1% gentamycin and 0.2% hygromycin. If many bacteria were expected, dilutions to the power of ten were made using 10X diluted LB medium and these dilutions were plated instead.

Protocol C: plating of the gel
The procedure for the plating of sodium polyacrylate gels was identical to protocol B, since the gel had a liquid-like consistency. For the sturdy gels the following procedure was used. The gel was removed from the medium, washed with PBS and deionized water and placed on a sterile Petri dish. Around 100 µL of the gel was cut off with a surgical blade and transferred to a 2 mL Eppendorf. For alginate beads a bead was removed, washed and put directly in the Eppendorf. To the eppendorf 900 µL of 10X diluted LB medium was added to the gel. The gel was crushed with tweezers and mixed in the vortex. A part of the solution, 100µL, was plated by the spread plating technique on a plate with solid LB medium. If many bacteria were expected, dilutions to the power of ten were made using 10X diluted LB medium and these dilutions were plated instead.

Protocol D: Scanning Electron Microscopy (SEM)
A small piece of the gel was cut off with a surgical blade and treated in a 36 well plate. Of a PBS solution 2.5mL was added to the 36 well plate and the well plate was shaken for ten minutes at 50 rpm. Consequently the samples were treated -for ten minutes per solution- with 70% ethanol, 80% ethanol, 90% ethanol, 95% ethanol and 100% ethanol, keeping the samples on the shaker at 50 rpm. The ethanol was removed afterwards and the samples were left to dry. After treating the samples, they were sputter coated resulting in a 20 nm gold layer on the gel fragments. Photos were made by Scanning Electron Microscopy (XL 30 TMP, Philips).

Protocol E: standard preparation of the alginate gels
A mixture of 2.5% alginate gel, 10X diluted medium and 0.1 OD or 1 OD bacterial suspension was made. In a syringe or tube 10% $CaCl_2$ was added with a cut out cleaning sponge and the gel was left to polymerise during fifteen minutes. The sponge was removed and a volume of 10% $CaCl_2$ was added and the gel was

left to polymerise for two hours. In an Erlenmeyer the 10% CaCl2 solution was added by gently sprinkling the gel with a pipette. The gel was washed on top with PBS and deionized water.

Solution preparations:
PSB solution: 8g/L NaCl, 0.2 g/L KCl, 1.44g/L Na_2HPO_4, 800 mL deionized water, adjust PH to PH 7.4, add up to one liter with deionized water.
Luria Broth (LB) solid medium: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 15 g/L granulated agar and up to one liter deionized water.
Luria Broth (LB) liquid medium: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl and up to one liter deionized water.

A.2 Figures and tables

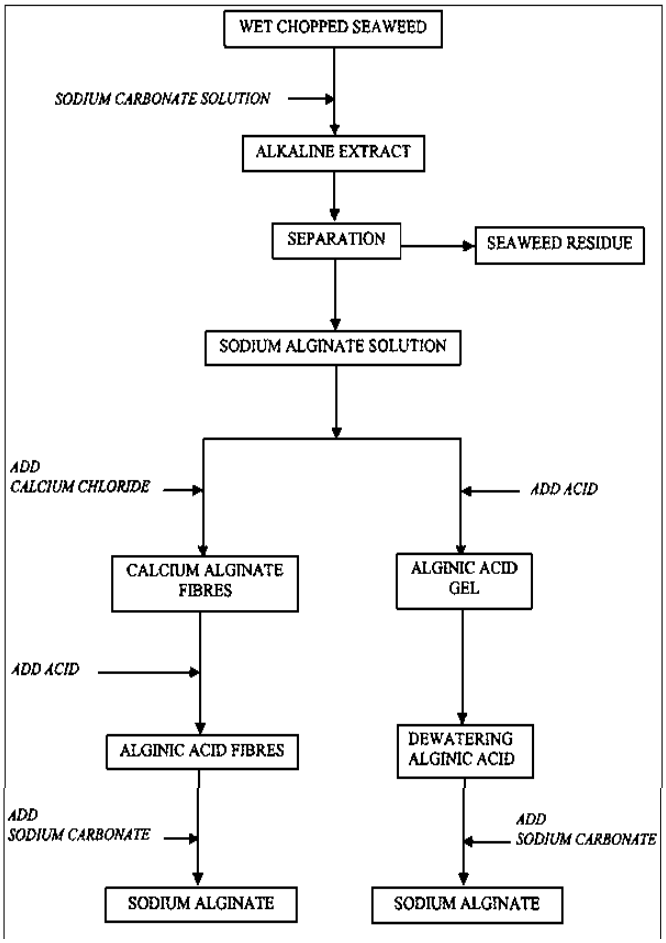

Figure 1: production line from raw material to utilizable alginate (McHugh 1987)

Table 1: difference in arsenic uptake per fresh weight plant for five different groups: plants with no bacteria and sodium arsenite in the medium (group 1), plants with no bacteria and 20 µM of sodium arsenite in the medium (group 2), plants with inoculated *O.tritici double mutant* bacteria without any sodium arsenite in the medium (group 3), plants with inoculated bacteria and 20 µM of sodium arsenite and plants with inoculated bacteria (group 4) and 33 µM of sodium arsenite (group 5). significance: p<0.05*,p<0.01**,p<0.001***

Difference in arsenic uptake per fresh weight (mg/g)					
Group	Mean (mg/g)	2	3	4	5
1	0	0.18	0	006	0.08
2	0.18		0.18	0.12***	0.10 ***
3				0.06	0.08
4	0.06				0.02
5	0.08				

Table 2: difference in arsenic uptake per dry weight plant for five different groups: plants with no bacteria and sodium arsenite in the medium (group 1), plants with no bacteria and 20 µM of sodium arsenite in the medium (group 2), plants with inoculated *O.tritici double mutant* bacteria without any sodium arsenite in the medium (group 3), plants with inoculated bacteria and 20 µM of sodium arsenite and plants with inoculated bacteria (group 4) and 33 µM of sodium arsenite (group 5). significance: p<0.05*,p<0.01**,p<0.001***

Arsenic uptake per dry weigh					
Group	Mean (mg/g)	2	3	4	5
1	0	1.60	0	0.46	0.64
2	1.60		1.60	1.13***	0.88 ***
3	0			.46	0.72
	0.46				0.26
5	0.72				

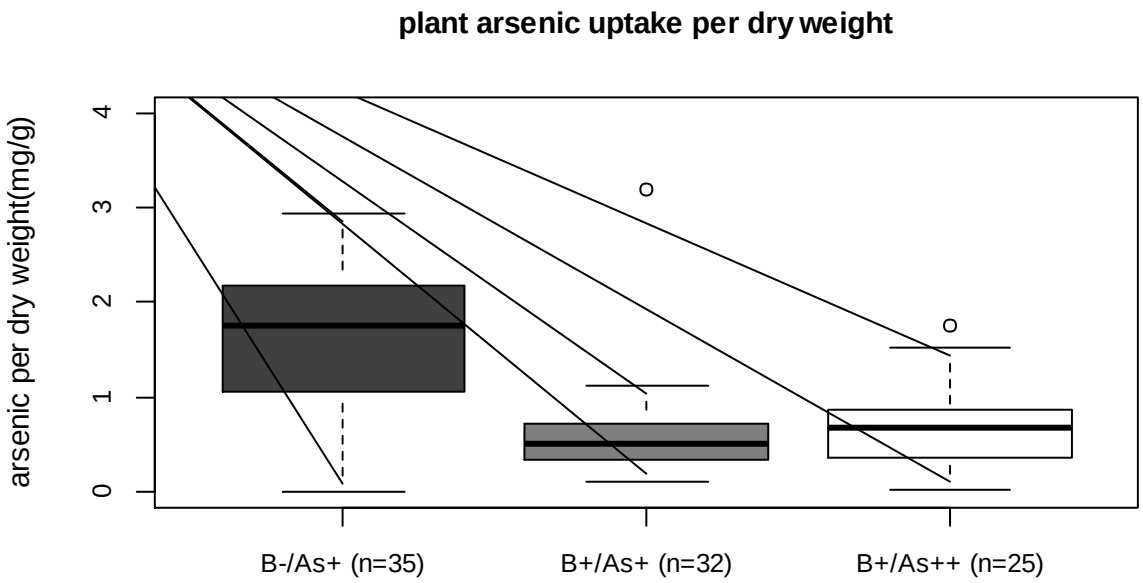

Figure 2: arsenic uptake per dry weight of plant for the three plant groups with arsenite sodium in the medium. The concentrations indicate the concentration of sodium arsenite in the medium.

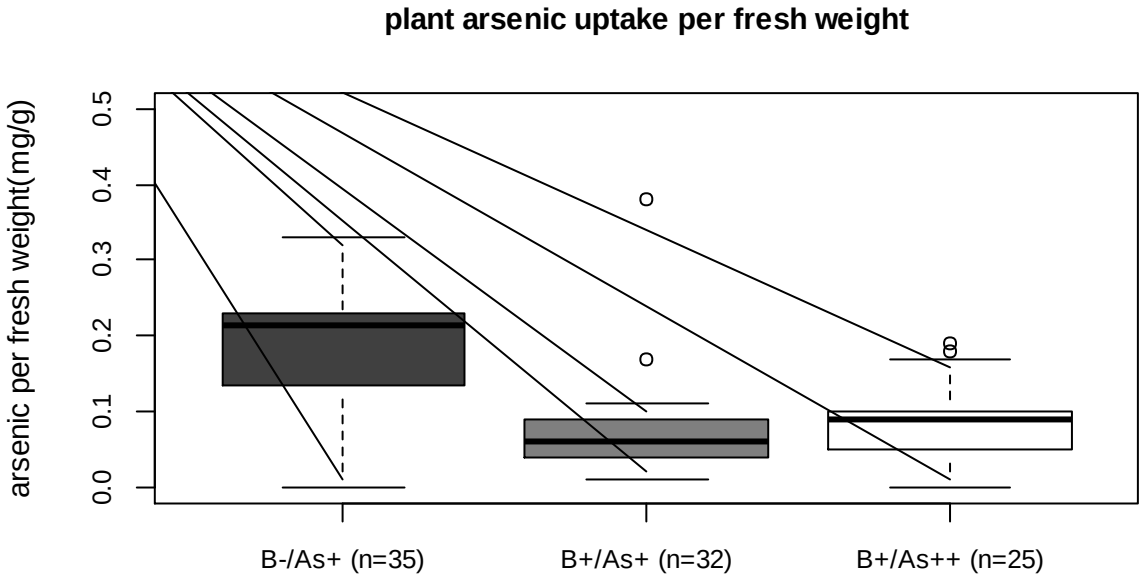

Figure 3: arsenic uptake per fresh weight of plant. The concentrations shown, are the concentrations of sodium arsenite present in the medium.

Table 3: morphological variables for the five different plant groups: plants with no bacteria and arsenite (B-/As-), plants with no bacteria and 20 µM of arsenite (B-/As+), plants with inoculated *O.tritici* double mutant without any arsenite in the medium (B+/As-), plants with inoculated bacteria and 20 µM of arsenite (B+/As+) and plants with inoculated bacteria and 33 µM of sodium arsenite (B+/As++). The differences were significant between all the groups in terms of stem length, number of roots and fresh weight. The hierarchy of values of different growth variables within groups is indicated with shades of gray.

Gou	Stem length (m)	Longest root length (cm)	Number of roots	Fresh weight (mg)	Dry weight (mg)
B-/As-	19.97	4.05	5.01	166.31	16.78
B-/As+	17.86	3.89	5.84	127.77	15.00
B+/As-	13.65	2.92	3.84	115.57	13.62
B+/As+	16.17	3.47	4.58	120.92	15.53
B+/As++	14.58	2.92	4.32	114.32	13.93